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A B S T R A C T

In recent years, there has been an increasing interest in identifying and characterizing the yeast flora associated with diverse types of habitat because of the many potential desirable technological properties of these microorganisms, especially in food applications. In this study, a total of 101 yeast strains were isolated from the skins of tropical fruits collected in several locations in the South West Indian Ocean. Sequence analysis of the D1/D2 domains of the large subunit (LSU) ribosomal RNA gene identified 26 different species. Among them, two species isolated from the skins of Cape gooseberry and cocoa beans appeared to represent putative new yeast species, as their LSU D1/D2 sequence was only 97.1% and 97.4% identical to that of the yeasts *Rhodotorula mucilaginosa* and *Candida pararugosa*, respectively. A total of 52 Volatile Organic Compounds (VOCs) were detected by Head Space Solid Phase Micro Extraction coupled to Gas Chromatography and Mass Spectroscopy (HS–SPME–GC/MS) from the 26 yeast species cultivated on a glucose rich medium. Among these VOCs, 6 uncommon compounds were identified, namely ethyl but-2-enoate, ethyl 2-methylbut-2-enoate (ethyl tiglate), ethyl 3-methylbut-2-enoate, 2-methylpropyl 2-methylbut-2-enoate, butyl 2-methylbut-2-enoate and 3-methylbutyl 2-methylbut-2-enoate, making them possible yeast species-specific markers. In addition, statistical methods such as Principal Component Analysis allowed to associate each yeast species with a specific flavor profile. Among them, *Saprochaete suaveolens* (syn: *Geotrichum fragrans*) turned to be the best producer of flavor compounds, with a total of 32 out of the 52 identified VOCs in its flavor profile.

1. Introduction

Flavors and fragrances play an important role in many of our daily product, finding applications in foods, cosmetics, perfumeries and phytosanitary product. The size of the market is ever increasing, with a sales revenue in US$8.6 billion to US 2000 to $23.9 billion in 2013 (http://www.leffingwell.com). Since 1923 (Omelianski, 1923), the microbial production of flavors has been extensively studied and many reviews on this field have been published (Abbas, 2006; Berger, 2009; Buzzini and Vaughan-Martini, 2006; Cheon et al., 2014; Dastager, 2009; Feron et al., 1996; Kim et al., 2014; Krings and Berger, 1998; Löser et al., 2014; Mdaini et al., 2006; Pires et al., 2014; Schrader, 2007; Styger et al., 2011). Yeasts and yeast-like fungi belonging to the genera *Saccharomyces*, *Yarrowia*, *Geotrichum*, *Saprochaete*, *Pichia*, *Candida*, *Williopsis*, and *Kluyveromyces* are recognized as reference yeast species for flavor production (Schrader, 2007; Willaert et al., 2005).

There are nowadays several biotechnological companies (Bell Flavors & Fragrances, Safisis, Givaudan, Firmenich, Isobionics among others) that can provide a large panel of flavor compounds elaborated from microbial transformation. Yet, the identification of new yeast species with high flavoring production potential remains a very attractive field of biotechnological interest. In this respect, Reunion Island and Madagascar, both located in the South West Indian Ocean, are

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recognized to be hot spots of biodiversity and present a high level of endemism (Hanson et al., 2009). A tropical climate provides organisms with ideal conditions for growth and proliferation. Furthermore, a rich diversity of tropical plants and fruits also provide suitable habitats for many microorganisms including yeasts (Barriga et al., 2014; Fleet, 2003; Nakase et al., 2006).

As part of a program aiming at the characterization of new yeasts species with high potential for aroma production, we isolated yeasts from a variety of different types of fruit collected in several locations in the South West Indian Ocean area. Head Space Solid Phase Micro Extraction coupled to Gas Chromatography and Mass Spectrometry (HS–SPME–GC/MS) was used to screen for microbial production of Volatile Organic Compounds (VOCs) of the isolated yeast species cultivated under conditions of glucose and amino acid excess. The results for each individual strain were compared as a whole using Principal Component Analysis (PCA) in order to group volatile molecules with yeast strains and to classify them as either low or high flavor producer.

2. Materials and methods

2.1. Yeast isolates from plants and culture media

Epiphytic yeasts were isolated at several locations in Madagascar (Province of Antsirabe) and Reunion Island (Area of Saint-Paul) from the skin of ripped fruits according to the method as described by Chanchaichaovivat et al. (2007), with some minor modifications. Wild tropical fruits, such as apple (*Malus* sp.), persimmon fruit (*Diospyros kaki and Diospyros* sp.), avocado (*Persea Americana*), passion fruit (*Passifora edulis and Passiflora* sp.), rose–apple (*Eugenia jambos*), pear (*Pyrus* sp.), pineapple (*Anana comosus*), Cape gooseberry (*Physalis peruviana*), dragon fruit (*Hylocereus polyrhizus*), peach (*Prunus persica*) and cocoa (*Theobroma cacao* var. *Criollo*) were collected in triplicate in natural environments where human activities are non-existent. Fruits were harvested aseptically from the trees and were stored at room temperature for less than 24 h in sterile bags. 50 mL of peptone water was added to each bag and the bags were shaken vigorously by hands for at least 5 min. 0.1 mL of this cellular suspension was then plated onto YEPD Chloramphenicol agar medium containing 20 g/L of glucose (alpha-D-glucose, anhydrous, Sigma-Aldrich), 20 g/L of peptone (Bacto™Peptone, Becton, Dickinson and company), 10 g/L of yeast extract (Biokar Diagnostics), 0.5 g/L Chloramphenicol (Calbiochem) and 15 g/L of agar (Agar-Agar for microbiology, Merck) and incubated at 28 °C for 48 h. Each extraction was carried out in three biological replicates (1 fruit per bag in triplicate).

All yeast colonies were examined on agar plate (global morphology) and under the microscope and individual colonies were selected on the basis of their differing visual characteristics (e.g. color, size, shape of the colony). The yeast isolates were re-streaked on YEPD to obtain pure cultures and were stored in YEGG broth (5 g/L yeast extract, 40 g/L glucose, 10 g/L glycerol) at −80 °C until analysis.

We acknowledge that the strains listed in Table 1 have been deposited at National Collection of Yeast Cultures (www.ncyc.co.uk) as frozen glycerol samples and are waiting to be formally characterized and accessioned.

2.2. Yeast identification by rDNA sequence analysis

The 101 isolated strains were first identified using API 20C AUX strips (bioMérieux). One representative of each species was then identified at molecular level by sequence analysis of the variable D1/D2 domains of the large subunit (LSU) ribosomal RNA gene. The large-subunit (LSU) D1/D2 domain was amplified and sequenced using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG) and NL4 (5′-GGTCCGTGTTTC AAGACGG) (Odonell, 1993). The amplified DNA was checked by 1.0% (w/v) agarose gel electrophoresis, purified and concentrated using QIAquick® PCR purification spin columns (Qiagen®). DNA concentration was measured using a spectrophotometer ND-1000 (Thermo Scientific) and the amplified products were sequenced by Eurofins MWG Operon (Germany). Sequence traces were edited manually and consensus sequences generated using the program SEQMAN®, version 7 (DNASTAR). Species identification was determined for each strain by using the resulting LSU D1/D2 sequence to search the EMBL database.

2.3. Qualitative analysis of volatile metabolites

For the qualitative study of flavors, a loop of freshly grown cells was transferred onto a YEPD agar slope into a glass tube and incubated at 28 °C for 48 h. After 24 h, the tubes were sealed to keep the volatile flavors inside and to characterize the profile by Solid Phase Micro Extraction (SPME) followed by GC/MS analysis. Prior to analysis, 5 μL of octanol (1 g/L in dichloromethane from SIGMA) was added as an internal standard into the sealed tubes. The head space of inclined cultures on YEPD was subjected to SPME analysis using a 2 cm fiber coated with 50/30 μm divinylbenzene/carboxen on polydimethylsiloxane (Buzzini et al., 2005) bonded to a flexible fused silica core (Supelco). Fiber was exposed to headspace for 15 min at 30 °C in a waterbath and inserted into the injection port of the GC/MS (Agilent technologies 6890N Network GC system) for thermal desorption at 270 °C for 15 min. Metabolites were separated by gas chromatography on a SPB5 column (60 m × 0.32 mm × 0.25 μm film thickness), coupled to a mass spectrometer (Agilent technologies 5973 Network mass selective detector). The carrier gas (He) was set at a flow rate of 0.8 mL/min. The column temperature was maintained at 45 °C for 2 min, raised to 230 °C at 4 °C/min and finally kept for 20 min. Injector and detector were set at 270 °C. The identification of volatile compounds was based upon comparison of their spectra and relative abundances with the NIST structure and Wiley database. The identity of the components was also confirmed by comparison of the calculated Relative Retention index with those of NIST database (http://webbook.nist.gov/chemistry). All analyses were performed in duplicate.

2.4. Statistical analysis

Factor analysis (Principal Component Analysis method) and cluster analysis (Ward's method) (Nurgel et al., 2002) were applied to check for similarities between the yeasts species with respect to the nature of the various volatile components produced by each yeast strains according to Oliveira et al. (2005). The analyses were performed using the PCA and hierarchical clustering programs developed in XLSTAT (Addinsoft, Inc.). A Pearson correlation was also performed using XLSTAT.

3. Results and discussion

3.1. Yeast identification and distribution

In this study, a total of 101 yeast strains, assigned to 26 different species by biochemical tests and LSU D1/D2 sequencing, were isolated from a triplicate experiment on 13 different types of tropical fruit collected in Reunion Island and Madagascar (Table 1 and Supplementary data A). The 26 species were classified into 15 genera and the most representatives were *Candida* (6 species) and *Pichia* (4 species). These genera were also found to be predominant in the screening carried out by Buzzini et al. (2005) on tropical ascomycetous yeasts. Among the isolated yeasts, Strain S6 (EB23) and S23 (EGPOC17) isolated from Cape gooseberry and cocoa beans, respectively, displayed only 97.4% sequence identity with *Candida pararugosa* for the former and 97.1% sequence identity to *Rhodotorula mucilaginosa* for the latter. As typically strains of the same species display less than 1% sequence divergence in the D1/D2 region of the LSU ribosomal RNA gene (Kurtzman and Robnett, 1998), we concluded that strain EB23 and EGPOC17 may be new yeast

Table 1
Epiphytic yeasts isolated from fruits in Madagascar and Reunion Island.

Strain number	Species name and percent sequence identity*	Fruit type and number of yeast species per fruit (into brackets)	Origin
S1	*Aureobasidium leucospermi* (100%)	Apple (4), avocado (5), pineapple (4)	M
S2	*Aureobasidium pullulans* (100%)	Persimmon fruit – sp. 2 (6)	M
S3	*Candida fermentati* (99.8%)	Apple (4), persimmon fruit – sp. 1 (4), persimmon fruit – sp. 2 (6)	M
S4	*Candida jaroonii* (99.8%)	Cacao beans (4)	M
S5	*Candida oleophila* (100%)	Rose-apple (4), passion fruit – sp. 1 (3)	M
S6**	*Candida pararugosa* (97.4%)	Cacao beans (4)	M
S7	*Candida quercitrusa* (99.8%)	Cape gooseberry (6)	M
S8	*Candida railenensis* (99.7%)	Pear (4)	M
S9	*Cryptococcus flavescens* (100%)	Apple (4), persimmon fruit – sp. 1 (4), Persimmon fruit – sp. 2 (6), avocado (5), Passion fruit – sp. 1 (3), pear (4), Cape gooseberry (6)	M
S10	*Cryptococcus laurentii* (100%)	Pineapple (4)	M
S11	*Cyberlindnera rhodanensis* (99.8%)	Dragon fruit (2)	M
S12	*Debaryomyces hansenii* (100%)	Apple (4), persimmon fruit – sp. 1 (4), persimmon fruit – sp. 2 (6), Avocado (5), passion fruit – sp. 2 (1), rose-apple (4), pear (4), Pineapple (4), Cape gooseberry (6)	M
S13	*Debaryomyces nepalensis* (100%)	Persimmon fruit – sp. 1 (4), passion fruit – sp. 1 (3), avocado (5), Cape gooseberry (6)	M
S14	*Saprochaete suaveolens* (*syn*: *Geotrichum fragrans*) (99.9%)	Dragon fruit (2)	R
S15	*Hanseniaspora uvarum* (99.7%)	Rose-apple (4), pineapple (4)	M
S16	*Kwoniella mangroviensis* (99.7%)	Avocado (5)	M
S17	*Lachancea fermentati* (100%)	Peach (3)	R
S18	*Pichia guillermondii* (100%)	Persimmon fruit – sp. 2 (6), rose-apple (4)	M
S19	*Pichia kluyveri* (99.8%)	Pear (4)	M
S20	*Pichia kudriavzevii* (99.8%)	Cacao beans (4)	M
S21	*Pichia manshurica* (100%)	Peach (3)	R
S22	*Rhodotorula glutinis* (99.5%)	Cape gooseberry (6)	M
S23**	*Rhodotorula mucilaginosa* (97.1%)	Cape gooseberry (6)	M
S24	*Saccharomyces cerevisiae* (99.5%)	Cacao beans (4)	M
S25	*Sporidiobolus pararoseus* (100%)	Persimmon fruit – sp. 2 (6)	M
S26	*Torulaspora delbrueckii* (100%)	Peach (3)	R

*Percentage of sequence identity of the strains when compared to reference strains from databases (into brackets); R: strain isolated in Reunion Island; M: strain isolated in Madagascar Island; **potentially new yeast species.

species but additional work will be required to resolve the taxonomic status of these two strains. For all other yeast isolates, the LSU D1/D2 sequence identity values were 99.5% or higher when compared with the type (reference) strains of known (i.e. described) yeast species (Table 1).

The distribution of the different yeast species on the collected fruits was highly variable and was likely dependent on the fruit composition (i.e. nature and sugar content, pH, etc.). For instance, we found an average of 4.5 different yeast species per fruit on Cape gooseberry, persimmon fruits, avocado, apple, rose-apple and pineapple; whereas *Saprochaete suaveolens* (syn: *Geotrichum fragrans*) and *C. jaroonii* were only found on one specific type of fruit, namely dragon fruit and cacao bean, respectively (Table 1 and Supplementary data A). The attractiveness of fruits to insects as a food source is well known and well documented. Indeed insects act as vectors for many different microorganisms including yeasts (Trindade et al., 2002) and the attractive effect of fruit could be linked to their overall palatability (flavor production) and chemical composition.

A review of the literature showed that some yeast species such as *Aureobasidium pullulans*, *Cryptococcus laurentii*, *Debaryomyces hansenii* and *R. mucilaginosa* are regarded as bioindicators of the general level of environmental pollution (Nagahama, 2006). For instance the (red) pigmented yeast *R. mucilaginosa* and *R. glutinis* are often found in higher numbers in the total yeast population of clean water whereas they are not found in polluted water (Nagahama, 2006). Thus, the presence of these yeast species may be a good indicator of a healthy environment.

3.2. Exploration of the flavor profile of the isolated strains

The volatile compounds produced by each representative of the 26 species isolated in Reunion Island and Madagascar were identified during growth on YEPD using GC/MS. A total of 52 different volatile

Table 2
VOCs produced by the isolated yeasts during growth on YEPD and identified by SPME–GC/MS.

Volatile compounds	Odor type[a]	Odor threshold[a,b]	RI exp[c]	RRI lit[d]	Yeast producing strains
ACIDS					
2-methylpropanoic acid	Sweaty, cheesy	32	751	744	*P. guillermondii; P. kluyveri*
Butanoic acid	Sweaty	240	771	769	*C. laurentii; D. hansenii; K. mangroviensis; P. guillermondii; P. kluyveri*
2-methylbutanoic acid	Fruity, sweaty	32–1580	849	843	*P. kluyveri; S. pararoseus*
3-methylbutanoic acid	Sweaty	120–700	839	828	*P. guillermondii; P. kluyveri; S. pararoseus*
ALCOHOLS					
Ethanol	Alcohol	100,000	nd	443	*A. pullulans; C. jaroonii; C. oleophila; C. pararugosa; C. railenensis; C. laurentii; D. hansenii; K. mangroviensis; L. fermentati; P. kudriavzevii; S. cerevisiae; T. delbrueckii*
3-methylthiopropanol	Cooked potato, cabbage	250	969	962	*T. delbrueckii*
Butan-1-ol	Malted, solvent	500	661	662	*A. pullulans; G. fragrans; L. fermentati*
2-methylbutanol	Malted, solvent	1200	770	739	*A. leucospermi; A. pullulans; C. fermentati; C. oleophila; C. quercitrusa; C. laurentii; D. hansenii; D. nepalensis; G. fragrans; K. mangroviensis; L. fermentati; P. guillermondii; P. kluyveri; P. manshurica; R. glutinis; R. mucilaginosa*
3-methylbutanol	Malted, alcohol, fruity	250–300	731	734	*A. leucospermi; A. pullulans; C. fermentati; C. jaroonii; C. oleophila; C. pararugosa; C. quercitrusa; C. railenensis; C. flavescens; C. laurentii; C. rhodanensis; D. hansenii; D. nepalensis; G. fragrans; H. uvarum; K. mangroviensis; L. fermentati; P. guillermondii; P. kluyveri; P. kudriavzevii; P. manshurica; R. mucilaginosa; S. cerevisiae; S. pararoseus; T. delbrueckii*
2-ethylhexanol		270,000	1021	1030	*A. leucospermi; A. pullulans; C. jaroonii; C. oleophila; C. quercitrusa; C. laurentii; D. hansenii; D. nepalensis; K. mangroviensis; L. fermentati; P. guillermondii; P. kluyveri; P. manshurica; R. glutinis; R. mucilaginosa; S. cerevisiae*
Volatile compounds	Odor type[a]	Odor threshold[a,b]	RRI exp[c]	RRI lit[d]	Yeast Producer
2-phenylethanol	Flowers, honey, rose	750–1100	1105	1114	*A. leucospermi; A. pullulans; C. fermentati; C. jaroonii; C. oleophila; C. pararugosa; C. quercitrusa; C. railenensis; C. flavescens; C. laurentii; C. rhodanensis; D. hansenii; D. nepalensis; G. fragrans; H. uvarum; K. mangroviensis; L. fermentati; P. guillermondii; P. kluyveri; P. kudriavzevii; P. manshurica; R. glutinis; R. mucilaginosa; S. cerevisiae; S. pararoseus; T. delbrueckii*
ALDEHYDES					
2-phenylethanal		4	1035	1041	*C. quercitrusa; P. kluyveri*
KETONES					
3-hydroxybutanone		800	703	703	*C. laurentii; D. hansenii; K. mangroviensis*
Heptan-2-one		140–3000	882	889	*C. quercitrusa*
Nonan-2-one		5–200	1079	1092	*C. fermentati; C. oleophila; C. quercitrusa*
ESTERS					
Ethyl ethanoate	Fruity	5	620	612	*C. rhodanensis; G. fragrans*
Ethyl propanoate	Banana, apple	10	709	709	*G. fragrans; H. uvarum*
Ethyl 2-methylpropanoate	Sweet, fruity, banana, apple	0,1	755	756	*G. fragrans*
Ethyl butanoate	Fruity, pear, pineapple	0.1–450,000	797	800	*G. fragrans*
Ethyl but-2-enoate			841	833	*G. fragrans*
Ethyl 2-methylbutanoate	Fruity, green, apple, floral	0,1–0,3	847	846	*G. fragrans*
Ethyl 2-methylbut-2-enoate	Fruity	65	935	936	*G. fragrans*
Ethyl 3-methylbutanoate	Fruity, blueberry	0023	850	849	*G. fragrans*
Butyl 3-methylbutanoate			1039	1040	*C. rhodanensis; G. fragrans*
2-methylbutyl ethanoate		1	903	874	*G. fragrans*
2-methylbutyl 2-methylpropanoate			1009	1001	*G. fragrans; R. mucilaginosa*
2-methylbutyl butanoate			1052	1052	*G. fragrans*
3-methylbutyl ethanoate	Pear, banana	2	873	871	*C. jaroonii; C. pararugosa; C. railenensis; C. flavescens; C. rhodanensis; G. fragrans; H. uvarum; K. mangroviensis; L. fermentati; P. kluyveri; P. kudriavzevii; P. manshurica; S. cerevisiae; S. pararoseus; T. delbrueckii*
3-methylbutyl propanoate	Apricot, pineapple		964	964	*C. rhodanensis; H. uvarum; S. pararoseus; T. delbrueckii*
3-methylbutyl butanoate	Apricot, pineapple		1049	1050	*G. fragrans; P. manshurica*
3-methylbutyl 2-methylbutanoate			1091	1101	*G. fragrans*
3-methylbutyl 2-methylbut-2-enoate			1185	1253	*G. fragrans*
3-methylbutyl 3-methylbutanoate			1096	1101	*G. fragrans*
Pentyl propanoate			964	964	*G. fragrans; P. kluyveri; P. manshurica*
Pentyl 3-methylbutanoate	Fruity		1098	1103	*G. fragrans*
2-ethylhexyl ethanoate			1194	1159	*C. railenensis; C. rhodanensis; H. uvarum; K. mangroviensis; P. kudriavzevii; R. mucilaginosa; S. cerevisiae; S. pararoseus*
2-phenylethyl ethanoate	Rose, honey, fruity	20	1249	1224	*C. rhodanensis; H. uvarum; P. kluyveri; P. kudriavzevii; P. manshurica; S. cerevisiae; S. pararoseus; T. delbrueckii*
Octyl ethanoate			1199	1211	*C. jaroonii*

Table 2 (*continued*)

Volatile compounds	Odor type[a]	Odor threshold[a,b]	RI exp[c]	RRI lit[d]	Yeast Producer
ESTERS					
2-phenylethyl propanoate	Floral, fruity		1337	1350	*C. rhodanensis; P. kluyveri; T. delbrueckii*
2-phenylethyl butanoate	Floral, rose, honey		1380	1405	*T. delbrueckii*
2-phenylethyl 3-methylbutanoate			1461	1495	*T. delbrueckii*

[a] Data from Chen et al. (2006), Czerny et al. (2008), Kirchhoff and Schieberle (2002), Leffingwell and Leffingwell (1991), Molimard and Spinnler (1996), and Takeoka et al. (1998).
[b] Odor threshold expressed in ng/mL of water.
[c] Relative Retention index on non-polar column experimentally determined.
[d] Relative Retention index on non-polar column (http://webbook.nist.gov/chemistry/).

molecules were detected and were classified into five categories namely acids, alcohols, ketones, aldehydes and esters (Table 2). According to the literature and the KEGG Pathway Database (http://www.genome.jp/kegg/pathway.html, see also Supplementary data B), these compounds are most likely derived from the metabolism of carbohydrate, amino acid, lipid, butanoate and propanoate. For instance, compounds like 2-methylbutanol, 3-methylbutanol and 2-methylpropanol were probably originated from isoleucine, leucine and valine catabolism using the Ehrlich pathway and ethyl hexanoate was likely obtained from fatty acids β-oxidation. We also identified 6 uncommon alpha unsaturated compounds with interesting chemical properties, which were but-2-enoate, ethyl 2-methylbut-2-enoate (ethyl tiglate), ethyl 3-methylbut-2-enoate, 2-methylpropyl 2-methylbut-2-enoate, butyl 2-methylbut-2-enoate and 3-methylbutyl 2-methylbut-2-enoate. We suggest that most of these unsaturated compounds are likely derived from incomplete β-oxidation of the branched chain amino acids present in excess in the growth medium, such as ethyl tiglate which originated from the β-oxidation of isoleucine (Grondin et al., 2015). Finally, ethyl butanoate and ethyl propanoate that were specifically identified in the *S. suaveolens* (*G. fragrans*) species could arise from either butanoate and propanoate metabolism, respectively, that have not been so far recognized in yeast species (see KEGG database and Supplementary data B) or from an incomplete function of the fatty acids β-oxidation.

With respect to their distribution, 5 alcohols (ethanol, 2-methylbutanol, 3-methylbutanol, 2-ethylhexanol and 2-phenylethanol) and 3 esters (3-methylbutyl ethanoate, 2-ethylhexyl ethanoate and 2-phenylethyl ethanoate) were the most frequently encountered molecules among the 26 isolated yeast species. Among them, 2-phenylethanol was produced by all 26 species (Table 2). Conversely, 25 of the 52 compounds (namely 3-methylthiopropanol, heptan-2-one and 23 esters) were produced by only one species as exemplified by *S. suaveolens* (syn: *G. fragrans*) which was found to be the unique producer of 20 of the 37 detected esters. Similarly, some acids (2-methylpropanoic acid and 2-methylbutanoic acid), aldehydes (2-phenylethanal), and esters (ethyl ethanoate, ethyl propanoate, butyl 3-methylbutanoate, 2-methylbutyl 2-methylpropanoate and 3-methylbutyl butanoate) were produced by only two yeast isolates (Table 2).

Yeasts are well known for their alcohol production (e.g. *Saccharomyces cerevisiae*) and our results are in accordance with previous published studies (Buzzini et al., 2003; Buzzini and Vaughan-Martini, 2006; Etschmann et al., 2003; Hazelwood et al., 2008). The literature also described strains which accumulate aldehydes, acids or esters but these molecules are less representative than alcohols (Buzzini et al., 2003; Suomalainen and Lehtonen, 1979). The production by *S. suaveolens* (syn: *G. fragrans*) and *Geotrichum candidum* of unsaturated compounds such as ethyl 2-methylbut-2-enoate (ethyl tiglate) was also described (Damasceno et al., 2003; Grondin et al., 2015; Pinotti et al., 2006). The production of these uncommon compounds is most likely linked to individual species and is potentially a characteristic of certain species or genera. Therefore, they may possibly be considered as potential chemotaxonomic markers for these species/genera. Results from Larsen and Frisvad (1995a,b), who demonstrated for the first time that fungal volatile metabolites can be used for classification of fungi at the species level are pointing in the same direction of this hypothesis.

From an economic perspective, some of these molecules such as 2-phenylethanol (rose-like odor), ethyl 2-methylbutanoate (apple-like odor), ethyl 3-methylbutanoate (blueberry-like odor), ethyl 2-methylpropanoate (banana-like odor), ethyl 2-methylbut-2-enoate (fruity odor), 3-methylbutyl propanoate (apricot-like odor) and 2-phenylethyl ethanoate (rose-like odor) harbored a pleasant odor and a high olfactory impact that may find applications in food and perfumery (Table 2). Also, ethyl tiglate (ethyl 2-methylbut-2-enoate) is a FEMA GRAS compound with a fruity odor which can be used in perfumery in the creation of unusual top-notes, particularly for the non-floral fragrance types (Arctander, 1969). This compound can also be used by the food industry in the production of alcoholic beverages like rum (http://www.perfumerflavorist.com). Similarly, 2-phenylethanol is a highly valuable compound. In 2010, the annual global production of 2-phenylethanol was estimated at ca. 10,000 t (Hua and Xu, 2011).

3.3. Comparison of the VOC profile of the strains by cluster and multivariate analysis

In order to visualize the grouping of the yeast species based on their flavoring characteristics and to investigate whether we can link in some way these molecules by groups of strains, a cluster and a multivariate analyses were performed using the VOC data that were classified according to their chemical classes and metabolic origins (Table 3). Each strain was associated with a number of variables including the concentration of VOCs (μg/L), the number of VOCs per chemical classes and the number of VOCs per hypothetical metabolic pathway (Table 3). Figs. 1 and 2 show the results of cluster analysis using the Ward's method and PCA analysis using the Pearson method (the Pearson correlation matrix used to construct Fig. 2 is presented in Supplementary data C). The first two principle components chosen for PCA analysis accounted for 51.35% and 16.15% of the variance and finally explained 67.5% of the total variance (Fig. 2; see also Supplementary data D and E). The dendrogram (Fig. 1) and the plot score (Fig. 2) both suggested the occurrence of four groups of yeasts.

According to PCA analysis, we found that group 1 included strains which produced mainly alcohols and esters whereas group 2 was constituted by strains which produced essential alcohols. *Candida quercitrusa* (Strain S7) and *Debaryomyces nepalensis* (Strain S13) only slightly diverged from group 2 and were included in this group (Figs. 1 and 2). The main difference of these two strains with the other strains of group 2 was the production of carbonyl compounds (aldehydes and ketones) for *C. quercitrusa* and a better ability to produce alcohol (985 μg/L) for *D. nepalensis*. The latter strain is further described for alcohol production. Kumar and Gummadi (2011) presented a production of 35.8 g/L for this species with an initial glucose concentration of 200 g/L. We also observed that *Sporidiobolus pararoseus* (Strain S25) diverged slightly from the other strains of group 1, probably due to its accumulation of acid compounds.

Pichia kluyveri (Strain S19) was better described in PC2 where principle component are defined mainly by an effect of aldehydes and acids' production. This strain alone was accounted for the group 3 and diverged significantly from group 1 because it accumulated acid and aldehyde compounds in addition to alcohols and esters also produced by strains of group 1. The accumulation of acids together with alcohol

Table 3
Classification of the VOCs produced by the isolated yeasts.

	Concentration of VOCs per chemical class (μg/L)						Number of VOCs per chemical class						Number of VOCs per type of hypothetical pathway						
Name of species	[Ac]*	[Al]*	[Ald]*	[Ke]*	[Es]*	[CTotal]*	Ac	Al	Ald	Ke	Es	Total	GP*	PP*	BP*	PeP*	B1P*	B2P*	EP*
A. leucospermi	0	282	0	0	0	282	0	4	0	0	0	4	0	0	0	0	1	0	3
A. pullulans	0	26	0	0	0	26	0	6	0	0	0	6	1	0	1	0	1	0	3
C. fermentati	0	187	0	8	0	194	0	3	0	1	0	4	0	0	0	0	1	0	3
C. jaroonii	0	16	0	0	3	19	0	4	0	0	3	7	4	0	0	0	3	0	3
C. oleophila	0	119	0	2	0	121	0	5	0	1	0	6	1	0	0	0	2	0	3
C. pararugosa	0	28	0	0	3	31	0	3	0	0	2	5	3	0	0	0	1	0	3
C. quercitrusa	0	122	3	14	0	138	0	4	1	2	0	7	0	0	0	0	3	0	4
C. railenensis	0	51	0	0	14	65	0	3	0	0	2	5	3	0	0	0	1	0	3
C. flavescens	0	36	0	0	12	48	0	2	0	0	1	3	1	0	0	0	0	0	3
C. laurentii	2	33	0	8	0	43	1	5	0	1	0	7	1	0	2	0	1	0	3
C. rhodanensis	0	100	0	0	1952	2052	0	2	0	0	9	11	7	2	2	0	1	0	8
D. hansenii	2	36	0	4	0	43	1	5	0	1	0	7	1	0	2	0	1	0	3
D. nepalensis	0	985	0	0	0	985	0	4	0	0	0	4	0	0	0	0	1	0	3
G. fragrans	0	268	0	0	3464	3732	0	4	0	0	28	32	13	2	9	2	0	5	34
H. uvarum	0	66	0	0	184	250	0	2	0	0	6	8	5	2	1	0	1	0	5
K. mangroviensis	3	30	0	18	5	55	1	5	0	1	2	9	3	0	2	0	2	0	4
L. fermentati	0	159	0	0	11	170	0	6	0	0	2	8	3	0	1	0	2	0	4
P. guillermondii	13	200	0	0	0	214	3	4	0	0	0	7	0	0	1	0	1	0	5
P. kluyveri	105	442	3	0	880	1430	4	4	1	0	5	14	3	2	1	1	2	0	10
P. kudriavzevii	0	33	0	0	87	120	0	3	0	0	4	7	5	0	0	0	1	0	5
P. manshurica	0	145	0	0	768	913	0	4	0	0	6	10	4	1	2	1	1	0	7
R. glutinis	0	38	0	0	0	38	0	3	0	0	0	3	0	0	0	0	1	0	2
R. mucilaginosa	0	46	0	0	8	53	0	4	0	0	2	6	1	0	0	0	2	0	5
S. cerevisiae	0	25	0	0	1	26	0	4	0	0	3	7	4	0	0	0	2	0	4
S. pararoseus	76	293	0	0	698	1067	2	2	0	0	4	8	3	1	0	0	1	0	7
T. delbrueckii	0	181	0	0	58	240	0	4	0	0	6	10	3	2	1	0	0	0	10

VOCs were classified in three categories (Concentration of VOCs per chemical class, Number of VOCs per chemical class and Number of VOCs per type of hypothetical pathway) for cluster and PCA analysis. *Variables used for PCA analysis. [Ac]: concentration of acids; [Al]: concentration of alcohols; [Ald]: concentration of aldehydes; [Ke]: concentration of ketones; [Es]: concentration of esters; [CTotal]: Concentration of VOCs produced; Ac: Number of acids; Al: number of alcohols; Ald: number of aldehydes; Es: number of ester; To: number of VOcs produced; Number of molecules derived from GP: Glycolysis pathway; PP: Propanoate pathway; BP: Butanoate pathway; Pep: Pentanoate pathway; B1P: β-oxidation pathway; B2P: unsaturated compounds from β-oxidation pathway; EP: Ehrlich pathway.

production in cultures of this strain indicated a good activity of the reductive and oxidative shunts of the Ehrlich pathway, whereas for the other yeasts, the reductive pathway was predominant. *P. kluyveri* was further described for its production of flavor compounds such as alcohols and esters (Amaya-Delgado et al., 2013). For the production of some compounds, namely ethyl lactate (not detected in our analysis) and alcohols, *P. kluyveri* was more efficient than *S. cerevisiae*. In addition, this species was also described for its accumulation of higher alcohols and aldehydes which correlated quite well with our results (Amaya-Delgado et al., 2013).

S. suaveolens (Strain S14), also known as *Geotrichum fragrans* (de Hoog and Smith, 2004) diverged significantly from groups 1, 2 and 3 and was therefore assimilated to group 4 due to its greater ability to produce VOCs. With a production of 32 VOCs, this yeast was by far the best

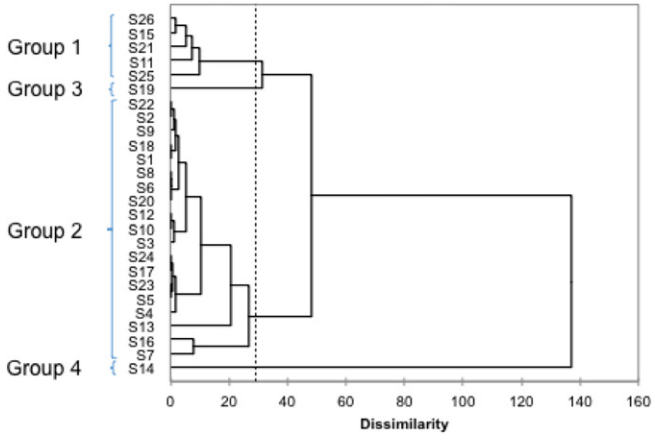

Fig. 1. Cluster analysis of the isolated yeast species. Strains S1 to S26 (Fig. 1) were grouped on the basis of their flavor production using Ward's method and performed using XLSTAT (Addinsoft). Automatic truncation based on entropy (dotted line) allowed identifying four consistent groups of yeasts. Most of the strains were classified into groups 1 and 2 as indicated by the dotted line (truncation). The dendrogram is more flattened for group 2 suggesting that this group of strains is more homogeneous than the first group. Species S19 and S14 were clearly out of groups 1 and 2 and were assigned to groups 3 and 4 respectively.

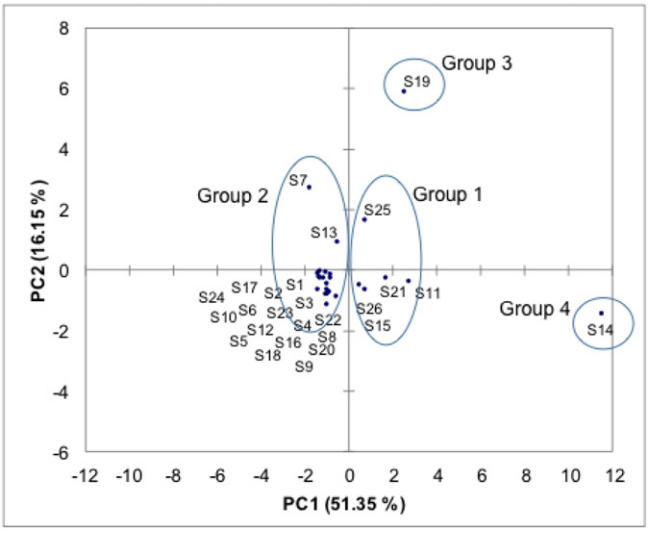

Fig. 2. Score plot of PC2 versus PC1 for yeast flavor analyzed by HS–SPME–GC/MS. Principal Component Analysis was performed using XLSTAT (Addinsoft). S1 to S26 are the analyzed strains. Group 1, including strains S11, S15, S21, S21 and S26 were strains producing mainly alcohols and esters. Group 2, including strains S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S12, S13, S16, S17, S18, S20, S22, S23 and S24 were strains producing mainly alcohols. The remaining strains (S19 and S14) constituted two other separated groups (groups 3 and 4 respectively).

producer of VOCs among all isolated strains (Table 3) and especially for ester compounds (28 of the 32 molecules were esters). This yeast was also the only strain which produced unsaturated compounds (Grondin et al., 2015). Damasceno et al. (2003) also described this yeast for production of alcohol and esters compounds in Cassava wastewater and Farbood et al. (1992) described a fermentation process for the production of unsaturated esters like ethyl tiglate using this yeast species.

To summarize, this work was a first approach to study yeasts isolated from Madagascar and Reunion Island and to investigate the relationship between microbial and chemical diversity. Statistical analysis allowed us to classify the yeasts according to their flavor productivity and four groups of yeasts were defined by this approach. Some yeasts preferentially produced alcohols whereas others produced aldehydes, ketones, acids or esters. Species such as *C. quercitrusa*, *D. nepalensis*, *P. kluyveri*, *S. pararoseus and S. suaveolens* showed a specific capacity to produce interesting flavors with potential interest for applications in food industry. Among them, *S. suaveolens*, with a production of 32 different flavors, could be considered the best flavor producer. This yeast was also the best producer of unsaturated compounds like ethyl tiglate. Further work was carried out on this strain and its related metabolism, with the aim of providing a more in-depth knowledge about the metabolic pathway of these unsaturated compounds (Grondin et al., 2015). The fact that these compounds were only produced by *S. suaveolens* would indicate a specific relationship between yeast and flavors. 'Yeast species-flavor' relationships clearly exist between strains and some specific flavor compounds could be used as potential chemotaxonomic markers. A study on flavor compounds produced by species of the genus *Saprochaete* and close relatives is currently underway and should provide a better description of this specific relationship between this taxonomic group of yeasts and their flavoring compounds.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2015.02.032.

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