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Effect of *Yarrowia lipolytica* RO25 cricket-based hydrolysates on sourdough quality parameters

Samantha Rossi[a], Luigi Parrotta[b,c], Stefano Del Duca[b,c], Marco Dalla Rosa[a,c], Francesca Patrignani[a,c] , Oliver Schluter*[a,d], and Rosalba Lanciotti*[a,c]

[a] Campus Food Science, Department of Agricultural and Food Sciences, Alma Mater Studiorum, University of Bologna, Cesena, Italy.

[b] Department of Biological, Geological and Environmental Sciences, University of Bologna, Bologna, Italy.

[c] Interdepartmental Centre for Agri-Food Industrial Research, University of Bologna, Bologna, Italy.

[d] Leibniz Institute for Agricultural Engineering and Bioeconomy, Quality and Safety of Food and Feed, Potsdam, Germany

*Corresponding Authors:

Rosalba Lanciotti: rosalba.lancotti@unibo.it, Campus of Food Science, p.zza Goidanich 60, 47521 Cesena (FC), Italy

Oliver Schluter: oschlueter@atb-potsdam.de, Leibniz-Institut für Agrartechnik und Bioökonomie e.v. Max-Eyth-Allee 100, 14469 Potsdam, Germany

Abstract

The principal aim of this research was to use *Yarrowia lipolytica* RO25 to obtain a cricket powder-based hydrolysate to produce sourdough for bread production. RO25 hydrolysed cricket sourdough (RO25H-CS) was compared with a control traditional sourdough and a control containing the no-hydrolysed cricket powder. Microbiological analyses evidenced a good growth of *Yarrowia lipolytica* in RO25 cricket hydrolysate and the RO25H-CS was characterized, in comparison with the controls, by a marked and peculiar total protein profile, attributed to the well-known proteolytic activities of *Yarrowia lipolytica*. RO25H-CS was also endowed with a specific profile in free fatty acids, including arachidonic and linolenic having a functional role. Moreover, RO25H-CS was characterized, compared with the control samples, by the highest releases of C18:2, C18:1 and C16:1, which are considered aroma precursors. In fact, the presence of highest proteolytic activity and the highest amount of free fatty acids detected in RO25H-CS sample underline a specific volatile molecules profile. The results obtained showed the great potential of *Yarrowia lipolytica* RO25 to produce sourdough characterized by specific sensory and functional fingerprints.

Keywords

Yeast cricket hydrolysate; Sourdough; Protein profile; Aroma profile; Fatty acid.

1. Introduction

Recently, insects have been gaining a lot of interest as nutritional sources to meet the need of more sustainable future food and feed demand. In fact, they constitute a valid source of proteins, fats, vitamins and minerals (Van Huis, 2013; Patrignani et al., 2020) and the growing interest in alternative protein fonts has led to a rapid expansion of industrial insect farms (Hanboonsong, Rattanapan, Waikakul, & Liwvanich, 2001). Moreover, insects can be considered a good source of polyunsaturated fatty acids, able to improve the consumer health, and of a great amount of essential amino acids such as threonine, tyrosine, valine, methionine and lysine, confirming their high

nutritional value (Pacetti, Mozzon, Lucci, & G Frega, 2013; Osimani et al., 2018). However, although they represent a high nutritional alternative to animal protein, their use is still limited in the culture of western countries because insects are not part of their alimentary diet model (Megido et al., 2016), and also because insects are characterized by high chitin content, which limits their application for food formulations. Yet, the European Food Safety Authority has proposed a list of insect species, including *Acheta domesticus* that could be used as food and/or feed in the EU (EFSA Scientific Committee, 2015) and to mitigate their negative perception, it has been suggested to use them in food formulation as flour or protein powder extract (Schouteten et al., 2016). For this, the attention of researchers and the food industry has been focused on the formulation of a wide range of insect powder-based foods with high nutritional value and sensory features, also gifted of functional characteristics. However, their use is subordinated to the authorization by EFSA, after evaluation of nutrient composition and its modification after processing, safety aspects related to the presence of potential allergens and contaminants, and hopefully, data on insect metabolism in human and potential cytotoxicity. In fact, it was found that insect-based foods pose potential risk to shellfish allergic patients due to homologous proteins including tropomyosin, an invertebrate cross-reactive allergen, with a hazard for shellfish sensitized patients to develop an allergy to edible crickets (Fernandez-Cassi, Supeanu, Jansson, Boqvist, & Vagsholm, 2018; Garino et al., 2020). However, Palmer (2016) showed that *A. domesticus* contained a lower amount of tropomyosin than other species of insect. Furthermore, EFSA (2018) declared that allergic reactions linked to *A. domesticus* are rarely reported in regions where cricket consumption is more common.

Depending on the considered country, also cricket powder was used in food formulations and these cricket powder-based food products are available in some EU countries. However, the use of cricket flour as ingredients involves some limitations. In fact, the flavour is not always considered pleasant and, therefore, it is necessary to mask it, or limit its percentage inside the formulation. In addition, cricket powder is subject to different forms of hydrolytic, oxidative, microbial and enzymatic deterioration (Patrignani et al., 2020). In fact, short chain fatty acids, deriving from hydrolytic and

oxidative phenomena, can be easily detected and considered responsible for bad smell and taste (Dave & Ghaly, 2011). The presence of water in food formulation acts as a catalyst for such hydrolytic phenomenon. From a microbiological point of view, deterioration of insect powder is caused by several bacteria, moulds and yeasts which can cause the appearance of viscosity, discoloration, acidification and gas formation in the matrix. Moreover, the matrix is also composed of chitin, which can represent a technological and toxicological problem for insect-based food products. A previous paper (Patrignani et al., 2020) has been highlighted the technological potential of selected strains of *Yarrowia lipolytica* and *Debaryomyces hansenii* to reduce the presence of chitin in cricket powder and improve the safety, functionality and sensory properties of the cricket powder. The use of the selected strains and optimised biotechnological process resulted in a cricket-based protein hydrolysates characterized by improved free amino acid and fatty acid profiles and the presence of bioactive compounds such as α-Aminobutyric acid (AABA), γ-Aminobutyric acid (GABA) and β-Aminobutyric acid (BABA) as well as by increased level of antimicrobials able to increase the safety and shelf-life of the cricket-based ingredients. Recently, the literature has pointed out the use of cricket powder for breadmaking (Osimani et al., 2018; Nissen, Samaei, Babini, & Gianotti, 2020) in order to increase consumer expectations towards this type of products.

In this context, the purpose of the present research was to evaluate the potential of a cricket powder hydrolysate, obtained by *Yarrowia lipolytica* RO25 according to Patrignani et al. (2020), to be used as ingredient in the formulation of a sourdough obtained using selected strains of yeasts and lactic acid bacteria (LAB) such as *Kazachstania unispora*, *Kazachstania servazzii* and *Fructilactobacillus sanfranciscensis* (formerly *Lactobacillus sanfranciscensis*). The sourdough obtained with the cricket hydrolysate was compared with the traditional wheat one and with a sourdough obtained using not hydrolysed cricket powder. For all the considered sourdoughs, the microbiological compositions, the pH values, the protein contents and the fatty acid and volatile profiles were evaluated.

2. Materials and methods

2.1 Growth conditions of yeast and bacteria strains

Yarrowia lipolytica RO25, *Kazachstania unispora* FM2, *Kazachstania servazzii* KAZ2, *Fructilactobacillus sanfranciscensis* DG1 belonging to the strain collection of the Department of Agricultural and Food Sciences, University of Bologna, were used.

Yarrowia lipolytica RO25 was used to hydrolyse the cricket powder. Before its use, *Yarrowia lipolytica* RO25 was grown twice in YPD (Oxoid, Basigstone, UK) broth incubated at 25 °C for 48h. *Kazachstania unispora* FM2, *Kazachstania servazzii* KAZ2, *Fructilactobacillus sanfranciscensis* DG1 were used for sourdough preparation. Before their use, *Kazachstania unispora* FM2 and *Kazachstania servazzii* KAZ2 were subcultured twice separately in YPD broth, while *Fructilactobacillus sanfranciscensis* DG1 was grown in MRS (Oxoid, Basigstone, UK) added of maltose (2 g/100 mL) broth and incubated at 30 °C for 12h.

2.2. Preparation of the cricket powder hydrolysate

The cricket powder hydrolysate was prepared according to literature (Patrignani et al., 2020) using the *Yarrowia lipolytica* RO25 strain inoculated at level of 5 Log10 CFU/g in cricket powder and water (ratio 1:3 w:w). A control ingredient was prepared in the same way, but without the *Yarrowia lipolytica* inoculation and named no hydrolysed cricket-based control. All the two samples were incubated at 25 °C for 72h with agitation.

2.3 Sourdough preparation

Kazachstania unispora FM2, *Kazachstania servazzii* KAZ2 and *Fructilactobacillus sanfranciscensis* DG1 were separately grown in YPD (Yeast Extract–Peptone–Dextrose Broth) and Maltose MRS (mMRS) broth, respectively, at 30 °C for 12h. Microbial cells were collected by centrifugation at 4000 rpm for 5 min, twice washed and resuspended in sterile physiological solution (0.9 g/100 mL NaCl w/v).

Initially, 200g of wheat flour and 100g of water were mixed and inoculated with fresh cultures of *Kazachstania unispora* FM2, *Kazachstania servazzii* KAZ2 and *Fructilactobacillus sanfranciscensis* DG1 at level of 4 Log10 CFU/g and 6 Log10 CFU/g, for yeasts and LAB, respectively.

After 24h of fermentation at 25 °C, the fermented wheat flour was subjected to the first refreshment adding flour and water as shown in Table 1. After further 24h at 25 °C, the preferment was divided in 3 aliquots, which were used to prepare three different set of samples (named second refreshment in the Table 1):

1. The first sample set was obtained by adding 77 g/100 mL of wheat flour to 23 g/100 mL of the preferment deriving from the first refreshment and 39.5 g/100 mL of tap water. This set of samples, without cricket as ingredient, was named wheat control sourdough (WCS).
2. The second set of samples was obtained by adding 30 g/100 mL (on dry weight) of no hydrolysed cricket to 23 g/100 mL of the preferment deriving from the first refreshment, 47 g/100 mL of wheat flour and 10 g/100 mL of bottle water. This set of samples was named no hydrolysed cricket powder sourdough (noH-CS).
3. The third set of samples was obtained by adding 30 g/100 mL (on dry weight) of RO25 cricket hydrolysate (30 g/100 mL on dry weight) to 23 g/100 mL of the preferment deriving from the first refreshment, 47 g/100 mL of wheat flour and 10 g/100 mL of bottle water. This set of samples was named RO25 hydrolysed cricket sourdough (RO25H-CS).

After a fermentation of 24h at 25 °C, the samples were analysed for their microbiological composition, the pH values, the protein contents, and fatty acid and volatile profiles.

2.5 Microbiological analyses and pH

The presence of *Listeria monocytogenes* and *Salmonella* spp. were investigated in cricket powder according to the ISO 11290 (2017) and ISO 6579 (2017) methods, respectively, and cricket-based hydrolysate.

Microbiological analyses were performed on: i) cricket powder immediately after *Yarrowia lipolytica* RO25 inoculation and during the incubation of samples at 25 °C (data not showed); ii) wheat flour immediately after yeast and LAB inoculation and over the incubation at 25 °C; iii) the preferment immediately after the first refreshment; iv) the three set of samples obtained after the second refreshment (at 0 and after 24h of incubation). The cell loads of yeasts and LAB in the different

157 samples were determined using, respectively, YPD Agar (Oxoid Ltd) and mMRS. Decimal dilutions
158 of the samples, performed in physiologic solution (0.9 g/100 mL NaCl w/v), were inoculated in Petri
159 dishes and incubated for 48h at 25 and 30 °C respectively for *Yarrowia lipolytica* RO25 and for the
160 remaining yeasts and bacteria.
161 The pH values of the samples were determined by pH meter (BASIC 20, Crison, Modena, Italy).
162 **2.6 Protein profiles**
163 Proteins were extracted from the different types of sourdough immediately after preparation (second
164 refreshment according to Table 1) and after 24h of fermentation at room temperature (RT) according
165 to the method proposed by Marco, Pérez, Ribotta, & Rosell (2007) with some modifications. Briefly,
166 total proteins were extracted under non-reducing and reducing conditions. Proteins were extracted
167 under reducing conditions by adding 5 mL of 100 mmol/L Tris–HCl pH 6.8, 4 g/100 mL (w/v),
168 sodium dodecyl sulphate (SDS), 20 g/100 mL glycerol, 200 mmol/L β-mercaptoethanol to 1 g of
169 sample then mixed by vortexing for at least 1 min. Then, samples were centrifuged at 5000 rpm for
170 10 min a 4 °C. Supernatants were precipitated overnight (O.N.) in 4 volumes of 20 g/100 mL TCA
171 in cold acetone with 0,007 g/100 mL β-mercaptoethanol at −20 °C; precipitated proteins were washed
172 in cold acetone containing 0,007 g/100 mL β-mercaptoethanol and then resuspended in suitable
173 buffers for 1-D electrophoresis. Proteins under non-reducing conditions were extracted following a
174 sequential extraction with following solvents: 5 g/100 mL of NaCl, 50 g/100 mL 1-propanol and 0.1
175 M NaOH, 0.5 g/100 mL SDS and 0.6 g/100 mL β-mercaptoethanol. Supernatants and pellet were
176 processed as described for reducing protein protocol. The protein concentration of samples was
177 determined using a commercial kit (2-D Quant Kit, GE HealthCare). The protocol was performed
178 exactly as described in the instruction manual using BSA as reference. Each sample was analysed in
179 three replicates.
180 Separation of proteins by 1-D electrophoresis was carried out on polyacrylamide gels as described by
181 Laemmli (1970). Gels (10 g/100 mL) were run using a Mini-Protean Tetra cell (Bio-Rad) equipped

182 with a Power Pac Bio- Rad 300 at 200 V for approximately 60 min. Gels were stained with Bio-Safe
183 Coomassie blue (Bio-Rad).

184 **2.7 Fatty acid analyses**

185 Lipid fractions were extracted from the different types of sourdough immediately after preparation
186 (second refreshment according to Table 1) and after 24h of fermentation at RT, according to the
187 method described by Boselli, Velazco, Caboni, & Lercker (2001) with some modifications. Briefly,
188 samples (6.0 g) were suspended in 75 mL of 1:1 (v/v) chloroform: methanol solution, incubated for
189 20 min at 60 °C, added with 30 mL of chloroform and filtered using medium flow filtering papers. In
190 order to remove polar solutes, in each sample, 30 mL of 1N KCl were added and incubated for 16 h
191 at 25 °C. The fatty acids in organic lower phase were recovered by filtration in presence of 5 g of
192 Na_2SO_4 anhydrous and evaporation at 40 °C using a Rotavapor (IKA RV8). The lipidic extracts were
193 resuspended in n-hexane and stored at -80 °C. Total fatty acids (FAs) methylation was carried out on
194 20 mg of lipidic extract using 2N methanolic KOH. For each sample, free fatty acids (FFAs) were
195 obtained from the total lipid extracts using aminopropyl bonded sorbent columns (SPE-NH2)
196 ISOLUTE (Biotage, UK). Columns were equilibrated with 10 mL of n-hexene, loaded with 200 mg
197 of total lipid extract and washed using 10 mL of chloroform / iso-propanol (2:1 v/v) mixture. The
198 FFAs fractions were recovered using 2 g/100 mL formic ethyl conveyed in diethyl ether. Free fatty
199 acids methyl esters were obtained by directly adding to each sample 50 µL of diazomethane. The
200 fatty acid composition was determined as fatty acid methyl esters (FAMEs). Methyl tridecanoate
201 (Sigma, Milan, Italy) (13:0, 0.02 mg/mL was used as internal standard and while Supelco FAME
202 MIX 37 (Sigma, Milan, Italy) was used as external reference. The total and free fatty acids methyl
203 esters profiles analyses were carried out on an Agilent 6890 gas chromatograph (Agilent
204 Technologies, Palo Alto, CA) coupled to an Agilent 5970 mass selective detector operating in
205 electron impact mode (ionization voltage, 70 eV). A Chrompack CP-Wax 52 CB capillary column
206 (50 m length, 0.32 mm i.d., 1.2 µm df) was used (Chrompack, Middelburg, The Netherlands). The
207 temperature program was 130 °C for 7 min, then programmed at 14 °C/min to 180 °C for 5 min and

finally at 8 °C/min to 240 °C, which was maintained for 27 min. Injector, interface, and ion source temperatures were 250, 250, and 230 °C, respectively. Injections were performed with a split ratio of 1:10 and helium (1 mL/min) as the carrier gas. The compounds were identified by use of the National Institute of Standards and Technology-United States Environmental Protection Agency-National Institute of Health (1998) and according to the Registry of Mass Spectral Data (1998), mass spectra libraries as well as literature MS data, whenever possible.

2.8 Volatile molecule profiles

Volatile molecules profiles were analysed for the different types of sourdough immediately after preparation (second refreshment according to Table 1) and after 24h of fermentation at RT. The analyses were performed using a GC-MS coupled with a solid phase microextraction (GC–MS-SPME) technique, according to Burns et al. (2008) with some modifications. Five g of each sample were placed in sterile vials and added with 10 ul of standard 4-methyl-2-pentanol at 10,000 mg/kg. Samples were heated for 10 minutes at 45 °C, after that a fibre (SPME Carboxen/PDMS, 85 μm, Stalleflex Supelco, Bellefonte, PA, USA) was introduced in the head-space for 40 min. Adsorbed molecules were desorbed for 10 min during the running in the gas-chromatograph column Chrompack CP-Wax 52 CB (Chrompack, Middelburg, Olanda) with the following characteristics: length 50 meters, internal diameter 0.32 mm. The analysis was performed with an Agilent Technology 7890N gas chromatograph, Network GC System combined with a Network Mass Selective detector HP 5975C mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The conditions were as follows: injection temperature 50 °C for 1 min; increase of 4.5 °C/min up to 65 °C and increase of 10 °C/min up to 230 °C, stay at 230 °C. The injector, interface and ion source temperatures were 250, 250 and 230 °C, respectively. The carrier gas was helium, with a flow rate of 1 mL/min. Ionic fragmentation occurred with an electronic impact at 70 eV.

Volatile peak identification was carried out by computer matching of mass spectral data with those of the compounds contained in the NIST library (NIST / EPA / NIH Mass spectral Library, Version 1.6, United States of America) of 2011 and WILEY (sixth edition, United States of America) of 1995.

2.9 Statistical analysis

The results are expressed as the mean of three different samples from three repeated experiments on different days. Fatty acid raw data were statistically analysed using the one-way ANOVA procedure of statistica 6.1 (StatSoft Italy srl, Vigonza, Italy). The differences between mean values were detected by the HSD Tukey test, and evaluations were based on a significance level of $P \leq 0.05$. Principal component analysis (PCA) was performed using Statistica software (version 8.0; StatSoft., Tulsa, OK) on raw data to highlight the statistical variance among the samples for free fatty acids and volatile profiles.

Protein profile raw data were analyzed using student-test of GraphPad Prism. In addition, differences among sample sets were determined by analysis of variance with two-way ANOVA, performed in RStudio with "anova" function, followed by a post-hoc "pairwise.t.test" function, with a threshold P= 0.05 and P=0.01.

3. Results

3.1 Microbiological quality and pH of RO25 cricket-based hydrolysate

The analyses of yeast cell loads, performed after 72h of incubation at 25 °C showed that *Yarrowia lipolytica* inoculated, at level of 5 Log10 CFU/g, was able to reach level of 7.4 Log10 CFU/g after 72h inducing a decreasing of pH from 6.64 to 5.74. On the other side, the control without the inoculation of *Yarrowia lipolityca* RO25 was characterized by a final pH of 7.0 and a yeast cell load of 3.7 Log10 CFU/g. These data were in agreement with those previously reported by Patrignani et al (2020) and they prove the ability of this species to grow competing with several microorganisms and to adapt to several strict conditions (Guerzoni et al., 2001; Lanciotti, Vannini, Lopez, Gobbetti, & Guerzoni, 2005). In addition, no foodborne pathogens were detected in the samples independently on the inoculation of the selected yeasts. Otherwise, the analyses performed on the initial cricket powder showed the absence of *Salmonella* spp. and *L. monocytogenes* in 25 g of product. However, according to a 2010 report by the Codex Alimentarius Commission, studies on the food safety aspects

of edible insects remain limited. In fact, although insects are rich in nutrients, they can create the perfect conditions for the survival and/or growth of pathogenic microorganisms. For this reason, the microbiological assessment of the initial insect-based raw material was performed (Walia, Kapoor & Farber, 2018).

3.2 Microbiological quality and pH of the wheat flour-based preferment and its first refreshment

The two strains of *Kazachstania* (FM2, KAZ2) and *Fructilactobacillus sanfranciscensis* DG1 were inoculated in flour and water (mixed according to the ratio reported in Table 1) at level of 4.5 log and 6.6 Log10 CFU/g, respectively. After 24 h of fermentation, yeasts and LAB reached a cell load of 6.5 Log10 CFU/g and 8.8 Log10 CFU/g, respectively. The pH of the preferment after 24h at 25 °C was 4.86, indicating a good acidifying capacity of the starters used, and mainly *Fructilactobacillus sanfranciscensis*, as indicated by the decrease from the initial pH value of 6.01 (Table 2). In fact, some positive effects of LAB in sourdough has been attributed to their acidifying activities (due to fast conversion of fermentable carbohydrates into mainly lactic acid, but also other organic acids such as acetic acid and formic acid) able to increase wheat bread quality and shelf-life and reducing the staling (Clarke, Schober, & Arendt, 2002; Corsetti et al., 2000). These positive effects are associated also with their many metabolic activities such as proteolysis, phytase activity, exopolysaccharide production and synthesis of volatile and antimicrobial compounds. These positive effects on the qualities of ingredients and final bakery products are particularly evident when tailored pure cultures are used in the composition of the sourdough (Scarnato et al., 2016).

The interaction between yeast and LAB in the preferment resulted already in an optimal ratio of 1:100 according to different authors after 24 h of fermentation (Gobetti, 1998; Ottogalli, Gallib, & Foschino, 1996). The obtained preferment was subjected to a further refreshment (named first refreshment), adding other water and wheat flour, according to the formulation reported in Table 1. After 24 h at 25 °C, the wheat-based preferment had a pH value of 4.66, confirming the fast acidification rate of the inoculated LAB strain and its good interaction with the selected yeast strains. The yeast and LAB cell loads were 6.9 and 8.8 Log10 CFU/g, indicating increases higher than 1 log-cycle after 24h from

286 the first refreshment (Table 2). Also, in this phase, the values of the ratio between yeast and LAB and
287 those of pH were in accordance with those recorded for wheat flour sourdough (Gobetti, 1998).
288 The preferment obtained was divided in 3 aliquots to prepare 3 different set of samples as reported in
289 Table 1.

290 ***3.3 Microbiological quality and pH of the three types of sourdough obtained after second***
291 ***refreshment***

292 The preferment from the first refreshment (used at 23 g/100 mL) was used to formulate three different
293 set of samples using wheat flour, RO25 cricket-based hydrolysate (30 g/100 mL) and no hydrolysed
294 cricket (30 g/100 mL) according to the formulations reported in Table 1. The WCS was initially
295 characterized by yeasts and LAB cell loads of 6.4 and 8.4 Log10 CFU/g, respectively, while after 24h
296 of incubation at 25 °C, they increased at level of 6.8 and 8.8, respectively. Regarding the pH, the
297 dough starting from 5.50 reached values of 3.88 (Table 2). In the RO25H-CS, yeasts and LAB,
298 initially present at cell loads of 6.8 and 8.3 Log10 CFU/g, respectively, reached, after 24h at 25 °C,
299 level of 7.1 and 9.1 Log10 CFU/g, respectively. The pH of this sourdough was initially 6.05 and
300 turned to 4.29 after 24h of fermentation at 25 °C (Table 2). Finally, the noH-CS was characterized by
301 initial cell loads of yeasts and LAB of 6.5 and 8.5, respectively, while, after 24h, they attained cell
302 loads of 7.5 and 9.3 Log10 CFU/g, respectively. In this last set of samples, the pH, initially
303 characterized by a value of 5.28, reached values of 4.07 after 24h at RT (Table 2). The final pH varied
304 according to the presence of cricket powder, hydrolysed or not. The highest values were detected in
305 the samples containing the RO25 cricket-based hydrolysate. On the other hand, the high proteolytic
306 activity and the capability to use organic acid such as lactic and acetic ones of *Yarrowia lipolytica*
307 are well documented determining an increase in the pH of the system (Lanciotti et al., 2005; Coelho,
308 Amaral, & Belo, 2010). Obviously, the different pH values resulted in different proteolytic patterns
309 since they can affect protease activities, protein solubilisation, which in its turn influences directly
310 the proteolysis process, and also the interaction of proteins with other molecules of the system (Van
311 den Tempel, & Jakobsen, 2000; Suzzi et al., 2001).

Also in this case, the final sourdoughs, independent on the presence of cricket powder, hydrolysed or not, were characterized by optimal ratio between yeasts and LAB able to potentially result in a significant enhancement of the final bread aroma profiles (Xu et al., 2020; Pétel, Onno, & Prost, 2017). In fact, notoriously the proteolytic system of LAB releases low-molecular-weight peptides and amino acids important for the final dough and baked product aroma, functionality, and quality (Scarnato et al., 2017).

3.5 Protein quantification and electrophoretic profile

In Table 3, the contents of Albumin/Globulin, Prolamins and Glutelin in three kinds of sourdoughs, detected immediately after the second refreshment (0h) and after 24h of fermentation at RT, are reported. Albumin/globulin proteins considerably increased in noH-CS after 24h of fermentation, while in the other samples remained constant. Regarding prolamin subfraction, all samples showed an increase in protein quantities after 24h. However, the most marked increase in prolamin fraction was evidenced for RO25H-CS and WCS. In fact, such samples, starting from 7.87 mg/g and 9.88 mg/g, respectively, final value of 13.94 and 16.65 were reached, respectively. Glutelin subfraction showed a different trend, increasing significantly in sourdough produced with RO25 cricket hydrolysate after 24 h of fermentation. In this sample, glutelin fraction increased from 13.61 mg/g to 17.61 mg/g. Differently, after 24h of fermentation, the sourdoughs obtained with wheat and no hydrolysed cricket showed a decrease of the glutelin fraction. Statistical analysis of all sample sets using RStudio, also indicated that the differences among samples was statistical significantly. In particular, the analysis indicated that: I) WCS sample and noH-CS sample for albumin/globulin fraction was statistical different (P= 0.0089), but this was not significantly different between T0 and T24. II) In prolamin fraction all data was statistically different between samples (P= 1.035^{e-07}) and between Time (P=3.321^{e-06}). III) In glutelin fraction all data was statistically different between sample (P=0.0006) and between Time (P=0.034). IV) Also, in total protein fraction all data was statistically different both between sample (P=2.948^{e-06}) and between Time (P=$1.082e^{-05}$). Glutelin fraction from WCS showed the highest immunoreactivity with sera of celiac disease patients representing the main

338 immunogenic fraction also for Non Celiac Gluten Sensitivity (Scarnato et al., 2019). Regarding the
339 total protein profiles extracted in reducing conditions, the electrophoretic profiles are reported in
340 Figure 1 and the analysis was performed on samples before (0h) and after 24 h of fermentation (24h).
341 The Figure 1 shows that in RO25H-CS, after 24 h of fermentation, an accumulation of proteins of 48
342 kDa, 25 kDa and 10 kDa occurred. The electrophoretic profile showed also that the sourdough
343 obtained from wheat (WCS) did not change significantly during the fermentation, while in the
344 sourdough obtained from no hydrolysed cricket powder (noH-CS) a decrease of intensity of the bands
345 corresponding to 25 and 10 KDa was observed. The difference in proteolytic patterns, considering
346 the dominance of the inoculated LAB strain in all the samples, can be attributed to the well-known
347 proteolytic activities of *Yarrowia lipolytica*, for which this species is recognized as having high
348 technological potential also in food sector. On the other hand, also Patrignani et al. (2020) observed
349 in the cricket-based hydrolysed ingredient a general protein content increase with respect to the
350 cricket powder itself, highlighting also for *Yarrowia lipolytica* RO25 an accumulation of BABA as
351 bioactive compounds.

352 **3.6 Fatty acid and free fatty acid profiles**

353 Tables 4 and 5 show the concentrations, expressed in mg/kg, of total fatty acids and free fatty acids,
354 respectively, of the three different types of sourdoughs obtained.
355 The total FAs detected in WCS, noH-CS and RO25H-CS, after 24 h of fermentation, were 733.76
356 mg/kg, 1632.23 and 607.76, respectively. In general, all the samples were characterized by the
357 presence of C14:0, C15:0, C16:0, C16:1, C17:0, C18:2 n-6, C18:1(9), C18:0, even if the minor
358 amounts were detected in sourdough obtained with *Yarrowia lipolityca* RO25 cricket hydrolysate.
359 On the other side these last samples were characterized also by the presence of C18:2 trans, C18:1
360 trans and C16:0-Ethyl while the sample from no hydrolysed cricket powder was also characterized
361 by the presence of C10:0 and C20:0.
362 To pinpoint the difference among samples, the data of total fatty acids were analysed by PCA. The
363 PCA loading plot of total fatty acids of the three different sourdoughs was mapped in the space

364 defined by PC1 and PC2, accounting for 72.42 g/100 mL and 18.89 g/100 mL of the total variance
365 (Figure 2 a and b).
366 In Figure 2 a, the RO25H-CS were mapped in the upper right side of the space and were separated
367 by WCS along PC1. However, the noH-CS was separated by RO25H-CS along the PC2, and this
368 grouping was principally affected by C18:1t, C18:2, and C16:0 ethyl ester (Figure 2 b).
369 The free fatty acids (FFAs) detected in WCS, noH-CS and RO25H-CS, after 24h of fermentation,
370 were 402.69 mg/kg, 1040.61 and 4698.57, respectively (Table 5). In general, all the samples were
371 characterized by the presence of C14:0, C15:0, C16:0, C16:1, C17:0, C18:2 n-6, C18:1(9), C18:0,
372 C18:3 n-3, even if the highest amounts were found in RO25H-CS. More specifically, RO25H-CS
373 samples were also characterized by the presence of a-C14:0, C 16:2, C16:1 (7), a-C16:0, C16:0 ethyl
374 ester, i-C16:0, C18:2 t, C18:1 ethyl ester, C18:0 ethyl ester; C19:0, C20:1 C20:4 and tetradecanedioic
375 acid, 3,6-epoxy-, dimethyl ester. The noH-CS samples were also characterized by the presence of
376 C9:0, C10:0, C20:0, C20:3 and nonanedioic acid, dimethyl ester.
377 Also, the FFAs were analysed by PCA and the results showed in Figure 2 c and d, described by PC1
378 (71.27 g/100 mL) and PC2 (20.64 g/100 mL). The RO25H-CS samples were separated by the
379 remaining samples along PC1 and PC2 (Figure 2 c). In particular, this grouping was affected by a-
380 C14:0, C16:0, a-C16:0, i-C16:0, HI-C16:0, C16:2, C16:1 (7), C18:0 ethyl ester, C18:1 ethyl ester,
381 C18:2, C19:0, C20:1 and tetradecanedioic acid, 3,6-epoxy-, dimethyl ester (Figure 2 d). The data
382 obtained regarding the FFAs are not surprising since in general, although with a high intraspecies
383 variability, *Yarrowia lipolytica* is endowed with high lipolytic activity documented in different
384 conditions and used, for this, at industrial level for lipase production (Guerzoni et al., 2001).
385 Moreover, the RO25H-CS was characterized, compared to the remaining samples, by the highest
386 releases of C18:2, C18:1 and C16:1, which are considered aroma precursors (Patrignani et al., 2008;
387 Birch, Petersen & Hansen, 2013; Maire, Rega, Cuvelier, Soto, & Giampaoli, 2013). The enrichment
388 in C20:4 of the sourdough coming from *Yarrowia lipolityca RO25* cricket hydrolysate is interesting

since, in similar way to C18:3, this unsaturated fatty acid is endowed with health promoting features (Patrignani et al., 2020).

3.7 Volatile molecules profiles of the three different types of sourdough obtained

Immediately after the second refreshment (0h) and after 24h of fermentation at 25 °C, the three types of sourdough obtained were analysed for their molecules profiles by using GC/MS/SPME. The detected molecules, belonging to different classes of compounds, including aldehydes, alcohols, ketones, acids and esters, are reported in Figure 3. About 60 molecules were identified in relation to the different samples highlighting specific aroma fingerprinting given by the initial ingredients used for the final sourdoughs. According to the obtained results, the RO25H-CS samples, after 24 h of fermentation, were characterized by the highest amounts of total aldehydes and ketones while the noH-CS samples were characterized by the highest amount of alcohols and acids.

Pyrazines were also sporadically detected in samples obtained from cricket powder independently on the presence of *Yarrowia lipolytica* RO25. On the other hand, this can be explained by the highest proteolysis occurrence and the highest amount of free fatty acids detected in samples deriving from RO25 cricket hydrolysate. On the other hand, it is well known that free AA and FFA are the main aroma precursors in fermented foods (Pétel et al., 2017; Scarnato et al., 2019). In fact, although precursors are present in wheat flour and the largest amount of aroma substances are formed during baking, sourdough fermentation is essential to achieve an acceptable flavour, since chemically acidified bread failed in the sensory quality (Czerny, & Schieberle, 2002; Hansen, & Schieberle, 2005). Yeast can convert free amino acids into alcohols through the Ehrlich pathway. In fact, free amino acids such as valine, leucine, isoleucine, methionine, and phenylalanine, after transamination, can be transformed into the corresponding alfa-keto acids, followed by a decarboxylation into the aldehyde and finally reduction into the alcohols. Asparagine as the precursor of 2,3-butanedione derives from homofermentative LAB fermentation and the Maillard reaction (Yan et al., 2019). Aldehydes and aliphatic hydrocarbons can also derive from lipid oxidation and fatty acid degradation. Among the compounds produced by lipid oxidation, carbonyls are the most important in the

development of the aromatic properties of a wide range of food products (Hansen, & Schieberle, 2005; Cho, & Peterson, 2010). However, Patrignani et al. (2020) also found similar results in cricket-based hydrolysate characterized by a high level of free fatty acids and free amino acids. Moreover, to pinpoint difference among the samples, raw data detected for volatile compounds were analysed by PCA. Figures 4a and 4b represent the projections of the samples and variables in the spaces enclosed by the first two main components PC1 and PC2, which account for 51.49 g/100 mL and 19.55 g/100 mL, respectively, of the total variance among the different samples. The WCS and noH-CS samples immediately after second refreshment (0h) were mapped together in the left top side of the factorial space while RO25H-CS was together with WCS after 24h of fermentation. After fermentation the RO25H-CS and noH-CS samples were separated from the samples immediately after second refreshment (0h) along the PC1 and between them along PC2. The molecules which contributed to the separation of noH-CS after 24 h of fermentation at 25 °C were principally acids, alcohols and esters while hexanal, 4-methyl-2-hexanone, 2,6-dimethyl-4-heptanone, 4-methyl-3-penten-2-one contributed to the separation of RO25H-CS.

4. Conclusion

The results obtained showed that *Yarrowia lipolytica* RO25 cricket powder hydrolysate can impart specific characteristics to sourdoughs intended for bread production. Specifically, the high technological potential of *Yarrowia lipolytica* imparts to sourdough produced with RO25 cricket hydrolysate, specific technological properties including specific proteolytic, fatty acid and volatile molecule profiles. Furthermore, the sourdough obtained from RO25 cricket powder hydrolysate was rich in health-promoting molecules like arachidonic and linolenic acids, contained in a higher concentration than noH-CS.

Therefore, the use of *Yarrowia lipolytica* RO25 cricket powder hydrolysate to produce sourdough could be useful for innovative bread production with high nutritional and functional value.

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561 **Caption**

562 **Figure 1.** Coomassie coloured SDS-PAGE containing extracted proteins in reducing conditions of
563 wheat control sourdough (WCS), no hydrolysed cricket powder sourdough (noH-CS) and RO25
564 hydrolysed cricket sourdough (RO25H-CS), immediately after second refreshment (0h) and after 24
565 h of fermentation (24h) at 25 °C. Molecular weights markers in the first lane and their values in kDa
566 on the left.

567 **Figure 2.** Projection of cases (a and c) and variables (b and d) obtained by PCA elaboration of total
568 and free fatty acids, respectively, characterizing wheat control sourdough (WCS), no hydrolysed
569 cricket powder sourdough (noH-CS) and RO25 hydrolysed cricket sourdough (RO25H-CS), after
570 24 h of fermentation at 25 °C. The data are shown in triplicate (A, B, C).

571 **Figure 3.** Principal classes of compounds (expressed in mg/kg) detected in wheat control sourdough
572 (WCS), no hydrolysed cricket powder sourdough (noH-CS) and RO25 hydrolysed cricket
573 sourdough (RO25H-CS), immediately after second refreshment (0h) and after 24 h of fermentation
574 at 25 °C.

575 **Figure 4.** Projection of cases (a) and variables (b) obtained by PCA elaboration of volatile molecule
576 profile characterizing wheat control sourdough (WCS), no hydrolysed cricket powder sourdough
577 (noH-CS) and RO25 hydrolysed cricket sourdough (RO25H-CS), immediately after second
578 refreshment and after 24 h of fermentation at 25 °C.