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Evaluation of non-*Saccharomyces* yeasts in the fermentation of wine, beer and cider for the development of new beverages

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Non-*Saccharomyces* yeasts were evaluated for their fermentation properties and the production of pleasant fruity aromas in three industrial media (beer, wine and cider). A total of 99 yeasts were screened for aroma production using a simple olfactory plate assay. Of these, 21 yeasts were further evaluated for their aroma profile and fermentation capacity using wort, grape and apple juice. The most promising yeasts were *Galactomyces geotrichum* (three strains), *Kazachstania zonata*, *Kluyveromyces lactis*, *Lindnera meyerae*, *Pichia kluyveri*, *Starmera caribae*, *Yarrowia lipolytica* and *Saccharomycodes ludwigii*. This study confirms the potential of non-conventional yeasts to produce pleasant aroma compounds under relevant fermentation conditions. In general, differences in the medium composition had less impact on the overall aroma profile than the choice of yeast. This study is the first to simultaneously evaluate multiple non-conventional yeasts in three different industrial media. The results reported here are a good starting point for the development of novel fermented beverages. © 2018 Carlsberg. *Journal of the Institute of Brewing* published by John Wiley & Sons Ltd on behalf of The Institute of Brewing & Distilling

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Introduction

Saccharomyces cerevisiae is the primary microorganism responsible for alcoholic fermentation. Through fermentation it transforms sugar into ethanol and carbon dioxide together with the production of metabolites that contribute to the sensorial properties of the product *(1–4)*. In the past, spontaneous fermentation was practised and characterised by non-*Saccharomyces* yeasts during the early stages of the fermentation, while *Saccharomyces* species dominated the later stages of the fermentation owing to the increased ethanol concentration *(5,6)*. Today, the inoculation of pure yeast cultures provides better control of the process, ensures a rapid and complete fermentation and, as a result, improves the reproducibility of the final product and gives a consistent quality *(5,7)*. However, this practice limits flavour complexity and does not produce the unique aromas and character generated from diverse strains and species. Accordingly, brewers are returning to spontaneous fermentation by local microflora in an attempt to introduce more character and complexity in their products *(8)*.

Non-conventional yeasts represent a large untapped reservoir, accounting for more than 1500 known species *(9)*, with a huge potential for industrial application. Recently, there has been increasing interest in the use of non-conventional yeasts in the beverage sector owing to their unusual properties and contributions of specific metabolites. For instance, they are exploited for their ability to produce lower ethanol yields *(10,11)* and to divert carbon metabolism away from ethanol production *(12,13)*. The increase in glycerol levels during fermentation, which contributes to the smoothness and complexity in wines, is also shown in several non-*Saccharomyces* strains *(14–16)*. Another well documented attribute is the range of aromatic compounds produced by these non-conventional yeasts *(17–23)*. The metabolic products resulting from their growth include higher alcohols, esters, acids, aldehydes, ketones, terpenoids and phenols. Such non-conventional yeasts are notable for the production of new aroma compounds, high concentration and novel combinations of flavours that add a unique character *(22–24)*. Owing to these traits, non-conventional yeasts may be applicable in new food products.

Many variables are known to affect aroma synthesis, including the yeast strain, the composition of the medium and the culture conditions *(25,26)*. Although the yeast strain is one of the most important factors, the sugar and nitrogen content of the medium also impact on the final aroma profile *(25,27)*. The total sugar concentration and the relative content of different fermentable sugar sources have an influence on the ester production. For instance, in brewing, fermentation of high-gravity worts can lead to an unbalanced flavour profile because of the overproduction of acetate esters *(25,28)*. Likewise, higher nitrogen concentrations in grape must are positively correlated with a higher concentration of acetate and ethyl esters *(29,30)*. Furthermore, the composition and

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combination of nitrogen sources available in the medium should be considered *(31–33)*.

Many studies with non-*Saccharomyces* yeast strains have been performed to determine the aroma profile after fermentation. Most use one medium, evaluate a few strains at the same time and focus on co-fermentation approaches with selected combinations of yeasts from the wine industry *(34–36)*. Recent screening projects *(23)* have characterised the formation of volatile compounds by a large number of non-conventional yeast strains. However, the traditional laboratory media used in these screenings are very different in composition from wort and fruit juices used in the production of beers and wines and do not reflect the production reality of different media compositions. To simulate real fermentation media and to study the impact of medium variation, this study presents for the first time a screening of non-conventional yeasts with the comparison of aroma production and fermentation properties between three different industrial media used for production of wine, beer and cider. The aims of the present study were (a) to identify yeast strains with interesting fruity or pleasant aroma features, (b) to determine the effect of the industrial medium on the aroma production and the fermentation performance by each yeast and (c) to compare the physiological behaviours and relationships between carbon/nitrogen assimilation of different yeast strains during alcoholic fermentations in the three different fermentation processes.

Materials and methods

Yeast strains

The strains used in this study are reported in Table 1. These non-*Saccharomyces* strains were obtained from CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands), Department of Cell and Organism Biology (Lund University, Sweden) and Carlsberg Research Laboratory (CRL; Copenhagen, Denmark). The yeast strains, belonging to different genera and isolated from different origins, were selected to represent yeast biodiversity. However, food origin was the primary consideration to ensure an appropriate biosafety level for consumption and food production *(8)*. Three reference strains, Lalvin EC1118 (Lallemand Inc., Montreal, Canada), Weihenstephan lager yeast W34/70 (Weihenstephan Institute, Freising, Germany) and English Cider Yeast WLP775 (White Labs, San Diego, CA, USA), were used as controls for each relevant medium type. All yeast strains were kept in YPD (1% *w/v* yeast extract, 2% w/v peptone, 2% w/v glucose) at 25°C.

Fermentation media

Glucose wort, grape juice and apple juice were used in order to mimic, simulate and evaluate the performance of non-conventional yeasts in three relevant beverage industries: brewing, wine making and cider making. As would be expected, the three media differed in their levels of sugar, nitrogen, salts and minerals (Table 2), which were analysed as described in the Materials and Methods section.

Glucose wort was provided by the Carlsberg Research Laboratory. Unhopped wort (14.5°P, pH 5) was prepared with 100% malted barley. This wort was enzymatically treated to convert the maltose to glucose and obtain better growth of the non-conventional strains, as many non-conventional yeasts cannot utilise maltose in worts under fermentative conditions. The glucose wort was kept at −20°C and was pasteurised at 80°C for 30 min before use.

Organic grape and apple juices were purchased at Irma's supermarket (Irma.dk). Grape juice (Biotta 100% pure organic red grape juice), squeezed from fresh and sun-ripened organic grapes (pH 3.5), and apple juice from selected varieties of Danish apples (Cox, Elstar and Jonagored), cold-pressed and unfiltered (pH 3.6), were already pasteurised and were used for fermentation under sterile conditions.

Agar plate screening for aroma production

Solid agar plates were prepared from the three industrial media to perform an initial aroma screening by a smelling or 'sniffing' approach. To make the solid media, 20 g/L agar was added and dissolved into pre-heated glucose wort, grape juice and apple juice at 80°C, then poured into Petri dishes. All non-conventional strains were streaked onto the agar plates and incubated at room temperature for 2–7 days. Plates inoculated with the different yeast strains were subjected to sensory analysis by a trained panel ($n = 4$). The aim of this screening was to identify diverse aroma compounds typically found in fermented beverages as desired compounds or off-flavours, or simply strains characterised by a strong and novel aroma profile. Plates were 'sniffed' directly and classified as: 'pleasant aroma', 'unpleasant aroma' or 'no growth'. Additionally, 'pleasant aroma' was ranked as 'intermediate' or 'strong' depending on the level of perception. 'Unpleasant aroma' was classified as 'phenolic' or 'acetic' as both are common undesirable compounds produced by non-conventional yeasts.

Laboratory scale fermentations

Yeasts were pre-cultured in 50 mL of YPD medium in 250 mL flasks with continuous shaking (150 rpm) for 48 h at room temperature. The cells were harvested by centrifugation at 1900 ***g*** for 10 min and resuspended in 10 mL of fresh fermentation medium (glucose wort, grape juice or apple juice). They were inoculated at ~1×10^7 viable cells/mL into fermentation cylinders. Cell viability was determined using Guava ViaCount Flex on a Guava ViaCount flow cytometer (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Fermentations were performed in 250 mL tall glass measuring cylinders (331 × 39 × 39 mm; Duran) containing 200 mL of fermentation medium and sealed with an inverted glass beaker (Duran) on the top of the cylinder to enable escape of carbon dioxide and easy sampling access for analyses. Fermentations were performed in duplicate with continuous stirring at 150 rpm using a magnetic bar on a stirring platform at 25°C. Fermentation performance was monitored by measuring the weight loss determined by CO_2 evolution. Samples were collected at the beginning and at the end of fermentation. Supernatant, obtained by centrifugation at 1900 ***g*** for 10 min, was stored at −20°C to analyse the concentration of fermentable sugars, ethanol, glycerol, amino acids and aroma compounds. Fermentation was completed when sugars were consumed and no further weight loss could be measured. Fermentation trials with strains unable to end-ferment were harvested after 15 days and compared with the reference control strains.

Metabolite analysis

Fermentable sugars (fructose, glucose, sucrose, maltose and maltotriose), ethanol and glycerol were determined by high-performance liquid chromatography (HPLC) using a Waters

Table 1. List of strains used in this study. Strains were classified depending on aroma profile in the agar plate 'sniff' assay. Strains highlighted in bold were selected for subsequent fermentation analysis owing to their strong and pleasant aroma.

Aroma	Strain	Taxon name	Substrate	Origin
P^{++}	**CBS 9164**	***Arthroascus schoenii***	**Insect**	**Mexico**
P	NRRL YB-4302	*Arxiozyme telluris*	Soil	South Africa
P^{+}	NRRL Y-1056	*Ashbya gossypii*		
—	CBS 9650	*Auriculibuller fuscus*	Plant	Portugal
P^{+}	CBS 5033	*Barnettozyma californica*	Plant	Unknown
P^{++}	**CBS 8860**	***Barnettozyma californica***	**Fruit**	**Russia**
up	CBS 6335	*Blastobotrys parvus*	Sea	Antarctica
up	CBS 9043	*Botryozyma mucatilis*	Animal	USA
up	CBS 5207	*Brettanomyces custersianus*	Beer	South Africa
—	CBS 8347	*Brettanomyces custersianus*	Fruit	Netherlands
—	CBS 6459	*Bullera dendrophila*		Unknown
—	CBS 2774	*Candida blankii*	Damp pulp	Sweden
up	CBS 4332	*Candida castellii*	Soil	Finland
P^{+}	CBS 1750	*Candida etchellsii*	Food	USA
P	NRRL Y-65	*Candida glabrata*	Man	
P	NRRL Y-17074	*Candida humilis*	Beer	South Africa
P^{+}	NRRL Y-7244	*Candida humilis*	Dough	USA
P^{++}	**NRRL Y-7245**	***Candida humilis***	**Dough**	**USA**
up	NRRL Y-7246	*Candida humilis*	Dough	USA
P^{++}	**NRRL Y-7248**	***Candida humilis***	**Dough**	**USA**
P^{+}	CBS 2875	*Candida membranifaciens*	Tannin	Spain
P	CBS 2016	*Candida rugosa var. rugosa*	Animal	UK
up	CBS 1925	*Cryptococcus albidus var. albidus*	Beer bottle	Sweden
up	CBS 8796	*Cryptococcus laurentii var. laurentii*	Man	Belgium
P	CBS 9463	*Cystofilobasidium capitatum*	Soil	USA
up	CBS 2330	*Debaryomyces fabryi*	Sake	Japan
ua	CBS 4210	*Dekkera anomala*	Cider	Unknown
ua	CBS 77	*Dekkera anomala*	Beer	UK
ua	CBS 2796	*Dekkera bruxellensis*	Wine	Germany
up	CBS 6714.1	*Dioszegia crocea*		Unknown
—	CBS 8282	*Fellomyces borneensis*	Lichen	Malaysia
—	CBS 7635	*Filobasidium floriforme*	Plant	USA
P^{++}	**CBS 469.93**	***Galactomyces geotrichum***		**Unknown**
P^{++}	**CBS 466.93**	***Galactomyces geotrichum***		**Unknown**
P^{++}	**CBS 772.71**	***Galactomyces geotrichum***	**Soil**	**Puerto Rico**
—	CBS 152.25	*Geotrichum fragrans*		Unknown
up	CBS 6321	*Guehomyces pullulans*	Man	Unknown
—	CBS 9456	*Hannaella oryzae*	Soil	USA
P^{+}	CBS 2567	*Hanseniaspora guilliermondii*	Juice	Israel
P	CBS 8031	*Hanseniaspora vineae*	Plant	Canada
ua	CBS 379	*Kazachstania exigua*		Unknown
P	CBS 10400	*Kazachstania gamospora*		Japan
P^{+}	NRRL Y-17920	*Kazachstania spencerorum*	Soil	South Africa
P^{++}	**CBS 10399**	***Kazachstania zonata***		**Japan**
P^{++}	**NRRL Y-10934**	***Kluyveromyces blattae***	**Insect**	
P^{++}	**CBS 2359**	***Kluyveromyces lactis***	**Dairy product**	**USA**
P	Y1058	*Kluyveromyces marxianus*	Kefir grains	France
ua	CBS 243	*Kregervanrija fluxuum*	Juice	Italy
P^{++}	**CBS 4728**	***Lanchancea thermotolerans***	**Fruit**	**Czechoslovakia**
P^{++}	**CBS 6292**	***Lanchancea thermotolerans***		**Australia**
—	CBS 9209	*Leucosporidiella fragaria*	Soil	Iceland
P^{++}	**CBS 7076**	***Lindnera meyerae***	**Plant**	**South Africa**
—	CBS 7027	*Lindnera mississippiensis*		Unknown
P^{+}	CBS 4148	*Lindnera misumaiensis*	Cider	UK
P	CBS 5841	*Lipomyces lipofer*		Unknown

(*Continues*)

Table 1. *(Continued)*

Aroma	Strain	Taxon name	Substrate	Origin
up	CBS 10300	*Lipomyces orientalis*	Soil	Vietnam
up	CBS 8728	*Lipomyces starkeyi*	Soil	Madagascar
—	CBS 7219	*Myxozyma geophila*	Soil	South Africa
P	CBS 4310	*Naumovia castellii*	Soil	Finland
P^{+}	CBS 4309	*Naumovozyma castellii*	Soil	Finland
P^{+}	CBS 6513	*Ogataea methanolica*	Soil	Japan
—	CBS 744	*Ogataea pini*	Insect	USA
up	CBS 7122	*Pichia deserticola*	Cactus	Haiti
up	CRL	*Pichia haplophila*		
up	CBS 6013	*Pichia holstii*	Soil	Unknown
P^{++}	**CRL**	***Pichia kluyveri***		
up	CBS 2284	*Pichia mandshurica*	Plant	USA
P^{++}	**IFO 10545**	***Pichia phylogasa***	**Soil**	**South Africa**
P	CBS 5254	*Priceomyces carsonii*	Alpech	Spain
up	CBS 9071	*Rhodosporidium babjevae*	Grain	USA
up	CBS 321	*Rhodosporidium kratochvilovae*	Air	Japan
up	CBS 9075	*Rhodosporidium sphaerocarpum*	Water	Bahamas
P^{++}	**CBS 7780**	***Saccharomycodes ludwigii var. ludwigii***	**Drink**	**Belgium**
ua	CBS 6806	*Sirobasidium magnum*	Plant	Unknown
P^{++}	**CBS 7692**	***Starmera caribae***	**Plant**	**West Indies**
P	CBS 6945	*Starmera pachycereana*		Unknown
P	CBS 7288	*Sterigmatomyces elviae*	Food	Japan
up	CBS 5657	*Sugiyamaella smithiae*	Insect	South Africa
P^{+}	NRRL Y-27309	*Tetrapisispora iriomotensis*	Soil	Japan
P^{+}	NRRL Y-866	*Torulaspora delbruecki*		Unknown
up	IFO 1160	*Torulaspora globosa*	Soil	West Indies
P^{++}	**CBS 6641**	***Torulaspora microellipsoides***	**Plant**	**USA**
—	CBS 8212	*Tremella brasiliensis*		Costa Rica
—	CBS 8207	*Tremella encephala*	Wood	Unknown
—	CBS 8472	*Tremella flava*	Plant	Taiwan
P^{+}	CBS 4878	*Trigonopsis cantarellii*	Juice	South Africa
P	CBS 4095	*Trigonopsis variabilis*	Fruit	Brazil
P^{++}	**CBS 5552**	***Wickerhamomyces subpelliculosus***	**Sugar**	**Unknown**
—	CBS 11316	*Xanthophyllomyces dendrorhous*		Unknown
P^{+}	CBS 6306	*Yamadazyma mexicana*	Insect	South Africa
P^{++}	**DSM 3286**	***Yarrowia lipolytica***	**Fuel**	
P	CBS 8625	*Zygoascus hellenicus*		Unknown
P^{+}	DSM 70492	*Zygosaccharomyces bailii*	Apple juice	
P	DSM 70834	*Zygosaccharomyces bailii*	Flour	
P	CBS 5681	*Zygosaccharomyces bailii var. bailii*	Wine	Unknown
up	NRRL Y-1560	*Zygosaccharomyces florentinus*	Grape must	Italy
P^{+}	CBS 8849	*Zygosaccharomyces kombuchaensis*	Fungi	Russia
up	NRRL Y-12654	*Zygosaccharomyces mrakii*	Silage	Italy
P^{++}	**CBS 9716**	***Zygosaccharomyces rouxii***	**Insect**	**Germany**

P, Pleasant aroma (+) intermediate (++) strong.
u, Unpleasant aroma (p) phenolic (a) acetic.
—, No growth

Alliance 2695, equipped with a Waters 2414 refractive index detector. Sample (10 μL) was injected into an Asahipak NH_2P-50-4E (Shodex™) with a particle size of 5 μm (250 × 4.6 mm) and the thermostat was set at 40°C. The mobile phase used was acetonitrile 730/H_2O 270 with a flux of 1 mL/min. The concentration of each metabolite was calculated using external standards and expressed as g/L (ethanol was expressed as % *v*/v).

Fifteen amino acids were analysed further to a derivatization step (fluoraldehyde OPA) followed by HPLC using the Agilent 1100 series HPLC equipped with UV detector (338 nm). The sample (0.5 μL sample + 20 μL fluoraldehyde OPA) was injected into a Hypersil ODS column with a particle size of 5 μm (100 × 2.1 mm) and the thermostat was set at 40°C. The composition of the mobile phases consisted of 0.1 mol/L sodium acetate, adjusted to pH 8 (A) and 100% methanol (B). The separation was carried out

Table 2. Media composition of glucose wort, grape juice and apple juice

	Glucose wort	Grape juice	Apple juice
Sugars (g/L)			
Fructose	3.1	83.4	82.1
Glucose	116.8	80.4	30.3
Sucrose	4.6	0	7.7
Maltose	3.7	0	0
Amino acids (mg/L)			
Aspartic acid	80	35	154
Glutamic acid	130	60	72
Serine	83	43	29
Histidine	56	39	4
Glycine	44	9	2
Threonine	86	52	10
Arginine	142	610	6
Alanine	132	75	16
Tyrosine	125	16	3
Methionine	45	3	3
Valine	156	26	10
Phenylalanine	155	16	5
Isoleucine	78	12	4
Leucine	187	25	6
Lysine	99	16	2
Minerals (mg/L)			
Calcium	36.4	147.3	27.9
Magnesium	106.3	90.2	37.7
Sodium	17.9	36	29.6
Potassium	785	1138	1184
Zinc	0.4	0.84	1.04
Copper	0.08	1.12	0.1
Iron	0.22	2.62	0.12
Aluminium	0	3.34	0.01
Manganese	0.08	1.16	0.31
Phosphorus	528.2	143	74
Silica	21	18.3	0.4
Chloride	292	31.7	11.7
Sulphate	47.2	204	47.9
Nitrate	0.6	11.2	1.5
Free oxalic acid	26.9	28.7	4.8
Phosphate	1299.3	352.5	163.9

in 21 min, under the following conditions: linear gradient starting at 5% B to 15% B in 5 min, to 25% B in 10 min, to 35% B in 13 min, to 55% B in 18 min, to 25% B in 20 min, and finally to 5% B in 21 min. The flow rate was 0.35 mL/min. The concentration of each amino acid was calculated using external standards and expressed as mg/L.

Aroma compounds

Volatile compounds were measured using a Thermo Scientific TSQ Quantum GC Triple Quadrupole GC/MS. An internal standard 2-octanol was added to each sample. This compound is not present in beer/wine/cider fermentations. Headspace aqueous solutions were prepared in 20 mL vials by mixing 1 mL of sample with 3.85 mL of deionised water, 2.2 g of sodium chloride, 0.1 g of ascorbic acid and 25 μL of internal standard. All samples were incubated for 10 min at 50°C. The volatile compounds were collected on divinylbenzene/carboxen/polydimethylsiloxane fibre (DVB-CAR-PDMS) for an extraction time of 40 min. A Solgel-wax column, 30 m/i.d. 0.25 mm/film 0.25 μm, was used for all analyses.

The oven was at 40°C for 4 min then the temperature was increased by 6°C/min to 250°C and maintained at this final temperature for 5 min. The injector and interface temperature were kept at 250°C. Helium was used as the carrier gas with a flow rate of 1.2 mL/min. The time for thermal desorption of analytes was 4 min. The MS detector was operated in full-scan mode at 70 eV and the scan range was from 35 to 350 *m/z*.

Data analysis was performed using the software ThermoExcalibur (version 1.0.1.03, Thermo Scientific). Identification of compounds was based on comparison with a mass spectral database (Nist 1.0.0.23). One characteristic quantifier ion and two to three qualifier ions were selected for each compound. The peak area of the quantifier ion was used for quantification. The concentration of each aroma compound was expressed as 2-octanol equivalents (μg/L).

Phenotype microarray

Phenotype microarray (PM) (Biolog, USA) was used to map differences in carbon and nitrogen assimilation of selected yeast strains. The Biolog system is based on redox technology, with cell respiration (NADH production) as a universal reporter. The wells contain a sensitive tetrazolium dye which is reduced when cells respire actively, resulting in the production of a purple colour (www.biolog.com). Biolog microplates PM1–PM2 (carbon sources) and PM3 (nitrogen sources) were used. Each commercially available PM microplate contains 95 test wells and one control well. The inoculum was prepared by suspending yeast cells from YPD plates in sterile distilled water to give 62% transmittance (%*T*) with the Biolog turbidimeter. Cell suspensions for the inocula in PM1–PM2 were prepared by mixing 500 μL of these cells, 20 mL of IFY-0 base (Biolog, USA), 320 μL dye mix D, 2 mL of PM additive (60 mmol/L L-glutamic acid monosodium, 60 mmol/L potassium phosphate pH 6.0 and 24 mmol/L sodium sulphate) and sterile water to adjust to a final volume of 24 mL. For PM3, 250 μL of cells were mixed with 10 mL of IFY-0 base (Biolog, USA), 160 μL dye mix D, 375 μL of D-glucose (3.2 M), 1 mL of PM additive (60 mmol/L potassium phosphate pH 6.0 and 24 mmol/L sodium sulphate) and sterile water to adjust to a final volume of 12 mL. A 100 μL aliquot of standardised cell suspension was inoculated into each well of a microplate, allowing simultaneous testing of different phenotypes in a single experiment.

Incubation and data recording were performed automatically at 25°C for 8 days. Digital images of microplates were captured at 15 min intervals to measure dye conversion, correlated with metabolic rate. On completion, the data was compiled and exported using Biolog software (File management/Kinetic plot and Parametric analysis version 1.6 build 21).

Statistical analysis

A heatmap of aroma compounds was used to reduce the dimensionality of the data and to find the best differentiation between yeast strains and media. The data obtained was converted to *Z*-scores to easily visualise which yeast strains are relevant aroma producers in relation to average production. *Z*-Scores were calculated as follows: $Z\text{-score} = (X - \mu)/\sigma$, where X is the concentration of the aroma functional group, μ is the mean value of all strains

per measured aroma functional group and media and σ is the standard deviation of values per tested aroma functional group and media of all strains. Hierarchical clustering was performed using MeV MultiExperiment Viewer (www.tm4.org), and Pearson correlation metrics and group clustering based on group averages (average linkage).

Two-way analysis of variance (ANOVA) was carried out with the Statistica 7.0 software to determine the influence of the media composition on aroma production.

Results and discussion

Large-scale screening of non-conventional yeasts for aroma production

A total of 99 non-conventional yeast strains, isolated from different natural niches and covering about 50 genera (Table 1) were screened for their ability to produce a pleasant and fruity aroma in three relevant fermentation media. First, the aroma production capacity was determined by the olfactory 'sniff' test on plates of glucose wort, grape must and apple juice. A wide range of aroma compounds from the yeasts were grouped and classified into three categories: no growth, unpleasant and pleasant aroma (Table 1). Unpleasant aroma was mainly characterised by phenolic notes, diacetyl (buttery), acetic or strong solvent-like aroma. Of the tested strains, 54% produced a pleasant aroma, distinguished by fruity, floral and/or sweet smells with different intensities (weak, intermediate or strong). The most promising genera were *Galactomyces*, *Kluyveromyces*, *Lanchancea* and *Lindnera*. The three *Galactomyces geotrichum* strains (CBS 469.93, CBS 466.93, CBS 772.71) produced a strong fruity aroma (passion fruit, pineapple, and banana) in glucose wort and grape juice agar plates but it was almost undetectable on apple medium. The *Kluyveromyces* sp., especially *Kluyveromyces lactis* CBS 2359, was distinguished by a strawberry fragrance with buttery/diacetyl notes. The *Lanchancea* and *Lindnera* yeasts were also fruity with a touch of sweet/honey/floral/marzipan fragrance. Of the strains identified with a pleasant aroma, 37% were isolated from food sources, whereas 43% originated from natural environments. In general, yeasts belonging to the taxonomic families of *Dekkera*, *Rhodosporidium* and *Cryptococcus* produced unpleasant flavours, such as acetic acid and phenol notes. *Pichia* spp. were characterised by producing phenolic-like notes and solvent smell, with the exception of *Pichia kluyveri*, with a strong and pleasant aroma of banana fruit. Curiously, strains belonging to the genus *Candida*, the anamorph of some *Pichia* spp., had a sweet/honey aroma. The *Zygosaccharomyces* yeasts were identified as pleasant aroma producers, with a few exceptions (*Z. florentinus* and *Z. mrakii*).

The 'sniff' assay provides a simple method where many yeast strains, media and different conditions can be easily analysed, thus avoiding time consuming, laborious and costly fermentation and aroma analyses. In addition, it could be used directly to select yeast strains adjusted to the preferences of winemakers/brewers/cidermakers.

In order to assess the diversity in aroma production of relevant compounds for fermented beverages, 21 strains were further selected for a more comprehensive evaluation by testing their performance in laboratory scale fermentation trials (strains highlighted in bold in Table 1). Strains were inoculated in three different fermentation media (Table 2) and the aroma profile measured at the end of fermentation. As controls, three yeast strains were used, each one characteristic for a fermentation medium: W34/70 (beer), EC1118 (wine) and WLP775 (cider). In total 80 volatile compounds were analysed, including 19 higher alcohols, seven acetate esters, 21 ethyl esters, seven volatile acids, 10 aldehydes, eight ketones, two pyrazines, four monoterpenes and three volatile phenols. In order to compare the contribution of yeast (and remove the media effect) *Z*-scores were calculated for each fermentation medium. As an overview, Fig. 1 shows the aromatic profile of all tested non-*Saccharomyces* strains per volatile functional group, revealing interesting trends. For instance, high levels of ethyl esters and volatile acids were produced by the control strains, in addition to *Yarrowia lipolytica* and two *G. geotrichum* (CBS 469.93, CBS 466.93). Whereas the control strains produced a mixture of these compounds, *Y. lipolytica* and *G. geotrichum* were characterised as producing esters such as ethyl decanoate, ethyl hexanoate and ethyl octanoate (Fig. 2a), and high levels of their respective hexanoic and octanoic acids precursors (Fig. 2b). Furthermore, *G. geotrichum* strains were uniquely capable of synthesising ethyl butanoate (Fig. 2a), a compound distinguished by fruity notes. The most notable production of acetate esters was produced by *K. zonata*, *K. lactis*, *P. kluyveri*, *S. caribae* and *L. meyerae* (Fig. 1). *K. zonata*, *P. kluyveri* and *K. lactis* were the highest phenylethyl acetate (rose, floral) producers (Fig. 2c). Remarkable amounts of butyl, hexyl and isoamyl acetate (banana, pear) were produced by *P. kluyveri*, *S. caribae* and *L. meyerae*. Similarly, ethyl butanoate by *G. geotrichum*, isobutyl acetate with a banana/fruity aroma was only produced by *S. caribae* (Fig. 2c). Ketones were produced by *Wickerhamomyces subpelliculosus* and *Candida humilis* NRRL Y-7245. Notably, *W. subpelliculosus* was the only yeast characterised by a strong phenolic profile, despite the fact that phenolic yeasts were discarded early in the olfactory screening assay, suggesting that strong fruity notes can mask phenolic aroma notes (Fig. 1). The right side of Fig. 1 shows the strains described by aldehyde production, such as *Lanchancea thermotolerans* and *C. humilis*. *K. zonata* was distinguished by releasing monoterpene. These exceptional aroma properties provided by specific non-conventional yeast strains open up a wide range of opportunities in the bioflavour field.

Media effect on yeast aroma production

To study the impact of media composition on aroma production, 10 yeast strains were chosen for further study, including three strains of *G. geotrichum* and single strains of *K. zonata*, *K. lactis*, *L. meyerae*, *P. kluyveri*, *S. caribae* and *Y. lipolytica*. *Saccharomycodes ludwigii* was also included for further study owing to its improved fermentation performance in beer compared with wine and cider (although the yeast did not overproduce any specific aromatic compound).

As shown in Fig. 2, strains characterised by certain volatile compounds in a specific fermentation medium displayed a similar trend in the other media. However, glucose wort, grape must and apple juice have different compositions (Table 2) which can impact on the aroma profile. For instance, phenolic compounds and pyrazines were more abundant in beer, whereas monoterpenes were more abundant in wine samples. This is linked to the availability of precursors in the fermentation medium. For example, malts used in beer production contain ferulic acid that can be converted to phenolic 4-vinyl guaiacol by yeasts expressing phenolic acid decarboxylases. Moreover, higher concentrations of esters and acids were produced in beer than wine and cider.

To determine the influence of the medium on yeast aroma production, control strains were used to normalise the data and

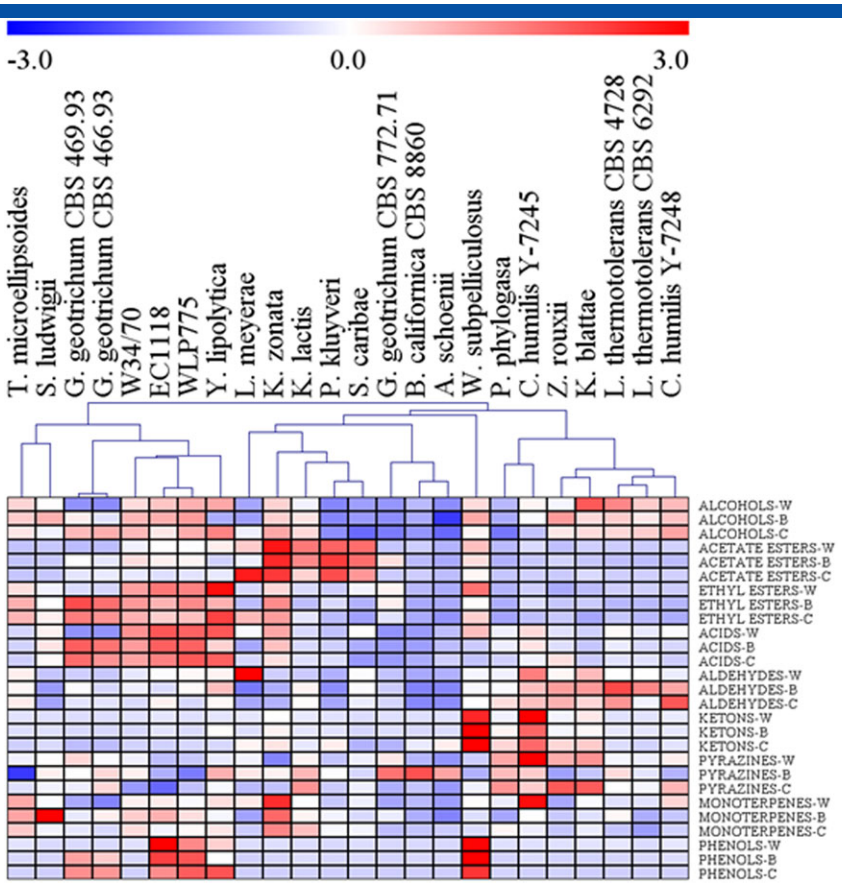

Figure 1. Overview of the aroma production of selected non-conventional strains in wine (W), beer (B) and cider (C) fermentations, together with their corresponding control strains. Colours represent the range of calculated *Z*-scores (calculated over the rows and per each fermentation medium), with blue indicating lower than average production, white indicating average production and red indicating higher than average production of the aroma functional group. Clustering of non-conventional yeast strains was performed on the basis of all the aroma functional groups and using a Pearson correlation coefficient. [Colour figure can be viewed at wileyonlinelibrary.com]

a two-way ANOVA analysis was performed (Table 3). When all of the aroma compounds were used for the analysis of each strain, the medium did not show a statistically significant impact in the majority of strains (Table 3). Therefore, these yeast strains had a greater impact on aroma production than the media composition. However, when the compounds were analysed separately, some were affected by the media (Table 3). For instance, propanol and butanol, two higher alcohols that yield solvent-like aroma, were significantly affected in cider (even if the values were normalised to the controls). In addition, cider also influences the production of fruity aroma flavours such as butyl acetate, ethyl butanoate and butyric acid. In beer, both isobutyl acetate and hexyl acetate stood out. This data reveals that there are aroma compounds more sensitive to their synthesis depending on the composition of the media, such as butanol, butyric acid and their respective esters. Previous work *(23)* identified non-conventional yeasts with potential for aroma production in food fermentation. However, such a study with laboratory media rather than industrial media did not consider the influence of the media composition on the final aroma formation. This procedure may have a subtle impact on the concentrations and ester profiles, which is important for the aroma complexity and balanced notes in the final product.

Fermentation contribution of non-*Saccharomyces* yeasts

To investigate fermentation activity of the selected strains, fermentation kinetics were monitored, and residual sugar, ethanol and glycerol concentration were measured at the end of the fermentation (Table 4). Grape must generated the highest carbon dioxide production together with highest ethanol and glycerol formation owing to the initial high levels of fermentable sugars. In glucose wort, the fermentation time was the shortest. This was expected as glucose wort showed the highest initial amino acid concentration and the highest utilisation of amino acids during fermentation. Apple juice supported the slowest fermentation rate, resulting in the lowest ethanol formation.

As might be expected, the fermentation kinetics of the non-conventional yeasts were typically lower than the control strains, but two groups were clearly identified: fermentative and non-fermentative strains (Table 4). *K. zonata*, *K. lactis*, *Y. lipolytica* and *S. ludwigii* were identified as fermentative strains as they consumed >80% of the sugars from the media. *K. zonata* and *K. lactis* were the most similar in fermentation performance to the control strains. This suggests that *K. zonata* could be used as a pure culture in fermentation of high alcohol beverages (adding its floral-rose note) and that *K. lactis* could be used as a pure culture or for mixed fermentation producing a strawberry-honey like beverage. *G. geotrichum* CBS 772.71 and *S. caribae* presented the lowest fermentation activity for all three media tested. The use of these non-fermentative yeast strains in mixed fermentations with *S. cerevisiae* may enhance aroma complexity. Alternatively, *G. geotrichum* CBS 772.71 and *S. caribae* as pure cultures may be interesting for the development of non-alcoholic beverages with fruity notes. *G. geotrichum* CBS 772.71 was the only non-ethanol

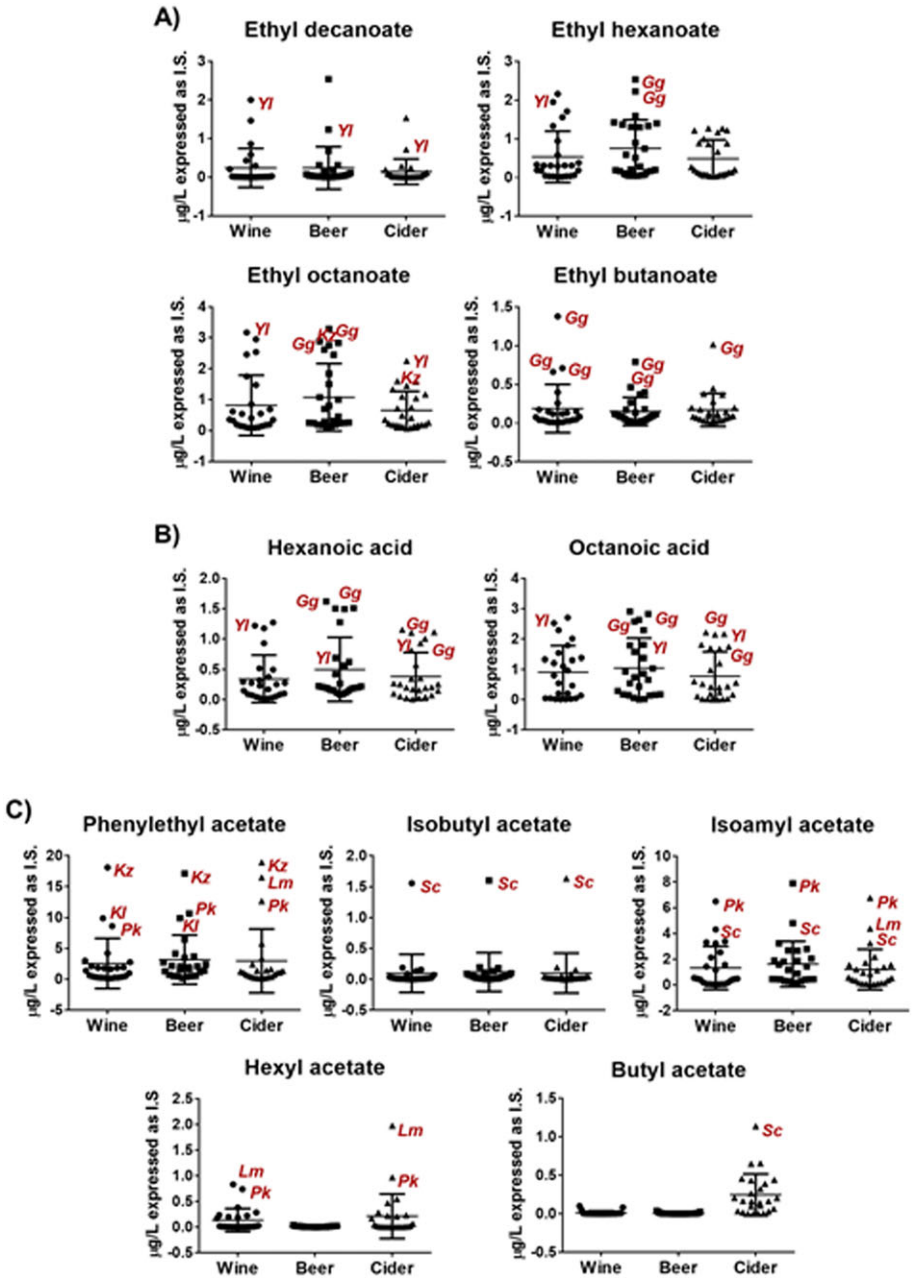

Figure 2. Aroma production of non-conventional yeast strains (pleasant) in wine, beer and cider fermentations. (a) Ethyl esters; (b) acids; and (c) acetate esters. Strains with significant levels of production are highlighted in red: *Galactomyces geotrichum* (Gg), *Kazachstania zonata* (Kz), *Kluyveromyces lactis* (Kl), *Lindnera meyerae* (Lm), *Pichia kluyveri* (Pk), *Starmera caribae* (Sc) and *Yarrowia lipolytica* (Yl). IS, Internal standards. Figure is reproduced in colour in online version. [Colour figure can be viewed at wileyonlinelibrary.com]

producer (Table 4), presenting an appropriate profile to develop low-alcoholic beverages with passion fruit aroma using, for example, cold contact as a bioflavouring strategy.

As would be expected, sugar utilisation and ethanol production were positively correlated (Fig. 3a). However, glycerol did not display the same profile (Fig. 3b). Although the trend was similar to that for ethanol production, some strains were more efficient at forming glycerol than the control strains. The most notable strain was *K. zonata*, which produced higher glycerol yields than the controls, using the same amount of sugars under the three fermentation conditions (Fig. 3c; Table 4). This could reflect enhanced glycerol metabolism, for example, high expression of glycerol-3-phosphate dehydrogenases. *S. ludwigii*, *K.lactis* and *P. kluyveri* also outperformed in glycerol production in beer fermentation (Fig. 3c; Table 4). Additionally, some of the non-fermenting strains showed lower consumption of sugar but were more efficient at producing glycerol (Table 4).

The effect of each yeast strain in the fermentation performance was greater than the influence of the media, as was also observed for the aroma production. However, some strains exhibited different fermentation profiles depending on the medium used (Fig. 3d; Table 4). For instance, *S. ludwigii* and *G. geotrichum* 466 and 469 showed lower fermentation capacity in grape juice than the other media. The performance of *S. ludwigii* in this study is contrary to

Table 3. Significant differences for volatile compounds on media composition for each non-conventional yeast strain obtained by a two-way ANOVA

	Strain	S. ludwigii	K. zonata	K. lactis	Y. lipolytica	P. kluyveri	L. meyerae	S. caribae	*G. geotrichum* 772.71	*G. geotrichum* 469.93	*G. geotrichum* 466.93
	Media	*				*					
Higher alcohols	Butanol	**	**	**	**	**	***	**	*	**	***
	Isoamyl alcohol	***									
	Propanol		***	***	***	***	***	***	***	***	***
	1-hexanol	**									
	2-phenylethanol	**									
Acetate esters	Butyl acetate	***	**			*	**				
	Ethyl acetate	**									
	Hexyl acetate	***		*					*	*	*
	Isoamyl acetate	***									
	Isobutyl acetate	***	*	**	*	**	*	*	**	**	**
	Phenethyl acetate	***									
Ethyl esters	Ethyl butanoate	**	**	***	*	**	**	**			
	Ethyl decanoate	***	**	**			*		**	***	***
	Ethyl dodecanoate	*									
	Ethyl hexadecanoate	***									*
	Ethyl hexanoate	**									
	Ethyl octanoate	***									
	Ethyl propanoate				**	**	*	**			
Acids	Butyric acid	***	**	**	***	**	**	**	**	**	**
	Hexanoic acid	**	**	*		*			**		
	Octanoic acid	*									

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

the findings of De Francesco *et al. (37)*, where *S. ludwigii* was identified for its potential to produce low-alcohol beers. However, many factors could be responsible of these differences, such as the strains employed and wort preparation. *Y. lipolytica* was not able to ferment glucose wort and *L. meyerae* showed an improvement by fermenting apple juice. Analysis by phenotypic microarray of carbon source utilisation by different yeast species showed that *Y. lipolytica* had a preference to utilise fructose rather than glucose (Fig. 3e). Such phenotype microarray trials on pure substrates help to explain the observed substrate utilisation and fermentation profile in wine and cider by *Y. lipolytica*, but the lack of substrate utilisation and low fermentation performance in beer where the media contains mainly glucose as the major carbon source. *S. ludwigii* also presents a similar trend in relation to fructose/glucose utilisation (Fig. 3e). The results presented in this study reconfirm that it is not always safe to assume that performance of a yeast in one medium will be the same in another medium.

Influence of amino acid consumption in fermentation and aroma production

In relation to nitrogen utilisation, there is a direct correlation between sugar and amino acid consumption (Fig. 4a) and fermentation capacity. The correlation was less pronounced in glucose wort (R^2 = 0.62). As expected, the control strains together with the fermentative strains utilised most of the amino acids present in the media. Surprisingly, *G. geotrichum* 772.71 was the only strain that consumed half of the initial amino acid content in glucose wort without fermentation (Fig. 4a). Glucose wort is the richest medium with respect to amino acid composition, although nitrogen consumption was not as efficient as that in grape must. This variation in nitrogen assimilation could be explained by the difference in carbon/nitrogen ratio between the two media.

The consumption of the amino acid sources was strain and media dependent. Nevertheless, all strains displayed the same order of assimilation of the nitrogen substrates. To determine the assimilation of the different amino acids in the three fermentation media, the average consumption of all the strains was used to establish a ranking in terms of wine, beer and cider (Fig. 4b). As previously reported for *Saccharomyces* strains *(38–40)*, classification of amino acids into 'good', 'intermediate' and 'poor' sources agreed reasonably well with the utilisation by the non-conventional yeasts in wine, beer and cider (Fig. 4b). Surprisingly, the intermediate nitrogen source methionine in beer was assimilated more efficiently than other amino acids whose absorption is considered as 'good' or 'fast' *(41)*. On the other hand, aspartic and glutamic acids supported less effective utilisation. In cider, alanine – classified as a poor nitrogen source – was assimilated more efficiently than 'good' sources such as

Table 4. Sugar, ethanol and glycerol composition of the wines, beers and ciders obtained after fermentation of grape juice, glucose wort and apple juice using different non-conventional yeasts

		***	Glucose (g/L)	Fructose (g/L)	Sucrose (g/L)	Maltose (g/L)	Total (g/L)	Ethanol (%v)	Glycerol (mg/L)
Grape juice	W34/70	F	0.0	0.7	0.4	0.3	1.4	8.9	7.6
	EC1118	F	0.0	1.0	0.5	0.4	1.9	9.2	7.6
	WLP775	F	0.0	1.0	0.4	1.4	2.8	9.1	8.1
	G. geotrichum 466.93	NF	75.2	82.1	0.4	0.5	158.2	0.0	1.5
	G. geotrichum 469.93	NF	74.8	82.1	0.4	0.4	157.7	0.0	1.7
	G. geotrichum 772.71	NF	72.1	81.4	0.4	0.4	154.3	0.0	1.2
	K. zonata	F	0.0	1.2	0.4	0.5	2.1	8.8	10.9
	K. lactis	F	0.2	16.2	0.3	0.0	16.7	8.0	7.0
	L. meyerae	NF	55.7	72.3	0.3	0.6	128.9	1.8	5.0
	P. kluyveri	NF	53.7	73.0	0.4	0.5	127.6	2.2	5.0
	S. ludwigii	NF	24.2	49.5	0.4	0.4	74.5	4.7	6.6
	S. caribae	NF	67.3	78.8	0.0	0.0	146.1	1.2	1.8
	Y. lipolytica	F	0.0	0.9	0.0	0.7	1.6	8.2	7.2
Glucose wort	W34/70	F	0.1	0.7	0.1	0.4	1.3	7.6	3.8
	EC1118	F	0.0	0.6	0.1	3.6	4.7	7.8	3.7
	WLP775	F	0.1	0.6	0.1	1.5	2.7	7.5	4.1
	G. geotrichum 466.93	NF	88.6	5.3	0.5	4.2	98.9	2.5	1.5
	G. geotrichum 469.93	NF	25.6	3.3	0.0	3.1	32.3	5.0	2.3
	G. geotrichum 772.71	NF	117.9	3.4	5.0	4.0	130.3	0.0	0.0
	K. zonata	F	0.2	0.7	0.2	3.8	5.3	7.0	6.1
	K. lactis	F	0.0	0.9	0.0	0.0	3.2	7.2	6.0
	L. meyerae	NF	97.1	3.1	4.9	3.9	109.3	1.9	1.0
	P. kluyveri	NF	66.1	2.9	4.8	3.9	78.1	3.4	5.0
	S. ludwigii	F	0.0	0.7	0.2	3.8	5.1	7.0	6.6
	S. caribae	NF	86.5	3.3	4.2	6.5	102.3	1.9	0.4
	Y. lipolytica	NF	92.2	4.8	0.1	5.4	102.5	1.1	1.5
Apple juice	W34/70	F	0.0	0.0	0.3	0.0	0.3	5.7	5.6
	EC1118	F	0.0	0.0	0.3	0.0	0.3	5.8	4.8
	WLP775	F	0.0	0.0	0.3	0.0	0.3	5.7	5.6
	G. geotrichum 466.93	F	0.0	0.0	1.4	0.0	1.4	5.3	4.2
	G. geotrichum 469.93	F	0.0	0.0	1.8	0.0	1.8	5.4	4.1
	G. geotrichum 772.71	NF	28.6	71.7	6.4	0.0	106.7	0.0	0.0
	K. zonata	F	0.0	0.0	0.3	0.0	0.3	5.0	7.8
	K. lactis	F	0.0	0.0	0.2	0.0	0.0	6.6	6.1
	L. meyerae	NF	4.8	40.5	0.3	0.0	45.6	3.1	4.5
	P. kluyveri	NF	2.6	30.7	6.3	0.0	39.6	3.2	5.2
	S. ludwigii	F	0.0	0.0	0.0	0.0	0.0	5.2	5.5
	S. caribae	NF	20.1	71.2	4.0	0.0	95.3	0.9	0.4
	Y. lipolytica	F	0.0	0.0	0.6	0.1	0.7	5.5	4.6

*** F, Fermentative (>80% sugars consumed).
NF, Non-fermentative.

arginine, threonine and lysine. Overall, the utilisation of amino acids in fermentative and non-fermentative strains followed a similar trend (Fig. 4c). However, non-fermentative strains had a preference for lysine and a reduced ability to assimilate alanine and phenylalanine compared with fermentative strains (Fig. 4c). In agreement with Crépin *et al. (39)*, nitrogen assimilation depends on the regulation of the transporters involved in uptake rather than the availability of a specific amino acid in the medium. Nevertheless, the results obtained in this study are difficult to compare with previous studies, given that non-conventional yeasts may have different transporters and regulatory pathways for nitrogen assimilation and that fermentation conditions together with media differ between the studies.

With cider, not only do the yeasts utilise histidine and tyrosine in the medium, but they also produce them, increasing their concentration at the end of fermentation. Tyrosine formation was also found in wine fermentation performed by *S. ludwigii*. Such amino acid release can be correlated with cell lysis at the end of fermentation or the presence of proteases which degrade smaller peptides into free amino acids. However, both amino acids are precursors of histamine and tyramine, toxic biogenic amines which are undesirable in fermented drinks *(42)*. Caruso *et al. (43)* tested 50 strains for the production

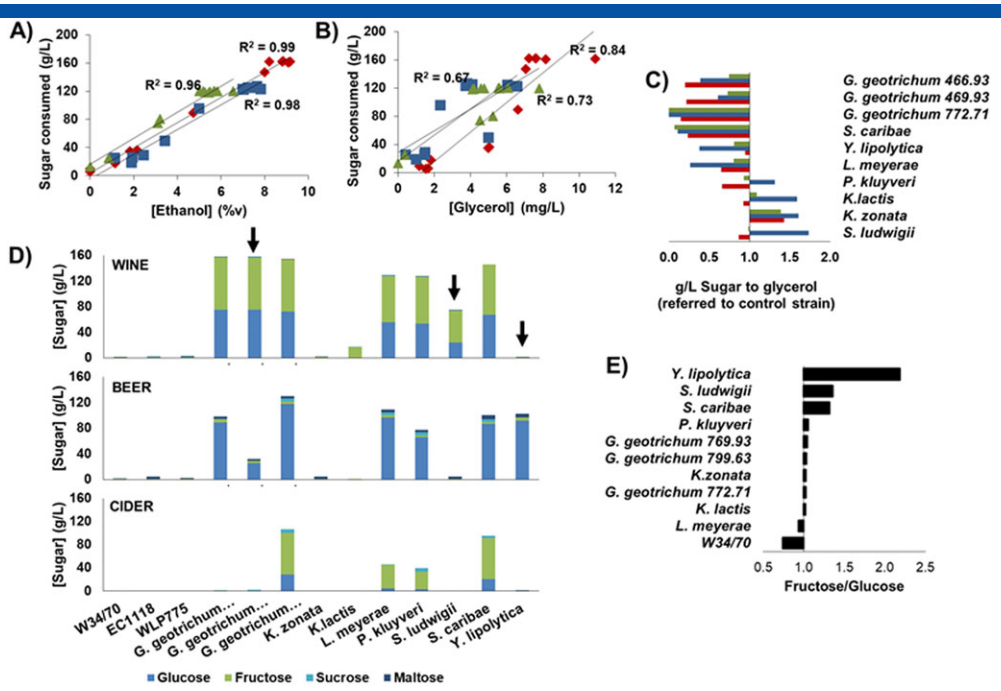

Figure 3. Fermentation of non-conventional strains. (a, b) Correlations of consumed sugar against ethanol and glycerol production in wine (red diamonds), beer (blue squares) and cider (green triangles). Linear regressions are displayed (black line) together with the regression coefficient (R^2). (c) Consumed sugar channelled to glycerol production in non-conventional strains. The different media are represented as: wine (red), beer (blue) and cider (green). Values are referenced to control strains. (d) Profile of sugar consumption in three different media: wine, beer and cider. Black arrows indicate the yeast strains with different fermentation performance depending on the media. (e) Fructose/glucose ratio utilization for the different yeast strains. Figure is reproduced in colour in online version. [Colour figure can be viewed at wileyonlinelibrary.com]

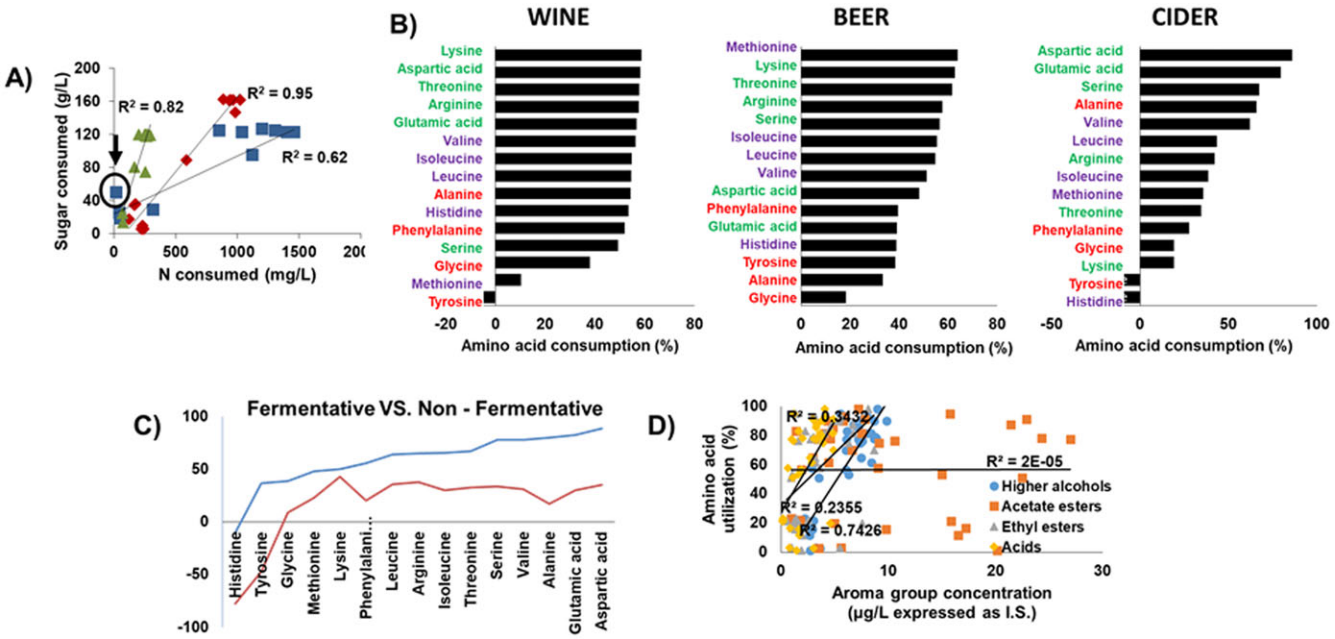

Figure 4. Influence of amino acid consumption in fermentation and aroma production. (a) Correlation between consumed sugar and amino acid assimilation in wine (red diamonds), beer (blue squares) and cider (green triangles). Linear regressions are displayed (black line) together with the regression coefficient (R^2). (b) Ranking of amino acid sources according to consumption percentage as average of all strains for each industrial media. Amino acids are coloured according to previous classifications as 'good' (green), 'intermediate' (purple) and 'bad' (red). (c) Nitrogen consumption in fermentative (blue line) and non-fermentative (red line) yeast strains. (d) Correlations of amino acid consumption against production of aroma functional groups. Linear regressions are displayed (black line) together with the regression coefficient (R^2). Figure is reproduced in colour in online version. [Colour figure can be viewed at wileyonlinelibrary.com]

of biogenic amines in wine, concluding that all non-conventional strains studied formed biogenic amines. Torrea and Ancín *(44)* found that inoculated wine musts produced a higher concentration of biogenic amines than spontaneous fermentations. They attribute this to the greater consumption of precursor amino acids during pure culture fermentation.

Although there are few studies on the formation of biogenic amines by yeasts, further work is needed to confirm whether non-conventional yeasts are involved.

To assess the effect of amino acid utilisation in aroma synthesis and identify their relationship with the non-conventional yeasts, correlation analysis was performed with the main aroma compounds (Fig. 4d). As was expected, the utilisation of amino acids did not always correlate with the synthesis of aroma compounds. The highest correlation was found for higher alcohols (R^2 = 0.74). This in accordance with the Ehrlich pathway *(45)*, where the catabolism of amino acids leads to the formation of higher alcohols, which contribute positively at low concentrations to the aroma of alcoholic beverages. Moreover, the production of ethyl esters showed some correlation with amino acid assimilation. Ethyl esters are produced by the condensation between ethanol and acyl-CoA, where acyl-CoA can originate from either pyruvic acid or fatty acids. Some of these fatty acids are derived from amino acid catabolism, which may explain these results. Interestingly, the content of ethyl esters and acids shows a positive correlation (R^2 = 0.72), as noted by acid production of *Y. lipolytica* and *G.geotrichum* strains. Surprisingly, acetate esters, formed by the combination of acetate and higher alcohol (from amino acid catabolism), did not show any correlation with amino acid consumption or higher alcohols. Ester formation depends on the concentration of the co-substrates, acyl-CoA and alcohol, and the activity of the acyltransferases and esterases *(24,46,47)*. Similar results were obtained by Gamero *et al. (23)*; the non-conventional yeast strains used in this study seem to resemble those of *S. cerevisiae*. However, the regulation and metabolism of ester synthesis may be different and explain differences in acetate ester production. For instance, *K. lactis* has the acyltransferase KlAtf, an orthologue to Atf1, Atf2 in *S. cerevisiae*, which plays a role in the aroma ester formation *(48)*. Some of the results presented here are in agreement with the results of Garde-Cerdán and Ancín-Azpilicueta *(30)*, who observed the effect of amino acid content on the production of some higher alcohols and acids. Acids and ethyl esters were also correlated, as seen with the high producers of acids, *G. geotrichum* and *Y. lipolytica*. Both *G. geotrichum* and *Y. lipolytica* show a very distant phylogenetic relationship with *S. cerevisiae* and have different genomic architectures *(49)*. They contain metabolic pathways and enzymes not found in *S. cerevisiae*, which may explain the high acid production and the different aroma produced *(50,51)*.

Phenotypic evaluation of *G. geotrichum*

In order to investigate in more detail why *Galactomyces* strains are able to synthesise fruity compounds resulting in a unique aroma profile, they were characterized using phenotype microarray technology. The results show that *Galactomyces* strains differ greatly from most strains in their utilisation of acids related to ethyl butanoate ester. Butyric acid, γ-amino butyric acid, γ- and β-butyric acid were assimilated by the three *Galactomyces* strains. Moreover, δ-amino valeric acid, α-keto valeric acid and caproic acid were also consumed efficiently by this species. In addition, *G. geotrichum* appears to be able to use branched chain and aromatic amino acids as a carbon source. Indeed, *G. geotrichum* is characterised by tropical/passion fruit aroma derived from the formation of ethyl butanoate. The ability to use carbon sources from the branched chain amino acid family is most likely the reason why this yeast produces this specific aroma compound at high levels.

Curiously, ethyl butanoate shows significant differences in all yeast strains studied, except for the *Galactomyces* strains (Table 3). Comparison between ethyl butanoate concentration at the beginning (after inoculation) and at the end of fermentation reveals that most of this aroma compound was already produced before inoculation. Ethyl butanoate synthesised by *Galactomyces* was the only compound for which the maximum production occurred before the inoculation, resulting in a carry-over flavouring effect (data not shown). These results indicate the importance of assessing each yeast strain individually to ensure the quality of desirable products.

G. geotrichum is known for its application in dairy products *(52,53)* and the presence of specific lipases which give a characteristic aroma *(54)*. Moreover, Zhu *et al. (55)* tested some *G. geotrichum* strains in wine fermentations, increasing the ester content. This study reveals that this yeast is a high producer of ethyl butanoate characterised by aromas of passion fruit, mango and pineapple. Moreover, these strains were the only ones able to use some of the precursor amino acids as a carbon source. Besides the diversity at species level, it was also observed that aroma production and fermentation performance are media and strain dependent in *G. geotrichum*. Although Gamero *et al. (23)* demonstrated that the three strains used in this study have conserved aroma and genetic profiles, the results presented here suggest that strain 772.71 shows a more divergent profile than the other two strains and performs differently depending on the fermentation media.

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