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Solid state lactic acid fermentation: A strategy to improve wheat bran functionality

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1 **Solid state lactic acid fermentation: a strategy to improve wheat bran functionality.**

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20

21 **Abstract**

22 Wheat bran, a by-product produced in huge amount during cereal milling, is today largely  
23 unexploited because of its poor suitability as food ingredient. Solid-state fermentation (SSF)  
24 using a *Lactobacillus rhamnosus strain* was applied to wheat bran and its influence on  
25 bioactive compounds (free and bound phenolic acids) and their antioxidant activity were  
26 evaluated. Moreover, the phytic acid (PAC) degradation and arabinoxylans (WEAX)  
27 solubilization properties were studied: the SSF treatment resulted in an almost 37 %  
28 decrement and a three times increment of PAC and WEAX, respectively. Finally, in order to  
29 get the bigger picture, microbial metabolites and the volatile profile of fermented wheat bran  
30 were characterized, showing amino acids and lipids metabolites and a complex aroma  
31 profile. Overall, lactic acid fermentation can be considered a valuable pre-treatment for the  
32 valorisation of cereal by-products.

33 **Key words:** Lactic acid bacteria (LAB), solid state fermentation, bioprocessing, wheat by-  
34 products, fermentation metabolites, nutritional improvement

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43 **Abbreviation Used**

44 <LOQ, below the limit of quantification; 4-HB, 4-hydroxybenzoic acid; ABTS, 2,2'-Azino-  
45 bis(3-ethylbenzothiazoline-6-sulfonic acid); AOA, anti-oxidant activity; AWB, autoclaved  
46 wheat bran; Caff, caffeic acid; CID, collision-induced dissociation; d.w., dry weight; Dif,  
47 diferulates; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EI, electronic impact; EIC, extracted ion  
48 chromatogram; ESI, electrospray ionization; FRAP, ferric reducing antioxidant power;  
49 FWB24, fermented wheat bran at 24 hours; FWB48, fermented wheat bran at 48 hours;  
50 GAE, gallic acid equivalent; GC-HS-SPME-MS, gas chromatography head space solid  
51 phase micro extraction mass spectrometry; LAB, lactic acid bacteria; MS, mass  
52 spectrometry; PA, phenolic acid; PAc, phytic acid; *p*-C, *para*-Coumaric acid; PCA, plate  
53 count agar; SD, standard deviation.; Sin, sinapic acid; SRM, single reaction monitoring;  
54 TBC, total microbial count; TEAC, trolox equivalent antioxidant capacity; t-Fer, *trans*-Ferulic  
55 acid; TPC, total phenolic content; TSC, total spore count; UHPLC, ultra-high performance  
56 liquid chromatography; UV, ultraviolet; Vis, visible; WB wheat bran; WEAX, water  
57 extractable arabinoxylan.

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## 65 **1. Introduction**

66 Wheat (*Triticum* spp.) is one of the most cultivated crops worldwide, and it is considered a  
67 staple food in both developed and developing countries. Wheat cereal grains are not usually  
68 consumed as whole seeds, but they undergo to several processes for the production of  
69 bread, pasta, and baked goods in general. These processes generate a huge amount of  
70 residue side-streams (Sozer, Nordlund, Ercili-Cura, & Poutanen, 2017), mainly used as  
71 ingredients in feed formulation and rarely directed to human nutrition. Despite that,  
72 nowadays bran and other cereal by-products are commonly used to increase the nutritional  
73 quality of foodstuffs, such as high-fiber bread or biscuits and whole grain pasta (Coda,  
74 Katina, & Rizzello, 2015). In fact, it is widely recognized that the most important  
75 macronutrients (protein, lipids and dietary fiber), micronutrients (vitamins and minerals) and  
76 bioactive compounds (polyphenols) are concentrated in seed outermost tissues (Hemdane  
77 et al., 2016). On the other hand, also undesired compounds occur in these fractions, such  
78 as phytic acid (inositol polyphosphate) and tannins, which are recognised anti-nutritive  
79 compounds (Kumar, Sinha, Makkar, & Becker, 2010). Moreover, it is worth noting that cereal  
80 bran or pericarp included as ingredients in a baked product often adversely affect the taste  
81 and flavour quality perceived by consumers. In particular, wheat bran confers a browner  
82 colour, an astringent and bitter taste and a poor consistency and texture to the final product  
83 (Heiniö et al., 2016). In addition, the poor technological properties of wheat bran,  
84 characterized by a low water binding capacity, low gas holding capacity and poor viscosity  
85 of dough (Hemdane et al., 2016), negatively influences the manufacturing process. For all  
86 these reasons, nowadays many innovative technologies are being studied and applied as  
87 pre-treatments to improve the nutritional and sensorial characteristics of wheat bran. Among  
88 them, the effects of lactic acid fermentation on the rheology (Messia et al., 2016) and, in a  
89 minor extent, on the nutritional value of bran-added products have been studied (Coda et

90 al., 2015). This technique has shown several positive effects such as the increase of the  
91 content and of the bioavailability of bioactive compounds (polyphenols), the release of  
92 arabinoxylans in their water-soluble form, the degradation of antinutritive compounds and  
93 the modification of sensorial properties (Coda, Rizzello, Curiel, Poutanen, & Katina, 2014;  
94 Filannino, Di Cagno, & Gobbetti, 2018). In this work, the ability of a dairy strain LAB to modify  
95 the overall characteristics of wheat bran was studied.

## 96 **2. Material and Methods**

### 97 **2.1 Raw materials and chemicals**

98 Wheat bran (WB) of *Triticum turgidum* subsp. *turanicum* whole grain (moisture 9.09 g/100g,  
99 ash 4.51 g/100g, protein 14.53 g/100 g, carbohydrates 68.57 g/100 g, lipids 3.3 g/100 g and  
100 total dietary fibre 40.7 g/100 g, average particle size 1 mm) was provided by durum wheat  
101 local industrial mills. Commercial lots of whole grain cereal were from Italy and came from  
102 the 2015-2016 crop year. Sampling for bran fraction was carried out by five sub-samples of  
103 the same lot collected at different times and combined into one during the milling process.  
104 HPLC-grade acetonitrile (>99.9%), ethyl acetate (>99.8%), formic acid (>95.0%), acetic  
105 acid, hydrochloric acid (HCl, 37.0%), methanol (>99.9%), sodium hydroxide (NaOH,  
106 >98.0%), phenolic acid standards (caffeic acid >98%, 4-hydroxybenzoic acid >99%, *p*-  
107 coumaric acid >98%, sinapic acid >98% and trans-ferulic acid >99%), chloridric acid (37 %),  
108 potassium persulfate (99,9%), iron (III) chloride, ( $\pm$ )-6-hydroxy-2,5,7,8-  
109 tetramethylchromane-2-carboxylic acid (97 %), gallic acid (>98%), Folin & Ciocalteu's  
110 phenol reagent (2 N), 2,2-diphenyl-1-picrylhydrazyl, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ,  
111 >98%), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt were all  
112 purchased from Sigma-Aldrich (St. Louis, Missouri, US).

### 113 **2.2 Fermentation process**

114 *Lactobacillus rhamnosus* 1473, a facultative hetero-fermentative strain isolated from  
115 Parmigiano Reggiano cheese (collection of Food and Drug Department, University of  
116 Parma, Italy) was singly used as starter for fermentation. The strain preparation and starter  
117 inoculum were prepared as described by Ricci et al., 2018. WB was sterilized and water was  
118 added (75%, AWB). *L. rhamnosus* 1473 was inoculated into AWB in order to reach 7 Log  
119 CFU mL<sup>-1</sup> and incubated at 37 °C for 24 h (FWB24) and 48 h (FWB48). Fermentation  
120 experiments were carried out in triplicate. Wheat bran samples without starter were  
121 incubated at 37 °C for 24 and 48 h and used as controls. Non-fermented sterilized wheat  
122 bran was also included in the sample set. Samples were lyophilized, accurately minced and  
123 stored at -80°C until extraction and analyses. The microbial count (TBC) was performed on  
124 WB, AWB and FWB48 on MRS agar (Oxoid, Milan, Italy) incubating at 37 °C for 48 h. The  
125 pH of WB, AWB, FWB24 and FWB48 samples was measured by pH meter (Mettler Toledo,  
126 Switzerland). Microbial counts and pH measurement were performed in triplicate.

## 127 **2.4 Phenolic compounds profiling**

### 128 **2.4.1. Sample preparation for free and total phenolic compounds**

129 Free and bound phenolic compounds were extracted from WB, AWB, FWB24 and FWB48.  
130 The extraction of free phenolic compounds was performed according to Verma et al., 2009,  
131 considering both the bound and free phenolic acid fractions. The extracts were also used  
132 for the UHPLC-MS/MS analysis and other assays.

### 133 **2.4.2. UHPLC-ESI-MS/MS profiling**

134 The UHPLC-ESI-MS/MS analysis was performed on WB, AWB, FWB24 and FWB48 using  
135 an UHPLC Dionex Ultimate 3000 instrument coupled with a triple quadrupole mass  
136 spectrometer (TSQ Vantage; Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped  
137 with an electrospray source (ESI), following the procedure of Verma et al., 2009. Detection

138 was carried out by Selected Reaction Monitoring (SRM), using the transitions reported in  
139 **Table S1**. Dimeric forms of ferulic acid with  $[M-H]^-$  value of  $m/z$  385 were analysed in full  
140 scan MS<sup>2</sup> mode and quantified as ferulic acid equivalents (sum in  $\mu\text{g}$  of ferulic acid  
141 equivalent per  $\text{g}^{-1}$ ). For quantification, two different calibration sets were prepared using  
142 acidified water as solvent (0.2% of formic acid): one with a calibration range of 0.05-5  $\mu\text{g g}^{-1}$   
143 <sup>-1</sup> and one in the range of 5-100  $\mu\text{g g}^{-1}$  for free and bound phenolic compounds respectively,  
144 obtaining a good linearity ( $R^2 > 0.99$ ) for both calibration ranges.

### 145 **2.4.3 Total phenolic content (TPC)**

146 Free and bound total phenolic content (TPC) of WB, AWB, FWB24 and FWB48 was  
147 analysed by the Folin–Ciocalteu’s method according to Singleton, Orthofer, & Lamuela-  
148 Raventós, 1998. Calibration curve was prepared using gallic acid as reference compound  
149 (100-1000  $\text{mg Kg}^{-1}$ ) and results were expressed as  $\text{mg}$  of gallic acid equivalents (GAE) per  
150  $\text{Kg}$  on dry weight basis.

### 151 **2.4.4 Determination of the antioxidant activity (AOA) using DPPH, FRAP and ABTS** 152 **assays**

153 The antioxidant activity of WB, AWB, FWB24 and FWB48 free and bound phenolic extracts  
154 were evaluated by the DPPH radical scavenging activity assay (Brand-Williams, Cuvelier, &  
155 Berset, 1995), by the FRAP assay (Pulido, Bravo, & Saura-Calixto, 2000) and by the ABTS+  
156 radical cation scavenging assay (Re et al., 1999). The % inhibition was calculated from the  
157 regression equation prepared using Trolox (0.1-1  $\text{mM}$ ) as reference standard and results  
158 were expressed as  $\text{mmol Trolox equivalent (TEAC) g}^{-1}$  dry weight.

### 159 **2.5 Quantification of phytic acid (PA)**

160 Phytic acid contents of WB, AWB, FWB24 and FWB48 were determined  
161 spectrophotometrically using Megazyme test kit KPHYT 05/07 (Megazyme International



162 Ireland Limited, Bray, Ireland). Results were expressed as g of phytic acid per 100 g<sup>-1</sup> dry  
163 weight.

## 164 **2.6 Quantification of water extractable arabinoxylans (WEAX)**

165 The WEAX of WB, AWB, FWB24 and FWB48 was determined according to Kiszonas et al.,  
166 2012. 0.4 g of samples were extracted with 20 mL of distilled water at room temperature  
167 under constant agitation. Extracts were centrifuged at 4000 rpm for 10 min at room  
168 temperature. Then, 2 mL of daily prepared working reaction solution (1 g of phloroglucinol  
169 dissolved in 5 mL of pure ethanol, 2 mL of hydrochloric acid, 110 mL of glacial acetic acid  
170 and 1 mL of a 17.5 g L<sup>-1</sup> glucose solution), 100 µL of supernatant and 100 µL of distilled  
171 water were added into stoppered glass tubes (12 mL, 16x100 mm). The tubes were then  
172 placed in a boiling water bath for 25 min and then cooled in ice. The absorbance was  
173 measured at 552 nm and 510 nm successively, using an UV-Vis spectrophotometer. D-(+)-  
174 Xylose was used as standard for the calibration curve (0.05-30 mg Kg<sup>-1</sup>). Finally, the WEAX  
175 content was calculated subtracting the absorbance value at 510 nm, which corresponds to  
176 hexose interferences, from the absorbance value at 552 nm and the obtained value was  
177 compared with the regression equation.

## 178 **2.7 Fermentation metabolites analysis with UPLC-ESI-LTQ/MS**

179 The aqueous methanolic (3/7, v/v) extracts derived from WB, AWB, FWB24 and FWB48  
180 were analysed using an Accela UHPLC 1250 equipped with a linear ion trap-mass  
181 spectrometer (MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) attached to  
182 a heated electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc., San Jose,  
183 CA, USA), using the protocol described by Ricci, Cirlini, Maoloni, et al., 2019.

## 184 **2.8 Volatile profile HS-SPME-GC-MS**

185 Volatile profiles of WB, AWB, FWB24 and FWB48 were carried out using head space solid-  
186 phase micro extraction (HS-SPME) and analysed by a gas chromatograph (Thermo  
187 Scientific Trace 1300 gas chromatograph) coupled to a Thermo Scientific ISQ single  
188 quadrupole mass spectrometer equipped with an electronic impact (EI) source, according  
189 to Dall'Asta et al., 2011. The main volatile compounds of wheat bran samples were identified  
190 on the basis of their mass spectra compared with the library (NIST 14) mass spectra. The  
191 semi-quantification of all detected gas-chromatographic peaks was carried out using toluene  
192 as internal standard.

## 193 **2.8 Statistical analysis**

194 One-way ANOVA was used to compare the different results obtained for WB, AWB, and  
195 FWB24 and FWB48. Results obtained from three fermentation replicates (n=3) and three  
196 experimental replicates (n=3) were analysed using *Tukey-b's* post-hoc test (significance  
197 level  $\alpha=0.05$ ). Statistical analyses were carried out using SPSS Statistics 21.0  
198 software (SPSS Inc., Chicago, IL). Moreover, *Pearson correlation* analysis was performed  
199 to measure the relationship between Folin-Ciocalteu's assay and the antioxidant activity  
200 tests.

## 201 **3.Results and Discussion**

### 202 ***3.1 L. rhamnosus 1473 growth and pH assessment***

203 Wheat bran fermentation is poorly reported in the literature and not extensively investigated.  
204 In particular, the employment of lactic acid bacteria is scarcely explored and only few studies  
205 were available (Arte et al., 2015; Messia et al., 2016; Prückler et al., 2015), and it is worthy  
206 of note that *L. rhamnosus* fermentation was never reported before. In this study, wheat bran  
207 microbial contamination was examined before fermentation, resulting in a total microbial  
208 count of ca. 5 Log CFU/g<sup>-1</sup>. Therefore, a sterilization step was necessary to eliminate the

209 endogenous microflora and to accurately evaluate the metabolic properties of *L. rhamnosus*  
210 1473. Its growth ability was monitored after 48h revealing the increase in microbial cells  
211 number ( $10.42 \pm 0.10$  Log CFU/g<sup>-1</sup>) from the original inoculum (ca. 7 Log CFU/g<sup>-1</sup>). A  
212 significant pH decrease (from  $6.53 \pm 0.22$  to  $4.70 \pm 0.10$ ) was also observed (**Table 1**).

### 213 **3.2 Free, bound and total phenolic content and antioxidant activity**

214 Results regarding the total phenolic content and the antioxidant activity of the free and bound  
215 extracts are reported in **Table 2**. Arguably, it was observed that free TPC decreased after  
216 autoclaving, but interestingly it increased during the fermentation process, with no significant  
217 difference between 24 and 48 hours. This phenomenon could be explained considering that  
218 phenolic compounds that are soluble in the matrix are also more sensitive to high  
219 temperature and can be degraded during the thermal treatment. On the other hand, the  
220 release of such compounds by the action of fermentation could occur. On the contrary, an  
221 opposite behaviour was observed for bound components. In fact, they increased after the  
222 thermal treatment and then decreased during the fermentation. This is possibly due to the  
223 neoformation of Maillard reaction's related compounds during the sterilization process, such  
224 as complex polyphenols (Ragae, Seetharaman, & Abdel-Aal, 2014). Since both processing  
225 (autoclaving and fermentation) modified the matrix composition by the solubilization and  
226 deconjugation of bound phenolic compounds, the ratio between total free and bound  
227 polyphenols was calculated (**Table 2**). Despite total phenolic content did not increase  
228 significantly during the fermentation in comparison to WB, the F/B ratio was higher in bran  
229 fermented for 24 and 48 hours. This means that *L. rhamnosus* metabolised the conjugated  
230 phenolic compounds, thus breaking the linkage between them and the cell-wall  
231 polysaccharides. These results are consistent with those previously reported by Zhao et al.,  
232 2017. Regarding the antioxidant activity (AOA), measured with the three different assays,  
233 significant differences were observed between the samples. In the case of DPPH, the

234 soluble antioxidant compounds decreased after the thermal treatment, while for ABTS and  
235 FRAP test no differences were found. Furthermore, a good positive correlation was found  
236 between total AOA measured with DPPH and ABTS tests and TPC method (r: 0.97; r: 0.22,  
237  $p < 0.05$ , respectively), while a negative correlation was found for the FRAP assay (r: -0.80,  
238  $p < 0.05$ ). This means that antioxidant activity is mainly due to phenolic compounds, although  
239 a minor contribution could be also due to other molecules which could have antioxidant  
240 potential such as peptides and amino acids, or also to newly formed/released bioactive  
241 compounds produced by the LAB metabolism. Finally, the F/B ratio (sum of free to sum of  
242 bound antioxidant activity assays ratio) of antioxidant activity also increased after  
243 fermentation, indicating an increased content of free and soluble antioxidant compounds.  
244 These compounds could exert a positive protection effect against the lipid oxidative process,  
245 known to be a cause of poor sensorial quality of finished food products (Calligaris,  
246 Manzocco, Anese, & Nicoli, 2016). Overall, the total AOA and TPC reported in this study are  
247 in line with other investigations (Nordlund, Katina, Aura, & Poutanen, 2013; Zhao et al.,  
248 2017).

### 249 **3.3 Phytic acid degradation**

250 Although rich in bioactive compounds, wheat bran and external layers of cereal grains in  
251 general have also high amount of phytic acid and phytates, which are recognised as anti-  
252 nutritive molecules negatively affecting the dietary bioavailability of important minerals such  
253 as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Zn}^{2+}$ , and amino acids (Carrizo et al., 2016). Consequently, from  
254 the nutritional point of view, phytate degradation is desirable to improve mineral  
255 bioavailability. Results are showed in **Table 2**. The sterilization step did not significantly  
256 modify the content of phytic acid in WB. On the contrary, wheat bran fermentation decreased  
257 the phytic acid content to 36,4% in comparison to WB, in agreement with results reported  
258 by Zhao et al., 2017. The hydrolysis of phytic acid is generally carried out by phytase and

259 phosphatase enzymes that can be found in microorganisms or food matrix. Being the  
260 endogenous phytases present in wheat bran probably inactivated during the thermal  
261 treatment, degradation of phytic acid is probably due to a phytate-degrading activity  
262 expressed in *Lactobacillus rhamnosus* 1473, as already reported in strains of the same  
263 species (Carrizo et al., 2016).

### 264 **3.4 Arabinoxylans solubilisation**

265 Arabinoxylans are important compounds that characterize the structure of vegetable cells,  
266 in particular those of cereals. They are present in both water-soluble and insoluble forms,  
267 and the former has recognised positive effects on the bread dough rheology (Courtin &  
268 Delcour, 2002). After thermal step and fermentation, the WEAX content of wheat bran  
269 increased significantly (**Table 2**). Sterilization induced a significant solubilization of these  
270 compounds, but LAB enhances WEAX content almost three times compared to WB. Specific  
271 enzymes, such as endoxylanases, can hydrolyse the backbone of high molecular weight  
272 arabinoxylans. These results are in agreement with those reported by Zhao et al., 2017.

### 273 **3.5 Free, bound and total phenolic acids profile**

274 Phenolic acids are the most abundant bioactive compounds present in wheat bran, and  
275 more in general in cereal grains. They can occur in soluble or insoluble forms. Thermal  
276 processing and fermentation of wheat bran significantly modified the composition of this  
277 matrix as shown in **Table 3**. Overall, a decreasing of the free phenolic acids was measured  
278 after the sterilization step, while a slight increase of the insoluble component was obtained.  
279 However, the free phenolic acids content significantly increased when wheat bran was  
280 submitted to lactic acid fermentation, albeit no difference was found between the 24 and 48  
281 hours of treatment. Nutritional improvement is not only related to the increased content of  
282 potentially bioactive compound but is determined also by their bioaccessibility. Thus, soluble

283 compounds are more likely to be absorbable in the human gastrointestinal tract and to be  
284 able to exert their beneficial functions (Mateo Anson et al., 2011). Several enzymes could  
285 be responsible for the solubilization of phenolic acids, such as endoxylanases, xylosidases,  
286 arabinofurosidases and ferulic acid esterases, especially related to fermentation processes  
287 (Faulds, Mandalari, LoCurto, Bisignano, & Waldron, 2004). This can be underlined by the  
288 F/B ratio (sum of free PAs to sum of bound PAs ratio), with a three-fold increase after lactic  
289 acid fermentation. Interestingly, among phenolic acids, a relatively high content of caffeic  
290 acid was found in fermented wheat bran, indicating that some metabolic activity of  
291 microorganism occurred. Indeed, previous studies pointed out that *Lactobacillus* spp. can  
292 produce caffeic acid starting from chlorogenic acids, which is present in wheat (Žilić et al.,  
293 2011), by hydrolysis, even if the metabolism of phenolics is LAB-specific (Filannino, Bai, Di  
294 Cagno, Gobbetti, & Gañzle, 2015). In addition, being caffeic acid a strong inhibitor of lipid  
295 peroxidation, as reported by the study of Khenouf et al., 2003, this is very important since  
296 wheat bran is a matrix particularly sensitive to the lipid oxidation. Moreover, the bound PAs  
297 component significantly diminished during fermentation, in particular the *p*-C, *t*-Fer and Sin  
298 acids. This is possibly correlated to the metabolic properties of *L. rhamnosus* 1473, which  
299 can convert these phenolic compounds to other microbial metabolites such as dihydroferulic  
300 acid or dihydrosinapic acid. Indeed, Filannino et al., 2015 demonstrated that strains  
301 belonging to *Lactobacillus* species can use hydroxycinnamic acids as external acceptor of  
302 electrons, thus exploiting an energy advantage. These modified forms have different  
303 absorption pathway and even an increased bioactivity compared to their parent form  
304 (Gobbetti et al., 2018). Moreover, the dimeric form of ferulic acid, Dif, was also detected at  
305 relevant concentration, although no significant differences were found among fermented and  
306 non-fermented wheat bran.

### 307 **3.6 Fermentation metabolites**

308 Fermented and raw wheat bran were also analysed using an LC-MS untargeted approach,  
309 with the aim to discover newly formed metabolites deriving from lactic acid fermentation. In  
310 **Table 4** the mass spectral characteristics of the putative fermentation metabolites found in  
311 fermented wheat bran and not in untreated wheat bran are reported. These compounds  
312 mainly derive from amino acids and fatty acids degradation. For example, 3-phenyllactic  
313 (**Figure 1B**) acid probably derives from the conversion of the amino acid phenylalanine into  
314 phenylpyruvic acid via transamination and successive degradation by specific enzymes  
315 (hydroxyl acid dehydrogenase) (Valerio, Di Biase, Lattanzio, & Lavermicocca, 2016).  
316 Consequently, as reported by other authors, 3-hydroxyphenyllactic (**Figure 1C**) acid could  
317 be a degradation metabolite of tyrosine, largely occurring in wheat cereal (Ricci, Cirlini,  
318 Calani, et al., 2019). Furthermore, other amino acids present in wheat bran can also be  
319 transformed by LAB metabolic pathways. In fact, 2-hydroxyvaleric (**Figure 1A**) acid can  
320 originate from valine, leucine and/or isoleucine and indole-3-lactic acid from tryptophan  
321 (Koistinen et al., 2018). Nowadays, these carboxylic acids are receiving attention due to  
322 their protective properties against pathogenic bacteria (Kim & Oh, 2013), fungi (Valerio et  
323 al., 2016) and also for their anti-mycotoxigenic features (Guimarães, Santiago, Teixeira,  
324 Venâncio, & Abrunhosa, 2018). In addition, amino acids are important precursor of several  
325 flavour such as aldehydes and alcohols that characterize sourdough fermentation and baked  
326 products (Corsetti & Settanni, 2007). Also fatty acids can be metabolised by LAB (Kim & Oh,  
327 2013). Indeed, *L. rhamnosus* 1473 appears to be able to convert fatty acids (FA) in their  
328 hydroxylated form, with one or more hydroxyl groups in different position of the hydrocarbon  
329 chain. Wheat bran has a relative high content of lipid and it is characterized by a complex  
330 mixture of triglycerides and free fatty acids. These are mainly constituted by mono- and  
331 polyunsaturated FA, such as oleic and linoleic acids. Lipid oxidation metabolism is governed  
332 by specific endogenous enzymes from both vegetable and bacterial origin. Also this class  
333 of compounds represents an interesting innovation point mainly because of their health-

334 related (Moreno, 2009), anti-fungal and technological (Metzger & Bornscheuer, 2006). It is  
335 also important to mention that these compounds could contribute to the sensorial and  
336 nutritional properties of wheat bran.

### 337 **3.7 Volatile profile of fermented wheat bran**

338 In **Table 5** the main volatile compounds detected in wheat bran samples by GC-HS-SPME-  
339 MS analysis are reported. A total of 47 compounds were identified, belonging to different  
340 classes: alcohols, aldehydes, ketones, carboxylic acids, furan derivatives and esters.  
341 Arguably, some compounds increased or decreased in terms of concentration, due to the *L.*  
342 *rhamnosus* 1473 metabolism. Alcohols were the most abundant compounds, both in terms  
343 of concentration and identified molecules (**Table S2**). These results are in agreement with  
344 the study by Ricci et al., 2018, in which the same strain was used to ferment elderberry juice.  
345 In the present study ethanol and ethyl acetate were not found in fermented wheat bran,  
346 probably because other reactions that use these molecules as precursor were involved.  
347 Certain aldehydes were found after autoclaving such as 5-ethylcyclopentene-1-  
348 carbaldehyde, benzaldehyde and 2,4-dimethylbenzaldehyde and were still present after  
349 fermentation. Then, furan derivatives, characteristic of bread aroma (Zhou & Therdthai,  
350 2012), were also identified in AWB. Other molecules such as nonanal could be formed by  
351 lipxygenase activity (Zhou & Therdthai, 2012). Globally, fermented wheat bran showed  
352 completely different aroma notes in comparison with unfermented bran: this is particularly  
353 important from the consumer point of view, leading to an improved acceptability of the  
354 sensorial quality of the product.

### 355 **4. Conclusion**

356 In conclusion, the aim of the present work was to give a complete overview on the ability of  
357 SSF, using lactic acid bacteria, to convert a low value matrix in a high functional food



358 ingredient. To the best of our knowledge this is the first study based on *L. rhamnosus*  
359 species wheat bran fermentation. In addition, differently from the currently available  
360 literature, free and bound phenolic components and antioxidant activity of fermented wheat  
361 bran were analysed. This bioprocess effectively improved the composition of wheat bran,  
362 resulting in an improved nutritional profile and complex structure modification. Phytic acids  
363 decreased almost three times while the soluble arabinoxylans triplicate their concentration.  
364 More important, beside the TPC slightly decrease, free components increased significantly  
365 after fermentation enhancing the soluble AOA of wheat bran. Then, microbial metabolites,  
366 deriving from amino acids and lipid metabolism, were identified in fermented wheat bran.  
367 These molecules are nowadays receiving great attention due to their multipurpose  
368 properties. Volatile profile was also evaluated, stressing the complexity of the aroma  
369 compounds created during fermentation. On the base of these results, lactic acid  
370 fermentation could be confirmed as an interesting innovative pre-treatment of wheat bran,  
371 capable to potentially enhance its health and sensorial properties.

#### 372 **Conflict of interest**

373 None.

#### 374 **Acknowledgment**

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#### 380 **Authors contribution**

381 C.D., G.G., C.L. and M.S. conceived and designed the experiments. M.S. performed all the  
382 experiments and analysed the data. M.S., A.R. and L.B. interpreted the results. M.S., C.D.,  
383 G.G. and C.L. drafted the paper. All the authors contributed to the critical review and revision  
384 of the manuscript.

### 385 **Supporting information description**

386 Appendix A.

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536 **Figure captions**

537 **Figure 1** Extracted ion chromatogram (EIC) of 2-Hydroxyvaleric (A), 3-Phenyllactic (B) 3-  
538 Hydroxyphenyllactic acids (C) and corresponding mass spectra, found in wheat bran after  
539 24 (FWB24) and 48 (FWB48) hours of fermentation. Specific fragments are surrounded by  
540 red circles. The figures also show the chromatograms wheat bran (WB), autoclaved wheat  
541 bran (AWB).

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556 **Tables**

557 **Table 1** pH, total microbial count (TBC) and total spore count (TSC) of native wheat bran  
 558 (WB), autoclaved wheat bran (AWB) and wheat bran after 24 (FWB24) and 48 (FWB48)  
 559 hours of fermentation.

Sample	pH	TBC	TSC
		Log CFU g <sup>-1</sup>	
WB	6.41±0.06b	5.32±0.14	2.56±0.66
AWB	6.53±0.22b	7.78±0.22 <sup>a</sup>	<Log <sup>-1</sup>
FWB24	4.67±0.08a	-	-
FWB48	4.70±0.10a	10.42±0.10	<Log <sup>-1</sup>

560 Results are reported as mean of three fermentation replicates and three experimental  
 561 replicates ± standard deviation (n=9). Different letters mean a significant difference  
 562 (<alpha>=0.05) between samples, following the *Tukey b*'s post-hoc test. – not measured.  
 563 NF not found. <sup>a</sup>after the inoculum.

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**Table 2** Changes in total phenolic content (TPC), overall antioxidant activity (DPPH, ABTS and ferric reducing ability of plasma (FRAP)), phytic acid (PA) and water extractable arabinoxylans (WEAX) of wheat bran (WB), autoclaved wheat bran (AWB) and wheat bran after 24 (FWB24) and 48 (FWB48) hours of fermentation.

Sampl e	TPC			F/B <sup>a</sup>	DPPH			ABTS			FRAP			F/B <sup>b</sup>	PA g 100 gr <sup>-1</sup>	WEAX mg g <sup>-1</sup>
	mg GAE Kg <sup>-1</sup>				mm TEAC g <sup>-1</sup>			mm TEAC g <sup>-1</sup>			mm TEAC g <sup>-1</sup>					
	Free	Bound	Tot		Free	Bound	Tot	Free	Bound	Tot	Free	Bound	Tot			
WB	1174.9±184.	2451.2±109.	4247.0±200.	0.4	3.6±0.1	17.7±0.2	20.0±0.	10.2±0.2	40.5±1.2	50.7±1.	11.0±0.4	34.5±0.4	48.9±0.	0.2	2.7±0.2	12.6±0.1
	7b	4b	6b	8	b	a	2	b	a	1	c	a	6	7	a	d
AWB	1043.5±0.7c	3203.5±0.0a	4599.6±90.4	0.3	2.2±0.0	17.9±1.0	21.5±0.	10.6±0.2	38.6±2.3	49.2±2.	11.6±0.8	36.3±0.3	45.5±0.	0.2	2.6±0.0	14.7±0.2
			a	3	c	a	6	b	a	4	b	a	3	6	a	c
FWB2 4	1447.2±178.	2343.8±315.	3791.1±241.	0.6	3.5±0.3	15.2±2.1	18.7±2.	10.7±0.2	40.2±2.7	50.9±3.	12.6±0.4	33.6±6.5	49.6±5.	0.3	2.3±0.2	22.7±2.9
	1a	0b	8b	2	b	a	2	b	a	5	b	a	7	0	b	b
FWB4 8	1553.3±70.4	2271.1±374.	3824.3±395.	0.6	4.0±0.2	13.9±1.1	17.8±1.	12.0±0.8	33.9±3.7	45.9±1.	19.2±3.8	29.0±3.6	48.1±1.	0.4	1.7±0.1	32.4±2.8
	a	1b	0b	8	a	b	1	a	b	8	a	a	0	6	c	a

Results are represented as mean of three fermentation replicates and three experimental replicates ± standard deviation (n=9). Data with different letters in the same column are significantly different ( $\alpha=0.05$ ), following the *Tukey b's* post-hoc test. <sup>a</sup> F/B: sum of free to sum of bound ratio. <sup>b</sup> sum of free antioxidant activity (AOA) assays to sum of bound AOA assays ratio.

**Table 3** Free and bound phenolic acids (PAs) content in wheat bran (WB), autoclaved wheat bran (AWB) and wheat bran after 24 (FWB24) and 48 (FWB48) hours of fermentation.

Sampl e	4-HB		p-C		Caff		t-Fer		Sin		Dif	F/B <sup>a</sup>
	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound		
WB	2.4±0.2	4.7±1.3	1.3±0.1	45.2±1.6c	<LOQ	<LOQ	31.0±1.4	3643.5±63.3a	7.1±1.1b	111.2±14.3	2324.7±38.3a	1,0
	b	a	b				b			a		4
AWB	1.4±0.5c	6.0±0.8	0.8±0.4c	60.2±4.2	<LOQ	<LOQ	12.7±4.7c	3870.4±245.2	5.8±1.2c	135.1±16.3	2364.7±76.5a	0,5
		a		a				a		a		4
FWB24	3.8±0.7	5.8±1.2	2.6±0.3	59.1±8.1	12.6±1.3	0.9±0.1	50.6±5.9	3786.0±562.3	12.7±1.8	81.6±13.8c	2489.0±339.9	2,1
	a	a	a	b	a	a	a	a	a		a	0
FWB48	4.0±0.6	5.8±1.1	2.8±0.2	38.3±3.6	15.0±1.3	0.8±0.2	47.9±3.8	2922.3±281.0	12.3±2.6	57.4±9.9d	2394.7±458.1	2,7
	a	a	a	d	a	a	a	b	a		a	1

Results are represented as mean of three fermentation replicates and two experimental replicates (n=6). Different letters in the same column mean a significant difference ( $p < 0.05$ ) between samples, following the *Tukey b's* post-hoc test. <LOQ 0.05  $\mu\text{g g}^{-1}$ . 4-HB: 4-hydroxybenzoic acid p-C: para-Coumaric, Caff: caffeic acid, t-Fer: trans-Ferulic acid, Dif: diferulates; acid; <sup>a</sup> F/B: sum of free PAs to sum of bound PAs ratio.

**Table 4** Mass spectral characteristics of compounds detected in fermented wheat bran.

Putative compound	[M-H] <sup>-</sup> ( <i>m/z</i> )	Rt (min)	MS <sup>2</sup>	Compound class	Ref
2-Hydroxyvaleric acid	117	3.9	71, 117		
3-Hydroxyphenyllactic acid	181	4.13	135, 163, 113	Amino acid degradation	(Kang,
3-Phenyllactic acid	165	6.2	119, 147		Price,
Indole-3-lactic acid	204	6.55	158, 116, 142, 128		Ashton,
Tetrahydroxy octadecenoic acid	345	9.04	327, 309		Tapsell, &
Trihydroxy octadecadienoic	327	9.13	309, 291, 239	Fatty acid hydroxylation	Johnson,
Trihydroxy octadecenoic acid	329	10.59	311, 293, 275, 211, 201, 171		2016;
Dihydroxy-octadecadienoic acid	313	10.71	293, 275		Koistinen et
Dihydroxy-octadecenoic acid	313	10.83	295, 277, 183		al., 2018)

Identified based on MS<sup>n</sup> data and retention time and their comparison with MS<sup>n</sup> and data from reference sources. Tentatively identified based on MS<sup>n</sup> and retention time and other literature evidence.

**Table 5** Volatile compounds, their relative abundance and corresponding odour perception according to GC–MS analysis of wheat bran (WB), autoclaved wheat bran (AWB) and wheat bran after 24 (FWB24) and 48 (FWB48) hours of fermentation.

<i>Class</i>	<i>Compound</i>	<i>Odour perception</i> <sup>a</sup>	<i>WB</i>	<i>AWB</i>	<i>FWB24</i>	<i>FWB48</i>
		Relative Abundance <sup>b</sup>				
Alcohols	Ethanol	Strong, alcohol	+	+	-	-
	Isoamyl alcohol	Pungent, fusel	+	+	-	-
	1-Pentanol	Pungent, fusel	+	+	-	NF
	2-Heptanol	Fruity	-	-	+	+
	1-Hexanol	Green grass	+	+	-	-
	4-Methylcyclohexanol	Woody	+	+	NF	NF
	1-Octen-3-ol	Fruity	-	-	+	+
	1-Heptanol	Solvent	+	+	NF	NF
	2,3-Butanediol	Butter cream	+	+	-	-
	1-Octanol	Waxy	+	NF	NF	NF
	2-Octen-1-ol	Fatty	+	NF	NF	NF
1-nonanol	Floral	+	NF	NF	NF	

	3-Nonen-1-ol	Fatty	+	NF	NF	NF
	2-Nonen-1-ol	Fatty	+	NF	NF	NF
	Phenethyl alcohol	Fruity	+	+	-	NF
	Deca-2,4-dien-1-ol	Fatty	+	NF	-	NF
		Camphor				
	Cyclohexanol	menthol	NF	NF	+	+
	1-Penten-3-ol	Fruity	NF	NF	+	+
	Cyclohexanol, 2 methyl 5	Fruity	NF	NF	+	+
	4-Ethylphenol	Smoky	+	+	NF	NF
	1-Nonen-4-ol	Sweet	NF	NF	NF	+
	4,4,6-Trimethylcyclohex-2-en-1-ol	Floral, balsamic	NF	NF	NF	+
	Acetoin	Sweet cream	+	+	NF	NF
Ketones	2-Heptanone	Cheesy	+	+	-	-
	3-Octanone	Green grass	+	+	-	-
	2-Octanone	Milky	+	+	-	-
	2-Nonanone	Fruity	+	+	-	NF

	6-Methyl-5-hepten-2-one	Fatty, green	+	-	NF	NF
	Camphor	Grass, woody	+	+	NF	NF
	5-Pentylloxolan-2-one	Floral	-	-	+	+
	3-Ethylcyclopentan-1-one	Vegetal, natural	NF	NF	+	+
	3-Octen-2-one	Melon	NF	NF	+	+
	2-Decanone	Floral	NF	NF	+	+
	2(3H)-Furanone	Grass	NF	NF	+	+
	trans-2-Octenal	Fatty	+	+	-	-
Aldehyde	5-Ethylcyclopentene-1-carbaldehyde	Fruity	NF	+	+	+
	Benzaldehyde	Fruity	NF	+	+	+
	2,4-Dimethylbenzaldehyde	Floral	NF	+	+	NF
	Pentanoic acid	Cheesy	+	+	-	-
Carboxylic acids	Octanoic acid	Cheesy	+	+	-	-
	Hexanoic acid	Cheesy	+	+	-	-
	Heptanoic acid	Cheesy	+	+	NF	NF

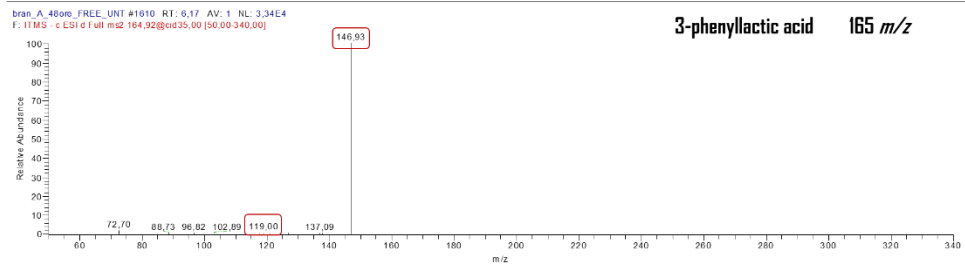
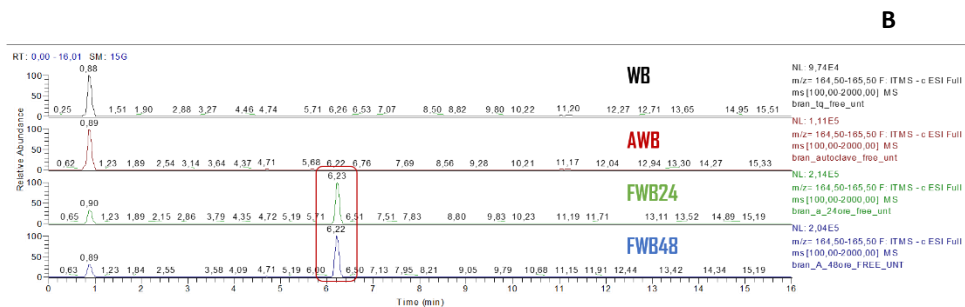
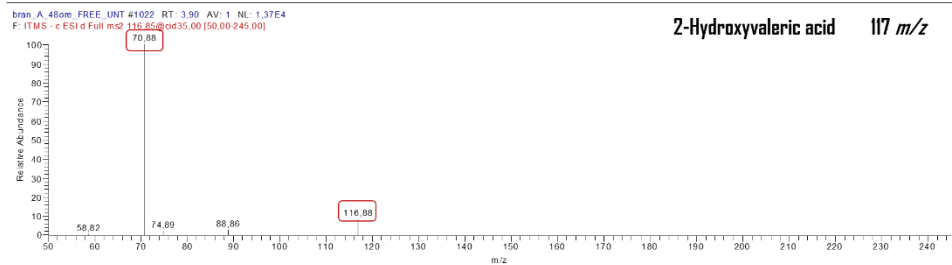
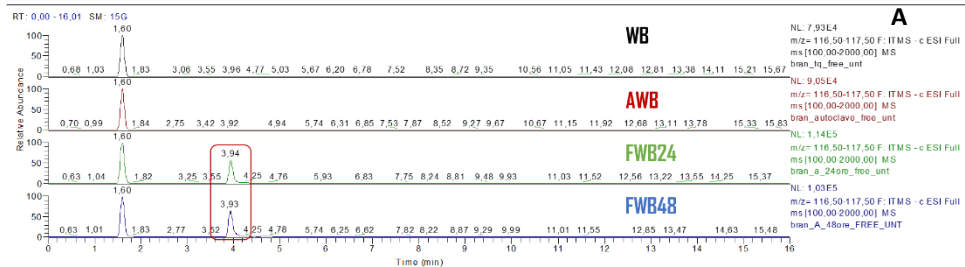


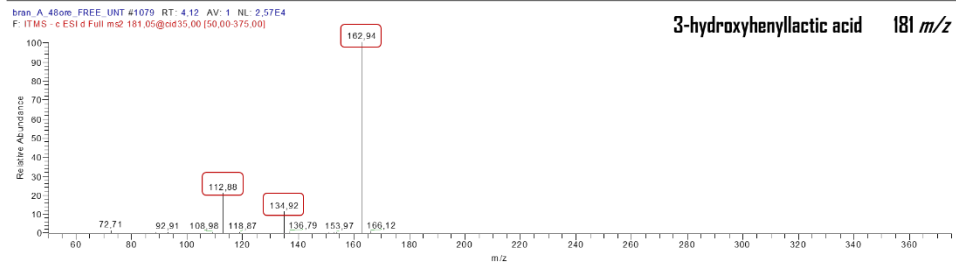
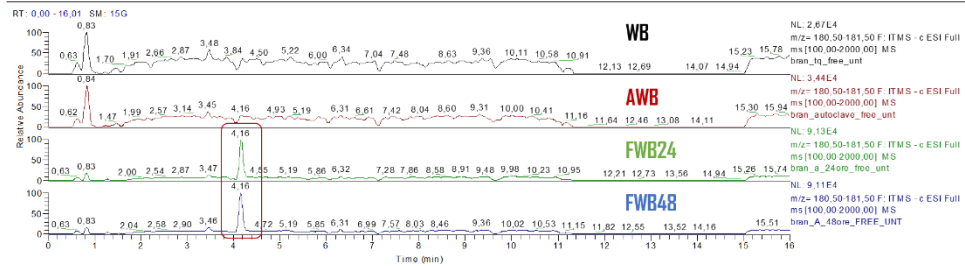
	2-Ethylfuran	Solvent, pungent	NF	+	+	+
Furan derivatives	2-Butylfuran	Fruity	NF	+	+	+
	cis-2-(2-Pentenyl) furan	Natural, floral	NF	+	+	NF
	2-(2-Pentenyl) furan	Fruity	NF	+	+	+
Esters	Ethyl Acetate	Fruity	+	+	NF	NF
	Acetic acid	Fruity	+	+	-	-

+, found in higher concentration; -, found in lower concentration; NF, not found. <sup>a</sup>Based on data reported in literature and information found at: <http://www.thegoodscentcompany.com/>, <sup>b</sup>, calculated on the basis of internal standard semi-quantification (see **Table A1**).

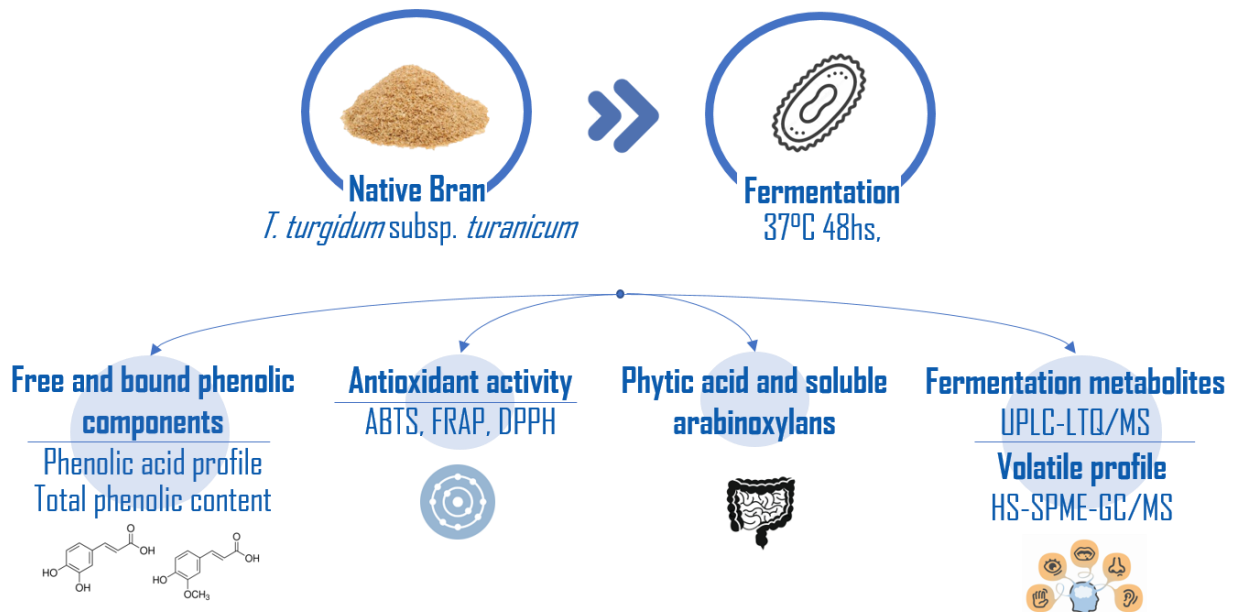
## Figure graphics

### Figure 1





## Graphic for table of contents



Parma, 23.08.2019

Dear Editor,

on behalf of my coauthors we are submitting the revised version of our manuscript coded, amended according to reviewers' comments.

Below you can find a detailed reply to reviewers.

We would like to thank them for the great job in improving the quality of our work.

Best Regards

Chiara Dall'Asta & Camilla Lazzi

#### REPLY TO REVIEWERS

Reviewer #1: In this article, solid-state fermentation (SSF) using a *Lactobacillus rhamnosus* was applied to wheat bran and its influence on bioactive compounds (free and bound phenolic acids) and their antioxidant activity were evaluated. This research had provided some implications for the application of wheat bran. Therefore, I would like to recommend the acceptance of this manuscript.

>> [We heartly thank the reviewer for appreciating our work.](#)

Reviewer #2: The manuscript deals with solid state lactic acid fermentation as a strategy to improve wheat bran functionality, using *Lactobacillus rhamnosus*, and analyzing its influence on bioactive compounds (free and bound phenolic acids) and their antioxidant activity. The aim of the paper is interesting and worth study. The technical quality, originality and experimental work of the manuscript are highly significant.

>> [We heartly thank the reviewer for appreciating our work.](#)

I recommend minor revisions:

1. Page 9, lines 199-203: Explain the experimental design and the number of repetitions.
2. Page 9, Line 201: The significance level is alfa ( $\alpha$ ). Use " $\alpha=0.05$ " instead of " $p<0.05$ ".
3. Page 10, line 214: Replace "increased" by "increase"
4. Page 10, lines 214-216: Unify decimals (number of significant figures) along the entire manuscript and tables.

>> [All the minor changes have been done](#)

#### 5. Tables

Correct tables according to the "Guide for authors": "...Include a short but informative title. Provide the experimental conditions, as far as they are necessary for understanding. The reader should not have to refer to the text in order to understand the tables. Place footnotes to tables below the table body and indicate them with superscript lowercase letters... If analytical data are reported, replicate analyses must have been carried out. State the number of replications and give standard error or other evidence of reliability of data..."

>> Table has been amended accordingly

Table 1. Caption: Explain each abbreviation: "TBC" and "TSC". Place abbreviations of each sample in parentheses (WB, AWB, FWB24, FWB48).

Unify decimals.

Footnote: Indicate the results presented and the number of repetitions, for example: means + - standard deviations (n = 3).

>> Table caption and footnote have been amended accordingly

Table 2. Caption: Explain abbreviations: chemical indicators (TPC, DPPH, ABTS, FRAP, etc.) and samples (WB, WB, FWB24, FWB48)

Footnote: "Biological replicates" and "experimental replicates" were not explained in Statistical analysis section. Explain "AOA" abbreviation.

>> Table caption and footnote have been amended accordingly

Table 3. Caption: Include abbreviations in parentheses.

Footnote: idem Table 2.

>> Table caption and footnote have been amended accordingly

Table 4. Caption: correct "...detected in fermented..."

Table 5. Caption: Include name of samples and abbreviations in parentheses.

>> Table captions have been amended accordingly

6. Figure 1. Complete figure caption: The figures also show the chromatograms of all samples (AW, AWD, FWB24 and FWB48). Include the name of samples, place abbreviations in parenthesis.

>> Figure caption has been amended accordingly