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Effect of fermentation with single and co-culture of lactic acid bacteria on okara: evaluation of bioactive compounds and volatile profile

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| 1  | Effect of fermentation with single and co-culture of lactic acid bacteria on okara: evaluation   |
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#### 27 Abstract

28 Okara is the main soybean by-product deriving from the processing of soy milk and tofu. Despite 29 being a product with a lot of potential, rich in many bioactive compounds such as polyphenols, it 30 presents an unpleasant, rancid aroma. For these reasons its use in food industry is limited. In this 31 study, we reported the integral use of okara in a solid state fermentation process, conducted with wild 32 strains of lactic acid bacteria, to evaluate the effect of bacterial metabolism on volatile and 33 polyphenolic profile. Strains belonging to Lactobacillus acidophilus, Lacticaseibacillus rhamnosus 34 and *Pediococcus acidilactici* species were used in mono-culture and, for the first time, in co-culture. 35 Results showed an improvement in the aromatic fraction showing a decrease of hexanal, responsible 36 of off-flavour, and an increase of ketones with fruity and buttery notes in fermented okara. 37 Polyphenols were also affected, and, in particular, a bioconversion of glucoside isoflavones to the 38 aglycone forms was highlighted in all fermented substrates. In addition, the appearance of both 39 phenyllactic and *p*-hydroxyphenyllactic acids as well as the increase of indole-3-lactic acids was 40 observed for the first time upon okara fermentation. Overall, the co-culture appears the most 41 promising for biovalorization of okara opening the possibility of its use in the development of 42 functional ingredients.

#### 43 Keywords:

44 Solid state fermentation, lactic acid bacteria, okara, by-product, waste, isoflavones, aroma.

#### 45 **1. Introduction**

Okara is the by-product resulting from the production of soy milk and tofu, after filtration of crushed soybeans. Following the increase in the demand for soy-based products in Europe and the habitual consumption in Asia, large quantities of okara are produced every year. The high production of okara currently represents a significant disposal problem for both industry and the environment, in fact each kilogram of dry soybeans generates about 1.1 kg of okara<sup>1</sup>. This byproduct is mainly used in the feed sector or discarded, although it is still rich in high quality

proteins, unsaturated fatty acids, dietary fiber, isoflavones, minerals and oligosaccharides<sup>2</sup>. The two main isoflavone glycosides, genistin and daidzin, are present in soybean in the form of  $\beta$ -Dglycoside. Some studies demonstrated that the corresponding aglycones genistein and daidzein, released by the action of  $\beta$ -glucosidase, exhibited higher biological activity and suggested that these aglycones can be better absorbed upon consumption, possibly because of the lower molecular weight and lower hydrophilicity<sup>3,4</sup>.

Some of the main drawbacks in the valorization of okara are its high degree of perishability, the presence of compounds with anti-nutritional effects and undesirable off-flavors and rancid aromas, caused by the oxidation of polyunsaturated lipids by the enzyme lipoxygenase, present in soybeans<sup>3</sup>.

62 Solid state fermentation (SSF) has been used, in last years, as a strategy to add value to okara. SSF 63 is defined as a bioprocess where microbial growth and product formation occur on the surface of 64 solid materials, almost in the absence of free water. Considering the limited amount of water and 65 the water activity values, only fungi and yeast should be suitable for this process, but also specific bacterial cultures can be employed, showing good performances<sup>5</sup> In this context, Lactic Acid 66 Bacteria (LAB), generally used as starter cultures to drive food fermentations, were recently used 67 for the SSF processes of waste and by-products<sup>6,7</sup>. Fermentation has recently been applied to 68 improve the flavor and texture of okara for food applications<sup>8</sup> but also to enhance the health 69 70 attributes, ideally through the production of functional ingredients<sup>9</sup>. Differently from these studies, 71 in this work we proposed the integral use of okara, without pre-treatments or additives, in a solid 72 state fermentation process conducted with LAB. As studies regarding the use of only probiotic strain of LAB<sup>10,11</sup> are present in the literature, we investigated the use of wild strains, isolated from 73 74 different niches, belonging to Lactobacillus acidophilus, Lacticaseibacillus rhamnosus and *Pediococcus acidilactici*. These species are reported to grow on soy and okara<sup>10,11</sup> or to reduce the 75 beany-flavor<sup>12,13</sup>. Considering that LAB-LAB co-cultures have not been widely studied, although 76 77 they seem advantageous compared to single cultures due to the synergistic action of the metabolic

pathways of the strains involved<sup>14</sup>, we carried out a comparison between mono and co-culture in
order to define the best conditions to improve phytochemical and aromatic features of okara.

#### 80 **2. Materials and methods**

81 2.1 Chemicals

Toluene used as reference for HS-SPME/GC-MS analyses was obtained from Sigma-Aldrich, USA. Phenyllactic acid, indole-3-lactic acid and genistein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Daidzein was from AASC Ltd. (Southampton, UK) while *p*-hydroxyphenyllactic acid from Santa Cruz Biotechnology (Dallas, TX, USA). Both HPLC-grade water and HPLC-grade acetonitrile were purchased from VWR International (Milan, Italy), as well as methanol and LC-MS grade formic acid.

88 2.2 Strains and cultures

89 Lacticaseibacillus rhamnosus 1473 from Parmigiano Reggiano cheese, reference strains of

90 Lactobacillus acidophilus LMG 8151, Pediococcus acidilactici 3992 from Grana Padano cheese

91 and a co-culture of these were used as starters for fermentation. 1473 and 3992 belong to the

92 collection of Food and Drug Department, University of Parma, while LMG 8151 was purchased

93 from BCCM (Belgian Co-ordinated Collections of Microorganisms) of Ghent University, Belgium.

94 All bacterial strains were maintained as frozen stocks (-80 °C) in Man Rogosa Sharpe (MRS)

95 medium (Oxoid, Milan, Italy) supplemented with 15% glycerol (w/v). Cultures were grown for one

- 96 week in MRS broth until their use for fermentation and incubated at 37 °C for 15 h.
- 97 2.3 Okara fermentation

98 The okara used for this work was provided by Sojasun company located in Fidenza (Parma, Italy)

99 and stored at -80 °C to avoid deterioration, due to the high activity of water. Before fermentation, the

100 substrate was autoclaved at 121 °C for 20 minutes.

101 The starter inoculum was prepared cultivating the revitalized strains until the late exponential phase

102 (ca. 15 h), harvesting the cell by centrifugation (12,857× g for 10 min at 4 °C), washing twice with

Ringer's solution (Oxoid, Milan, Italy), and finally re-suspending in sterile distilled water to a final concentration of 9.0 Log CFU/mL. Each culture was inoculated into 30 g of okara in order to reach 6 -7 Log CFU/g. The inoculum was homogenized in the sample by mixing for 2 minutes with a sterile loop. Co-culture was obtained by mixing single revitalized strains in equal volume and further diluting the mixture to reach 6-7 Log CFU/g in the product. The okara was fermented at 37 °C with all the strains and co-culture for 72 h.

Each fermentation was performed in duplicate. Samples were analyzed after inoculum ( $T_0$ ) and at the end of fermentation process ( $T_{72}$ ) by viable cell counts, carried out by plate count on MRS agar (Oxoid, Milan, Italy), incubating at 37 °C for 48 h.

112 2.4 Investigation of the volatile composition of fermented okara by HS-SPME/GC-MS technique 113 The volatile profile of fermented and unfermented okara, was analysed by HS-SPME/GC-MS 114 technique following the protocol reported by Ricci, Cirlini, et al.  $(2018)^{15}$  with slight modifications. 115 In particular, 3 g of okara and 10 µL of an aqueous toluene standard solution (100 µg/mL in 10 mL) 116 were used for the analyses.

117 GC-MS analyses were performed on a Thermo Scientific Trace 1300 gas chromatograph interfaced 118 with a Thermo Scientific ISO single quadrupole mass spectrometer, equipped with an electronic 119 impact (EI) source (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The column used for 120 the analytes separation, as GC and MS parameters, HS-SPME sampling conditions in terms of 121 equilibration and extraction time and temperature, and fiber type were the same described Ricci, Cirlini, et al. (2018)<sup>15</sup>. After the analyses, the gas-chromatographic detected signals were identified 122 123 by both the comparison of the obtained mass spectra with those present in the instrument library 124 (NIST-14), as by calculation of their linear retention indexes (LRIs). In addition, the semi-125 quantification of the identified compounds was performed on the basis of a reference (Toluene).

126 2.5 Extraction of polyphenolic compounds and LAB-derived metabolites

127 To carry out the extraction of polyphenols, fermented and unfermented samples were firstly freeze-128 dried with a lyophilizer (Lio 5PDGT, Cinquepascal, Italy). The samples were subjected to the 129 extraction of free polyphenols, in particular 3 mL of a methanol/water solution (80:20 v/v) acidified 130 with 0.2% of formic acid were added to 0.3 g of each sample. All samples were stirred for 1 min with 131 vortex and 10 min shaker then treated for 15 min in an ultrasonic bath, other 10 min in shaker and finally centrifuged for 25 min at 12,857× g, 4 °C. The supernatant was collected while the pellet 132 133 obtained was re-suspended in 0.3 mL of methanol-water solution and subjected to a second and 134 consequently to a third extraction, performed as described before. The supernatants were pooled and 135 then diluted with a solution of water/methanol (80:20 v/v) acidified with 0.1% formic acid, then 136 centrifuged at 14,462 g for 5 min before UHPLC-MS<sup>n</sup> analyses.

137 2.6 Determination of polyphenolic profile and LAB-derived metabolites through UHPLC MS<sup>n</sup>

138 The samples were analyzed with an Accela UHPLC 1250 interfaced with an ion trap mass 139 spectrometer (LTQ-MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with 140 a ESI interface (H-ESI-II). Chromatographic separation was carried out through an Acquity UPLC 141 HSS T3 column (2.1X 100 mm), 1.8 µm particle size equipped with an Acquity UPLC HSS T3 142 VanGuard pre-column (2.1 x 5 mm) was used (Water, Ireland). The chromatographic and mass spectrometer conditions were the same reported by Ricci, Cirlini, Calani, et al. (2019)<sup>16</sup>. Analyses of 143 144 okara samples were carried out in negative ionization mode using full-scan, data-dependant MS<sup>3</sup> 145 scanning from m/z 100 to 2000. Phenyllactic acid, indole-3-lactic acid, p-hydroxyphenyllactic acid, 146 genistein and daidzein aglycones were quantified with their authentic standard compounds by 147 extracting the corresponding deprotonated molecule ([M-H]<sup>-</sup>) in the full scan chromatograms. 148 Calibration curves of phenyllactic, indole-3-lactic and p-hydroxyphenyllactic acids ranged from 0.5 149 to 50 µmol/L, while calibration curves of both genistein and daidzein ranged from 0.05 to 20 µmol/L. 150 Instead, the O-glycosylated isoflavones, especially the O-acetylglycosides, showed a very high 151 fragmentation behavior in the negative ESI source, leading thus inadequate the monitoring of their

152 corresponding [M-H]<sup>-</sup> in order to avoid a loss of sensitivity. Thus, all O-glycosylated daidzein and 153 genistein at each retention time were quantified by extracting the corresponding ion of daidzein and genistein at m/z 253 and 269, respectively. Glycitein aglycone and glycitein-O-glycoside were 154 155 quantified as genistein equivalent by using the same approach reported for genistein and daidzein glycosides. The identification of compounds listed in the Table 3 was performed by comparison of 156 MS<sup>n</sup> ion spectra with the MS<sup>n</sup> data stored in several online libraries as: PubChem 157 158 (https://pubchem.ncbi.nlm.nih.gov/); mzCloud (www.mzcloud.org/home); Metlin 159 of (http://metlin.scripps.edu); MoNA Mass Bank North America (https://mona.fiehnlab.ucdavis.edu/). Additional MS<sup>n</sup> information was obtained through previous 160 works<sup>17,18</sup>. 161

162 2.7 Statistical analyses

The data obtained from the analysis of volatile and polyphenolic profile were analyzed using the analysis of variance (one way ANOVA) and significant differences among the means (p <0.05) were determined applying Tukey post hoc test using SPSS 21.0 software (SPSS Inc., Chicago, IL, USA) for different samples. Heat map was carried out using Heatmapper (www.heatmapper.ca)

167 while alluvial diagram using Rawgraph (<u>https://rawgraphs.io/</u>).

168

### 169 **3. Results and discussion**

170 3.1 Evaluation of lactic acid bacteria growth

SSF of okara was carried out by inoculating three different LAB strains, namely *L. acidophilus* (8151), *L. rhamnosus* (1473), *Pediococcus acidilactici* (3992), and their co-coculture, at the concentration of 6-7 Log CFU/g. The microbial growth ability was assessed immediately after inoculation (T<sub>0</sub>) and after 72 h of fermentation (T<sub>72</sub>) at 37 °C, by plate counting on MRS agar. Results (Figure 1) highlighted that not all the tested strains were able to grow. Differences in growth performance may be ascribed to the different adaptability of the strains in

177 stressful matrices, such as integral okara without pre-treatment and nutrient addition. Contrary to the

observation of Moraes et al. (2016)<sup>19</sup>, where okara was added only in low percentage to soymilk, the 178 179 strains of L. acidophilus used in this work did not show growth, probably due to the different composition of substrate and nutrients. According to Perreira et al. (2011)<sup>20</sup> the growth of 180 microorganisms depends on various factors, such as the substrate used and the strain employed 181 In agreement with Voss et al., (2018)<sup>10</sup>, *L. rhamnosus* shows the ability to grow in okara samples, 182 183 demonstrating a higher adaptability increase the microbial load of 1 Log cfu/g. Also in the case of 184 co-culture, an increase of bacterial concentration was observed, probably due to a synergistc effect 185 of strains. Interactions in LAB-LAB co-cultures in SSF process had never been studied, but the 186 knowledge acquired in food industry shows that metabolic interactions among bacteria can be 187 useful to modify the substrate. In particular, the use of co-cultures seems advantageous compared to the single culture, due to the synergistic action of the metabolic pathways of the strains involved<sup>14</sup>, 188 leading to increased degradation of the substrates<sup>21</sup>, with a consequent increase of peptides and 189 amino acids<sup>22</sup>, organic acids<sup>23</sup>, and volatile compounds<sup>24</sup>. 190

191 3.2 Volatile profile of fermented and unfermented okara

192 The characterization of the volatile composition of fermented and unfermented okara was performed 193 by HS-SPME/GC-MS technique. A total of 42 different compounds, belonging to different classes 194 (aldehydes, alcohols, ketones and furan compounds) were detected. The full identification of all 195 detected volatile compounds is reported in Table S1.

Significant differences between fermented and unfermented samples were recorded mainly for aldehydes. A high concentration of aldehydes was observed both in the control (1450.64±296.01  $\mu$ g/g) and in the sample inoculated with *L. acidophilus* (866.12±70.59  $\mu$ g/g), while a decrease was recorded in okara fermented with *P. acidilactici* (282.92±37.79  $\mu$ g/g), co-culture (68.28±5.86  $\mu$ g/g) and *L. rhamnosus* (25.29±5.66  $\mu$ g/g).

The compound responsible for these variations is mainly hexanal, with a persistent herbaceous aroma, generated by lipid oxidation<sup>25</sup>, resulting in the unpleasant smell of the soybean-based products. Notably, a significant decrease of its concentration was observed (Table 1) after 72 hours in *P*. 204 *acidilactici* fermented sample, while in *L. rhamnosus* and co-culture fermented samples this 205 compound was undetected. The decrease of aldehydes, and specifically of hexanal, upon fermentation 206 was previously reported with the use of different strains of yeast<sup>26</sup>. Among the most abundant 207 aldehydes present in unfermented sample, also benzaldehyde, with bitter almond notes, was detected 208 (114.58±51.76 µg/g). Although no significant differences were highlighted, this compound seemed 209 to decrease in fermented okara, as observed in Figure 2, contrary to what observed in other soy 210 products, in which benzaldehyde increase upon fermentation<sup>27,28</sup>.

A higher concentration of nonanal was found in the unfermented samples in comparison to the fermented ones. Nonanal is an aldehyde deriving from lipid degradation, that contributes to the beany aroma of legumes<sup>29</sup>. A significant difference was observed between the control ( $208.27\pm117.35 \mu g/g$ ) and fermented samples with co-culture and *L. rhamnosus* 1473, where nonanal was completely absent. The overall decrease of aldehydes, observed in all fermented samples, may be related to contemporary formation of alcohols, via reduction mechanisms during fermentation, as shown in Table 1.

Alcohols were the second major class in the volatile fraction of okara and the most abundant compound is 1-octen-3-ol (green and mushroom notes); its formation in soy has been attributed to enzymatic reactions in soaked soybeans, a pre-treatment for soy milk manufacture<sup>26</sup>. Although no significant differences among the samples were observed, SSF process with *P. acidilactici* and *L. rhamnosus* induced a decrease of about 50% of 1-octen-3-ol concentration. It is possible to hypothesize that the lower concentration of 1-octen-3-ol is associated with a lower enzymatic activity of the two species.

One of the main components of the aroma of soybean is 1-hexanol<sup>30</sup>. A statistically significant increase of this volatile was observed in samples fermented with *P. acidilactici* (217.53 $\pm$ 10.72 µg/g), *L. acidophilus* (206.74 $\pm$ 46.22 µg/g), and *L. rhamnosus* (216.96 $\pm$ 25.33 µg/g), while an opposite behavior was observed in okara fermented with co-culture (16.01 $\pm$ 3.58 µg/g). Stress conditions cause different cellular responses, depending on the strain which may translate into the formation of

secondary metabolites, such as aromatic compounds<sup>31</sup>. As the synthetic mechanisms for alcohol and other volatile compounds are strain specific, it is possible to hypothesize that, when strains are present as monoculture there is a reduction of unstable aldehydes and ketones to primary and secondary alcohols, while the synergic interaction between the strains in the coculture may instead lead to a production of higher levels of ketones, that could be correlated to the oxidation of alcohols.

235 After 72 hours of incubation, a significant increase of ketones (Figure 2) was recorded in the sample 236 fermented with co-culture, mainly ascribed to 2-nonanone and 2-heptanone (461.54±9.53 µg/g and 237  $1581.64\pm61.19 \,\mu\text{g/g}$ , respectively). This increase could be related to the combined metabolic activity 238 of the strains that leads to the degradation and metabolization of the substrates, thus increasing the 239 concentration of volatile compounds. Ketones flavor notes are generally described as desirable, and associated with sweet, fruity and creamy sensations<sup>4</sup>. In particular, 2-butanone-3-hydroxy (acetoin), 240 241 detected in sample fermented with co-culture and characterized by fatty butter taste, is widely used as flavor and fragrance in the food industry<sup>32</sup>. 242

Our results were in agreement with previous studies where an increase in ketones concentration was observed after fermentation of soy-based products with  $Bacillus^{26}$  and yeast<sup>28</sup>.

Solid state fermentation did not significantly affect the total concentration of furan compounds, which are present at high concentrations in the control sample although a general decreasing trend was observed in all fermented samples. This class is mainly represented by furan 2-pentyl, a product deriving from the oxidation of unsaturated fatty acids, often used as a food additive due its caramel notes. For this component, a decrease in concentration in all the fermented samples was observed.

250 Finally, the presence of two hydrocarbons was also observed, with no significant differences among

the analyzed samples.

252 3.3 Phytochemical profile and LAB-derived metabolites

253 The fermentation effect on non-volatile organic acids and polyphenolic compounds using different

254 LAB strains and co-culture towards okara-derived phytochemicals was evaluated through UHPLC-

MS<sup>n</sup>. At least 45 different compounds were identified, even some components specifically occurred in certain samples as a consequence of metabolic biotransformation by LAB strains (Table 2).

257 The first identification step allowed the subsequent quantification of the most abundant isoflavones

and some LAB-derived metabolites to unravel the putative role of LAB in the production of

259 bioactives upon okara fermentation (Table 3).

260 Unfermented okara contained several polyphenols, most notably isoflavones, which mainly

261 occurred as O-glycosides. Isoflavone-O-glycosides were converted by LAB  $\beta$ -glucosidases in their

aglycone forms. Indeed, in all fermented samples a decrease of glycosides and the consequent

263 increase of each respective aglycone was observed, in comparison to the control (Figure 3).

264 Notably, daidzein-O-glycosides were mainly converted into free daidzein in all fermented samples

265 with significant differences between LAB strains, reaching the highest concentration after

266 fermentation with *L. rhamnosus* (500.77±20.47 μg/g) and co-culture (520.49±27.12 μg/g). A similar

trend was observed for genistein, although fermented samples with co-culture strains showed a

significant higher concentration (532.60 $\pm$ 16.61  $\mu$ g/g) of this isoflavone with respect to samples

biotransformed by *L. rhamnosus* (494.79 $\pm$ 15.67 µg/g). Genistein and daidzein were by far the most

abundant isoflavones upon fermentation, while glycitein was barely recovered, contributing to ~ 1%

271 of the overall isoflavone aglycones upon SSF.

272 The deglycosylation of isoflavones was previously observed in okara and soy products after

273 fermentation with different monoculture of yeast and LAB<sup>3,33</sup>, but, the current study reveals the

high potential of LAB co-culture to convert isoflavones for the first time. A synergistic effect of co-

culture, corresponding to a high bioconversion of isoflavones, was observed in okara fermented

with fungi<sup>34</sup>.

277 The capability of both *L. rhamnosus* and co-culture to produce higher levels of aglycones could

278 represent a basis to investigate okara as a functional ingredient, given the putative better absorption

of aglyconic isoflavones in the upper gastrointestinal (GI) tract with respect to the corresponding

280 glycosides, even if some literature works didn't reach the same conclusion<sup>35–38</sup>. Results of these

studies are difficult to compare as different delivery forms of isoflavones, such as pure compounds, tablets or soy-based products, were investigated. However, focusing only on soy-based products, several human feeding studies highlighted the improvement of isoflavone bioavailability in the first GI tract upon soy fermentation as a result of the higher aglycone content than the unfermented counterparts<sup>36,37</sup>.

286 Although isoflavones were the main polyphenols in the okara samples investigated in the present 287 study, single and co-culture LAB strains similarly interacted with other minor flavonoids and 288 phenolic acids (Table S2). The flavanone naringenin was significantly higher (as chromatographic 289 area) in both L. rhamnosus and co-culture okara samples with respect to the other fermentations, 290 whereas all three *O*-glycosylated isomers of naringenin dropped upon SSF, two of these reaching 291 non detectable levels in the okara fermented with co-culture strains. Besides glycosylated 292 flavonoids, unfermented okara contained phenolic acids such as vanillic and syringic acids, both in 293 O-glycosidic form, which significantly decreased after SSF only in L. rhamnosus and co-culture 294 strains (Table S2).

295 Besides the increase of aglycone isoflavone and naringenin, released through LAB-mediated 296 deglycosylation, the SSF of okara led to the formation of LAB-derived smaller phenolic metabolites 297 such as indol-3-lactic, phenyllactic and *p*-hydroxyphenyllactic acids, which were almost completely 298 absent in the unfermented samples. The capacity of LAB to produce phenyllactic acid during fermentation had been previously reported, in particular for *L. rhamnosus* 1473<sup>16</sup>, and in the current 299 300 study these compounds reached the highest concentration after co-culture fermentation, i.e. 301 191.79 $\pm$ 15.05 for phenyllactic acid and 133.95 $\pm$ 4.42 µg/g for *p*-hydroxyphenyllactic acid. Their 302 production may be ascribed to the metabolism of amino acids by LAB. In particular, the former is 303 produced from the metabolism of phenylalanine, while the latter from tyrosine metabolism. The 304 recovery of phenyllactic acid in fermented okara could be interesting, since previous studies have 305 shown that this phenolic acid has antimicrobial activity against both Gram-positive and Gram-306 negative bacteria, and inhibitory activity against a wide range of fungi, isolated from baked goods,

307 flours and cereals, including some mycotoxigenic species. Many strains of the *Lacticaseibacillus* 308 genus are able to produce phenyllactic and *p*-hydroxyphenyllactic acids, which contribute to 309 preserving the quality of food, maintaining the sensorial characteristics typical of fermented 310 products<sup>39,40</sup>. The presence of these compounds could be useful in the case of okara, which presents 311 itself as an easily perishable and microbiologically unstable material.

312 The SSF led to a further increase of another LAB-derived metabolite, namely indole-3-lactic acid, produced *via* tryptophan catabolism<sup>41</sup>. This catabolite can be produced by yeasts and bacterial 313 314 species, and is able to inhibit the growth of Gram positive and Gram negative bacteria<sup>42</sup>, as well as by acting as antifungal compound against *Penicillium* strains<sup>43</sup>. Indole-3-lactic acid reached the 315 316 highest concentration in the okara fermented with co-culture ( $84.91\pm4.89 \mu g/g$ ), displaying 317 significant differences when compared to single LAB strains and control (Table 3). Accordingly, an 318 opposite trend was observed for the precursor tryptophan, which showed a significant prominent 319 drop (as chromatographic peak area) in the co-culture fermented samples (Table S2). Several 320 promising studies have highlighted the putative bioactivity of indole-3-lactic acid through in vitro and *in vivo* experiments<sup>41</sup>. Some *in vitro* experiments showed that indole-3-lactic acid is able to 321 reduce the inflammation<sup>44,45</sup> and this behavior was partially confirmed in human studies. Moreover, 322 323 circulating indole-3-lactic acid was significantly lower in plasma of obese subjects than in nonobese ones, and was paralleled by lower serum levels of inflammatory markers<sup>46</sup>. The putative anti-324 325 inflammatory activity elicited by indole-3-lactic acid was further supported in an intervention study, 326 as its plasma levels significantly increased in humans that followed a Mediterranean diet of four days when compared to a control fast food diet in a crossover design<sup>47</sup>. 327 328 Another amino acid-derived metabolite, specifically from leucine, namely 2-hydroxyisocaproic acid

329 (leucic acid), was undetectable in the control sample while it was recovered in all fermented okara

330 samples, even if differences emerged between LAB strains. In detail, okara fermented with *L*.

331 *acidophilus* showed the highest recovery of this  $\alpha$ -hydroxy acid after SSF with co-culture (Table

332 S2), in agreement with previous works on leucic acid production by different LAB strains<sup>17,48</sup>. The

ability to biotransform compounds presents in substrates even in absence of replication, like that
 occurred with *L. acidophilus*, was recently reported<sup>49</sup>.

#### 335 **4. Conclusions**

The present study explored the use of LAB to ferment okara, ÷ we carried out a comparison between mono and co-culture in order to define the best conditions to improve phytochemical and aromatic features of okara. T the metabolic activity of LAB resulted in fermented final products with different chemical composition and biological activity. Although the bioprocess occurred especially at a high replication rate, also in the case of non-multiplying bacterial cell an increase of specific metabolites was observed.

342 Exploring different strains and their combinations, the co-culture containing *L. acidophilus*, *L.* 

343 *rhamnosus* and *P. acidilactici* was the best starter candidate due to its ability to significantly modify

344 the aromatic and polyphenolic profile of raw material. Besides the optimal growth performance, a

345 decrease of off-flavor (hexanal, nonanal) and a large conversion of isoflavones in their aglycone

346 forms were obtained. Moreover, a notable production of LAB-derived metabolites such as indol-3-

347 lactic, phenyllactic and *p*-hydroxyphenyllactic acids, that can exert a human biological activity or

348 antimicrobial activity, was observed.

349 On the basis of the obtained results, solid state fermentation may represent an innovative strategy

350 for the reuse of okara with the final goal of the recovery of possible functional ingredients.

#### 351 Abbreviations

352 ANOVA, analysis of variance; BCCM, Belgian Co-ordinated Collections of Microorganisms; CFU,

353 colony-forming unit; EI, electronic impact; GI, upper gastrointestinal tract; HPLC, high

354 performance liquid chromatography; HS-SPME/GC-MS, headspace solid phase microextraction

and gas chromatography-mass spectrometry; LAB lactic acid bacteria; LC-MS, liquid

356 chromatography-mass spectrometry; LRIs, linear retention indexes; MRS, Man Rogosa Sharpe; SD,

357 standard deviation; ND, not detected; SD, standard deviation; RT, retention time; SSF, solid state

358 fermentation; UHPLC-MS<sup>n</sup>, ultrahigh-performance liquid chromatography-mass spectrometry;

359 UPLC, ultra-performance liquid chromatography.

360 Author Contributions: Conceptualization, Camilla Lazzi; Data curation, Jasmine Hadj Saadoun,

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- 366 Martina Cirlini, Valentina Bernini, Erasmo Neviani, Daniele Del Rio, Gianni Galaverna and
- 367 Camilla Lazzi.

#### 368 **Conflict of Interest**

369 There are no conflicts to declare.

### 370 Electronic Supplementary Information

371 Electronic Supplementary Information (ESI) available: Assignment of GC-MS signals (Table S1),
372 Chromatographic area of minor components in unfermented and fermented okara samples analyzed
373 through UHPLC MS<sup>n</sup> (Table S2).

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# **Table 1.** Concentration ( $\mu g/g$ ) of volatile compounds found in unfermented (control) and fermented

542 okara with different strains for 72 hours

|                            | Cortal                      | L. acidophilus P. acidilactici |                           | L. rhamnosus             | Co. an literation       |
|----------------------------|-----------------------------|--------------------------------|---------------------------|--------------------------|-------------------------|
|                            | Control                     | 8151                           | 3992                      | 1473                     | Co-culture              |
| Aldehydes                  |                             |                                |                           |                          |                         |
| Pentanal                   | 46.37±28.73ª                | 29.08±5.93ª                    | 9.84±2.01ª                | ND                       | ND                      |
| Hexanal                    | 494.83±8.57ª                | 492.95±103.37ª                 | 147.64±38.95 <sup>b</sup> | ND                       | ND                      |
| Heptanal                   | 139.49±48.09ª               | 76.79±31.43 <sup>a,b</sup>     | 27.37±4.06 <sup>b</sup>   | ND                       | ND                      |
| Octanal                    | 124.31±66.77 <sup>a</sup>   | 35.18±2.96 ª                   | 19.66±3.89 °              | ND                       | ND                      |
| 2-Heptenal                 | 127.74±10.71ª               | 15.33±1.70 <sup>b</sup>        | 6.35±3.39 <sup>b</sup>    | ND                       | 9.21±1.10 <sup>b</sup>  |
| Nonanal                    | 208.27±117.35ª              | 49.64±11.51ª                   | 17.47±1.52 <sup>a</sup>   | ND                       | ND                      |
| 2-Octenal (E)              | 95.87±46.06ª                | 19.33±2.25 <sup>b</sup>        | 8.84±1.93 <sup>b</sup>    | ND                       | 12.91±3.88 <sup>b</sup> |
| Furfural                   | 23.66±7.87ª                 | 3.73±1.46 <sup>b</sup>         | ND                        | ND                       | ND                      |
| Decanal                    | 46.73±21.40 <sup>a</sup>    | 15.74±2.56ª                    | ND                        | ND                       | ND                      |
| Benzaldehyde               | 114.58±51.76 <sup>a</sup>   | 109.88±18.37 <sup>a</sup>      | 44.96±4.09 <sup>a</sup>   | 22.44±4.50 <sup>a</sup>  | 46.16±3.31 <sup>a</sup> |
| Dodecanal                  | 5.83±2.98ª                  | ND                             | ND                        | ND                       | ND                      |
| Benzaldehyde, 4-ethyl      | 9.95±3.52ª                  | 5.80±0.25 <sup>a</sup>         | 1.80±0.11 <sup>b</sup>    | ND                       | ND                      |
| 2,4-Decadienal             | 13.01±4.83ª                 | 12.67±1.62 <sup>a</sup>        | 4.62±1.46 <sup>b</sup>    | ND                       | ND                      |
| Benzaldehyde, 2,5-dimethyl | ND                          | ND                             | ND                        | 4.09±1.75 <sup>a</sup>   | ND                      |
| Total                      | 1450.64±296.01ª             | 866.12±70.59 <sup>a</sup>      | 282.92±37.79 <sup>b</sup> | 25.29±5.66 <sup>b</sup>  | 68.28±5.86 <sup>b</sup> |
|                            |                             |                                |                           |                          |                         |
| Alcohols                   |                             |                                |                           |                          |                         |
| Propan-2-ol                | 131.81±10.15 <sup>a,b</sup> | 235.48±93.96 <sup>a</sup>      | 86.83±7.90 <sup>a,b</sup> | 37.29±11.86 <sup>b</sup> | $65.44 \pm 20.80^{b}$   |
| Ethanol                    | 44.03±42.39ª                | 57.44±13.89 <sup>a</sup>       | 20.38±2.75ª               | 13.76±1.37ª              | 19.62±5.50ª             |
| 1-Butanol,3-methyl         | ND                          | 7.64±2.29 <sup>a</sup>         | $9.81\pm0.68^{a}$         | 2.09±0.15 <sup>b</sup>   | ND                      |
| 1-Pentanol                 | 90.17 ±50.94ª               | 42.71±15.78ª                   | 18.90±1.55ª               | 42.33±3.58ª              | ND                      |
| 1-Hexanol                  | 64.21±27.45 <sup>b</sup>    | 206.74±46.22ª                  | 217.53±10.72ª             | 216.96±25.33ª            | 16.01±3.58 <sup>b</sup> |
| 3-Octanol                  | ND                          | ND                             | 6.88±2.33ª                | 8.08±0.01ª               | ND                      |
| 1-Octen-3-ol               | 235.68±124.19ª              | 225.55±67.58 <sup>a</sup>      | 107.51±1.73 <sup>a</sup>  | 101.03±1.89 <sup>a</sup> | 225.51±20.94ª           |

| 1-Heptanol                | 33.33±16.08 <sup>a</sup>   | 19.81±2.25ª                | 15.56±1.34ª               | 12.48±2.10ª               | ND                         |
|---------------------------|----------------------------|----------------------------|---------------------------|---------------------------|----------------------------|
| 4-Ethylcyclohexanol       | 64.03±25.17 <sup>a</sup>   | 0.84±0.34°                 | 5.21±0.87 <sup>b</sup>    | 8.92±2.53 <sup>b</sup>    | 8.50±0.66 <sup>b</sup>     |
| 1-Octanol                 | 29.98±7.43 <sup>a</sup>    | 34.67±7.54 <sup>a</sup>    | 17.81±3.64 <sup>a,b</sup> | 11.67±3.90 <sup>b</sup>   | 1.68±0.01 <sup>b</sup>     |
| 2-Octen-1-ol              | $3.43 \pm 2.72^{b}$        | 13.41±1.85 <sup>a</sup>    | 3.60±0.62 <sup>b</sup>    | 3.15±0.99 <sup>b</sup>    | 5.86±0.67 <sup>b</sup>     |
| Benzyl Alcohol            | 5.57±1.97 <sup>a</sup>     | 8.01±3.21ª                 | 5.26±1.70 <sup>a</sup>    | 4.96±0.20 <sup>a</sup>    | 6.27±1.56 <sup>a</sup>     |
| Phenylethyl Alcohol       | 1.11±1.09 <sup>a</sup>     | 1.82±0.77 <sup>a</sup>     | ND                        | 1.66±0.93ª                | 2.06±0.73ª                 |
| Total                     | 666.46±167.67 <sup>a</sup> | 853.95±178.20 <sup>a</sup> | 636.94±115.19ª            | 464.38±36.57 <sup>a</sup> | 365.63±4.67 <sup>a</sup>   |
| Ketones                   |                            |                            |                           |                           |                            |
| Acetone                   | 45.09±20.99 <sup>a,b</sup> | 48.17±12.86 <sup>a,b</sup> | 18.75±0.09 <sup>b</sup>   | 20.99±0.26 <sup>b</sup>   | 84.39±4.69 <sup>a</sup>    |
| 2-Butanone                | 1.35±0.16 <sup>a</sup>     | $0.95 \pm 0.48^{a}$        | ND                        | ND                        | ND                         |
| 2-Heptanone               | 49.53±0.30 <sup>b</sup>    | 29.55±5.56 <sup>b</sup>    | 27.08±1.57 <sup>b</sup>   | 36.21±1.15 <sup>b</sup>   | 1581.64±61.19 <sup>a</sup> |
| 2-Octanone                | 1.49±1.18 <sup>a</sup>     | 1.54±0.61ª                 | 1.16±0.61ª                | 1.14±0.25ª                | 3.81±0.99ª                 |
| 2-Butanone-3-hydroxy      | ND                         | ND                         | ND                        | ND                        | 166.30±3.62ª               |
| 2-Nonanone                | ND                         | ND                         | ND                        | ND                        | 461.54±9.53ª               |
| 3-Octen-2-one             | 13.93±2.88ª                | $8.52{\pm}1.16^{a,b}$      | 5.01±2.11 <sup>b</sup>    | $2.31 \pm 0.58^{b}$       | 18.70±0.97ª                |
| 3,5-Octadien-2-one        | 4.72±2.60 <sup>a</sup>     | 10.07±5.76 <sup>a</sup>    | 5.84±1.39ª                | 3.61±0.10 <sup>a</sup>    | 11.05±2.80 <sup>a</sup>    |
| 2-Undecanone              | ND                         | ND                         | ND                        | ND                        | 28.18±2.94 <sup>a</sup>    |
| Total                     | 116.10±19.46 <sup>b</sup>  | 98.81±18.68 <sup>b</sup>   | 57.84±1.00 <sup>b</sup>   | 64.26±0.03 <sup>b</sup>   | 2355.62±46.69ª             |
| Furanic compounds         |                            |                            |                           |                           |                            |
| Furan, 2-ethyl            | 13.63±0.48 <sup>a</sup>    | 41.62±12.71ª               | 20.02±2.09 <sup>a</sup>   | 37.01±8.01ª               | 27.33±0.74 <sup>a</sup>    |
| 2-n-Buthyl furan          | 6.37±2.68 <sup>b</sup>     | 16.55±3.54 <sup>a</sup>    | 7.19±0.20 <sup>b</sup>    | $5.66 \pm 1.25^{b}$       | $8.67 \pm 0.08^{b}$        |
| Furan, 2-pentyl           | 802.27±302.31ª             | 461.91±1.64 <sup>a</sup>   | 315.37±7.84 <sup>a</sup>  | $352.87{\pm}5.05^{a}$     | 435.61±73.73 <sup>a</sup>  |
| Furan, 2-(1-pentenyl)-(E) | 27.85±5.85 <sup>a</sup>    | 20.50±1.31ª                | ND                        | 5.76±0.46 <sup>b</sup>    | 21.23±2.85 <sup>a</sup>    |
| Total                     | 848.05±218.07 <sup>a</sup> | 540.58±11.26 <sup>a</sup>  | 342.59±7.17 <sup>a</sup>  | 401.29±1.53ª              | 483.28±61.79 <sup>a</sup>  |
| Other                     |                            |                            |                           |                           |                            |
| Heptane                   | 27.06±4.62ª                | 8.97±3.91 <sup>b</sup>     | 6.48±0.23 <sup>b</sup>    | 28.38±7.42 ª              | 27.82±0.96 <sup>a</sup>    |
| Octane                    | 41.95±27.62 <sup>a</sup>   | 7.67±1.31ª                 | 10.94±3.43ª               | 17.22±3.26 <sup>a</sup>   | 26.71±1.17 <sup>a</sup>    |
| Total                     | 69.01±22.80 <sup>a</sup>   | 16.64±3.69 <sup>a</sup>    | 17.42±2.26 <sup>a</sup>   | 45.61±2.95 <sup>a</sup>   | 54.54±1.51ª                |

543 Data are expressed as mean  $\pm$  standard deviation. Different letters indicate significantly different values (p<0.05); ND: not detected

# **Table 2.** UHPLC-MS<sup>n</sup> characteristics of compounds detected in unfermented and fermented okara

| Compound                               | RT   | [M-H]- (m/z) | MS <sup>2</sup> ions (m/z)                      | MS <sup>3</sup> ions (m/z)                               |
|--|------|--------------|---|--|
| Succinic acid                          | 1.57 | 117          | 73, 99  |  |
| 2-Hydroxyvaleric acid or               | 2.04 | 117          | 71  |  |
| 2-Hydroxyisovaleric acid               | 3.94 | 11/          | /1  |  |
| Hydroxycaproic acid isomer             | 5.50 | 131          | 85  |  |
| 2-Hydroxyisocaproic acid (Leucic acid) | 5.60 | 131          | <b>85</b> , 87, 113, 59                         | 69   |
| Malic acid                             | 0.99 | 133          | 115   | 71   |
| 2-Hydroxy-4-(methylthio)butyric acid   | 3.94 | 149          | 101, 103, 107                                   |  |
| <i>p</i> -Coumaric acid                | 6.00 | 163          | 119   |  |
| Phenylalanine                          | 2.87 | 164          |   |  |
| Phenyllactic acid                      | 6.22 | 165          | 147, 119  |  |
| Tyrosine                               | 1.48 | 180          | <b>163</b> , 119, 136                           | 119  |
| <i>p</i> -Hydroxyphenyllactic acid     | 4.16 | 181          | 163, 135, 113                                   |  |
| Azelaic acid                           | 7.11 | 187          | <b>125</b> , 169, 97                            | 97, 105, 83  |
| Citric acid                            | 1.32 | 191          | 111, 173, 129, 87                               |  |
| Tryptophan                             | 4.10 | 203          | 159, 116, 142, 173, 129                         |  |
| Indole-3-lactic acid                   | 6.54 | 204          | <b>186</b> , 158, 142, 160, 116                 | 142, 158, 116, 130                                       |
| Pantothenic acid                       | 3.26 | 218          | <b>88</b> , 146                                 | 59   |
| Doidzoin                               | 7 05 | 252          | 209, 197, 224, 225, 226, 169,                   |  |
| Daiuzem                                | 7.85 | 235          | 182, 195, 145                                   |  |
| Conjetein                              | 0.10 | 2(0          | <b>225</b> , 224, 201, 241, 181, 197,           | 191 192 196 105 107 109                                  |
| Genistem                               | 9.10 | 209          | 199, 213, 169, 133, 159, 107                    | 181, 182, 180, 193, 197, 198                             |
| Naringenin                             | 9.00 | 271          | 151, 177, 125, 107, 165                         |  |
| Glycitein                              | 8.10 | 283          | 268   | 240  |
| Kaempferol                             | 9.18 | 285          | 241, 239, 189, 257                              |  |
| Vanillic acid-O-hexoside               | 3.42 | 329          | <b>167</b> , 123, 209                           | 152, 123, 108  |
| Syringic acid-O-hexoside               | 3.80 | 359          | <b>197</b> , 182                                | 182, 153, 138  |
| Daidzein-O-hexoside                    | 5.57 | 415          | <b>253</b> , 295                                | 209, 225, 180, 212, 208,<br>207, 196                     |
| Genistein-O-hexoside                   | 6.37 | 431          | <b>269</b> , 268, 311                           | 224, 201, 241, 225, 240,<br>226, 213, 180, 160, 157, 123 |
| Naringanin-O-havasida I                | 5 80 | 133          | 271   | 151 177  |
| Naringenin-O-hexoside II               | 6.78 | 433          | 271   | 151, 177   |
| Naringenin-O-hexoside III              | 7 30 | 433          | <b>271</b> 313                                  | 151,177  |
| Glycitein- <i>O</i> -hexoside          | 5 70 | 445          | 283   | 268  |
| Kaempferol- <i>Q</i> -hexoside         | 6.60 | 447          | <b>285</b> 327 363 256 241                      | 241 257 213 167 151 256                                  |
| Dihydrokaempferol- <i>O</i> -heyoside  | 5.00 | 449          | <b>287</b> 269 259                              | 259 243 269  |
| Daidzein- <i>O</i> -acetylbevoside I   | 6.20 | 457          | 397 253   | 239, 213, 209  |
| Daidzein-O-acetylhexoside II           | 6.30 | 457          | 253   |  |
| Daidzein- <i>O</i> -acetylhevoside III | 6 40 | 457          | 253   |  |
| Daidzein- <i>O</i> -acetylhexoside IV  | 6 94 | 457          | <b>253</b> 252 295 397                          | 224, 225, 197, 209, 208, 135                             |
| Genistein-O-acetylhexoside I           | 7.03 | 473          | 269   | <i>221, 223, 171, 207, 200, 133</i>                      |
| Genistein-O-acetylhexoside II          | 7.12 | 473          | <b>4</b> 73 <b>269</b> , 413 225, 240, 227, 181 |  |
|  |      |              | ,   | 227, 225, 224, 251, 250.                                 |
| nistein-O-acetylhexoside III           | 7.83 | 473          | <b>269</b> , 268, 311                           | 241, 133   |

|              | Clycitain Q-acatylhayosida           | 6.41 | 187 | 283              | 268                                  |
|--------------|--------------------------------------|------|-----|------------------|--------------------------------------|
|              | Naringenin- <i>O</i> -acetylhexoside | 7 35 | 487 | 283              | 151 177 107                          |
|              | Pinoresinol- <i>O</i> -hexoside      | 6.37 | 519 | <b>357</b> , 475 | 151, 136, 327, 295, 311              |
|              | Kaempferol-O-dihexoside              | 5.74 | 609 | <b>285</b> , 429 | 257, 213, 151, 229, 241,<br>197, 200 |
|              | Daidzein-O-hexoside derivative       | 4.35 | 623 | 415              | 253, 295                             |
|              | Genistein-O-hexoside derivative      | 4.78 | 639 | <b>431</b> , 593 | 268, 269                             |
| 549          |                                      |      |     |                  |                                      |
|              |                                      |      |     |                  |                                      |
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| 558          |                                      |      |     |                  |                                      |
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| 561          |                                      |      |     |                  |                                      |
| 501          |                                      |      |     |                  |                                      |
| 562          |                                      |      |     |                  |                                      |
| 5.00         |                                      |      |     |                  |                                      |
| 563          |                                      |      |     |                  |                                      |
| 564          |                                      |      |     |                  |                                      |
|              |                                      |      |     |                  |                                      |
| 565          |                                      |      |     |                  |                                      |
| 566          |                                      |      |     |                  |                                      |
| 500          |                                      |      |     |                  |                                      |
| 567          |                                      |      |     |                  |                                      |
| <b>5</b> < 0 |                                      |      |     |                  |                                      |
| 268          |                                      |      |     |                  |                                      |
| 569          |                                      |      |     |                  |                                      |
|              |                                      |      |     |                  |                                      |
| 570          |                                      |      |     |                  |                                      |

- **Table 3.** Concentration  $(\mu g/g)$  of isoflavones and main LAB-derived metabolites recorded in 572 unfermented (control) and fermented okara with different strains after 72 hours.

|  |                          |                           | Okara sample                    |                           |                           |
|--|--------------------------|---------------------------|---------------------------------|---------------------------|---------------------------|
| Compound                               | Control                  | L. acidophilus<br>8151    | L. rhamnosus<br>1473            | P. acidilactici<br>3992   | Co-culture                |
| <i>p</i> -Hydroxy-phenyllactic acid    | ND                       | $48.54 \pm 3.58^{b}$      | 30.82 ±1.93 <sup>c</sup>        | 37.98±4.17 <sup>c</sup>   | 133.95±4.42 <sup>a</sup>  |
| Phenyllactic acid                      | ND                       | 96.24±9.10 <sup>b</sup>   | 32.58±2.40°                     | 48.83±2.81 <sup>c</sup>   | 191.79±15.05 <sup>a</sup> |
| Indole-3-lactic acid                   | $9.99 \pm 1.53^{d}$      | 30.65±1.60 <sup>b</sup>   | 18.65±0.57 <sup>c</sup>         | 19.77±1.45 <sup>c</sup>   | 84.91±4.89 <sup>a</sup>   |
| Daidzein                               | 109.67±5.76 <sup>d</sup> | 277.41±25.50°             | 500.77±20.47 <sup>a</sup>       | 358.62±5.52 <sup>b</sup>  | 520.49±27.12 <sup>a</sup> |
| Daidzein-O-hexoside                    | 33.24±2.79 <sup>a</sup>  | 26.30±2.24 <sup>b</sup>   | ND                              | 13.36±2.01°               | ND                        |
| Daidzein-O-acetvlhexoside I            | 7.53±1.12 <sup>a</sup>   | $2.12 \pm 0.33^{b}$       | $1.92 \pm 0.27^{b}$             | 2.10±0.54 <sup>b</sup>    | 1.63±0.35 <sup>b</sup>    |
| Daidzein- <i>O</i> -acetvlhexoside II  | $6.22 \pm 0.75^{a}$      | $1.74 \pm 0.30^{b}$       | $1.40 \pm 0.17^{b}$             | $1.42 \pm 0.27^{b}$       | $1.62 \pm 0.35^{b}$       |
| Daidzein- <i>O</i> -acetvlhexoside III | 136.76±9.76 <sup>a</sup> | $1.94 \pm 0.58^{\circ}$   | 59.55±3.45 <sup>b</sup>         | 56.40±4.76 <sup>b</sup>   | 3.76±1.24 <sup>c</sup>    |
| Genistein                              | 108.01±2.80 <sup>e</sup> | 376.37±22.62 <sup>c</sup> | 494.79±15.67 <sup>b</sup>       | $303.28 \pm 7.72^{d}$     | 532.60±16.61 <sup>a</sup> |
| Genistein-O-hexoside                   | 25.64±1.20 <sup>a</sup>  | 13.45±0.49°               | ND                              | 16.09±2.20 <sup>b</sup>   | ND                        |
| Genistein-O-acetylhexoside I           | 11.11±0.14 <sup>a</sup>  | $4.20{\pm}0.44^{b}$       | $4.77 \pm 0.52^{b}$             | 4.83±0.42 <sup>b</sup>    | 11.10±0.78 <sup>a</sup>   |
| Genistein- <i>O</i> -acetylhexoside II | 152.17±1.56 <sup>a</sup> | 59.87±6.63 <sup>c</sup>   | 71.64±3.78 <sup>b</sup>         | 68.98±5.91 <sup>b,c</sup> | 11.03±0.84 <sup>e</sup>   |
| Glycitein                              | $0.52{\pm}0.07^{d}$      | 1.55±0.32 <sup>c</sup>    | 6.48±0.41 <sup><i>a,b</i></sup> | 1.22±0.16 <sup>c,d</sup>  | 5.85±0.60 <sup>a</sup>    |
| Glycitein-O-bevoside                   | $6.60 \pm 0.24^{a}$      | $6.97 \pm 0.74^{a}$       | ND                              | 6.76±0.32 <sup>a</sup>    | ND                        |
| Glycitein-O-acetylhexoside             | 12.61±0.26 <sup>a</sup>  | 6.03±0.53 <sup>b</sup>    | 5.04±0.26 <sup>c</sup>          | 5.02±0.46 <sup>c</sup>    | $0.85 \pm 0.04^{d}$       |

Mean values  $\pm$  SD, n=3 for control and n= 4 for fermented samples. Different letters indicate significantly different values (p<0.05); ND: not detected

| 579 | Figure captions  |
|-----|--|
| 580 | Figure 1. Box plot representing viable cell concentration (Log CFU/g) of strains in okara after      |
| 581 | inoculum (T <sub>0</sub> ) and after 72 hours (T <sub>72</sub> ) of fermentation at $37^{\circ}$ C   |
| 582 | Figure 2. Alluvial diagram showing the most representative volatile compounds for each class in      |
| 583 | fermented and unfermented (control) okara  |
| 584 | Figure 3. Heatmap visualization of the phytochemical compounds of fermented and unfermented          |
| 585 | (control) okara, based on the Euclidean distance. The color scale represents the scaled abundance of |
| 586 | each variable, with yellow color indicating high abundance and blue color indicating low             |
| 587 | abundance.   |
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# **Figure 2.**



### **Figure 3.**

