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Effects of fermentation and rye flour on microstructure and volatile compounds of chestnut flour based sourdoughs

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The study is aimed at developing a new cereal-based product, with increased nutritional quality, by using natural fermentation of blends of chestnut and rye flour. In spite of the remarkable similarity, the technological potential of combinations of both flours has never been explored before. Three spontaneous chestnut/rye sourdough fermentations were performed over a period of twelve days with daily back-slopping. Samples taken at all refreshment steps were used for culture-dependent and culture-independent evaluation of the microbiota present. Dominant species basically overlapped to those associated to sourdoughs strengthened with chestnut flour, such as *Pediococcus pentosaceus* or *Weissella paramesenteroides*. Microstructures, evaluated by means of Scanning Electron Microscopy, revealed the presence in chestnut sourdoughs of a distinguishable network surrounding starch granules, while rye flour-added sourdoughs showed a less structured matrix. By gas chromatography coupled to mass spectrometry, 51 volatile organic compounds were identified at 24 h and after prolonged fermentation. Within volatile organic compounds, alcohols, esters, acids, aldehydes and ketones, all well-known flavour compounds in sourdough fermentation, appeared as dominant. The PCA discriminated the sourdoughs into three distinct clusters and highlighted a clear influence of fermentation time on the volatile composition of sourdoughs.

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1. Introduction

The use of the sourdough process as a means of leavening is one of the oldest biotechnological processes in cereal food production. Sourdough is a mixture of mainly cereal flour and water, which is made metabolically active by a heterogeneous population of lactic acid bacteria (LAB) and yeasts, either by spontaneous fermentation or by fermentation initiated through the addition of a sourdough starter culture, whether or not involving backslopping (De Vuyst, Vrancken, Ravyts, Rimaux, & Weckx, 2009). Sourdough fermentation has proved to be essential in rye (*Secale cereale* L.) bread making by ensuring the dough acidification and inhibition of amylases activity, as well as by improving the water binding capacity of starch (Weckx et al., 2010). Furthermore, rye sourdough fermentation improves the nutritional value of rye bread by increasing the levels of bioactive (Weckx et al., 2010) and odour active compounds (Kirchoff & Schieberle, 2002).

Rye flour (RF) is known to contain many essential and nonessential dietary components and B-complex vitamins (Mihhalevski, Nisamedtinov, Hälvin, Ošeka, & Paalme, 2013). As rye does not contain enough gluten, the structure of rye bread depends on the starch in the rye flour, as well as other carbohydrates known as pentosans (Wing & Scott, 1999 , p. 255).

In recent times, there has been a growing interest in the use of chestnut (*Castanea sativa* Mill) flour for the production of leavened bakery. Chemical composition of chestnut flour (CF) is close to that of many cereals, with starch (50–60%) as main component. The high quality proteins with essential amino acids (4–7%), as well as the content in mineral salts and vitamins (B1, E and C) make CF a worthy ingredient for healthy bakery products.

Moreover, CF is a rich source of phytochemicals and polyphenolics, with gallic and ellagic acid as predominant among hydrolysable and condensed tannins (De Vasconcelos, Bennett, Rosa, & Ferreira-Cardoso, 2010; Durazzo, Turfani, Azzini, Maiani,

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& Carcea, 2013). CF contains interesting levels of lignans, compounds known to exert significant positive effects on human health (Durazzo et al., 2013). On the other hand, phenolic compounds, particularly phenolic acids and tannins, exhibit antimicrobial activity (De Vasconcelos et al., 2010). As matter of fact, in sourdoughs realized with the sole chestnut flour, the achievement of the microbial equilibrium may require a longer time (Aponte et al., 2013). CF needs to be mixed with other flours and the definition of the exact content of CF in the blend becomes crucial. Encouraging results were achieved when moderate levels of CF are added to wheat (Dall'Asta et al., 2013) or to rice flour (Demirkesen, Mert, Sumnu, & Sahin, 2010). Indeed, in several traditional recipes to prepare breads, other than cakes, and pancakes, the percentage of CF in the dough is generally around 40%. A further confirmation about this chestnut ratio may be retrieved in surveys of Demirkesen et al. (2010); Demirkesen, Sumnu, Sahin, and Uysal (2011): according to authors, a ratio 40/60 chestnut/rice flour, appeared to be a good compromise to obtain bread with fair firmness, density and colour, but still characterized by a good fibre content.

With respect to the increasing demand for functional cereal-based products with high nutritional quality, this study focuses on the potential use of blends of CF and RF, since both flours are characterized by a fair content in dietary fibre, polyphenolic compounds and essential amino acids. Moreover, the cultivations of chestnut and rye, share the same mountain environment, because of their ability to withstand cold. In detail, the impact of natural fermentation on the volatile organic compounds (VOCs) formation, by means of gas chromatography coupled to mass spectrometry (GC/MS), and on the microstructure, as revealed by Scanning Electron Microscopy (SEM) analysis, was evaluated during sourdoughs maturation of blends of RF and CF. Microbial dynamics were followed by means of a culture-independent PCR-DGGE-based method.

2. Materials and methods

2.1. Samples

RF and CF, used in the preparation of laboratory sourdoughs, were kindly provided by Ipafood (Avellino, Italy). Nutritional characteristics (g/100 g) can be reassumed as follows: CF – 10.7 moisture, 69.3 carbohydrates, 4.6 proteins, 9.5 fibre, 3.8 fat, 1.99 minerals; RF – 12.5 moisture, 73.5 carbohydrates, 9.5 proteins, 2.5 fibre, 1.5 fat, 0.5 minerals.

2.2. Laboratory mature sourdough preparation

Sourdoughs from chestnut flour (100% wt/wt) (A) and chestnut and rye flours B (60/40% wt/wt) or C (40/60% wt/wt) were prepared according to a procedure previously described (Aponte et al., 2013). Briefly, sourdoughs were prepared from 100 g of flour and about 100 g of sterile water (yield dough about 200), in sterile 500 mL containers. Bakery yeast isolated in pure culture was added (about 10^7 CFU/g) at the sole first step. After 24 h of incubation, backslopping was performed with 10% of the ripe sourdough and repeated every 24 h. Fermentations were propagated, in asepsis and at room temperature, until a stable microbiota was established. Each trial was performed in triplicate. At each refreshment step, samples, taken from the ripe sourdough, were submitted to pH and total titratable acids (TTA) determination according to a standard method (Aponte et al., 2013). LAB and yeast loads were evaluated by counting on MRS modified and YGC according to Aponte et al. (2013), respectively. 10-g samples were taken from the sourdoughs C at seven selected times (24, 48, 72, 96, 120, 168 and 288 h) and submitted to DNA extraction according to a protocol previously described (Aponte et al., 2013). Amplicons of hypervariable region V3 within 16S rDNA were obtained by nested PCR according to a routinely procedure previously detailed (Aponte et al., 2013). PCR products were, then, analysed by DGGE using a Bio-Rad D-code apparatus. Parallel electrophoresis experiments were performed at 60 °C in 1× TAE buffer, by using polyacrylamide gels (8% wt/vol) containing 30–60% urea-formamide denaturing gradient (100% corresponded to 7 mol/L urea and 40%, wt/vol, formamide) increasing in the direction of the electrophoresis. The gels were run for 10 min at 50 V and 4 h at 200 V, stained with ethidium bromide for 5 min and rinsed for 20 min in distilled water. DGGE bands to be sequenced were purified and sequenced according to Aponte et al. (2013). Research for DNA similarity was performed with the National Centre of Biotechnology Information GenBank.

2.3. Scanning Electron Microscopy (SEM) analysis

Small portions of each sample were cut, fixed in 10% glutaraldehyde and sequentially embedded in acetone solutions of increasing concentration to ensure full dehydration. Samples were dried at the critical point and coated with gold particles. Microstructure was examined by means of Scanning Electron Microscopy (LEO EVO 40 SEM, Zeiss, Germany) with a 20 kV acceleration voltage and a magnification of × 1.000.

2.4. Characterization of volatile organic compounds (VOCs)

2.4.1. Head space solid phase microextraction (HS-SPME) analysis

The volatile fraction of samples was analysed by headspace sampling, using the solid phase microextraction technique (HS-SPME). For each SPME analysis, 2 g of sourdough (24 and 288 h) were placed into a 20 mL headspace vial, and added of 2 mL of distilled water and 5 μL of 4-methyl-2 pentanol (internal standard, 100 mg/L standard solution). The vial was placed in a thermostatic block (40 °C) on a stirrer and the fibre was inserted and maintained in the sample head space for 30 min, than it was removed and immediately inserted into the GC–MS injector for the desorption of compounds. For the analyses, a silica fibre, coated with 85 μm of Carboxen–Polydimethylsiloxane (Carboxen/PDMS) according to Aponte et al. (2013) was used (Supelco, Bellefonte, PA, USA).

2.4.2. Gas chromatography–mass spectrometry (GC–MS) analysis

For VOCs evaluation, an Agilent Technologies (Agilent Technologies, USA) 7890A gas-chromatograph coupled to an Agilent Technologies 5975 mass spectrometer equipped with a 30 m × 0.25 mm ID, film thickness 0.25 μm capillary column (HP-INNOWAX, Agilent Technologies, USA) was used. Gas carrier was Helium (flow 1.5 mL/min) and SPME injections were splitless (straight glass line, 0.75 mm I.D.) at 240 °C for 20 min during which time thermal desorption of analytes from the fibre occurred. The oven parameters were as follows: initial temperature was 40 °C held for 3 min, followed by an increase to 240 °C at a rate of 5 °C/min, then held for 10 min. Injector temperature was 240 °C. Mass spectrometer operated in scan mode over mass range from 33 to 300 amu (2 s/scan) at an ionization potential of 70 eV. Mass spectral matches were made by comparison of mass spectra and retention time with those of MS database (Wiley7, Nist 05). A semi-quantitive analysis was obtained by comparison of the VOCs peak areas with that of internal standard (4-methyl-2 pentanol), obtained from the total ion chromatograms, using a response factor of 1. Blank experiments were conducted in two different modalities: blank of the fibre and blank of the empty vial. These types of control were carried out every 20 analyses. All analyses were performed in triplicate.

2.5. Statistical analysis

Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). In order to evaluate the effect of fermentation, analysis of variance was carried on physico–chemical and microbial parameters as well on VOCs groups. Significant differences between the detected parameters were compared by means of Duncan's multiple comparison test at the 95% confidence level ($p \leq 0.05$). For each VOCs group and type of sourdough, statistical differences were evaluated for data at fermentation times by using a t-Student test where $p < 0.05$ was considered as statistical significant. Furthermore, a principal component analysis (PCA) was performed to visualize possible relationships within the data matrix. To decide the number of principal components (PCs), the eigen values of the correlation matrix, indicating the percentage of variability explained by each component, were tabulated and a scree plot was constructed.

3. Results and discussion

3.1. Sourdoughs characteristics

During monitoring, the three kinds of sourdoughs, namely 100% CF (A), 40/60% RF/CF (B), and 60/40% RF/CF (C), exhibited a different behaviour (Fig. 1). In sourdoughs obtained by mixing of flours, pH dropped of about two units (from 5.57 ± 0.02 to 4.74 ± 0.01 and from 5.97 ± 0.01 to 4.56 ± 0.04 for B and C, respectively) within the first 48 h, and then stayed almost stable up to the end of monitoring (288 h) (Fig. 1). Conversely, pH of A sourdoughs entirely realized with CF, never dropped below 5.00 (Fig. 1). As expected, sourdoughs produced by mixing RF and CF in ratio 40/60 (B) exhibited the highest TTA levels (Fig. 1). At any rate, acidity of the renewed sourdoughs quickly increased over 15 mL of NaOH per 10 g sourdough after the first 24 h of incubation (Fig. 1).

By the day two, a stabilization of LAB populations was reached in C sourdoughs, while three days more were required for sourdoughs A and B, to reach the same population level (Fig. 1). After, one week, the three sourdoughs exhibited a perfectly comparable trend (Fig. 1). LAB loads at the equilibrium perfectly matched those reported by Viiard, Mihhalevski, Rühka, Paalme, and Sarand (2012) for aerobic LAB counts in rye industrial sourdoughs.

A stable yeast microbiota was achieved by the day six in sourdoughs based on mixing of RF and CF (B and C), while an unusual trend was recorded in trial A: yeast loads stayed almost one log higher for the entire period of monitoring. Yeast/LAB ratio was around 1:10 in almost all the samples, reflecting the typical proportion existing in mature sourdoughs (Aponte et al., 2013).

Fluctuations within the bacterial population of samples of sourdoughs obtained by mixing RF and CF in ratio 60/40% (C) were evaluated by means of a culture independent approach PCR-DGGE based. Samples of C sourdoughs were collected at 24, 48, 72, 96, 120, 168 and 288 h and submitted to DNA extraction followed by DGGE analysis of V3 amplicons. Major bands were excised, checked for co-migration, sequenced and submitted to BLAST comparison

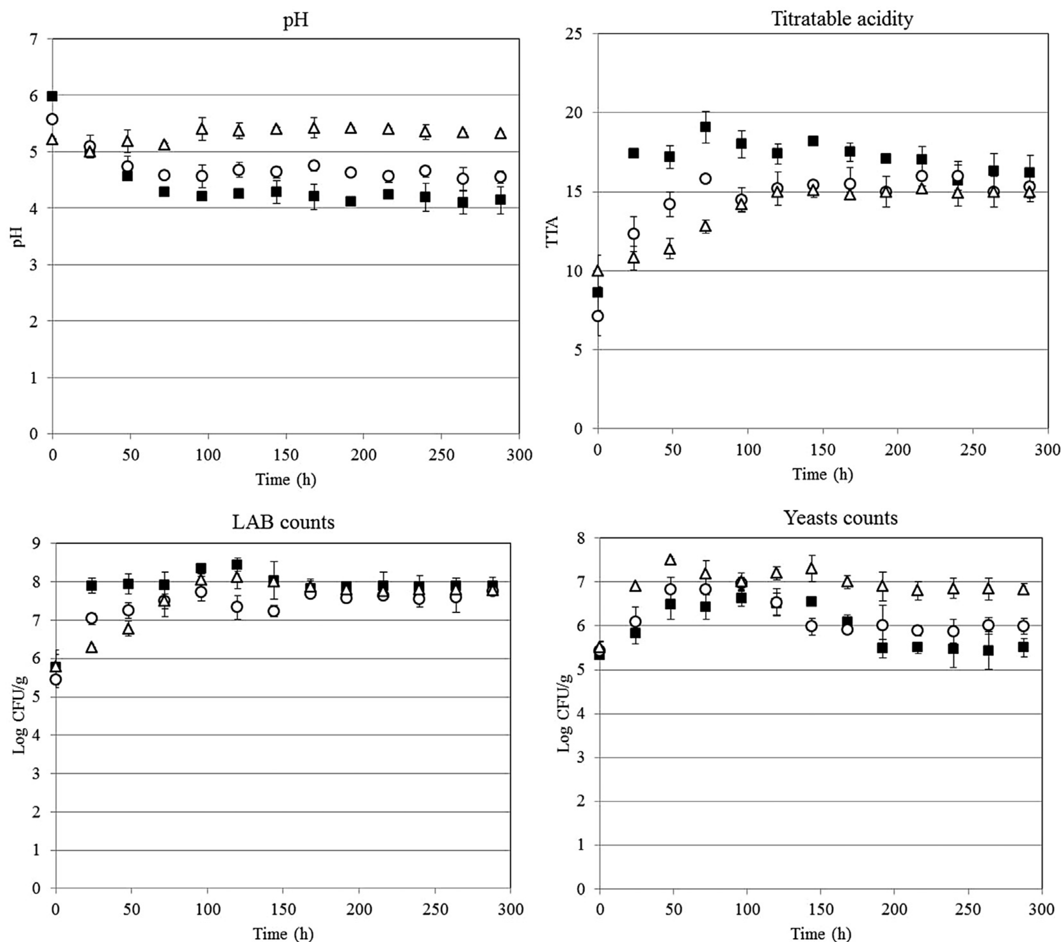

Fig. 1. Evolution of LAB, yeasts and measured chemical parameters at each refreshment step during the course of propagation of sourdough fermentation batches A (△), B (○) and C (■).

with the nucleotide database of the NCBI. According to data, sourdoughs appeared characterized by an extremely poor bacterial variety throughout the ripening (Fig. 2). Dominant species could be related to the genera *Lactobacillus* spp. and *Pediococcus* spp. Bands referable to the genus *Weissella* were retrieved as well. Actually, these genera dominate the microbial ecosystem of type I sourdoughs (Scheirlinck et al., 2007). In detail, one major band, common to all sourdoughs, was reported to *Pediococcus* (*Pd.*) *pentosaceus* (similarity level of 100% to accession number NR042058.1). Weckx et al. (2010) detected members of this species in rye sourdoughs by DGGE analysis as well as by strains identification. A couplet of faint bands, visible in all samples, could be reported (99% of similarity at least) to the following *Lactobacillus* (*Lb.*) species: *Lactobacillus fabifermentans* (GenBank: accession no. NR042676.1), *Lactobacillus plantarum* (NR042394.1), *Lactobacillus plantarum* subsp *argentoratensis* (NR042254.1), *Lactobacillus paraplantarum* (NR042676.1) and *Lactobacillus pentosus* (NR029133.1). These species, referable to the so-called *Lb. plantarum* group, have a 16S rRNA sequence similarity of over 99% and may be distinguish by *hsp60* sequencing or *hsp60* PCR-RFLP analysis (Blaiotta et al., 2008). Actually, members of the *Lb. plantarum* group are considered the main species dominating sourdough fermentation processes that are characterized by low incubation temperatures and continuous backslopping (De Vuyst et al., 2009). *Lactobacillus farciminis*, a typical sourdough LAB (De Vuyst et al., 2009), was visible above all at 48 and 72 h of ripening. Results are in agreement with other studies on the bacterial population in Italian sourdoughs (Aponte et al., 2013). *Lactobacillus sanfranciscensis*, a key organism in traditionally produced wheat and rye sourdoughs (Sekwati-Monang, Valcheva, & Gänzle, 2012), was not found in our samples, even if a selective substrate to isolate this species was used. Likely, antimicrobially active phenolic compounds may act against *Lb. sanfranciscensis.* Such phenomenon has already been recorded in sorghum (Sekwati-Monang et al., 2012).

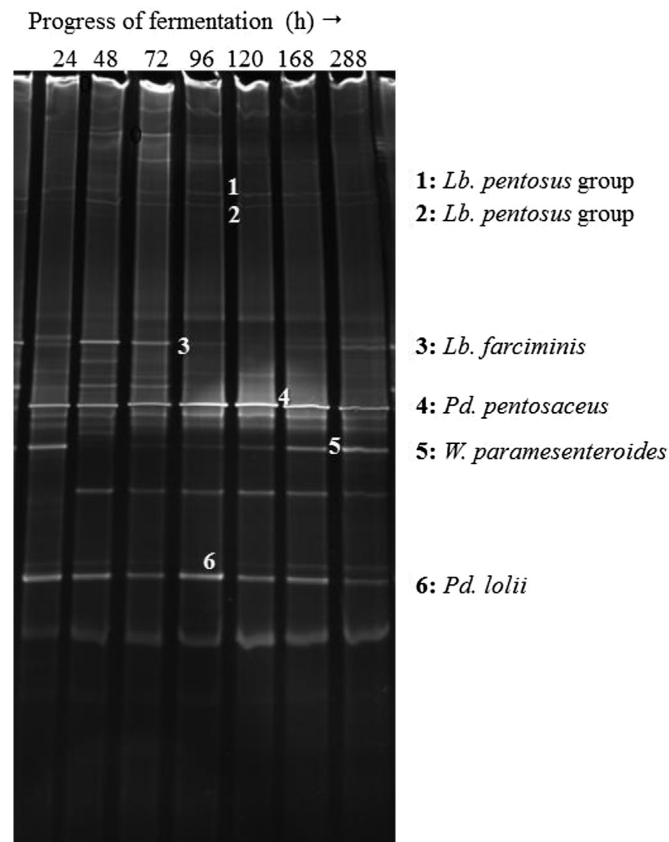

Fig. 2. V3 16S rRNA-PCR-DGGE analysis of rye/chestnut sourdough fermentations (C), daily back-slopped, and sampled after 24, 48, 72, 96, 120, 144, 168, and 288 h of fermentation.

Microbiota of sourdoughs propagated in wheat or rye flours do not exhibit characteristic differences (Sekwati-Monang et al., 2012). However, the use of other cereal flours or pseudocereals selects for fermentation microbiota that differ from wheat and rye sourdoughs (Sekwati-Monang et al., 2012). In this perspective, obtained results do not surprise. Microbiota of back-slopped rye/chestnut sourdoughs only partially overlap with the conventional microbiota of wheat and rye sourdoughs. More evident similarities may be found with the microbiota of blends of different flours in combination with a 40% of CF, as reported in a previous study (Aponte et al., 2013).

3.2. Microscopic observation of sourdoughs

The SEM images of sourdoughs A, B and C after 0, 24, and 288 h of fermentation are shown in Fig. 3. All sourdoughs contained small as well as large starch granules of lenticular and spherical shapes that were distributed throughout the protein matrix. This observation agrees with the report of Kim, Morita, Lee, and Moon (2003) on dough and sourdough bread. Starch granules present in A sourdoughs (Fig. 3, panel A_0) were smooth, oval and irregular. The sizes of granules ranged from small to medium, having diameters from 5 to 30 μm. These values are in accordance with previous results obtained for starch granules isolated from *Castanea sativa* (Correia, Cruz-Lopes, & Beirao-da-Costa, 2012). The SEM images of B (40% RF) and C (60% RF) exhibited a proteic matrix in which the starch granules were clearly visible (Fig. 3, panel B_0 and C_0). No significant effects of RF addition on the microstructure of the three sourdoughs could be observed after mixing ($t = 0$ h) (Fig. 3, panel A_0–C_0). With progress of fermentation, the microstructure of A sourdoughs was characterized by a compact network including starch granules (Fig. 3, panel A_{24} and A_{288}). Since CF does not contain gluten, the observed structure of A sourdoughs should depend on the starch, the protein as well as the high fibre content. The fibre content of CF has already proved to be responsible for emulsifying, stabilising, texturising and thickening properties of dough (Demirkesen, Sumnu, Sahin, & Uysal, 2011). Changes became more evident at the end of fermentation (288 h), as revealed by comparison of A micrographs along monitoring (Fig. 3, panel A_{0-288}). On the other hand, in RF-added sourdoughs, the matrix appeared less structured and organized during fermentation (Fig. 3, panel B_{24-288} and C_{24-288}) likely because proteins of rye dough are not capable of forming three dimensional structures (Beck, Jekle, Selmair, Koehler, & Becker, 2011). The starch granules appeared entrapped within the compact matrix, which mainly depends on the starch in the RF, as well as on other carbohydrates known as pentosans. Actually, at low pH, the pentosan fraction forms a gel-like layer on the flour particles hindering protein aggregation (Wang, van Vliet, & Hamera, 2004). Significant changes in surface appearance of B sourdoughs were visible at ending of fermentation (288 h), while no significant effect of fermentation time was observed on microstructure of C samples. Results appeared in agreement with those reported by Wing and Scott (1999) , p. 255 for rye bread.

3.3. Identification and quantification of volatile organic compounds

Although the main part of the aroma is produced upon cooking, many aroma precursors are formed during the fermentation process. The generation of VOCs occurred in sourdough mainly because of microbial metabolism (e.g. alcohols, 2,3-butanedione, esters and

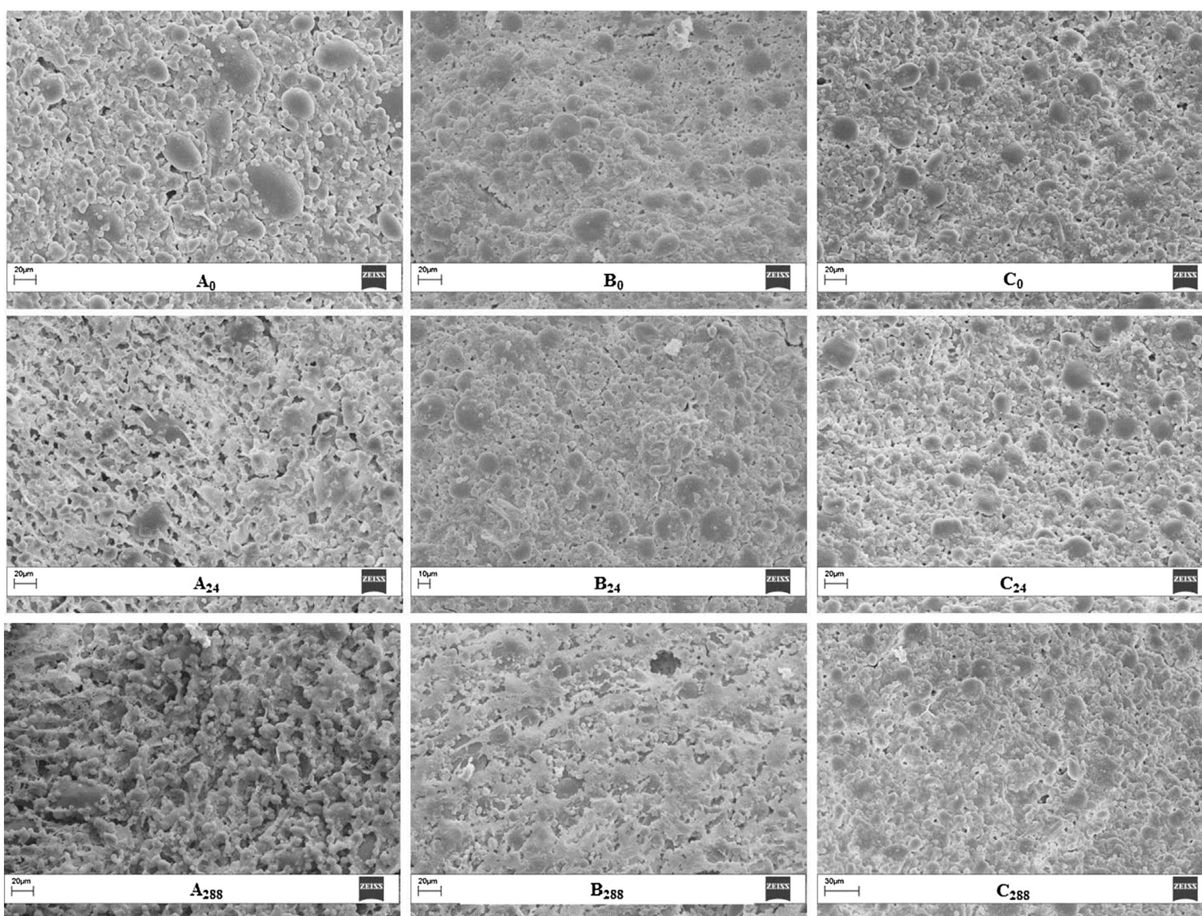

Fig. 3. Scanning Electron Microscopy (SEM) micrographs of A, B and C sourdoughs after 0 h, 24 h and 288 h of fermentation.

acids), enzymatic oxidation or autoxidation of flour lipids (e.g. aldehydes, ketones and 2-pentylfuran), caramelization process under drying conditions and rearrangement of carbohydrates via Maillard reaction (e.g. furans, pyrazines, phenolics); however, some compounds are already present in the RF (Kirchoff & Schieberle, 2002) and the CF (Cirlini et al., 2012). To describe VOCs profiles, sourdoughs A, B and C were analysed at 24 and 288 h. A total of 51 volatile flavour compounds was identified through SPME-GC/MS analysis, including aldehydes (6), alcohols (13), esters (13), ketones (6), acids (2), phenolics (4), alkanes (1), furans (1), terpenes (1), lactones (1) and pyrazines (3), (Table 1). Analysis of variance was carried on all results of rye/chestnut sourdoughs (B and C) VOCs (this study) and on 100% chestnut sourdough (Aponte et al., 2013). Duncan's multiple comparison test revealed significant differences ($p \leq 0.05$) within main chemical classes.

The appreciable quantities of aldehydes may be due to lipoxygenase reactions initiated by ruptured vegetal cells during milling into flour (Peppard & Halsey, 1981). In the case of CF and RF, all the aldehydes presenting a linear chain, such as hexanal and acetaldehyde, could result from the degradative oxidation of fatty acids and of the corresponding alcohols, pentanol and hexanol (Pires Borges, Soeiro Carvalho, Reis Correia, & Silva, 2007). Specifically, hexanal is reported to be salient in RF (Kirchoff & Schieberle, 2002). As matter of fact, the higher concentration of hexanal recorded in B and C if compared to A, is likely to be related to the high susceptibility to rancidity of the rye grain and to the industrial processes (i.e. heat treatments) required for fine-tuning the rye flavour (Seitz, Chung, & Rengarajan, 1998). Rancidity in rye products is due as more to the change of the hexanal concentration, than its absolute presence, since hexanal may partly evaporate or be quenched into non-volatile compounds. 2 methylpropanal, the most abundant aldehyde found in B and C, has been associated with the rye-like flavour (Hougen, Quilliam, & Curran, 1971). While nonanal, the most abundant aldehyde found in A, could be a byproduct from the decomposition of ethyl oleate (Peppard & Halsey, 1981). Aromatic aldehydes, such as benzaldehyde, were also found in all sourdoughs at 24 h and especially in A (39.2 ± 3.7 µg/kg) and B (40.0 ± 0.9 µg/kg), probably as result of aromatic amino acids degradation. Furfural could be attributed to the caramelization process under drying conditions.

The development of alcohols can be attributed to the activities of high LAB levels occurring during steeping, as well as to yeasts activity (Rehman, Paterson, & Piggott, 2006). Apart from ethanol, the most abundant alcohols were 3-metil-1-butanol (isoamylalcohol) and 2-methyl-1-propanol (isobutylalcohol) reported as the most important flavour active compounds produced by yeast fermentation in homofermentative culture (Lund, Hansen, & Lewis, 1988). 1-pentanol, 2-phenylethyilalcohol and 1-butanol were also found. Such results appeared in agreement with those reported by Lund et al. (1988) for rye flour. 2-furfurylalcohol could be ascribed to the caramelization process under drying conditions, which may involve free sugars naturally occurring in fresh chestnut fruits (Cirlini et al., 2012). However, the origin of most other aliphatic aldehydes and alcohols, as well as of aliphatic hydrocarbons and 2-pentylfuran, is the oxidative and/or thermal degradation of unsaturated lipids (Grosch, 1982). As matter of fact, 2-pentylfuran in high concentrations is related to rancidity. Esters such as acetates, propionates, hexanoates, lactates and octanoates, have been reported

Table 1
Compounds detected in A (100% CF), B (40/60% RF/CF) and C (60/40% RF/CF) samples after 24 and 288 h of fermentation. Each value is given in μg/kg (mean ± SD). Retention Index (RI) identification by comparison with RI database.

Compound	RI	A_{24}	B_{24}	C_{24}	A_{288}	B_{288}	C_{288}
Aldehydes		**1930.7**$_c$	**513.7**$_a$	**540.0**$_b$	**559.7**$_c$	**221.3**$_b$	**166.8**$_a$
Acetaldehyde	719	457 ± 10	125 ± 1	63 ± 2	492 ± 4	112 ± 1	30 ± 1
2-Methylpropanal	853	99 ± 9	195 ± 2	227 ± 8	38 ± 1	74 ± 6	91 ± 7
Hexanal	1138	30 ± 1	38 ± 5	157 ± 13	10 ± 1	22 ± 4	29 ± 3
Nonanal	1396	1268 ± 14	103 ± 3	51 ± 1	nd	nd	nd
Furfural	1509	38 ± 4	13 ± 6	23 ± 4	20 ± 1	10 ± 2	nd
Benzaldehyde	1573	39 ± 4	40 ± 1	20 ± 1	nd	4 ± 0	17 ± 0
Alcohols		**78,428.4**$_c$	**23,563.4**$_b$	**19,903.8**$_a$	**37,579.0**$_c$	**15,491.1**$_b$	**11,635.4**$_a$
Ethanol	1007	31,890 ± 142	14,995 ± 94	1068 ± 10	11,946 ± 56	9462 ± 191	5906 ± 5
1-Propanol	1104	777 ± 6	248 ± 5	nd	719 ± 5	105 ± 2	nd
1-Butanol	1196	321 ± 7	23 ± 12	62 ± 17	149 ± 5	38 ± 6	nd
1-Penten-3-ol	1209	nd	11 ± 1	55 ± 3	nd	13 ± 0	35 ± 3
isobutylalcohol	1097	5757 ± 15	833 ± 2	640 ± 19	674 ± 4	316 ± 7	364 ± 6
Isoamylalcohol	1253	38,829 ± 35	6792 ± 34	7811 ± 1	22,794 ± 10	4978 ± 8	4828 ± 75
1-Pentanol	1291	367 ± 6	262 ± 15	221 ± 10	254 ± 2	87 ± 7	201 ± 25
Cis-2-pentenol	1323	nd	9 ± 1	46 ± 3	nd	nd	42 ± 2
1-Hexanol	1395	38 ± 4	123 ± 6	25 ± 1	109 ± 4	239 ± 11	20 ± 2
1-Octanol	1606	nd	nd	8 ± 0	nd	nd	nd
2-Furfuryl alcohol	1707	57 ± 6	12 ± 2	24 ± 1	61 ± 2	26 ± 3	20 ± 4
2-Phenylethylalcohol	1970	356 ± 5	242 ± 12	324 ± 4	1051 ± 4	217 ± 9	220 ± 9
3-Ethoxy-1-propanol	1418	35 ± 3	14 ± 0	9 ± 2	78 ± 3	10 ± 0	nd
Esters		**18,943.8**$_c$	**11,623.1**$_a$	**16,735.3**$_b$	**11,615.2**$_c$	**22,164.3**$_b$	**33,651.2**$_c$
Ethyl acetate	924	14,106 ± 10	8438 ± 15	11,049 ± 1	10,490 ± 37	18,245 ± 165	26,324 ± 117
Methyl acetate	849	nd	31 ± 4	19 ± 7	nd	72 ± 8	162 ± 5
Propyl acetate	1041	117 ± 11	253 ± 26	685 ± 25	nd	363 ± 13	736 ± 43
2-Methyl propyl acetate	1018	193 ± 13	114 ± 7	38 ± 6	36 ± 1	193 ± 9	314 ± 7
Ethyl butyrate	1103	243 ± 23	238 ± 5	466 ± 8	nd	22 ± 4	33 ± 6
Isoamyl acetate[a]	1170	1601 ± 30	242 ± 1	167 ± 6	410 ± 1	1360 ± 4	2035 ± 7
Ethyl pentanoate	1181	nd	204 ± 15	655 ± 29	nd	90 ± 9	638 ± 10
Ethyl hexanoate	1275	700 ± 13	1703 ± 11	3263 ± 10	368 ± 7	521 ± 8	1540 ± 20
Ethyl lactate	1386	110 ± 7	180 ± 6.9	69 ± 14	189 ± 3	1215 ± 4	1719 ± 10
Ethyl heptanoate	1377	19 ± 1	26 ± 1	113 ± 6	27 ± 1	32 ± 4	45 ± 9
Ethyl octanoate	1481	444 ± 11	148 ± 6	93 ± 11	102 ± 4	40 ± 4	42 ± 9
Ethyl 2-methylpropanoate	1060	770 ± 7	39 ± 5	97 ± 1	nd	13 ± 3	56 ± 7
Ethyl 2-furancarboxylate	1675	640 ± 8	7 ± 1	22 ± 1	52 ± 2	nd	7 ± 3
Ketones		**2066.3**$_c$	**657.7**$_b$	**553.3**$_a$	**864.9**$_c$	**229.2**$_b$	**32.9**$_a$
2,3-Butanedione	1032	338 ± 6	175 ± 16	120 ± 1	nd	nd	nd
2-Pentanone	1042	56 ± 5	62 ± 8	118 ± 19	nd	nd	nd
2-heptanone	1227	16 ± 1	nd	nd	nd	nd	nd
Acetoin	1329	1401 ± 1	420 ± 14	315 ± 5	842 ± 11	229 ± 4	33 ± 4
2,3-Pentanedione	1121	232 ± 22	nd	nd	nd	nd	nd
2-Methylciclopentanone	1229	18 ± 1	nd	nd	23 ± 1	nd	nd
Alkanes			**12.9**	**37.4**		**98.1**	**208.8**
2,6-Dimethylundecane	1055	nd	13 ± 3	37 ± 5	nd	98 ± 3	209 ± 9
Acids		**1492.6**$_c$	**131.5**$_a$	**165.5**$_b$	**768.8**$_a$	**1940.2**$_b$	**2938.2**$_c$
Acetic acid	1498	1006 ± 11	132 ± 2	166 ± 10	721 ± 5	1940 ± 22	2938 ± 4
2-Methylpropanoic acid	1547	486 ± 7	nd	nd	48 ± 1	nd	nd
Phenolics		**1068.2**$_c$	**54.6**$_b$	**39.1**$_a$	**774.7**$_c$	**42.3**$_b$	**38.8**$_a$
Guaiacol[b]	1917	986 ± 13	37 ± 1	19 ± 1	194 ± 7	22 ± 1	14 ± 3
Phenol	2065	47 ± 4	18 ± 2	20 ± 3	48 ± 3	21 ± 2	25 ± 3
m-Cresol[c]	2152	18 ± 3	nd	nd	57 ± 2	nd	nd
p-Cresol[d]	2018	18 ± 1	nd	nd	42 ± 2	nd	nd
Oxygenous heterocyclic compounds		**5.4**$_a$	**3.9**$_a$	**12.5**$_b$	**7.8**$_a$	**9.1**$_a$	**14.6**$_b$
2-Pentylfuran	1271	5 ± 1	4 ± 1	13 ± 4	8 ± 0	9 ± 0	15 ± 3
Terpenes		**105.1**$_a$	**219.8**$_b$	**426.1**$_c$	**5.0**$_a$	**6.9**$_{a.b}$	**13.4**$_b$
Limonene	1235	105 ± 6	220 ± 7	426 ± 7	5 ± 0	7 ± 0	13 ± 1
Lactones		**18.4**$_a$	**16.9**$_a$	**16.6**$_a$	**8.2**$_a$	**13.2**$_b$	**14.6**$_b$
γ-Butyrolactone	1685	18 ± 0	17 ± 2	17 ± 7	8 ± 0	13 ± 3	15 ± 3
Pyrazines			**7.3**	**8.8**		**19.8**	**50.8**
2-Methylpyrazine	1308	nd	nd	nd	nd	10 ± 0	21 ± 5
2,5-Dimethylpyrazine	1367	nd	7 ± 3	9 ± 2	nd	4 ± 0	23 ± 4
Ethyl-3,6-dimethylpyrazine	1451	nd	nd	nd	nd	6 ± 0	7 ± 0

nd: not detected.
Internal Standard: 4-methyl-2 pentanol.
Within the VOCs group, means with different letters for the same fermentation time (24 or 288 h) are significantly different ($p < 0.05$).
[a] 3-methylbutil acetate.
[b] 2-methoxyphenol.
[c] 3-methylphenol.
[d] 2-methoxy-4 methyl phenol.

as sourdough constituents (Kirchoff & Schieberle, 2002). The most abundant ester was ethyl acetate. Actually, this compound was found mostly in rye sourdoughs obtained by using *Lb. plantarum* strains as starter (Ravyts & De Vuyst, 2011), so its occurrence, in the light of the DGGE results, does not surprise. 3-methylbutil acetate and ethyl lactate that are presumed to be produced by yeast esterase activity, increased with fermentation time in B and C. Results for 3-methylbutil acetate are in good agreement with those reported by Ravyts and De Vuyst (2011) in rye sourdoughs. The content of ethyl hexanoate was almost halved at the end of the monitoring in all trials.

Ketones were widely represented in A, B and C. Especially, acetoin and 2,3-butanedione, important fermentation indexes, were detected in all sourdoughs at 24 h. Alkanes were present only in rye/chestnut flour sourdough (B and C). As expected, the level of acetic acid increased in trials B and C (Table 1) but not in trial A, in agreement with the recorded pH and TTA trends (Fig. 1).

Among phenols, 2-methoxyphenol (guaiacol) and phenol were found in all sourdoughs (A–C). While p- and m-cresol were identified only in A. Phenolic compounds, formed during the thermal degradation of lignin under pyrolysis conditions, could be ascribed to the smoke generated by burning chestnut wood or by thermal degradation of barks (Gruber, 2010).

Limonene was lost with prolonged fermentation process. γ-butyrolactone, characteristic compound of chestnut fruit and flour (Cirlini et al., 2012), was found in all samples. Pyrazines were detected in low amount only in rye samples: B and C. The derivatives of pyrazines and pyridines are often identified in rye products as a result of heat treatments. Furthermore, their amount increased concurrently with fermentation time, in accordance with previous results obtained for pyrazines originating from amino acids in rye bread (Schieberle & Grosch, 1985).

VOCs profiles appeared affected by the length of the sourdough maturation process. In order to emphathize the effect of fermentation time, VOCs results were grouped into chemical classes (Fig. 4) and compared at both fermentation times (t-Student test, $^*p < 0.05$). No difference in the VOCs profiles was recorded in the three trials: alcohols, ketones, aldehydes and terpenes significantly decreased ($p < 0.05$), while esters and acids significantly increased ($p < 0.05$). Furans and lactones were not affected by prolonged fermentation (Fig. 4).

In general terms, the evolution of some VOCs classes along time appeared strongly affected by the type of flour: esters slightly increased with maturation in trial A (actually, in absolute values, these compounds decreased), but when the CF content is reduced (B and C), their increase appeared more evident; the same trend could be even observed for acids. On the other hand, the marked decrease recorded for alcohols, aldehydes and ketones in the trial C seemed to be related to the RF content; as matter of fact, their variation appeared more restrained when the RF was reduced (B) or absent (A) (Fig. 4).

3.4. Principal component analysis

On the basis of the VOCs results (Table 1), relationships between sourdoughs (Trials A-C) at different fermentation times were obtained using factorial principal component analysis (PCA). The two first principal components (PCs) were sufficient to explain the maximum variation in all original data. Fig. 5 shows plots of loadings (Fig. 5a) and scores (Fig. 5b) obtained from PCs, where the first two principal components (PC1 and PC2) accounted for 79% of the total variance of the data. In particular, PC1 explained 54% of the variation of the data, while PC2 explained 25%.

The PC1 on the positive axis of the plane was positively correlated to ketones, aldehydes, alcohols, phenolics, lactones and

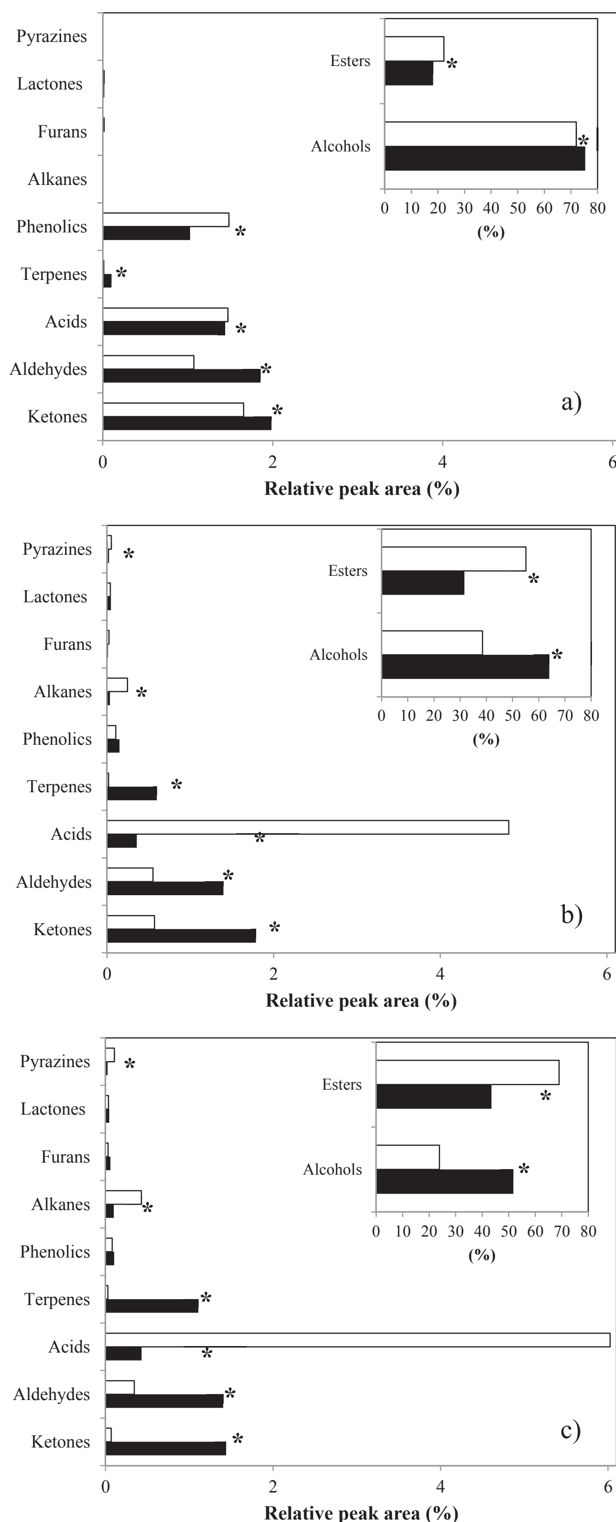

Fig. 4. Changes of relative amounts of VOCs in sourdoughs A (a), B (b) and C (c) after 24 (■) and 288 h (□) of fermentation. For each VOCs group, significant differences (*: $p < 0.05$) were shown at 24 and 288 h.

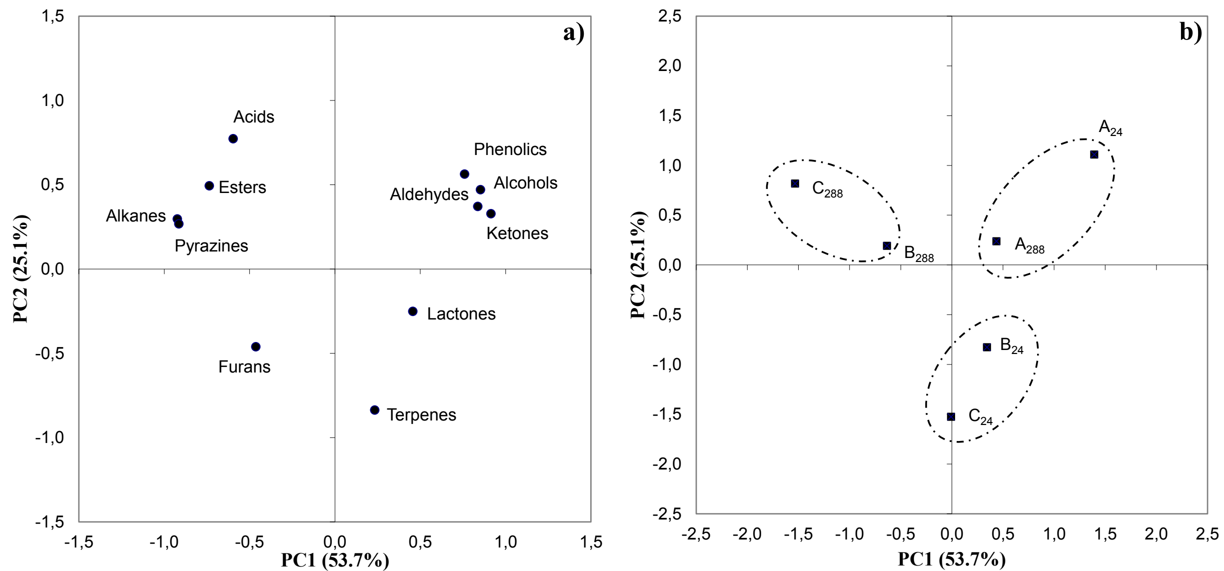

Fig. 5. Results of PCA - loading (a) and score (b) biplot for A, B and C after 24 and 288 h of fermentation.

terpenes. Regarding PC2, the main positive contribution was due to acids, esters, alkanes and pyrazines, while furans showed negative eigenvalues (Fig 5a). The scores distribution (Fig 5b) allowed for clustering of the samples into three groups. In particular, A sourdoughs (A_{24} and A_{288}) were positively scored on PC1 and proved to be strongly characterized by aldehydes, ketones, phenolics, alcohols (eigenvalues > 0.9) during fermentation, while B and C at 24 h of fermentation (B_{24} and C_{24}) were linked to lactones (>0.5) and terpenes. The last group, showing negative scores on the PC1 and entirely located in the positive part of PC2, was composed of samples B and C at 288 h of fermentation (B_{288} and C_{288}) and was characterized by acids, esters, alkanes and pyrazines. As expected, sourdoughs B were located more proximate to C rather than A (Fig. 5) at the same fermentation time. Results of PCA revealed the influence of fermentation time and of rye flour on the volatile composition of sourdoughs.

4. Conclusions

The work describes the development of a stable biota in spontaneously fermented sourdoughs of blends of chestnut and rye flour. In the considered conditions, dominant species basically overlap to those associated to sourdoughs strengthened with CF, such as *Pd. pentosaceus* or *Weissella paramesenteroides*. Microstructures of sourdoughs realized with only CF appeared as a compact network including starch granules, while RF-added sourdoughs showed a less organized matrix. The existence of interactions between components of the CF dough, a typical gluten-free flour, has been associated to its high fibre content. By SPME, 51 volatile organic compounds were identified in sourdoughs obtained by 100% CF and RF/CF blends during fermentation. The VOCs profiles appeared mainly dominated by alcohols, esters, acids, aldehydes and ketones, all well-known flavour compounds in sourdough fermentation. The PCA discriminated the sourdoughs into three distinct clusters.

Although the interactions of the metabolites or flavour compounds produced by microorganisms with the microstructure of the food matrix should be more systematically investigated, the findings of this work could have useful implications. Firstly, the outcomes may support technologists in the development and improvement of highly nutritional bakery products based on chestnut and rye flour. In spite of the remarkable similarity between the selected flour types, the technological potential of combinations of both has never been explored before.

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