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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Abstract

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

Keywords:

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

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[1]. This by-product 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[2]. The two main isoflavone glycosides, genistin and daidzin, are present in soybean in the form of β-D-glycoside. Some studies demonstrated that the corresponding aglycones genistein and daidzein, released by the action of β-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[3,4].

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[3].

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

pathways of the strains involved[14], 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.

2. Materials and methods

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.

2.2 Strains and cultures

Lacticaseibacillus rhamnosus 1473 from Parmigiano Reggiano cheese, reference strains of *Lactobacillus acidophilus* LMG 8151, *Pediococcus acidilactici* 3992 from Grana Padano cheese and a co-culture of these were used as starters for fermentation. 1473 and 3992 belong to the collection of Food and Drug Department, University of Parma, while LMG 8151 was purchased from BCCM (Belgian Co-ordinated Collections of Microorganisms) of Ghent University, Belgium. All bacterial strains were maintained as frozen stocks (-80 °C) in Man Rogosa Sharpe (MRS) medium (Oxoid, Milan, Italy) supplemented with 15% glycerol (w/v). Cultures were grown for one week in MRS broth until their use for fermentation and incubated at 37 °C for 15 h.

2.3 Okara fermentation

The okara used for this work was provided by Sojasun company located in Fidenza (Parma, Italy) and stored at -80 °C to avoid deterioration, due to the high activity of water. Before fermentation, the substrate was autoclaved at 121 °C for 20 minutes.

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

2.4 Investigation of the volatile composition of fermented okara by HS-SPME/GC-MS technique

The volatile profile of fermented and unfermented okara, was analysed by HS-SPME/GC-MS technique following the protocol reported by Ricci, Cirlini, et al. (2018)[15] with slight modifications. In particular, 3 g of okara and 10 μL of an aqueous toluene standard solution (100 μg/mL in 10 mL) were used for the analyses.

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

2.5 Extraction of polyphenolic compounds and LAB-derived metabolites

To carry out the extraction of polyphenols, fermented and unfermented samples were firstly freeze-dried with a lyophilizer (Lio 5PDGT, Cinquepascal, Italy). The samples were subjected to the extraction of free polyphenols, in particular 3 mL of a methanol/water solution (80:20 v/v) acidified with 0.2% of formic acid were added to 0.3 g of each sample. All samples were stirred for 1 min with 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 obtained was re-suspended in 0.3 mL of methanol-water solution and subjected to a second and consequently to a third extraction, performed as described before. The supernatants were pooled and then diluted with a solution of water/methanol (80:20 v/v) acidified with 0.1% formic acid, then centrifuged at 14,462 g for 5 min before UHPLC-MS^n analyses.

2.6 Determination of polyphenolic profile and LAB-derived metabolites through UHPLC MS^n

The samples were analyzed with an Accela UHPLC 1250 interfaced with an ion trap mass spectrometer (LTQ-MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with a ESI interface (H-ESI-II). Chromatographic separation was carried out through an Acquity UPLC HSS T3 column (2.1X 100 mm), 1.8 µm particle size equipped with an Acquity UPLC HSS T3 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)[16]. Analyses of okara samples were carried out in negative ionization mode using full-scan, data-dependant MS^3 scanning from m/z 100 to 2000. Phenyllactic acid, indole-3-lactic acid, *p*-hydroxyphenyllactic acid, genistein and daidzein aglycones were quantified with their authentic standard compounds by extracting the corresponding deprotonated molecule ($[M-H]^-$) in the full scan chromatograms. Calibration curves of phenyllactic, indole-3-lactic and *p*-hydroxyphenyllactic acids ranged from 0.5 to 50 µmol/L, while calibration curves of both genistein and daidzein ranged from 0.05 to 20 µmol/L. Instead, the *O*-glycosylated isoflavones, especially the *O*-acetylglycosides, showed a very high fragmentation behavior in the negative ESI source, leading thus inadequate the monitoring of their

corresponding $[M-H]^-$ in order to avoid a loss of sensitivity. Thus, all *O*-glycosylated daidzein and 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 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 MS^n ion spectra with the MS^n data stored in several online libraries as: PubChem (https://pubchem.ncbi.nlm.nih.gov/); mzCloud (www.mzcloud.org/home); Metlin (http://metlin.scripps.edu); MoNA – Mass Bank of North America (https://mona.fiehnlab.ucdavis.edu/). Additional MS^n information was obtained through previous works[17,18].

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) while alluvial diagram using Rawgraph (https://rawgraphs.io/).

3. Results and discussion

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-~~co~~culture, at the concentration of 6-7 Log CFU/g. The microbial growth ability was assessed immediately after inoculation (T_0) and after 72 h of fermentation (T_{72}) 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 stressful matrices, such as integral okara without pre-treatment and nutrient addition. Contrary to the

178 observation of Moraes et al. (2016)[19], where okara was added only in low percentage to soymilk, the
179 strains of *L. acidophilus* used in this work did not show growth, probably due to the different
180 composition of substrate and nutrients. According to Perreira et al. (2011)[20] the growth of
181 microorganisms depends on various factors, such as the substrate used and the strain employed
182 In agreement with Voss et al., (2018)[10], *L. rhamnosus* shows the ability to grow in okara samples,
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
188 the single culture, due to the synergistic action of the metabolic pathways of the strains involved[14],
189 leading to increased degradation of the substrates[21], with a consequent increase of peptides and
190 amino acids[22], organic acids[23], and volatile compounds[24].
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.
196 Significant differences between fermented and unfermented samples were recorded mainly for
197 aldehydes. A high concentration of aldehydes was observed both in the control (1450.64±296.01
198 μg/g) and in the sample inoculated with *L. acidophilus* (866.12±70.59 μg/g), while a decrease was
199 recorded in okara fermented with *P. acidilactici* (282.92±37.79 μg/g), co-culture (68.28±5.86 μg/g)
200 and *L. rhamnosus* (25.29±5.66 μg/g).
201 The compound responsible for these variations is mainly hexanal, with a persistent herbaceous aroma,
202 generated by lipid oxidation[25], resulting in the unpleasant smell of the soybean-based products.
203 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[26]. 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[27,28].
211 A higher concentration of nonanal was found in the unfermented samples in comparison to the
212 fermented ones. Nonanal is an aldehyde deriving from lipid degradation, that contributes to the beany
213 aroma of legumes[29]. A significant difference was observed between the control (208.27±117.35 μg/g)
214 and fermented samples with co-culture and *L. rhamnosus* 1473, where nonanal was completely
215 absent. The overall decrease of aldehydes, observed in all fermented samples, may be related to
216 contemporary formation of alcohols, via reduction mechanisms during fermentation, as shown in
217 Table 1.
218 Alcohols were the second major class in the volatile fraction of okara and the most abundant
219 compound is 1-octen-3-ol (green and mushroom notes); its formation in soy has been attributed to
220 enzymatic reactions in soaked soybeans, a pre-treatment for soy milk manufacture[26]. Although no
221 significant differences among the samples were observed, SSF process with *P. acidilactici* and *L.*
222 *rhamnosus* induced a decrease of about 50% of 1-octen-3-ol concentration. It is possible to
223 hypothesize that the lower concentration of 1-octen-3-ol is associated with a lower enzymatic activity
224 of the two species.
225 One of the main components of the aroma of soybean is 1-hexanol[30]. A statistically significant
226 increase of this volatile was observed in samples fermented with *P. acidilactici* (217.53±10.72 μg/g),
227 *L. acidophilus* (206.74±46.22 μg/g), and *L. rhamnosus* (216.96±25.33 μg/g), while an opposite
228 behavior was observed in okara fermented with co-culture (16.01±3.58 μg/g). Stress conditions cause
229 different cellular responses, depending on the strain which may translate into the formation of

secondary metabolites, such as aromatic compounds[31]. 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.

After 72 hours of incubation, a significant increase of ketones (Figure 2) was recorded in the sample fermented with co-culture, mainly ascribed to 2-nonanone and 2-heptanone (461.54±9.53 μg/g and 1581.64±61.19 μg/g, respectively). This increase could be related to the combined metabolic activity of the strains that leads to the degradation and metabolization of the substrates, thus increasing the concentration of volatile compounds. Ketones flavor notes are generally described as desirable, and associated with sweet, fruity and creamy sensations[4]. In particular, 2-butanone-3-hydroxy (acetoin), detected in sample fermented with co-culture and characterized by fatty butter taste, is widely used as flavor and fragrance in the food industry[32].

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[28].

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.

Finally, the presence of two hydrocarbons was also observed, with no significant differences among the analyzed samples.

3.3 Phytochemical profile and LAB-derived metabolites

The fermentation effect on non-volatile organic acids and polyphenolic compounds using different LAB strains and co-culture towards okara-derived phytochemicals was evaluated through UHPLC-

255 MS^n. At least 45 different compounds were identified, even some components specifically occurred
256 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
258 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 *β*-glucosidases in their
262 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
267 trend was observed for genistein, although fermented samples with co-culture strains showed a
268 significant higher concentration (532.60±16.61 μg/g) of this isoflavone with respect to samples
269 biotransformed by *L. rhamnosu*s (494.79±15.67 μg/g). Genistein and daidzein were by far the most
270 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[3,33], but, the current study reveals the
274 high potential of LAB co-culture to convert isoflavones for the first time. A synergistic effect of co-
275 culture, corresponding to a high bioconversion of isoflavones, was observed in okara fermented
276 with fungi[34].
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
279 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[35–38]. Results of these

281 studies are difficult to compare as different delivery forms of isoflavones, such as pure compounds,
282 tablets or soy-based products, were investigated. However, focusing only on soy-based products,
283 several human feeding studies highlighted the improvement of isoflavone bioavailability in the first
284 GI tract upon soy fermentation as a result of the higher aglycone content than the unfermented
285 counterparts[36,37].
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
299 fermentation had been previously reported, in particular for *L. rhamnosus* 1473[16], and in the current
300 study these compounds reached the highest concentration after co-culture fermentation, i.e.
301 191.79±15.05 for phenyllactic acid and 133.95±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[39,40]. 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,
313 produced *via* tryptophan catabolism[41]. This catabolite can be produced by yeasts and bacterial
314 species, and is able to inhibit the growth of Gram positive and Gram negative bacteria[42], as well as
315 by acting as antifungal compound against *Penicillium* strains[43]. Indole-3-lactic acid reached the
316 highest concentration in the okara fermented with co-culture (84.91±4.89 μg/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*
321 and *in vivo* experiments[41]. Some *in vitro* experiments showed that indole-3-lactic acid is able to
322 reduce the inflammation[44,45] and this behavior was partially confirmed in human studies. Moreover,
323 circulating indole-3-lactic acid was significantly lower in plasma of obese subjects than in non-
324 obese ones, and was paralleled by lower serum levels of inflammatory markers[46]. The putative anti-
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
327 days when compared to a control fast food diet in a crossover design[47].
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 α-hydroxy acid after SSF with co-culture (Table
332 S2), in agreement with previous works on leucic acid production by different LAB strains[17,48]. The

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

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~~t~~he 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.

Exploring different strains and their combinations, the co-culture containing *L. acidophilus*, *L. rhamnosus* and *P. acidilactici* was the best starter candidate due to its ability to significantly modify the aromatic and polyphenolic profile of raw material. Besides the optimal growth performance, a decrease of off-flavor (hexanal, nonanal) and a large conversion of isoflavones in their aglycone forms were obtained. Moreover, a notable production of LAB-derived metabolites such as indol-3-lactic, phenyllactic and *p*-hydroxyphenyllactic acids, that can exert a human biological activity or antimicrobial activity, was observed.

On the basis of the obtained results, solid state fermentation may represent an innovative strategy for the reuse of okara with the final goal of the recovery of possible functional ingredients.

Abbreviations

ANOVA, analysis of variance; BCCM, Belgian Co-ordinated Collections of Microorganisms; CFU, colony-forming unit; EI, electronic impact; GI, upper gastrointestinal tract; HPLC, high performance liquid chromatography; HS-SPME/GC-MS, headspace solid phase microextraction and gas chromatography-mass spectrometry; LAB lactic acid bacteria; LC-MS, liquid chromatography-mass spectrometry; LRIs, linear retention indexes; MRS, Man Rogosa Sharpe; SD, standard deviation; ND, not detected; SD, standard deviation; RT, retention time; SSF, solid state

fermentation; UHPLC-MS^n, ultrahigh-performance liquid chromatography-mass spectrometry; UPLC, ultra-performance liquid chromatography.

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Conflict of Interest

There are no conflicts to declare.

Electronic Supplementary Information

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

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541 **Table 1.** Concentration (μg/g) of volatile compounds found in unfermented (control) and fermented

542 okara with different strains for 72 hours

	Control	*L. acidophilus* 8151	*P. acidilactici* 3992	*L. rhamnosus* 1473	Co-culture
Aldehydes					
Pentanal	46.37±28.73[a]	29.08±5.93[a]	9.84±2.01[a]	ND	ND
Hexanal	494.83±8.57[a]	492.95±103.37[a]	147.64±38.95[b]	ND	ND
Heptanal	139.49±48.09[a]	76.79±31.43[a,b]	27.37±4.06[b]	ND	ND
Octanal	124.31±66.77[a]	35.18±2.96 [a]	19.66±3.89 [a]	ND	ND
2-Heptenal	127.74±10.71[a]	15.33±1.70[b]	6.35±3.39[b]	ND	9.21±1.10[b]
Nonanal	208.27±117.35[a]	49.64±11.51[a]	17.47±1.52[a]	ND	ND
2-Octenal (E)	95.87±46.06[a]	19.33±2.25[b]	8.84±1.93[b]	ND	12.91±3.88[b]
Furfural	23.66±7.87[a]	3.73±1.46[b]	ND	ND	ND
Decanal	46.73±21.40[a]	15.74±2.56[a]	ND	ND	ND
Benzaldehyde	114.58±51.76[a]	109.88±18.37[a]	44.96±4.09[a]	22.44±4.50[a]	46.16±3.31[a]
Dodecanal	5.83±2.98[a]	ND	ND	ND	ND
Benzaldehyde, 4-ethyl	9.95±3.52[a]	5.80±0.25[a]	1.80±0.11[b]	ND	ND
2,4-Decadienal	13.01±4.83[a]	12.67±1.62[a]	4.62±1.46[b]	ND	ND
Benzaldehyde, 2,5-dimethyl	ND	ND	ND	4.09±1.75[a]	ND
Total	1450.64±296.01[a]	866.12±70.59[a]	282.92±37.79[b]	25.29±5.66[b]	68.28±5.86[b]
Alcohols					
Propan-2-ol	131.81±10.15[a,b]	235.48±93.96[a]	86.83±7.90[a,b]	37.29±11.86[b]	65.44±20.80[b]
Ethanol	44.03±42.39[a]	57.44±13.89[a]	20.38±2.75[a]	13.76±1.37[a]	19.62±5.50[a]
1-Butanol,3-methyl	ND	7.64±2.29[a]	9.81±0.68[a]	2.09±0.15[b]	ND
1-Pentanol	90.17 ±50.94[a]	42.71±15.78[a]	18.90±1.55[a]	42.33±3.58[a]	ND
1-Hexanol	64.21±27.45[b]	206.74±46.22[a]	217.53±10.72[a]	216.96±25.33[a]	16.01±3.58[b]
3-Octanol	ND	ND	6.88±2.33[a]	8.08±0.01[a]	ND
1-Octen-3-ol	235.68±124.19[a]	225.55±67.58[a]	107.51±1.73[a]	101.03±1.89[a]	225.51±20.94[a]

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

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

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547 **Table 2.** UHPLC-MS^n characteristics of compounds detected in unfermented and fermented okara

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Compound	RT	[M-H]- (m/z)	MS^2 ions (m/z)	MS^3 ions (m/z)
Succinic acid	1.57	117	73, 99	
2-Hydroxyvaleric acid or 2-Hydroxyisovaleric acid	3.94	117	71	
Hydroxycaproic acid isomer	5.50	131	85	
2-Hydroxyisocaproic acid (Leucic acid)	5.60	131	**85**, 87, 113, 59	69
Malic acid	0.99	133	115	71
2-Hydroxy-4-(methylthio)butyric acid	3.94	149	101, 103, 107	
p-Coumaric acid	6.00	163	119	
Phenylalanine	2.87	164		
Phenyllactic acid	6.22	165	147, 119	
Tyrosine	1.48	180	**163**, 119, 136	119
***p*-Hydroxyphenyllactic acid**	4.16	181	163, 135, 113	
Azelaic acid	7.11	187	**125**, 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	**186**, 158, 142, 160, 116	142, 158, 116, 130
Pantothenic acid	3.26	218	**88**, 146	59
Daidzein	7.85	253	209, 197, 224, 225, 226, 169, 182, 195, 145	
Genistein	9.10	269	**225**, 224, 201, 241, 181, 197, 199, 213, 169, 133, 159, 107	181, 182, 186, 195, 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	**167**, 123, 209	152, 123, 108
Syringic acid-*O*-hexoside	3.80	359	**197**, 182	182, 153, 138
Daidzein-*O*-hexoside	5.57	415	**253**, 295	209, 225, 180, 212, 208, 207, 196
Genistein-*O*-hexoside	6.37	431	**269**, 268, 311	224, 201, 241, 225, 240, 226, 213, 180, 169, 157, 133
Naringenin-*O*-hexoside I	5.89	433	271	151, 177
Naringenin-*O*-hexoside II	6.78	433	271	151, 177
Naringenin-O-hexoside III	7.30	433	**271**, 313	151, 177
Glycitein-*O*-hexoside	5.70	445	283	268
Kaempferol-*O*-hexoside	6.60	447	**285**, 327, 363, 256, 241	241, 257, 213, 167, 151, 256
Dihydrokaempferol-*O*-hexoside	5.25	449	**287**, 269, 259	259, 243, 269
Daidzein-*O*-acetylhexoside I	6.20	457	397, 253	
Daidzein-O-acetylhexoside II	6.30	457	253	
Daidzein-*O*-acetylhexoside III	6.40	457	253	
Daidzein-*O*-acetylhexoside IV	6.94	457	**253**, 252, 295, 397	224, 225, 197, 209, 208, 135
Genistein-*O*-acetylhexoside I	7.03	473	269	
Genistein-*O*-acetylhexoside II	7.12	473	**269**, 413	225, 240, 227, 181
Genistein-*O*-acetylhexoside III	7.83	473	**269**, 268, 311	227, 225, 224, 251, 250, 241, 133

Glycitein-*O*-acetylhexoside	6.41	487	283	268
Naringenin-*O*-acetylhexoside	7.35	475	271	151, 177, 107
Pinoresinol-*O*-hexoside	6.37	519	**357**, 475	151, 136, 327, 295, 311
Kaempferol-*O*-dihexoside	5.74	609	**285**, 429	257, 213, 151, 229, 241, 197, 200
Daidzein-*O*-hexoside derivative	4.35	623	415	253, 295
Genistein-*O*-hexoside derivative	4.78	639	**431**, 593	268, 269

571 **Table 3.** Concentration (μg/g) of isoflavones and main LAB-derived metabolites recorded in
572 unfermented (control) and fermented okara with different strains after 72 hours.
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	Okara sample				
Compound	**Control**	***L. acidophilus* 8151**	***L. rhamnosus* 1473**	***P. acidilactici* 3992**	**Co-culture**
p-Hydroxy-phenyllactic acid	ND	48.54 ±3.58^{b}	30.82 ±1.93^{c}	37.98±4.17^{c}	133.95±4.42^{a}
Phenyllactic acid	ND	96.24±9.10^{b}	32.58±2.40^{c}	48.83±2.81^{c}	191.79±15.05^{a}
Indole-3-lactic acid	9.99±1.53^{d}	30.65±1.60^{b}	18.65±0.57^{c}	19.77±1.45^{c}	84.91±4.89^{a}
Daidzein	109.67±5.76^{d}	277.41±25.50^{c}	500.77±20.47^{a}	358.62±5.52^{b}	520.49±27.12^{a}
Daidzein-*O*-hexoside	33.24±2.79^{a}	26.30±2.24^{b}	ND	13.36±2.01^{c}	ND
Daidzein-*O*-acetylhexoside I	7.53±1.12^{a}	2.12±0.33^{b}	1.92±0.27^{b}	2.10±0.54^{b}	1.63±0.35^{b}
Daidzein-*O*-acetylhexoside II	6.22±0.75^{a}	1.74±0.30^{b}	1.40±0.17^{b}	1.42±0.27^{b}	1.62±0.35^{b}
Daidzein-*O*-acetylhexoside III	136.76±9.76^{a}	1.94±0.58^{c}	59.55±3.45^{b}	56.40±4.76^{b}	3.76±1.24^{c}
Genistein	108.01±2.80^{e}	376.37±22.62^{c}	494.79±15.67^{b}	303.28±7.72^{d}	532.60±16.61^{a}
Genistein-*O*-hexoside	25.64±1.20^{a}	13.45±0.49^{c}	ND	16.09±2.20^{b}	ND
Genistein-*O*-acetylhexoside I	11.11±0.14^{a}	4.20±0.44^{b}	4.77±0.52^{b}	4.83±0.42^{b}	11.10±0.78^{a}
Genistein-*O*-acetylhexoside II	152.17±1.56^{a}	59.87±6.63^{c}	71.64±3.78^{b}	68.98±5.91^{b,c}	11.03±0.84^{e}
Glycitein	0.52±0.07^{d}	1.55±0.32^{c}	6.48±0.41^{a,b}	1.22±0.16^{c,d}	5.85±0.60^{a}
Glycitein-*O*-hexoside	6.60±0.24^{a}	6.97±0.74^{a}	ND	6.76±0.32^{a}	ND
Glycitein-*O*-acetylhexoside	12.61±0.26^{a}	6.03±0.53^{b}	5.04±0.26^{c}	5.02±0.46^{c}	0.85±0.04^{d}

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

574
575
576
577
578

Figure captions

Figure 1. Box plot representing viable cell concentration (Log CFU/g) of strains in okara after inoculum (T_0) and after 72 hours (T_{72}) of fermentation at 37° C

Figure 2. Alluvial diagram showing the most representative volatile compounds for each class in fermented and unfermented (control) okara

Figure 3. Heatmap visualization of the phytochemical compounds of fermented and unfermented (control) okara, based on the Euclidean distance. The color scale represents the scaled abundance of each variable, with yellow color indicating high abundance and blue color indicating low abundance**.**

Figure 1.

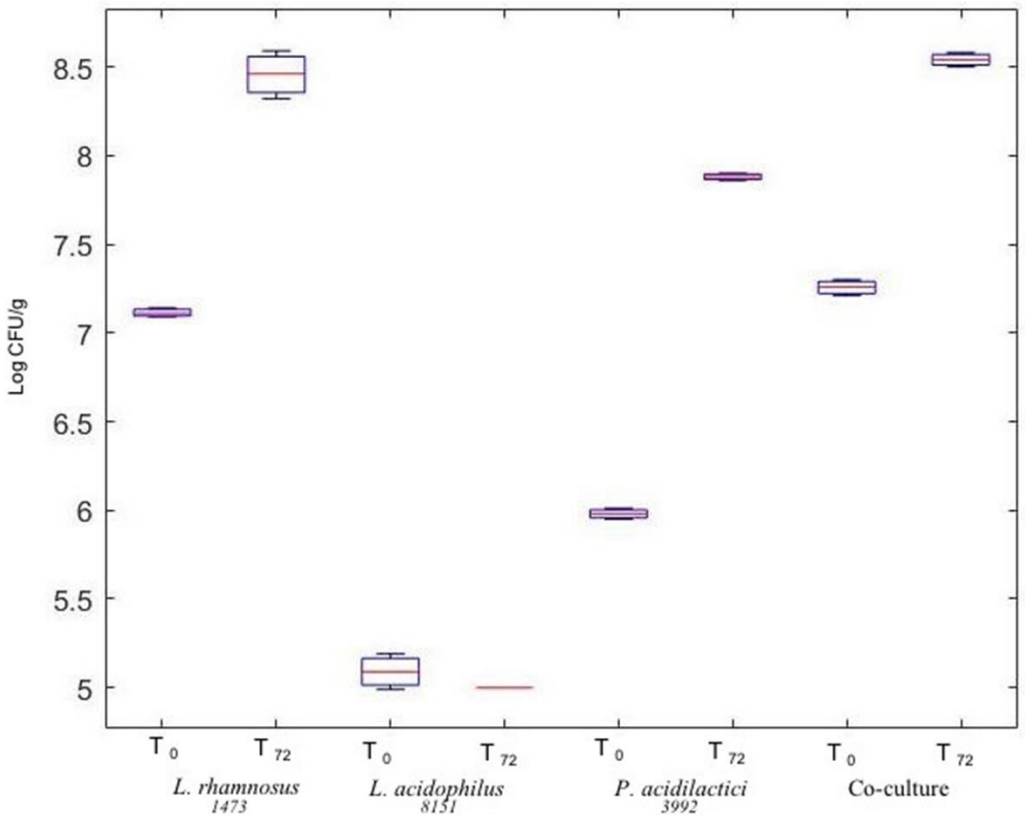

Log CFU/g
8.5
8
7.5
7
6.5
6
5.5
5
T 0
T 72
T 0
T 72
T 0
T 72
T 0
T 72
L. rhamnosus 1473
L. acidophilus 8151
P. acidilactici 3992
Co-culture

Figure 2.

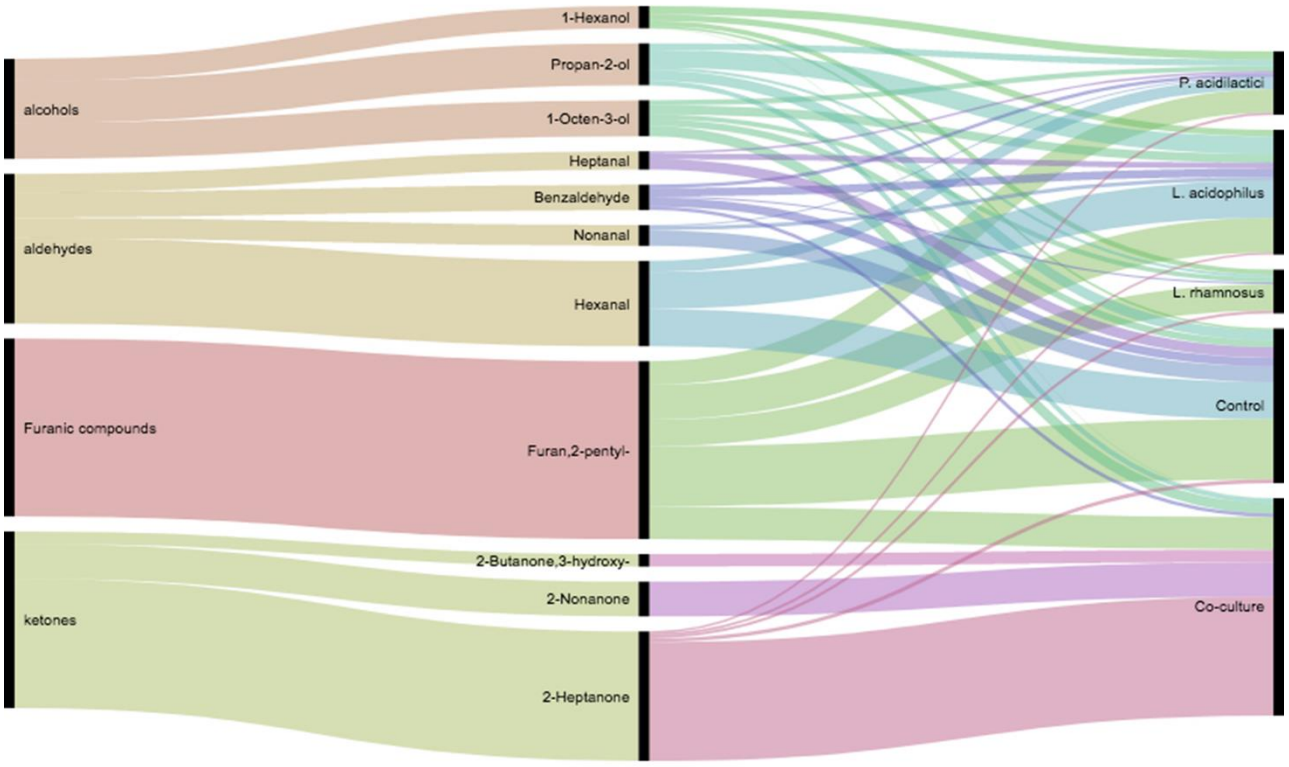
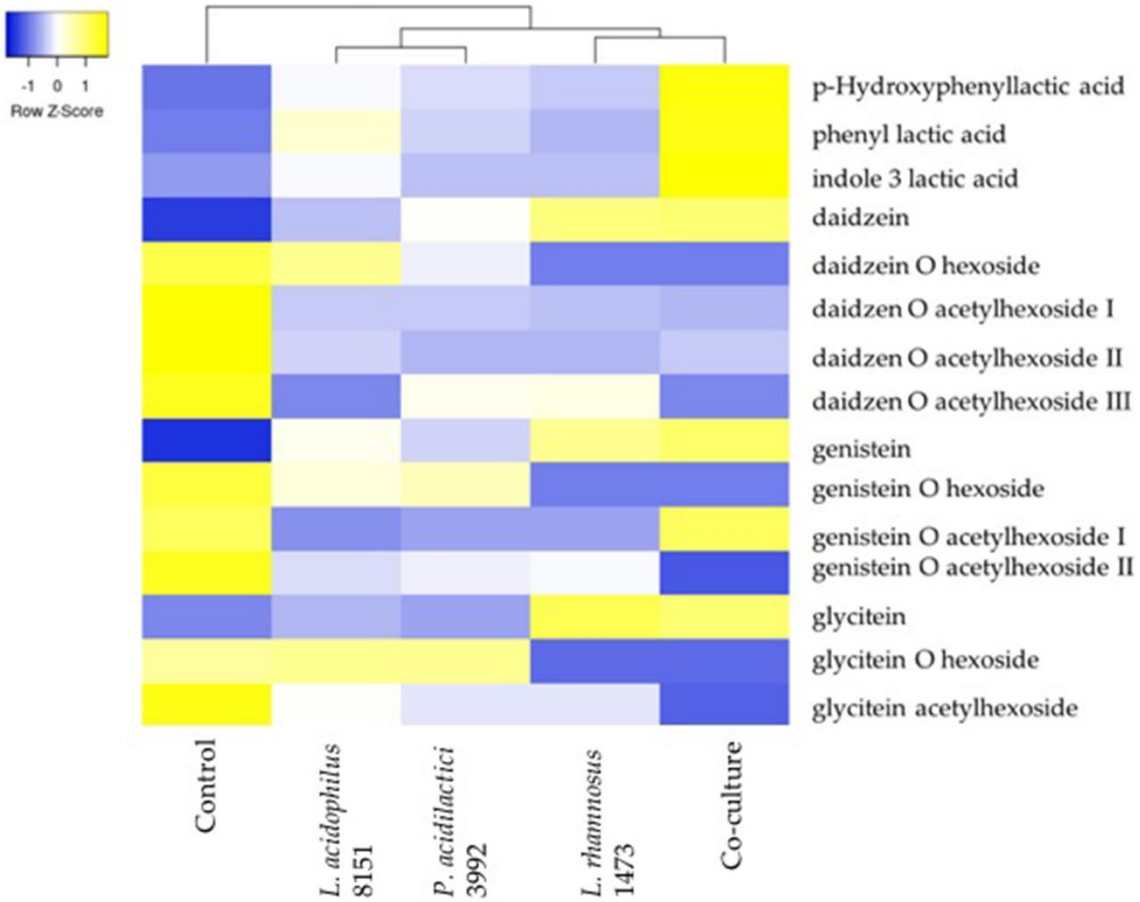

alcohols
aldehydes
Furanic compounds
ketones
1-Hexanol
Propan-2-ol
1-Octen-3-ol
Heptanal
Benzaldehyde
Nonanal
Hexanal
Furan,2-pentyl-
2-Butanone,3-hydroxy-
2-Nonanone
2-Heptanone
P. acidilactici
L. acidophilus
L. rhamnosus
Control
Co-culture

Figure 3.


-1 0 1
Row Z-Score
p-Hydroxyphenyllactic acid
phenyl lactic acid
indole 3 lactic acid
daidzein
daidzein O hexoside
daidzen O acetylhexoside I
daidzen O acetylhexoside II
daidzen O acetylhexoside III
genistein
genistein O hexoside
genistein O acetylhexoside I
genistein O acetylhexoside II
glycitein
glycitein O hexoside
glycitein acetylhexoside
Control
L. acidophilus 8151
P. acidilactici 3992
L. rhamnosus 1473
Co-culture