

Non-conventional yeasts from fermented honey by-products: Focus on *Hanseniaspora uvarum* strains for craft beer production



Michele Matraxia^a, Antonio Alfonzo^a, Rosario Prestianni^a, Nicola Francesca^{a,*}, Raimondo Gaglio^a, Aldo Todaro^a, Vincenzo Alfeo^b, Giuseppe Perretti^b, Pietro Columba^a, Luca Settanni^a, Giancarlo Moschetti^a

^a Dipartimento Scienze Agrarie, Alimentari e Forestali, Università degli Studi di Palermo, Viale delle Scienze 4, 90128, Palermo, Italy

^b Italian Brewing Research Centre, Department of Agricultural, Food and Environmental Science, University of Perugia, 06126, Perugia, Italy

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ABSTRACT

The increasing interest in novel beer productions focused on non-*Saccharomyces* yeasts in order to pursue their potential in generating groundbreaking sensory profiles. Traditional fermented beverages represent an important source of yeast strains which could express interesting features during brewing. A total of 404 yeasts were isolated from fermented honey by-products and identified as *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii* and *Hanseniaspora uvarum*. Five *H. uvarum* strains were screened for their brewing capability. Interestingly, *Hanseniaspora uvarum* strains showed growth in presence of ethanol and hop and a more rapid growth than the control strain *S. cerevisiae* US-05. Even though all strains showed a very low fermentation power, their concentrations ranged between 7 and 8 Log cycles during fermentation. The statistical analyses showed significant differences among the strains and underlined the ability of YGA2 and YGA34 to grow rapidly in presence of ethanol and hop. The strain YGA34 showed the best technological properties and was selected for beer production. Its presence in mixed- and sequential-culture fermentations with US-05 did not influence attenuation and ethanol concentration but had a significant impact on glycerol and acetic acid concentrations, with a higher sensory complexity and intensity, representing promising co-starters during craft beer production.

1. Introduction

Beer is one of the oldest and most widely consumed alcoholic drink in the world. The first record of the production of a beer-like drink is reported by the Sumerians, about 6000 years ago (Katz and Maytag, 1991). The modern brewing process is the result of continuous adjustments made over time. Many kinds of beer were born and established worldwide, each with its own distinctive feature, often linked to the production area (Glover, 2001; Protz, 1995). In recent years, the manufacture of craft beers included local cereal varieties as well as fruits in order to provide territorial ties.

Together with *Saccharomyces* other yeasts such as *Candida*, *Brettanomyces/Dekkera*, *Debaryomyces*, *Rhodotorula*, *Cryptococcus* and *Pichia* are detected in traditional beer styles, including Lambic, Gueuze, Belgian red-brown acidic ale and American coolship ale, subjected to spontaneous fermentations (Bokulich et al., 2012; Snauwaert et al.,

2016; Spitaels et al., 2014). Although the interest on conventional *Saccharomyces* strains isolated from non-brewing matrices, like sourdough (Catallo et al., 2020; Marongiu et al., 2015), or distilleries (Araújo et al., 2018) is still strong, the application of non-*Saccharomyces* yeasts in beer is increasing. This phenomenon depends on their enzymatic activities that influence the aromatic profiles of the beers, mainly by the modulation of esters influenced by the activity of esterase enzymes (Pires et al., 2014) or through the shaping of terpenes and higher alcohols (Lappe-Oliveras et al., 2008). Furthermore, the reduction of calories, the production of low levels of alcohol, the souring, the production of lactic acid and the generation of glycerol during fermentation by non-*Saccharomyces* yeasts might differentiate the existing beers or even create new styles (Cubillos et al., 2019; Michel et al., 2016). Due to specific substrate assimilation patterns, non-*Saccharomyces* yeasts produce several compounds (i.e. glycerol, lactic acid, acetaldehyde and ethyl acetate) useful to the productions of typical and distinguishable

* Corresponding author.

E-mail address: nicola.francesca@unipa.it (N. Francesca).

Table 1

Microbial loads (Log CFU/g or mL) of fermented honey by-products (FHP) samples.

FHP Samples	Media			
	DWA	TGYA	WL	LA
1-a	2.98 ± 0.27 ^{ab}	3.01 ± 0.32 ^{bcd}	5.45 ± 0.22 ^{ab}	4.88 ± 0.22 ^d
1-b	2.73 ± 0.17 ^{abc}	3.47 ± 0.11 ^{ab}	4.75 ± 0.41 ^{bcd}	5.02 ± 0.33 ^{cd}
1-c	2.29 ± 0.28 ^c	2.8 ± 0.17 ^{cde}	4.88 ± 0.49 ^{bcd}	5.17 ± 0.21 ^{bcd}
2-a	2.44 ± 0.31 ^{bc}	2.22 ± 0.19 ^e	5.33 ± 0.31 ^{abc}	2.88 ± 0.28 ^e
2-b	3.04 ± 0.21 ^{ab}	2.74 ± 0.34 ^{cde}	5.87 ± 0.31 ^a	3.01 ± 0.21 ^e
2-c	2.72 ± 0.20 ^{abc}	2.69 ± 0.24 ^{de}	5.92 ± 0.37 ^a	2.23 ± 0.21 ^e
3-a	n.d. ^d	n.d. ^f	5.45 ± 0.40 ^{ab}	6.01 ± 0.30 ^a
3-b	n.d. ^d	n.d. ^f	5.17 ± 0.32 ^{abcd}	5.72 ± 0.48 ^{abc}
3-c	n.d. ^d	n.d. ^f	5.08 ± 0.17 ^{abcde}	5.89 ± 0.20 ^{ab}
4-a	3.18 ± 0.17 ^a	3.33 ± 0.38 ^{abc}	4.15 ± 0.20 ^e	4.88 ± 0.22 ^d
4-b	2.95 ± 0.41 ^{ab}	3.87 ± 0.15 ^a	4.25 ± 0.31 ^{de}	5.02 ± 0.33 ^{cd}
4-c	3.14 ± 0.20 ^a	3.8 ± 0.11 ^a	4.38 ± 0.31 ^{cde}	5.17 ± 0.21 ^{bcd}
Statistical significance	***	***	**	***

Abbreviations: DWA, de whalley agar for total osmophilic yeasts; TGYA, tryptone glucose yeast extract agar for total osmotolerant yeasts; WL, wallerstein laboratory nutrient agar for total yeasts; LA, lysine agar for *Saccharomyces* yeast growth inhibition; n.d., not detected (value < detection limit of method). Results indicate mean values ± SD of three determinations.

Data within a column followed by the same letter are not significantly different according to Tukey's test.

P value: **, P < 0.01; ***, P < 0.001.

beers (Ciani and Comitini, 2011; Cordero-Bueso et al., 2013; Gonzlez et al., 2013; Johnson, 2013) or even assimilate sugars like maltose or maltotriose, which are not commonly assimilated by non-domesticated yeasts (Nikulin et al., 2020).

As reported by Sannino et al. (2019), great attention has been paid to some non-*Saccharomyces* yeasts, mainly Ascomycota genera, namely, *Pichia*, *Saccharomyces*, *Zygosaccharomyces*, *Hanseniaspora* and *Torulaspora* for their possible exploitation as starter cultures in brewing. Also the common wine spoilage yeasts *Dekkera/Brettanomyces* have been exploited for their brewing potential (Lentz et al., 2014). They generally

present low fermentation yields and are more sensitivity to ethanol but, in some cases, they can improve other characteristics such as texture and integration of aroma for the creation of a final peculiar bouquet. Actually, the application of non-*Saccharomyces* strains characterized by an intense production of enzymes, mainly hydrolases, such as glucosidase capable of releasing aroma precursors or aroma-active substances, but unable to perform the alcoholic fermentation (AF), represents an encouraging strategy for bio-flavoring fermented beverages (Rodriguez et al., 2007, 2010; Ruiz et al., 2018; Sadineni et al., 2012). Recent studies showed that *Hanseniaspora* genus can play several roles during a brewing process. Bellut et al. (2018) evaluated two different strains of *Hanseniaspora vineae* and *Hanseniaspora valbyensis* during alcohol-free beer production: both strains showed high viability rates, but only marginal differences, in terms of volatile profiles, with the control production carried out with *S. cerevisiae* were registered. Larroque et al. (2021) successfully applied a strain of *H. vineae*, previously used in co-fermentation of wine, for the improvement of the fruity organoleptic characteristics of beer.

From this perspective, non-*Saccharomyces* strains are usually used in co-fermentation because ethanol production is performed by *Saccharomyces* species (Cubillo et al., 2019). According to Cubillo et al. (2019), the yeast diversity of several traditional fermented beverages is still uninvestigated and they represent potential ecological niches to isolate novel strains for brewery technologies.

With regards to uninvestigated traditional alcoholic beverages, several fermented alcoholic products that are typical of restricted geographical areas are produced in Sicily. Often, these beverages are expression of the local culture, history and folklore. Among these, "Spiritu Re' Fascitrari" (SRF) is a distillate liqueur produced from honey by-products at Sortino (Syracuse province, Sicily region, Italy). The term "fascitrari" comes from the ancient hives made with stems of the plant *Ferula communis* called "fascitro" in the local dialect (Ajovalasit and Columba, 1998).

SRF is obtained from the recovery process of beeswax from opercula and residual substances from honey production. Residual water from wax recovery process, after fermentation, is distilled and blended with a decoction of honey and various aromas. Gaglio et al. (2017) described the main yeast species active during SRF spontaneous AF as belonging to the species *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Wickerhamomyces anomalus* and *Lachancea fermentati*. SRF matrices certainly represent a microbial source of starter strains to be used in fermented beverage industries (Varela, 2016). Sinacori et al. (2014) who investigated the microbial ecology associated to honey of different geographical and botanical origins, highlighted the presence of various yeast species of interest as beer starter cultures.

Table 2

Molecular identification of yeast species isolated from FHP samples.

Species	Restriction profile	5.8 S-ITS PCR	Size of restriction fragment ^a				Accession Number ^b	No. of isolates ^c (%) ^d
			CfoI	HaeIII	HinfI	DdeI		
<i>Hanseniaspora uvarum</i>	I	750	320 + 105	750	350 + 200+180 + 90	300 + 180+95	MT362721; MT362722	145 (36)
<i>Saccharomyces cerevisiae</i>	II	850	370 + 340	320 + 220+180 + 130	380 + 120	n.d.	MT364261; MT364262	51 (13)
<i>Wickerhamomyces anomalus</i>	III	650	575	600 + 50	310 + 310	n.d.	MT364263; MT364264	43 (11)
<i>Zygosaccharomyces bailii</i>	IV	775	330 + 295	700	340 + 230+175	n.d.	MT364265	88 (22)
<i>Zygosaccharomyces rouxii</i>	V	735	295 + 205+175	420 + 175	350 + 250+130	n.d.	MT364266	77 (19)

Abbreviation: n.d., not determined according to Esteve Zarzoso et al. (1999).

^a Values refer to the number of base pairs (bp) per fragment.

^b Accession number of D1/D2 region of the 26 S rRNA gene of isolates deposited into Genbank database.

^c Number of isolates per each yeast species.

^d Percentage based on the total number of isolates.

Table 3Technological screening of *Hanseniaspora uvarum* strains.

Strain code	Resistance to ethanol		Resistance to hop			Cross resistance			H ₂ S assay ^a	Flocculation assay ^b	Sedimentation volume ^c
	5% (v/v)	10% (v/v)	25 IBU	50 IBU	90 IBU	25 IBU/5% ethanol	50 IBU/5% ethanol	90 IBU/5% ethanol			
YGA2	+	+/-	+	+	+	+/-	-	-	0	0	0.55
YGA34	+	+/-	+	+	+	+	+/-	-	0	0	0.65
YGA36	+	+/-	+	+	+	+	+/-	+/-	1	0	0.60
YGA38	+	+/-	+	+	+	+	+/-	+/-	0	0	0.35
YGA73	+	-	+	+	+	+	-	-	0	0	0.45

Symbols: +, positive growth; -, no growth; +/-, weak growth.

Abbreviations: IBU, International Bitterness Unit; H₂S, Hydrogen sulfide.^a Color of colony on Biggy agar plates: 0 = white; 1 = beige; 2 = light brown; 3 = brown; 4 = dark brown; 5 = black.^b Flocculation degree after 22 days of incubation.^c Mean sedimentation volume (mL) expressed according to Helm's Assay.

For this reason, the present study aimed to: (i), isolate and identify yeasts from fermented honey by-products (FHP); (ii), characterize the yeasts strains for their main brewing traits; (iii), select non-*Saccharomyces* yeasts as potential co-starter for fermenting wort and/or green beer; (iv), evaluate the effect of *Hanseniaspora uvarum* inoculum during beer fermentation and sensory quality of bottled products.

2. Materials and methods

2.1. Sampling and microbiological analysis of fermented honey by-products

A total of 12 samples of FHP were collected during four independent productions of SRF distillate, with three replicates for each sampling. In details, the watery solution of pressed watery decoction was spontaneously fermented at the honey company “G. Pagliaro” located in Sortino (Siracusa, Sicily, Italy) and the samples were collected at the end of the AF. All samples were transferred into sterile plastic bags (BagLight® 400 Multilayer® bags, Interscience, Saint Nom, France) and transported with a portable fridge to the laboratory of Agricultural Microbiology (Department of Agricultural, Food and Forest Science, University of Palermo, Italy).

The microbiological analysis was performed to investigate the presence of yeasts associated to FHP following the same procedures published by Gaglio et al. (2017) to count and isolate total osmophilic yeasts, total osmotolerant yeasts and total yeasts. Briefly, samples associated with SRF production were homogenized in glucose solution to avoid cell damages. Liquid samples were subjected to decimal serial dilution in 30% (w/v) glucose solution. Total yeast counts were performed after sample dilution in peptone water solution (Sinacori et al., 2014). Total osmophilic yeasts were cultivated on De Walley Agar (DWA), total osmotolerant yeasts on tryptone glucose yeast extract agar (TGY), total yeasts were on Wallerstein laboratory (WL) nutrient agar, while non-*Saccharomyces* yeasts on Lysine Agar (LA).

When no colony developed, the following enrichment procedure was applied: 50 g or 50 mL of each sample were added to 50 mL of yeast extract peptone dextrose (YPD) and incubated statically for 5 and 10 d at 25 °C. All media were purchased from Oxoid (Rodano, Milan, Italy). Microbiological counts were performed in triplicate.

2.2. Isolation and genotypical identification of yeasts

After growth, all isolates were picked up from agar plates and purified by successive sub-culturing onto Malt Extract Agar (MEA). Yeast isolates were identified by molecular methods. DNA was extracted by cell lysis using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. To perform a first discrimination of the yeasts, all isolates were analyzed by restriction fragment length polymorphism (RFLP) of the region spanning the

internal transcribed spacers (ITS1 and ITS2) and the 5.8 S rRNA gene. DNA amplification occurred with the primer pair ITS1/ITS4 according to Esteve-Zarzoso et al. (1999). The generated amplicons were then digested with the endonucleases *Cfo*I, *Hae*III and *Hinf*I (MBI Fermentas, St. Leon-Rot, Germany) at 37 °C for 8 h. ITS amplicons, as well as the corresponding restriction fragments, were analyzed on agarose gel using 1.5% and 3% (w/v) agarose in 1 × TBE (89 mM Tris-borate, 2 mM EDTA pH 8) buffer, stained with SYBR safe DNA gel stain (Invitrogen, Milan, Italy), visualized by UV transillumination and acquired by Gel Doc 1000 Video Gel Documentation System (BioRad, Richmond, CA). Standard DNA ladders were 1 kb Plus and 50 pb (Invitrogen). At least one isolate per group was further processed by sequencing of the D1/D2 region of the 26 S rRNA gene (Gaglio et al., 2017). The identities of the generated sequences were determined by BLASTN (<http://www.ncbi.nlm.nih.gov>).

2.3. Genotypic and technological characterization of *Hanseniaspora uvarum* strains

2.3.1. Strain typing of *Hanseniaspora uvarum* isolates

The intraspecific characterization of the isolates was carried out by different RAPD-PCR assays with primers M13 (Francesca et al., 2014) and P80 (Capice et al., 2005). The same isolates were subjected to tandem repeat-tRNA (TRtRNA)-PCR method for the molecular typing of non-*Saccharomyces* subspecies, combining TtRNAsC primer with either ISS-MB or (CAG)₅ primers, as reported by Barquet et al. (2012). All patterns were analyzed using the Gelcompare II software version 6.5 (Applied-Maths, Sin Marten Latem, Belgium).

2.3.2. Hydrogen sulphide production

To evaluate the production of hydrogen sulphide (H₂S), the strains of *H. uvarum* were cultured onto bismuth sulphite agar (Biggy Agar), Wilson-Blair medium (Merck, Darmstadt, Germany; Jiranek et al., 1995). H₂S production was estimated by colony blackening after 3 d of incubation at 28 °C, using a five-level scale: 0 = white, 1 = beige, 2 = light brown, 3 = brown, 4 = dark brown, 5 = black. *S. cerevisiae* US-05 (Fermentis, Lesaffre, France) and *S. cerevisiae* GR1 (from the collection of SAAF Department) were used as negative (0 = white) and positive controls (3 = brown), as described by Araújo et al. (2018) and Francesca et al. (2010).

2.3.3. Ethanol tolerance

For ethanol tolerance assay, dilutions of exponential pure cultures were spotted onto Petri dishes containing MEA solid medium added with ethanol at 5 and 10% (v/v).

2.3.4. Cross resistance to hop and ethanol

The tolerance of the *H. uvarum* strains to hop was evaluated applying the procedure illustrated by Michel et al. (2016) with the following

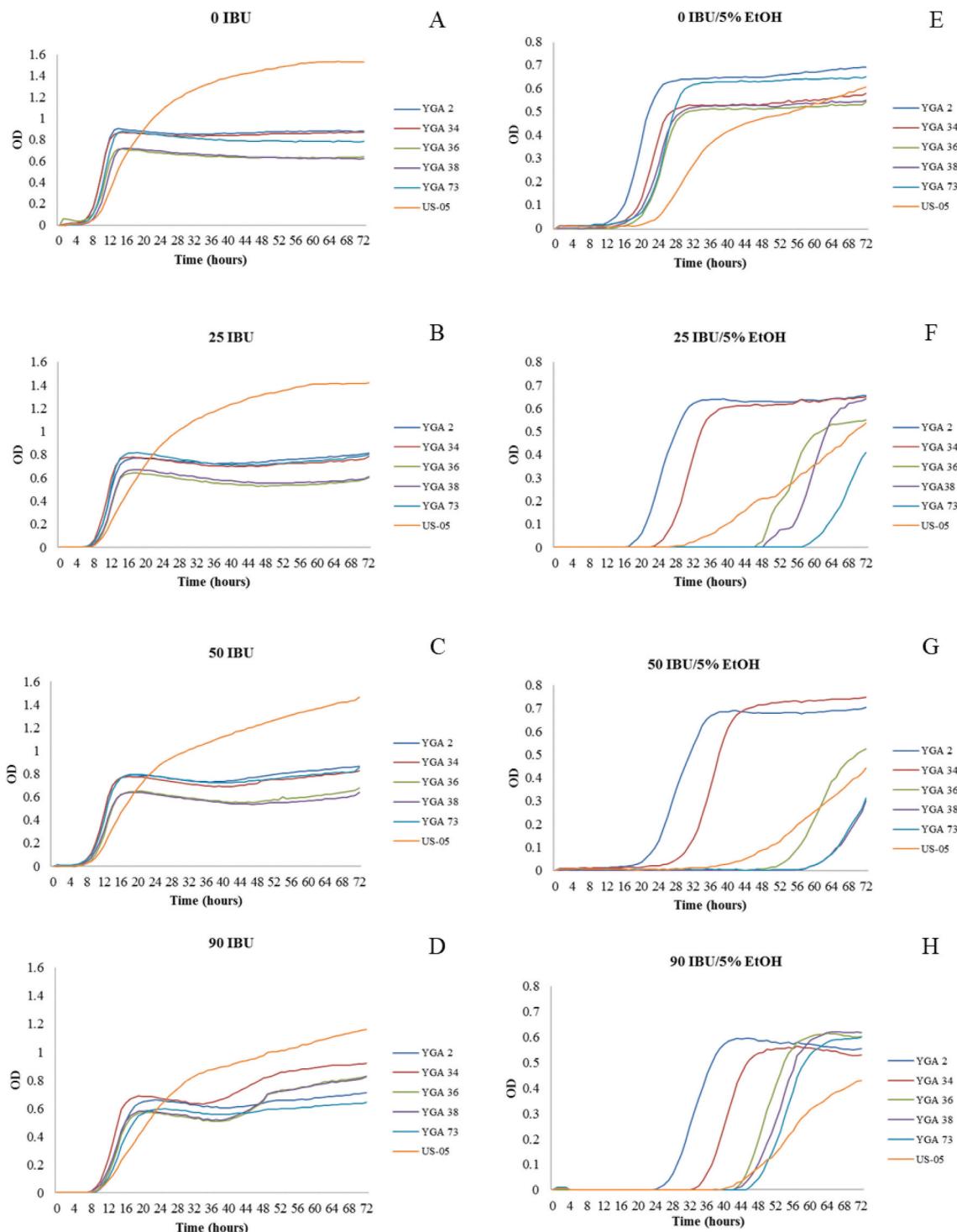


Fig. 1. Growth of *Hanseniaspora uvarum* strains at different IBU and ethanol concentrations. Abbreviation: IBU, International Bitterness Unit; OD, optical density. The sensitivity to different IBU and ethanol concentrations were measured by OD values at 600 nm in triplicate. Values of standard deviations ranged between 0 and 0.315 and are not showed for a better graphical visualization of figures.

modifications: the pure strain cultures were inoculated into a set of four 10 mL tubes containing 5 mL of sterile wort (45 min at 100 °C) prepared by mixing 10% of malt extract (Oxoid, Milan, Italy) to distilled water with a final pH 5.5; the wort was adjusted to iso- α -acid concentrations of 0, 25, 50 and 90 ppm (same value in IBU) and incubated for 72 h at of 27 °C. Additional set of four tubes containing sterile wort were adjusted to 0, 25, 50 and 90 ppm iso- α -acid and added with 5% (v/v) filter-sterilized ethanol. The growth was assessed by visual inspection

(Kurtzman et al., 2011; Michel et al., 2016).

2.3.5. Growth kinetics in presence of hop and ethanol

The growth of the pure strain cultures in wort was further investigated by optical density (OD) measurement at 600 nm wavelength into a 96-well microtitre plate (Michel et al., 2016). The strains for growth kinetics experiments were cultivated as reported by Hall et al. (2014). The measurement was performed at 1 h interval for the 72 h by using the

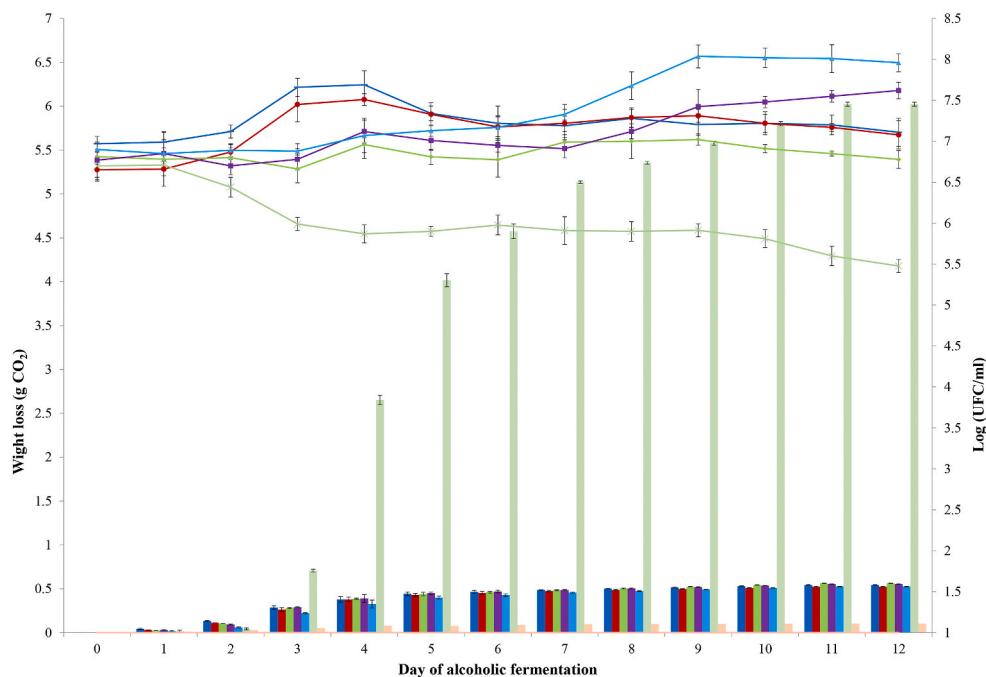


Fig. 2. Fermentation kinetics of wort beer inoculated with *Hanseniaspora uvarum* strains. Axes: values of principal vertical axis (left side) refer to histogram graph and represent the weight loss (g CO₂) of wort beer during AF; values of secondary vertical axis (right side) refers to growth curve of strains inoculated into wort beer during AF.

Codes: YGA2, YGA34, YGA36, YGA38 and YGA73, are codes of *Hanseniaspora uvarum* inoculated into wort representing the experimental trials; US-05, code of *Saccharomyces cerevisiae* commercial strain inoculated into wort representing the positive control trial; NC, negative control trial subjected to spontaneous fermentation.

ScanReady Microplate photometer P-800 (Life Real Biotechnology Co., Ltd, Hangzhou, China). The temperature was set at 27 °C (Salvadò et al., 2011). All analyses were performed in triplicates in two independent experiments.

The variables describing the growth curves were: total growth calculated as the integrated area underlying the curve up to 72 h; lag phase time was statistically estimated as the duration of the growth lag phase (Hall et al., 2014); the slope of exponential phase was determined as described by Hall et al. (2014); exponential phase refers to the integrated area underlying the curve and the time of exponential phase was statistically determined; the maximum growth was represented by the highest values of OD measured up to 72 h of incubation.

2.3.6. Flocculation assay

Flocculation assay was carried out as previously described by Tofalo et al. (2014). The medium used was Yeast Nitrogen Base (YNB, Difco Laboratories, Detroit, Mich., USA) with 2% glucose added and prepared according to manufacturer's instructions. A volume of 5 mL of this medium was inoculated with a loopful of the pure yeast in exponential growth phase and incubated at 28 °C under stationary conditions. Flocculation ability, evaluated by visual inspection and compared with appropriate controls, was graded on a scale from 0 (non flocculent) to 5 (highly flocculent). The visual inspection was performed after 2, 15 and 20 d from strain culture inoculation. Flocculation measurement with Helm's assay was also performed. Briefly, the sedimentation of yeasts was observed in a calcium sulfate solution buffered at pH 4.5, quantifying the sedimentation volume and flocculation type according to Casey et al. (1994). All analyses were performed in triplicates.

2.3.7. Wort fermentation: monitoring of weight loss and strain concentration

A laboratory-scale fermentation was performed to evaluate the ability of the strains to ferment a beer wort. To guarantee standardized conditions for all trials, wort fermentation medium was prepared as

described by Holt et al. (2018), with some variations: 150 g of dry Malt Extract (Munton spraymalt 6.5 EBC, Suffolk, England) and 50 ppm of calcium carbonate were added to 1 L of distilled water. The pH value was set at 5.2 with 1 mol/L HCl. Hopping was performed after autoclaving by adding an aliquot of iso- α -acid to reach a final concentration of 25 ppm (same value in IBU) (Michel et al., 2016). The final gravity of the malt extract medium was 13.3 °P. Aliquots of 150 mL of wort were put into 300 mL flasks, sealed with a Müller valve to allow the CO₂ produced during fermentation to leave the system and autoclaved at 110 °C for 15 min. After autoclaving, the malt extract wort was let to cool down to 18 °C and, subsequently, inoculated with each yeast strains. The fermentation was performed at 18 °C under static conditions and was monitored daily by measuring the weight loss up to day 12. Beer wort inoculated with *S. cerevisiae* strain US-05 was the positive control trial. The un-inoculated beer wort represented the negative control trial. According to Ciani and Maccarelli (1998), fermentation rate (FR) was calculated as daily CO₂ production after 3 d and at the end of AF. All beer wort fermentations were performed in triplicates in two independent experiments.

All experimental trials were subjected to sampling before and after the inoculation of the strains, as well as at day 3, 6, 9, and 12 of fermentation. The samples were immediately subjected to microbial counts, performed on WL nutrient agar as previously described. After growth, all isolates were picked up from the agar plates, purified to homogeneity and subjected to genotypic identification at species and strain level as reported above. All analyses were performed in duplicates.

2.4. Beer production

2.4.1. Experimental design and sample collection

To better understand the effect of *H. uvarum* inoculum during fermentation, experimental top-fermented beers were produced at a medium-scale level (10 L batch) using four different inoculum

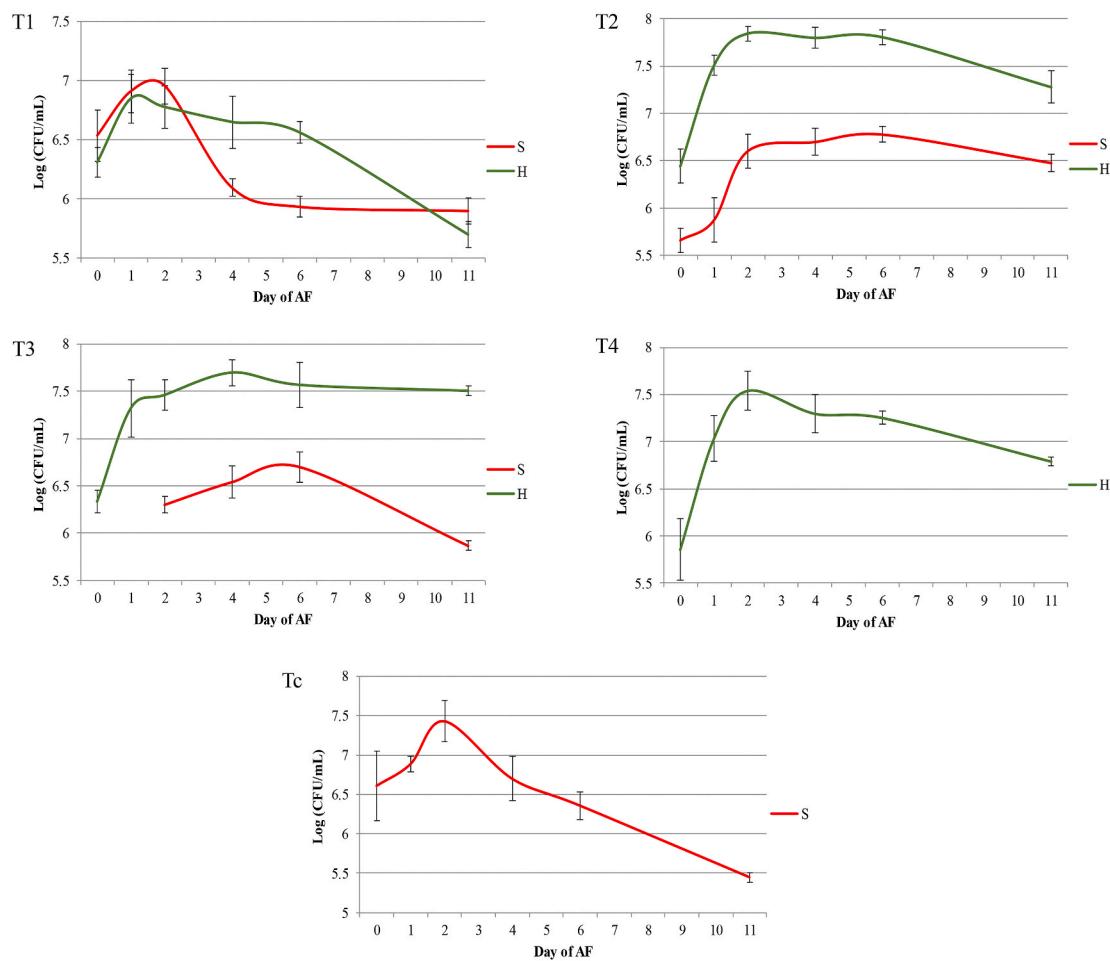


Fig. 3. Evolution of yeast populations of *S. cerevisiae* and *H. uvarum* populations during trials fermentation. Codes: T1 refers to co-inoculated fermentation with 1:1 ratio between YGA34 and US-05; T2 refers to co-inoculated fermentation with 10:1 ratio between YGA34 and US-05; T3 refers to sequentially inoculated fermentation with YGA34 and US-05; T4 refers to fermentation trial inoculated with monoculture of YGA34; Tc refers to fermentation trial inoculated with monoculture of US-05. Legend: –S refers to *S. cerevisiae* population; –H refers to *H. uvarum* population.

Table 4
Mean percentage of sugar consumption during AF calculated for each trial.

	D-glucose (%)	D-fructose (%)	Sucrose (%)	Maltose (%)
T1	99.40 ± 0.02	97.30 ± 0.05	100.00 ± 0.00	82.00 ± 0.04
T2	99.50 ± 0.05	97.90 ± 0.01	100.00 ± 0.00	89.20 ± 0.10
T3	99.50 ± 0.02	98.10 ± 0.07	100.00 ± 0.00	84.60 ± 0.03
T4	99.20 ± 0.03	96.80 ± 0.02	37.90 ± 0.39	2.20 ± 0.07
Tc	99.40 ± 0.05	96.80 ± 0.08	100.00 ± 0.00	84.30 ± 0.01

Results indicate mean values ± SD of three determinations.

combinations for *H. uvarum* YGA34 and *S. cerevisiae* commercial strain US-05. Four experimental trials (T1, T2, T3 and T4) and one control trial (Tc) were inoculated as follows: co-inoculation of *H. uvarum* strain YGA34 and *S. cerevisiae* strain US-05 with 1:1 ratio (trial T1); co-inoculation of *H. uvarum* strain YGA34 and *S. cerevisiae* strain US-05 with 10:1 ratio (trial T2); sequential fermentation starting from YGA34 strain and, after 48 h, inoculation of US-05 strain (trial T3); monoculture of *H. uvarum* strain YGA34 strain (trial T4); monoculture of *S. cerevisiae* US-05 strain (trial Tc). The inoculation rate was planned with a cell density of 2.0×10^6 cells/mL of each yeast strain for the trials T1, T3 and T4, while *H. uvarum* YGA34 and *S. cerevisiae* US-05 were inoculated at 2.0×10^6 and 2.0×10^5 cells/mL, respectively, in trial T2.

The beers were produced at the pilot plant of SAAF, Department - University of Palermo (Italy). Brewing was performed using an “all-in-one” microbrewery plant Klarstein mod. 10031629 (Chal-Tec GmbH

Table 5
Final concentration of ethanol, glycerol and acetic acid in green beers produced with different combinations of inoculum.

	Ethanol (%)	Ethanol yield (g/g)	Glycerol (g/L)	Acetic acid (g/L)
T1	5.05 ± 0.16 ^{ab}	0.50 ± 0.06 ^a	3.20 ± 0.18 ^b	0.03 ± 0.02 ^c
T2	5.16 ± 0.13 ^a	0.48 ± 0.04 ^a	3.09 ± 0.08 ^b	0.17 ± 0.03 ^b
T3	4.80 ± 0.07 ^c	0.46 ± 0.04 ^a	3.80 ± 0.06 ^a	0.26 ± 0.04 ^a
T4	0.52 ± 0.01 ^d	0.31 ± 0.02 ^b	1.28 ± 0.40 ^c	0.27 ± 0.01 ^a
Tc	4.90 ± 0.11 ^{bc}	0.47 ± 0.05 ^a	3.08 ± 0.04 ^b	0.03 ± 0.02 ^c
Statistical significance	***	*	***	***

Results indicate mean values ± SD of three determinations.

Data within a column followed by the same letter are not significantly different according to Tukey's test.

P value: *; P < 0.05; ***, P < 0.001.

Berlin, Germany). Nine kilograms of Pilsen malt (BestMalz, Heidelberg, Germany), previously ground through a two roller mill (Brouwland, Beverlo, Belgium), were added to 30 L of water with 10 g of calcium chloride (CaCl_2) for pH correction (Marconi et al., 2016). The mash was heated to 70 °C for 40 min for the single-step mash, until the complete

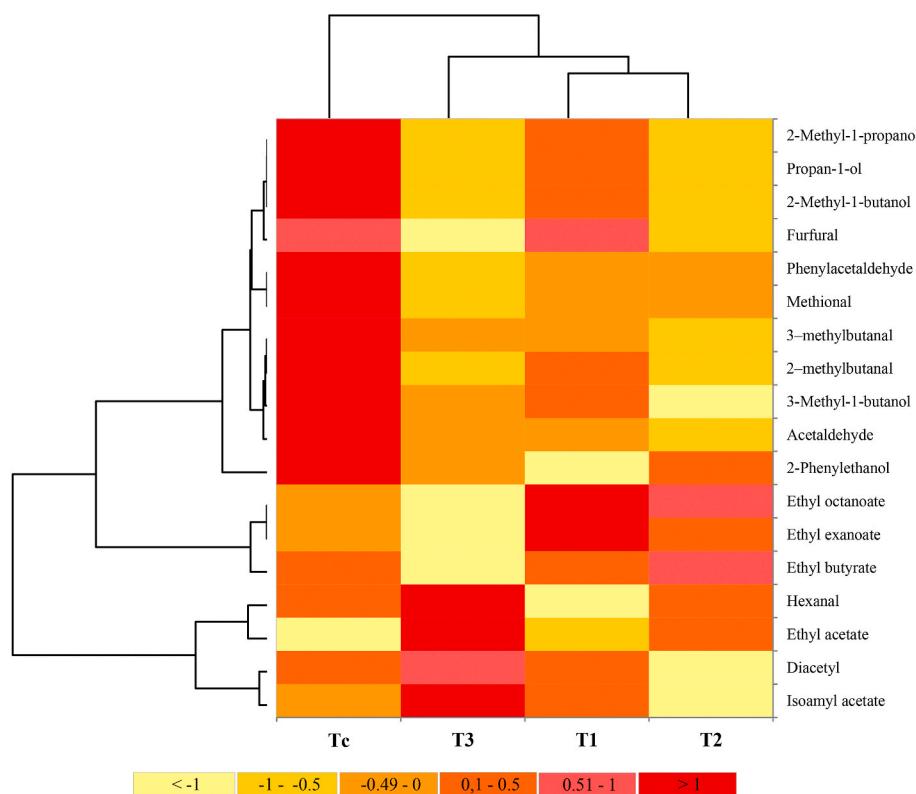


Fig. 4. Distribution of volatile organic compounds among experimental beers. The heat map plot depicts the relative concentration of each VOCs (variables clustering on the Y-axis) within each sample (X-axis clustering). Codes: T1 refers to co-inoculated fermentation with 1:1 ratio between YGA34 and US-05; T2 refers to co-inoculated fermentation with 10:1 ratio between YGA34 and US-05; T3 refers to sequentially inoculated fermentation with YGA34 and US-05; Tc refers to fermentation trial inoculated with monoculture of US-05.

conversion of sugars, verified with iodine solution. Subsequently, the mixture was heated to 78 °C for 10 min. The grains were rinsed (sparging) using 20 L H₂O, resulting in a total volume of 45 L. The resulting wort was boiled for 60 min, during which the hops (pellets, 40 g, 11.5% of α-acids) were added to reach a final concentration of 25 IBU. After boiling, the resulting volume was 42 L, with 13.5 °Bx (degrees Brix). The wort was then clarified through a whirlpool consisting of 10 min of recirculation and 10 min of resting (Marconi et al., 2016). The must was finally cooled to 21 °C in preparation for the inoculation of the selected yeast strains. Standard quality parameters of beer wort were: 5.62 pH, 13.5° Bx (Brix degree), 1053 SG (Specific Gravity), 7.16 g/L d-glucose, 0.94 g/L d-fructose, 10.02 g/L sucrose, 75.63 g/L maltose, 71.4 mg/L free α-amino nitrogen, 106.6 mg/L ammonia nitrogen.

Strains were reactivated from -80 °C glycerol stocks and plated onto YPD agar (10 g/L yeast extract, 20 g/L bacteriological peptone, 15 g/L agar, and 20 g/L d-glucose). After 3–5 d at 25 °C, the strains were pre-cultured overnight in 5 mL YPD and then re-inoculated into sterile flasks containing YPD, where cells were allowed to grow for 3 d at 25 °C (Holt et al., 2018). The cells were washed twice with Ringer solution, and inoculated as described above.

At the end of AF, the beer samples were bottle conditioned for 16 d by dextrose addition (4.5 g/L) and US-05 with a pitching rate of 5.0 × 10⁶ cells/mL.

All experimental fermentation trials were performed in duplicate. Samples were collected at different stages of beer production: wort, after the inoculum of yeast strains, during the AF (day 1, 2, 4, 6 and 11), at the end of AF (green beer), at the end of bottle conditioning. All samples were subjected to analysis within 24 h from collection. All analyses were performed in triplicate.

2.4.2. Microbiological counts, isolation and genotype monitoring of the added strains

The concentration of yeasts (total yeast, *Saccharomyces* spp. and *Hanseniaspora* spp. populations) were evaluated onto WL nutrient agar (Martin et al., 2018). After growth, all isolates were picked up from the

agar plates, purified to homogeneity and subjected to genotypic identification as reported above (paragraph 2.2.). In order to verify the dominance of *H. uvarum* strain YGA34, all isolates within *Hanseniaspora* spp. group were characterized at strain level as reported above (paragraph 2.3.1).

The genetic diversity of *Saccharomyces* isolates was assessed by Interdelta analysis (Legras and Karst, 2003). Interdelta patterns were analyzed using the Gel Compar II software (v. 6.1. Applied Maths NV. Sint-Martens-Latem. Belgium) and similarities among patterns were assessed. Profiles showing more than 95% of similarity were considered identical.

2.4.3. Determination of physicochemical parameters

The pH measurement was conducted with a pH meter (Mod.70 XS/50010162) while °Brix were determined with a refractometer (DBR Salt). The analyzer iCubio iMagic M9 (Shenzhen iCubio Biomedical Technology Co.. Ltd.. Shenzhen, China) was used and run with full automation for the enzymatic determination of glucose, fructose, sucrose, maltose, ethanol, glycerol, and acetic acid. It automatically pipetted reagents and samples into the cuvette, allowed incubation at a controlled temperature, read absorbance at the specific wavelength, and calculated the concentration of the analyses with a calibration curve. The parameters used in the automated photometric systems were: temperature, 37 °C; wavelengths, 340 nm and 415 nm (bichromatic); optical path. 1 cm. The reagents used were: Enzytec™ Liquid d-Glucose/d-Fructose Cod. E8160. Enzytec™ Liquid Sucrose/d-Glucose Cod. E8180. Enzytec™ Liquid Ethanol Cod. E8340. Enzytec™ Fluid Glycerol Cod. E5360. Enzytec™ Acetic acid Cod. E2580. The standard used for the calibrations of the apparatus were: Enzytec™ Multi-acid standard automation Cod. E1241 for acetic acid; Enzytec™ Sugar standard Cod. E5450 for glucose and fructose; Enzytec™ Alcohol standard Cod. E5420 for ethanol; Enzytec™ Sugar standard manual Cod. E1242 for glycerol. All reagents and standards were purchased from R-Biopharm AG (Darmstadt. Germany). All samples were diluted to the optimal concentration with respect to the calibration curve of the apparatus.

A

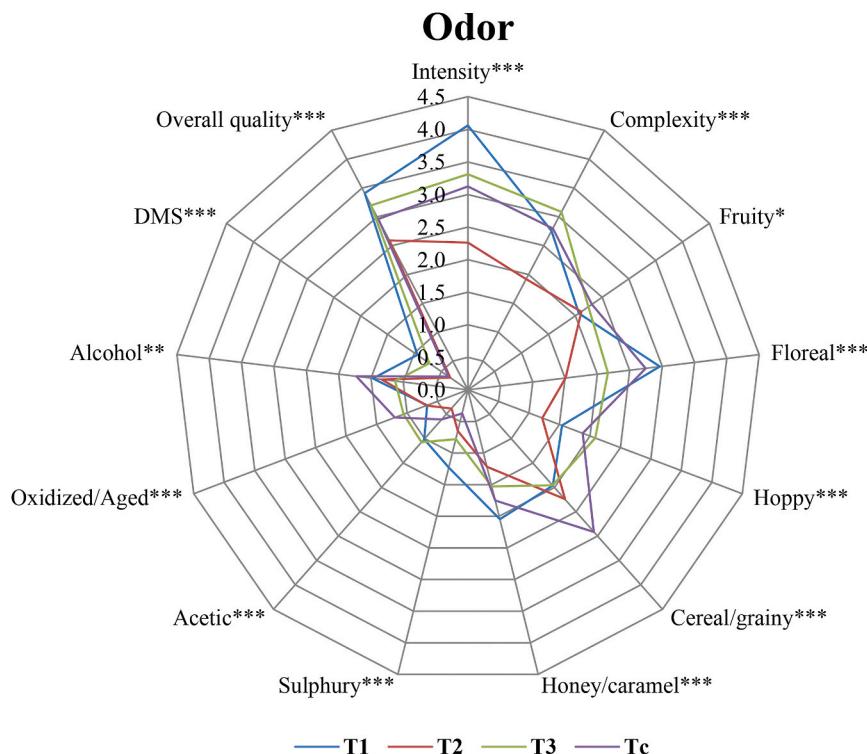
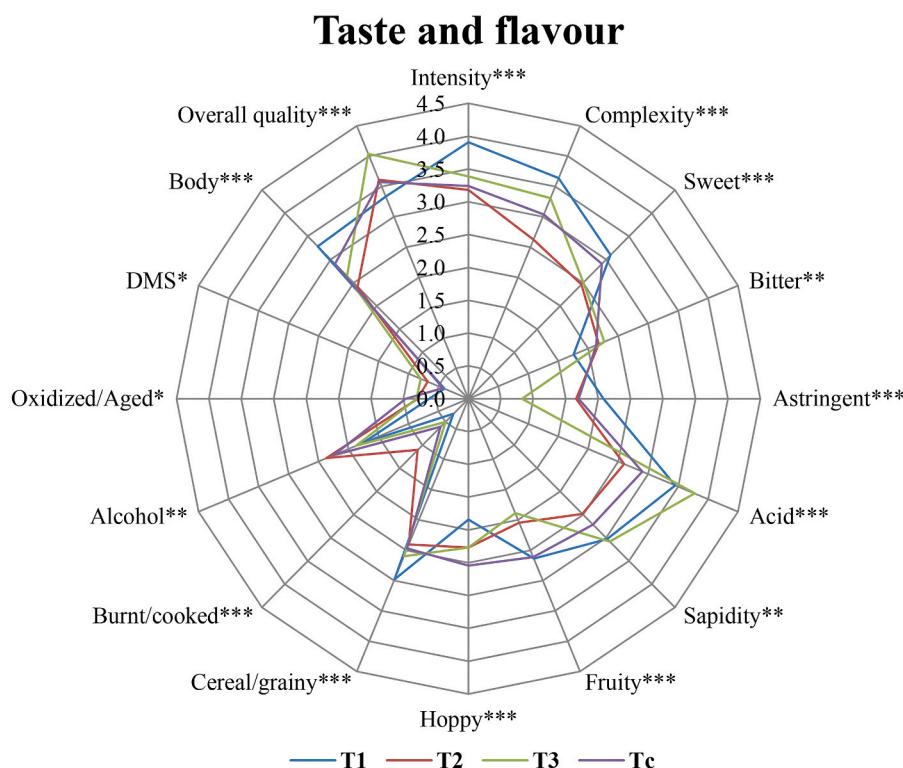


Fig. 5. Spider-plot of the sensory analysis performed on beers. a) odor; b) taste and flavour. Codes: T1 refers to co-inoculated fermentation with 1:1 ratio between YGA34 and US-05; T2 refers to co-inoculated fermentation with 10:1 ratio between YGA34 and US-05; T3 refers to sequentially inoculated fermentation with YGA34 and US-05; Tc refers to fermentation trial inoculated with monoculture of US-05. P value: *, P < 0.05; **, P < 0,01; ***, P < 0.001; not significant values are not reported in figure.

B



2.4.4. Determination of volatile organic compounds

The volatile compounds of the experimental beers were determined using an Agilent Technologies 6850 gas chromatograph (GC) equipped with an Agilent Technologies Mass Spectrometer (MS) 5975C (Santa

Clara, CA, USA), The GC-MS with a glass direct inlet liner (1.5 mm inner diameter and 140 μ L volume) and a DB-5MS capillary column of 60 m \times 0.32 mm \times 1 μ m (J&W Scientific, Inc., Folsom, CA, USA) consisting of cross-linked 5% phenyl methyl siloxane was used. Typical beer volatile

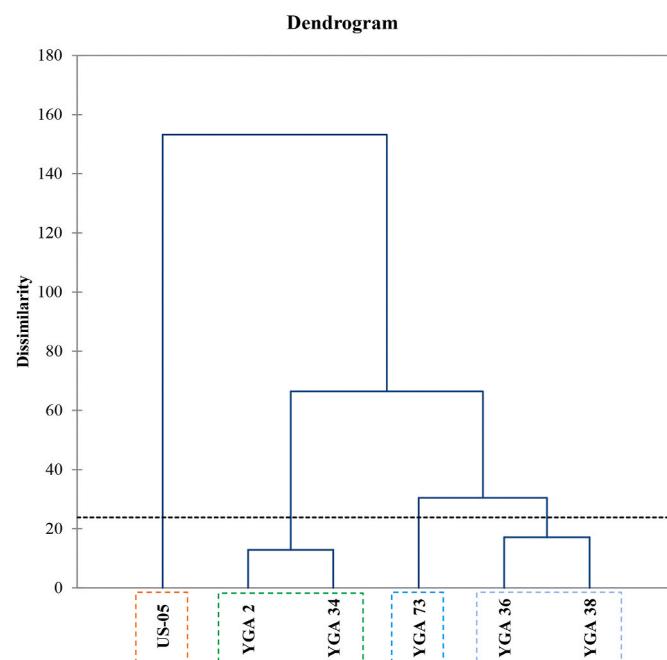


Fig. 6. Dendrogram of strains per each class resulting from AHC analysis based on values of technological screening of *Hanseniaspora uvarum* strains Dissimilarity is calculated by Euclidean distance and Ward's agglomeration method. The analysis is based on values of technological variables reported at paragraph 2.5. Codes: YGA2, YGA34, YGA36, YGA38 and YGA73 refer to *Hanseniaspora uvarum* strains; US-05 refers to *S. cerevisiae* commercial strain.

compounds were determined according to Vesely et al. (2003) and Malfliet et al. (2009) based on solid-phase microextraction (SPME) with on-fibre derivatization using a 65 µm PDMS/DVB fiber coating (Supelco, Bellefonte, PA, USA; catalogue no. 57328-U). An aqueous solution (4 g/L) of the derivatization agent O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBOA, Sigma-Aldrich, Milwaukee, WI, US) was prepared each week. The internal standard for the determination of aldehydes and vicinal diketones, 2-chlorobenzaldehyde (Sigma-Aldrich Milwaukee, WI, US), was prepared weekly in a solution of 5% ethanol with a concentration of 10 mg/L. The internal standard for the determination of higher alcohols and esters was 1-butanol (Sigma-Aldrich), which was prepared each week in water at a concentration of 60 mg/L. The analyses were conducted in triplicate.

2.4.5. Sensory analysis

The designed sensory evaluation of experimental beers consisted of quantitative descriptive analysis carried out by panelists to define color, odor, taste and overall quality.

Eleven judges (ranging from 23 to 52 years old) were recruited from University of Palermo, beer associations and professional brewers. All had experience in brewing and participated in previous studies as sensory judges. The judges were submitted to preliminary tests to determine their sensory performance on basic tastes and the aromas associated with beers. The sensory analysis of beers was conducted following the methodology reported by Marconi et al. (2016) and ISO regulations: visual perception (appearance), olfactory sensations based on odor (via the nostril, orthonasally) and flavour (via the back of the throat, retro-nasally), oral sensations based on taste, mouth-feel and overall quality.

The odor was explained as perception of volatile compounds with the beverage outside the mouth [orthonasal (in-glass) odor]; the flavor as perception of volatile compounds with the beverage inside the mouth and back of the throat [retro-nasal (mouth-derived) odor]; basic taste as gustatory sensations and mouth-feel sensations as chemical feeling factors with beverage inside and after the mouth and overall quality based on global evaluation of odor, taste, mouth-feel and flavour (Issa-Issa

et al., 2020; Jackson, 2017).

The panelists consensually generated 28 sensory descriptive attributes regarding appearance, odor, flavor, taste and overall quality in several sessions. The set of attributes were: appearance (yellow color); odor (intensity, complexity, fruity, citrus, floral, hoppy, cereal/grainy, malty, honey/caramel, roasted/burnt, sulphury, acetic, oxidized/aged, alcohol, and off-odor); gustatory taste (sweet, bitter, sour and salty); mouth-feel (body and astringent); flavor (intensity, complexity, fruity, citrus, hoppy, cereal/grainy, malty, honey/caramel, roasted/burnt, alcohol, sulphury, and off-flavor). The descriptor overall quality was also included for both odor and flavour.

The panelists also generated a consensual descriptive ballot for the experimental beers in which the descriptors were associated with a 9 cm unstructured scale anchored at the left and right extremes with the terms "none/weak" and "strong", respectively (Jackson, 2017).

The sensory assessments were performed in blind tasting conditions at the tasting room of University of Palermo (Palermo, Italy). The experimental beers samples (50 mL) were served monadically at 15 °C in standard ISO type tasting glasses, labelled with three-digit random codes. Water was provided for rinsing between beers. All evaluations were made between 10.00 and 12.00 a.m. in individual booths (ISO 8589, 234 2007). The final scores were obtained as a mean of three evaluations with the respective statistical analysis.

2.5. Statistical and explorative multivariate analyses

Data were investigated using a generalized linear model (GLM) based on ANOVA model that included effects of *in vitro* tests and strain, as well as the interaction between tests and strains. The post-hoc Tukey's method was applied for pairwise comparison. Statistical significance was attributed to $p < 0.05$ (Mazzei et al., 2010).

In addition, explorative multivariate analysis was conducted to better differentiate the strain combinations on the basis of the results from *in vitro* technological screening, micro-fermentations and laboratory-scale beer productions.

In details, the statistical analyses were performed on the data set including a total of 58 variables: total growth, exponential growth, maximum growth, lag phase time, slope of exponential phase calculated in absence of hop and ethanol, as well as in presence of different concentrations of hop (25, 50 and 90 IBU) and ethanol (5% v/v); resistance to ethanol (5, 10% v/v); resistance to hop (25, 50 and 90 IBU); cross resistance both to ethanol (5% v/v) and hop (25, 50 and 90 IBU); production of H₂S and flocculation growth pattern. The agglomerative hierarchical cluster analysis (AHC) and principal component analysis (PCA), based on correlation matrix by Pearson (n), and implemented by symmetric biplot representation were performed in order to investigate relationships among the samples as reported by Martorana et al. (2015). Due to high number of variables describing the technological screening of strains, a graphical representation of individual values and distribution of technological characteristics among strains was performed by using a heat map clustered (HMC). This analysis was based on double hierarchical dendrogram with heat map plot, the individual content values contained in the data matrix as colors. The relative values of the technological characteristics were depicted by color intensity from yellow (lowest value) to red (highest value). HMC analysis of values was performed using the autoscaled data.

Data set resulted from VOCs and sensory analysis of experimental beers produced at laboratory-scale was also subjected to explorative multivariate analysis (HCA, PCA and HMC) following the same methodology reported above.

All data subjected to multivariate analysis were preliminary evaluated by using the Barlett's sphericity test (Dillon and Goldstein, 1984; Mazzei et al., 2013) in order to check the statistically significant difference among strains within each data set.

STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA) was used for data processing and graphic construction of HCA and PCA

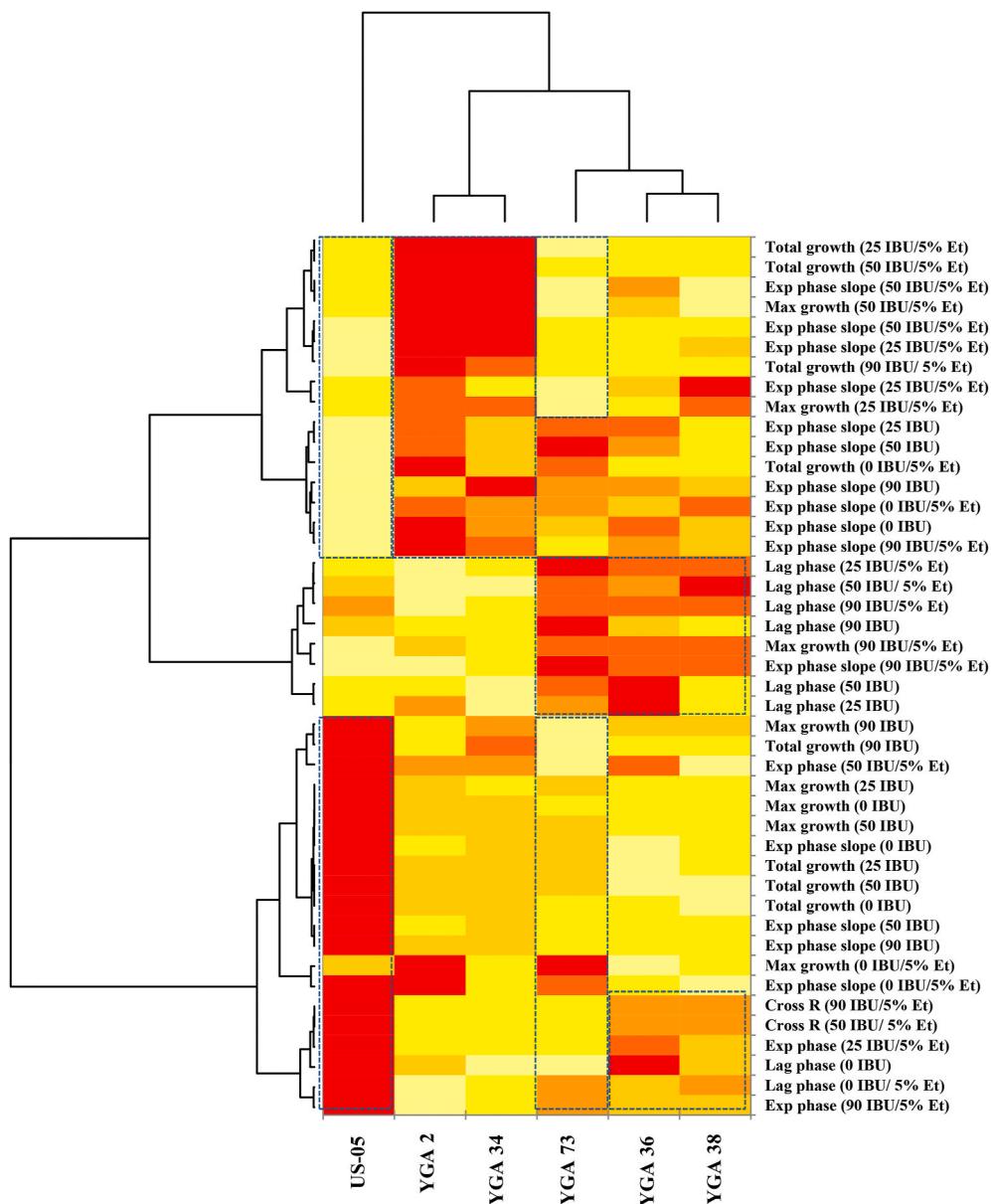


Fig. 7. Distribution of technological variables among strains. The heat map plot depicts the relative percentage of values per each technological variable (Y-axis clustering) within each strain (X-axis clustering). The analysis is based on values of technological variables reported at paragraph 2.5. Codes: YGA2, YGA34, YGA36, YGA38 and YGA73 refer to *Hanseniaspora uvarum* strains; US-05 refers to *Saccharomyces cerevisiae* commercial strain.

analyses. The XLStat software version 7.5.2 (Addinsoft, New York, USA) was applied for HMCA and biplot representation.

3. Results

3.1. Microbiological analysis of FHP samples

Yeast loads in the 12 FHP samples analyzed are reported in Table 1. Significant differences in terms of yeast cell densities were registered among samples and media used for colony enumeration. The highest yeast counts were found on WL agar (5.9 Log CFU/mL). The levels of non-*Saccharomyces* population detected on LA was quite variable ranging between 2.23 and 6.01 Log CFU/mL. In particular, sample FHP-3 showed the highest non-*Saccharomyces* levels on both media. In general, the lowest levels were registered for total osmophilic and osmo-tolerant yeasts on DWA and TGYA media, respectively.

3.2. Genotypic identification of yeasts

A total of 404 yeasts were isolated from FHP samples and subjected to genotypic characterization. The restriction analysis of ITS1-5.8S-ITS2 separated the isolates into five groups (Table 2); five groups were preliminary identified at species level by comparison of the restriction profiles with those reported in literature (Esteve Zarzoso et al., 1999; Francesca et al., 2014; Sannino et al., 2013). Specifically, the isolates were identified as *Hanseniaspora uvarum* (group I), *Saccharomyces cerevisiae* (group II), *Wickerhamomyces anomalus* (group III), *Zygosaccharomyces bailii* (group IV) and *Zygosaccharomyces rouxii* (group V). The genotypic identification of yeasts was completed by pairwise alignment of D1/D2 sequence of the type strain of each species (Table 2).

With regards to genera/species distribution (Table 2) among samples, the majority of isolates belonged to the *H. uvarum* group. The species *Z. bailii* and *Z. rouxii* were also isolated from several samples and their presence reached 22 and 19% of total number of isolates, respectively.

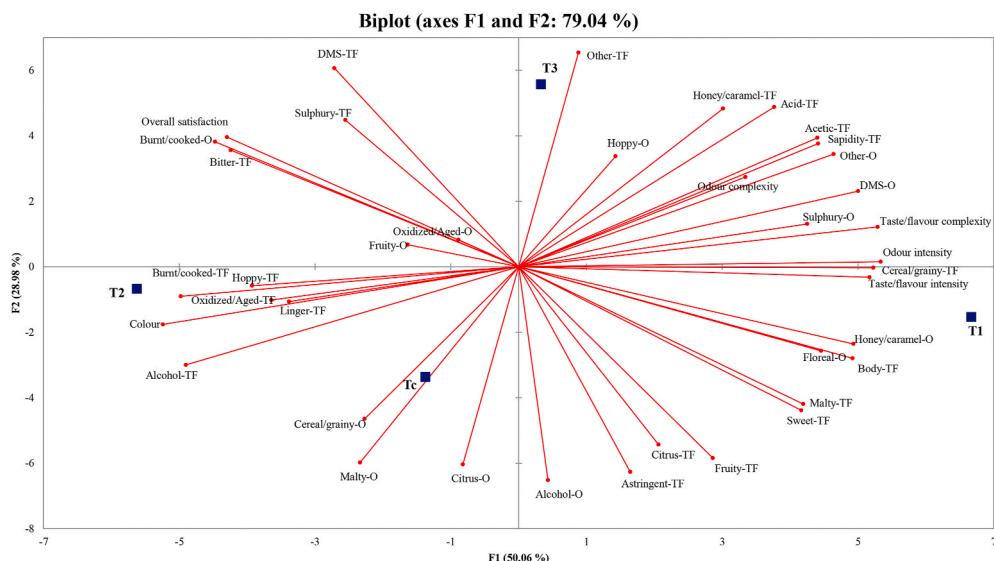


Fig. 8. PCA for sensory data of beers. Biplot graphs show relationships among factors, variables and trials. Codes: T1 refers to co-inoculated fermentation with 1:1 ratio between YGA34 and US-05; T2 refers to co-inoculated fermentation with 10:1 ratio between YGA34 and US-05; T3 refers to sequentially inoculated fermentation with YGA34 and US-05; Tc refers to fermentation trial inoculated with monoculture of US-05.

All isolates of *H. uvarum* were further investigated at strain level by RAPD-PCR and TRtRNA-PCR analysis. The dendrogram resulting from these analyses (Supplementary Fig. 1) showed that these isolates represented five distinct clusters corresponding to five different strains (YGA2, YGA34, YGA36, YGA38 and YGA73).

3.3. Technological characteristics of *Hanseniaspora uvarum* strains for beer production

3.3.1. H_2S production and flocculation tests

The five *H. uvarum* strains were screened for their brewing characters (Table 3). All strains were characterized by a very low production of H_2S on Biggy agar plates (white – light brown colony) and non-flocculent behavior. The mean sedimentation volume measured with Helm's assay ranged between 0.35 and 0.7 mL. According to Casey et al. (1994), all yeasts showed a type II flocculation with a rising interface near the bottom of the testing tubes, typical of non-flocculent yeasts.

3.3.2. Resistance to ethanol and hop

Intense growth at 5% (v/v) of ethanol was observed for all strains, while only a weak growth was found for the strains YGA2, YGA34, YGA36 and YGA38 at 10% (v/v). Only the strain YGA73 was unable to grow at 10% (v/v) of ethanol (Table 3).

The growth in presence of iso- α -acid was also evaluated (Table 3). All strains were able to grow in liquid medium containing 0, 25, 50 and 90 IBU. In terms of cross-resistance to ethanol and hop, all *H. uvarum* strains were able to grow in presence of 5% ethanol and up to 25 IBU (Table 3). The strains YGA36 and YGA38 showed growth at 5% (v/v) ethanol and up to 90 IBU.

3.3.3. Growth kinetics in presence of hop and ethanol

The resistance to ethanol and hop was further investigated by dynamic measurement of cell growth kinetics at 600 nm within 72 h from inoculation (Fig. 1). The strain growth curves were analyzed in terms of time of lag phase (LP), slope of exponential phase (EP). The highest scores reached during the stationary phase (SP) were also included in the analysis.

When the tests were performed at different concentrations of iso- α -acids without ethanol (Fig. 1A–D), all *H. uvarum* strains showed LP times comparable to control *S. cerevisiae* US-05, with values between 4 and 9 h. The values of EP slope in *H. uvarum* strains resulted significantly

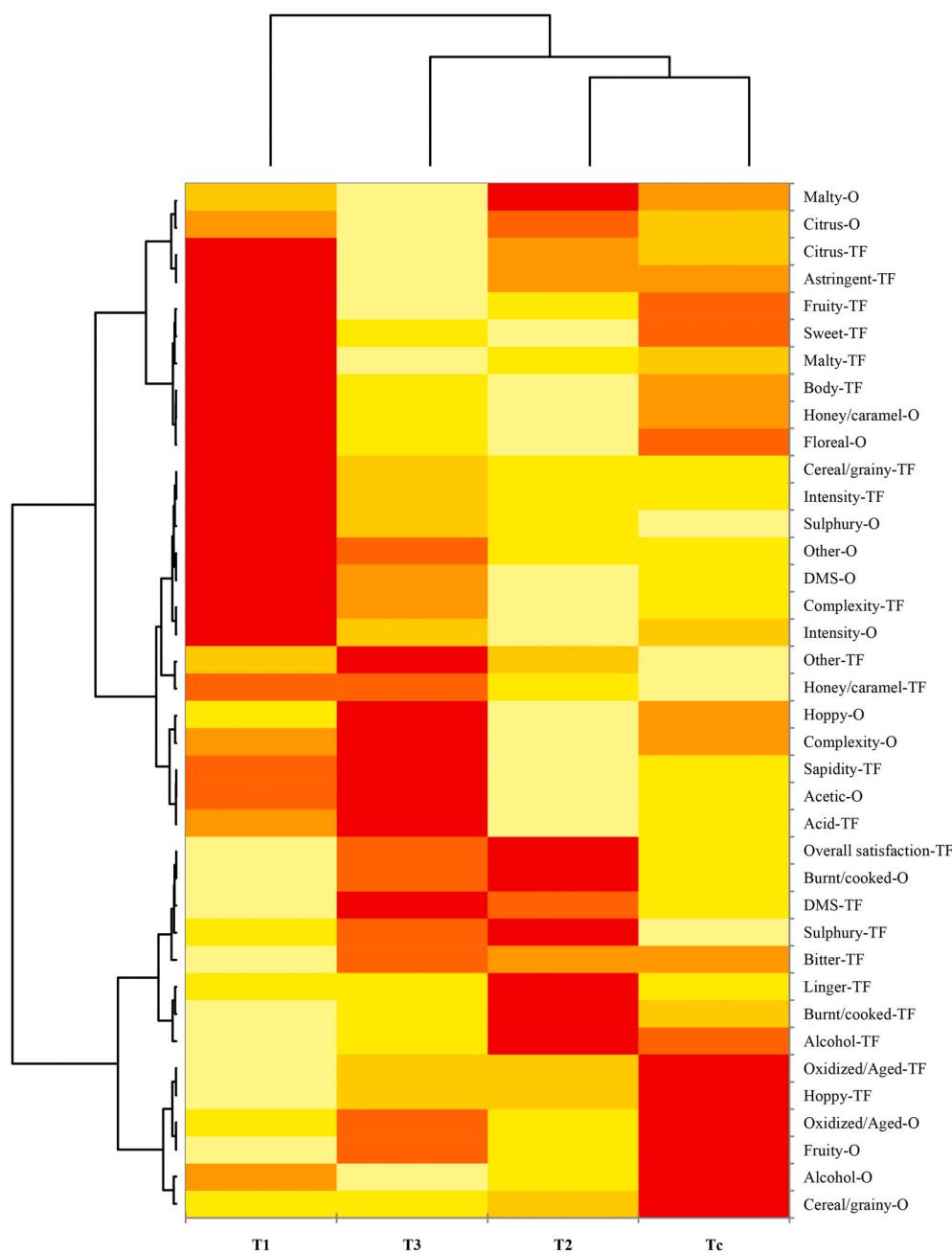
higher than control trial; the EP for the strain US-05 was, on average, 2.4%, while the *H. uvarum* strains reached values between 6.77% (YGA38) and 10.04% (YGA2). Between the 12th and 16th hour of incubation, all *H. uvarum* strains reached SP (0.72), while the strain US-05 reached 0.49 OD. From the 24th hour onwards, the control US-05 showed higher values of SP than *H. uvarum* strains, with a maximum value of 1.50 registered at the end of the monitoring. In addition, significant differences were found among the five *H. uvarum* strains when the growth curves were calculated in presence of different IBU without ethanol. The strains YG34, YGA2 and YGA73 showed the best growth within the 16th hour of incubation, with a SP value 0.2 units higher than that recorded for the strains YGA36 and YGA38. At 90 IBU, the strain YGA34 reached the highest value of EP slope (6.23%).

When the strains grew in presence of both 5% (v/v) ethanol and different IBU, their LP time significantly increased, from an average of 5.9 h (without ethanol) to an average of 32 h (with 5% ethanol) (Fig. 1E–H). Once again, the best values of both LP time and EP slope were found for the strains YGA2 and YGA34. In detail, LP time for these strains ranged between 10 and 33 h, while that of the control strain ranged between 19 and 39 h, depending on IBU concentration. When, the IBU concentration increased up to 50 and 90 the differences between the strains YGA2 and YGA34 and the control strain increased significantly (Fig. 1G–H), with a LP time 13 h shorter and SP values higher (0.74 for YGA34 and 0.68 for YGA2) than those observed for the strain US-05, which obtained values of 0.44. Statistical significance was attributed to $p < 0.05$ (Mazzei et al., 2010).

3.3.4. Wort fermentation

The kinetic of weight loss due to CO_2 production is graphically presented in Fig. 2. The values of FR at 3 d for *H. uvarum* strains ranged between 0.22 and 0.29 g. The positive control trial, inoculated with *S. cerevisiae* strain US-05, showed the highest FR (0.71 g). At day 12 of AF, all *H. uvarum* strains showed a FR between 0.52 and 0.56 g. The highest value of FR was found for the control strain US-05 (6.02 g).

During fermentation, the strains were also monitored by microbiological analysis (Fig. 2). Just after inoculation, all strains were found at concentrations ranging between 6 and 7 Log CFU/mL. At day 3 of AF, all *H. uvarum* strains reached levels higher than those of the control strain US-05; the highest counts (7.40 Log CFU/mL) were displayed by the strains YGA2 and YGA34. From day 6 onward, the levels of all *H. uvarum* strains were about 6.5 Log CFU/mL which was higher than the level



registered for the control strain US-05. At day 12, the end of monitoring, all experimental trials showed an increase of yeast levels; the highest values, 7.42 and 8.03 Log CFU/mL, were found for the strains YGA38 and YGA73, respectively.

The persistence of the strains inoculated was phenotypically and genotypically investigated. All isolates collected from the experimental trials showed shape of colony and cellular morphology typical of *Hanseniaspora* genus (Cadez et al., 2014; Jindamorakot et al., 2009; Kurtzman et al., 2011). In addition, the dendrogram resulting from RAPD-PCR analysis showed that all isolates were divided into five clusters for the five strains inoculated. All isolates collected from control trial shared the same RAPD pattern of *S. cerevisiae* strain US-05 (data not shown).

3.4. Beer production

3.4.1. Yeast growth during fermentation

The theoretical inoculum ratio resulted as planned, with one Log

cycle difference between the two yeast species for the trial T2 and an inoculum of about 6 Log cycle for the other trials. The evolution of yeast populations during the fermentation is reported in Fig. 3.

Trial T1, inoculated with *S. cerevisiae/H. uvarum* ratio of 1:1, showed similar levels of the two species (6.5 Log CFU/mL for US-05 and 6.3 Log CFU/mL for YGA34) at t_0 . This ratio was maintained also at 24 h after inoculum, but a 0.5 Log cycles increase of *S. cerevisiae* over *H. uvarum* was registered at 48 h. After 4 d of AF, a decrease of cell density was registered for *S. cerevisiae*, while the count resulting for *H. uvarum* were quite constant.

Trial T2 revealed a complete dominance of the strain YGA34 during fermentation. For this trial, the levels of *H. uvarum* were 0.8–1.6 Log cycles higher than those of *S. cerevisiae* for the entire fermentation. The highest levels of *H. uvarum* YGA34 was reached at 2 and 6 d of AF, with 7.8 Log CFU/mL, while the strain US-05 reached 6.8 Log CFU/mL at the 6th day.

Regarding trial T3, the strain YGA34 showed an increase of cell

Fig. 9. Distribution of sensory descriptors among experimental beers. The heat map plot depicts the relative score of each aroma, taste and overall satisfaction (variables clustering on the Y-axis) within each trial (X-axis clustering). Codes: T1 refers to co-inoculated fermentation with 1:1 ratio between YGA34 and US-05; T2 refers to co-inoculated fermentation with 10:1 ratio between YGA34 and US-05; T3 refers to sequentially inoculated fermentation with YGA34 and US-05; Tc refers to fermentation trial inoculated with monoculture of US-05.

density during the first 4 d of AF, with values from 6.3 to 7.7 Log CFU/mL; after day 4, a slight decrease to 7.5 Log CFU/mL at day 11 was registered. After the inoculum of US-05 at 48 h, *S. cerevisiae* population showed the maximum cell density at day 6 of AF (6.7 Log CFU/mL) while the lower value was registered at day 11 of AF (5.9 Log CFU/mL).

Trial T4, inoculated only with *H. uvarum*, although characterized by an initial load of 5.5 Log CFU/mL, grew rapidly up to 7 Log cycles 24 h after the inoculum and maintained these values until day 6 of AF; at day 11 of monitoring, the yeast population of *H. uvarum* was 6.8 Log CFU/mL.

The control trial displayed a classic fermentation kinetics.

3.4.2. pH, total soluble solids and sugar changes during fermentation

At the end of AF, pH values ranged between 3.95 and 4.08 for all trials except for T4 and un-inoculated control, which showed higher values of 4.87 and 5.48 respectively.

The final gravity (FG) of the beers indicated similar value for thesis T1, T2, T3 and Tc, with 1.015, while a value of 1.050 was registered for T4, with a decrease of 0.002 SG points respect to initial wort. The SG of the non-inoculated must did not show any detectable decrease during fermentation.

The percentages of the residual sugars are reported in Table 4. Fructose and glucose were fermented to over 96% in all experimental trials. Except in T4, sucrose was completely fermented for all other trials. Maltose consumption ranged between 82.0 and 89.2% for the trials T1, T2, T3 and Tc. The trial T4, inoculated only with monoculture of *H. uvarum*, did not show any maltose fermentation due to the inability of this species to use maltose; for this reason, bottle conditioning was not carried out for trial T4.

In terms of ethanol, glycerol and acetic acid production measured at the end of AF, the values are reported in Table 5. The highest value of ethanol was reached in T2, with 5.16% (v/v). Trial T4, which did not complete the AF, showed a final ethanol value of 0.52%.

Regarding glycerol content, except for T4 showing 1.3 g/L, this parameter ranged between 3.1 for T2 and Tc and 3.8 g/L for in T3. The acetic acid content detected at the end of fermentation was below 0.27 g/L for all trials. The highest value for this parameter was registered in trials T3 and T4, while the lowest value (0.03 g/L) was recorded for T1 and Tc.

3.4.3. Determination of volatile compounds

The assessment allowed the identification of 18 compounds, as higher alcohols, aldehydes, esters and vicinal diketones. The values of total VOCs were 198.36 mg/L (T1), 169.61 mg/L (T2), 186.28 mg/L (T3) and 225.98 mg/L (Tc).

The experimental beers were characterized by different flavour profile, as represented in heat-map (Fig. 4), in which the relationships among beers are based on the amount of each VOC. Among these volatile compound class, 2-methylpropan-1-ol dominated especially in Tc and T1 trials, followed by 3-methylbutan-1-ol and propanol. The second most abundant VOC class is represented by esters. With this regard, substantial differences were found in the overall level of these VOCs group, with maximum values recorded for trial T3 (32.7 mg/L), while the minimum value was recorded by Tc (9.2 mg/L). Except for trial T3, which reached values above this threshold (32.2 mg/L), all other trials showed lower values, with the minimum value obtained by the control trial (8.7 mg/L). In particular, isoamyl acetate ranged between 0.16 mg/L in T2 and 0.31 in T3, while ethyl hexanoate exhibited values always lower than 0.08 mg/L.

3.4.4. Sensory analysis

The sensory evaluation on the final products showed several statistically significant differences among trials, which are shown in Fig. 5. None of the experimental beers showed off-odors and/or off-flavours. No differences were found in terms of color appearance. The highest score for overall quality was found for beers of trials T2 and T3. Beers

fermented in presence of *H. uvarum* YGA34 strain, especially trials T1 and T3, showed highest score for intensity, complexity, floral descriptors in odorous profile as well as for intensity, complexity, sour, sapidity in taste-flavour profile. Sensory differences were also recognized within the trials fermented with YGA34 strain; the trial T3, which involved the inoculum of *H. uvarum* 48 h before that of *S. cerevisiae*, showed the highest value of sour and sapidity descriptors in the taste profile, whereas trial T2 showed the lowest values for the same attributes.

3.5. Statistical and explorative multivariate analyses

The Barlett's sphericity test was applied to all data matrix inputs and differences statistically ($p < 0.0001$) significant were found among trials.

AHC analysis discriminated all technological variables into four clusters (classes) as reported by dendograms in Supplementary Fig. S2. The variance between classes was significantly higher (73.29%) than that found within classes (26.71%). Dendrogram (Fig. 6) and bar chart (Fig. S3) show a deeper analysis of classes by focusing on dissimilarity (%) found among strains (objects). The strains YGA2, YGA34 and YGA36, YGA38 clustered into two classes with values of within-class variance lower than 12.81% and 17.10%, respectively. The strain YGA73 significantly differed from the other *H. uvarum* strains; the control strain *S. cerevisiae* US-05 was out of groups and reached values of dissimilarity higher than 150.

PCA was used to condense all technological information into a reduced number of Factors. The results of PCA (Figs. S4A and S4B) showed that all five eigen-values were higher than 1. Even though, Factor 1 and Factor 2 (Fig. S4A) explained very high values (58.08 and 23.92%, respectively) of total variance; the classes represented by strains the YGA36, YGA38 and YGA73 were not statistically discriminated. A deeper differentiation among strains was achieved by PCA (Fig. S4B) based on Factor 2 and Factor 3 accounting for a 23.92% and 10.91% of total variability, respectively.

The components of PCA were correlated to variables as shown in Figs. S5A and S5B. By biplot analysis based on F1 and F2 (Fig. S5A) the strains YGA2 and YGA34 were closely related to the best growth in presence of ethanol (5% v/v) and high concentration of hop (50 and 90 IBU). Even though, the components F2 and F3 contributed to differentiate strain YGA73 from the strains YGA36 and YGA38, all these strains were correlated to the highest values of lag phase and maximum growth in presence of ethanol and hop (Figs. S5A and S5B). The control strain US-05 was clearly discriminated from *H. uvarum* strains.

Further insights on correlation between strains and technological variables were provided by HMC analysis (Fig. 7). This analysis was performed to obtain a deeper strain differentiation on the basis of the technological variable values. Since a total of 58 variables were analyzed, the HMC color intensity clearly showed the main differences among strains. To confirm this trend, the double dendrogram divided the variables in six mega-clusters based on the values of technological characters of the strains (Fig. S6).

Regarding statistical multivariate analysis on VOCs, the double hierarchical dendrogram combined with heat map plot (Fig. S7 and Fig. 4) showed that all trials significantly affected the VOCs composition of the beers. Interestingly, control trial fermented with *S. cerevisiae* US-05 strain Tc resulted in a separate single cluster, while the trials performed with *H. uvarum* inoculums grouped into a different branch, with T1 and T2 clustering together and trial T3 forming a separate single cluster. Thus, the biplot in Fig. S8 represents the final distribution of trials with respect to the different VOCs detected. In particular, trial T3 was mostly affected by ethyl acetate, isoamyl acetate and hexanal variables.

The statistical multivariate investigation was carried out also on data from sensory analysis. Due to high numbers of sensory attributes and trials, the HCA was applied to identify significant differences among trials. The level of dissimilarity among trials is reported in Fig. S9.

Interestingly, control trial clustered together with trial T2, while T1 and T3 trials formed single clusters. The biplot reported in Fig. 8 and heatmap in Fig. 9 represent the final distribution of all experimental trials and the control. The trials T1 and T3 produced the best beers in terms of odor intensity and complexity, and acetic, sapidity and acid notes for the taste profile. Trial T1 was characterized by high values of floral, honey/caramel, malty, sweet and body aroma level. On the other hand, the trials T2 and Tc were mostly influenced by the parameters of alcohol and burnt/cooked, with a high persistency of taste.

4. Discussion

The present research has two main focuses: to characterize the yeast ecology associated to honey by-products subjected to the spontaneous AF; and to select novel non-*Saccharomyces* strains to be used as co-starter culture for craft beer production.

The quality of beer depends on several ingredients, such as malt, hop, water. Moreover, the yeasts used as fermenting starters are of paramount relevance to improve shelf life and sensory characteristics of beer. The majority of beers are fermented by using strains of *S. cerevisiae* and *S. pastorianus* species commercially available worldwide. Recently, non-conventional yeasts gained popularity among brewers in order to obtain distinctive products thanks to the diversity of substrate assimilation patterns that may be displayed. Non-conventional yeasts in brewing are represented by *Saccharomyces* and non-*Saccharomyces* yeasts isolated from non-brewing environment (Catallo et al., 2020; Cubillos et al., 2019). With these regards, Cubillos et al. (2019) published an updated review pertaining to the use of wild *Saccharomyces* and non-*Saccharomyces* in brewing system. Those authors focused the research on the diversity of spontaneously fermented food systems (wine, fruit, dough), as well as on fermented sugary matrixes (cachaça, kombucha) (Araújo et al., 2018; Bellut et al., 2018; Gutiérrez et al., 2018; van Rijswijck et al., 2017), showing that several matrices are promising for yeast isolation for brewing application.

With regards to unconventional yeasts, the use of non-*Saccharomyces* strains in controlled fermentations is a key factor to gain market share (Ciani and Comitini, 2011; Cordero-Bueso et al., 2013; González et al., 2013; Johnson, 2013; van Dijken, 2002). Different strains of *Torulaspora delbrueckii* isolated from different matrixes including wine, soil, fruits, sugar cane, papaya leaves, sugar cane juice, grapes, fig fruit, coconut palm and corosol fruit have been screened and applied for beer production (Canonico et al., 2016). Furthermore, *Zygosaccharomyces rouxii* was used by De Francesco et al. (2015) as starter culture for low-alcohol beer production and *Brettanomyces bruxellensis*, isolated from fruit by Lentz et al. (2014), was tested for the ability to ferment wort sugars and for ethanol tolerance. Then, it is now widely accepted that traditional fermented beverages and non-conventional yeasts represent a novelty for brewers (Cubillos et al., 2019).

Up to date, the use of honey in beer industry has been only related to the addition as ingredients for wort and/or green beer production, or as food adjuvant to improve sensory profile of bottled beer (Lu et al., 2011).

With this in mind, the present paper focused on yeast ecology of fermented honey niches as novel sources for non-*Saccharomyces* yeasts. Sinacori et al. (2014) find out that *Zygosaccharomyces mellis* and *Zygosaccharomyces rouxii* were isolated from different samples of honey from different geographical and botanical origins. Gaglio et al. (2017) highlighted the presence of different species of non-*Saccharomyces* including *Lachancea fermentati*, *Wickerhamomyces anomalus* and *Zygosaccharomyces* spp., mainly. In the present study, contrarily to the works of Sinacori et al. (2014) and Gaglio et al. (2017), yeast ecology of FHP was mainly represented by isolates ascribable to *H. uvarum*. Actually, the role of this species in brewing system is still uninvestigated. As widely reported in scientific literature, *H. uvarum* is mostly associated to the winery industry. This species is associated with the grape surface and is characterized by the ability to assimilate glucose rapidly (Pretorius,

2000) and for its high production of β-glycosidase (Arévalo Villena et al., 2005; Fia et al., 2005). Several species within *Hanseniaspora* genus have been recently investigated for their ability to improve aroma profile of wine, cider and cachaça (Grijalva-Vallejos et al., 2020).

Regarding to the use of *Hanseniaspora* spp. strains in brewing, so far very limited data have been published. To our knowledge, a few *Hanseniaspora* spp. were isolated from spontaneously fermented Lambic beer (Spitaels et al., 2014), but their role still remains unrevealed. The species *Hanseniaspora valbyensis* and *Hanseniaspora vineae* were screened to evaluate their application for the production of alcohol-free beer (Bellut et al., 2018), and *H. vineae* strain YH72, isolated from ash bark was tested for the ability to acidify wort in sour beer production (Osburn et al., 2018). The description of *Hanseniaspora gamundiae* from sugar-rich fungal stromata of *Cytaria hariotii* highlights the importance of matrices containing high concentrations of carbohydrates to isolate yeasts to be tested in beverage fermentations (Cadez et al., 2019).

Focusing on the technological performances of the five strains of *H. uvarum* of our study, an interesting tolerance to ethanol was observed. Except strain YGA73, all other strains showed growth on medium containing 10% (v/v) ethanol, even though weak. Several species of non-*Saccharomyces* yeasts are not able to grow at this ethanol concentration. In literature it is reported that non-*Saccharomyces* yeasts like *Hanseniaspora* spp. are known as low resistant to ethanol. *Hanseniaspora vineae* (Bellut et al., 2018; Benito et al., 2019; Osburn et al., 2018), for example, does not tolerate ethanol concentrations higher than 4% (v/v), although this trait is not genus or species dependent but strain-dependent (Martin et al., 2018).

Based on these considerations, *H. uvarum* strains selected in this study might be applied as co-starters for producing beer up to 10% (v/v) ethanol. Regarding the cross hop/ethanol resistance, our strains showed a remarkable growth kinetics in presence of high concentration of iso-α-acids and ethanol concentrations. The five strains showed growth phases better than the commercial *S. cerevisiae* strain US-05 used as positive control. These findings indicated that the tested strains possess useful characteristics to be used as co-starters for the production of a wide range of beer worts, including highly hopped worts. The results of FR showed a very low ability of *H. uvarum* strains to drive an AF in beer wort, but plate counts demonstrated the presence of all strains at high levels (7.5–8.2 Log CFU/mL) up to 12 d of wort fermentation. The low FR does not represent a limitation for beer production; several species of non-*Saccharomyces* are characterized by very low fermentation power and/or vigor, but most of them are able to improve the sensory characteristics of fermented beverages such as beer and wine. For example, the strain *Metschnikowia pulcherrima* Flavia® (Lallemand Inc., Montreal, Canada), does not ferment grape sugars into ethanol but, it is one of the most largely used strains in winemaking as co-starter due to its intense production of β-glucosidase which changes the wine aromatic profile by increasing smoky and flowery notes (Rodriguez et al., 2007, 2010; Ruiz et al., 2018; Sadineni et al., 2012; Su et al., 2020). Even *Hanseniaspora/Kloeckera* yeast could affect the wine fermentation results, by modeling flavor profile and the metabolism of *S. cerevisiae* commercial strains, due to a different secondary metabolic pathways and an increased enzymatic activity (Martin et al., 2018).

Multivariate data analysis has been widely applied in food processes (Berrueta et al., 2007) and, recently, extensively applied in beer research (Snauwaert et al., 2016). In our study, an extensive data analysis based on 58 technological variables was performed. Both PCA and HMC analyses were able to find the significant correlation among strains, variable and values confirming their usefulness to underline the technological properties of yeast strains.

Even though in trial T1 a dominance of *S. cerevisiae* populations was registered after 48 h, *H. uvarum* developed at higher cell densities than *S. cerevisiae* in trial T2. When the inoculum of *H. uvarum* and *S. cerevisiae* occurred sequentially (trial T3), the dominance of *H. uvarum* persisted throughout the fermentation process, with the populations of *S. cerevisiae* being below 6.7 Log CFU/mL. In trial T4, despite the

inability of *H. uvarum* to consume the complex sugars of the wort, its levels were close to 7.5 Log CFU/mL.

Actually, it has been not reported maltose fermentation for the *Hanseniaspora* genus and genes for maltose assimilation were not identified in public available genomes of *Hanseniaspora* spp. (Cadez and Smith, 2011; Cadez et al., 2019).

The strain *H. uvarum* YGA34 was able to produce considerable amounts of acetic acid, although at lower concentrations than the control trial, already during the first 48 h of AF. Regarding glycerol production, the trial showing the highest amounts was that involving the sequential inoculation, while no substantial differences among the trials were detected for ethanol production.

Despite the metabolic inability of *H. uvarum* YGA34 strain to assimilate and ferment maltose, the effect of its application during the earlier stage of beer fermentation was highlighted by sensory and VOC analyses.

The most abundant VOC category was alcohols that are known for enhancing flowery, solvent like or alcoholic flavours, however their presence above certain threshold could be undesired (Eßlinger, 2009). The second most abundant VOC class has been represented by esters, whose level depend on the yeast strain-specific activity of synthesis and breakdown enzymes (Pires et al., 2014). The main aroma-active ester was ethyl acetate, a secondary metabolite of alcoholic fermentation, responsible of fruity aroma of beers and whose perception threshold is equal to 25 mg/L. Ethyl hexanoate and isoamyl acetate, which are liable for banana, apple and anise aroma, showed values below the perception threshold (Meilgaard, 1975).

Thus, the use of this unconventional strain for beer production led to a novel product mainly under sensory profiles. *H. uvarum* YGA34 showed a certain degree of dominance also in combination with *S. cerevisiae*. The three different inoculation methods highlighted a good co-existence between the two yeast species that generated three different final products, all without any detectable defect. The organoleptic quality of the experimental beers fermented with *H. uvarum* YGA34 were confirmed by sensory analysis that showed high scores for taste complexity and intensity, aroma intensity, acid, sapid descriptors, as well as overall satisfaction.

5. Conclusions

In conclusion, for the first time, the yeast diversity of FHP, an ancient honey-based beverage produced in Sicily (Italy) was explored for the selection of food starters and provided scientific data on the technological relevance of yeasts from honey and/or FHP for brewing application. This work enriches the very limited scientific knowledge on the role of *Hanseniaspora* yeasts as potential co-starter for beer production. Throughout a genotypic and phenotypic polyphasic approach it was possible to identify and characterize five *H. uvarum* strains. For the first time a high resistance to ethanol and hop in beer wort has been reported for *H. uvarum* strains. The application of these strains during brewing showed differences in terms of physico-chemical parameters, VOCs and sensory traits indicating that *H. uvarum* strains are promising as co-starter in a wide range of beer productions. However, further investigations are needed to evaluate the role of these strains during wort fermentation in presence of different strains of *S. cerevisiae* and with different beer wort composition.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fm.2021.103806>.

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