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Article

Lactic Acid Bacterial Culture Selection for Orange Pomace Fermentation and Its Potential Use in Functional Orange Juice

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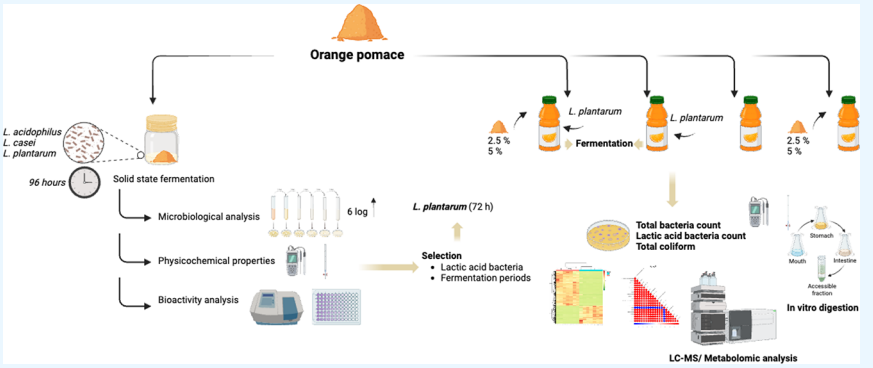

ABSTRACT: The main goal of this study is to improve the bioactivity of citrus pomace by subjecting it to solid-state fermentation by *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus plantarum* over varying periods of time. The viability of *Lactobacillus*, as well as the total phenolic content (TPC) and total antioxidant capacity (TAC) values of orange pomace (OP), varied depending on the *Lactobacillus* species and fermentation period. The incorporation of pomace into orange juice samples at a ratio of 5% considerably enhanced the viability of probiotics. The TPC and TAC of orange juice samples were reduced after fermentation. The addition of orange pomace to orange juice significantly increased prostaglandin H2 and improved antioxidant capacity with more pronounced effects at increased pomace concentrations. After postfermentation with *L. plantarum*, key bioactive compounds such as corynoxeine and phenolics were upregulated, while picroside III and allocryptopine levels were decreased. On the other hand, metabolomics analysis revealed significant changes after fermentation in amino acid, sphingolipid, and fatty acid metabolism and the synthesis of secondary metabolites improving the nutritional profile and bioactivity of fermented orange juice. These findings highlight the potential of pomace addition and fermentation to improve the health benefits and quality of orange juice products. Therefore, the combined use of fermentation and fortification with OP could be a promising approach to creating new functional foods and promoting the use of edible food waste and byproducts.

1. INTRODUCTION

Orange (*Citrus sinensis*) juice, having a wide range of consumers from children to adults due to its good taste and nutritional value, is the most industrially produced fruit juice in the world.[1,2] Orange fruits and juices are rich in several bioactive compounds, including phenolic compounds, flavonoids, carotenoids, vitamins (especially, vitamin C), and pectin, which are important for human nutrition and health due to their health-promoting effects.[3,4] Epidemiological studies have shown that regular consumption of orange juice reduces the incidence of several diseases such as cardiovascular disease, diabetes, obesity, and cancer due to the antioxidant, hypotensive, vasodilator, anti-inflammatory, and hypolipidemic activity of its bioactives, especially phenolic compounds.[5] Thus, the consumption of fruit juices is increasing among health-conscious consumers worldwide, and natural fruit juices are becoming more popular than fruit-based drinks due to their perceived health benefits.[2]

When considering the production amount, approximately one-fourth of citrus fruits and 45% of oranges are used for industrial juice production. During juice production, a significant amount of solid waste is generated, comprising of peels, pomace, and seeds, which represent approximately 50−

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60% of the whole fruit.[6−8] These orange peels and pomace, as a consumable food waste, are rich sources of phenolic compounds, flavonoids, organic acids, pectin, and limonene, which have been proven to have several health benefits.[8,9]

However, dealing with the byproducts of citrus processing has become an urgent issue in processing units due to the lack of reasonable and efficient disposal methods.[9] Today, various management strategies have been investigated in a wide range of upcycling methods from pectin and cellulose extraction to bioethanol production to solve this problem and utilize food waste/byproducts. However, the primary destinations of orange processing wastes are still limited to animal feed, composting, or incineration/landfill.[10] Additionally, within the management strategies, the integration of such consumable food wastes and byproducts with high phytochemical content and good nutritional value into food formulations is the best option to meet consumer expectations and develop new functional foods.

Lactic acid bacteria (LAB) fermentation, a traditional processing technique, can improve the nutritional value and digestibility of food components as well as enhance and produce functional bioactive compounds. These effects of LAB fermentation are mainly attributed to the activity of microorganisms such as the ability to produce enzymes including peptidase, proteinase, amylase, decarboxylase, dehydrogenases, and β-glucosidase.[11,12] Various strains of LAB had distinct impacts on the physicochemical characteristics, taste, and biological functions.[13]

On the other hand, it has been demonstrated that the utilization of simulated saliva fluid (SSF) together with lactic acid bacteria, specifically *Lactobacillus plantarum*, can enhance the overall phenolic content of citrus pomace,[9] *Lactobacillus casei* on mulberry pomace,[14] and *Lactobacillus acidophilus* on grape pomace.[15] Based on previous studies, these three types of LAB were selected for fermentation of orange pomace, including *L. acidophilus*, *L. casei*, and *L. plantarum*. Additionally, the time during fermentation is a crucial determinant of the fermentation quality. This duration can impact the concentration and composition of bioactive compounds in citrus juice.[12]

Therefore, there is a growing trend toward fermentation for functional fruit juice production due to the potential of improving bioactive compounds through fermentation. For example, in the literature, there are several studies demonstrating that LAB fermentation improved the total phenolic content (TPC) and total antioxidant capacity (TAC) of strawberry juice, jujube juice,[8] citrus juice,[12] kiwifruit juice,[16] and jujube-wolfberry composite juice.[17] However, the study by Multari et al.[18] gave different results on the final phenolic content of fermented juices prepared from two orange varieties, leading to the conclusion that the effects of fermentation were highly dependent on the substrate type. Furthermore, for orange juice, it has been shown that fermentation with LAB is highly effective in improving the bioavailability of phenolic compounds[19,20] and in promoting health effects like protection against cardiovascular disease.[21,22] Fermentation is also a promising technique for improving the nutritional value and phytochemical content of fruit waste and byproducts including citrus pomace,[9] orange peels,[10] and orange pomace.[23] Thus, these results on fermented orange juice and orange pomace show that the application of LAB fermentation combined with the use of orange pomace (OP) can be a good strategy for producing functional orange beverages.

Even though the effects of fermentation on the bioactive content of some fruit juices or their wastes have been investigated, information on the bioaccessibility of these bioactives is limited; thus, food applications with the use of these materials are scarce. Considering these, the aim of this study was to produce a novel fermented orange juice by utilizing OP as the primary ingredient, thereby leading to the minimization of orange juice processing wastes. In the first step, probiotics (*L. acidophilus*, *L. casei*, and *L. plantarum*) were chosen as cultures to ferment orange pomaces throughout varying periods of fermentation, and their biological activity and cell survival were investigated. On the other hand, the changes in TPC and TAC before and after *in vitro* digestion, as well as the metabolite profiles of orange juice samples were investigated.

2. MATERIALS AND METHODS

2.1. Materials. Fresh orange pomace samples were obtained from Türkiye (Döhler Gıda San. A.Ş.). Pomace samples were ground, freeze-dried (Christ Alpha 1-2 LSCbasic, Osterode am Harz, Germany), and stored at −20 °C.

2.2. Culture Preparation. *L. acidophilus* LA-5, *L. casei* 431, and *L. plantarum* Harvest-LB1 were obtained from the CHR Hansen probiotic culture collection. The cultures were placed in 100 mL Erlenmeyer flasks with 50 mL of deMan, Rogosa, and Sharpe (MRS) broth (Merck: 110661) and incubated at 37 °C for 24 h. The cells were harvested by centrifugation at 8000 rpm for 10 min at 4 °C. Then, they were washed twice in 50 mM sterile potassium phosphate buffer (pH 7.4) and resuspended in sterile distilled water to achieve a final lactic acid bacteria concentration of 8 log colony-forming unit (CFU)/mL.

2.3. Fermentation of Pomace. Freeze-dried orange pomace was mixed with distilled water at a ratio of 1:4 (w:w), and before fermentation, the mixture was pasteurized at 90 °C for 10 min and inoculated individually with the culture (5%, v/w). The groups, consisting of both inoculated and control samples, were placed in an incubator and fermented at 37 °C for 1−4 days without agitation.

All the fermentation experiments were performed in triplicate.[24] The control sample was prepared by keeping the mixture (orange pomace and water, 1:4, w:w) for 4 days without fermentation. For the treated samples, the mixture was inoculated with either 5% *L. acidophilus* (coded as FOP_LA), 5% *L. casei* (coded as FOP_LC), or 5% *L. plantarum* (coded as FOP_LP). After fermentation, samples were freeze-dried and stored at −20 °C for bioactivity analysis. Optimum fermentation conditions and culture have been determined according to the results of the bioactivity analysis.

2.4. Preparation of Fermented Orange Juice. The juice was prepared using a household extractor from Valencia oranges obtained from a local market in Türkiye. The extracted juice was pasteurized at 75 °C for 5 min to inactivate the natural microbiota present and then cooled to room temperature. The fermented juices were prepared with the inoculation of 5% (v/v) *L. plantarum* (8 log CFU/mL), and prepared juices were maintained at a constant temperature of 37 °C for 72 h.[25] Samples were prepared according to the details provided in Table 1 under optimum fermentation conditions with three replicates.

Furthermore, pH values were measured with a pH meter (Hanna, HI 9321). Total titratable acidity (TTA) was measured by titrating the samples with 0.1 N NaOH until a

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Table 1. Codes and Description of Juice Samples

code	samples
OJ	orange juice
FOJ	fermented orange juice
OJ-2.5OP	orange juice added with 2.5% w/w orange pomace
F-OJ-2.5OP	after adding 2.5% orange pomace to the orange juice, fermentation was applied
OJ-5OP	orange juice added with 5% w/w orange pomace
F-OJ-5OP	after adding 5% orange pomace to the orange juice, fermentation was applied

pH of 8.1. The results were expressed as the amount of citric acid equivalents (AOAC, 2000).

2.5. Microbiological Analysis. Pomace samples (1 g) or fruit juices (1 mL) were homogenized with sterile 0.1% peptone water. Lactic acid bacteria counts in fermented pomace and juice samples were enumerated on MRS agar by the pour plating method after aerobic incubation at 37 °C for 48 h. The total number of aerobic mesophilic bacteria and Enterobacteria in orange juices was determined by pour plating on Plate Count Agar (Merck) at 30 °C for 48 h and on Violet Red Bile Glucose Agar (Merck) at 37 °C for 24 h, respectively.

2.6. Extraction of Polyphenols. The solvent extraction method described by Capanoglu et al.[26] was performed to extract polyphenols from each sample. In short, approximately 2 g of fresh weight from each sample was extracted using 5 mL of 75% aqueous methanol containing 0.1% formic acid, followed by sonication for 15 min. The samples were then centrifuged for 10 min at 2700*g*, and the supernatant was collected. An additional 5 mL of 75% aqueous methanol with 0.1% formic acid was added to the pellet, and the extraction process was repeated two more times. Then, the supernatants were combined and used for further analyses.

2.7. *In Vitro* Gastrointestinal Digestion Procedure. An *in vitro* gastrointestinal model was carried out according to the standardized method described by Minekus et al.[30] to mimic the conditions that food undergoes after ingestion in the mouth, stomach, and small intestine. The temperatures of all solutions were adjusted to 37 °C before use, and the incubation of all samples was performed at this temperature throughout the simulated gastrointestinal tract (GIT) procedure. For the mouth phase, 5 g of sample was sequentially mixed with 3.5 mL of SSF, 0.5 mL of mucin stock solution containing 30.0 mg/mL mucin, 25 μL of 0.3 M $CaCl_2$ solution, and 975 μL of Milli-Q water. The obtained mixture (pH 7.0) was then incubated for 2 min in an incubator shaker with continuous agitation to simulate the mouth phase. In the stomach phase, 10 mL of sample from the mouth phase was mixed in sequence with 7.5 mL of simulated gastric fluid, 1.6 mL of porcine pepsin stock solution containing 36.6 mg/mL porcine pepsin, and 5 μL of 0.3 M $CaCl_2$ solution, and the pH of the obtained mixture was adjusted to 3.0 by adding 0.2 mL of 1 M HCl solution. Then, 0.695 mL of Milli-Q water was added to complete the total volume to 20 mL. The obtained mixture (pH 3.0) was then incubated for 2 h in an incubator shaker with continuous agitation to simulate the stomach phase. For the small intestine phase, 20 mL of sample from the stomach phase was mixed in sequence with 11 mL of simulated intestinal fluid, 5 mL of pancreatin stock solution containing 8.0 mg/mL pancreatin, 2.5 mL of fresh bile solution (160 mM in fresh bile), and 40 μL of 0.3 M $CaCl_2$ solution, and the pH of the obtained mixture was adjusted to 7.0 by adding 0.15 mL of NaOH solution. Then, 1.31 mL of Milli-Q water was added to complete the total volume to 40 mL. The obtained mixture (pH 7.0) was then incubated for 2 h in an incubator shaker with continuous agitation to simulate the small intestine phase. After all stages of the *in vitro* gastrointestinal digestion procedure, all samples were collected and centrifuged at 4 °C and 4000 rpm for 30 min to remove any large particles. Then, the supernatants were stored at −20 °C until further analyses.

2.8. Total Phenolic Content (TPC) and Total Antioxidant Capacity (TAC). The TPC and TAC of the samples were measured spectrophotometrically using a single-beam UV-3100PC spectrophotometer (VWR, Belgium). Before analyses, all extracted samples and the fractions collected from the *in vitro* GIT procedure were passed through a polyethylene terephthalate (PET) filter (Chromafil PET-45/25) to remove any particulate matter that could cause light scattering.

The TPC was determined according to the Folin-Ciocalteu method described by Singleton and Rossi (1965).[27] The TPC results of the samples were expressed as grams of gallic acid equivalent (GAE) per kilogram of fresh weight (FW) sample. The total antioxidant capacity (TAC) assays were performed by two different assays including cupric ion reducing antioxidant capacity (CUPRAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, which were performed according to Apak et al. (2004)[28] and Blois (1958),[29] respectively. The results of these assays were expressed as grams of Trolox equivalent (TE) per kilogram of FW sample.

2.9. Sample Preparation and Untargeted Metabolomic Analysis. *2.9.1. Sample Extraction.* For the solid samples, 50 mg of the sample was weighed, 1000 μL of extraction solvent containing an internal standard (methanol:acetonitrile:water at a volume ratio of 2:2:1, with the internal standard concentration of 20 mg/L) was added, the mixture was vortexed for 30 s, and then steel beads were added and treated with a ball mill at 45 Hz for 10 min, followed by ultrasonication for 10 min (in an ice-water bath). On the other hand, for the liquid samples, 100 μL of the sample was weighed, 500 μL of extraction solvent containing an internal standard (methanol:acetonitrile at a volume ratio of 1:1, with the internal standard concentration of 20 mg/L) was added, and the mixture was vortexed for 30 s and ultrasonicated for 10 min (in an ice-water bath). For the cell samples, 1000 μL of extraction solvent containing an internal standard (methanol:acetonitrile at a volume ratio of 1:1, with the internal standard concentration of 20 mg/L) was used to transfer the sample into an EP tube in three portions (300, 300, and 400 μL) and vortexed for 30 s each time, and steel beads were added and treated with a ball mill at 45 Hz for 10 min, followed by ultrasonication for 10 min (in an ice-water bath). Subsequent steps are consistent for all sample types, and the procedure was as follows: incubation at −20 °C for 1 h, centrifugation of the samples at 4 °C at 12,000 rpm for 15 min, and collection of 500 μL of the supernatant into an EP tube. Then, the extracts were dried in a vacuum concentrator and reconstituted in 160 μL of extraction solvent (acetonitrile:water at a volume ratio of 1:1). Samples were vortexed for 30 s and then ultrasonicated in an ice-water bath for 10 min. The samples were centrifuged at 4 °C at 12,000 rpm for 15 min, and 120 μL of the supernatant was transferred into a 2 mL autosampler vial, taking 10 μL from each sample to mix into a QC (quality control) sample for machine analysis.[31]

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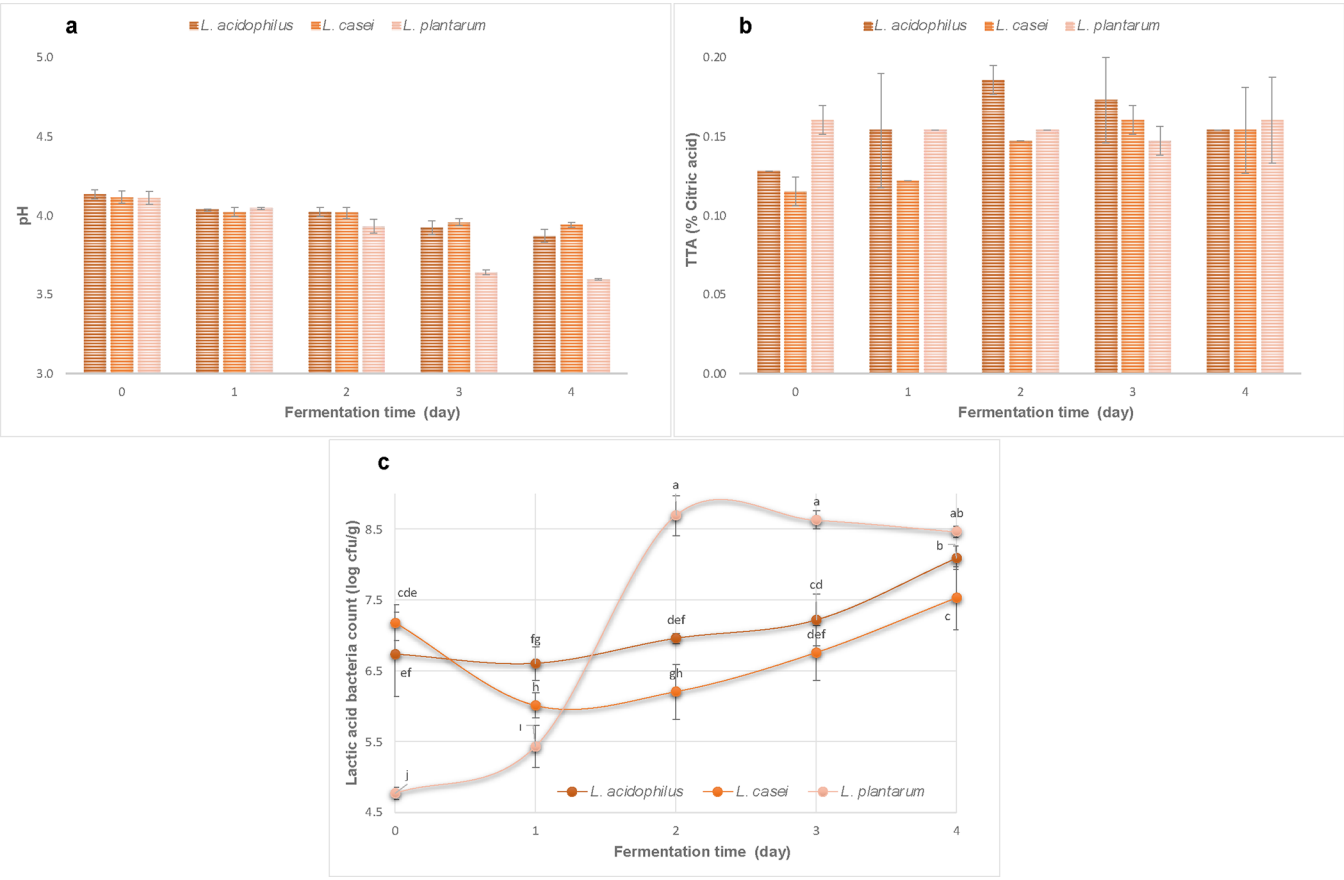

Figure 1. pH (a), TTA (b), and total viable lactic acid bacteria counts (c) during lactic acid fermentation of orange pomace.

2.9.2. Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) Analysis. The Waters Xevo G2-XS QTOF is a high-resolution mass spectrometer that can capture both primary and secondary mass spectrometry data in MSe mode, controlled by MassLynx V4.2 software. Each acquisition cycle allows for dual-channel data collection at both low and high collision energies simultaneously. The low collision energy is set at 2 V, while the high collision energy varies between 10 and 40 V, with a scanning frequency of 0.2 s per mass spectrum. The electrospray ionization ion source is configured with the following parameters: a capillary voltage of 2000 V for positive ion mode and −1500 V for negative ion mode, a cone voltage of 30 V, an ion source temperature of 150 °C, a desolvent gas temperature of 500 °C, a backflush gas flow rate of 50 L/h, and a desolventizing gas flow rate of 800 L/h.

2.9.3. Data Preprocessing, Annotation, and Analysis. The raw data obtained via MassLynx V4.2 were processed with Progenesis QI software, which facilitated peak extraction, alignment, and other data processing tasks. These processes were supported by the METLIN database integrated within Progenesis QI and Biomark's proprietary library for compound identification, along with theoretical fragment identification and mass deviation analysis. After the peak area data were normalized against the total peak area, further analyses were conducted. To assess the consistency of samples within groups and quality control samples, principal component analysis and Spearman correlation analysis were employed. Identified compounds were then explored for classification and pathway information using the Kyoto Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB), and LIPID MAPS databases. Based on groupings, the fold changes were calculated and compared, and the significance of differences for each compound was determined using a *t* test to compute the *p*-value. Orthogonal partial least squares discriminant analysis (OPLS-DA) modeling was performed using the R language package, and the model's reliability was validated through 200 permutation tests. The variable importance in projection (VIP) value was calculated through multiple cross-validation methods.[32,33] To identify differential metabolites, a combined approach using fold change, *p*-value, and VIP values from the OPLS-DA model was adopted. The criteria for selection were a fold change (FC) greater than 1, a *p*-value less than 0.01, and a VIP value greater than 1. The significance of the KEGG pathway enrichment for the differential metabolites was evaluated using the hypergeometric distribution test.

2.10. Statistical Analysis. All samples were prepared independently in triplicate, and each sample was analyzed twice. The means and standard deviation were calculated by combining the results of these analyses. For the statistical analysis of data, SPSS 20.0 version software was used. The analysis of variance (ANOVA) and Duncan test with a 95% confidence level ($p < 0.05$) were taken as the basis for analyzing differences between treatments.

3. RESULTS AND DISCUSSION

3.1. Effect of LAB Fermentation on Bacterial Count and Physicochemical Properties in Orange Pomace. The change in pH value and titratable acidity of the orange pomace samples is shown in Figure 1a,b. At the end of *L.*

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Table 2. Changes in TPC and TAC Values during Lactic Acid Fermentation of Orange Pomace Samples[a]

	fermentation time (day)	TPC(mg GAE/100 g DW)	DPPH(mg TE/100 g DW)	CUPRA(mg TE/100 g DW)
OP	0	344.03 ± 35.74^{hi}	283.04 ± 18.42^{abc}	313.54 ± 40.59^{bcdefghi}
	1	448.81 ± 33.63^{bcdef}	231.40 ± 26.66^{d}	235.06 ± 15.38^{i}
	2	387.34 ± 30.03^{fgh}	277.41 ± 29.87^{abc}	296.63 ± 49.80^{efg}
	3	489.31 ± 35.58^{ab}	262.09 ± 26.80^{bcd}	365.05 ± 30.70^{abc}
	4	479.67 ± 34.64^{abc}	252.32 ± 12.18^{cd}	334.00 ± 20.87^{bcde}
FOP-LA	0	428.83 ± 53.73^{bcdefg}	266.73 ± 22.35^{bc}	298.61 ± 16.92^{efg}
	1	397.67 ± 24.00^{efgh}	267.54 ± 11.31^{bc}	304.94 ± 21.26^{efg}
	2	395.89 ± 17.05^{defgh}	273.98 ± 28.81^{abc}	315.48 ± 6.77^{ghi}
	3	467.15 ± 25.09^{bcd}	300.98 ± 24.18^{a}	423.01 ± 53.32^{a}
	4	491.29 ± 44.67^{ab}	276.19 ± 17.71^{abc}	387.39 ± 21.20^{ab}
FOP-LC	0	369.00 ± 17.20^{gh}	282.73 ± 19.30^{abc}	367.73 ± 28.14^{bcd}
	1	461.06 ± 20.85^{bcde}	286.65 ± 17.60^{abc}	370.24 ± 11.53^{bc}
	2	373.35 ± 38.68^{gh}	285.16 ± 18.13^{abc}	334.72 ± 28.70^{cdef}
	3	487.72 ± 18.87^{ab}	288.54 ± 16.95^{ab}	369.46 ± 14.86^{bc}
	4	412.89 ± 59.32^{defgh}	284.35 ± 16.85^{abc}	339.35 ± 30.67^{cde}
FOP-LP	0	292.71 ± 52.34^{i}	280.56 ± 9.81^{abc}	318.50 ± 34.17^{defg}
	1	416.76 ± 71.46^{cdefg}	277.00 ± 27.28^{abc}	254.97 ± 32.77^{hi}
	2	347.48 ± 14.10^{hi}	275.79 ± 25.08^{abc}	250.58 ± 16.48^{hi}
	3	540.67 ± 94.85^{a}	273.67 ± 27.72^{abc}	370.49 ± 20.20^{bc}
	4	424.22 ± 51.62^{bcdefg}	275.61 ± 20.24^{abc}	290.20 ± 33.06^{fgh}

[a]Data represent average quantities ± standard deviation (n = 3). Different letters in the columns represent statistically significant differences (p < 0.05).

acidophilus and *L. casei* fermentation, the pH value of the pomaces reached 3.8, whereas the pH of the *L. plantarum*-fermented pomaces dropped to approximately 3.6. The titratable acidity of the pomaces was increased after the fermentation process. However, the samples fermented with *L. plantarum* showed a drop in acidity during the first 72 h of fermentation, indicating that the bacteria may have used the acids in the pomaces as a source of energy early in the fermentation process to make other, less acidic compounds.[12]

The growth potential of *Lactobacillus* cultures in orange pomaces was assessed by monitoring their viable counts throughout the fermentation process (Figure 1c). The viable cell count of *L. plantarum*-fermented pomaces exceeded 8.0 log CFU/mL after 2 days of fermentation. In contrast, *L. casei*-fermented pomaces only reached 8 log CFU/mL after 4 days of incubation, while *L. acidophilus*-fermented pomaces did not reach a viability of 8 log CFU/mL during the fermentation period. The highest *Lactobacillus* viability was observed in the second (8.69 log CFU/mL) and third (8.62 log CFU/mL) days of fermentation with *L. plantarum* (Figure 1c). Results showed that the *Lactobacillus* cultures rapidly multiplied upon addition into pomaces. The decline in *Lactobacillus* growth was observed after 2 days of fermentation. The low pH level in pomaces may be directly linked to the observed reduction in viable cell numbers.[24] In accordance with our results, Cheng et al.[14] and Tang et al.[24] reported that a longer fermentation period caused reduction in LAB growth in blueberry and mulberry pomace.

In another study, Xu et al.[12] examined the number of colonies of four *Lactobacillus* spp at various intervals (0, 24, 48, 72, and 96 h) during the fermentation of citrus juices. They observed that the viability of the cultures initially increased and then decreased. Moreover, Yan et al.[35] examined the impact of fermentation duration on the viability of LAB and found that the number of viable bacteria initially increased and subsequently declined as the glucose level increased. The variations in growth performance among the tested LABs may be attributed to their varying levels of responsiveness to the fermentation substrate, such as the accumulation of organic acids, lower pH in the pomace, or complete utilization of nutrients.[12] Similar patterns have also been reported by Xu et al.[12] The cultures performing the best that were recommended as starters for the fermentation of orange pomaces were *L. plantarum* according to the findings of the viability test. All LAB exhibited a cell density over 7.0 log CFU/mL, suggesting that orange pomace is a favorable substrate for the cultivation of LAB bacteria. However, after 2 days of fermentation, the viability of LAB was lost.

3.2. Effect of LAB Fermentation on the Total Phenolic Content and Antioxidant Activity of Orange Pomace.

Microbial fermentation is a biological process that can increase the release of bioactive compounds in plants.[36] The TPC and TAC of orange pomaces with different fermentation periods and laboratories are shown in Table 2. The highest TPC (540.7 ± 94.9 mg GAE/100 g dry weight (DW)) was observed in orange pomaces fermented with *L. plantarum* for 3 days (p < 0.05).

The total phenolic content (TPC) of orange pomaces showed a substantial increase during the fermentation period. The total phenolic contents obtained in this work were lower than those reported by Shahram et al.[37] and Benelli et al.[38] but in accordance with those of Hu et al.[9] Hu et al.[9] revealed that the citrus pomace exhibited the maximum phenolic content at 6.75 and 7.05 mg GAE/g DW when fermented with *L. plantarum* M14 and *L. plantarum* P10, respectively. Various laboratories produce distinct enzymes throughout the fermentation process, and their impact on phenolic and flavonoid compounds varies as well.[39]

During fermentation, LAB release specialized enzymes called polyphenol oxidases.[40] These enzymes break down and convert phenolic compounds that are either bound or in a polymerized form. Thus, several studies suggested that the observed increase in the level of TPC could be attributed to the synthesis of glycosidases and polyphenol oxidases by

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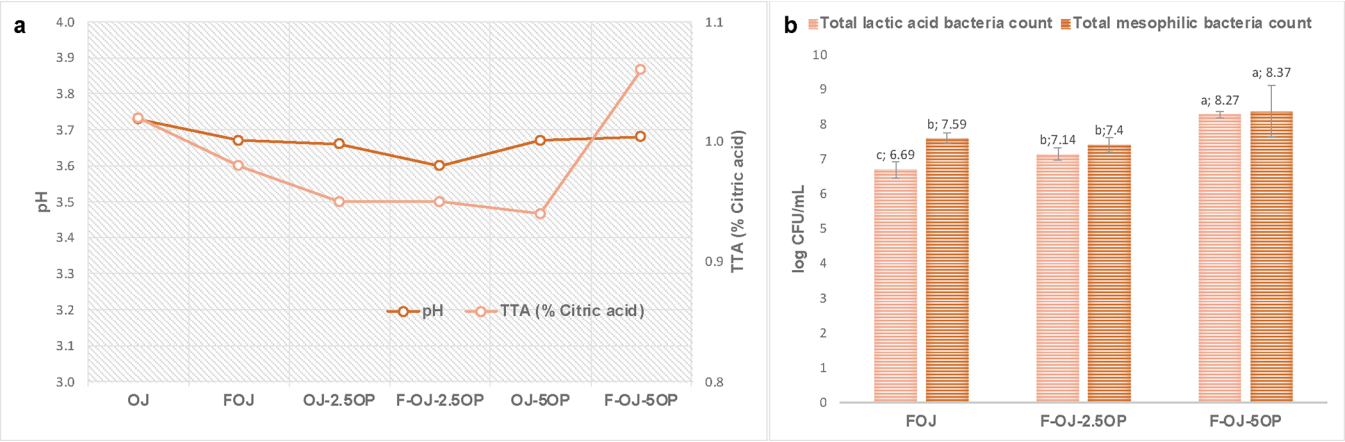

Figure 2. pH and TTA (a) values and total viable cell counts (b) of orange juice samples. Data represent average quantities ± standard deviation (n = 3). Different letters in each column (b) represent statistically significant differences ($p < 0.05$).

culture during fermentation.[41] The fermented orange pomace showed decreases first and then increases, indicating that TAC and TPC changed during fermentation; similar patterns have also been reported by Xu et al.[12] at different fermentation periods.

The principle regulating various antioxidant indexes varies; hence, a full evaluation of antioxidant activity/capacity must take into account numerous methods.[42] Only a small rise was observed with the DPPH assay; however, the CUPRAC method showed a greater increase. The DPPH and CUPRAC values in the orange pomaces were found to be the highest as 301.0 and 423.0 mg TE/100 g DW, respectively, with *L. acidophilus* in 3 day-fermented samples ($p < 0.05$), which are similar to the results of another relevant study.[9] There may be several variables that could contribute to the increase in TAC following fermentation. There is a correlation between the antioxidant ability and the amounts of phenolic compounds. Furthermore, novel antioxidant compounds might be produced during the process of fermentation.[42] The potential causes for the increase in antioxidant capacity are attributed to the hydrolytic ability to release active compounds during fermentation such as free phenolic compounds and organic acids. The observed increase in TPC stated in our study may perhaps be the primary factor contributing to the improvement in TAC. Hence, the utilization of LAB fermentation is a significant approach in the production of functional beverages.[39,43] The increase in DPPH antioxidant activity indicated that the OP fermented by *Lactobacillus* could be a suitable hydrogen donor, while the anomalous single electron of the nitrogen atom in DPPH$^{\bullet}$ was paired by accepting a hydrogen atom bearing an electron so as to be converted to the corresponding hydrazine.[24] Regarding the analysis of fermented pomace, the preparation of orange juices was carried out using *L. plantarum* fermentation for a duration of 3 days based on the results of *Lactobacillus* viability and TPC.

3.3. Effects of Fermentation and Pomace Fortification on the Viable Cell Count of Orange Juices. The pH values of all prepared juices after the fermentation process are shown in Figure 2a. However, there was no significant difference between the prepared juices. There was no change in the pH of orange juices, attributed to its reduced acidity. The pH did not change in fermented orange juices, and the value was found to be similar (3.46) in accordance with our results.[22] The total titratable acidity of the juices was the highest in the fermented juice containing 5% orange pomace. However, fermentation did not have an impact on the TTA of the orange juices with a 2.5% concentration.

The colony counts of fermented juices are listed in Figure 2b. Coliform bacteria were not detected in any of the prepared juices. The obtained viability exceeded the minimum requirement of 6−7 log CFU/mL for products containing probiotic microorganisms. The highest *Lactobacillus* count of 8.27 log CFU/mL was observed with fermented orange juice containing 5% orange pomace ($p < 0.05$). The reason for this could be that LABs have the ability to use orange pomace nutrients in juice to grow and produce high levels of acids.[16] In a study conducted by Dias et al.,[44] it was found that grape beverages supplemented with grape pomace had higher *Lactobacillus* viability after 24 h of fermentation. This could be attributed to the utilization of fruit pomace in the production of functional food, which has the potential to improve the stability and growth of probiotic cells.

According to *Lactobacillus* viability results, 2.5 and 5% pomace addition in orange juice significantly increased the *Lactobacillus* viability (Figure 2b). In line with our viability findings, Xu et al.[12] discovered that the average viability after 72 h of fermentation in orange juice was found as 8.0 log CFU/mL. There was no significant difference among the total mesophilic bacteria counts in FOJ and F-OJ-2.5OP. Thus, our findings indicated that orange pomace had no detrimental impact on the growth of *L. plantarum*.

3.4. Effects of Fermentation and Pomace Fortification on the Total Phenolic Content and Antioxidant Activity of Orange Juices. Today, it is well-known that long-term consumption of polyphenol-rich foods, such as fruits and vegetables, provides positive health effects, including protection against various diseases, and these health benefits are mainly attributed to their high phenolic content.[45] Therefore, the TPC of all fermented and unfermented orange juices with or without orange pomace was examined before and after *in vitro* gastrointestinal digestion to observe the effects of the fermentation process, the addition of orange pomace, and its addition levels on the final phenolic content (Table 3).

The results indicated that the addition of orange pomace increased the TPC values of both fermented and unfermented orange juice samples before and after *in vitro* digestion. The general trend in fermented samples was F-OJ-5OP > F-OJ-2.5OP > FOJ, like in the unfermented samples. More recently,

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Table 3. TPC Values of Initial Extract and Samples after *In Vitro* Digestion of Orange Juice[a]

code	TPC (mg GAE/100 g FW)	
	initial	after *in vitro* digestion
OJ	87.5 ± 0.5^{bc}	88.2 ± 3.3^{b}
FOJ	67.7 ± 5.0^{d}	80.0 ± 6.0^{b}
OJ-2.5OP	91.6 ± 1.9^{b}	96.6 ± 12.6^{ab}
F-OJ-2.5OP	82.4 ± 6.4^{c}	95.6 ± 4.6^{ab}
OJ-5OP	103.2 ± 4.2^{a}	112.1 ± 17.2^{a}
F-OJ-5OP	85.5 ± 5.4^{bc}	89.6 ± 15.7^{b}

[a]Data represent average quantities ± standard deviation (n = 3). Different letters in the columns represent statistically significant differences (p < 0.05).

Xu et al.,[46] showed that fermentation improved the contents of total phenolics and total flavonoids of orange juice after the addition of pomace. The increase in phenolic compounds in fermented orange juice containing orange pomace can mainly be attributed to the release of soluble conjugated phenolics or insoluble combined phenolics from the plant cell wall due to the ability of microorganisms to hydrolyze glycosylated phenolic compounds from orange peel, right along with the promotion of polyphenols in orange juice by the addition of pomace.[46] The TPC values were reduced in all fermented orange juice samples, with or without orange pomace, compared with their corresponding unfermented samples before and after *in vitro* digestion. For example, in the initial part, the TPC values of the fermented samples ranged from 67.7 to 85.5 mg GAE/100 g FW, while those of the corresponding unfermented samples ranged from 87.5 to 103.2 mg GAE/100 g FW. Similarly, a decrease in TPC was observed in apple juice[47] and sweet lemon juice[34] after LAB fermentation and in orange juice after alcoholic fermentation.[48] These observed reductions in the total phenolic content of fermented fruit juices may be due to the depolymerization of macromolecular phenolic compounds and/or the conversion of individual phenolic compounds resulting from the metabolic activity of LAB.[34,47] In contrast to our results, LAB fermentation improved the total phenolic content of strawberry juice,[49] jujube juice,[11] pomegranate juice,[50] citrus juice,[46] and citrus fruit extract.[51] Multari et al.[18] also reported that fermentation with *Lactobacillus rhamnosus* improved the phenolic content of "Washington navel" juice but reduced the phenolic content of "Tarocco" juice and concluded that the fermentation process was matrix-specific. Thus, it can be deduced that the effect of fermentation on the total phenolic content is highly dependent on the type and conditions of the fermentation process, the species and strains of microorganisms, and the variety of substrate. Moreover, all orange juice samples had higher levels of total phenolics after *in vitro* digestion than before. Fermented orange juice samples had between 4.8 and 18.1% higher TPC values after *in vitro* digestion than before, while unfermented orange juice samples had between 0.8 and 8.6% higher TPC values after *in vitro* digestion than before. Thus, this result indicates that fermentation provided positive effects on the total phenolic content of all orange juice samples with or without orange pomace during *in vitro* digestion, and this is in concordance with the study by Wang et al.,[16] which demonstrated that retention of phenolic compounds in blueberry juice was improved by LAB fermentation after *in vitro*-simulated digestion.

The beneficial effects of phenolic compounds on human health are mainly associated with their direct or indirect antioxidant activities.[52] Thus, the effect of LAB fermentation and enrichment with orange pomace on the bioactivity of antioxidants in the orange juice samples was also studied. The total antioxidant capacity of the samples was determined using the CUPRAC and DPPH methods, and the results are presented in Table 4. Based on the CUPRAC and DPPH assays, fortification with orange pomace provided improvement in the total antioxidant capacity of both fermented and unfermented orange juice samples before and after *in vitro* digestion.

For instance, in the initial part, the CUPRAC analysis results of F-OJ-5OP and F-OJ-2.5OP samples were 4.3 and 3.5 times that of the FOP sample, respectively. This improvement in the TAC values can be attributed to the release of phenolic compounds from orange pomace and the formation of new phenolic compounds with more potent antioxidant properties through transformation due to the activity of LAB. In concordance with this result, Xu et al.[46] also demonstrated a significant increase in the antioxidant activity of fermented orange juice containing pomace. Similar results were also observed in the initial part for fermented samples based on the DPPH assay. The TAC values were decreased in all undigested samples after fermentation based on the DPPH assay. Considering the CUPRAC results, the same trend as for the DPPH results was observed in all samples in the initial part, but fermentation increased the TAC values in digested samples without or with 2.5% orange pomace, except for orange juice with 5% orange pomace. A study in which the effects of fermentation on the bioactive compounds and bioactivity of different fruit juices (sweet orange, crown pear, tomato, watermelon, and pineapple) were investigated indicated that a

Table 4. TAC Values of Initial Extract and Samples after *In Vitro* Digestion of Orange Juice[a]

code	TAC (mg TE/100 g FW)			
	CUPRAC		DPPH	
	initial	after *in vitro* digestion	initial	after *in vitro* digestion
OJ	13.8 ± 2.6^{c}	29.7 ± 11.9^{c}	92.1 ± 3.8^{a}	44.9 ± 1.1^{cd}
FOJ	5.9 ± 2.6^{d}	32.5 ± 8.5^{bc}	57.3 ± 1.9^{b}	44.2 ± 1.2^{d}
OJ-2.5OP	21.8 ± 2.7^{b}	39.3 ± 3.4^{abc}	86.8 ± 7.5^{a}	46.9 ± 1.0^{bc}
F-OJ-2.5OP	20.5 ± 2.6^{b}	48.8 ± 1.4^{a}	64.6 ± 11.9^{b}	45.8 ± 1.4^{cd}
OJ-5OP	27.3 ± 4.0^{a}	49.2 ± 7.0^{a}	100.0 ± 7.8^{a}	52.3 ± 1.5^{a}
F-OJ-5OP	25.5 ± 1.0^{ab}	44.6 ± 4.8^{ab}	64.3 ± 6.4^{b}	48.6 ± 2.0^{b}

[a]Data represent average quantities ± standard deviation (n = 3). Different letters in the columns represent statistically significant differences (p < 0.05).

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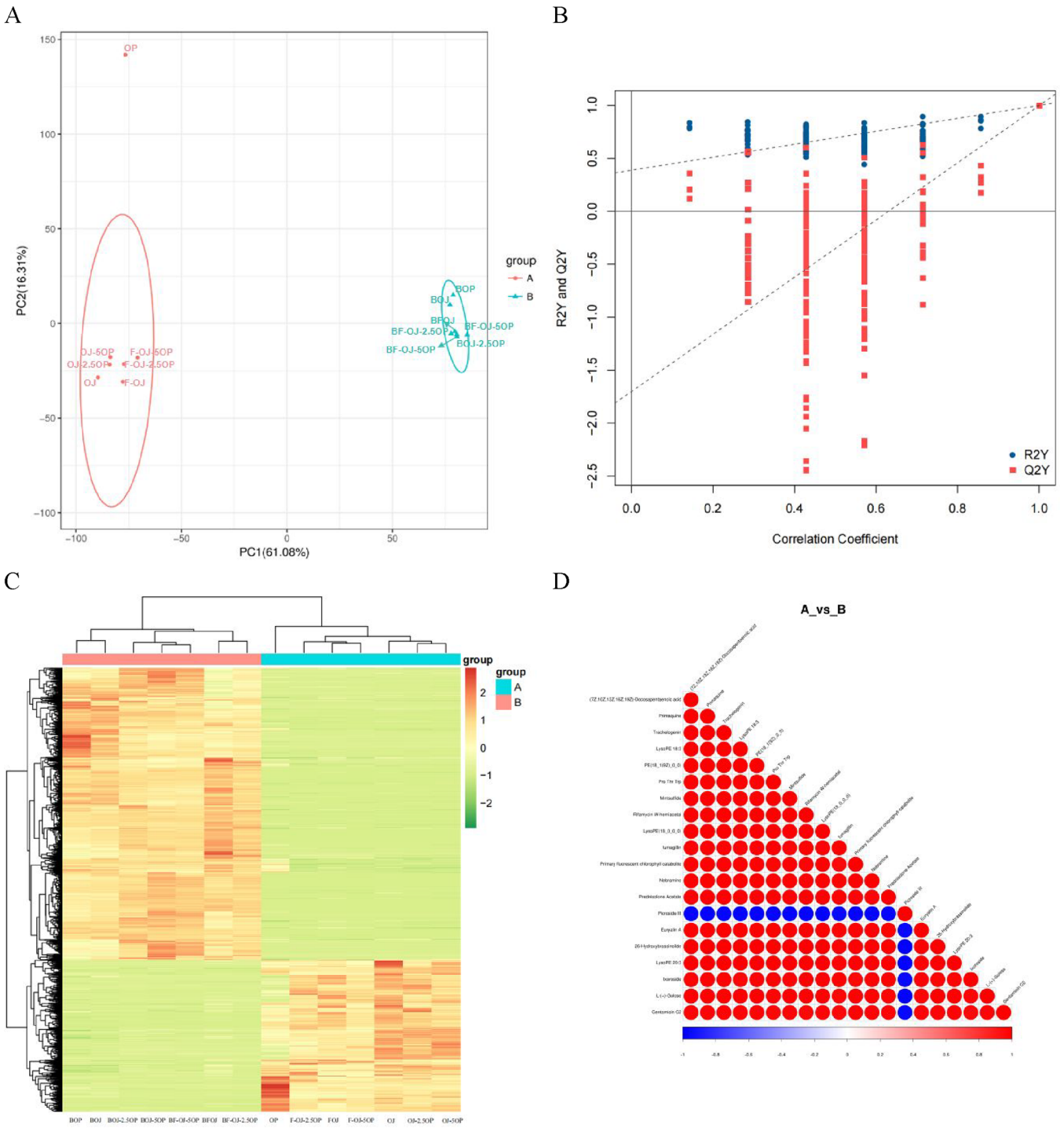

Figure 3. Multivariate statistical analysis before and after fermentation by *L. plantarum*: (A) PCA analysis before and after fermentation in positive and negative ion modes; (B) OPLS-DA analysis before and after fermentation in positive and negative ion modes; (C) heatmap of metabolite correlation clustering before and after fermentation in positive and negative ion modes; (D) correlation graphs of differential metabolites before and after fermentation in positive and negative ion modes. The letter B, brought to the head of the encoded samples, refers to the samples obtained at the end of the *in vitro* digestion.

significant decrease was obtained in both DPPH and ABTS scavenging capacities of all juices after 48 h of fermentation, with some exceptions, and the fermentation effects were highly dependent on the substrate.[53] In contrast, there are also several studies in the literature showing that the antioxidant capacity of kiwifruit juice,[16] strawberry juice,[49] loquat juice,[43] *etc.*, was improved by fermentation. Thus, these different results of fermentation can be mainly due to the differences in microorganisms, substrate, fermentation conditions, and also application of different methods. After *in vitro* digestion, the CUPRAC results of all samples were higher than those of the corresponding undigested samples. This can be attributed to the extension of biotransformation resulting from the concomitant formation of more potent antioxidants[54] and the good retention of bioactive compounds[16] during digestion. In contrast to the CUPRAC results, the TAC values of the

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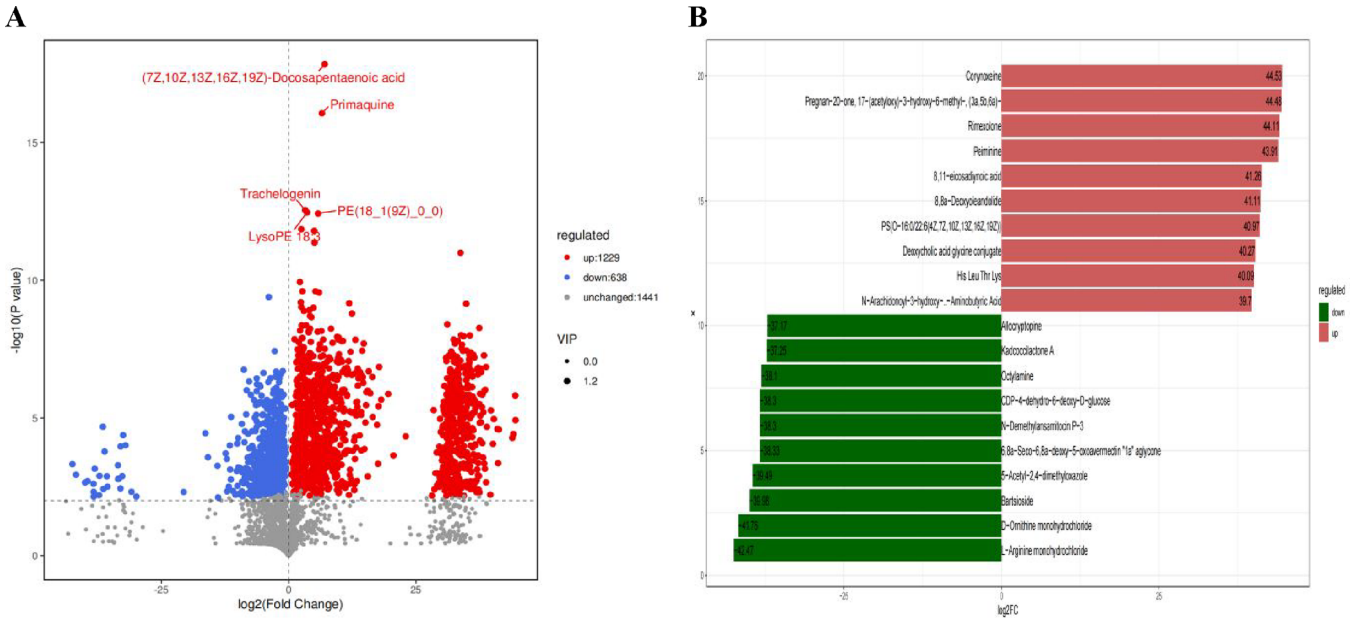

Figure 4. Differential statistical analysis before and after fermentation by *L. plantarum*: (A) volcano plot of differential metabolites before and after fermentation in positive and negative ion modes; (B) bar graph of the top 10 differential fold changes before and after fermentation in positive and negative ion modes.

digested orange juice samples were lower than those of their corresponding undigested samples based on the DPPH assay. This could be due to possible interactions among the food matrix, other dietary components, and the reagent used in the DPPH assay. Furthermore, the different mechanisms of the methods used and the effects of digestion conditions, such as pH, on the reagents should be considered. Unlike the definitely electron transfer (ET)-based CUPRAC assay, DPPH is a mixed mode (ET- and hydrogen atom transfer (HAT)-based) assay, and DPPH results are more severely affected from environmental conditions such as pH, daylight, and dissolved oxygen. Moreover, the steric accessibility of the DPPH reagent toward phenolic polymers may be different from that of the CUPRAC reagent acting as an outer-sphere electron transfer agent. Another factor that needs to be considered here is bioconversion; solid-state fermentation of agri-wastes may cause conversion of phenolics to products having less antioxidative ability, e.g., microbial conversion in the solid-state fermentation of ferulic acid may produce vanillin,[55] for which Kumar et al.[56] have shown that ferulic acid has higher antioxidant activity than vanillin in all tests performed.

Overall, the addition of orange pomace as a food ingredient to orange juices, followed by LAB fermentation, improved the total phenolic content and antioxidant capacity of the final products. This combination represents an effective strategy for developing new functional beverages with health-promoting benefits while contributing to the valorization of food waste and byproducts.

3.5. Untargeted LC-QTOF Metabolomic Profiling: Effects of Fermentation and Simulated *In Vitro* Digestion on the Metabolites of Orange Juices. *L. plantarum*, widely applied in food fermentation, can alter the chemical composition of the host environment through its metabolic pathways, producing or consuming specific metabolites.[57] These changes may reflect on the nutritional value, bioactivity, flavor, and food safety.[58] The metabolites upregulated and downregulated after fermentation by *L. plantarum* not only impact the nutritional value and bioactivity of the product but may also modify its flavor, texture, and shelf life.[59] For instance, the increase in antioxidant alkaloids may enhance the product's health benefits, while reducing the content of specific organic amines could optimize flavor and taste.[60] In-depth studies on the mechanisms and impacts of specific metabolite changes can reveal more about the effects of microbial fermentation on food chemical components, providing a scientific basis for the development of fermented food.[61]

From the metabolomics data, we can see that the addition of orange pomace also has a great effect on the function of orange juice. The content of prostaglandin H2 in orange juice increased significantly after adding orange pomace and increased with the increase in adding orange pomace. The content of anamorelin decreased, and the content of OJ-2.5OP was lower than that of OJ-5OP. The addition of orange pomace also increased the content of glycerol 1-myristate and (−)hydroxydihydrobovolide in samples, and the content of orange juice with 5.5% orange pomace addition was much higher than that with 2.5% addition.

The experimental results showed that the addition of orange pomace can improve the antioxidant capacity of orange juice, which is a valuable source of total phenols in natural foods. The addition of fruit pomaces or byproducts to fruit juice has significant health benefits, and the experimental results provide a new idea for the utilization of byproducts in the food industry in the future.

3.5.1. Gene Expression Quantitative Analysis. As illustrated through LC−quadrupole time-of-flight (LC-QTOF) analysis, a total of 3308 metabolites were identified, and their biological roles were annotated using the KEGG database.[62] Based on this, both unsupervised principal component analysis (PCA) and supervised OPLS-DA were conducted. From the PCA score plots (Figure 3A), it can be observed that the samples naturally cluster into two distinctly separated groups, the red sample group (Group A) and the blue sample group

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Table 5. Results of Differential Metabolite Area-Under-the-Curve Analysis

number	name	FC	*p*-value	VIP	regulated
1	prostaglandin H2	−2.83023	0.00378	1.03103	down
2	gibberellin A53	−7.95511	0.00041	1.18834	down
3	neoruscogenin	−1.70270	3.84675	1.24077	down
4	anamorelin	−8.24214	0.00072	1.16394	down
5	octadecanoic acid pyrrolidide	35.88659	7.34075	1.28823	up
6	(−)-hydroxydihydrobovolide	−3.50754	0.00579	1.00730	down
7	cannabigerolate	35.87033	0.00090	1.15147	up
8	glycerol 1-myristate	−3.96450	0.00072	1.15576	down
9	ergotamine	32.53263	3.29829	1.29599	up
10	2-hydroxy-6-oxoocta-2,4,7-trienoate	−7.65146	0.00292	1.07468	down

(Group B), corresponding to the prefermentation and postfermentation sample groups, respectively. PC1 accounts for 61.08% of the variance, explaining the differences in the compositional changes of oranges before and after fermentation with *L. plantarum* with a cumulative contribution rate of 77.38% for PC1 and PC2. Furthermore, supervised OPLS-DA analysis (Figure 3B) shows the sample points distributed in different regions, indicating significant differences before and after fermentation by *L. plantarum*, with the model being stable and reliable without overfitting. Following PCA analysis, a heatmap representing the metabolites shows differences before and after fermentation by *L. plantarum* (as shown in Figure 3C), with clear separation between pre- and postfermentation and obvious upregulation of metabolites. As depicted in Figure 3D, among the positively and negatively correlated metabolites, 20 metabolites with the strongest differential correlation were selected for illustration, showing strong correlations. Among these, the metabolite picroside III displayed a strong negative correlation with the rest of the metabolites.

The data showed that the content of picroside III decreased after LAB fermentation; picroside III has anti-inflammatory and antiapoptotic effects, is an antioxidant, and has neuroprotective effects. Although the content of picroside III decreased after LAB fermentation, the change trend of other metabolites was the same, which confirmed the experimental results of 3.2 and 3.3; the total phenol content in orange juice increased after LAB fermentation, and the antioxidant capacity was enhanced.

3.5.2. Differential Gene Screening. VIP reflects the impact and explanatory power of each variable on the classification and discrimination of sample groups. Generally, a VIP value greater than 1 is considered to indicate that the variable significantly contributes to the separation between groups. Based on a criterion of VIP value >1 and *p*-value <0.05, according to the OPLS-DA analysis results, a total of 3308 metabolomic components were screened (among which 1867 showed significant differences, with 1229 upregulated and 638 downregulated), detailed in Figure 4.

Using open metabolomics databases such as KEGG, HMDB, LIPID MAPS, *etc.*, the top 10 compounds in terms of single-dimensional statistical content were selected as the metabolites with significant differences, as shown in Table 5.

This study specifically conducted identification analysis on the top 10 upregulated and downregulated differential components, with results detailed in Figure 3B. Among these 20 components, nos. 1−10 are the dominant components after fermentation by *L. plantarum*, mainly including 3 types of steroidal compounds, 2 types of alkaloids, lipids and fatty acids, peptides and amino acid derivatives, and plant derivatives. Nos. 11−20 are the dominant components before fermentation by *L. plantarum*, mainly including 2 types of amino acids and their derivatives, 2 types of lactone compounds, 2 types of organic compounds, 2 types of alkaloids, amino acids and peptides, and nucleoside ribose sugar derivatives.

To filter out important metabolites and visually present the changes before and after fermentation by *L. plantarum*, we set criteria for metabolite screening and generated volcano plots, as detailed in Figure 3A. In this study, several marked compounds such as (7*Z*,10*Z*,13*Z*,16*Z*)-docosapentaenoic acid, primaquine, trachelogenin, PE(18-1(9*Z*)-0-0), and LysoPE (18:3) stood out significantly in terms of their significance (*p*-values) and fold changes.

After qualitative and quantitative analysis of the detected metabolites, we first compared the fold changes in the quantitative information on metabolites across different groups. For analysis, the top 10 compounds with respect to their content were selected, as shown in Figure 4B. After fermentation by *L. plantarum*, some new metabolic products were generated, enriching the chemical diversity of the orange. These include phenolics and specific organic acids with strong antioxidant capabilities. The downregulated metabolites mainly reflect the significant changes in the internal environment of the orange during fermentation and the biotransformation pressure exerted by *L. plantarum*. These fermentation-induced changes may help improve the taste of the orange, increase its nutritional value or bioactivity, and have significant implications for the food industry and the development of health products.

For the phenomenon of metabolites upregulated after fermentation of oranges by *L. plantarum*, the upregulation mechanisms of several key metabolites and their potential impacts can be analyzed in more detail. The upregulation of corynoxeine, an alkaloid, during fermentation may be related to *L. plantarum*'s influence on specific enzyme activities.[63] This microbe may promote the expression of specific biosynthetic enzymes in oranges such as hydroxylases and methyltransferases. Additionally, the fermentation environment might activate secondary metabolic pathways in oranges, leading to more efficient conversion of corynoxeine's precursors into the target compound.[64] Corynoxeine possesses significant bioactivities, including neuroprotective and anti-inflammatory effects.[65] Its increase in fermented products could provide additional health benefits to consumers, especially potential support for neurological health and anti-inflammatory effects.

The upregulation of peiminine might originate from *L. plantarum*'s effect on specific enzymes in oranges, leading to the enhanced activity of alkaloid biosynthesis pathways. Peiminine typically exists in plants at low concentrations,

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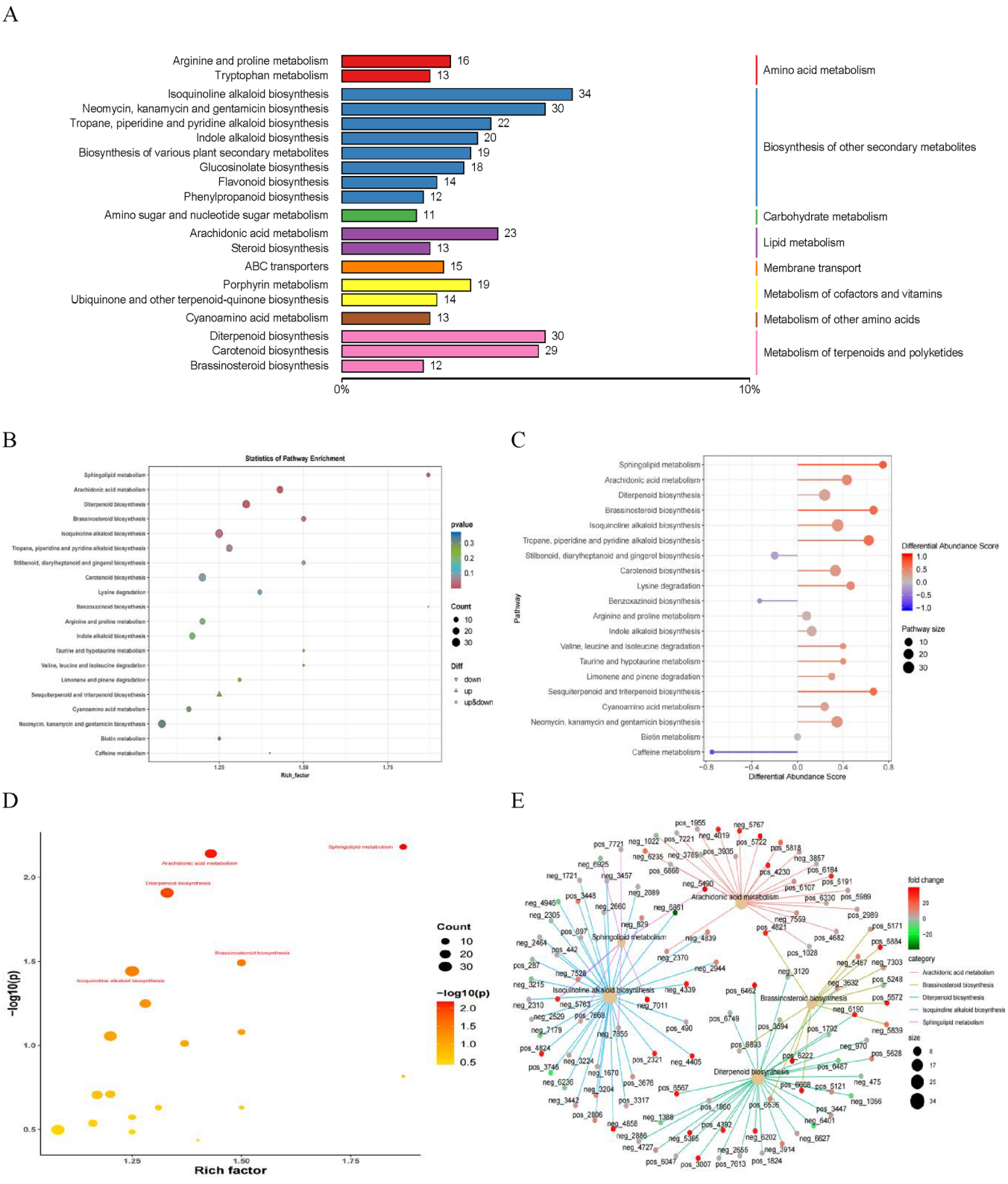

Figure 5. Differential metabolites' KEGG functional annotation and enrichment analysis: (A) top 20 pathway entries with the most annotated differential metabolites; (B) enrichment dot plot; (C) differential abundance score plot of differential metabolites; (D) KEGG enrichment factor bubble chart of differential metabolites; (E) enrichment network diagram.

and its upregulation reflects that microbial fermentation can promote the synthesis of certain secondary metabolites. Peiminine is known for its potential anti-inflammatory, analgesic, and antitumor effects.[66] Its increased content could enhance the health-related value of fermented orange products, especially in terms of anti-inflammatory and analgesic effects. Besides potential health benefits, the increase in peiminine could also affect the product's flavor characteristics, which requires further study through sensory evaluation.

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The downregulation of allocryptopine might be due to *L. plantarum* using this compound as an energy source or substrate for specific metabolic pathways, leading to its consumption during fermentation.[67] Moreover, changes in environmental pH could affect allocryptopine's stability, promoting its breakdown.[68] Although allocryptopine has certain bioactivities, its downregulation could reduce the bitterness in fermented orange products, thereby improving taste and flavor.[69] Octylamine, an organic amine, might be downregulated due to *L. plantarum*'s metabolic action, utilizing it as a nitrogen source, or due to chemical instability under acidic conditions, leading to its degradation.[70] High concentrations of octylamine could adversely affect the flavor of food; its downregulation helps to improve the sensory quality of fermented products.

3.5.3. Differential Gene Function Annotation and Enrichment Analysis. The annotation results of the significantly differential metabolites in the KEGG pathways were categorized and displayed. The information on the KEGG pathway classification was sourced from the KEGG database.[71] The classification diagram is shown in Figure 5A, where differential metabolites are primarily enriched in amino acid metabolism, the synthesis of other secondary metabolites, carbohydrate metabolism, and lipid metabolism. Changes in metabolites to various extents were also observed in membrane transport, cofactors, and vitamin metabolism, as well as terpenoid and polyketide metabolism. A comparison before and after fermentation by *L. plantarum* reveals that the pathway with the most changes in metabolites within amino acid metabolism is arginine and proline metabolism with changes in 16 metabolites. This indicates that fermentation might significantly affect the metabolism of these amino acids. In the biosynthesis of other secondary metabolites, this pathway includes various biosynthetic pathways of different plant alkaloids, such as isoquinoline and tropane alkaloids, showing a higher number of changed metabolites. In carbohydrate metabolism, there are 11 changed metabolites in the amino sugar and nucleotide sugar metabolism pathway. In lipid metabolism, the arachidonic acid metabolism pathway has 21 changed metabolites, indicating that the metabolism of sugars and fatty acids was also affected during fermentation.

Based on the results of metabolite differences, a KEGG pathway enrichment analysis was performed. In the KEGG pathway enrichment bubble chart, the *x*-axis represents the rich factor for each pathway, the *y*-axis lists various pathway names (arranged in descending order of *p*-values), the color of the dots reflects the *p*-value size—with redder colors indicating more significant enrichment—and the size of the dot represents the number of differential metabolites enriched.[71] This study displays the top 20 pathways ranked by *p*-value from the smallest to the largest.

According to Figure 5B, a comparison before and after fermentation by *L. plantarum* shows that sphingolipid metabolism displays a higher enrichment factor, indicating that this pathway is significant. Diterpenoid biosynthesis shows a high number of significantly enriched genes under these conditions, suggesting that these pathways are significantly enriched and possibly closely related to the biochemical changes in oranges after fermentation.

Figure 5C presents the differential abundance analysis, typically used to compare changes in metabolite abundance across metabolic pathways under different conditions. The *x*-axis represents the degree of change in metabolite abundance within each metabolic pathway.[72] Higher scores indicate a more significant increase in the relative abundance of the metabolites under experimental conditions. Significant increases in metabolite abundance in pathways such as sphingolipid metabolism, brassinosteroid biosynthesis, and sesquiterpenoid and triterpenoid biosynthesis may indicate that these pathways are activated during fermentation, possibly due to enhanced metabolic activity under fermentation conditions or accumulation caused by the utilization and transformation of metabolites in these pathways by *L. plantarum*.

From Figure 5D, it can be indicated that some pathways, such as arachidonic acid metabolism and sphingolipid metabolism, show higher enrichment factors and significance in oranges after fermentation, meaning that a relatively larger number of genes in these metabolic pathways exhibit significant expression changes. This indicates that these pathways are more active during fermentation, which may be related to the nutritional characteristics of oranges or the biochemical changes mediated by *L. plantarum*. The enrichment network diagram in Figure 5E displays the correlations and degrees of change among metabolites in different metabolic pathways with each node representing a specific metabolite. Large red nodes in the arachidonic acid metabolism pathway indicate significant upregulation of related metabolites after fermentation; similarly, larger purple nodes in the sphingolipid metabolism pathway suggest increased activity in this pathway during fermentation.

In summary, the enrichment analysis of oranges before and after fermentation by *L. plantarum* reveals key biochemical changes, further exploring how fermentation affects the nutritional value, bioactivity, and final product quality of oranges, especially in pathways such as arachidonic acid metabolism and sphingolipid metabolism, which are related to the health benefits of fermented orange products.

Through the optimization of the OPLS-DA model, this study conducted multivariate data analysis to explore the impact of fermentation by *L. plantarum* on the metabolomic profile of oranges. The robustness of the OPLS-DA model was validated through 200 permutation tests, ensuring the credibility of the analysis results. In the aspect of differential significance analysis, a combination of fold change (FC), *p*-value, and variable importance in projection (VIP) was utilized to filter metabolites with significant changes. Fold change quantifies the extent of change in metabolite abundance under experimental conditions, while the *p*-value assesses the statistical significance of these changes. The VIP value reflects the contribution of metabolites in the model and their ability to distinguish between different experimental groups. A metabolite is considered significantly changed only if it has an FC greater than 1, a *p*-value less than 0.01, and a VIP value greater than 1.[73]

A hypergeometric distribution test was used to perform the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on significantly changed metabolites, thereby revealing the profound impact of fermentation treatment on the metabolic activities of oranges. The enrichment analysis results unveiled which metabolic pathways were significantly affected during the fermentation process, thus providing insights into how *L. plantarum* regulates the biochemical processes in oranges. These findings offer a solid statistical foundation for subsequent biological interpretation and functional research.

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4. CONCLUSIONS

The utilization of solid-state fermentation with *Lactobacillus* demonstrated a substantial enhancement in the total phenolic content and total antioxidant capacity of orange pomace samples. The impact of fermentation was dependent on the use of different strains and fermentation period. Functional orange beverages were designed by preparing fermented and unfermented orange juices fortified with orange pomace at different ratios. The addition of orange pomace to orange juice significantly increased prostaglandin H2 and enhanced antioxidant capacity with more pronounced effects at increased pomace concentrations. Postfermentation with *L. plantarum* led to positive changes in key bioactive compounds such as corynoxeine and phenolics. Metabolomics analysis revealed significant changes in amino acid, sphingolipid, and fatty acid metabolism, improving the nutritional profile, bioactivity, and health benefits of fermented orange juice products. Orange pomace addition had an impact on the *Lactobacillus* viability. The total phenolic content and antioxidant capacity of all orange juice samples were found to be reduced after fermentation. However, the presence of orange pomace led to an improvement in the total phenolic content and antioxidant capacity. The fermentation process greatly boosted important biochemical pathways including amino acid metabolism, lipid metabolism, and the synthesis of other secondary metabolites. The upregulation of these metabolites not only reflects the significant changes in the chemical composition of oranges brought about by fermentation with *L. plantarum* but also highlights the potential of the fermentation process in enhancing the functionality of food products. Thus, enrichment of orange juice with orange pomace, especially in a fermented form, could be a good alternative to develop new functional foods and valorization of consumable food waste and byproducts.

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Notes

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