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1 **Effect of leavening agent on Maillard reaction and the bifidogenic effect of**  
2 **traditional French bread**

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5 Romane Troadec<sup>a</sup>, Sofia Nestora<sup>a</sup>, Céline Niquet-Léridon<sup>a</sup>, David Marier<sup>a</sup>, Philippe Jacolot<sup>a</sup>,  
6 Elodie Sarron<sup>a</sup>, Stéphanie Regnault<sup>a</sup>, Pauline M. Anton<sup>a</sup>, Céline Jouquand<sup>a\*</sup>

7 Institut Polytechnique UniLaSalle, Université d'Artois, ULR 7519, Beauvais, France <sup>a</sup>

8 \*Corresponding author : Céline Jouquand (Celine.Jouquand@unilasalle.fr) – Institut  
9 Polytechnique UniLaSalle, Université d'Artois, ULR 7519, 19 rue Pierre Waguet, BP 30313,  
10 60026 Beauvais, France

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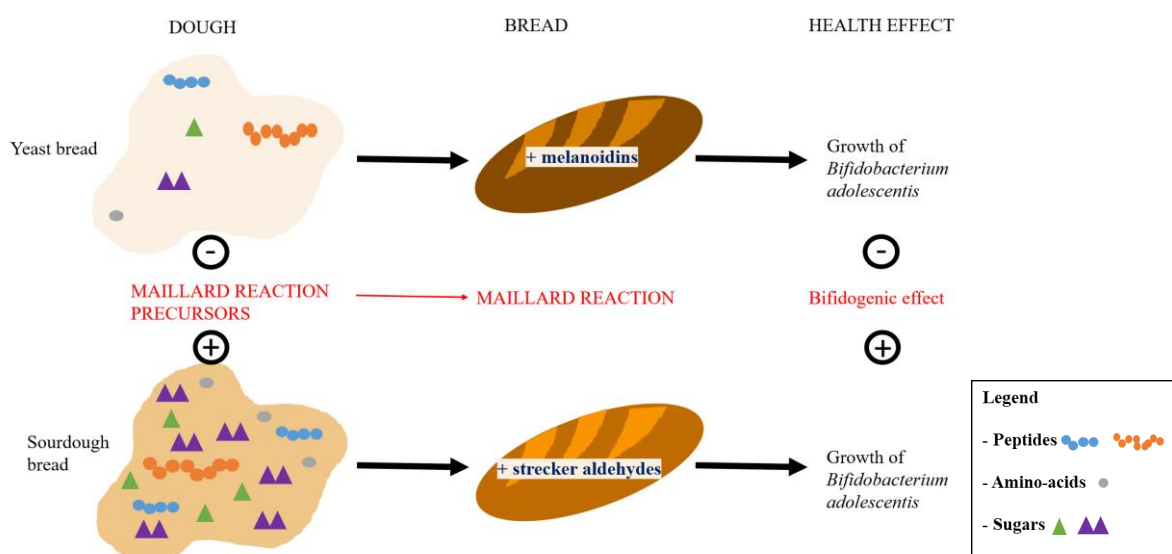
21 **Abstract:**

22 The study aimed to evaluate the effect of using sourdough or yeast as a leavening agent for  
23 French bread making on the Maillard reaction and the bifidogenic effect. Sugars, proteins, and  
24 free asparagine were quantified in bread dough before and after fermentation. The levels of the  
25 Maillard reaction precursors were very different depending on the leavening agent used, which  
26 affected the Maillard reaction during baking. Strecker degradation was favored in the crust of  
27 sourdough bread (SB), generating about 7 times more aldehydes than in the crust of yeast bread  
28 (YB), thus improving the sensory quality of the bread. In the YB crust, the melanoidization  
29 pathway was predominant. The bifidogenic effect of crust and crumb from both breads was  
30 evaluated through the *in vitro* growth of *Bifidobacterium adolescentis*. SB showed a higher  
31 bifidogenic effect, probably due to its composition more favorable for bacteria growth.

32 **Keywords:** French bread; Maillard reaction products; Strecker degradation; melanoidins;  
33 bifidogenic effect

34

35 **Graphical abstract:**



36

## 37 **1. Introduction**

38 Bread is widely consumed throughout the world, and considered as a staple food in many  
39 countries, including France, where each inhabitant consumes about 38 kg per year (FEB, 2021).

40 Bread is composed of simple ingredients - flour, water, salt, and a leavening agent - and  
41 provides to consumers great nutritional properties, being a source of carbohydrates, fibers,  
42 vitamins, and minerals (Hidalgo & Brandolini, 2014). Owing to this important bread  
43 consumption, it is crucial to understand the impact of the bread making process on its sensory  
44 and nutritional properties, in order to guide the bakery industry to maintain or improve bread  
45 quality.

46 Bread quality and nutritional properties are mainly dependent of bread making processes and  
47 may be improved by the use of additives. Among the multiple French bread recipes, traditional  
48 French bread is very popular, and is obtained from a specific baking process, protected by a  
49 decree, which forbid to use additives, or preservatives, and to freeze the bread (Ministère de  
50 l'Economie, Ministère de la Justice, Ministère de l'Agriculture et de la Pêche, 1993). In this  
51 study, we focused on the traditional French bread, on one hand to investigate a widely  
52 consumed bread, and on the other hand to avoid bias of using additives. The quality of the  
53 French bread is unique and the specific process confers to breads authentic quality and sensory  
54 properties. In numerous studies, authors have investigated on the impact of the process on  
55 sensory (Arendt et al., 2007; Pico et al., 2015) and nutritional properties (Dewettinck et al.,  
56 2008 ; Canesin & Cazarin, 2021) of bread or bread models, but only a few focused on the  
57 traditional French bread (Jouquand et al., 2018).

58 The type of leavening agent used in bread making process is crucial in the development of  
59 sensory and nutritional properties of the bread. Yeast fermentation is the most commonly used  
60 in France, but nowadays sourdough is increasingly employed for bread making. Sourdough is

61 either obtained by natural flour fermentation, with both yeasts and lactic acid bacteria, or made  
62 using starter culture, which is a mix of selected sourdough microorganisms. For yeast leavened  
63 bread, the most common yeast species used in French bakeries is *Saccharomyces cerevisiae*  
64 (commonly known as the baker's yeast), which mostly performs alcoholic fermentation, while  
65 sourdough fermentation involves, in addition to alcoholic fermentation, acetic and lactic  
66 fermentation (Gobbetti, 1998). During fermentation, microorganisms generate volatile  
67 compounds, such as alcohols, acids, ketones and esters involved in the sensory quality of the  
68 crumb via the degradation of sugars like glucose, fructose and maltose (Frasse et al., 1993). As  
69 thus, while the fermentation process largely contributes to the development of aromas in the  
70 crumb, the aromas in the crust mainly come from the Maillard reaction (MR) during baking.  
71 However, sugars and protein being precursors of the Maillard reaction, generation of aromas in  
72 the crust is highly dependent on the precursors still available after fermentation.

73 The Maillard reaction is a complex chain of reactions initiated by a condensation of amino  
74 groups of proteins or amino acids with carbonyl groups on reducing sugars. Then, the  
75 condensation products are rearranged into Amadori products which are fragmented or modified  
76 to reactive  $\alpha$ -dicarbonyl compounds. These intermediates may undergo Strecker degradation  
77 by condensation with free amino acids. The resulting Strecker aldehydes are highly correlated  
78 with the pleasant notes of the crust and their formation depends on the availability of free amino  
79 acids in the dough (Zehentbauer & Grosch, 1998). Sourdough fermentation significantly  
80 increases the content of free amino acids in the dough compared to yeast fermentation,  
81 providing flavor precursors for the Strecker degradation (Kati et al., 2004). Heterocyclic  
82 compounds such as furans and pyrazines are also formed during the intermediate stages of MR,  
83 and participate with Strecker aldehydes in the sensory perception of the crust (Pico et al., 2015).  
84 At the last stages of MR, brown pigment, named melanoidins are produced in the crust of bread.  
85 Melanoidins are high-molecular-weight (HMW) heterogenous polymers, but knowledge about

86 their exact structure is scarce due to their complexity and diversity. As for their formation  
87 mechanisms, Hofmann assumed that HMW melanoidins could be formed by linking low-  
88 molecular-weight chromophores Maillard reaction products (MRPs) to proteins via reactive  
89 side chains of amino acids (Hofmann, 1998). These chromophores usually contain imidazoles,  
90 pyrroles and their nitrogen-containing derivatives. In 2002, Lindenmeier et al. identified a  
91 protein-bound pyrrolinone reductonyl -lysine, also named pronyl-L-lysine, responsible for the  
92 brown color of bread crust (Lindenmeier et al., 2002). In 2005, Borrelli et Fogliano confirmed  
93 that bread melanoidins had a protein skeleton, and were constituted by colored compounds  
94 linked to the gluten polymer (Borrelli & Fogliano, 2005).

95 Apart from their physicochemical properties, melanoidins have gained a lot of attention in the  
96 past years owing to their putative beneficial effects. For instance, the anti-oxidant properties of  
97 melanoidins have been demonstrated *in vitro* (Rufián-Henares & Morales, 2007), and their anti-  
98 inflammatory effect was also suggested *in vitro*, through the inhibition of Enterobacteria growth  
99 (Helou et al., 2015). Moreover, Borrelli & Fogliano, suggested that bread melanoidins have a  
100 positive effect on the growth of Bifidobacteria strains (Borrelli & Fogliano, 2005), thus a  
101 bifidogenic effect. The bifidogenic effect is characterized by the ability of an exogenous factor  
102 such as a food component to promote the growth of Bifidobacteria in the gut microbiota. The  
103 growth of these Bifidobacteria is correlated with a balanced microbiota thus reducing the risk  
104 of dysbiosis (Tojo, 2014). We recently studied *in vitro* the effect of yeast bread on  
105 *Bifidobacterium adolescentis* (Jouquand et al., 2018), and showed that its growth was not  
106 improved by a higher melanoidin content in the crust compared to the crumb where melanoidins  
107 content is absent.

108 Since the formation of Maillard compounds is dependent on the composition of precursors in  
109 the dough, differences between sourdough bread and yeast bread are expected in terms of

110 aromas and melanoidins, which may affect the bifidogenic effect of these two types of bread.  
111 In this study, we evaluated the impact of two types of leavening agents (yeast / sourdough) on  
112 the precursors of the Maillard reaction and its products. The focus was on the formation  
113 pathways of Strecker aldehydes and melanoidins. The bifidogenic effect of these traditional  
114 French breads was also investigated by monitoring the *in vitro* growth of *Bifidobacterium*  
115 *adolescentis*.

116

117

118

## 119 **2. Materials and Methods**

120

### 121 2.1. Bread making process

122 The aim of the study was to work on breads that are acceptable to consumers and that could be  
123 found in French bakeries. Therefore, we have chosen bread-making parameters traditionally  
124 used by French bakers. Processes chosen for this study have been developed according to  
125 Myhrvold and Migoya and validated by a certified French baker (Myhrvold & Migoya, 2017).

126

#### 127 2.1.1. Yeast bread

128 Yeast bread (YB) dough was prepared using wheat flour type T65 (Farine Label Rouge T65,  
129 Paulic, Saint-Gérard, France), water (36.7%), salt (1.3%), yeast (0.85%) as a leavening agent  
130 (Lesaffre, Marcq-en-Baroeul, France), for a final hydration rate of 60%. Ingredients were  
131 kneaded 10 min at low speed, and 4 min at medium speed in a variable mixer (BV22 Haut, VMI  
132 mixing company, Montaigu, France). Then, the dough was fermented for 1 h at 23 °C and then

133 for 24 h at 6 °C in a growth chamber at 70% humidity. Dough was separated into 250 g pieces,  
134 ball-shaped by hand, and fermented for 30 min at room temperature. Loafs were wrought into  
135 bread-shape using a vertical moulder (Unic tradi forma, JAC, Liège, Belgique) and were  
136 subsequently placed for 1 h at 23 °C and 70% of humidity. Before baking, the loafs were  
137 scarified at the top using a cutter. Breads were baked in a deck oven with steam injection (110  
138 mL) at 250 °C for 25 min. After 30 min of cooling at room temperature, the breads were stored  
139 at -20 °C in sealed plastic bags for further analysis. Three batches of 9 breads were made at 3  
140 different days to evaluate the repeatability of the process.

141

#### 142 2.1.2. Sourdough bread

143 Sourdough bread (SB) dough was prepared using wheat flour type T65 (Farine Label Rouge  
144 T65, Paulic, Saint-Gérard, France), water (26.6%), salt (1.2%), and sourdough (21.2%) as a  
145 leavening agent, for a final hydration rate of 60%. Sourdough was prepared one day before, by  
146 mixing sourdough starter (0.4%) (LV4 starter, LeSaffre, Marcq-en-Baroeul, France), T65 flour  
147 (50%) and water at 38 °C (50%). The sourdough was left 24 h at 30 °C before being used.  
148 Ingredients were kneaded 10 min at low speed, and 4 min at medium speed using a variable  
149 mixer (BV22 Haut, VMI mixing company, Montaigu, France). Then, the dough was fermented  
150 for 2 h at 23 °C and 70% of humidity in a growth chamber. The dough was separated into 250  
151 g pieces, ball-shaped by hand, and fermented for 30 min at room temperature ( $\pm 20$  °C). Loafs  
152 were wrought in bread shape using a vertical moulder (Unic tradi forma, JAC, Liège, Belgique).  
153 Loafs were put in the growth chamber for 24 h at 6 °C, 70% humidity. Before baking, loafs  
154 were scarified at the top using a cutter. Breads were baked in a deck oven (Ebo 68, Schiltigheim,  
155 France) with steam injection (110 mL) at 250 °C for 25 min. Three batches of 9 breads were



156 made at 3 different days to evaluate the repeatability of the process. After 30 min of cooling at  
157 room temperature, the breads were stored at -20 °C in sealed plastic bags for further analysis.

158

## 159 2.2. Chemicals, reagents, and standard solutions

160 Methanol HPLC Grade (MeOH), Water HPLC Grade, Acetonitrile HPLC Grade (ACN),  
161 Nonfluoropentanoic acid 97% (NFPA), Sodium dodecyl sulfate > 99% (SDS), Trizma® base,  
162 Ethanol 100% (EtOH), Acetic acid > 99%, Trichloroacetic acid > 99% (TCA), 2-methyl  
163 propanol, Hydrochloric acid 37% (HCl), Sodium hydroxide pellets (NaOH), Carrez solutions,  
164 β-mercaptoethanol, sugar standards and enzymes were purchased at Sigma-Aldrich (Saint-  
165 Quentin Fallavier, France). Internal standard ASN <sup>15</sup>N<sub>2</sub> and ASN were from Euriso-top (Saint  
166 Aubin, France). Buffer solution Tris/Glycine/SDS 1X, Mini PROTEAN TGX Gels® and 2X  
167 Laemmli buffer solution were provided by BioRad (BioRad, Hercules, USA). At last, tryptone,  
168 yeast extract, cysteine hydrochloride and agar were from Becton Dickison, France. Blue  
169 brilliant Coomassie blue was from Merk Millipore (USA).

170

## 171 2.3. Reducing sugar content:

172 The reducing sugar content was determined in the flour, dough and bread crust and crumb. The  
173 method used for this analysis was adapted from the method of Epriliati et al. (2010). Briefly,  
174 500 mg of freeze-dried and ground sample were added to 4 mL of a MeOH/water (80/20 v/v)  
175 mix. Samples were mixed for 15 min and centrifuged at 20 °C during 10min at 8000 x g.  
176 Supernatants were collected in a 10 mL flask, and the same steps were repeated once again.  
177 Flasks were completed at 10 mL with ultra-pure water. Samples were filtered through a 0.45 μm  
178 nylon membrane and placed in HPLC vials. Extracts were injected into a HPLC U3000 system

179 (ThermoFisher Scientific, Courtaboeuf, France) coupled to an ELSD LT 90 detector (Sedere,  
180 Olivet, France) The separation was carried out with a Water/ACN 25/75 (v:v) at a 0.8 mL/min  
181 isocratic elution flow using a Prevail Carbohydrate ES (250 mm x 4.6 mm, 5 $\mu$ m) column and  
182 a precolumn (Grace Alltech). A calibration range containing maltose, glucose and fructose was  
183 also injected to calculate concentrations of the samples.

184

#### 185 2.4.SDS PAGE analysis

186 Freeze-dried and ground samples (0.028 g of bread crust, crumb or dough) or frozen samples  
187 of bread digestates (166  $\mu$ L) were suspended in tris HCl buffer (50 mM; pH 7.5) in presence of  
188 denaturing agents (1% (w/v) SDS and 0.2% (v/v)  $\beta$ -mercaptoethanol). Each sample was  
189 vortexed for 1 min, then placed in ultrasonic bath (Ultrasonic cleaner VWR) for 30 s and finally  
190 shaken at 40 rpm for 40 min (Rotator SB3 Stuart). Samples were centrifuged at 10000 x g for  
191 5 min and the supernatants were collected. An amount of 500  $\mu$ L of supernatant were diluted  
192 in 500  $\mu$ L of 2X Laemmli buffer solution pH 6.8. containing 65.8 mM Tris–HCl, 2% SDS, 25%  
193 glycerol, 0.01% bromophenol blue. Samples were then heated at 95 °C for 5 min in a heat block  
194 (Memmert, Schwabach, Germany). For this analysis, mini-PROTEAN TGX Gels, 12%  
195 polyacrylamide gel, 12 wells (BioRad, Hercules, USA) were used. Proteins were separated  
196 using 4% stacking gel and 12% resolving gel. Electrophoresis was performed at 40 mA for 45  
197 min using Tris/Glycine/SDS 1X solution (BioRad, Hercules, USA). Gels were fixed in TCA  
198 10% for 10 min, stained overnight with Coomassie Brilliant Blue-R250 before destaining using  
199 30% ethanol and 10% acetic acid. The molecular weights were determined thanks to a  
200 molecular ladder (Precision Plus Protein<sup>TM</sup> Unstained Protein Standard, Strep-tagged  
201 recombinant, BioRad, Hercules, USA).

202

203

204 2.5. Free asparagine content:

205 Free asparagine content was measured in bread dough at the beginning and at the end of  
206 fermentation time with a validated method used routinely in our laboratory. The asparagine  
207 content was measured from 3 batches of dough produced on 3 different days. Two hundred  
208 milligrams of freeze-dried and ground dough were suspended in 7 mL of ultra-pure water. An  
209 internal standard Asn <sup>15</sup>N<sub>2</sub> was added at a concentration corresponding to the calibration range.  
210 Samples were vortexed and centrifuged at 2500 x g for 10 min. Supernatants were collected  
211 and the previous steps were repeated twice, pooling supernatants. Obtained samples were  
212 clarified with 0.5 mL of Carrez I (15% (w/v) potassium ferrocyanide) and 0.5 mL Carrez II  
213 (30% (w/v) zinc acetate), and centrifuged 10 min at 2500 x g. Supernatants were poured in a  
214 25 mL flask and completed with ultra-pure water, then filtered through a 0.45 µm nylon  
215 membrane before analysis. These extracts were then injected into a HPLC-MS/MS  
216 (ThermoFisher Scientific, Courtaboeuf, France). Twenty microliters were injected into a  
217 Hypercarb (10 mm x 2.1 mm, 5µm) column (ThermoFisher Scientific, Courtaboeuf, France).  
218 The separation was carried out with Nonfluoropentanoic acid (NFPA) (20mM) / Acetonitrile  
219 (ACN) at 200 µL/min. The percentage of ACN increased from 0 to 10% at 10 min and reached  
220 50% at 12 min. After 5 min at 50% ACN, the column was equilibrated with 100% NFPA for  
221 5min. Column oven temperature was set at 25°C. Tandem MS analyses were performed using  
222 a TSQ Quantum Ultra Triple stage quadrupole mass spectrometer (ThermoFisher Scientific,  
223 Courtaboeuf, France). Specific transitions m/z 133.0 m/z → m/z 87.0 and m/z 135.0 → m/z  
224 89.0 were used for the detection and quantification of asparagine and <sup>15</sup>N<sub>2</sub> respectively.  
225 Asparagine concentrations were calculated using a calibration range with ratio between Asn  
226 and Asn <sup>15</sup>N<sub>2</sub>.

227

## 228 2.6. Analysis of volatile compounds

229 Analysis of volatile compounds was adapted from the method described in the study of  
230 Jouquand et al. (Jouquand et al., 2018). An amount of 1 g of ground crust or crumb was placed  
231 into a 20-mL vial. Then, 3 mL of a 2-methyl 1-propanol solution (12.5 ppm) were added as an  
232 internal standard. The vials were sealed with PTFE/silicone caps (Sigma-Aldrich, U.S.A.) and  
233 vortexed. The extraction of volatile compounds was performed using SPME (solid phase micro-  
234 extraction). After a sample equilibration time of 15 min at 40 °C, a 2-cm fiber  
235 (divinylbenzene/carboxen/polydimethylsiloxane) was exposed to the headspace for 1 h at 40  
236 °C (Sigma-Aldrich). The fiber was then put into the injector of the GC/MS, in splitless mode,  
237 for 2 min at 250 °C. For each sample, extractions were carried out in triplicate. Separation and  
238 identification of volatile compounds were carried out using a Focus GC (ThermoFisher  
239 Scientific, Courtaboeuf, France) coupled with a PolarisQ detector (ThermoFisher Scientific,  
240 Courtaboeuf, France). Inlet, ionizing sources, and transfer line temperatures were kept at,  
241 respectively, 250, 300, and 250 °C. Mass units were monitored from m/z 40 to m/z 250 and  
242 ionized at 70 eV. A column of Zebron™ ZB wax plus™ (30m × 0.25 mm × 0.25 μm)  
243 (Phenomenex, France.) was used to separate the volatile compounds. Helium was used as  
244 carrier gas at a flow rate of 1 mL/min. Tentative identifications were based on matching mass  
245 spectra of unknowns with those in the NIST mass spectral library or published data. The  
246 identification of volatile compounds was confirmed using retention indices calculated using  
247 retention time data from a series of alkane standards (C7 to C30) run under the same  
248 chromatographic conditions. The relative concentration of volatile compounds was determined  
249 by normalizing the peak area of each compound to the peak area of internal standard, and  
250 normalized peak areas were compared between samples.

251

252

## 253 2.7. Colorimetric analysis

254 Twenty colorimetric measurements were performed on each bread of each batch, using a  
255 portable colorimeter (Model CR-400; Konica Minolta, Japan), adjusted to operate with an  
256 aperture of 10mm, illuminant D65. Ten measures evenly distributed were carried out at the top  
257 of the bread, and 10 at the bottom, always at the same points. Results were expressed in the  
258 international color space CIE L\* a\* b\*. The Lightness (L\*) was used to evaluate the level of  
259 baking.

260

## 261 2.8. Analysis of water-soluble melanoidins

262 To estimate melanoidins in bread crust, the method used was the semi-quantitative method  
263 developed by Helou et al. (2016). As the extraction protocol did not consider the insoluble  
264 fraction of melanoidins, the water-soluble melanoidins extracted were used as markers of the  
265 total melanoidins in bread. Half a gram of grinded bread crust was suspended in 4.5 mL of ultra-  
266 pure water. The suspensions were mixed for 30 min and then centrifuged at 10000 x g for 10  
267 min at 4 °C. Supernatants were collected and filtered through a 0.45- $\mu$ m nylon membrane.  
268 Extracts were then injected into a HPLC system coupled to a PDA detector and a fluorescence  
269 detector (ThermoFisher Scientific, Courtaboeuf, France). The separation was carried out with  
270 phosphate buffer (50 mM) containing 150 mM sodium sulfate (pH 7.0) at a 0.6 mL/min  
271 isocratic elution flow using a KW-802.5 (300  $\times$  8 mm, 5  $\mu$ m, 400 Å) column coupled to a KW-  
272 G guard column (50  $\times$  6 mm, 7  $\mu$ m) (Shodex, Munich, Germany). The detection was made at  
273 the excitation–emission wavelengths of 350 - 430 nm. An external standard molecular weight  
274 calibration curve was used for molecular weight determination using different proteins or  
275 peptides (MW 0.5 to 17 kDa). The detection was made at 280 nm. The group of peaks having  
276 a retention time between 17.5 and 27.5 min corresponding to the melanoidins molecular weight

277 were used for the interpretation. This group of peaks represents fluorescent MRPs, used as  
278 markers of the total melanoidins formed in the crust.

279

#### 280 2.9. Bread digestion:

281 Bread digestion was adapted from the procedure described in a previous study (Helou et al.,  
282 2015). Crust or crumb powders (10 g each) were suspended in 55 mL of ultra-pure water pH  
283 3.0 (using HCl 6 M). As a control, the powders were replaced using 10 g of ultra-pure water.  
284 Enzymes, purchased from Sigma Aldrich, were added: 3.9 mg lipase from *Burkholderia*  
285 *cepacia* (30 U/mg<sup>-1</sup>) and 3.7 mg of porcine pepsin (2900 U/mg<sup>-1</sup>). After pH readjustment at 3.0  
286 (using HCl 6 M), the suspensions were incubated at 37 °C for 90 min under shaking at 140 rpm  
287 (water bath model SW23, Julabo, Allentown, PA, U.S.A.). Porcine bile extract and porcine  
288 pancreatin were added at 0.1 and 0.7 g, respectively. The pH was adjusted to 6.5 using NaOH  
289 6 M. The suspensions were then incubated at 37 °C for 150 min under agitation at 140 rpm in  
290 the same water bath. Finally, the digested materials were separated into aliquots (8 g) and kept  
291 frozen at -20 °C until use. This procedure was repeated for each of the 3 batches of bread.

292

#### 293 2.10. Batch fermentation:

294 Batch fermentation experiment was adapted from the procedure described in a previous study  
295 (Jouquand et al., 2018). All experiments were performed under anaerobic conditions (90% N<sub>2</sub>,  
296 5% H<sub>2</sub> and 5% CO<sub>2</sub>) in a Bactron Shel Lab II-1 anaerobic chamber (Sheldon Manufacturing  
297 Inc., Cornelius OR, U.S.A.) as previously described by (Helou et al., 2015). To determine the  
298 bifidogenic effect of digested crust or crumb, a strain of *Bifidobacterium adolescentis* (CRBIP  
299 64.59T) isolated from human feces was studied in the presence of different bread digestates in  
300 M20 medium for 36 h at 37 °C. Batch fermentations were carried out in 100-mL flasks  
301 containing fermentation medium (50 mL). The medium culture was composed of 30 mL of

302 modified M20 medium, 9 g of digested bread crust or bread crumb or control (digestion carried  
303 out with water), 1.25 mL of hemin solution, 20.79 mL of distilled water and 175  $\mu$ L of  
304 inoculum. The modified M20 medium was prepared using 60 g/L tryptone (Becton Dickinson,  
305 France), 40 g/L yeast extract (Becton Dickinson, France), 1 g/L cysteine hydrochloride, and  
306 9.78 g/L glucose in phosphate buffer (0.2 M, pH 7.0) and autoclaved at 110 °C for 30 min.  
307 Directly after inoculation, and during the bacteria growth, 2 mL were collected every 2 h for  
308 pH measurements with a microsonde Sentix MIC-D connected to WTW INOLAB 730 pH  
309 meter and to follow the bacterial growth by plating on M20 agar. Serial dilutions were carried  
310 out and 1 mL of appropriate dilutions was plated in triplicate on M20 medium agar. The number  
311 of CFU per mL was determined after 48 h at 37 °C under anaerobic conditions in a  
312 bacteriological incubator. The growth rate of the bacteria was calculated according to the slope  
313 of the exponential phase of growth.

314

#### 315 2.11. Statistics

316 Instrumental data were presented as means  $\pm$  SD. Differences between means were analyzed  
317 by a Student's t-test for unpaired series with the Graph Pad Prism software (V8.4.3, San Diego,  
318 CA, USA). Data from *Bifidobacterium adolescentis* batch fermentation were analyzed by a  
319 two-ways ANOVA (SB/YB ; Crust/Crumb) followed by Tukey-Kramer post-hoc test with the  
320 XLSTAT software (Addinsoft, Paris, France). A P value <0.05 was considered to be significant.

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### 327 **3. Results and discussion**

#### 328 3.1. Effect of leavening agent on Maillard reaction precursors

329 Since the generation of aromas in the crust is dependent of MR precursors, this study first  
330 focused on the effect of yeast or sourdough on sugars and proteins levels in the dough. The  
331 effect of leavening agent was evaluated in the dough at the beginning and after 26.5 h of  
332 fermentation (**Table 1**). In both dough types (YB and SB), maltose was the main sugar resulting  
333 from the hydrolysis of damaged starch (DS) by endogenous flour amylases (Barber et al., 1991).  
334 At the beginning of fermentation, maltose content was + 1.3 times higher in SB dough  
335 ( $P < 0.0001$ ), probably due to its presence in the sourdough. After 26.5 h, maltose content  
336 increased significantly ( $P < 0,05$ ) in both doughs (YB: + 1.7 times and SB: + 1.5 times),  
337 indicating that it was not the main substrate for yeast and lactic acid bacteria at this stage.

338 Glucose and fructose were present at higher concentrations in YB at the beginning of  
339 fermentation ( $P = 0.003$  ;  $P < 0.0001$ , respectively), which could be explained by the  
340 predominance of yeast cells in YB dough compared to SB dough. Indeed, glucose and fructose  
341 are fermentable sugars resulting from the hydrolysis of fructan by yeast invertase (Perlman &  
342 Halvorson, 1981). After 26.5 h, glucose and fructose content decreased dramatically in YB  
343 dough ( $P < 0.0001$ ), due to the preference of yeast for glucose and fructose over maltose as  
344 fermentation substrate (Barber et al., 1991). In the SB dough, their concentration did not change  
345 significantly after 26.5 h, probably because of the release of these sugars into the dough by the  
346 action of yeasts invertase, that was compensated for their consumption by lactic acid bacteria.

347 These results highlight the difference in sugars' metabolism depending on the leavening agent,  
348 leading to a different pattern of the Maillard reaction precursors in the two doughs which results  
349 in breads with distinct nutritional properties.



350 The effect of the leavening agent on protein composition was assessed by SDS PAGE analysis  
351 on each dough type, aiming to investigate the protein patterns at the beginning and after 26.5 h  
352 of fermentation (**Figure 1**). The main protein fractions of wheat flour: HMW-GS (67-100 kDa)  
353 and LMW-GS (32-39 kDa),  $\omega$ -gliadins (39-44 kDa), and  $\alpha/\beta$ - and  $\gamma$ -gliadins (between 28 and  
354 35 kDa) were found in both bread doughs. Bands corresponding to globulin (around 30 kDa)  
355 and albumin (15 kDa) were also found, in accordance with the Osborne classification (Osborne,  
356 1924). Therefore, the two bread doughs did not differ in terms of protein fractions. In the YB  
357 dough, no substantial variation in the intensity of the bands of protein fractions was observed  
358 before and after fermentation suggesting limited protein hydrolysis by proteases during  
359 fermentation. Indeed, the fermentation by *Saccharomyces cerevisiae* does not favor protein  
360 hydrolysis due to the pH of the dough (pH > 4.5) (Gobbetti, 1998), even though a slight  
361 degradation occurs through flour proteases in the presence of water (Kawamura & Yonezawa,  
362 1982). In the SB dough, after 26.5 h of fermentation, the band corresponding to HMW-GS (100  
363 kDa) was less intense compared to the beginning of fermentation, whereas the band  
364 corresponding to albumin (15 kDa) was more intense. This result seems to indicate a protein  
365 hydrolysis during the fermentation process by Lactobacilli, leading to the formation of lower  
366 molecular weight proteins. Our results are corroborated by previous studies showing that  
367 sourdough fermentation promoted wheat protein degradation through microbial proteases  
368 (Gobbetti et al., 1996). In addition, the acidification of dough by Lactobacilli could enhance  
369 flour proteases activity responsible for the degradation of gluten proteins into peptides and/or  
370 free amino acids (Kawamura & Yonezawa, 1982). Overall, sourdough fermentation with lactic  
371 acid bacteria leads to an increase in free amino acids during fermentation, while dough  
372 fermentation with yeast reduces the free amino acids content (Thiele et al., 2002). Therefore,  
373 the addition of sourdough at the beginning of SB making process provides additional amino  
374 acids to the dough.

375 Among free amino-acids, we focused on asparagine, the main precursor of acrylamide, a  
376 carcinogenic MR product (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2015)  
377 detected in low moisture food products heated at temperatures above 120 °C such as in the case  
378 of bread crust (Semla et al., 2017). The decrease in free asparagine content after fermentation  
379 was much important in the YB than in the SB (93.64% and 73.57% respectively (P<0.0001)),  
380 leading to a higher asparagine content in SB dough. This difference is certainly due to the ability  
381 of *Saccharomyces cerevisiae* to metabolize asparagine thanks to its extracellular L-  
382 asparaginase as described in previous studies (Dunlop et al., 1976). Based on this result, a  
383 higher amount of acrylamide is expected in SB crust, nevertheless it has been shown that French  
384 bread was not a main contributor of dietary acrylamide (Jouquand et al., 2018).

385

### 386 3.2. Formation of Maillard reaction products in YB and SB

387 The overall aroma of bread is complex and depends on different reactions occurring in the  
388 crumb, the crust or both. In the crumb, aroma compounds are mainly generated during the  
389 fermentation of sugars in the dough by yeast and lactic acid bacteria. Some volatile compounds  
390 such as aldehydes, ketones and alcohols can also be generated by the lipid oxidation. During  
391 baking the Maillard reaction produces other volatile compounds that contribute to the typical  
392 aroma in the crust. Few MRPs are formed in the crumb because the internal temperature and  
393 the hydration rate of the dough are not favorable for the initiation of the Maillard reaction. Some  
394 MRPs can be found in the crumb and probably come from a transfer from the crust to the crumb  
395 (Pico et al., 2015).

396 Strecker degradation is the main pathway that generates key odorants during Maillard reaction  
397 by converting  $\alpha$ -amino acids into structurally related aldehydes (Pico et al., 2015). In this study,  
398 54 and 49 compounds were respectively identified in the SB and YB crust with their probable

399 biological source (**Table 2**). Volatile compounds from the Maillard reaction, especially Strecker  
400 aldehydes, were much more concentrated in SB than in YB. For instance, the relative  
401 concentrations of 2-methylbutanal and 3-methylbutanal were respectively 7.7 and 14.1 times  
402 higher in SB than in YB (**Table 2**). This result could be explained by protein hydrolysis during  
403 SB fermentation, observed by SDS-PAGE (**Figure 1**), thus releasing free amino-acids, the main  
404 reactants of the Strecker degradation (Zehentbauer & Grosch, 1998 ; Thiele et al., 2002).  
405 Alcohols (3-methyl-butanol; ethanol) were more represented in the YB crust, due to yeasts  
406 metabolism during fermentation (Pico et al., 2015).

407 Melanoidins were also quantified to evaluate the effect of leavening agent on their formation.  
408 As shown in **Figure 2**, the levels of melanoidins was evaluated, according to the lightness of  
409 the crust. YB crust contained more water-soluble melanoidins than the SB crust (P=0.04).  
410 Therefore, the melanoidins formation pathway seemed to be predominant in YB, probably due  
411 to the presence of more HMW proteins in YB dough after fermentation than in SB (**Figure 1**),  
412 thus confirming the hypothesis of a protein skeleton for bread melanoidins.

413 Based on this study, we can say that both types of leavening agent had a significant effect on  
414 the Maillard reaction pathways. In SB, the Strecker degradation could direct the Maillard  
415 reaction towards aroma generation, rather than melanoidins formation. In YB, the higher  
416 amounts of melanoidins lead to a “well-baked” aspect particularly appreciated by consumers  
417 which is consistent with our previous findings (Jouquand et al., 2018). Consequently, we can  
418 speculate that the difference in melanoidin content could potentially have an effect on the  
419 nutritional properties of bread.

420

421

### 422 3.3. Effect of leavening agent on *in vitro* bifidogenic effect

423 Since the melanoidins were suggested to have bifidogenic properties *in vitro* (Borrelli &  
424 Fogliano, 2005), we have chosen to investigate their bifidogenic effect on *Bifidobacterium*  
425 *adolescentis*, a commensal bacterium of healthy human tract and known for its ability to  
426 metabolize diet-derived glycans (Duranti et al., 2016). *Bifidobacterium adolescentis* was grown  
427 during 36 h in M20 medium supplemented with *in vitro* digested YB or SB crust and crumb, or  
428 control digestate. The growth of *Bifidobacterium adolescentis* was always enhanced by the  
429 addition of bread digestate, compared to the control, which is in agreement with the bifidogenic  
430 effect of bread shown in previous studies (Costabile et al., 2014; Jouquand et al., 2018) (**Figure**  
431 **3**). In this study, the two-ways ANOVA showed that the bifidogenic effect of SB was  
432 significantly higher than the one of YB ( $P=0.015$ ), which is in agreement with the findings of  
433 Costabile et al. (Costabile et al., 2014). The growth rate of the bacteria in contact with the  
434 digested SB crust ( $0.296 \text{ h}^{-1} \pm 0.04$ ) was 1.24 times higher than that of the YB crust ( $0.239 \text{ h}^{-1}$   
435  $\pm 0.02$ ), and the growth rate of the bacteria in contact with the digested SB crumb ( $0.264 \text{ h}^{-1} \pm$   
436  $0.05$ ) was 1.21 times higher than that of the YB crumb ( $0.217 \text{ h}^{-1} \pm 0.02$ ). Such an increase is  
437 significant given the fact that *Bifidobacterium adolescentis* growth rate ranges from  $0.17 \text{ h}^{-1}$  to  
438  $0.30 \text{ h}^{-1}$  according to literature (Amaretti et al., 2006). However, for each bread, there was no  
439 significant difference between crust and crumb on the growth rate of the bacteria ( $P=0.165$ )  
440 which is consistent with our previous findings (Helou et al., 2017). Based on these results, we  
441 suggest that melanoidins from the bread crust were not responsible for the bifidogenic effect  
442 observed, but at the same time they did not have a negative impact on *Bifidobacterium*  
443 *adolescentis* growth. Moreover, the lack of difference between crust and crumb suggests no  
444 implication of any other MRPs from the crust in the bifidogenic effect.

445 The higher bifidogenic effect of SB compared to YB was shown by Costabile and co-workers  
446 (Costabile et al., 2014), however the causative agent associated with this phenomenon has yet  
447 to be elucidated. Considering the complexity of bread matrix as a substrate for the bacteria, we  
448 hypothesize that the bifidogenic effect observed in SB was the consequence of a synergistic  
449 effect of several factors. For instance, our results could be partially explained by the difference  
450 in the availability of nutrients in both breads, in particular that of sugars (**Table 3**). Indeed,  
451 since the concentrations in maltose, glucose and fructose are dependent on the fermentation  
452 process and on the MR, total sugar levels present in SB and YB are different (**Table 3**). Before  
453 *in vitro* digestion, the SB crust contained 1.32 times more glucose than the YB crust. In the SB  
454 crumb, glucose was 3.64 times higher than in YB crumb. As for fructose, it was more  
455 represented in YB crust and crumb than in SB. To date, there is no consensus on the preferred  
456 sugar media for the growth of *Bifidobacterium adolescentis*. A couple of studies reported a  
457 preference for glucose as a substrate for Bifidobacteria (Degnan & Macfarlane, 1991; Perrin et  
458 al., 2001), which is in agreement with our results, and could partly explain the higher effect of  
459 SB which contains more glucose. Additionally, it has been shown that some strains of  
460 Lactobacilli from sourdough are capable of producing exopolysaccharides (EPS), which are  
461 known to be a good substrate for the growth of *Bifidobacterium adolescentis* (Korakli et al.,  
462 2002 ; Arendt et al., 2007). However, the exact composition of microbial species in the starter  
463 culture used for sourdough making is unknown. In conclusion, our hypothesis is that the  
464 bifidogenic effect observed in the SB could be induced by a more favorable sugar composition,  
465 as well as by the EPS potentially present in the SB digestate. Further analysis needs to be  
466 conducted in order to determine the origin of the bifidogenic effect of SB bread.

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468

#### 469 **4. Conclusions**

470 This study aimed to highlight the effect of leavening agents (yeast and sourdough) used for  
471 traditional bread making on sensory and nutritional markers. The Strecker degradation was  
472 predominant in the sourdough bread crust, while melanoidization was favored in the yeast  
473 leavened bread. These differences were due to the availability of the precursors of the Maillard  
474 reaction, which changed according to the leavening agent used. Moreover, an enhanced  
475 bifidogenic effect was observed for sourdough bread, which could be explained by a number  
476 of associated factors such as the variations of the substrates available for gut bacteria growth, a  
477 more favorable sugar composition as well as the potential exopolysaccharides secretion by  
478 sourdough *Lactobacillus*. Conversely, melanoidins did not seem to impact the bifidogenic  
479 effect of the sourdough bread. In conclusion, sourdough fermentation of traditional French  
480 bread is breadmaking method that shall be privileged by food industries aiming to improve the  
481 sensory and nutritional properties of bread. To that end, this work shall be completed in order  
482 to determine the ideal fermentation conditions for sourdough bread making resulting to better  
483 formulated breads with an improved nutritional value.

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495 The authors declare no conflict of interest.

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499 **CRedit author statement**

500 Romane Troadec: Conceptualization, investigation, writing – original draft, visualization.

501 Sofia Nestora: Writing – reviewing & editing

502 Céline Niquet-Léridon: Methodology, resources, reviewing

503 David Marier: Investigation, resources.

504 Philippe Jacolot: Methodology, resources

505 Elodie Sarron: Reviewing

506 Stéphanie Regnault: Methodology, resources, writing – reviewing & editing

507 Pauline M. Anton: Conceptualization, writing – reviewing & editing, supervision, project  
508 administration.

509 Céline Jouquand: Conceptualization, resources, writing – original draft, reviewing & editing,  
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629 **Table captions:**

630 **Table 1.** Reducing sugar content (mg/100g of dry matter) in yeast bread and sourdough bread  
631 doughs before and after fermentation.

632 **Table 2.** Relative concentrations of volatile compounds identified in the crust of yeast and  
633 sourdough bread.

634 **Table 3.** Reducing sugar content (mg/100g of dry matter) in the crust and crumb of yeast bread  
635 and sourdough bread.

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648 **Table 1.** Reducing sugar content (mg/100g of dry matter) in yeast bread and sourdough bread  
649 doughs before and after fermentation.

<b>SUGARS</b> (mg/100g of dry matter)	<b>TYPE OF BREAD</b>	<b>BEGINNING OF FERMENTATION</b>	<b>AFTER 26.5 h OF FERMENTATION</b>
<b>Maltose</b>	<b>YB</b>	1654.6 ± 84.2 a	2814.5 ± 326.6 b
	<b>SB</b>	2245.1 ± 62.7 a	3336.0 ± 108.1 b
<b>Glucose</b>	<b>YB</b>	217.2 ± 34.3 a	78.2 ± 7.2 b
	<b>SB</b>	176.7 ± 21.6 a	267.0 ± 34.4 b
<b>Fructose</b>	<b>YB</b>	316.9 ± 33.0 a	176.6 ± 42.2 b
	<b>SB</b>	170.5 ± 24.9 a	126.9 ± 27.1 b

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651 Different letters in the same row indicate significant differences between groups ( $p < 0.05$ ).

652 Student t-test for unpaired samples ( $n=9$ ).

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661 **Table 2.** Relative concentrations of volatile compounds identified in the crust of yeast (YB)  
 662 and sourdough bread (SB).

<i>N</i> <sup>o</sup>	<i>Compound</i>	<i>YB</i>	<i>SB</i>	<i>RI</i>	<i>Origin</i>
1	3-Methylfuran	0.05 ± 0.001	0.20± 0.09	.*	M
2	2-Methylpropanal	0.05 ± 0.01	0.92 ± 0.21	-	M
3	2-Methylbutanal	0.38 ± 0.16	2.93 ± 0.53	951	M
4	3-Methylbutanal	0.36 ± 0.12	5.08 ± 0.38	953	M, F
5	Ethanol	1.98 ± 0.27	ND	961	F
6	2,3-pentanedione	0.04 ± 0.001	0.15 ± 0.07	1049	M
7	Dimethyl disulfide	ND	0.13 ± 0.04	1060	-
8	Hexanal	0.32 ± 0.05	0.76 ± 0.13	1069	L
9	Isoamyl acetate	ND	0.13 ± 0.05	1106	F
10	Heptanal	0.10 ± 0.03	0.34 ± 0.08.	1165	L
11	Pyridine	ND	0.03 ± 0.02	1179	M
12	3-Methyl-1-butanol	3.04 ± 0.22	0.98± 0.13	1201	F
13	2-Pentylfuran	0.21± 0.07	0.70 ± 0.17	1210	M, L
14	1-Pentanol	0.03 ± 0.005	0.09 ± 0.01	1239	L
15	Methylpyrazine	0.03 ± 0.003	ND	1252	M
16	2-(Ethoxymethyl)-furan	0.32 ± 0.21	0.14± 0.06	1271	M
17	1-Octen-3-one	0.05± 0.01	0.07 ± 0.02	1277	L
18	1-Hydroxy-2-propanone	0.02± 0.01	0.05± 0.02	1293	M, F
19	( <i>Z</i> )-2-Heptenal	0.01 ± 0.001	0.05 ± 0.01	1304	L, F
20	Ethylpyrazine	0.02 ± 0.001	0.06 ± 0.02	1316	M
21	6-Methyl-5-hepten-2-one	ND	0.04 ± 0.01	1320	-
22	Ethyl lactate	ND	0.09 ± 0.02	1330	F
23	1-Hexanol	0.07± 0.02	0.34± 0.05	1339	L
24	Dimethyl trisulfide	0.01 ± 0.007	0.06 ± 0.01	1361	-
25	2-Ethyl-6-methyl-pyrazine	0.01 ± 0.001	0.03 ± 0.01	1368	M
26	Nonanal	0.05± 0.007	0.07± 0.008	1376	L
27	( <i>E</i> )-2-Octenal	0.02± 0.002	0.06± 0.009	1407	L
28	Ethyl octanoate	0.08 ± 0.01	0.09 ± 0.02	1419	F
29	1-Octen-3-ol	0.03 ± 0.002	0.09± 0.01	1439	L
30	1-Heptanol	0.02 ± 0.002	0.07 ± 0.02	1444	F
31	Acetic acid	0.16 ± 0.006	1.21± 0.06	1450	F
32	Furfural	0.34 ± 0.12	1.98 ± 0.16	1456	M, F
33	2-Ethylhexanol	0.02 ± 0.001	0.03± 0.01	1480	F
34	2-Acetylfuran	0.06 ± 0.01	0.36 ± 0.14	1497	M
35	Benzaldehyde	0.09 ± 0.02	0.36 ± 0.09	1513	M, L, F
36	( <i>E</i> )-2-Nonenal	0.07 ± 0.001	0.19 ± 0.03	1524	L
37	Furfuryl acetate	0.03 ± 0.01	0.05 ± 0.03	1530	M
38	5-Methyl-2-furancarboxaldehyde	0.05 ± 0.02	0.21± 0.13	1567	M
39	2,2'-methylenebis-furan	0.03 ± 0.01	ND	1604	M
40	Isomaltol	0.02 ± 0.01	0.10± 0.07	1613	F
41	Butanoic acid	ND	0.03 ± 0.005	1628	F
42	Phenylacetaldehyde	ND	0.25± 0.05	1636	M, F
43	2-Furanmethanol	1.24 ± 0.40	1.37 ± 0.31	1656	M
44	3-Methylbutanoic acid	ND	0.05 ± 0.01	1658	F
45	( <i>Z</i> )-4-Decen-1-ol	0.01 ± 0.01	0.04 ± 0.01	1746	F
46	2,4-Decadienal	ND	0.05 ± 0.01	1753	L
47	1-(2-furanylmethyl)-1H-pyrrole	0.03± 0.001	0.05 ± 0.02	1820	M
48	Hexanoic acid	0.02 ± 0.001	0.15 ± 0.03	1826	F, L
49	3-Phenylfuran	0.12 ± 0.01	0.04± 0.01	1846	M
50	Benzyl alcohol	0.01± 0.001	0.12± 0.04	1849	M, L
51	Phenylethanol	0.24 ± 0.08	0.49 ± 0.06	1873	F
52	Maltol	0.13± 0.05	0.22± 0.08	1959	M
53	2-Acetylpyrrole	0.03 ± 0.01	0.06 ± 0.02	1969	M
54	1H-Pyrrole-2-carboxaldehyde	0.02 ± 0.01	0.07 ± 0.03	2009	M
55	Octanoic acid	0.10 ± 0.01	0.07± 0.01	2043	F, L
56	Nonanoic acid	0.09 ± 0.02	0.04 ± 0.02	2119	F
57	2-Methoxy-4-vinylphenol	0.02 ± 0.002	0.04 ± 0.02	2141	M
58	5-Hydroxymethylfurfural	0.01 ± 0.002	0.02 ± 0.003	-	M

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664 RI= Retention index on ZB wax plus column ; Origin = Origin of the compound formation

665 (M=Maillard reaction ; F = Fermentation ; L= Lipid oxidation) \* - : Not determined

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683 **Table 3.** Reducing sugar content (mg/100g of dry matter) in the crust and crumb of yeast bread  
 684 and sourdough bread.

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<b>Sugars</b> (mg/100g of dry matter)	<b>TYPE OF BREAD</b>	<b>CRUST</b>	<b>CRUMB</b>
	<b>YB</b>	516.2 ± 90.5 a	3265.8 ± 625.2 a
	<b>SB</b>	1714.9 ± 232.9 b	3040.0 ± 235.2 a
<b>Maltose</b>			
	<b>YB</b>	91.2 ± 6.6 a	64.8 ± 11.4 a
<b>Glucose</b>	<b>SB</b>	177.0 ± 22.7 b	233.6 ± 35.4 b
<b>Fructose</b>	<b>YB</b>	124.9 ± 7.1 a	175.8 ± 52.1 a
	<b>SB</b>	89.3 ± 20.3 b	91.5 ± 14.2 b

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687 Different letters in the same column indicate significant differences between the two groups for  
 688 each sugar studied (p<0.05). Student t-test for unpaired samples (n=9).

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697 **Figure captions:**

698 **Figure 1.** Doughs of yeast bread and sourdough bread at the beginning and after fermentation  
699 analyzed by SDS-PAGE. Protein marker is on the left side. The molecular weight of  
700 the markers ranges from 5 kDa up to 245 kDa.

701 **Figure 2.** Melanoidins in the crust of yeast bread (YB – white mark) and sourdough bread (SB  
702 – black mark) according to the lightness (L\*) of the crust (n=9).

703 **Figure 3.** Growth of *Bifidobacterium adolescentis* in M20 medium supplemented with *in vitro*  
704 digested yeast bread (YB) and sourdough bread (SB) crust or crumb (n=4). Control corresponds  
705 to *in vitro* digested water.

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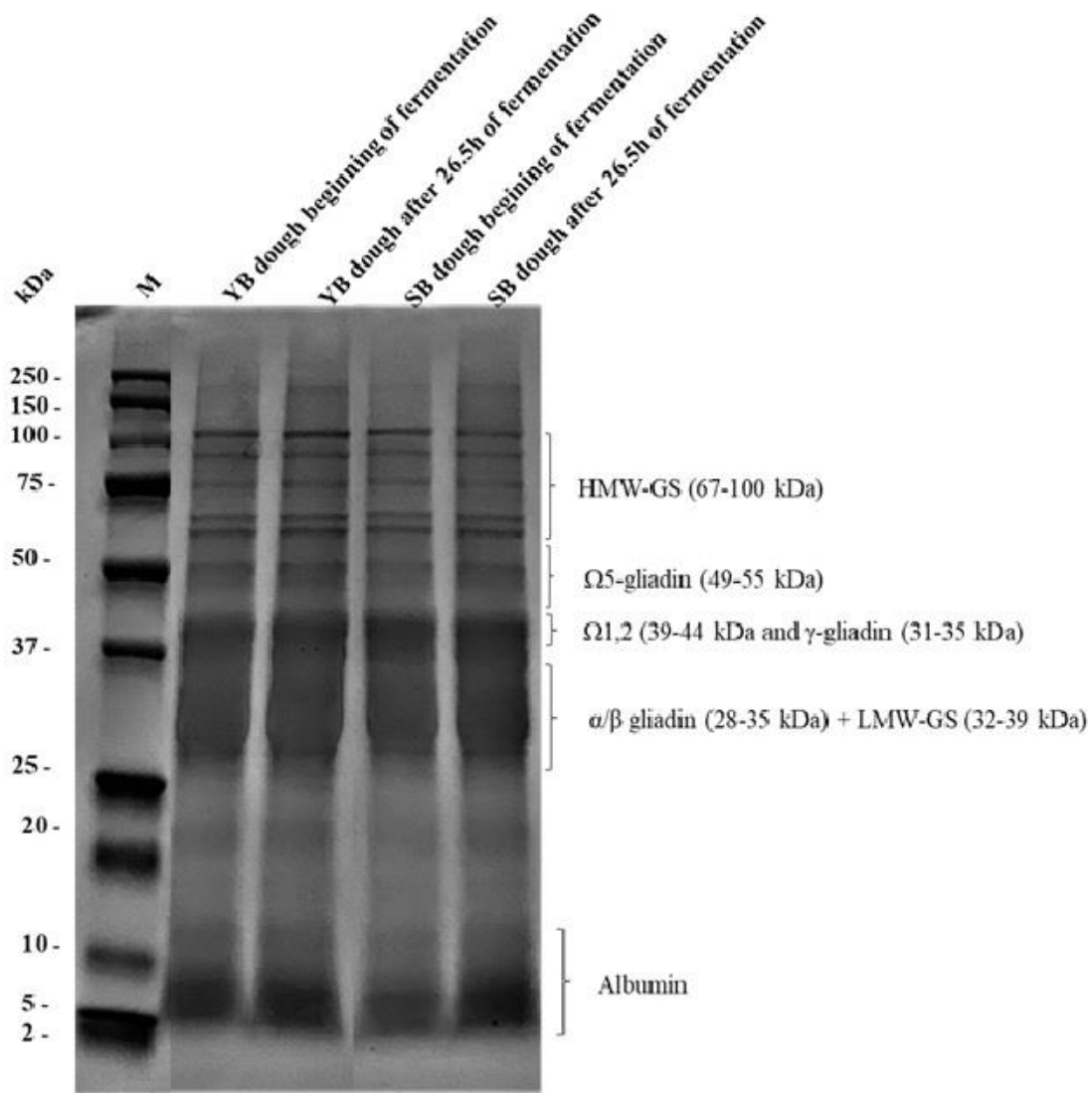
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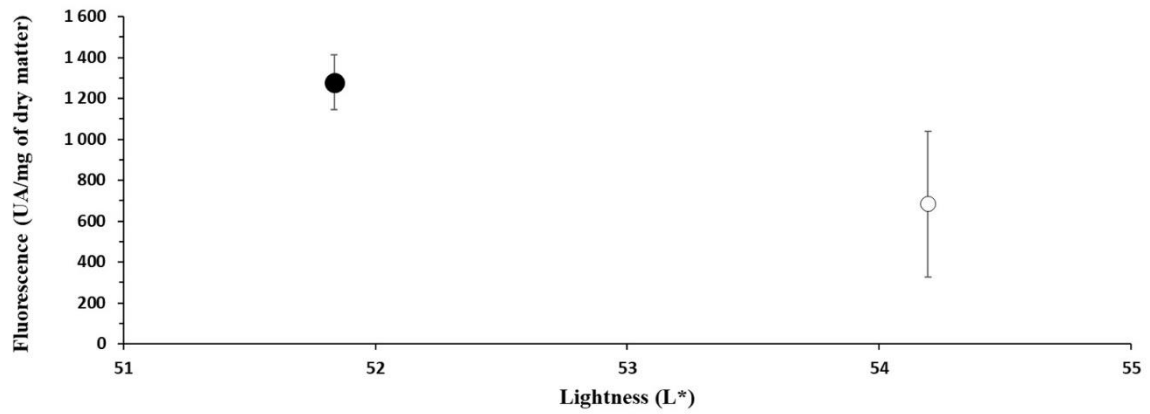
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727 **Fig. 2.** Melanoidins in the crust of yeast bread (YB – white mark) and sourdough bread (SB –  
728 black mark) according to the lightness (L\*) of the crust (n=9).

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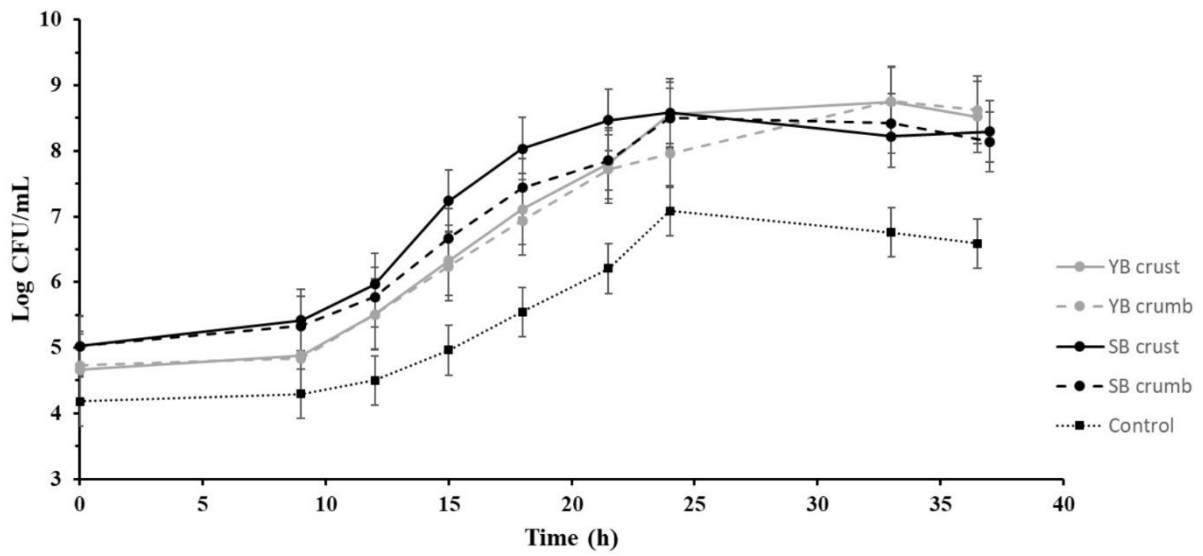
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743 **Fig. 3.** Growth of *Bifidobacterium adolescentis* in M20 medium supplemented with *in vitro*  
744 digested yeast bread (YB) and sourdough bread (SB) crust or crumb (n=4). Control corresponds  
745 to *in vitro* digested water.

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