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

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Abstract

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

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

Abbreviation Used

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

1. Introduction

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

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

2. Material and Methods

2.1 Raw materials and chemicals

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

2.2 Fermentation process

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

2.4 Phenolic compounds profiling

2.4.1. Sample preparation for free and total phenolic compounds

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

2.4.2. UHPLC-ESI-MS/MS profiling

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

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

2.4.3 Total phenolic content (TPC)

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

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

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

2.5 Quantification of phytic acid (PA)

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

Ireland Limited, Bray, Ireland). Results were expressed as g of phytic acid per 100 g $^{-1}$ dry weight.

2.6 Quantification of water extractable arabinoxylans (WEAX)

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

2.7 Fermentation metabolites analysis with UPLC-ESI-LTQ/MS

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

2.8 Volatile profile HS-SPME-GC-MS

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

2.8 Statistical analysis

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

3.Results and Discussion

3.1 L. rhamnosus 1473 growth and pH assessment

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

endogenous microflora and to accurately evaluate the metabolic properties of *L. rhamnosus 1473*. Its growth ability was monitored after 48h revealing the increase in microbial cells number (10.42±0.10 Log CFU/g^{-1}) from the original inoculum (ca. 7 Log CFU/g^{-1}). A significant pH decrease (from 6.53±0.22 to 4.70±0.10) was also observed (**Table 1**).

3.2 Free, bound and total phenolic content and antioxidant activity

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

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

3.3 Phytic acid degradation

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

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

3.4 Arabinoxylans solubilisation

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

3.5 Free, bound and total phenolic acids profile

Phenolic acids are the most abundant bioactive compounds present in wheat bran, and more in general in cereal grains. They can occur in soluble or insoluble forms. Thermal processing and fermentation of wheat bran significantly modified the composition of this matrix as shown in **Table 3**. Overall, a decreasing of the free phenolic acids was measured after the sterilization step, while a slight increase of the insoluble component was obtained. However, the free phenolic acids content significantly increased when wheat bran was submitted to lactic acid fermentation, albeit no difference was found between the 24 and 48 hours of treatment. Nutritional improvement is not only related to the increased content of 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, & Ga¨nzle, 2015). In addition, being caffeic acid a strong inhibitor of lipid
295 peroxidation, as reported by the study of Khennouf 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***

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

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

3.7 Volatile profile of fermented wheat bran

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

4. Conclusion

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

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

Conflict of interest

None.

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Authors contribution

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

Supporting information description

Appendix A.

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534

535

Figure captions

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

Tables

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

Sample	***pH***	***TBC***	***TSC***
		Log CFU g^{-1}	
WB	6.41±0.06b	5.32±0.14	2.56±0.66
AWB	6.53±0.22b	7.78±0.22[a]	<Log $^{-1}$
FWB24	4.67±0.08a	-	-
FWB48	4.70±0.10a	10.42±0.10	<Log $^{-1}$

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

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.

Sample	***TPC*** mg GAE Kg^{-1}			***F/B*** [a]	***DPPH*** mm TEAC g^{-1}			***ABTS*** mm TEAC g^{-1}			***FRAP*** mm TEAC g^{-1}			***F/B*** [b]	***PA*** g 100 gr^{-1}	***WEAX*** mg g^{-1}
	Free	Bound	Tot		Free	Bound	Tot	Free	Bound	Tot	Free	Bound	Tot			
WB	1174.9±184.7b	2451.2±109.4b	4247.0±200.6b	0.48	3.6±0.1b	17.7±0.2a	20.0±0.2	10.2±0.2b	40.5±1.2a	50.7±1.1	11.0±0.4c	34.5±0.4a	48.9±0.6	0.27	2.7±0.2a	12.6±0.1d
AWB	1043.5±0.7c	3203.5±0.0a	4599.6±90.4a	0.33	2.2±0.0c	17.9±1.0a	21.5±0.6	10.6±0.2b	38.6±2.3a	49.2±2.4	11.6±0.8b	36.3±0.3a	45.5±0.3	0.26	2.6±0.0a	14.7±0.2c
FWB24	1447.2±178.1a	2343.8±315.0b	3791.1±241.8b	0.62	3.5±0.3b	15.2±2.1a	18.7±2.2	10.7±0.2b	40.2±2.7a	50.9±3.5	12.6±0.4b	33.6±6.5a	49.6±5.7	0.30	2.3±0.2b	22.7±2.9b
FWB48	1553.3±70.4a	2271.1±374.1b	3824.3±395.0b	0.68	4.0±0.2a	13.9±1.1b	17.8±1.1	12.0±0.8a	33.9±3.7b	45.9±1.8	19.2±3.8a	29.0±3.6a	48.1±1.0	0.46	1.7±0.1c	32.4±2.8a

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. [a] F/B: sum of free to sum of bound ratio. [b] 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.

Sample	*4-HB*		*p-C*		*Caff*		*t-Fer*		*Sin*		*Dif*	*F/B* [a]
	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound		
	µg g^{-1} d.w.											
WB	2.4±0.2 b	4.7±1.3 a	1.3±0.1 b	45.2±1.6c	<LOQ	<LOQ	31.0±1.4 b	3643.5±63.3a	7.1±1.1b	111.2±14.3 a	2324.7±38.3a	1,04
AWB	1.4±0.5c	6.0±0.8 a	0.8±0.4c	60.2±4.2 a	<LOQ	<LOQ	12.7±4.7c	3870.4±245.2 a	5.8±1.2c	135.1±16.3 a	2364.7±76.5a	0,54
FWB24	3.8±0.7 a	5.8±1.2 a	2.6±0.3 a	59.1±8.1 b	12.6±1.3 a	0.9±0.1 a	50.6±5.9 a	3786.0±562.3 a	12.7±1.8 a	81.6±13.8c	2489.0±339.9 a	2,10
FWB48	4.0±0.6 a	5.8±1.1 a	2.8±0.2 a	38.3±3.6 d	15.0±1.3 a	0.8±0.2 a	47.9±3.8 a	2922.3±281.0 b	12.3±2.6 a	57.4±9.9d	2394.7±458.1 a	2,71

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 µg g^{-1}. 4-HB: 4-hydroxybenzoic acid p-C: para-Coumaric, Caff: caffeic acid, t-Fer: trans-Ferulic acid, Dif: diferulates; acid; [a] 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]- (*m/z*)	Rt (min)	MS^2	Compound class	Ref
2-Hydroxyvaleric acid	117	3.9	71, 117	Amino acid degradation	(Kang, Price, Ashton, Tapsell, & Johnson, 2016; Koistinen et al., 2018)
3-Hydroxyphenyllactic acid	181	4.13	135, 163, 113		
3-Phenyllactic acid	165	6.2	119, 147		
Indole-3-lactic acid	204	6.55	158, 116, 142, 128		
Tetrahydroxy octadecenoic acid	345	9.04	327, 309	Fatty acid hydroxylation	
Trihydroxy octadecadienoic	327	9.13	309, 291, 239		
Trihydroxy octadecenoic acid	329	10.59	311, 293, 275, 211, 201, 171		
Dihydroxy-octadecadienoic acid	313	10.71	293, 275		
Dihydroxy-octadecenoic acid	313	10.83	295, 277, 183		

Identified based on MS^n data and retention time and their comparison with MS^n and data from reference sources. Tentatively identified based on MS^n 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.

Class	*Compound*	*Odour perception* [a]	*WB*	*AWB*	*FWB24*	*FWB48*
			Relative Abundance [b]			
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
	Cyclohexanol	Camphor 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	Sweety	NF	NF	NF	+
	4,4,6-Trimethylcyclohex-2-en-1-ol	Floral, balsamic	NF	NF	NF	+
Ketones	Acetoin	Sweet cream	+	+	NF	NF
	2-Heptanone	Cheesy	+	+	-	-
	3-Octanone	Green grass	+	+	-	-
	2-Octanone	Milky	+	+	-	-
	2-Nonanone	Fruity	+	+	-	NF

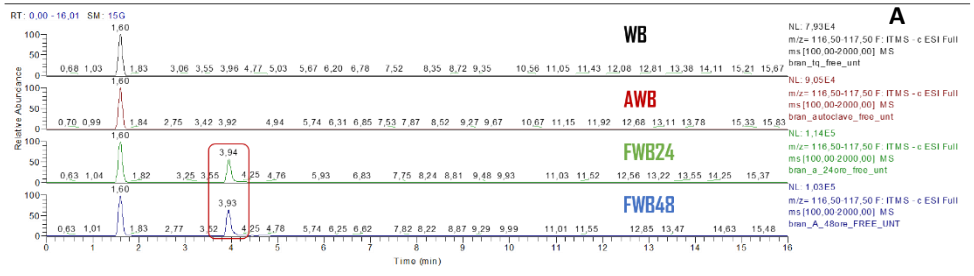
	6-Methyl-5-hepten-2-one	Fatty, green	+	-	NF	NF
	Camphor	Grass, woody	+	+	NF	NF
	5-Pentyloxolan-2-one	Floral	-	-	+	+
	3-Ethylcyclopentan-1-one	Vegetal, natural	NF	NF	+	+
	3-Octen-2-one	Melon	NF	NF	+	+
	2-Decanone	Floral	NF	NF	+	+
	2(3H)-Furanone	Grass	NF	NF	+	+
Aldehyde	trans-2-Octenal	Fatty	+	+	-	-
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

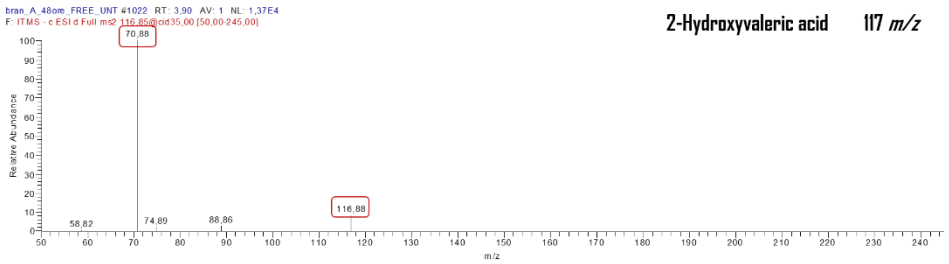
Furan derivatives	2-Ethylfuran	Solvent, pungent	NF	+	+	+
	2-Butylfuran	Fruity	NF	+	+	+
	cis-2-(2-Pentenyl) furan	Natural, floral	NF	+	+	NF
	2-(2-Pentenyl) furan	Fruity	NF	+	+	+
Esters	Ethyl Acetate	Fruity	+	+	NF	NF
	Acetic acid	Fruity	+	+	-	-

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

Figure graphics

Figure 1


A
WB
AWB
FWB24
FWB48
Time (min)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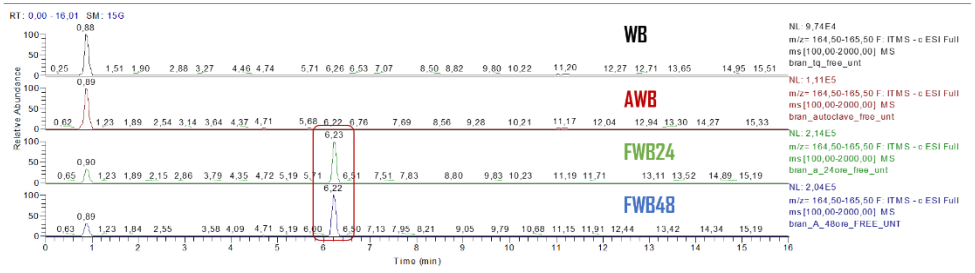
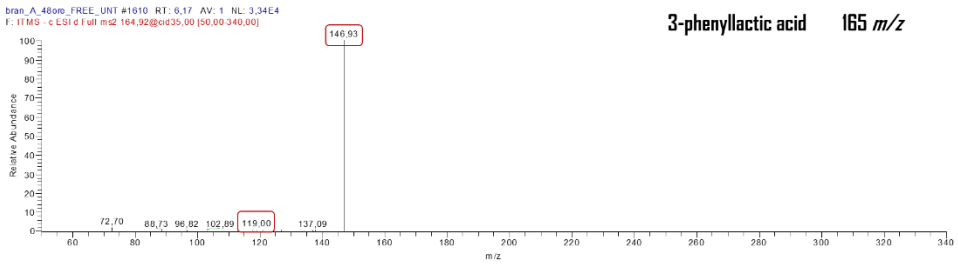
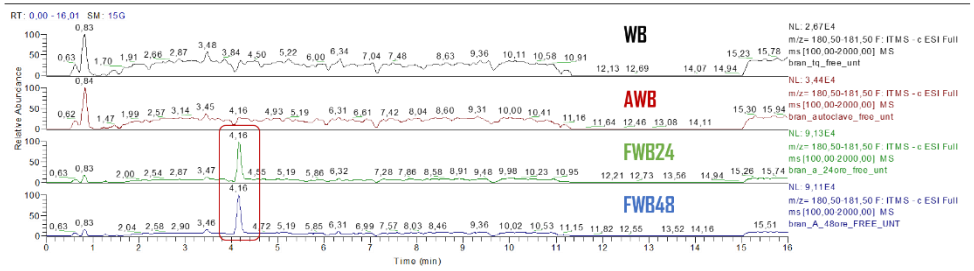
m/z

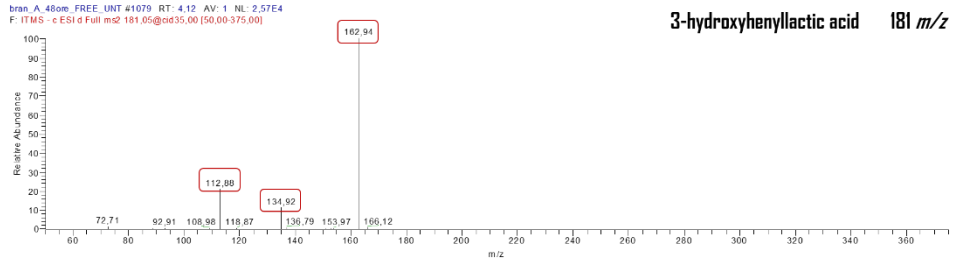
B


WB
AWB
FWB24
FWB48
Time (min)

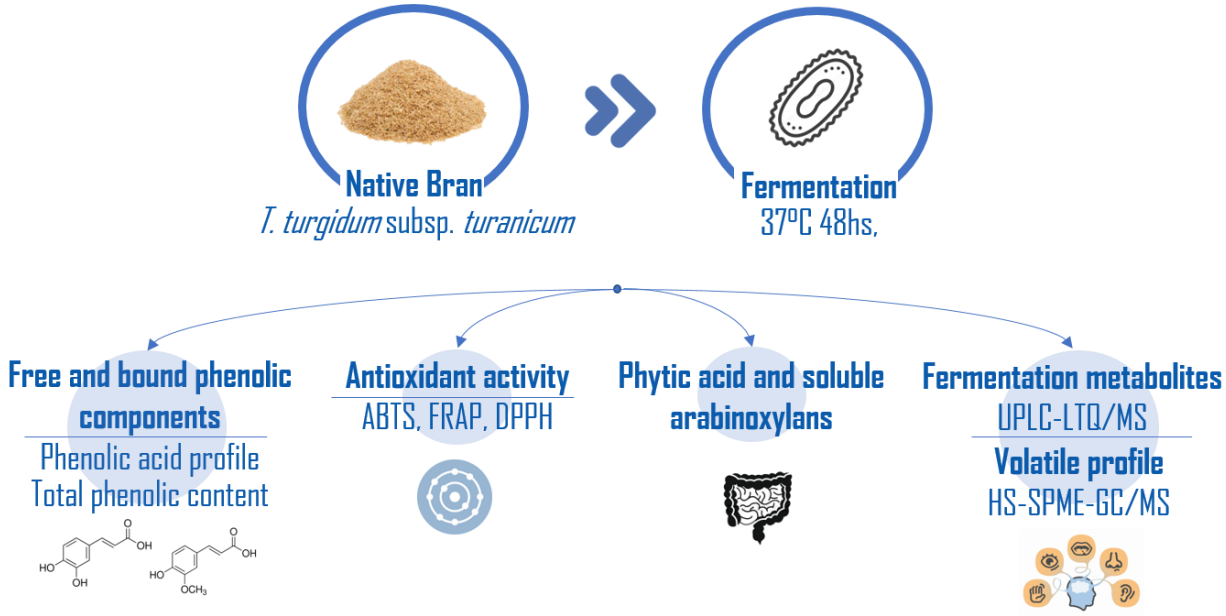

3-phenyllactic acid 165 m/z
m/z

C


WB
AWB
FWB24
FWB48
Relative Abundance
Time (min)


3-hydroxyhenyllactic acid 181 m/z
Relative Abundance
m/z

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