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Microbiological characterisation and volatiles profile of model, *ex-novo*, and traditional Italian white wheat sourdoughs

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ABSTRACT

The interplay of sourdough microbiology and generated volatile compounds that define its sensory characteristics was studied. In order to detail the flavour generating potential of microorganisms, eight single-strain dough fermentations were studied, four of them never investigated before. Moreover, for the first time, both *ex-novo* and traditional wheat sourdoughs were investigated and compared to chemically acidified dough. HS-SPME-GC-MS was used to sample and analyse volatile compounds, some of which have never been detected before in sourdoughs. Alcohols, esters, carbonyl compounds, and acids mainly characterised the volatile profiles. Different sourdough microbiota resulted in different volatile profiles. PCA indicated that samples could be clustered according to their specific microbiota. Production of aroma compounds was strain-specific, confirming previous findings. This study can contribute to the management of desirable features and differentiate specialty products, as well as selecting new, suitable, sourdoughs after microbial screening.

1. Introduction

Sourdough has been used in bread production since ancient times, mixing flour, water and other ingredients according to recipe. Sourdoughs are ecosystems composed of specific microbiota, mostly yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) (Minervini, De Angelis, Di Cagno, & Gobbetti, 2014). Sourdough use is increasing because it augments preservation time, due to lower crumb pH, as compared to commercial baker's yeast breads; also, it often meets consumers' preferences, not only for artisanal specialties, but also for foods without chemical preservatives. Sourdough breads are also more digestible and richer in nutritional values (Corsetti & Settanni, 2007).

Taste and flavour depend strongly on dough fermentation microbiota, which produce extensive metabolite repertoires, including volatile flavour compounds. These molecules play a crucial role in specialty product identification and consumer acceptance. Only a few studies deal with the sourdough flavour/microbiota relationship (Czerny & Schieberle, 2002; Damiani et al., 1996; Hansen & Hansen, 1994; Ravyts & De Vuyst, 2011). In all cases laboratory sourdough fermentation was performed with different starters. Volatile profiles were studied *via* a number of different analytical techniques. Usually the most complex volatile profiles were obtained with microbial associations and with the addition of yeasts. Aldehydes, alcohols, ketones, and carboxylic acids were often detected.

Sour-bread sensorial quality appears positively influenced by microbial fermentations. In sourdough mixtures, different yeast and bacterial strains can not only increase the quantity of desirable volatile compounds, but also produce aromatic precursors that will react at cooking temperature. Moreover some sourdough microorganisms are able to degrade undesirable compounds (Czerny & Schieberle, 2002). Ravyts and De Vuyst (2011) recently emphasized that, despite its commercial importance, the relation between different microbiota and dough flavour is not fully understood; therefore, they suggest more attention should be paid to the influence of sourdough strains on aroma production.

Here we describe (i) the volatile profile in many different single-strain sourdoughs (model sourdoughs), in order to study the flavour generating potential of newly isolated microorganisms; (ii) the volatile profile of *ex-novo* (EN) sourdoughs, prepared by inoculation with plant materials, such as berries, etc., together with traditional (T) sourdoughs, used in family or local artisan bakeries; (iii) the relation of the observed flavours with specific microbial associations. Headspace solid-phase micro-extraction gas chromatography mass spectrometry (HS-SPME-GC-MS) was

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employed to sample wheat sourdough volatiles, in order to correlate their volatile profile to their microbiota.

Usually, the unique and delicate sourdough flavour depends on the interaction of many different volatile compounds that have differences, among other physicochemical properties, in volatility and polarity, hence the sampling procedure is crucial for reliable results. Compared to several sampling techniques of aroma compounds, HS-SPME shows a number of advantages: it is a solvent-free extraction, furthermore it is cheap and it needs low sample volumes moreover the substances are concentrated on absorbing fibers and they are directly desorbed into the gas chromatograph injector, this way laborious processing of the sample fraught with potential problems is avoided; moreover the fibre we selected (DVB/CAR/PDMS) proved to be the most universal assembly for sufficient isolation of compounds with different physicochemical properties and it is efficient and able to sample both flavour and off-flavour compounds (Cecchi, Passamonti, & Cecchi, 2010). It has to be underlined that this technique has never been used before to describe traditional wheat sourdoughs aromatic profile, while it was used only once to sample volatiles from laboratory-made wheat sourdoughs.

2. Materials and methods

2.1. Starter culture

The bacterial strains and wild *S. cerevisiae*, used in single strain sourdough, were isolated from Italian traditional dough samples. The *S. cerevisiae* starter culture (baker's yeast) comes from Lievital (Lesaffre Italia Spa, Parma, Italy).

2.2. Single strain model dough preparation

The studied model monoculture sourdough sample names are detailed in Table 1. *Lactobacillus crustorum* (strain SGL10), *Lactobacillus plantarum* (strain CA1), *Lactobacillus spicheri* (strain CP2), *Leuconostoc holzapfelii* (strain SP1), *Weissella confusa* (strain PA2), *Acetobacter cerevisiae* (strain AC4), *S. cerevisiae* (baker's yeast) and wild *S. cerevisiae* (strain UPRA) were inoculated singularly in a white wheat flour dough (with 200 dough yield (*DY* = dough weight × 100/flour weight) and 10^7 CFU/g) using bacterial cells from mMRS broth (modified De Man Rogosa and Sharpe medium) sub-cultured (30 °C, 12 h). Yeast model dough was prepared in a

Table 1
Samples analysed in this study. Characteristics of acidified control, single strains (SS), traditional (T), and *ex-novo* (EN) samples. Bacterial (with LAB metabolism) and yeast composition in samples. Natural microbial source of *ex-novo* samples.

Sample Name	Type	Strain used in the inoculum in SS sourdough	Natural microbial source	Bacterial identifications	LAB Metabolism	Yeast identifications
CA	Acidified control	None (negative control)				
LdB	SS	*S. cerevisiae* (Baker's yeast also used as positive control)				
SC	SS	wild *S. cerevisiae*				
SGL10	SS	*Lactobacillus crustorum*			HO	
CA1	SS	*Lactobacillus plantarum*			FHE	
CP2	SS	*Lactobacillus spicheri*			FHE	
SP1	SS	*Leuconostoc holzapfelii*			FHE	
PA2	SS	*Weissella confusa*			HE	
AC4	SS	*Acetobacter cerevisiae*				
MG	EN		*Punica granatum* fruits	*L. plantarum* group	FHE	*Saccharomyces cerevisiae*
VP	EN		*Veronica persica* flowers	*L. sanfranciscensis*	HE	*S. cerevisiae*
SA	EN		*Senapis alba flowers*	*L. graminis*	FHE	*S. cerevisiae*
FdM	EN		*Malus domestica* flowers	*L. plantarum* group	FHE	*S. cerevisiae*
				L. rossiae	HE	
My. C.	EN		*Myrtus communis* berries	*L. sanfranciscensis*	HE	*S. cerevisiae*
AM	EN		Mother of vinegar	*L. sanfranciscnsis*	HE	*S. cerevisiae*
AS	T			*P. pentosaceus*	FHE	*S. cerevisiae*
				L. brevis,	HE	
				L. sanfranciscensis,	HE	
				Leuc. holzapfelii,	HE	
				L. sakei	FHE	
K2	T			*L. sanfranciscensis*	HE	*S. cerevisiae*
AM	T			*P. pentosaceus*	FHE	*S. cerevisiae*
				L. rossiae	HE	
AA	T			*L. plantarum* group	FHE	*Wickerhamomyces anomalus*
				P. pentosaceus	FHE	*S. cerevisiae*
				W. cibaria	HE	
CP	T			*L. plantarum* group	FHE	*S. cerevisiae*
				L. spicheri	FHE	
				Leuc. holzapfelii	HE	
VA	T			*L. plantarum* group	FHE	*S. cerevisiae*
				L. sanfranciscensis	HE	
GFR	T			*P. pentosaceus*	FHE	*S. cerevisiae*
				L. graminis	FHE	*Saccharomyces barnettii*

SS = single strain
EN = *ex-novo*
T = traditional
HO = homofermentative
FHE = facultative heterofermentative
HE = heterofermentative

white wheat flour dough (200 *DY*; 10^5 CFU/gr), using yeast cells from YPD broth (YPD is yeast dextrose peptone medium) sub-cultured (30 °C, 12 h).

Model dough was fermented 24 h at 30 °C. A cell count and morphological evaluation of colonies was performed.

2.3. Traditional and ex-novo sourdough samples preparation

The studied traditional and *ex-novo* sourdough sample names are detailed in Table 1. The former were collected from local bakeries and restaurants, the latter were produced using the microbial natural source detailed in Table 1 as described in Ripari (2013). Traditional and *ex-novo* sourdoughs were refreshed 3 times for three consecutive days with white wheat flour, that was mixed with water and dough sample in a ratio 2:1:1 (w/v/w). The dough yield was 200. Forty grams of this sample were placed in a graduated cylinder (100 mL); the rest of the dough was placed in sterile beaker. Fermentation was carried out at 30 °C for 24 h. Leavening and pH were monitored during fermentation time. After 24 h cell counts and volatile compounds were investigated. A positive control was obtained by inoculation of baker's yeast as leavening agent. A negative control was obtained by acidification of dough with acetic acid and lactic acid (1:2) without any microbial starter.

2.4. Determination of pH and leavening of traditional sourdough

Each 2 h, 10 g of traditional sourdough were diluted in 90 mL of sterile distilled water. The pH was determined by a pHmeter Basic 20 (Crison Instruments SA, Barcelona, Spain). These measures were performed during fermentation. The leavening was monitored, observing the increase of dough volume during fermentation time.

2.5. Cell counts and isolations of microbial population for all sourdough samples

LAB and yeast populations were revealed by homogenising samples of 10 g in 90 ml peptone–salt water (peptone 0.1% w/v, NaCl 0.8% w/v) and plating of 8–9-fold dilutions (for LAB) and 4–5-fold dilutions (for yeasts) on appropriate agar media. LAB count number was estimated on mMRS agar, AAB count number on GYC medium (Minervini, Lattanzi, De Angelis, Di Cagno, & Gobbetti, 2012), and yeast count number on YPD agar (pH 4.3).

After incubation at 30 °C for 48–72 h the number of colonies (CFU/g) was estimated. LAB and yeast with different cell and colony morphologies were isolated for identification.

2.6. Identification of yeast and bacterial isolated for all sourdough samples

After extraction of yeast and bacteria DNA was isolated from all colonies and not only from the dominant strain, using "Blood & Tissue" kit. Yeast and Bacterial DNA was amplified using primer M13 (Huey & Hall, 1989). Strains with different random amplification of polymorphic DNA (RAPD) pattern were identified using P0/P6 primers for bacteria (Di Cello, Ventura, Fani, & Guckert, 1999) and P1/P2 for yeasts (Sandhu, Kline, Stockman, & Roberts, 1995). Purification of P0/P6 and P1/P2 fragments was performed using high purity PCR product purification kit (Quiagen). After sequencing (MWG-Biotech, Milan. Italy) identification of strains using database (blast sequence alignment and ribosomal database project) were obtained.

2.7. Volatile profile analysis for all sourdough samples

All samples were analysed in duplicate with the same procedure. Sourdough samples were weighed (2.0000 ± 0.0010 g) in 5-mL headspace vials which were closed with PTFE/silicone septa. Volatiles in the headspace were measured through solid-phase microextraction followed by gas chromatography coupled to mass spectrometry (HS-SPME-GC-MS).

SPME fibres were obtained from Supelco (Bellefonte, PA). The fibre was divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm. The fibre was conditioned before use, as recommended by the manufacturer. Before extraction, stabilisation of the headspace in the vial was reached by equilibration for 30 min at room temperature (thermostatted 20 ± 0.1 °C), to reach thermal equilibrium before extraction. The extraction efficiency, at a given temperature is a function of the extraction time, and usually increases with increasing time. Different volatile compounds are expected to possess different equilibrium times. As HS-SPME is a multiphase equilibrium process, maximum sensitivity is obtained by allowing the analyte to reach equilibrium (Ribeiro, Costa Freitas, & Gomes da Silva, 2008). At equilibration times greater than 2 h only minor changes of the volatile profile of sourdough samples occurred. After sampling, the fibre was inserted manually into the GC injection port of a Hewlett Packard GC–MS, G1800C GCD Series II (Palo Alto, CA), set at 270 °C in the splitless mode for 1 min and desorbed for 4 min. The GC–MS was equipped with a 0.75 mm i.d. inlet liner and a HP-5MS column (30 m × 0.25 mm I. D. × 0.25 μm film thickness; Agilent). Before sampling, the fibre was conditioned for 5 min at 270 °C and blank runs were done periodically during the study to reveal possible carry-over. The carrier gas was helium with a constant flow of 1 mL/min; the oven temperature was held at 30 °C for 15 min, then programmed from 30 to 260 °C at 10 °C/min and then held at 260 °C for 1 min. Mass spectra were acquired in electron impact mode (70 eV), using full scan with mass analysis in the range m/z 30–400. The transfer line temperature was set at 270 °C; the ion source and the quadrupole were heated by conduction. The identification of the analytes detailed in Table 2 was based on comparison of their retention times with those of standards obtained from Sigma Aldrich (Milan, Italy). In the absence of the commercial standard, that is for (*E*)-2-hepten-1-ol, 2-pentylfuran, *trans*-(2-ethylcyclopentyl)methanol, 1,3-bis(1,1-dimethylethyl)benzene and dihydro-5-pentyl-2(3*H*)-furanone, peak identification was carried out by computer matching of mass spectral data with those of compounds contained in NIST 1998 library; match quality of above 98% was needed for a positive identification (Cecchi & Alfei, 2013). Volatile compounds eluting later than hexane were also identified by comparison of their linear retention indices (Van den Dool & Kratz, 1963) relative to *n*-alkanes, calculated using a straight-chain alkanes mixture (C6–C19), with literature values obtained using chromatographic phases similar to that used (Acree & Arn, 2007; Linstrom & Mallard, 2013).

The relative proportions of the constituents were obtained by peak area percentages (Cecchi, 2014). Only compounds with a signal to noise ratio higher than 5 were considered. To be able to compare the quantities of each compound in different samples from the area percentage of that compound in their chromatograms all the compounds contributing to the total area should have similar response factors, so that higher area percentage means higher amount. Volatile compounds of different functionality, like alcohols, aldehydes, acids, esters or ketones, present different response factors; hence this procedure enables only a semi-quantitative estimate of volatile compounds. Nevertheless the statistical analysis of those data is meaningful as detailed at Section 3.6.

2.8. Statistical analysis

To visualise a possible effect of the microbiota on the volatile profile of sourdough, statistical analysis was applied to raw data using Tanagra 1.4.50 software, as well as PCA and varimax as factor

rotation methods. HAC cluster analysis was also employed. Any value whose signal-to-noise ratio was lower than 5 was considered not detectable and it was set to a value of zero, in order to avoid missing data in the dataset. The Pearson (n) pre-treatment was applied before performing the PCA in order to standardise the variables.

3. Results and discussion

3.1. Microbiological dough results

Traditional sourdough samples show an increase of volume and a decrease of pH during fermentation (Fig. 1). Dough obtained with baker's yeast revealed a very rapid rising without a change of pH. After 8 h, these samples show 0.7 as an average variation of pH (pH 4.0–4.4). No rising and no variation of pH occurred for the negative acidified control (flour and water *DY* = 200).

The microbial counts for all samples show that, on average, bacteria population is 1.6×10^9 CFU/g, while yeast population is 1.6×10^7 CFU/g. The ratio between yeasts and bacteria is 1:100, in agreement with other previous studies (Lönner & Ahrnè, 1995). Microbial interactions led to interesting bacteria–yeast association and selections. Microbial identifications confirm previous results (Ripari, 2013) and reveal that all traditional sourdoughs have a specific community of yeasts and bacteria, as detailed in Table 1.

No homofermentative strains were identified in both traditional and *ex-novo* samples. *S. cerevisiae* was found in all samples and in 2 traditional samples (AA-GFR) *Wickerhamomyces anomalus* and *Saccharomyces barnettii* were identified. In almost all *ex-novo* sourdough samples the presence of *Lactobacillus sanfranciscensis* and *S. cerevisiae* is common, as detailed in Table 1. In the case of

Table 2
Sourdoughs volatile compounds (percent area) obtained by HS-SPME/GC-MS, with their odour type and chemical class. Sample names are explained in Table 1.

		acetaldehyde	ethanol	ethyl acetate	diacetyl	acetic acid	acetoin	1-butanol, 3-methyl	1-pentanol	hexanal	butanoic acid, ethyl ester	ethyl lactate	butanoic acid	2-hexenal, (E)	1-hexanol	1-butanol, 3-methyl acetate	2-heptanone	acetic acid, pentyl ester	2-heptenal- (Z)	benzaldehyde	2-hepten-1-ol, (E)	1-heptanol	1-octen-3-one	1-octen-3-ol	3-octanone	furan, 2-pentyl	2-octanone	3-octanol
RI_{lit}		412	440	605	612	622	715	736	764	799	805	815	844	858	865	878	891	922	964	967	970	973	980	985	990	993	999	1002
RI_{exp}		-	-	602	616	625	710	740	769	793	800	815	850	855	869	880	890	916	959	969	972	978	986	989	995	998	1001	1007
times detected		1	20	20	5	4	1	16	18	8	1	13	1	1	21	12	1	11	4	1	1	19	1	18	11	17	3	6
CA	control	nd	nd	nd	nd	57.09	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
LdB	SS	nd	18.38	34.78	3.01	nd	nd	29.10	nd	nd	nd	nd	nd	nd	0.39	3.39	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
S.C.	SS	nd	20.35	25.34	1.67	nd	nd	37.55	nd	nd	nd	nd	nd	nd	1.52	1.60	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AC4	SS	4.46	nd	nd	5.89	7.73	12.69	nd	nd	nd	nd	nd	23.24	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.06	nd	nd
SGL10	SS	nd	nd	nd	3.91	nd	nd	nd	3.15	4.94	nd	nd	nd	nd	27.61	nd	nd	nd	3.38	nd	nd	0.96	nd	3.36	nd	1.50	nd	nd
CP2	SS	nd	19.84	24.75	nd	nd	nd	nd	1.45	nd	nd	1.91	nd	nd	17.31	nd	nd	nd	nd	nd	nd	1.72	nd	1.43	nd	0.23	nd	nd
SP1	SS	nd	0.80	6.20	4.32	nd	nd	nd	2.48	31.96	nd	nd	nd	nd	8.16	nd	nd	nd	2.67	nd	nd	0.35	nd	1.85	nd	0.90	nd	nd
PA2	SS	nd	3.47	6.15	nd	5.63	nd	nd	3.56	3.96	nd	nd	nd	nd	23.92	nd	nd	nd	nd	0.90	nd	2.45	nd	2.27	0.28	0.57	nd	nd
CA1	SS	nd	0.93	6.87	nd	nd	nd	nd	2.27	9.86	nd	nd	nd	0.43	5.92	nd	0.42	nd	5.96	nd	nd	1.15	0.68	3.77	0.47	1.70	nd	1.15
VP	EN	nd	19.19	25.61	nd	nd	nd	6.10	1.29	1.41	nd	1.28	nd	nd	13.59	nd	nd	0.14	nd	nd	nd	2.14	nd	1.32	0.22	0.29	0.22	0.16
FdM	EN	nd	8.70	22.60	nd	nd	nd	4.31	0.05	nd	nd	nd	nd	nd	12.20	nd	nd	nd	nd	nd	nd	2.62	nd	1.03	nd	0.07	nd	nd
MG	EN	nd	20.17	18.16	nd	nd	nd	7.23	1.55	nd	nd	0.89	nd	nd	15.99	nd	nd	0.10	nd	nd	nd	2.93	nd	1.90	0.35	0.37	0.42	0.31
SA	EN	nd	22.86	31.60	nd	nd	nd	8.54	0.07	nd	nd	1.18	nd	nd	4.45	4.85	nd	0.22	nd	nd	nd	1.59	nd	0.81	0.32	0.41	nd	0.29
AM	EN	nd	15.58	20.50	nd	1.96	nd	6.46	1.13	nd	nd	0.73	nd	nd	13.89	nd	nd	0.21	nd	nd	nd	2.40	nd	1.53	0.26	0.89	nd	0.14
My.c.	EN	nd	8.27	71.33	nd	nd	nd	2.63	nd	nd	nd	nd	nd	nd	1.26	2.87	nd	0.46	nd	nd	nd	0.36	nd	0.25	nd	0.19	nd	nd
AMT	T	nd	13.39	45.62	nd	nd	nd	9.64	3.20	0.79	nd	3.38	nd	nd	8.65	7.57	nd	0.25	nd	nd	nd	1.47	nd	0.44	0.08	0.07	0.24	nd
AST	T	nd	10.56	35.70	nd	nd	nd	8.18	4.95	4.32	nd	4.49	nd	nd	21.18	0.04	nd	0.13	0.08	nd	0.23	1.88	nd	0.57	0.39	0.08	nd	nd
CPT	T	nd	16.89	35.86	nd	nd	nd	16.02	3.33	nd	nd	1.76	nd	nd	5.34	7.39	nd	0.19	nd	nd	nd	0.96	nd	0.28	nd	nd	nd	nd
AAT	T	nd	12.24	44.88	nd	nd	nd	9.03	2.44	0.67	nd	7.42	nd	nd	6.16	5.79	nd	0.10	nd	nd	nd	1.21	nd	0.46	0.17	0.30	nd	nd
VAT	T	nd	20.14	41.57	nd	nd	nd	23.81	1.20	nd	nd	0.82	nd	nd	1.80	3.54	nd	nd	nd	nd	nd	0.12	nd	0.07	nd	nd	nd	nd
GRFT	T	nd	14.33	48.19	nd	nd	nd	15.15	3.25	nd	nd	3.89	nd	nd	2.75	6.49	nd	nd	nd	nd	nd	0.37	nd	nd	0.29	nd	nd	nd
MT	T	nd	10.36	50.28	nd	nd	nd	8.60	2.49	nd	nd	3.65	nd	nd	6.63	5.57	nd	0.13	nd	nd	nd	1.30	nd	0.10	nd	0.07	nd	0.11
K2T	T	nd	15.90	49.64	nd	nd	nd	7.55	2.47	nd	1.10	3.40	nd	nd	6.62	4.59	nd	0.26	nd	nd	nd	1.29	nd	0.57	0.20	0.34	nd	nd
odour type		na	alcoholic	ethereal	na	acid	na	fermented	fermented	green	na	fruity	cheesy	green	herbal	fruity	cheesy	fruity	green	fruity	na	green	earthy	earthy	herbal	fruity	earthy	earthy
chemical class		Aldehyde	Alcohol	Ester	ketone	Acid	ketone	Alcohol	Alcohol	Aldehyde	Ester	Ester	Acid	Aldehyde	Alcohol	Ester	ketone	Ester	Aldehyde	Aldehyde	Alcohol	Alcohol	ketone	Alcohol	ketone	Furan	ketone	Alcohol

(*continued on next page*)

Table 2 (*continued*)

		octanal	hexanoic acid, ethyl ester	hexanoic acid	acetic acid, hexyl ester	p-cymene	2-octenal (E)	trans-(2-Ethylcyclopentyl)methanol	2-octen-1-ol (Z)	1-octanol	formic acid, octyl ester	chalcogran	heptanoic acid, ethyl ester	nonanal	2,4-octadienal, (E,E)	phenylethyl alcohol	1-nonanol	2,4-nonadienal, (E,E)	octanoic acid, ethyl ester	decanal	acetic acid, octyl ester	Benzene, 1,3-bis(1,1-dimethylethyl)	acetic acid, 2-phenylethyl ester	nonanoic acid, ethyl ester	tridecane	2-undecenal	2(3H)-Furanone, dihydro-5-pentyl	decanoic acid, ethyl ester
RI_{lit}		1006	1007	1010	1015	1024	1055	1058	1071	1075	1104		1107	1108	1112	1120	1178	1190	1195	1206	1210	1249	1255	1292	1300	1355	1366	1384
RI_{exp}		1011	1013	1015	1018	1023	1050	1060	1076	1081	1090	1094	1112	1115	1120	1127	1181	1200	1202	1209	1215	1254	1257	1290	1295	1351	1355	1378
times detected		1	15	3	16	1	3	5	9	17	3	5	10	10	1	15	9	1	14	5	2	1	7	7	8	1	4	5
CA	control	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.29	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
LdB	SS	nd	0.18	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.59	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
S.C.	SS	nd	0.99	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.34	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AC4	SS	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
SGL10	SS	nd	nd	0.22	nd	nd	3.00	nd	0.23	0.40	nd	nd	nd	0.32	nd	nd	0.29	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CP2	SS	nd	nd	nd	0.22	nd	nd	nd	0.25	0.27	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
SP1	SS	nd	nd	nd	nd	nd	2.33	nd	nd	0.48	0.48	nd	nd	0.56	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
PA2	SS	nd	nd	nd	nd	nd	nd	nd	1.12	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CA1	SS	nd	nd	0.29	0.83	nd	8.64	nd	0.46	1.37	1.37	0.37	nd	nd	0.38	nd	0.27	0.31	nd	0.17	nd	nd	nd	0.28	nd	0.30	nd	nd
VP	EN	nd	1.06	nd	1.43	nd	nd	nd	0.27	2.56	nd	0.26	0.37	2.29	nd	3.35	0.37	nd	1.16	0.07	nd	nd	0.28	0.27	nd	nd	0.27	0.09
FdM	EN	3.90	nd	nd	1.48	nd	nd	nd	nd	4.52	nd	nd	nd	0.53	nd	4.66	1.02	nd	3.32	6.53	nd	nd	nd	2.79	nd	nd	0.67	nd
MG	EN	nd	1.04	nd	1.51	nd	nd	nd	0.30	3.60	nd	0.16	0.26	0.75	nd	4.53	0.42	nd	1.24	0.10	nd	nd	0.32	0.20	nd	nd	0.39	0.09
SA	EN	nd	1.55	nd	1.69	nd	nd	nd	0.25	1.70	nd	nd	0.51	nd	nd	6.79	nd	nd	2.35	nd	nd	nd	0.61	0.31	nd	nd	nd	0.20
AM	EN	nd	1.79	0.22	2.27	nd	nd	nd	0.24	3.06	nd	0.18	0.50	0.27	nd	4.36	0.34	nd	2.16	nd	0.10	0.12	0.44	0.44	nd	nd	0.36	0.15
My.c.	EN	nd	0.30	nd	2.77	nd	nd	nd	nd	0.69	nd	nd	nd	1.74	nd	1.39	0.10	nd	0.44	nd	0.12	nd	0.46	0.14	nd	nd	nd	nd
AMT	T	nd	0.37	nd	0.48	nd	nd	0.34	nd	0.89	nd	nd	0.12	0.00	nd	0.06	0.03	nd	0.77	nd	nd	nd	0.03	nd	0.15	nd	nd	nd
AST	T	nd	0.62	nd	0.16	0.05	nd	nd	0.30	1.10	nd	nd	0.09	0.17	nd	0.10	0.05	nd	0.17	0.04	nd	nd	nd	nd	0.05	nd	nd	nd
CPT	T	nd	1.16	nd	0.37	nd	nd	0.24	nd	0.51	nd	nd	0.18	nd	nd	0.31	nd	nd	0.40	nd	nd	nd	nd	nd	0.06	nd	nd	nd
AAT	T	nd	0.75	nd	0.29	nd	nd	nd	nd	0.67	nd	nd	0.13	nd	nd	0.31	nd	nd	0.53	nd	nd	nd	0.02	nd	0.07	nd	nd	nd
VAT	T	nd	0.36	nd	0.06	nd	nd	nd	nd	0.11	nd	nd	nd	nd	nd	0.37	nd	nd	0.29	nd	nd	nd	nd	nd	0.04	nd	nd	nd
GRFT	T	nd	0.60	nd	0.16	nd	nd	0.11	nd	0.27	nd	nd	nd	nd	nd	nd	nd	nd	0.18	nd	nd	nd	nd	nd	0.04	nd	nd	nd
MT	T	nd	0.33	nd	0.30	nd	nd	0.18	nd	0.61	nd	nd	0.08	nd	nd	0.05	nd	nd	0.50	nd	nd	nd	nd	nd	0.06	nd	nd	nd
K2T	T	nd	0.61	nd	0.41	nd	nd	0.17	nd	nd	0.91	0.10	0.06	nd	nd	0.07	nd	nd	0.32	nd	nd	nd	nd	nd	0.11	nd	nd	0.04
odour type		aldehydic	fruity	fatty	fruity	nd	fatty	nd	green	fruity	nd	-	fruity	aldehydic	fatty	floral	floral	fatty	fruity	aldehydic	floral	na	floral	waxy fruity	na	fruity	coconut	waxy fruity
chemical class		Aldehyde	Ester	Acid	Ester	Alkane	Aldehyde	Alcohol	Alcohol	Alcohol	Ester	Alkane	Ester	Aldehyde	Aldehyde	Alcohol	Alcohol	Aldehyde	Ester	Aldehyde	Ester	Alkane	Ester	Ester	Alkane	Aldehyde	Furan	Ester

nd = not detectable.
SS, EN, T same meaning as in Table 1.

mono-culture model dough only one kind of colony was observed on plates after incubation, to confirm the non-contamination of dough. All LAB strains grew until 10^9 CFU/g, while in the negative control, no colony was visible on the plates.

3.2. Volatiles profile of sourdough samples

Regarding the composition of aroma volatile compounds in sourdoughs, just a few studies on its correlations with the fermenting microbiota have been so far provided. Damiani et al. (1996) studied volatile compounds produced by laboratory sourdough microbiota and concluded that *Lactobacillus brevis* subsp. *linderi* and *L. plantarum* strains yielded most complex volatile profiles; they also found that sourdoughs started with microbial associations generate an even larger range of volatiles. Their study did not consider traditional, artisanal sourdoughs, but only model, inoculated sourdoughs. In addition, their experimental design was based on purge-and-trap extraction of a sourdough–water extract; thus, many volatiles may not have been sampled, since most of them are not water soluble. This is also suggested by the absence of compounds eluting later than nonanal from an HP5MS column.

During laboratory sourdough fermentation with a commercial starter, Czerny and Schieberle (2002) investigated flavour compounds. In this case, the resident microbiota remained unidentified and no relationship between volatile profile and starter species could be drawn. Moreover volatiles were obtained from a laborious Soxhlet extraction followed by a concentration of the extract by distilling off the solvent, fraught with potential loss of the most volatile compounds.

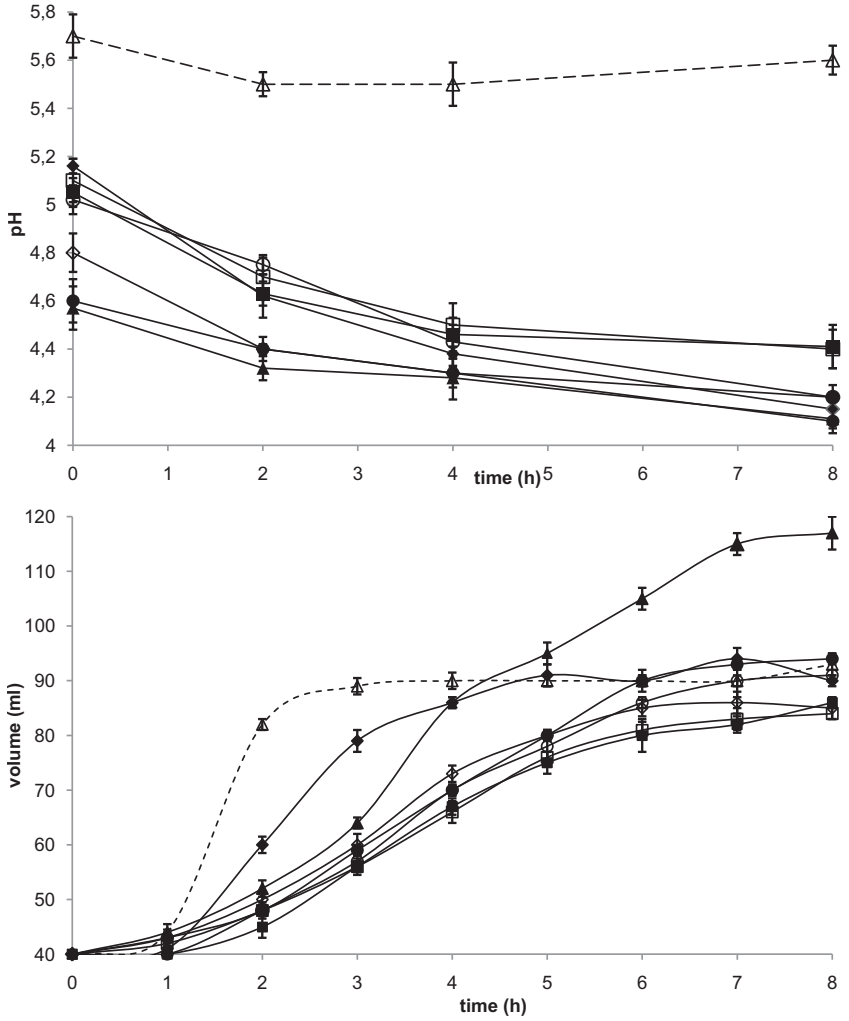

Fig. 1. Raising and evolution of pH of traditional sourdough samples (■ = CP, ◆ = AS, ▲ = AM, ● = K2, □ = VA, ◇ = AA, ○ = GFR) and positive control (△ = baker's yeast). Sample names are explained in Table 1.

Hansen and Hansen (1994) investigated volatile compounds in laboratory sourdoughs fermented with four different *Lactobacillus* starters and examined the effect of yeast addition on volatiles production by dynamic headspace extraction–gas chromatography/mass spectrometry (DHE–GC/MS). When heterofermentative culture was used, ethanol and ethyl acetate were the major compounds. In addition, the content of other volatiles was extremely low. Finally, when yeasts were added, number and amount of volatile compounds increased. Ravyts and De Vuyst (2011) detailed the volatile profile of laboratory sourdoughs prepared using different LAB starter cultures. They found multiple aldehydes, alcohols, ketones, and carboxylic acids. As they focused on LABs, they did not study yeasts. Therefore, their volatile profile might have been influenced also by yeast communities, to an unknown extent. Alfonzo et al. (2013) studied the LAB population in wheat flours. They analysed volatile organic compounds emitted from sterile flour extract broth inoculated with flour LAB; they detected 18 compounds, mainly alcohols, aldehydes, esters and acids. Their interesting procedure parallels ours even if they did not test volatiles from sourdough.

In this context results of HS-SPME-GC-MS analysis presented in Table 2 are particularly interesting. All sample names are explained in Table 1. Average percent area of each identified compound give the percentage distribution of volatile substances in each sourdough samples.

Table 2 also details the chemical class and odour type of each molecule (http://bioinformatics.charite.de/superscent, 2016 accessed February 2016). Volatile compounds have different odour activities (Reiners & Grosch, 1998) and compounds present at higher concentrations may not be the main contributors to a specific aroma (Erickson & Covey, 1980); furthermore the presence of a certain volatile does not consequently imply that it contributes to the final aroma.

A laboratory air control sample was analysed and limonene, camphor, pinene, longifolene, geranyl acetone, *p*-cymene and isobornyl acetate were detected. Since they were also occasionally found in some sourdough sample, their non-microbiological origin could be clearly assessed and they were not included in Table 2. In this context it is important to recall that terpenes and related compounds are widespread airborne contaminants, hence caution must be exercised when terpenes production is enthusiastically ascribed for the first time to LAB (Belviso, Giordano, Dolci, & Zeppa, 2011).

In order to study the influence of wheat flour volatiles on the volatile profile of sourdoughs, we studied a control sample obtained from the simple acidification of the dough with lactic acid and acetic acid (Hansen & Hansen, 1994). Acetic acid is the main peak in the chromatogram of the chemically acidified control sample. We also detected nonanal, a lipid oxidation volatile compound, that is formed during storage, due to lipoxygenase activity. This

indicates that almost all aroma compounds in Table 2 come from yeasts and/or LAB activity. At variance with this outcome, pentanal, hexanal, heptanal or 1-hexanol were reported by Czerny and Schieberle (2002), who found that LAB do not produce new flavour compounds. However they did not use an acidified control dough and it can be expected that HS-SPME of wheat flour is different from the HS-SPME of chemically acidified dough. It is highly probable that the presence of acetic acid may saturate the fibre or may impair the release of the small amounts of other flour oxidation products. Acidification was needed to mimic the acidification of the dough during LAB fermentation and to avoid contamination by endogenous flour bacteria that are not suitable for the leavening process. Surely, the type of flour and the extraction procedure may influence this outcome.

3.2.1. Volatile profiles of mono-strain model sourdoughs

Yeasts have the primary leavening role and for this reasons sourdoughs obtained using both baker's yeast and wild *S. cerevisiae* were studied. Unfortunately, using only yeasts, sourdough flavour is due to very few compounds, as already observed (Rehman, Paterson, & Piggott, 2006). 3-Methyl-1-butanol was found only in the presence of yeasts, even if it was reported that LAB may also produce it in a very strain-specific manner (Settanni et al., 2013). In yeast model doughs no acids or aldehydes and ketones were detected, at variance with previous results (Damiani et al., 1996) where a different sampling procedure was used. Of note, diacetyl was detected in the volatile profile of dough fermented by baker's yeast and wild *S. cerevisiae*.

Since acetic bacteria may eventually be isolated from sourdoughs (Minervini et al., 2012), we investigated the volatiles of dough inoculated by *A. cerevisiae*. Acetic fermentation also results in a simple volatiles profile, mainly characterised by acetic acid, butanoic acid, and acetaldehyde. 2-Pentylfuran, produced by all other studied LABs but not by yeasts, was also found. We were also able to detect both acetoin (that was not found in any other sourdough) and diacetyl (De Ley, 1959). However, *A. cerevisiae* was not isolated from any traditional or *ex-novo* sourdoughs.

Volatile profiles of sourdoughs obtained from mono-strain LAB reflect their specific metabolism: ethanol and ethyl acetate are absent for homofermentative *L. crustorum* that however produces, as expected, diacetyl and many other carbonyl compounds. Ethanol and ethyl acetate are always present for heterofermentative and facultative heterofermentative lactic acid bacteria, even if to a different extent. For example, the abundance of ethanol was very low for heterofermentative *Leuc. holzapfelii* and heterofermentative facultative *L. plantarum*; probably, part of the free ethanol may have reacted to give ethyl acetate. Apart from ethanol, 1-hexanol was the dominating alcohol in all these sourdoughs, as already reported (Ravyts & De Vuyst, 2011).

Esters were always detected except for the homofermentative *L. crustorum*. Similarly, only heterofermentative facultative *L. spicheri* does not appreciably give aldehydes and ketones. Acetic acid was only detected for heterofermentative *W. confusa*, even if esters of acetic acid were found for heterofermentative facultative *L. spicheri* and *L. plantarum*, while hexanoic acid was found for homofermentative *L. crustorum* and for heterofermentative facultative *L. plantarum*. The latter gave a very rich volatiles profile thereby confirming what Damiani et al. (1996) found; 26 different compounds were identified most of them being alcohols, aldehydes and ketones. Many of the compounds detailed in Table 2 were not previously reported, probably because of the different sampling techniques or fermentation conditions used by other authors.

L. plantarum and *L. crustorum* have already been investigated as regards their flavour generating potential. Ravyts and De Vuyst (2011) studied both strains but, again, they did not find many of the volatiles in Table 2. The volatiles profiles of dough fermented by *A. cerevisiae*, *W. confusa*, *Leuc. holzapfelii*, and *L. spicheri* have not been studied previously.

3.2.2. Volatile profiles of ex-novo and traditional sourdoughs

The volatile profile of both *ex-novo* and traditional sourdoughs that comprise both yeasts and LAB is more complex that mono-strain sourdoughs, as shown in Table 2. First of all, it can be observed that the most abundant compounds are alcohols and esters that are much better represented than in mono-strain sourdoughs.

Ex-novo sourdoughs, produced using microorganisms present in different flowers, berries, fruits, and the mother of vinegar, are characterised by more volatile compounds than traditional sourdoughs. *Ex-novo* sourdoughs always gave a similar microbiota composition, while traditional sourdoughs have a very rich microbiota, but their volatile profiles are surprisingly less complex than those of *ex-novo* sourdoughs. *trans*-(2-Ethylcyclopentyl)methanol was only found in the volatiles profile of some traditional sourdoughs; tridecane was always absent in the headspace of *ex-novo* sourdoughs and always present in that of traditional samples while for ethyl nonanoate the opposite happens. It follows that these two compounds may be useful to discriminate between *ex-novo* and traditional sourdoughs. Ethyl lactate and isoamyl acetate that are always present in traditional sourdoughs were much less frequently encountered in *ex-novo* samples. Conversely, 2-pentylfuran, always present in *ex-novo* sourdoughs, was less found in traditional samples. Acetoin, diacetyl, butanoic acid, (*E*)-2-hexenal, 2-heptanone, benzaldehyde, 1-octen-3-one, (*E*)-2-octenal (*E,E*)-2,4-octadienal, (*E,E*)-2,4-nonadienal, and 2-undecenal that were occasionally present in single strain sourdoughs, were never present in both *ex-novo* and traditional sourdoughs. The difference between traditional and *ex-novo* samples may in part be due to the fact that the former were propagated for decades in local bakeries and restaurants; hence domesticated strains are selected. On the converse *ex-novo* samples are newly produced and analysed after propagating them only for 30 days and this may imply the presence of wilder strains; even if their microbiota is poorer it may be more active towards the synthesis of volatiles.

3.3. Significance of the presence of specific volatile compounds

Major volatile compounds of sourdough were ethanol and ethyl acetate. Alcohols are the most represented class of compounds. 3-Methyl-1-butanol, that gives the "fermented" flavour, is a clear marker of the yeast's presence. Common alcohols, in order of decreasing volatility, are 1-pentanol, 1-hexanol, 1-heptanol, 1-octen-3-ol, 3-octen-1-ol, 1-octanol, phenylethyl alcohol, and 1-nonanol. None of them were found for AC4, whereas only 1-hexanol and phenylethyl alcohol were found for baker's yeast. Amino acid degradation during dough fermentation by the Ehrlich mechanism leads to odour-active aldehydes or the corresponding alcohols. This flavour-forming pathway catalysed by yeasts (Hansen & Schieberle, 2005) rationalises the conversion of phenylalanine to phenylethyl alcohol. In *S. cerevisiae*, phenylethyl alcohol was found to be a quorum sensing molecule that is a molecule involved in a system of response related to population density: it enables communication dependent on cell density that can regulate several behaviours. Fungal quorum sensing systems research is still in its infancy, and it could eventually lead to the development of new antifungal therapeutics (Albuquerque & Casadevall, 2012). Detection of quorum sensing molecules is a challenging and new concept in microbial ecology: in this context the procedure we put forth (HS-SPME-GC-MS) can be a valid alternative to expensive and laborious extraction of the target compounds 1-Octen-3-ol, known as a mushroom-like flavour, is obtained from

1-octen-3-one, due to the yeast's enone-reductase activity. It may come from the oxidative breakdown of linoleic acid (Assaf, Hadar, & Dosoretz, 1997). The odour types of the found alcohols are fermented, herbal, green, earthy, fruity and floral (http://bioinformatics.charite.de/superscent, 2016 accessed February 2016; http://www.flavornet.org/d_kovats_db5.html accessed February 2016)

As regards esters, we only found either ethyl esters of a specific organic acid or acetates of a specific alcohol. Only *Acetobacter* and *L. crustorum* do not appreciably produce esters. Both *ex-novo* and traditional sourdoughs, characterised by a complex microbiota, give more esters than simple mono-strain sourdoughs. Ethyl acetate apart, ethyl lactate and the ethyl octanoate are the most represented esters.

Aldehydes and ketones are not appreciably produced by *S. cerevisiae* and *Acetobacter*. *L. plantarum* (FE) produces the largest number of different carbonyl compounds. Hexanal, 3-octanone, and nonanal are the most common carbonyl compounds. A very widespread volatile compound is 2-pentylfuran; it could be formed from 2,4-decadienal (Mandin, Duckham, & Ames, 1999) during the autoxidation of linoleate. It is used as a food flavouring agent with floral notes (Wang & Kays, 2000) and it was found to belong, e.g., to the *S. cerevisiae* metabolome, even if its biological properties are still not known (http://www.ymdb.ca, 2016 accessed February 2016).

Dihydro-5-pentyl-2-(3H)-furanone (γ-nonalactone), which seems to be typical of *ex-novo* sourdoughs, was also found in alcoholic beverages, fruits, wheat bread, black tea and other foodstuffs, as well as in rye sourdough fermentations (Ravyts & De Vuyst, 2011). The production of benzaldehyde from phenylalanine using a cell extract of *L. plantarum* has already been described (Masja, Groot, & De Bont, 1998); results obtained with our model sourdough reveal that among all the only sample that produces it is *W. confusa*.

3.4. Compounds identified for the first time or only once in wheat sourdoughs volatile profile

2-Heptanone, pentyl acetate, 1-octen-3-one, 3-octanone, 3-octanol, ethyl hexanoate, hexyl acetate, 2-ethyl-1,6-dioxaspiro[4,4]nonane (chalcogran), ethyl heptanoate, 2,4-octadienal, bicyclo[3.3.1]nonane, 1-nonanol, (*E,E*)-2,4-nonadienal, ethyl octanoate, octyl acetate, 1,3-bis(1,1-dimethylethyl)benzene, *trans*-(2-ethylcyclopentyl)methanol, 2-phenylethyl acetate, ethyl nonanoate, 2-undecenal and ethyl decanoate were found in the headspace of sourdough for the first time, to the best of our knowledge. We detected 2-hexenal, which was already found in the aromatic profile of sourdough bread but not in that of sourdough (Chung & Rengarajan, 1998). *trans*-(2-Ethylcyclopentyl)methanol is another interesting analyte since, along with tridecane, it seems to be specific to traditional sourdoughs: it was found to be emitted by truffle (Splivallo, Bossi, Maffei, & Bonfante, 2007) but was never detected before in sourdoughs. The presence of tridecane (Settanni et al., 2013) may be rationalised taking into account the presence of yeast alkane-signalling gene; some specialised yeasts can use alkanes as a source of carbon and/or energy. Similarly certain types of bacteria can metabolise alkanes. (Rojo, 2009).

It is worth noticing that even though the primary metabolic products of LAB are lactic acids and acetic acid, we never detected lactic acid, probably due to the non-volatility of this compound or to its catabolism to pyruvate. Similarly most acetic acid can be found in the form of acetates.

Table 3
Cluster analysis of all sourdough samples. Sample names are explained in Table 1.

Sample	Type	Cluster
CA	Model	C2
LdB	Model	C3
S.C.	Model	C3
AC4	Model	C2
SGL10	Model	C2
CP2	Model	C2
SP1	Model	C2
PA2	Model	C2
CA1	Model	C2
VP	EN	C1
FdM	EN	C1
MG	EN	C1
SA	EN	C1
AM	EN	C1
My.c.	EN	C3
AMT	T	C3
AST	T	C3
CPT	T	C3
AAT	T	C3
VAT	T	C3
GRFT	T	C3
MT	T	C3
K2T	T	C3

3.5. Correlations among common volatile analytes

Many interesting correlations among volatile compounds in Table 2 were evidenced through the statistical analysis. Table S1 represents the Spearman correlation matrix among common analytes (found at least 6 times in the analysed samples). The meaning of many correlations is obviously an open question but some clear trends will be detailed in the following.

The strongest correlations ($r = 0.9$) were found between the ethyl esters of hexanoic and heptanoic acids and between 1-octen-3-ol and 2-pentyl furan.

Strong correlations ($r \geqslant 0.7$) were also found between

(i) Esters (e.g., ethyl acetate *vs*. isoamyl acetate, ethyl heptanoate *vs*. 2-phenylethyl acetate, ethyl heptanoate *vs*. ethyl octanoate, ethyl octanoate *vs*. ethyl nonanoate, pentyl acetate *vs*. ethyl hexanoate, pentyl acetate *vs*. hexyl acetate, ethyl octanoate *vs*. 2-phenylethyl acetate, hexyl acetate *vs*. ethyl octanoate, hexyl acetate *vs*. ethyl nonanoate), thereby indicating similar biosynthetic pathways for different esters.
(ii) Alcohols (e.g., 1-hexanol *vs*. 1-heptanol; 1-hexanol *vs*. 1-octen-3-ol; 1-octanol *vs* 1-nonanol; 1-octen-3-ol *vs*. 2-octen-1-ol), thereby indicating similar biosynthetic pathways for different alcohols.
(iii) Aldehyde and the corresponding alcohol (nonanal *vs*. 1-nonanol), since they represent an oxidised and reduced pair of the same molecule.
(iv) Alcohol and the corresponding ester (e.g. phenylethyl alcohol *vs*. 2-phenylethyl acetate,, 3-methyl-1-butanol (isoamyl alcohol) *vs*. isoamyl acetate), since the alcohol is necessary for the biosynthesis of the ester.
(v) Between an alcohol and a generic ester (e.g., 3-methyl-1-butanol *vs*. ethyl acetate, 1-octanol *vs*. ethyl octanoate, 1-octanol *vs*. ethyl nonanoate, phenylethyl alcohol and ethyl octanoate, 1-octanol *vs*. hexyl acetate).

All in all this evidence indicates that the biosynthetic pathways of these classes of compounds interlock with each other. However, the reason for the strong correlation between tridecane and esters (ethyl lactate and isoamyl acetate) needs to be clarified. Other significant, if not so strong, correlations exist between other esters, e.g., ethyl lactate *vs*. ethyl acetate. It is also interesting to observe that ethanol is slightly positively correlated to 3-methyl-1-butanol and phenylethyl alcohol and, to a less extent, to various ethyl esters, whereas 1-pentanol is correlated to 1-hexanol.

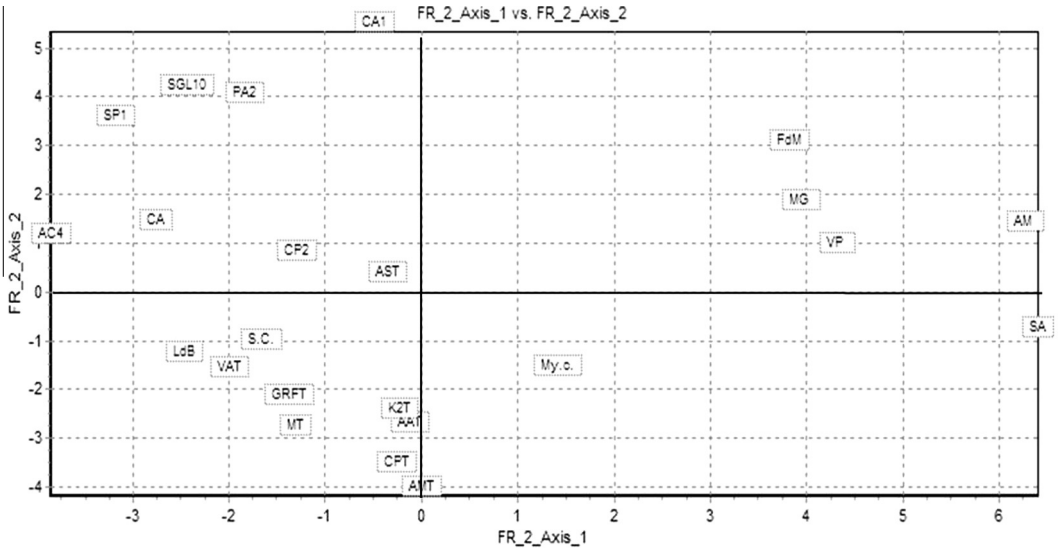

Fig. 2. Score plot of first and second principal components after PCA based on volatile components of all sourdough samples. Sample names are explained in Table 1.

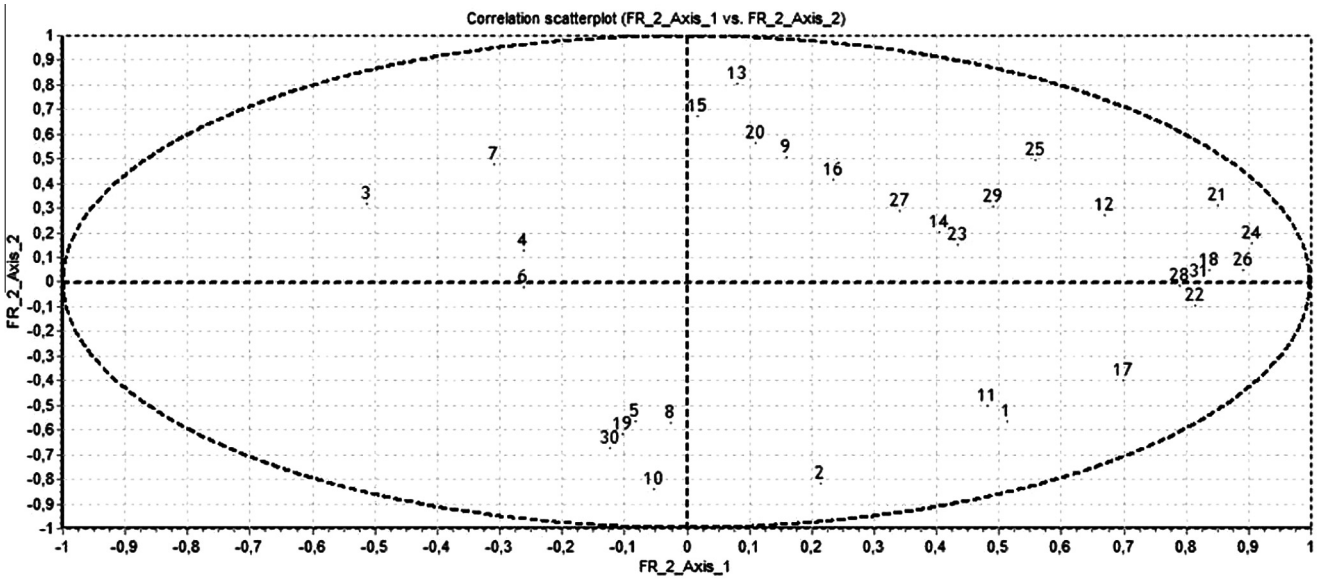

Fig. 3. Loading plot of first and second principal components PCA analysis based on volatile components of all sourdough sample. Volatile compounds: (1) ethanol, (2) ethyl acetate, (3) diacetyl, (4) acetic acid, (5), 3-methyl-1-butanol, (6) 1-pentanol, (7) hexanal, (8) ethyl lactate, (9) 1-hexanol, (10) isoamyl acetate, (11) pentyl acetate, (12) 1-heptanol, (13) 1-octen-3-ol, 14) 3-octanone, (15) 2-pentylfuran, (16) 3-octanol, (17) ethyl hexanoate, (18) hexyl acetate, (19) *trans*-(2-ethylcyclopentyl)methanol, (20) (*Z*)-2-octen-1-ol, (21) 1-octanol, (22) ethyl heptanoate, (23) nonanal, (24) phenylethyl alcohol, (25) 1-nonanol, (26) ethyl octanoate, (27) decanal, (28) acid, 2-phenylethyl acetate, (29) ethyl nonanoate, (30) tridecane, (31) ethyl decanoate.

Significant negative correlations ($r \leqslant -0.5$) are not as numerous as the positive ones. An important negative correlation can be found between 3-methyl-1-butanol and 2-pentylfuran, and since 1-octen-3-ol and 2-pentylfuran are strongly correlated, 3-methyl-1-butanol and 1-octen-3-ol are negatively correlated. Interestingly, ethanol is negatively correlated to hexanal.

3.6. Principal Component Analysis (PCA) and clustering of sourdough samples

In this study, PCA was used as an exploratory technique to identify groups among samples based on their volatile profile and to find the discriminating power of the variables. PCA provided an overview of the capacity of the variables (analytes found by the HS-SPME/GC-MS measurements) to discriminate single strain, laboratory-made, and traditional sourdough samples. PCA highlighted statistically significant differences among these groups.

After applying PCA to the raw data set, two principal components (Axis 1 and Axis 2) were extracted according to the Spearman algorithm. The percentages of variance explained were, respectively 32.14% and 24.30%, hence the cumulative explained variance was 56.44%. Visual clustering of sourdoughs was apparent when the scores of the samples were displayed with respect to the first two principal components (Fig. 2). Cluster analysis detailed in Table 3 aims at assigning individual samples into groups (C1, C2, C3) based on their volatiles profiles molecular fingerprint. In the present study, C1 contains *ex-novo* samples, C2 is characterised by single strain samples, and C3 groups traditional samples. The negative control is grouped with single strain samples. The clustering model, however, goes wrong in two cases: it classifies baker's yeast and wild *S. cerevisiae* as traditional samples (this is not

surprising given the relevance of yeasts in traditional sourdough) and it classifies an *ex-novo* sample, namely My.c., as a traditional one probably because of the lower amounts of heavier esters and alcohols.

Fig. 2 can be rationalised on the basis of the correlation scatter plot shown in Fig. 3, hence the two figures have to be analysed comparatively. Fig. 3 indicates that Axis 1 discriminates, for positive values, the presence of alcohol compounds (e.g. 1-octanol) and high molecular weight esters (e.g. hexyl acetate) and, for negative values, the presence of acetic acid. Axis 2 discriminates, for negative values, the presence of low molecular weight esters (e.g., ethyl acetate) and for positive values aldehydes (e.g., hexanal) and high molecular weight alcohols (e.g., 1-octen-3-ol).

The fact that traditional sourdoughs are more prone to the production of ethanol and of low molecular weight esters rather than heavier esters (such as ethyl decanoate) and heavier alcohols (such as (*Z*)-2-octen-1-ol) explains the location of their cluster. In this context it is worth noticing that sample AST is slightly singled out from other traditional samples; this can be rationalized taking into account the fact that its microbiota is the most complex and two LAB strains present in its microbiota, namely *L. sakei* and *L. brevis*, were not isolated from any other sourdough sample. The closeness between the samples fermented by the wild *S. cerevisiae* (SC) and *S. cerevisiae* from the baker's yeast (LdB) is rewarding and indicates the similarity of their flavour profile. The fact that both these samples are grouped with traditional sourdough samples in this lower left quadrant confirms the main role of yeasts in the leavening process of traditional sourdough.

The vectors of tridecane and *trans*-(2-ethylcyclopentyl) methanol in the lower left direction (third quadrant of Fig. 3) are distributed in the negative region of Axis 1 and in the negative region of Axis 2 in Fig. 2 because both analytes were only found in the headspace of traditional sourdoughs. The second quadrant in Fig. 3 contains the vectors of acetic acid and diacetyl associated with AC4, fermented by *A. cerevisiae,* and negative control (CA) acidified via acetic acid; acetic acid dominates the volatile profile of both samples. In Fig. 2, in this quadrant we also find single-bacteria model dough samples, maybe for their incapacity of producing high levels of esters and for their ability to yield aldehydic compounds. The only model dough far from other single strain doughs is CA1, that is the sample fermented by the facultative heterofermentative *L. plantarum* (CA1), the volatile profile of which is very complex. Interestingly, the presence of the homofermentative strain (SGL10) in the most positive position of Axis 2 indicates its higher ability to produce aldehydes (in agreement with the results in literature). In this context it has to be emphasized that all samples fermented with at least a homofermentative strain (namely traditional sourdough samples AMT, AST, AAT, GRFT, and MT) accumulate close to Axis 2. The complexity of their microbiota scatters their score on Axis 2, but none of them is able to give considerable amounts of heavier esters or heavier alcohols.

Most vectors representing alcohols and carbonyl compounds point upper right in Fig. 3 and are in the first quadrant, with aldehydes characterised by a positive Axis 2. All ex novo sourdough samples are found in the corresponding first quadrant in Fig. 2. This indicates that, at variance with traditional samples, *ex-novo* ones are able to produce many alcohols and esters, including the heavier ones. They actually produce the highest number of volatile compounds. The same microbiota, in different sourdough samples, does not generate identical volatile profiles: It is well known that the proteolytic activity of LAB to give amino acids in dough is strain-specific (Gänzle, Loponen, & Gobbetti, 2008). Amino acid degradation during dough fermentation by the flavour-forming Ehrlich mechanism, catalysed by yeasts (Hansen & Schieberle, 2005), leads to odour-active aldehydes or the corresponding alcohols. It follows that since the first step is strain-specific the resulting final flavour compounds are strain-specific too as already observed. This rationalizes our experimental evidence: for example, *ex-novo* samples VP, MG, AM, My.c., and traditional sample K2T all share the same microbiota; nevertheless the traditional sample K2T, propagated for decades, is quite separate in Fig. 2 from *ex-novo* samples with the same microbiota. This further confirms that volatile compounds production is not species-specific but strain-specific (Hansen & Hansen, 1994; Settanni et al., 2013).

It can be concluded that PCA of the volatile compounds can clearly differentiate laboratory-made model doughs, traditional and ex novo sourdoughs.

4. Conclusions

In sourdoughs, flavour-active compounds are produced by yeasts and LABs both individually and via their interaction in a strain-specific manner. HS-SPME-GC-MS along with the identification of the isolated yeasts and bacteria proved to be useful to describe diverse volatile profiles on the basis of different metabolisms. This study can contribute to the management of desirable metabolites, *via* a flavour fine-tuning fermentation, in an effort to optimise and differentiate specialty products. It is clear that the next step in this research will be the evaluation of the volatile compounds emitted by breads manufactured from the studied sourdough samples.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.02.150.

References

Acree, T. E., & Arn, H. (2007). *Cornell University*.Geneva, NY: NYSAES.

Albuquerque, P., & Casadevall, A. (2012). Quorum sensing in fungi, a review. *Medical Mycology, 50*(4), 337–345.

Alfonzo, A., Ventimiglia, G., Corona, O., Di Gerlando, R., Gaglio, R., Francesca, N., ... Settanni, L. (2013). Diversity and technological potential of lactic acid bacteria of wheat flours. *Food Microbiology, 36*, 343–354.

Assaf, S., Hadar, Y., & Dosoretz, C. G. (1997). 1-Octen-3-01 and 13-hydroperoxylinoleate are products of distinct pathways in the oxidative breakdown of linoleic acid by Pleurotus pulmonurius. *Enzyme and Microbial Technology, 21*, 484–490.

Belviso, S., Giordano, M., Dolci, P., & Zeppa, G. (2011). Degradation and biosynthesis of terpenoids by lactic acid bacteria isolated from cheese: first evidence. *Dairy Science & Technology, 91*, 227–236.

Cecchi, T. (2014). Head spaced solid phase micro extraction profile of volatile organic compounds emitted from parquet samples. *Journal of Wood Chemistry and Technology, 34*, 211–224.

Cecchi, T., & Alfei, B. (2013). Volatile profiles of Italian monovarietal extra virgin olive oils via HS-SPME-GC-MS: newly identified compounds, flavours molecular markers, and terpenic profile. *Food Chemistry, 141*, 2025–2035.

Cecchi, T., Passamonti, P., & Cecchi, P. (2010). Study of the quality of extra virgin olive oil stored in PET bottles with or without an oxygen scavenger. *Food Chemistry, 120*, 730–735.

Chung, L. M., & Rengarajan, R. (1998). Volatiles in selected commercial breads. *Cereal Chemistry, 75*(6), 847–853.

Corsetti, A., & Settanni, L. (2007). Lactobacilli in sourdough fermentation, Review. *Food Research International, 40*, 539–558.

Czerny, M., & Schieberle, P. (2002). Important aroma compounds in freshly ground wholemeal and white wheat flour-identification and quantitative changes during sourdough fermentation. *Journal of Agricultural and Food Chemistry, 50*, 6835–6840.

Damiani, P., Gobbetti, M., Cossignani, L., Corsetti, A., Simonetti, M. S., & Rossi, J. (1996). The Sourdough Microflora. Characterization of hetero- and homofermentative lactic acid bacteria, yeasts and their interactions on the basis of the volatile compounds produced. *Food Science and Technology, 29*, 63–70.

De Ley, J. (1959). On the formation of acetoin by *Acetobacter*. *Journal of General Microbiology, 21*, 352–365.

Di Cello, P. C., Ventura, F., Fani, M. R., & Guckert, A. (1999). Frequency and biodiversity of 2,4-diacetylphloroglucinol-producing bacteria isolated from the maize rhizosphere at different stages of plant growth. *Applied and Environmental Microbiology, 66*, 948–955.

Erickson, R. P., & Covey, E. (1980). On the singularity of taste sensations: What is a taste primary? *Physiology and Behaviour, 25*, 527–533.
Gänzle, M., Loponen, J., & Gobbetti, M. (2008). Proteolysis in sourdough fermentations: Mechanisms and potential for improved bread quality. *Trends in Food Science and Technology, 19*, 513–521.
Hansen, B., & Hansen, A. (1994). Volatile compounds in wheat sourdoughs produced by lactic-acid bacteria and sourdough yeasts. *Zeitschrift fur lebensmittel-untersuchung und-forschung, 198*, 202–209.
Hansen, Å. S., & Schieberle, P. (2005). Generation of aroma compounds during sourdough fermentation: applied and fundamental aspects. *Trends in Food Science & Technology, 16*, 85–94.
<http://bioinformatics.charite.de/superscent> Accessed February 2016 Acree, T., E., & Arn, H.
<http://www.flavornet.org/d_kovats_db5.html,> Accessed February 2016 . Cornell University, NYSAES, Geneva, NY, 2007.
<http://www.ymdb.ca> Accessed February 2016.
Huey, B., & Hall, J. (1989). Hypervariable DNA fingerprinting in *E. coli* minisatellite probe from bacteriophage M13. *Journal of Bacteriology, 171*, 2528–2532.
Linstrom, P. J., & Mallard, W. G. (Eds.). (2013). *NIST Chemistry Webbok. NIST Standard Reference Database Number 69. Gas Chromatography-Retention Indices by NIST Mass Spectrometry Data Center, Stein SE director, versions: 2005, 2008 and 2011 NIST, Gaithersburg, MD*.
Lönner, C., & Ahrné, S. (1995). Lactobacillus: Baking. In Y. H. Hui & G. G. Khachatourians (Eds.), *Food biotechnology microorganisms* (pp. 797–844). Eureka, California: VCH Publishers Inc.
Mandin, O., Duckham, S. C., & Ames, J. M. (1999). Volatile components from potato-like model systems. *Journal of Agricultural and Food Chemistry, 47*, 2355–2359.
Masja, N., Groot, N., & De Bont, J. A. M. (1998). Conversion of phenylalanine to benzaldehyde initiated by an aminotransferase in *Lactobacillus plantarum*. *Applied and Environmental Microbiology, 64*, 3009–3013.
Minervini, F., De Angelis, M., Di Cagno, R., & Gobbetti, M. (2014). Ecological parameters influencing microbial diversity and stability of traditional sourdough. *International Journal of Food Microbiology, 3*(171), 136–146.
Minervini, F., Lattanzi, A., De Angelis, M., Di Cagno, R., & Gobbetti, M. (2012). Influence of artisan bakery- or laboratory-propagated sourdoughs on the diversity of lactic acid bacterium and yeast microbiotas. *Applied Environmental Microbiology, 78*, 5328–5340.
Ravyts, F., & De Vuyst, L. (2011). Prevalence and impact of single-strain starter cultures of LAB on metabolite formation in sourdough. *Food Microbiology, 28*, 1129–1139.
Rehman, S., Paterson, A., & Piggott, J. R. (2006). Flavour in sourdough breads: a review. *Trends in Food Science and Technology, 17*(10), 557–566.
Reiners, J., & Grosch, W. (1998). Odorants of virgin olive oils with different flavor profiles. *Journal of Agricultural and Food Chemistry, 46*, 2754–2763.
Ribeiro, L. H., Costa Freitas, A. M., & Gomes da Silva, M. D. R. (2008). The use of headspace solid phase microextraction for the characterization of volatile compounds in olive oil matrices. *Talanta, 77*, 110–117.
Ripari, V. (2013). *Pasta madre: tradizione e modernità* (Ph.D. thesis). Università Politecnica delle Marche.
Rojo, F. (2009). Degradation of alkanes by bacteria. *Environmental Microbiology, 11*, 2477–2490.
Sandhu, G. S., Kline, B. C., Stockman, L., & Roberts, G. D. (1995). Molecular probes for diagnosis of fungal infections. *Journal of Clinical Microbiology, 33*, 2913–2919.
Settanni, L., Ventimiglia, G., Alfonzo, A., Corona, A. O., Miceli, A., & Moschetti, G. (2013). An integrated technological approach to the selection of lactic acid bacteria of flour origin for sourdough production. *Food Research International, 54*(2), 1569–1578.
Splivallo, R., Bossi, S., Maffei, M., & Bonfante, P. (2007). Discrimination of truffle fruiting body versus mycelial aromas by stir bar sorptive extraction. *Phytochemistry, 68*(20), 2584–2598.
Van Den Dool, H., & Kratz, P. D. (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography, 11*, 463–471.
Wang, Y., & Kays, S. J. (2000). Contribution of volatile compounds to the characteristic aroma of baked 'jewel' sweetpotatoes. *Journal of the American Society for Horticultural Science, 125*, 638–643.