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Application of microbial cross-over for the production of Italian Grape Ale (IGA), a fruit beer obtained by grape must addition

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Abstract:	The addition of fruits or their derivate to malt wort is one of the most prominent trends due to growing consumer interest towards new beer styles. In this scenario, many Italian brewers, during the last years, have produced Italian Grape Ale (IGA) beers, beverage that represents a communion between beer and wine. Moreover, an emerging trend in modern brewing is the microbial cross-over, a novel technological approach in food microbiology where a microorganism from one traditional fermentation process is used to improve quality and safety in another agri-food product. Thus, three autochthonous Saccharomyces cerevisiae strains (CHE-3, P4, TA4-10) previously isolated from different foods, and the commercial strain US-05, were tested at laboratory scale in fermentation media obtained from malt extract added with two grape must amounts (15 and 25%) in comparison with malt wort. At the end of the fermentation, the three strains showed significantly higher CO2 production than the commercial one. The main analytical parameters (organic acids, real and apparent attenuation, ethanol content, glycerol level and carbohydrate profile) and by-products related to the organoleptic quality of the experimental beers were strongly influenced both by the grape must addition to the fermentation medium and the used strains. However, in all the tested strains the highest aromatic expression was observed in the beers obtained by adding 15% grape must. The selection of media composition and the high potential of innovation related to microbial biodiversity represent a pivotal tool for contributing to product differentiation, particularly desired in the craft beers sector.
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Cover Letter

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Dear Editor,

Enclosed please find a copy of the manuscript "Application of microbial cross-over for the production of Italian Grape Ale (IGA), a fruit beer obtained by grape must addition" by Gabriella Siesto,Rocchina Pietrafesa, Maria Tufariello, Carmela Gerardi, Francesco Grieco and Angela Capece, that we would like to submit to the Board of Editors to be considered for publication in Food Bioscience.

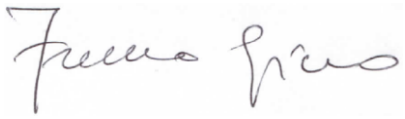
We state the novelty of the manuscript and that it has not been published neither previously nor is under consideration for publication elsewhere. We believe that our work is very current as new publications appear in journals on this area of work in the last years. In particular, our review focuses on the potential of using pigmented cereals and legumes for renewing the sensorial quality of beers and for improving their health-promoting skills.

We declare to do not have actual or potential conflict of interest concerning the submitted manuscript.

With many thanks for your attention and with kindest regards

Sincerely

Dr. Francesco Grieco



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Application of microbial cross-over for the production of Italian Grape Ale (IGA), a fruit beer obtained by grape must addition

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ABSTRACT

The addition of fruits or their derivate to malt wort is one of the most prominent trends due to growing consumer interest towards new beer styles. In this scenario, many Italian brewers, during the last years, have produced Italian Grape Ale (IGA) beers, beverage that represents a communion between beer and wine.

Moreover, an emerging trend in modern brewing is the microbial cross-over, a novel technological approach in food microbiology where a microorganism from one traditional fermentation process is used to improve quality and safety in another agri-food product.

Thus, three autochthonous *Saccharomyces cerevisiae* strains (CHE-3, P4, TA4-10) previously isolated from different foods, and the commercial strain US-05, were tested at laboratory scale in fermentation media obtained from malt extract added with two grape must amounts (15 and 25%) in comparison with malt wort. At the end of the fermentation, the three strains showed significantly higher CO_2 production than the commercial one. The main analytical parameters (organic acids, real and apparent attenuation, ethanol content, glycerol level and carbohydrate profile) and by-products related to the organoleptic quality of the experimental beers were strongly influenced both by the grape must addition to the fermentation medium and the used strains. However, in all the tested strains the highest aromatic expression was observed in the beers obtained by adding 15% grape must. The selection of media composition and the high potential of innovation related to microbial biodiversity represent a pivotal tool for contributing to product differentiation, particularly desired in the craft beers sector.

Keywords: IGA beer; autochthonous *Saccharomyces cerevisiae* strains; starter culture; microbial cross-over; aromatic compounds.

INTRODUCTION

Beer is one of the most ancient and pleasant consumed beverages in the world. Different types and styles of beer are available on the market in response to consumers demand, increasingly focused on artisanal products (Postigo et al., 2021). Traditionally, beer is brewed from four basic ingredients: water, malt, hops and yeast and the most popular categories of industrial products are 'Ale' and 'Lager' beers, obtained at different fermentation temperatures (16-24°C and 6-15°C, respectively). Actually, there is a global growing interest towards novel beer styles that differ from those traditional for some operating conditions (wort production, microorganisms used, addition of salt, herbs, spices or fruit) (Denby et al., 2018; Ducruet et al., 2017; Mayer et al., 2016). In particular, the special-type beers, obtained by adding different fruits, show a complexity sensory profile, with a harmonious and balanced fruity character (Martínez et al., 2017). The fruit beer is one of the most attractive products of micro- and craft breweries (Nardini and Garaguso, 2020) and, at same time, it presents some interesting properties as high amount of polyphenols (mainly phenoloic acids), aromatic compounds and good antioxidant capability (Gasínski et al., 2020; Kawa-Rygielska et al., 2019; Salanță et al., 2020). In recent years, Beer Judge Certification Program (BJCP, 2015) has established a new original fruit beer made in Italy and well known as Italian Grape Ale (IGA), resulting from the union between beer and wine. This style beer brewed by many Italian craft breweries could better express the connection with the territory, biodiversity of Italian grape cultivars as well as enhancing the brewer's creativity (Garavaglia et al., 2020). IGA beers are produced utilizing pilsener/pils or other pale base malts with the addition of grapes or grape must in a range from 5% to 40% (the maximum amount permitted by the law) of the wort composition (De Simone et al., 2021) and added at different fermentation stages (boiling, primary or secondary fermentation or bottling). Other than the addition of fruits, herbs, and spices, the choice of yeast starter culture is a further tool available to craft brewers to obtain products characterized by distinctive aromatic profiles (Capece et al., 2021; Postigo et al., 2021). An emerging trend in food microbiology is the application of microbial cross-over, a

technological approach where a microorganism from one traditional specific fermentation process is used to improve quality in another agri-food production/chain (Dank et al., 2021).

Indeed, several studies reported that *Saccharomyces cerevisiae* strains, isolated from different foods (as wine and bread) can produce typical and strain-specific fermentative aroma profiles in beer (Cubillos et al., 2019; Marongiu et al., 2015; Rossi et al., 2018). In beer production technology, the use of starter cultures is addressed to increase the efficiency of fermentation process, to realize new beers, and to enhance the overall quality of the produced beer (Aquilani et al., 2015; Canonico et al., 2014, 2021; Capece et al., 2018). The sugars naturally occurring in the raw materials are converted into CO_2 and ethanol by yeasts metabolism which is also responsible for the production of many secondary compound during fermentation, as higher alcohols and esters (Lodolo et al., 2008; Pires et al., 2014), desirable volatile molecules for a pleasant beer.

Typically, a considerable number of different volatile compounds determines beer flavour, but only several of these are recognized as aroma-active compounds (Olaniran et al., 2011, 2017), belonging mainly to the classes of esters, alcohols, (higher or fusel alcohols), aldehydes and acids (Biazon et al., 2009).

In this study, three indigenous *S. cerevisiae* strains, isolated from different food matrices, in comparison to a commercial *S. cerevisiae* strain frequently used in brewing, were tested in fermentation trials at laboratory scale with aim to select a starter culture potentially useful for production of IGA craft beer. The four strains were tested in fermentation media obtained from malt extract added with two different grape must amounts (15 and 25%) in comparison with a malt wort without grape must addition. To the best of our knowledge, this investigation is the first study concerning the employment of the microbial cross-over for the production of IGA craft beer in Southern Italy.

2. Materials and methods

2.1 Yeast strains

Three indigenous *Saccharomyces cerevisiae* strains, belonging to UNIBAS Yeast Collection (UBYC), University of Basilicata (Potenza, Italy), were used in this study: CHE-3, isolated from fruit; P4, isolated from sourdough (Capece et al., 2018); TA4-10, isolated during spontaneous fermentation of Inzolia grape must (Capece et al., 2011). These yeast strains were previously selected based on their phenotypic characteristics, such as ability to ferment glucose, maltose, sucrose and fructose, and high fermentative power in laboratory scale trials performed in 100 mL of commercial malt extract (data not shown). The commercial starter US-05 was used as the control. The strains were grown on YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone; 2% (w/v) glucose; 2% (w/v) agar, Oxoid, Hampshire, UK), and maintained at 4°C for further analysis.

2.2 Fermentation trials at laboratory scale

A commercial extract malt for Pale Ale beer (MrMalt®, Udine, Italy) has been treated according to provider instructions. The malt syrup was dissolved in 12 L of sterilized water and the mixture was subdivided in three batches: the control, without any addition (M); IGA15 and IGA25, with addition of grape must up 15 and 25%, respectively, of the total volume. Before addition, white grape must heated up to 80°C and rapidly cooled to room temperature. Experiments were carried out in flasks containing 400 mL of wort and the flasks were inoculated with the yeast strains at a concentration of 1×10^7 cells/mL, starting from a pre-culture grown in liquid YPD at 26°C for 24 hours. The fermentation trials were performed at 20°C in duplicate under static conditions. Fermentation kinetics were monitored by measuring the weight loss of flasks and reduction of total soluble solids (°Plato) with a refractometer. Once weight loss remained constant for 3 consecutive days, the secondary

fermentation was carried out by disposing samples in 250 mL bottles in the presence of 5 g/L of sucrose for two weeks at 20°C and, after storage at 4°C for one month, the samples were analyzed.

2.3 *HPLC analysis*

Organic acids (tartaric acid, succinic acid and acetic acid) were identified onto an Agilent Hi-Plex H (300 × 7.7 mm) with internal particles of 8.0 μm (Agilent Technologies, Santa Clara, CA, USA). The temperature of the column compartment was maintained at 70°C. The flow rate applied was 0.4 mL/min with a run time of 30 min. The phase was 4.0 mM /L H_2SO_4 in ultrapure water (Coelho et al., 2018). Standard solutions were injected to obtain the retention time for each compound. For the determination of tartaric, acetic, and succinic acids detection was conducted in the DAD at 210 nm. The maltodextrin, sucrose, maltose, maltotriose, glucose, fructose, glycerol and ethanol concentration were quantified using an Agilent Hi-Plex Ca column (300 mm x 7.7 mm) with internal particles of 8.0 μm (Agilent Technologies, Santa Clara, CA, USA). The mobile phase used was deionized water and a constant flow rate of 0.6 mL/min for a run time of 30 min. Sugars detection was carried out by refractive index detection (RID). Quantification of individual organic acids and sugar were performed directly by Chem-Station software (Agilent) using a five-point regression curve ($R^2 \geq 0.99$) because of authentic standards.

2.4 Volatilomic assay

The contents of main secondary compounds, such as acetaldehyde, ethyl acetate, acetoin, n-propanol, isobutanol, n-butanol, 2-methyl-1-butanol and 3-methyl-1-butanol, were quantified by direct injection into a gas-chromatographic system (GC Agilent 7890). The sample was prepared and analyzed following the procedure described by Capece et al. (2021).

The analysis of volatilomic profile was carried out by a solid phase micro extraction in combination with a gas chromatography coupled to mass spectrometry (HS-SPME-GC/MS). According to the

methods of Tufariello et al. (2019), 100 μL of internal standard solution (ISTD, 4-methyl-2-pentanol, 300 mg/L) was added to a volume of 5 mL of wine in a 20 mL headspace vial (Alltech Corp., Deerfield, IL, USA). A 50/30 DVB-CAR-PDMS solid phase microextraction (SPME) fiber (Supelco, Bellofonte, PA) was inserted into the vial and let to adsorb volatiles for 30 min at 40°C and then transferred to the injector port (250°C) where desorption occurred in 2 min. Splittless mode was selected as injection mode. GC-MS analyses were performed on a GC 6890 (Agilent Technologies, Palo Alto, CA) coupled to an Agilent MSD 5973 Network detector using a HP-INNOWAX capillary column (60 m × 0.25 mm, 0.25 μm, J&W Scientific Inc., Folsom, CA, USA) as reported by Tufariello, et al. (2019). The annotation of the volatile compounds was achieved by comparing mass spectra with those of the data system library (NIST 98, P > 90%), with the retention data of commercially available standards and MS data reported in the literature. Concentration of each volatile compound was assessed by the internal standard method following this equation:

VOCs concentration = (VOC GC peak area/IS GC peak area) × IS concentration as reported in previous scientific studies (Kang et al., 2010; Perestrelo et al., 2018).

2.5 Statistical analysis

One-way analysis of variance (ANOVA) was carried out to determine the differences among the four tested strains in the fermentative performance for each medium used: malt wort, malt wort added with 15% and 25% of grape must. Two-way ANOVA was carried out to evaluate the influence of the fermenting yeast strain (first independent variable) and the fermenting media (second independent variable on each of the analytical parameters (dependent variable).

Data of aromatic compounds were submitted to classical two-way clustering analysis, by using Ward's algorithm and Euclidean similarity index. The Z-scores were used to reduce the dimensionality of the data and to find the best differentiation between yeast strains and type of the

media used to produce beers (Capece et al., 2021). The data obtained were converted to Z-scores, calculated as follows:

$$\text{Z-score} = (X - \mu)/\sigma,$$

where X = concentration of the aromatic compound, μ = mean value of all strains per measured compound and σ = standard deviation of values per tested compound.

All statistical analysis were carried out using the Past free software ver. 4.2 (Natural History Museum-University of Oslo, Oslo, Norway) (Hammer, 2001).

3. Results and discussion

3.1 Fermentative aptitude of Saccharomyces cerevisiae strains

The three *S. cerevisiae* strains CHE-3, P4 and TA4-10, isolated from fruit, sourdough and grape must, respectively, were for the first time tested as starter for the production of IGA craft beer. The first step of the our cross-over strategy aimed to the assessment of the brewing potential of the above strains, in order to design novel starter cultures, capable to positively modulate the quality properties of beer.

The first step of our procedure was addressed to evaluate the fermentative performance (measured as CO_2 grams produced during the process) of the four strains on brewing worts with or without the addition of grape must up 15 and 25% (Figure 1 A-C). By comparing the CO_2 produced in the three fermentation media for all the strains, the CO_2 production increases with the addition of grape must, with the highest level in beers obtained with wort added with 25% of grape must (Figure 1C). These data are in line with a recent research by Castro Marin et al. (2021), in which fermentations performed in samples supplied with 10 and 20% of Cv. Lambrusco Grapes Must showed faster kinetics than

control wort. By analyzing the different strain behaviour in each condition, in wort without grape must addition (sample M, Figure 1A), P4 strain showed the highest release of CO_2 during the first fermentation days, with an increase of CO_2 production from 7.77 to 14.07 g CO_2/400 mL, whereas the lowest values were observed for CHE-3 strain (from 4.98 to 12.60 g CO_2/400 mL). After the sixth fermentation day and until the end of the monitored period (13 days), the highest fermentative activity was exhibited by the commercial starter US-05. At the end of the process, no statistical differences were found among the three selected strains.

As regards the fermentative performance in wort added with 15% of grape must (IGA15, Figure 1B), the trend was similar to that observed in the control fermentation. In the first three days of fermentation, P4 yielded the highest CO_2 production (from 10.82 to 17.78 g CO_2/400 mL), whereas US-05 produced the lowest CO_2 amount (ranged from 7.43 to 15.32 g CO_2/400 mL). After the sixth fermentation day, the commercial starter showed CO_2 production levels very similar to the three indigenous strains; indeed, after sixth fermentation day no significant differences were registered among all the strains.

Finally, in wort added with 25% of grape must (IGA25, Figure 1C), during the first three days the same results observed both in M and in IGA15 fermentations were found, with the highest CO_2 production for P4 strain. Moreover, in this condition (IGA25), after the eighth fermentation day, the strain showing the highest CO_2 production was CHE-3, reaching 26.71±0.74 g CO_2/400 mL at the end of fermentation process. However, at the end of the process the three indigenous strains showed significantly higher CO_2 production than the commercial one, indicating that these strains probably are less influenced by the high percentage of grape must in the fermentation medium. These results confirmed that the exploitation of the brewing potential of different groups of microorganisms, such as *Saccharomyces* strains isolated from other fermented food and beverage, is an emerging trend to contribute to beer differentiation, in particular for craft beer production (De Simone et al., 2021).

Recently, the brewing potential of yeasts isolated from other traditional fermented beverages, such as cachaça (Araújo et al., 2018), tequila (Cubillos et al., 2019), or African alcoholic spirits (Johansen

et al., 2019) has been assessed. In addition to alcoholic beverages, other fermented foods could also serve as a source for novel yeast isolation (Tamang, 2010). Moreover, numerous *S. cerevisiae* strains isolated from Brazilian distilleries have been employed in high gravity beer production, undoubtedly displaying to be novel starter cultures for the brewing industry (Christofoleti-Furlan et al., 2020).

3.2. Beer analytical profiles

At the end of secondary fermentation, the experimental beers produced by the four *S. cerevisiae* strains in the three different media (M, IGA15 and IGA25) were analyzed for analytical parameters and by-products related to organoleptic quality.

The carbohydrate profile of wort samples and experimental beers are illustrated in Table 1. As regards the different fermentation media, maltose, maltotriose and maltodextrine were found to be the most abundant saccharides in malt wort, whereas the highest content of glucose and fructose was found in wort added with grape must, in direct proportion with grape must content. As regards the beers, no residual maltose was found in the samples fermented with CHE-3, P4 and TA4-10 strains (with exception of the low content detected in beer from malt wort inoculated with TA4-10 strain), whereas all the samples inoculated with commercial strain US-05 contained low maltose amounts, without difference statistically significant among the three experimental beers. No fructose was detected in all the samples, while a glucose content ranging from 1.67 and 3.47 mg/mL were detected in all the IGA samples, with exception of CHE-3 IGA15. As already reported for the fermentation media, the beers obtained by malt worth contained the highest content of maltotriose and maltodextrine. The results of the two-way ANOVA showed that the strain influenced mainly maltotriose, maltose content, the type of fermentation media significantly influenced the content of all fermentable sugars, and the interaction between media and strain affected mainly maltose and glucose content.

The varieties and concentrations of carbohydrates varied among the different types of experimental beer. The sugars present in finished beers reflected the utilization of yeasts; the monosaccharides are usually virtually completely fermented; in fact, only traces of glucose are present in the final beers,

242 whereas the effect of brewer's yeast on oligosaccharides, mainly for maltotriose, varies for different
243 yeasts. The ability to metabolize maltose and maltotriose is not widespread, thus restricting the
244 brewing potential only to a few species (Cubillos et al., 2019). Maltotriose is fermented slowly and
245 sometimes incompletely, so traces may remain in beer (Li et al., 2020). Our results confirmed that
246 the utilization degree as well as the ability to utilize these sugars is strain dependent (Meier-Dörnberg
247 et al., 2017). Two wild strains, CHE-3 and P4 (isolated from fruit and sourdough, respectively) were
248 more efficient in maltose fermentation than the commercial strain US-05. Other authors (Rossi et al.,
249 2018) by comparing *S. cerevisiae* strains isolated from different sources, such as grape must, bakery,
250 wine, during laboratory-scale fermentation, selected a baking yeast as the most promising strain,
251 showing that sourdough could represent an important source of biodiversity for selection of
252 autochthonous strains suitable for craft beer production.
253 The data regarding to the main analytical parameters detected in the experimental beers are reported
254 in Table 2. As shown by the real and apparent attenuation (RA and AA, respectively), fermentation
255 progressed to a greater extent in the IGA25 samples, in particular in beer obtained by US-05, with
256 RA and AA values of 72.19 and 88.08%, respectively, whereas the lowest values were detected in
257 the M beers for all the tested strains. Consequently, the highest amount of ethanol content was found
258 in IGA25 beers, with exception of sample fermented with commercial strain US-05. This beer
259 contained the lowest ethanol amount among the three samples fermented with this strain (3.76% v/v),
260 thus justifying the higher amount of residual of fermentable sugars glucose and maltose found in US-
261 05 IGA25 than those detected in US-05 M and US-05 IGA15 beers (Table 1).
262 Glycerol is the by-product of ethanol fermentation carried out by *S. cerevisiae* that contributes to
263 body and mouthfeel, with influence on beer flavor (Langstaff et al., 1991; Zhao et al., 2015). The
264 lowest levels of glycerol were found in beers from malt wort, ranging between 2.05 and 3.75 g/L,
265 whereas the highest amounts were detected in samples obtained by wort added with 25% of grape
266 must (between 7.14 and 3.70 g/L). The increase in glycerol level seen in IGA25 beers, which were
267 the samples with the highest ethanol content (with the exception of US-05 beers) could be related to

the defensive mechanism adopted by *S. cerevisiae* yeast, which produces glycerol to mitigate the toxic effects of high ethanol concentrations in the environment (Udom et al., 2019).

The addition of grape must increase the content of acetic acid for CH3 and US-05 samples, while for the experimental beers obtained by P4 and TA4-10 fermentations the level of acetic acid was higher in beers from malt wort than IGA beers, although in beers fermented with P4 strain the differences in acetic acid content were not statistically significant. Otherwise, TA4-10 M beer contained a level of acetic acid significantly higher than acetic acid detected in TA4-10 IGA25 (594.89 and 399.78 mg/L, respectively).

With the addition of grape must, the concentration of tartaric acid increased significantly; this acid is naturally abundant in grapes, so an increase in that acid in IGA beers was not surprising (dos Santos Lima et al., 2015). The same trend was not observed for succinic acid content. In fact, although grapes contain a small amount of succinic acid, the content of this acid was higher in beer from malt wort than IGA beers, with the exception of sample obtained by P4 fermentation, although no differences statistically significant for succinic acid content were found among the three experimental beers obtained by inoculating this strain. Similar result was observed for experimental beers obtained by inoculating the commercial strain US-05.

The two-way ANOVA analysis showed that the strains influenced mainly AA and RA values, the type of beer wort affected mainly tartaric acid content, AA and RAA values, whereas the interaction affected mainly AA, RAA and acetic acid content.

3.3. Beer volatilomic profile

Thirty-nine volatile compounds were detected by HS-SPME-GC/MS of the experimental beers produced by the four different strains in three different fermentation media, including 6 alcohols, 4 volatile acids, 19 esters, 3 aldehydes and ketones, 4 terpenes, 1 phenol, 1 hydrocarbon, and 1 lactone (Table S1). In Table 3, we have reported principal higher alcohols and their esters identified and

293 quantified by GC-FID and GC-MS. It is worth noting the significantly ($p < 0.05$) positive interaction
294 Strains (A) x Type of beers (B) on the volatile concentrations among different samples.
295 The Figure 2 reports, for each fermentation media, the summation of the aromatic compounds,
296 subdivided in the main aromatic classes, detected in the experimental beers fermented with the four
297 different starters. The greatest aromatic expression, for all the strains studied, was observed in the
298 beers obtained by adding 15% grape must to the malt wort.
299 The highest level of aroma compounds was found in IGA15 beer, brewed with the indigenous strain
300 CHE-3, with levels around 580 mg/L, a level very similar to the amount found in beer fermented with
301 the commercial strain US-05. However, also in the other two types of produced beers, M and IGA25,
302 these strains showed similar levels of total content of volatile compounds. The beers brewed with P4
303 strain contained the lowest levels of volatiles in all the fermentation media used (280 mg/L in M, 450
304 mg/l in IGA25 and 400 mg/L in IGA25, respectively).
305 Among the different compounds, esters and alcohols play a very important role in the organoleptic
306 characteristics of the finished product (aroma, taste, tactile sensations). In particular, esters, thanks to
307 their low perception threshold, positively influence the aroma with fruity notes; for example, isoamyl
308 acetate, ethyl hexanoate and ethyl octanoate (Table 3) have been associated with banana, sweet and
309 sour apple notes, respectively (Ocvirk et al., 2018; Pires et al., 2014; Thompson-Witrick et al., 2015).
310 The addition of grape must at 15% increased ester concentration; in fact, the highest values were
311 found in all the IGA15 beers, ranging from a minimum of 77.57 mg/L for the strain TA4-10 to a
312 maximum of 141.71 mg/L for the strain CHE-3. The addition of 25% of grape must increased the
313 ester concentration in comparison to beers from malt wort for CHE-3 and P4, whereas for beers
314 obtained with TA4-10 and US-05 starters, esters level was lower than beer from malt wort. The
315 lowest esters concentrations were found in the M and IGA25 beers brewed with the strain P4 (44.10
316 and 55.28 mg/L, respectively).
317 Different studies (Piddocke et al., 2009; Younis and Stewart, 2000) reported that the type of sugars
318 may influence the changes in the aromatic profile of the final beer; in fact, worts rich in easily

assimilable glucose and fructose usually produce beers characterized by higher contents of esters than those rich in maltose. The mechanisms explaining the effect of individual assimilable sugar on ester production are not yet fully understood and controversial results are reported. Younis and Stewart (1998) suggested that higher levels of glucose increase acetyl-CoA, which is the main substrate for acetate ester synthesis, whereas maltose-rich worts may weakly induce acetyl-CoA formation for acetate ester production (Shindo et al., 1992). Conversely, other authors found that an increase of maltose levels as a sole carbon source in a synthetic medium determined an increasing tendency to accumulate acetate esters (Saerens et al., 2008).

Higher alcohols, also known as fusel alcohols, are the most abundant organoleptic compounds present in beer (Baiano et al., 2023). In fact, previous reports reported that beers contain more than forty higher alcohols, including n-propanol, isobutanol, benzyl alcohol, 2- phenylethanol, amyl alcohol and isoamyl alcohol (Loviso and Libkind, 2019; Thompson-Witrick et al., 2015). Concentrations below 300 mg/L confer flower, pleasant notes, refreshing and impart desirable warming character, which gives complexity to the beers, whereas excessive amounts cause a burning sensation and bring an alcohol or solvent aroma to the nose.

The most abundant alcohols were isobutanol, especially in beers brewed only with malt wort (M), and isoamyl alcohol, especially in beers added with 25% of grape must. The total amount of alcohols was highest in beers with 25% grape must addition, with values ranging from 230.69 mg/l for strain P4 (showing the lowest values in all three produced beers) to 331.40 mg/L for US-05. In general, the addition of grape must increase the content of amyl alcohols, in particular of isoamyl alcohol. These two alcohols are synthesized by yeast from amino acids through Ehrlich pathway; the substrates for production of isoamyl and amyl alcohols are leucine and isoleucine, respectively (Hazelwood et al., 2008). It has been reported that high fermentative activity, as is the case of IGA15 and 25 samples (Figure 1), increases the synthesis of leucine or isoleucine by yeasts (Castro Marin et al., 2021), which might be responsible for the higher content of these alcohols in IGA samples than the control.

During IGA beer brewing, terpenes may originate from both hops and grape must and they can play an important role on to determine the aroma profile of final product (Dietz et al., 2021). In these samples, only four terpenes were identified: linalool, α-terpineol, citronellol, and geraniol, as shown in table S1. The highest concentration of terpenes was found in beer added with 15% of grape must fermented with the indigenous strain CHE-3.

Among the aldehydes determined by GC-FID analysis, the most abundant was acetaldehyde, as expected, whereas among the ketones, the most abundant one was acetoin. Even for these categories of compounds, as for the others already described, the concentration was different depending on both the strain used and the type of fermentation medium (malt wort with or without the addition of grape must). High values of acetaldehyde were found in IGA15 beer brewed with CHE-3 strain (97.97 mg/L) and in IGA25 brewed with the commercial strain US-05 (93.09 mg/L). Low concentrations of acetaldehyde were detected in all three types of beer produced with the strain TA4-10 (values ranging from 33.37 mg/L in M to 44.56 mg/L in IGA25). The addition of grape must increase acetaldehyde content in all the samples; this result can be correlated to the higher content of glucose of IGA samples in comparison to malt wort (Table 1). Other authors observed an increase in the acetaldehyde formation in fermentation with high glucose concentrations, such as glucose syrup-supplemented fermentations (Piddocke et al., 2009) or the use of adjuncts with high glucose concentrations (Briggs et al., 2004).

Among the volatile acids analysed, the most abundant was octanoic acid followed by decanoic acid (table S1). Total volatile acids were found in higher concentrations in all four IGA15 beers, with values ranging from 11.72 mg/L for TA4-10 to 19.34 mg/L for the commercial strain US-05.

Only y-nonalattone was identified as lactones, as depicted in Table S1. This compound was absent in control beer (M) present in a concentration of 0.13 mg/L in beer IGA15 produced with the strains P4 and TA4-10, whereas it was present in all beers IGA25 with lower concentrations (0.05 mg/L) for P4 and higher (0.15 mg/L) for TA4-10. Among the phenols, 4-vinyl guaiacol was found in greater

amounts in beers brewed with TA4-10 strain in malt wort (18.22 mg/L) and P4 strain in wort supplemented with 15% of grape must (14.85 mg/L).

The differences among the four yeast strains and three different fermentation media were further analysed by performing a clustering analysis (Figure 3). The major volatile compounds, including higher alcohols, acetate esters, ethyl esters, volatile acids, aldehydes, ketones, pyrazines, terpenes and volatile phenols, was normalized on the basis of the Z-score transform. Red colours indicate that the amount of the volatile compounds was less than the average value, whereas the blue colours indicate that the volatile compound levels were higher than average level. The aroma compound levels in the beers produced with the strain CHE-3 in the 3 different media were similar; in fact, these beers were clustered in the same group. The strains P4 and US-05 showed a similar trend; in fact, both the starters produced beers characterized by similar aroma levels using malt wort and malt supplemented with 15% of grape must, which clustered together and were separated by beers produced with malt wort added with 25% of grape must. All the beers produced by TA4-10 strains were separated, indicating that the metabolic behaviour of this strain is strongly influenced by the composition of fermentation medium.

4. Conclusions

The results obtained in this study have shown that both fermentation substrate and yeast strain significantly affect process kinetics and chemical characteristics of IGA beers. The three selected *S. cerevisiae* strains showed higher fermentative activity and higher ability to utilize sugars present in the different fermentation media than the commercial brewing strain. This study indicated that yeast strains from other traditional environment can produce quality-enhanced IGA beer, thus confirming the potential of cross-over fermentation for novel beverages

As regards the analytical composition of the experimental beers, the addition of grape must significantly increase the content of ethanol, as expected, glycerol and volatile compounds, such as esters and some higher alcohols. However, in all the tested strains the highest aromatic expression was observed in the beers obtained by adding 15% grape must.

In recent years, the attention of researchers has been focused only on the exploitation of new raw materials to obtain a distinctive beer. Moreover, these findings confirm that wild microorganisms, isolated from different food sources and maintained in the microbial collections, represent a very interesting reservoir of novel starter cultures which contribute to product differentiation, particularly desired in the craft beers sector. Further investigations at brewery scale are at the present underway to validate these strains as novel starters for IGA production.

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Declaration of competing interests

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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Appendix A. Supplementary data

The following is the Supplementary data to this article:

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Captions to figure

Figure 1. Fermentative performance of 4 *S. cerevisiae* strains, reported as g of CO_2 produced during the fermentation of conventional wort (A) and wort added with 15 and 25% of grape must (B and C, respectively). Letters on plot bars indicate significant differences ($p < 0.05$) among the four strains. Data are expressed as the means of duplicate experiments ±standard deviation.

Figure 2. Classes of principal volatile compounds of the experimental beers produced with 4 different yeast strains (CHE-3, P4, TA4-10, and US-05) using malt wort and malt wort supplemented with 15 and 25% of grape must (M, IGA 15 and IGA 25, respectively).

Figure 3. Heat map visualization and clustering results of volatile compounds detected in the experimental beers produced with 4 different yeast strains (CHE-3, P4, TA4-10, and US-05) using malt wort and malt wort supplemented with 15 and 25% of grape must (M, IGA15 and IGA25, respectively). The data were converted to Z-scores to easily visualize which yeast strains are relevant aroma producers in relation to average production. For each fermentation substrate, Z-Scores were calculated as follows: Z-score = $(X - \mu)/\sigma$, where X is the concentration of the aroma compound, μ is the mean value of all strains per measured aroma and σ is the standard deviation of values per tested aroma.

Figure 1

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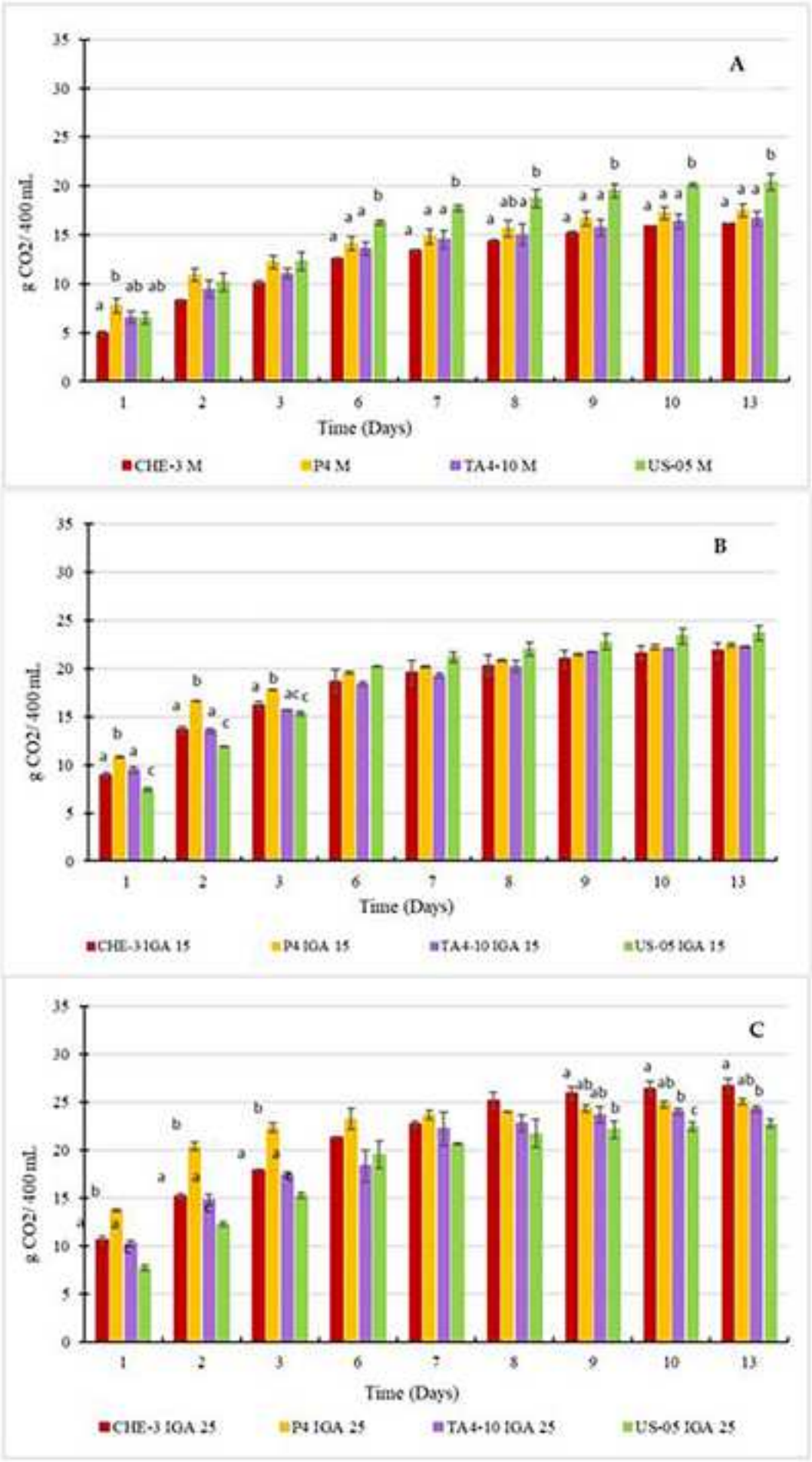

Figure 2

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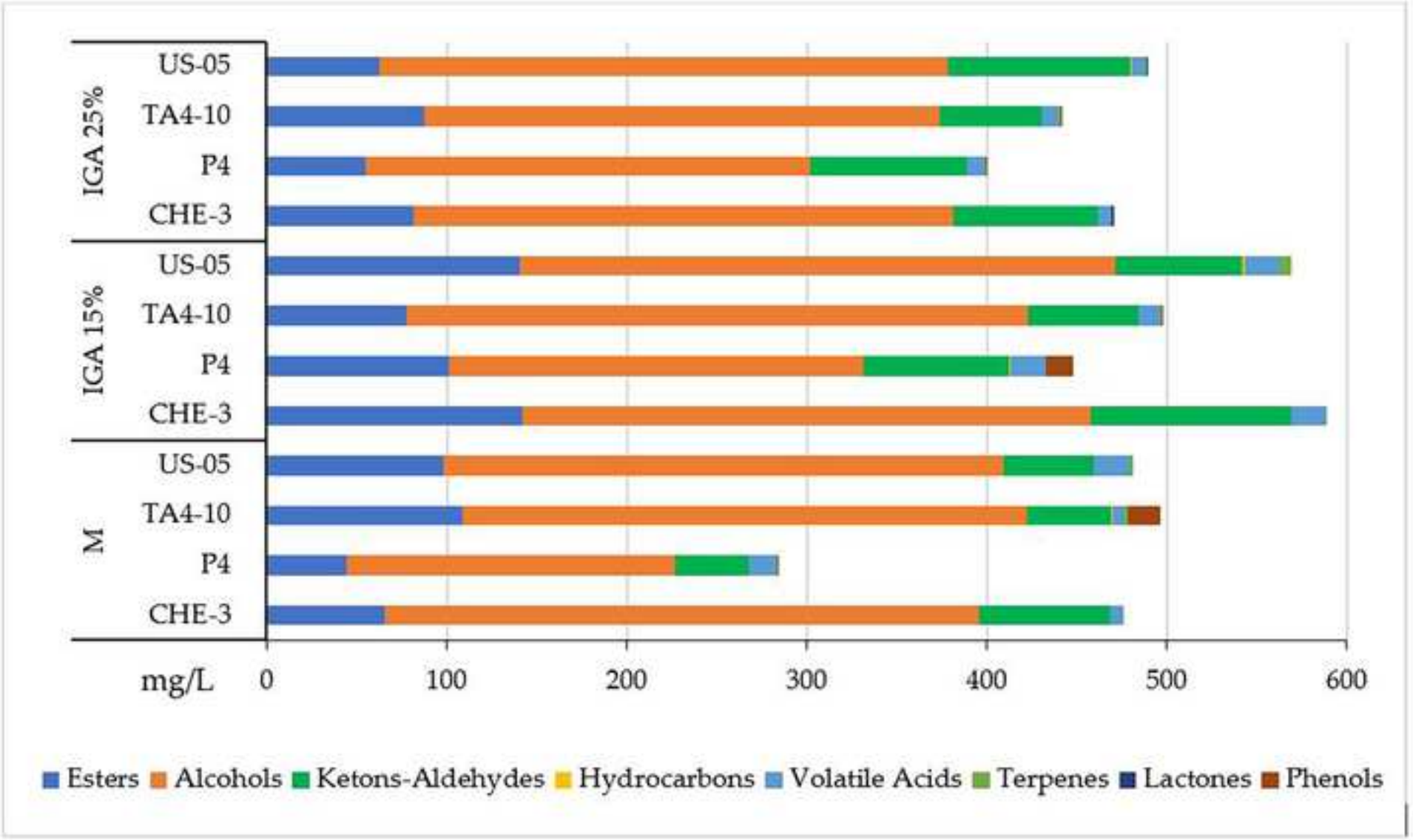

Figure 3

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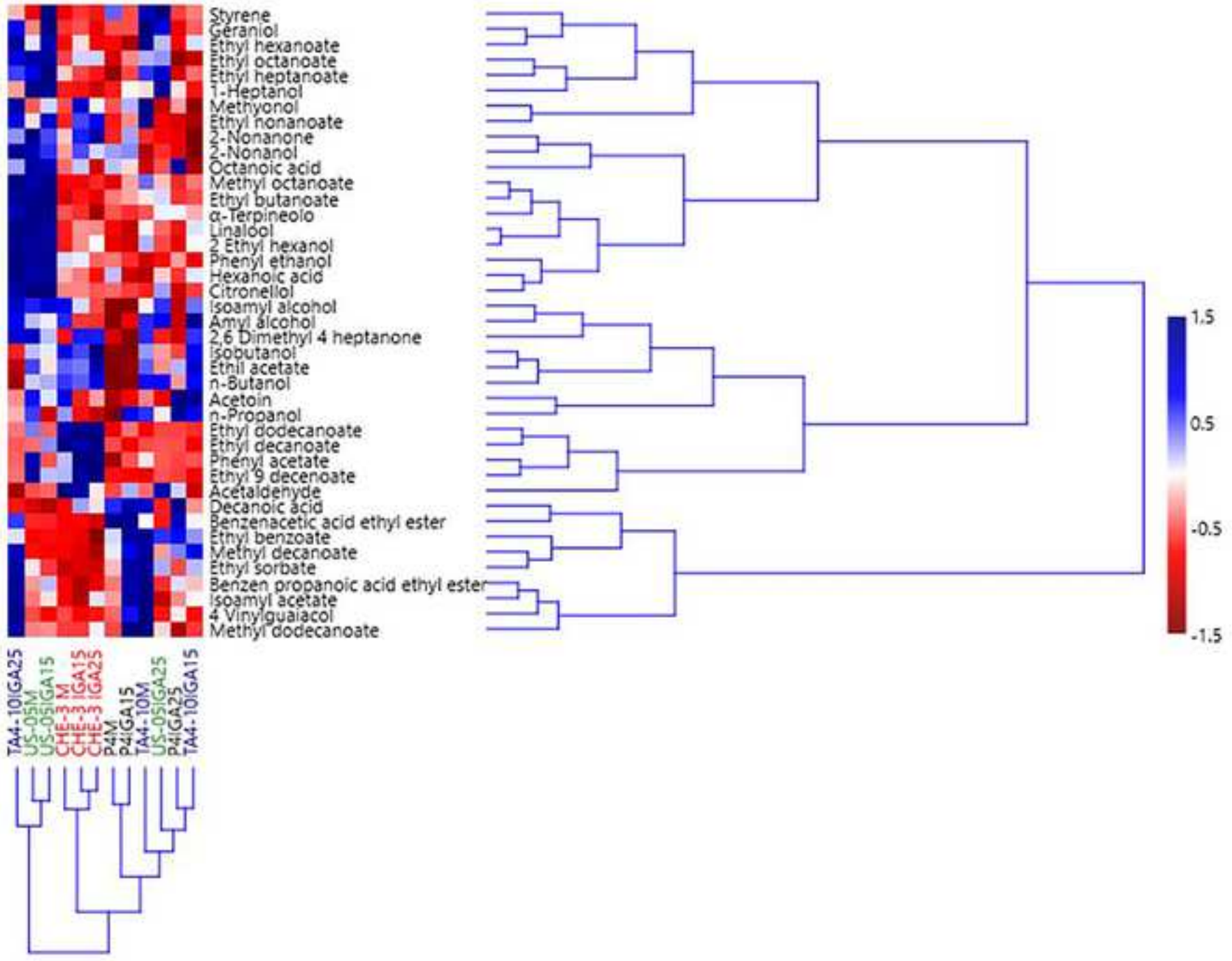

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Table 1. Carbohydrate content of fermentation media and experimental beers (g/L).

Samples	Maltodextrin	Maltotriose	Maltose	Glucose	Fructose
Malt wort	19.12	26.38	50.22	10.8	3.27
IGA15	17.14	20.17	34.56	17.14	13.16
IGA25	17.37	18.61	38.18	28.63	25.41
CHE-3 M	16.99±0.88^{a}	25.91±2.12^{a}	ND	ND	ND
CHE-3 IGA15	14.07±0.20^{a}	19.03±0.01^{a}	ND	ND	ND
CHE-3 IGA25	12.71±0.07^{b}	16.21±0.78^{b}	ND	3.47±0.02	ND
P4 M	18.30±0.12^{a}	18.82±0.76^{a}	ND	ND	ND
P4 IGA15	16.12±0.13^{b}	15.58±0.11^{b}	ND	1.67±0.05^{a}	ND
P4 IGA25	14.37±0.14^{c}	15.88±0.09^{b}	ND	1.78±0.12^{a}	ND
TA4-10 M	17.96±0.06^{a}	23.05±0.01^{a}	2.08±0.03	ND	ND
TA4-10 IGA15	15.96±0.10^{a}	20.00±0.09^{b}	ND	2.47±0.10^{a}	ND
TA4-10 IGA25	16.01±1,66^{a}	18.01±0.05^{c}	ND	2.82±0.12^{a}	ND
US-05 M	17.00±0-17^{a}	10.65±0.14^{a}	0.88±0.01^{a}	ND	ND
US-05 IGA15	15.70±0.25^{a}	7.32±0.91^{b}	0.67±0.01^{a}	2.80±0.01^{a}	ND
US-05 IGA25	14.31±0.79^{a}	7.62±0.16^{b}	1.08±0.29^{a}	2.06±0.58^{a}	ND
p Strains (A)	6.89E-3	4.30E-10	1.80E-08	1.72E-3	/
p Type of beers (B)	2.38E-05	9.62E-07	1.16E-06	4.22E-10	/
p Interaction A x B	4.48E-1	9.83E-3	6.65E-08	1.68E-06	/

Data are reported as mean ± standard deviation of two independent replicates, ND = not detected. Superscript letters indicate significant differences ($p < 0.05$) among each strain in three different types of beers (M, IGA15, IGA25) for each parameter. *p* Strains (A), type of beers (B) and their Interaction (A x B) = result of Two-ways factorial ANOVA performed on the experimental beers using as factors the yeast strains and the type of wort used for the production of beers.

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Table 2. Main analytical parameters of the laboratory scale beers.

	AA#	RA##	Ethanol (%v/v)	Glycerol (g/L)	Acetic acid (g/L)	Tartaric acid (g/L)	Succinic acid (g/L)
CHE-3 M	65.55±0.71^{a}	53.69±0.58^{a}	3.72±0.02^{a}	2.95±0.31^{a}	0.45±0.02^{a}	ND	7.53±0.05^{a}
CHE-3 IGA15	74.58±0.59^{b}	61.13±0.48^{b}	5.44±0.11^{b}	4.61±0.03^{b}	0.49±0.001^{a}	2.13±0.01^{a}	6.88±0.02^{b}
CHE-3 IGA25	84.23±0.54^{c}	69.04±0.45^{c}	6.40±0.03^{b}	5.30±0.01^{b}	0.58±0.006^{b}	2.46±0.01^{b}	6.49±0.03^{c}
P4 M	70.50±0.71^{a}	57.79±0.68^{a}	4.25±0.12^{a}	3.75±0.15^{a}	0.49±0.01^{a}	ND	7.43±0.12^{a}
P4 IGA15	78.96±0.29^{b}	64.72±0.24^{b}	5.78±0.10^{b}	6.04±0.13^{b}	0.45±0.05^{a}	2.13±0.05^{a}	7.28±0.04^{a}
P4 IGA25	84.81±0.37^{c}	69.51±0.22^{c}	6.60±0.33^{b}	7.14±0.02^{c}	0.46±0.007^{a}	2.43±0.22^{a}	7.91±0.31^{a}
TA4-10 M	69.50±0.71^{a}	56.97±0.58^{a}	3.85±0.02^{a}	3.09±0.16^{a}	0.40±0.01^{a}	ND	7.07±0.08^{a}
TA4-10 IGA15	79.58±0.59^{b}	65.23±0.48^{b}	4.80±0.10^{b}	5.02±0.09^{b}	0.55±0.03^{b}	2.09±0.05^{a}	5.92±0.03^{b}
TA4-10 IGA25	85.00±0.54^{c}	69.67±0.45^{c}	5.67±0.03^{c}	5.86±0.09^{c}	0.59±0.01^{b}	2.60±0.16^{b}	5.65±0.03^{b}
US-05 M	79.50±0.81^{a}	65.16±0.58^{a}	4.70±0.01^{a}	2.05±0.00^{a}	0.35±0.015^{a}	ND	7.53±0.14^{a}
US-05 IGA15	87.92±0.59^{b}	72.06±0.48^{b}	4.60±0.59^{a}	3.63±0.38^{ab}	0.47±0.02^{b}	2.01±0.11^{a}	6.12±0.53^{a}
US-05 IGA25	88.08±0.64^{b}	72.19±0.45^{b}	3.76±0.07^{a}	3.70±0.30^{b}	0.55±0.03^{b}	1.92±0.07^{a}	6.56±0.18^{a}
p Strains (A)	7.30E-12	7.45E-12	1.19E-02	1.19E-08	7.99E-04	1.76E-03	2.28E-05
p Type of beers (B)	1.32E-14	1.34E-14	1.03E-04	5.32E-10	1.27E-01	2.45E-15	8.83E-05
p Interaction A x B	8.58E-07	8.81E-07	3.07E-03	1.78E-02	6.48E-07	3.59E-03	5.66E-03

Data are means ± sd of two independent replicates. # = apparent attenuation, ## = real attenuation. ND = not detected. Superscript letters indicate significant differences ($p < 0.05$) among each strain in three different types of beers (M, IGA15, IGA25) for each parameter. *p* Strains (A), type of beers (B) and their Interaction (A x B) = result of Two-ways factorial ANOVA performed on the experimental beers using as factors the yeast strains and the type of wort used for the production of beers.

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Table 3. Concentration (mg/L) of most important aroma compounds in laboratory-scale beers produced with 4 different yeast strains (CHE-3, P4, TA4-10, and US-05) using malt wort and malt wort supplemented with 15 and 25% of grape must (M, IGA15 and IGA25, respectively).

	Ethyl octanoate	**Ethyl hexanoate**	**Ethyl acetate**	**Isoamyl acetate**	**Phenyl acetate**	**Phenyl-ethanol**	***n*-Propanol**	**Isobutanol**	**Amyl alcohol**	**Isoamyl alcohol**	**Acetaldehyde**	**Acetoin**
Aroma	Rose, honey	Apple, fruity	Fruity, solvent	Banana	Apple, aniseed	Rose	Alcohol, sweet	Solvent	Alcoholic, banana	Alcoholic, banana, sweet	Green apple, sweet	Butter
CHE-3 M	19.47±4.21^{a}	ND	20.19±1.34^{a}	0.72±0.14^{a}	1.95±0.41^{a}	12.82±3.54^{a}	14.62±1.16^{a}	77.50±1.49^{a}	31.76±0.49^{a}	66.26±5.23^{a}	57.63±0.11^{a}	13.62±0.35^{a}
CHE-3 IGA15	49.63±5.14^{b}	3.61±0.45	16.16±0.25^{b}	2.33±0.12^{b}	8.12±1.17^{b}	16.31±4.17^{a}	14.27±0.39^{a}	62.90±2.72^{b}	29.80±2.34^{a}	89.17±1.88^{b}	97.97±3.97^{b}	8.35±0.55^{b}
CHE-3 IGA25	27.92±4.22^{a}	ND	15.97±0.76^{b}	2.79±0.21^{b}	5.92±0.85^{b}	10.12±2.94^{a}	11.54±0.11^{b}	59.08±0.81^{b}	33.34±0.53^{a}	82.90±2.54^{b}	68.37±2.79^{a}	8.53±0.20^{b}
P4 M	12.45±3.55^{a}	ND	20.56±1.40^{a}	1.28±0.28^{a}	5.32±0.36^{a}	8.90±2.55^{a}	14.91±0.07^{a}	65.43±2.25^{a}	29.41±3.86^{a}	64.88±4.97^{a}	32.30±3.48^{a}	12.95±1.15^{a}
P4 IGA15	39.18±6.11^{b}	ND	14.65±3.07^{ab}	2.63±0.64^{b}	13.88±5.24^{b}	12.57±3.18^{a}	13.83±0.68^{a}	58.84±2.90^{ab}	36.08±4.41^{ab}	100.42±7.12^{b}	54.12±3.63^{b}	11.93±1.37^{a}
P4 IGA25	14.86±4.25^{a}	ND	11.39±1.71^{b}	2.24±0.33^{b}	10.39±2.41^{b}	11.50±3.25^{a}	14.58±1.19^{a}	43.98±0.22^{b}	46.85±2.98^{b}	102.79±4.46^{b}	93.09±7.01^{c}	6.87±0.25^{b}
TA4-10 M	31.91±5.22^{a}	3.97±0.15^{a}	20.20±1.84^{a}	3.21±0.52^{a}	1.89±0.35^{a}	4.14±0.24^{a}	14.95±1.17^{a}	67.24±2.40^{a}	31.73±1.01^{a}	56.82±5.73^{a}	33.37±2.91^{a}	9.68±1.37^{a}
TA4-10 IGA15	23.87±4.15^{a}	4.24±0.22^{a}	18.13±2.05^{a}	2.47±0.42^{a}	4.51±0.24^{b}	11.38±2.57^{b}	16.85±0.55^{a}	65.01±4.07^{a}	49.33±1.32^{b}	93.67±1.05^{b}	38.39±1.02^{ab}	18.15±0.47^{b}
TA4-10 IGA25	34.79±4.88a	8.90±0.28b	9.02±0.11b	4.39±0.64^{a}	7.86±2.18^{b}	25.85±4.18^{c}	14.13±2.15^{a}	40.93±3.42^{b}	46.17±2.06^{b}	110.84±2.26^{c}	44.56±3.54^{b}	8.39±1.89^{a}
US-05 M	49.23±5.31a	1.52±0.01a	11.33±1.76a	1.17±0.14^{a}	3.19±0.27^{a}	22.01±5.01^{a}	12.67±1.89^{a}	38.70±6.58^{a}	20.43±3.21^{a}	41.45±6.69^{a}	31.99±1.98^{a}	7.03±1.74^{a}
US-05 IGA15	74.76±5.84b	7.87±1.25b	10.66±0.87a	2.46±0.18^{b}	ND	23.75±3.58^{a}	16.91±3.65^{a}	46.45±5.75^{a}	27.33±2.82^{a}	67.36±3.25^{ab}	71.49±2.51^{b}	7.77±1.11^{ab}
US-05 IGA25	29.33±6.32c	4.11±0.11c	12.99±2.15a	1.30±0.31^{a}	1.68±0.44^{b}	7.97±2.15^{b}	18.95±0.63^{b}	41.76±2.32^{a}	28.98±0.68^{a}	72.57±5.04^{b}	72.46±7.06^{b}	13.72±1.58^{b}
p Strains (A)	2.82E-06	6.06E-11	1.92E-3	2.03E-05	4.12E-10	2.35E-2	9.70E-2	1.30E-06	5.16E-07	2.14E-06	1.02E-07	1.48E-2
p Type of beers (B)	4.83E-06	1.45E-07	3.22E-4	1.92E-4	1.72E-1	9.02E-2	4.27E-1	7.05E-06	8.69E-06	2.44E-08	1.65E-08	8.58E-3
p Interaction A x B	1.00E-4	4.73E-08	6.69E-3	9.45E-4	1.72E-07	2.06E-4	4.71E-2	1.24E-3	1.59E-3	1.13E-2	2.18E-06	2.26E-06

Data are means ± sd of two independent replicates. # = apparent attenuation, ## = real attenuation. ND = not detected. Superscript letters indicate significant differences ($p < 0.05$) among each strain in three different types of beers (M, IGA15, IGA25) for each parameter. *p* Strains (A), type of beers (B) and their Interaction (A x B) = result of Two-ways factorial ANOVA performed on the experimental beers using as factors the yeast strains and the type of wort used for the production of beers.

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Gabriella Siesto: Investigation, Methodology, Writing- original draft; **Rocchina Pietrafesa**: Investigation, Methodology, Writing- original draft; **Maria Tufariello:** Investigation, validation; **Carmela Gerardi:** Investigation; validation; **Francesco Grieco:** Supervision, Writing-review and editing; **Angela Capece:** Conceptualization, Supervision, Writing- original draft, Writing-review and editing.

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