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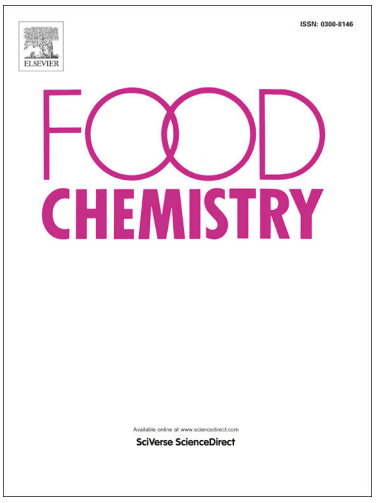

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ABSTRACT

Insects represent a novel source of edible high nutritional value proteins which are gaining increasing interest as an alternative to traditional animal foods. In this work, cricket flour was used to produce gluten-free sourdough breads, suitable for celiac people and “source of proteins”. The doughs were fermented by different methods and pH and microbial growth, volatile compounds, protein profile, and antioxidant activity, before and after baking, were analyzed and compared to standard gluten-free doughs. The results showed that cricket-enriched doughs and the standard had similar fermentation processes. Cricket enrichment conferred to the breads a typical flavoring profile, characterized by a unique bouquet of volatile compounds, made by nonanoic acid, 2,4-nonadienal (E,E), 1-hexanol, 1-heptanol, and 3-octen-2-one, expressed in different amounts depending on the

type of inoculum. Finally, antioxidant activities were significantly enhanced in cricket breads, indicating that cricket powder provides to bakery gluten-free goods high nutritional value proteins and antioxidant properties.

Keywords: novel food; SPME-GC-MS; *Lactobacillus* spp.; *Saccharomyces cerevisiae*; antioxidant activity; multivariate analysis; cricket.

Chemical compounds studied in this article

Hexanoic acid (PubChem CID: 8892); nonanoic acid (PubChem CID: 8158); 2,4-nonadienal, (E,E) (PubChem CID: 5283339); 1-hexanol (PubChem CID: 8103), 1-heptanol (PubChem CID: 8129); 1-octen-3-ol (PubChem CID: 18827); 2,4-butanedione (PubChem CID: 650); 2-heptanone (PubChem CID: 8051); 3-octen-2-one (PubChem CID: 15475); 1,4-Butanediol (PubChem CID: 8064).

1 INTRODUCTION

FAO estimated that by the year 2050 world's population will rise up to over 9 billion and consequently food demand will hardly be satisfied. Currently, 40% of earth's land is used to produce food, but agriculture and grazing lands are on shortage, thus more efficient and sustainable methods are necessary to face this challenge. In developing countries there is a need to supply enriched foods, particularly with protein, vitamins, and microelements for child and adolescent daily consumption. On the other hand, in the western world there is an increasing request for high-quality proteins to be used as supplement for sportsmen, or in special diets for older people, or for people suffering from specific diseases, like celiac disease. The latter is a chronic systemic autoimmune disorder caused by a permanent intolerance to gluten proteins in genetically susceptible individuals. Their diet entails the substitution of gluten-containing products with gluten-free products, which are often inadequate for the low protein content and a high fat and salt content (Melini & Melini, 2019). Proteins for food enrichment are currently produced from vegetable or

animal sources, with limited sustainability, and the stocks are not enough for the market demands. For this purpose, insect proteins are strongly investigated as a practical, cost-effective alternative to animal source proteins, e.g. meat and poultry (Alston, Beddow, & Pardey, 2009). Specifically insects, such as house cricket (*Acheta domesticus* L.), have more than 65% of protein content, 80% of their body can generate food (in contrast to 40% of cattle and 55% of pork), and can be harvested from farms that have minimal land requirements and do not need persistent chemicals. The use of insects in food industry was recently allowed and addressed in the EU under EUs regulation (EC) No 258/97 on novel foods (European Parliament, 2016). Recent studies have proved that consumers find insects more appealing when used as an ingredient to prepare foods with familiar flavors and textures or, alternatively, by masking their appearance in commonly accepted products, e.g. snack bars, chips, shakes and pastry products (Tan, Fisher, van Trijp, & Stieger, 2016). So, the use of insect flour instead of the whole organism, can be a way to promote the spread and consumption of this sustainable protein source even in those countries where insects are not part of the food tradition. Introduction of insect flour into bakery products resulted not only in the improvement of the content of protein, fat and fiber and minerals but also of the antihypertensive and antioxidant potential coming from peptides encrypted in their protein sequences (Zielińska, Kaaras & Jakubczyk, 2017). Also *in vitro* enzymatic hydrolysis of protein extracts from other edible insects, contributed to increase their antioxidant activity (Hall, Johnson & Liceaga, 2018). Moreover, cricket flour increased availability of water to the biopolymers in dough with a consequent reduction of hardness and an improvement of bread consistency (Kowalczewski et al. 2019). Furthermore, insect flour represents an interesting ingredient for the sourdough fermented bakery products with possible beneficial effects. Indeed, lactic acid bacteria generate biotransformations of flour ingredients both by releasing health related compounds and degrading antinutritional compounds (Guerzoni, Gianotti & Serrazanetti, 2011; Terefe & Augustin, 2019).

In this study, cricket flour was used to produce cricket-fortified gluten-free sourdough bread. This novel food could be a good source of proteins, responding both to the growing demand for protein from sustainable sources and to the market request for foods suitable for people with celiac disease. The antioxidant properties of bread were analyzed by radical scavenging, ferrous ion-chelating, and ferric reducing antioxidant power assays. A complete volatilome characterization was performed by solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS). In fermented foods, the metabolite profiling was applied to observe metabolite modifications during fermentation and to predict the sensorial and nutritional quality in different fermented food matrices including dairy, (Mozzi, Ortiz, Bleckwedel, De Vuyst, & Pescuma, 2013), baked goods (Taneyo-Saa, Nissen & Gianotti, 2019), tomato (Kim, Park, Seo, Kweon & Son, 2019), and vegetable drinks (Nissen, Demircan, Taneyo-Saa & Gianotti, 2019). The volatilome analysis of cricket bread performed in this work, is suggested as a new approach to improve microbial fermentation during the preparation of bakery products and increase the quality of bakery goods.

2 MATERIALS and METHODS

2.1 Microbial strains and culture conditions

All the microbial strains tested belong to the microbial collection of DISTAL (Department of Agricultural and Food Sciences), University of Bologna, and were previously isolated from sourdough and extensively studied (Taneyo-Saa, Di Silvestro, Nissen, Dinelli & Gianotti, 2018; Taneyo-Saa, Nissen & Gianotti, 2019). *Lactobacillus plantarum* 98a, *Lb. sanfranciscensis* Bb12 and *Saccharomyces cerevisiae* LBS were obtained from 30% (v/v) glycerol stocks stored at -80°C. *Lb. plantarum* was propagated in de Man Rugosa Sharpe (MRS) (Oxoid, Thermo Scientific, Waltham, MA, USA) broth, while *Lb. sanfranciscensis* in MRS containing 0.05% (w/v) of L-cysteine (Sigma, Saint Louis, MO, USA), applying jars with catalyst for anaerobiosis (Thermo Scientific, USA). Both strains were incubated at 37 °C for at least 48 h. *S. cerevisiae* LBS was propagated in Sabouraud broth (Oxoid, Thermo Scientific, USA) at 30 °C for 24 h.

2.2 Doughs and bread preparation

The flours used in this research are commercial organic certified products, whose proximate compositions are supplied in a supplementary table (Table S1, supplementary material). Four types of doughs were prepared, approximately weighting 700 g. Two were used for direct fermentations and the others for sourdough fermentations. To produce the doughs a Bimby mixer (Vorverk, Wuppertal, Germany) was employed setting the "bread" program for 4 minutes. The control and cricket-enriched doughs for direct fermentation were made, respectively, with: i) 319.4 g of corn flour (Molino Rossetto SpA, Pontelongo, Italy), 80.96 g of rice flour (Molino Rossetto, Italy), 5.6 g of hydroxy propyl methyl cellulose (HPMC) (Bioline Integratori, Canaro, Italy) 4 g of NaCl, and 340 mL of sterile water; ii) 302.32 g of corn flour, 75.2 g of rice flour, 22.16 g of cricket powder (Kreca Ento-Food BV, Ermelo, The Netherlands), 5.6 g of HPMC, 4 g of NaCl, and 340 mL of sterile water. The standard doughs were; not inoculated (samples SX and CX), inoculated with 7 Log CFU/mL of an equal mix of *L. sanfrancisciensis* and *L. plantarum* (samples SL and CL), and inoculated with 8 Log CFU/mL of *S. cerevisiae* LBS (samples SY and CY). Doughs were then leavened in an air incubator (C3TE model, Cavallo s.r.l., Buccinasco, Italy) for 18 h at 31 °C in three independent batches, generating samples SX18, CX18, SL18, CL18, SY18, and CY18. The doughs for control and cricket-based sourdough fermentation were made, respectively, with: iii) 255.52 g of corn flour, 64.77 g of rice flour, 4.48 g of HPMC, 3.2 g of NaCl, 272 mL of sterile water and 140 g of dough SL18; iv) 241.86 g of corn flour, 60.16 g of rice flour, 17.73 g of cricket powder, 4.48 g of HPMC, 3.2 g of NaCl, 272 mL of sterile water and 140 g of dough CL18. The total content of cricket flour therefore resulted from dough formulation and the cricket flour portion present in sourdough (CL18) and attained to the same amount of dough directly fermented (ii). The sourdoughs so far obtained (samples YS+ and YC+) were inoculated with 7 Log CFU/mL of *S. cerevisiae* LBS and fermented for 6 h at 31 °C producing samples YS+6 and YC+6. Portions of 50 grams of dough were put in small pans and baked at 180 °C for 20 min, using an electric ventilated

oven with a steam program for bread (E0B9S21WX model, Electrolux, Stockholm, Sweden). Bread samples were marked with a B placed after the previously mentioned abbreviations. Sampling of breads for analyses were done with homogeneous proportion of crust and crumb. For complete sample code description, a supplementary table is provided (Table S2, supplementary material).

2.3 Microbial quantification and pH values during the process

Microbial quantification was obtained by both culture-dependent and culture-independent protocols. The culture-dependent was obtained by plating serial dilutions of the samples made in physiological solution (0.9% NaCl), for LAB on MRS agar containing cycloheximide (0.1 g/L) (Oxoid, Thermo Fisher Scientific, USA) incubated aerobically for 24 h at 37 °C, while for yeast on Sabouraud dextrose agar (Oxoid, Thermo Fisher Scientific, USA) and chloramphenicol (0.4 g/L) (Sigma, Saint Louis, MO, USA) incubated aerobically for 48 h at 30 °C. Culture independent quantifications were obtained by qPCR with the SYBR Green I chemistry, applying genus specific primers as Lac1 for *Lactobacillus* spp., then named LAB, (forward:5'-GCAGCAGTAGGGAATCTTCCA-3' and reverse: 5'-GCATTYCACCGCTACACATG-3') (Nissen, Demircan, Taneyo-Saa & Gianotti, 2019) and ITS 23S for *S. cerevisiae*, then named yeasts, (forward: 5'-GTTTCCGTAGGTGAACCTGC-3' and reverse: 5'-ATATGCTTAAGTTCAGCGGGT-3') (Foschino, Gallina, Andrighetto, Rossetti & Galli, 2004). Genetic standards were prepared from relative PCR amplicons from pure cultures of the target bacterial species as described previously (Nissen, Demircan, Taneyo-Saa & Gianotti, 2019). Extraction of bacterial DNA was obtained with Pure Link Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Foster City, CA, USA). For both the targets, qPCR reaction on a RotorGene 6000 (Qiagen, Hilden, Germany) was set as follow: an holding stage at 98 °C for 6 min, and a cycling stage made of 95 °C for 20 sec and 60 °C for 60 sec, repeated for 45 times, followed by melting curves analysis. Quantifications were made with a five-points standards of RecA and Lac1, separately. Reactions were prepared with 1 ng of DNA, 2x Power up SYBR Green (Thermo Scientific, USA) and 250 nM of each primers (Eurofins Genomics, Ebersberg, Germany).

pH was determined with a pHmeter (Crison, Alella, Spain) at 20 °C appropriately calibrated with three standard buffer solutions at pH 9.21, pH 4.00, and pH 2.00.

2.5 SPME-GC-MS

Volatile organic compounds (VOCs) evaluation was carried out on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent Technologies 5975 mass spectrometer operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The SPME-GC-MS (Solid Phase Micro-Extraction Gas Chromatography-Mass Spectrometry) protocol and the identification of volatile compounds were done accordingly to previous reports, with minor modifications (Taneyo-Saa, Di Silvestro, Nissen, Dinelli & Gianotti, 2018; Taneyo-Saa, Nissen & Gianotti, 2019). Briefly, before each head space sampling the fiber was exposed to the GC inlet for 10 min for thermal desorption at 250 °C in a blank sample. The samples were then equilibrated for 10 min at 40 °C. The SPME fiber was exposed to each sample for 40 min and finally the fiber was inserted into the injection port of the GC for a 10 min sample desorption. The temperature program was: 50 °C for 1 min, then programmed at 1.5 °C/min to 65 °C, and finally at 3.5 °C/min to 220 °C, which was maintained for 25 min. Injector, interface, and ion source temperatures were 250, 250, and 230 °C, respectively. Injections were carried out in splitless mode and helium (3 mL/min) was used as carrier gas. Identification of molecules was carried out by comparing their retention times with those of pure compounds (Sigma, USA) and confirmed by searching mass spectra in the available databases (NIST version 2005 and Wiley version 1996) and literature (Taneyo-Saa, Di Silvestro, Nissen, Dinelli & Gianotti, 2018; Taneyo-Saa, Nissen & Gianotti, 2019). Ethyl alcohol and acetate were absolutely quantified in mg/kg, while all other VOCs where relatively quantified in percentage. All results are expressed as mean values obtained at least from duplicate batches in two independent experiments.

2.6 Protein characterization

Reagents were from Sigma (Saint Louis, MO, USA) and Merck (Darmstadt, Germany). All reagents were analytical grade. Pre-cast gels, the MW marker for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), bovine serum albumin (BSA), and related reagents were from Bio-Rad (Hercules, CA, USA).

2.6.1 Sample preparation for the Bradford, SDS-PAGE and antioxidant activity assays.

Dough and bread samples (50 g) were freeze-dried in a Heto Power Dry LL 3000 lyophilizer (Thermo Scientific, USA) and finely ground with M20 universal mill (IKA, Werke, Germany). Aliquots of 0.1 g of lyophilized powder were mixed with 1 mL Tris buffer (20 mM) pH 8 and shook well for 1 h at 100 rpm in a rotary shaker (709/R, ASAL S.r.l., Cernusco s/N, Italy). After centrifugation at 10.518 x g for 10 min the supernatant was collected, and the pH was adjusted to 7. Samples were kept at -80 ºC until use.

2.6.2 Concentration of soluble proteins

The concentration of soluble proteins was measured by the Bradford assay using the Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). Reference calibration curve was made with BSA, from 0 to 10 mg/L. All analyses were performed on a microplate scale (SPARK 10M microplate reader, TECAN, Mannedorf, CH) and results are expressed as mean values of three replicates.

2.6.3 Protein pattern analysis by SDS-PAGE

Protein pattern was analyzed on 14% (v/v) Tris SDS-PAGE, using Mini-PROTEAN equipment (Bio-Rad, USA). The Precision Plus Protein Standard from the same company was selected as MW

marker. All analyses were performed on a microplate scale (TECAN, CH) and results are expressed as mean values of three replicates.

2.7. Antioxidant activity assays

Antioxidant activity was analyzed by ABTS (2,20-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay, ferrous ion-chelating ability assay, and ferric reducing antioxidant power assay (FRAP). All analyses were performed on a microplate scale (TECAN, CH) and results are expressed as mean values of three replicates.

2.7.1. ABTS radical scavenging assay

The ABTS radical scavenging activity was determined according to the procedure of Ozgen, Reese, Tulio, Sheerens & Miller (2006). An $ABTS^{\cdot+}$ solution was prepared by mixing an ABTS stock solution (7 mM in water), with 2.45 mM $K_2S_2O_8$ (potassium persulphate). The mixture was left overnight in the dark and then diluted with sodium acetate 20 mM pH 4.5, to an absorbance of 0.70 ± 0.01 at 734 nm. The solution (198 µL) was mixed with the sample (2 µL) and incubated at room temperature in the dark for 30 min, before reading the absorbance at 734 nm. The calibration curve was made with ascorbic acid (AA), from 0 to 5 mg/L. Results were expressed as mg AA equivalents/ L of sample solution.

2.7.2. DPPH radical scavenging assay

The scavenging activity of DPPH radical was determined according to the method of Sharma & Bhat (2009). Aliquots (20 µL) of sample at different concentrations were mixed with 180 µL of 50 µM DPPH solution in methanol. The reaction mixture was incubated for 30 min in the dark at room temperature and the absorbance was then read at 517 nm. Results were expressed as mg AA

equivalents/L of sample solution by means of a dose-response calibration curve of AA (from 0 to 5 mg/L).

2.7.3. Ferrous ion-chelating ability assay

Ferrous ion-chelating activity was measured according to the method of Decker & Welch (1990). Aliquots of samples (25 µL), at different protein concentration, where mixed with 100 µL of 50 µM ferrous sulphate ($FeSO_4$) and 100 µL of 300 µM ferrozine. After incubation at room temperature for 10 min, the absorbance was measured at 562 nm. Results were expressed as µg of EDTA (ethylenediaminetetraacetic acid) equivalents/L of sample solution by means of a dose-response calibration curve of EDTA (from 0 to 10 µg/L).

2.7.4. Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was performed following the method of Benzie & Strain (1996). Briefly, the FRAP reagent was prepared freshly mixing 1 mL of 10 mM TPTZ (2,4,6- tripyridy-s-triazine) solution in 40 mM HCl, 1 mL of 20 mM $FeCl_3$ (iron trichloride) and 10 mL of 300 mM acetate buffer, pH 3.6. Aliquots of samples (10 µL) were mixed with 300 µL FRAP reagent and the absorbance was measured at 593 nm after incubation at room temperature for 10 min. Results were expressed as mg AA eq/ L of sample solution by means of a dose-response calibration curve of AA (from 0 to 2 mg/L).

2.8 Sensory evaluation

The breads were evaluated after 3 h from baking by 20 semi-trained testers (consumers), that scored the produced breads according to a preference protocol with a scale from 0 (unacceptable) to 7 (excellent) (Plessas, Fisher, et al., 2008). Two independent consumers' tests were performed, and results were marked in a spider chart as average scores for color, aftertaste, smell, taste, crispiness and overall appreciation of the breads.

2.9 Statistical analyses

Statistical analyses regarding the volatilome and its correlations with physiological parameter were performed using TIBCO Statistica 8.0 (Tibco Inc., Palo Alto, CA, USA). Normality was checked with the Shapiro-Wilk's test, while homoscedasticity was evaluated with the Levene's test (Granato, de Araujo Calado & Jarvis, 2014). Differences between all samples were evaluated with Analysis of Variance (ANOVA), while Principal Component Analysis (PCA), K-mean clustering, Spearman Rank Correlations, and two-way joining heatmap were used to study the relationship between the variables (Nissen, Demircan, Taneyo-Saa & Gianotti, 2019). For post-hoc test a Tukey HSD was employed. For PCA and Spearman Rank Correlations, the data was normalized using the mean centering method. All results are expressed as mean values obtained at least from duplicates batches in two independent experiments. Statistical tests for the Bradford and antioxidant assays were carried out using Statsdirect software 3 (Statsdirect Ltd., Merseyside, UK). For Bradford results, Kruskal-Wallis nonparametric test with Conover-Iman post hoc test in a significant level of $p < 0.05$ was applied. For antioxidant results, statistical significance of a difference between treatments of antioxidant activity was analyzed using one-way analysis of variance (ANOVA) with Tukey's post hoc test in a significant level of $p < 0.05$.

3 RESULTS

3.1 Volatilome analysis

To describe the volatilome through SPME GC-MS among 54 cases, four steps were trod: i) a data set of 73 significant molecules ($p<0.05$) was generated prior normalization with the mean centering method and expressed as a quantification heatmap (Figure S1 supplementary material); ii) a dataset containing the main fermentation metabolites quantified in mg/kg; iii) multivariate analyses obtained from four super-normalized data sets organized by different VOCs chemical classes excluding the main fermentation metabolites; iv) Spearman Rank correlations made from

independent chemical class sorted VOCs and a normalized dataset obtained from mg/kg of main metabolites (acetate, acetoin, ethanol, lactate, 1,4-butandiol) pH values, and microbial growth (yeasts and LAB) (Nissen, Demircan, Taneyo-Saa & Gianotti, 2019). This approach was necessary in order to exalt the discrimination of cases made even by those variables descriptors that were present in small or very small quantity. Lastly, protein content and antioxidant activity results were discussed apart.

3.1.1 Quantification of ethanol, acetic acid, lactic acid, acetoin, and 1,4-butanediol

Considering major molecular compounds such as acetate, ethanol, lactate, 1,4-butanediol, and acetoin, results of quantification in mg/kg of fermented matrices are reported in Table 1 as means of two replicates and two different batches. Ethanol was produced massively in cricket enriched dough directly fermented with yeast for 18 hours (CY18), scoring approximately 3.5 mg/kg more than the control (SY18) and almost doubled the quantity found in cricket enriched sourdough (YC+6). Acetate in cricket enriched doughs scored the highest concentration when directly fermented with lactobacilli for 18 h (CL18) accounting for around 11 mg/kg, almost 1.5 mg/kg more than the relative control (SL18), and more than 2 mg/kg of cricket enriched sourdough (YC+6). Results from lactate quantifications (Table 1) had a similar trend, still showing CL18 as the best performer with 3.58 mg/kg, that were three-fold more than the relative control (SL18), and more than two-fold higher than YC+6. Opposite are the outputs recorded for 1,4-butanediol and acetoin which are shown either to be produced more in the standard doughs. For example, 1,4-butanediol hit the top on standard dough directly fermented with yeast (SY18), slightly higher, but not significantly, than the related cricket enriched dough. This comparison became significant ($p < 0.05$) when the sourdoughs were deemed, indeed YC+6 recorded approximately 4 mg/kg less than the control (YS+6). Considering acetoin, the most interesting results are those obtained from sourdough fermentation, where the cricket samples had almost the half quantity of this compound in respect to the sourdough control.

3.1.2 Multivariate analysis of organic acids

Peak area of GCMS chromatograms were transformed in relative values and then normalized with the mean centering method. From analysis of variance including all samples (n = 54), 16 organic acids resulted significant ($p < 0.05$). Applying K-means clustering on PCA loadings (Figure 1A and 1B), five clusters of cases were drawn (Figure 1C). For the dataset of each of them a normality test, Shapiro Wilk's W test and normal probability plot showed a proper distribution for all except cluster 2 (data not shown). Cluster 1 was an heterogeneous set on PCA's quadrant IV, whose members (n = 12) were all fermented samples but with no distinction over matrices (cricket or standard), nor for fermentation type (direct or sourdough). Anyway, there was a clear sign that distinguished this cluster, that is the LAB contribute. In fact, 8 samples were those fermented with the sourdough or the sole lactobacilli mix, while 4 samples were those fermented spontaneously. This cluster was described by top concentration of acetic, hexanoic, lactic, nonanoic, and phthalic acids, here reported in a descending quantity order. Acetate was more than six-folds higher than in other clusters, while lactate was just quantified here. Hexanoate abundance was the highest, but no significant differences were found in respect to those clusters containing either not fermented or bread cases.

Cluster 2 occupied PCA's quadrant II including four cases of direct fermented breads and four cases of standard flours. This cluster was described by the highest quantitation of sorbic and hexadecanoic acid; the former was just found in this cluster and the latter was doubling the others.

Cluster 3 was on quadrant I and was the largest (n = 18) comprising almost all not fermented samples with similar influence of both the standard and the cricket doughs. The quantitation of compounds was the lowest for this cluster and no particular compound has emerged.

Cluster 4 was centered left on the PCAs plane and was a large (n = 12) and heterogeneous group, that can be considered as the opponent of cluster 1, because it included all yeast-fermented samples. It was the sole that presented a more intense contribution from the matrix, because 8 out of 12

samples were related to cricket. This cluster was described by top abundances of propanoic, butanoic, heptanoic, and octanoic acid. Typical signatures of cluster 3 were butanoic acid that was more than 3-fold higher than the opponent cluster 1 and heptanoic acid that was just here quantified. Cluster 5 contained breads obtained with sourdough fermentation, without distinction from cricket or standard recipes. This cluster was described by top concentration of decanoic, 2-hydroxy-tetradecanoic acid, and pentanoic acid, tallied in descending quantity order. It can be considered as the opponent of cluster 2, which contained the direct fermented breads. Considering Spearman Rank significant correlations ($p < 0,05$) (Figure 1D), propanoic, hexadecanoic, and phthalic acid showed a similar trend, being positively correlated to LAB growth, whereas propanoic and hexadecenoic acid were more linked to the LAB effect as seen by positive correlations with pH decrease, acetate, and lactate. Phthalic acid seemed bound to yeast fermentation as demonstrated by positive correlations with yeast growth, 1,4-butanediol, and ethanol productions. Hexanoic, 2-hexenoic, heptanoic, and nonanoic acids correlated similarly in a positive way with pH decrease and acetate production. Moreover, heptanoic and nonanoic acids correlated even with lactate, indicating that these VOCs are linked to the fermentation environment generated by LAB metabolites, but not directly to LAB growth. An opposite trend was instead that presented by sorbic, pentanoic, and decanoic acid, that were negatively correlated to LAB growth and to yeast growth, 1,4-butanediol and ethanol production, for sorbic and pentanoic acid, and to lactate just for sorbic acid.

3.1.3 Multivariate analysis of aldehydes

From analysis of variance of all samples ($n = 54$), 15 aldehydes resulted significant ($p < 0.05$). PCA loadings (Figure 2A and 2B) were clustered in four sets by K-means analysis (Figure 2C) and a proper distribution was seen for all, but cluster 2 (data not shown). Cluster 1 was centered left on the PCAs plane and was a large ($n = 12$) set, heterogeneous for the type of process but homogeneous for the matrix. In fact, its members were all cricket fermented doughs and breads, made directly or via sourdough. This cluster was described by top concentrations of acetaldehyde,

3-methyl butanal, and 2,4-nonadienal, (E,E), reported in descending order of quantity. Acetaldehyde was found to be about 4-fold higher than what found in the cluster with standard cases and more than 3-fold higher than the cluster containing not fermented cricket doughs. 3-Methyl butanal was found just in fermented cases, while 2,4-nonadienal (E,E) was a signature of this cluster. Cluster 2 was centered right on the PCAs plane and was a large (n = 13) set, heterogeneous for the type of process but homogeneous for the matrix. It bore 9 out of 10 cases of not fermented cricket doughs, two of cricket flour, and two directly fermented cricket doughs. In this cluster dodecanal, octanal, and 9-hexadecenal, (Z), largest to the smallest, emerged over all dataset. In particular, the two latter were unique of this set. Cluster 3 was the largest (n = 17) group occupying quadrant III, heterogeneous for the process, but homogeneous for the matrix. It contained all standard samples, but one. This cluster was characterized by top abundance among other clusters of nonanal, benzaldehyde, heptanal, 2-methyl-2-butenal, and 9-octadecenal, reported in descending concentration order. Nonanal was a unique signature of this cluster. Cluster 4 was an homogeneous small set (n = 11) positioned in quadrant II. Its members were all standard fermented samples, but two. It was described by top concentration among others of furfural and 2,4-decadienal, then by high levels of hexanal and benzaldehyde.

From Spearman Rank (Figure 2D), acetaldehyde, hexanal, heptanal, nonanal, and benzaldehyde showed a similar trend, being positively correlated to 1,4-butanediol production. These compounds were inversely correlated to both microbial growth variables, but not significantly for acetaldehyde, to ethanol production, but not significantly for acetaldehyde and benzaldehyde, and to acetate and lactate production, but not significantly for hexanal and nonanal. An opposite trend was instead that presented by 2-methyl-2-butenal, furfural, 2,4-decadienal, dodecanal, and 9-octadecenal. 2-methyl-2-butenal was positively correlated to yeast growth and ethanol production; furfural to pH decrease and acetate production; 2,4-nonadienal, (E,E) was positively correlated to yeast growth and 1,4-butanediol; 2,4-decadienal to pH decrease, lactate, and acetate production; dodecanal to LAB growth and lactate; while 9-octadecenal to LAB growth, yeast growth, lactate, and 1,4-butanediol.

3.1.4 Multivariate analysis of alcohols.

In order to exalt more differences among the alcohol variables, the normalization of the dataset and the statistical analysis was done excluding ethanol and 1,4-butandiol, which represented a too large part of total alcohols. Moreover, the cases related to flours and not fermented doughs were not included (n = 28), due to the scarce abundance. From analysis of variance, 18 alcohols resulted significant ($p < 0.05$) and on PCA (Figure 3A and 3B) the cases were clustered in four sets (Figure 3C), owing a proper distribution, except for cluster 1 (data not shown). Cluster 1 was a large (n = 10) set, occupying quadrant III, homogeneous for the matrix and the process, but not for fermentation. In fact, its members were all standard either directly fermented doughs or sourdough. It was described by top concentrations among other of 1-hexanol, ethanol, 2,2'-[oxybis(methylenethio)]bis-, 1-pentanol, and 1-hexadecanol, reported in descending order of quantity. In respect to the cluster containing the fermented cricket doughs, cluster 1 had three times more ethanol, 2,2'-[oxybis(methylenethio)]bis- and the double of 1-pentanol and phenylethyl alcohol. Cluster 2 was a small group (n = 4) occupying quadrant II, made by standard breads, indifferently from fermentation. The samples herein were described by top amounts of 2-ethylhexanol, 1-undecanol, 3-furanmethanol, and butyl-1-octanol, reported in descending magnitude. Cluster 3 was similar to the previous, but comprised the cricket breads with top amounts of 1-decanol, 2-methyl and isotridecanol, with the former as a unique mark. Residues of ethanol in the form of ethanol, 2,2'-[oxybis(methylenethio)]bis- were higher when compared to standard breads. Cluster 4 was a large set (n = 10) that included all the fermented cricket samples. This cluster was described by top values of 1-hexanol, 1-octen-3-ol, and 1-heptanol, presented in descending magnitude. In comparison to cluster 1 it had double amount of 1-hexanol and five time more 1-octen-3-ol, while 1-heptanol was found just here. From Spearman Rank (Figure 3D), 1-pentanol, 1-octen-3-ol, 1-dodecanol, 1-hexadecanol, and 2,4-decadienol, (E,E) had similar trend. Four out of five of these compounds were bound to LAB growth, while three to yeast growth.

While 1-pentanol was linked both to LAB growth and lactate, 1-hexadecanol was bound to yeast growth and 1,4-butanediol. On an opposite fashion were correlated 3-furanmethanol, butyl-1-octanol, and octadienol. For example, 3-furanmethanol was negatively correlated to all microbial variables.

3.1.5 Multivariate analysis of ketones

From analysis of variance that included just fermented samples and the breads (n = 28), 23 ketones from C2 to C15 resulted significant ($p < 0.05$). On PCA (Figure 4A and 4B) three clusters of cases properly distributed were spotted (Figure 4C). Cluster 1 was the largest (n = 12) set, positioned in quadrant IV (Figure 4A), comprising all standard fermented samples and the sourdough breads. It was described by twenty different ketones, with top concentration of 2-butanone, hex-3-en-2-one, and 2-nonanone. Cluster 2 was a large (n = 10) and homogeneous set, positioned in quadrant I, including cricket fermented doughs and cricket sourdough breads. It had top concentrations of 2,4-butanedione, 5-decanone, 2-heptanone, 2-dodecanone, oct-3-en-2-one, and 2,2-dimethylcyclohexanone. 2,4-butanedione was quantified almost ten times higher than the opponent cluster containing similar standard cases, while oct-3-en-2-one, 5-decanone, and 2-dodecanone were a unique signature of cricket fermented doughs and sourdoughs breads. Lastly, cluster 3 was that set on the left sector of the PCAs plane, distant to the other clusters, and was the smallest group (n = 6), comprising cases related to control fermented doughs and breads. It was described mainly by 2,6-dimethyl-4-heptanone, 5,9-undecadien-2-one, 6,10-dimethyl-, (E)-6,10-dimethylundec-3-en-2-one, gamma dodecalactone, and 2-octanone. From Spearman Rank correlation (Figure 4D), 2-nonanone and 2-hydroxy-3,3-dimethyl-cyclopentenone were positively correlated to microbial growth or to microbial metabolite, while 2-undecanone, oct-3-en-2-one, (E)-6,10-dimethylundec-3-en-2-one, and gamma dodecalactone were inversely correlated to the aforementioned independent variables.

3.2 Soluble protein concentration and profile

The dough and bread soluble protein content measured by the Bradford assay, and their protein profile analyzed by SDS-PAGE, are reported in supplementary material (Figure S2 and S3). The amount of soluble proteins in cricket dough before fermentation was significantly higher than in the standard sample (respectively, 1.3 g/L for sample CX, and 0.93 g/L for sample SX). After 18 h fermentation with LAB, yeasts and sourdough the amount of soluble proteins remained basically unchanged in standard doughs (samples SL18, SY8, and YS+6), while in cricket doughs decreased of about 28% in samples CL18, CY18, and of about 16% in sample YC+6. This decrease could be explained by hydrolysis of soluble flour proteins to smaller peptides, not detectable by the Bradford assay, due to the activity of microbial proteolytic enzymes. The presence of proteolytic activity was confirmed by the protein profile observed on SDS-PAGE. The profile of the initial samples (SX and CX), was made of a main band around 15 kDa, and some minor bands with higher MW. After fermentation, particularly with LAB (samples SL18 and CL18), an increase of peptides with MW around and below 10 kDa, was clearly visible on gel. After baking of doughs obtained with the three fermentation processes, the amount of soluble proteins strongly decreased (samples SLB, SYB, YS+B for standard bread and CLB, CYB, YC+B for cricket bread) (Figure S3). In parallel, in these samples the 15 kDa band almost disappeared, suggesting a further protein/peptide reduction to smaller peptides.

3.3 Antioxidant activity

The antioxidant activity of control and cricket bread was measured by four different assays: DPPH, ABTS, Fe^{2+} chelating activity and FRAP. All of them showed that cricket dough had initial antioxidant activity significantly higher than the standard dough (samples CX and SX, Fig. 5). ABTS and DPPH activities after fermentation and baking were very low or totally absent for standard samples, while in cricket doughs and breads the trend looked different. In particular, the ABTS and DPPH values decreased after fermentation (samples CL18, CY18, and YC+6), but

significantly increased after baking, in comparison to the initial value (samples CLB, 146.44 and 56.11 mg AA eq/L, CYB, 116.45 and 32.57 mg AA eq/L, YC+B, 123.54 and 43.98 mg AA eq/L, compared to initial sample CX, 78.33 and 30.83 mg AA eq/L, Fig. 5A and 5B, $p < 0.05$). A similar trend (decrease of the values after fermentation and increase after baking) was observed also for Fe^{2+} chelating activity, in particular for cricket samples. After fermentation this activity was present only in cricket dough fermented with sourdough, but at lower level than the initial value (samples CYB, 26.00 µg EDTA eq/L, and CX, 86.04 µg EDTA eq/L, respectively). After baking it was detectable in all cricket breads, with the highest value in LAB fermented bread (sample CLB, 79.42 µg EDTA eq/L, Fig. 5C). Cricket doughs and breads also showed iron reducing capacity, while in standard samples it was detectable only in two baked samples. FRAP initial value was 28.65 mg AA eq/L in cricket doughs (sample CX) and reached the highest levels in LAB fermented cricket dough and bread (samples CL18, 34.74 mg AA eq/L, and CLB, 35.77 mg AA eq/L, Fig.5D). Considering the all set of results, cricket bread obtained by LAB fermentation had the highest antioxidant activity within the four applied methods.

4 DISCUSSION

4.1 Organic acids

From multivariate analysis a first distinction between fermented doughs and breads was seen, a second between inocula, and a third between sourdough and direct breads. At this level of analysis, the sole distinction among the matrices was seen for the standard and the cricket samples that were not clustered along. Notwithstanding, considering that acetic, hexanoic, lactic, and nonanoic acids are bound to the performance and quality of LAB fermentation, the cricket samples have shown to be likely effective as the standard samples, either when fermented directly by lactobacilli or by sourdough. This signature was evident as a cluster was mainly made by the sourdough or the sole LAB fermented samples and described by top concentration of the aforementioned acids.

Acetic and lactic acid are related to the fermentation performance of any given LAB inoculum, in fact are assigned as the main metabolites of food fermentation by heterofermentative lactobacilli, and contribute with great impact to both the sensorial and the quality and safety of a fermented food. Indeed, these VOCs determine a typical sharp, acrid, vinegar, sour taste, with a buttery nuance given by lactic acid. (Petel, Onno & Prost, 2017). Both have biological properties, inhibiting ubiquitous bacilli, spoilage microbes and food-borne pathogens, generating an environment for competitive exclusion and acting mechanistically on Gram negative membrane (Bartkiene et al., 2019). Acetate fits the new definition of prebiotics (Gibson et al., 2017), stating that a prebiotic must stimulate selectively the growth and/or activity of intestinal bacteria associated with health and wellbeing, thus excluding opportunistic or pathogens. In fact, acetate is renown to foster the selective growth of probiotics in the gut (Esgalhado, Kemp, Damasceno, Fouque & Mafra, 2017), and is essential for a healthy host intestinal epithelium, stimulating epithelial immune function (Goverse et al., 2107).

Hexanoic acid (caproic acid) and nonanoic acid (pelargonic acid) are medium chain fatty acids renown as effective on excessive calorie burning, inducing weight loss (Rego Costa, Rosado & Soares-Mota, 2012). Contrastingly, caproic acid has a cheesy, waxy, fatty, goat scents that result sensorially unpleasant. It is a fermentation metabolite produced by lactobacilli or yeast originated by sourdough fermentation and responsible of the inhibition of moulds in bread (Petel, Onno & Prost, 2017). Similar effect and aroma are generated by pelargonic acid, a microbial metabolite found in whey fermented alcoholic beverages (Dragone, Mussatto, Oliveira & Teixeira, 2009). Acetic acid is even found in conventional bread made from wheat and leavened by commercial baker's yeast (Pasqualone, Caponio, Pagani, Summo, & Paradiso, 2019) otherwise lactic acid is typical of LAB or sourdough fermentation (Petel, Onno & Prost, 2017). Hexanoic and nonanoic acids are reported to be present in conventional bread and in semolina bread made by fermentation with natural sourdough (Petel, Onno & Prost, 2017; Giannone, Giarnetti, Spina, Todaro, et al.,

2018). Recently, these organic acids were described in bread fortified with insect other than cricket, i.e. palm weevil larvae (Ayensu, Lutterodt, Annan, Edusei, & Loh, 2019).

4.2 Aldehydes

For the dataset of aldehydes distinctions based on the matrix and on the process have occurred, but not depending on the type of fermentation. The production of 2-methyl-2-butenal, furfural, 2,4-decadienal, dodecanal, and 9-octadecenal seemed triggered by microbes or by their metabolites. From the multivariate analysis the main descriptors of cricket goods were acetaldehyde, 3-methyl butanal, dodecanal, and 2,4-nonadienal, (E,E). Acetaldehyde was described to have a pungent, ethereal, fruity, floral, green, roasted, malty odor and is derived by Maillard reaction, lipid oxidation and yeast fermentation (Petel, Onno & Prost, 2017). It was found in fermented sourdough and in breads directly fermented with baker's yeast (Petel, Onno & Prost, 2017; Pasqualone, Caponio, Pagani, Summo, & Paradiso, 2019). 2,3-methyl butanal is derived from yeast isoleucine conversion, conferring a pleasant nuance (musty, cocoa, coffee, nut sweet roasted) to sourdough breads (Petel, Onno & Prost, 2017), but can also be found in conventional wheat breads fermented by baker's yeast (Pasqualone, Caponio, Pagani, Summo, & Paradiso, 2019). 2,4-nonadienal, (E,E) is used in perfumes and in foods, giving off floral notes to all odor types, and was found in sour pickles fermented by lactic acid bacteria (Zieliński, Surma & Zielińska, 2017).

4.3 Alcohols

Among the dataset of alcohols, PCA described that samples distinction was due to the matrix and to the process effects. Two opponent clusters, the cricket and the standard, related to fermentation were positioned in distant plane quadrants, thus net differences for their descriptors were seen. From multivariate analysis, the main descriptors of cricket samples were 1-hexanol, 1-octen-3-ol, and 1-heptanol in fermented doughs, while 1-decanol, 2-methyl and isotridecanol in breads.

1-hexanol, 1-heptanol, and 1-octen-3-ol were reported to be present in rice, soybean, rye and wheat flours or products, conferring a typical olfactory nuance described as: musty, pungent, leafy green, and mushroom taste, respectively (Chen, Xu & Qian, 2013). 1-hexanol is common and abundant in sourdough, mainly produced by LAB fermentation and in line with our results, uses to persist after baking (Petel, Onno & Prost, 2017; Giannone, Giarnetti, Spina, Todaro, et al., 2018). 1-heptanol instead is still associated to sourdough, but alike our result is not detectable in breads (Petel, Onno & Prost, 2017). 1-octen-3-ol was described in different plant and mushrooms and is derived from linoleic acid during oxidative breakdown, owning antimicrobial activity against spoilage and pathogenic microbes (Mishra et al., 2017).

4.4 Ketones

Among ketones the main influence on distinction of samples was promoted by food matrix and by the process. On PCA two big sets were containing all the fermented doughs separated for the matrix and the sourdough breads, while a third cluster contained not sourdough bread. Bearing in mind that the overall content of ketones and the speciation of compounds were much lower in this third cluster, it was possible to consider that the production of ketones was pushed by prolonged fermentation. In fact, just 9 ketones were seen in the third cluster in comparison to 20 in the other two clusters. 2,4-butanedione, 2-heptanone, 2-dodecanone, and 3-octen-2-one were those ketones solid descriptors of cricket goods. 2,4-butanedione is found abundant in balsamic vinegars and in beers (Nissen, Demircan, Taneyo-Saa & Gianotti, 2019). It is produced mainly by homofermentative LAB (Petel, Onno & Prost, 2017) and is used to improve aroma and texture, conferring a scent of butter or making beer lighter to the palate (Nissen, Demircan, Taneyo-Saa & Gianotti, 2019). 2-heptanone is found in wheat or rye sourdough and confers a typical aroma described as fruity, spicy, sweet, coconut. (Ripari, Cecchi & Berardi, 2016). 3-octen-2-one has an earthy, mushroom, spicy, sweet, grass odor and was detected just in wheat sourdough (Ventimiglia et al., 2015).

4.5 Protein characterization

The formulation of cricket dough (i.e. the percentage of rice, corn and cricket flour) was defined to allow the claim "source of proteins" which can be assigned, according to the Regulation (EU) No 1924/2006 and No 1047/2012, to a food in which proteins contribute at least 12%, to the total energy. In the control dough, that is dough devoid of cricket flour, the amount of the latter was replaced by rice and corn flour, maintaining the same proportion of the two, as in cricket dough. Due to the higher percentage of proteins (w/w) in cricket flour (66.1%), with respect to rice (7%) and corn (8.5%) flours, the total proteins in cricket dough (10.7 %, of which 5.3 from rice, 1,6 from corn and 3.8 from cricket), were higher than in control dough (7,3%, of which 5.6 from rice and 1.7 from corn). Coherently, the amount of soluble proteins in cricket dough was initially higher than in control samples. Fermentation brought to a decrease of soluble proteins to low MW peptides, probably due to the hydrolytic activity of microbial enzymes. The amount of soluble proteins further decreased after heating. Heating could bring to thermal denaturation and aggregation of proteins but also to partial degradation of proteins to smaller peptides and amino acids (not detectable by the Bradford assay and SDS-PAGE). Other authors (You, Zheng, Regenstein, Zhao & Dong, 2012) showed indeed that the content of peptides above 5 kDa of loach protein hydrolysates decreased significantly (and peptides 1-5 kDa increased significantly) after heat treatment.

4.6 Antioxidant activity

Mixture of substances can contribute to antioxidant activity of a foodstuff so, in order to examine its antioxidant potential, it is important to consider several assays, rather than reckon on a single analysis. In this light, the antioxidant activity of control and cricket doughs and breads was measured by four different assays. $ABTS^{-}$ and $DPPH^{-}$ radical scavenging assays measure the scavenging capacity of hydrogen donating-antioxidants towards free radicals $ABTS^{\cdot+}$ and $DPPH^{\cdot}$. Ferrous ion- chelating ability assay measure the chelating capacity of substances, towards ferrous

ion (Fe^{2+}). Finally, the FRAP method measures the antioxidant potential in samples through the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). All these assays showed that cricket dough had initial antioxidant activity significantly higher than the control dough. These results are in agreement with the recent findings of other authors on the antioxidant properties of flour from *Acheta domesticus* (Lucas-Gonzalez, Fernandez-Lopez, Perez-Alvarez & Viuda-Martos, 2019; Di Mattia, Battista, Sacchetti & Serafini, 2019; David-Birman, Raften & Lesmes, 2018).

After fermentation of cricket doughs, a general decrease of antioxidant capacity was observed, with the only exception of ferric reducing capacity for LAB fermented cricket dough. No data are currently available in the literature for fermented and bakery products obtained with *Acheta domesticus* flour. It was reported that hydrolysis of protein extracts of other cricket species and edible insects contribute to increase their antioxidant activity (Hall, Johnson & Liceaga, 2018; Zielińska, Baraniak & Karas, 2017; Zielińska, Karas & Jakubczyk, 2017). In our samples, protein hydrolysis observed after fermentation, did not positively influenced the antioxidant properties of doughs. Conversely, baking significantly increased antioxidant activities, particularly the ABTS, DPPH and Fe^{2+} chelating outputs. Similarly, Zielińska, Baraniak & Karas (2017) observed that heat treatment and particularly baking, increased the antiradical activity of hydrolysates and peptide fractions from another cricket specie, *Grillodes sigillatus*. The protein component could affect the antioxidant activity after baking by generating smaller peptides with stronger activity, by protein structure changes, but also acting as substrate for Maillard reaction. Antioxidant activity of thermal treated cricket flour examined by FRAP and ORAC assays, revealed improved antioxidant activity which was attributed to conformational changes in cricket proteins (David-Birman, Raften & Lesmes, 2018). It is also well known that Maillard products formed during baking process, such as complex melanoidin, have antioxidant activity (Borrelli et al., 2003). Antioxidant compounds originated by Maillard reaction in baked products were also able to decrease lipid oxidation (Wijewickreme & Kitts, 1998).

Overall, the results of antioxidant activity indicate that doughs prepared with cricket flour have a strong antioxidant activity *in vitro*, asserted by different mechanisms, which is maintained and even increased after baking. In particular, cricket bread obtained by LAB fermentation had the highest activity by all the four assays applied. Despite the molecular basis of these findings still need to be clarified, they add value to this cricket-based food, which can become a source of antioxidant compounds, aside from high quality proteins.

5 CONCLUSIONS

In this study, a cricket-fortified gluten-free sourdough bread "source of proteins" was produced and analyzed for volatilome composition and antioxidant properties. Multivariate analysis on VOCs portrayed a robust landscape, where samples made with the addition of cricket powder were described mainly by: i) similar fermentation performance to a standard dough, with no difference in respect to abundances of acetoin and acetate, little higher abundances of ethanol and lactate, and little lower abundances of 1,4-butanediol; ii) a typical flavoring profile made by nuances of hexanoic and nonanoic acid, 2,4-nonadienal, (E,E), 1-hexanol, 1-heptanol, and 1-octen-3-ol, 2,4-butanedione, 2-heptanone, and 3-octen-2-one. The use of cricket powder ultimately conferred to the bakery goods a typical aroma, characterized by a unique bouquet of volatile organic compounds, differently expressed when different types of inoculum were applied. Bread containing cricket flour had antioxidant properties significantly higher in comparison to bread produced with only gluten-free flours, particularly after LAB fermentation. Furthermore, sensorial evaluations of cricket enriched breads recorded similar scores than standard ones (Figure S4). So, cricket flour addition to bakery food did not only provide sustainable proteins with high nutritional value, but also exerted beneficial effects on human organism by prevention of oxidative damage.

In conclusion, this study offers for the first time a complete aromatic profile of a cricket-enriched bakery gluten-free product obtained by a sourdough process. These results contribute to substantiate

cricket flour as a source of sustainable proteins with high quality, nutritional value and antioxidant properties.

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Supplementary material

Table S1. Description of the proximate composition of flours

Table S2. Description of samples produced and analyzed.

Figure S1. Heatmap of relative quantification of significant molecules.

Figure S2. Soluble protein concentration (mg/L) of standard and cricket doughs and breads.

Figure S3. SDS-PAGE 14% of standard (A) and cricket (B) doughs and breads.

Figure S4. Spider chart of two independent consumer's tests performed by 20 semi-trained volunteers.

Figure captions

Figure 1.A) PCA of cases and B) variables on organic acids; C) K-means clustering; D) Spearman rank correlations on alcohols. Blue line = cluster 1; orange line = cluster 2; green line = cluster 3; fuchsia line = cluster 4; black line = cluster 5. *p < 0.05; **2-hydroxy tetradecanoic acid. Samples abbreviations: FC = Flour from cricket; FR = flour from rice; FM = Flour form maize; CX = cricket enriched dough not inoculated; CL = cricket enriched dough inoculated with lactobacilli; CY = cricket enriched dough inoculated with yeast; SX = standard dough not inoculated; SL = standard dough inoculated with lactobacilli; SY = standard dough inoculated with yeast; CX18 = CX fermented 18 h; CL18 = CL fermented 18 h; CY18 = CY fermented 18 h; SX18 = SX fermented 18 h; SL18 = SL fermented 18 h; SY18 = SY fermented 18 h; YC+ = cricket enriched sourdough; YS+ = standard sourdough; YC+6 = YC+ fermented 6 h; YS+6 = YS+ fermented 6 h; YC = cricket enriched dough inoculated with yeast; YS = standard dough inoculated with yeast; YC6 = YC fermented 6 h; YS6 = YS fermented 6 h; YCB = YC bread; YSB = YS bread; YC+B = YC+ bread; YS+B = YS+ bread.

Figure 2. A) PCA of cases and B) variables on aldehydes; C) K-means clustering; D) Spearman rank correlations on aldehydes. Blue line = cluster 1; orange line = cluster 2; green line = cluster 3; fuchsia line = cluster 4. *p < 0.05; For samples abbreviations of cases see Figure 1.

Figure 3. A) PCA of cases and B) variables on alcohols; C) K-means clustering; D) Spearman rank correlations on ketones. Blue line = cluster 1; orange line = cluster 2; green line = cluster 3; fuchsia line = cluster 4. *p < 0.05; **ethanol, 2,2'-[oxybis(methylenethio)]bis-. Samples abbreviations: CX18 = CX fermented 18 h; CL18 = CL fermented 18 h; CY18 = CY fermented 18 h; SX18 = SX fermented 18 h; SL18 = SL fermented 18 h; SY18 = SY fermented 18 h; YC+ = cricket enriched sourdough; YS+ = standard sourdough; YC+6 = YC+ fermented 6 h; YS+6 = YS+ fermented 6 h; YC = cricket enriched dough inoculated with yeast; YS = standard dough inoculated with yeast; YC6 = YC fermented 6 h; YS6 = YS fermented 6 h; YCB = YC bread; YSB = YS bread; YC+B = YC+ bread; YS+B = YS+ bread.

Figure 4. A) PCA of cases and B) variables on ketones; C) K-means clustering; D) Spearman rank correlations on alkanes. Blue line = cluster 1; orange line = cluster 2; green line = cluster 3. *p < 0.05; **2-hydroxy-3,3-dimethyl-cyclopentenone; †(E)-6,10-dimethylundec-3-en-2-one; *†5,9-Undecadien-2-one, 6,10-dimethyl-. For samples abbreviations see Figure 3.

Figure 5. Antioxidant activity of control and cricket bread measured by: A) ABTS radical scavenging activity assay (mg AA eq/L); B) DPPH radical scavenging activity assay (mg AA eq/L); C) ferrous ion-chelating ability assay (μg EDTA eq/L); D) ferric reducing antioxidant power (FRAP) assay (mg AA eq/L). $^{a-j}$ = Means with different letters are significantly different at p < 0.05 by Tukey's test. Samples abbreviations: SX = standard dough not inoculated; SX18 = SX fermented 18 h; SL18 = standard dough inoculated with lactobacilli fermented 18 h; SLB = SL18 bread; SY18 = standard dough inoculated with yeast fermented 18 h; SYB = SY18 bread; YS+6 = standard

sourdough fermented 6 h; YS+B = YS+6 bread; CX = cricket enriched dough not inoculated; CX18 = CX fermented 18 h; CL18 = cricket enriched dough inoculated with lactobacilli fermented 18 h; CLB = CL18 bread; CY18 = cricket enriched dough inoculated with yeast fermented 18 h; CYB = CY18 bread; YC+6 = cricket enriched sourdough fermented 6 h; YC+B = YC+6 bread.

Supplementary material figure captions.

Table S1. Description of the proximate composition of flours.

Table S2. Description of samples produced and analyzed.

Figure S1. Heatmap of relative quantification of VOCs. *ethanol, 2,2'-[oxybis(methylenethio)]bis-; **2-hydroxy-3,3-dimethyl-cyclopentenone; †(E)-6,10-dimethylundec-3-en-2-one; *†5,9-undecadien-2-one, 6,10-dimethyl-.

Figure S2. Soluble protein concentration (mg/L) of standard and cricket samples measured by the Bradford assay. $^{a-j}$ = Means with different letters are significantly different at $p < 0.05$ by Conover-Iman's test Samples abbreviations: SX = standard dough not inoculated; SX18 = SX fermented 18 h; SL18 = standard dough inoculated with lactobacilli fermented 18 h; SLB = SL18 bread; SY18 = standard dough inoculated with yeast fermented 18 h; SYB = SY18 bread; YS+6 = standard sourdough fermented 6 h; YS+B = YS+6 bread; CX = cricket enriched dough not inoculated; CX18 = CX fermented 18 h; CL18 = cricket enriched dough inoculated with lactobacilli fermented 18 h; CLB = CL18 bread; CY18 = cricket enriched dough inoculated with yeast fermented 18 h; CYB = CY18 bread; YC+6 = cricket enriched sourdough fermented 6 h; YC+B = YC+6 bread.

Figure S3. Protein pattern of standard (A) and cricket (B) samples on SDS-PAGE 14%. Samples abbreviations: SX = standard dough not inoculated; SX18 = SX fermented 18 h; SL18 = standard

dough inoculated with lactobacilli fermented 18 h; SLB = SL18 bread; SY18 = standard dough inoculated with yeast fermented 18 h; SYB = SY18 bread; YS+6 = standard sourdough fermented 6 h; YS+B = YS+6 bread; CX = cricket enriched dough not inoculated; CX18 = CX fermented 18 h; CL18 = cricket enriched dough inoculated with lactobacilli fermented 18 h; CLB = CL18 bread; CY18 = cricket enriched dough inoculated with yeast fermented 18 h; CYB = CY18 bread; YC+6 = cricket enriched sourdough fermented 6 h; YC+B = YC+6 bread.

Figure S4. Spider chart of two independent consumer's tests. Black plot = CLB (cricket enriched bread fermented 18 h by lactobacilli); gray plot = CYB (cricket enriched bread fermented 18 h by yeast); blue plot = YC+B (cricket enriched sourdough bread); light blue plot = YCB (cricket enriched bread fermented 6 h by yeast); red plot = SLB (standard bread fermented 18 h by lactobacilli); orange plot = SYB (standard bread fermented 18 h by yeast); fuchsia plot = YS+B (standard sourdough bread); yellow plot = YSB (standard bread fermented 6 h by yeast).

Table 1. Quantification (mg/kg) of main fermentation metabolites.

Sample	Ethanol	Acetate	Lactate	1,4-butanediol	Acetoin
SX18	$2.91^{a} \pm 0.15$	$3.12^{b} \pm 0.19$	0.00 ± 0.00	$8.90^{a} \pm 0.16$	$6.10^{a} \pm 0.16$
SL18	$8.35^{b} \pm 0.13$	$9.52^{c} \pm 0.12$	$1.13^{a} \pm 0.07$	$12.54^{b} \pm 0.27$	$22.67^{c} \pm 0.32$
SY18	$12.83^{b} \pm 0.13$	$1.92^{b} \pm 0.21$	0.00 ± 0.00	$14.90^{b} \pm 0.16$	$15.87^{b} \pm 0.31$
CX18	$5.39^{a} \pm 0.31$	$0.24^{a} \pm 0.04$	$0.27^{a} \pm 0.02$	$7.10^{a} \pm 0.02$	$8.44^{a} \pm 0.32$
CL18	$9.26^{b} \pm 0.54$	$11.10^{c} \pm 0.47$	$3.58^{b} \pm 0.11$	$12.38^{b} \pm 0.11$	$19.98^{c} \pm 0.22$
CY18	$16.45^{c} \pm 0.26$	$0.88^{a} \pm 0.07$	0.00 ± 0.00	$14.26^{b} \pm 0.17$	$15.59^{b} \pm 0.67$
YC+6	$9.69^{b} \pm 0.22$	$8.81^{c} \pm 0.21$	$1.58^{a} \pm 0.04$	$7.43^{a} \pm 0.28$	$13.74^{b} \pm 0.35$
YS+6	$8.89^{b} \pm 0.16$	$6.84^{c} \pm 0.13$	$1.02^{a} \pm 0.14$	$11.60^{b} \pm 0.30$	$24.42^{c} \pm 0.23$

SX18 = Standard dough not inoculated and fermented for 18 h; SL18 = Standard dough inoculated with lactobacilli and fermented for 18 h; SY18 = Standard dough inoculated with *S. cerevisiae* LBS and fermented for 18 h; CX18 = Cricket enriched dough not inoculated and fermented for 18 h; CL18 = Cricket enriched dough inoculated with lactobacilli and fermented for 18 h; CY18 = Cricket enriched dough inoculated with *S. cerevisiae* LBS and fermented for 18 h; YC+6 = Cricket enriched sourdough fermented for 6 h; YS+6 = Standard sourdough fermented for 6 h. ^{a,b,c}: different letters indicate statistical significance among each dependent variables by Tukey's test.

Highlights

1: cricket flour was used to produce cricket-fortified gluten-free sourdough bread

2: antioxidant properties and volatilome of fermented and baked cricket breads were studied

3: cricket-enriched doughs and a standard dough had similar fermentation processes

4: the use of cricket powder ultimately conferred to the bakery products a typical aroma

5: bread containing cricket powder fermented with LAB had higher antioxidant properties

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