separations

MDPI

Article

Optimization of Fermentation Conditions for Ellagic Acid Production from *Juglans mandshurica* Fermentation Using *Weissella confusa*: Evaluation of Antioxidant Activity

Min Hong [1,2], Jong-Hui Kim [1,3], Da-Hye Choi [4], Joon-Hee Han [1], Jin-Hee Han [5] and Tae-Hyung Kwon [1,6,*]

1 Institute of Biological Resources, Chuncheon Bioindustry Foundation, Chuncheon 24232, Republic of Korea; fabre_min@cbf.or.kr (M.H.)
2 Department of Plant Medicine, College of Agriculture and Life Sciences, Kangwon National University, Chuncheon 24341, Republic of Korea
3 Department of Physiology, School of Medicine, Kangwon National University, Chuncheon 24341, Republic of Korea
4 Crop Research Div., Gangwon State Agricultural Research & Extension Services, 40 Jangbon-gil, Sinbuk-eup, Chuncheon 24203, Republic of Korea
5 Department of Tropical Medicine, School of Medicine, Kangwon National University, Chuncheon 24341, Republic of Korea
6 Department of Barista and Bakery, Gangwon State University, Gangneung 25425, Republic of Korea
* Correspondence: taehyung0218@naver.com; Tel.: +82-33-258-6991

Abstract

This study aimed to optimize fermentation conditions for maximizing ellagic acid accumulation/release from *Juglans mandshurica* (Manchurian walnut) fruit extract using *Weissella confusa*. A 20% ethanol extract was fermented across a central composite design spanning temperature (26.59–43.41 °C), pH (2.98–8.02), and time (3.91–24.09 h), and response surface methodology was used to determine the optimal conditions. The predicted optimum was 36.61 °C, initial pH 6.70, and 24.09 h, under which the ellagic acid yield reached 64.05 μg/mL, experimentally validated at this single optimum condition using three independent replicate fermentations (n = 3), giving 64.04 ± 0.95 μg/mL by HPLC. Antioxidant activity at the optimized condition was evaluated using total phenolic content, DPPH, and ABTS radical scavenging assays. The fermented extract showed markedly enhanced radical scavenging activity, with DPPH inhibition reaching 85.47% at 400 μg/mL and ABTS exceeding 99.99%, while the total phenolic content was 141.41 ± 2.32 mg gallic acid equivalents (GAE)/g. These results indicate that *W. confusa*-mediated fermentation enhances the ellagic acid content and antioxidant potential of *J. mandshurica* extract, presumably through enzymatic hydrolysis of ellagitannins involving tannase and β-glucosidase, although these enzyme activities were not directly measured. This approach offers a sustainable strategy for developing functional ingredients with natural antioxidant properties for food and nutraceutical applications.

Keywords: ellagic acid; *Juglans mandshurica*; fermentation; antioxidant; biotransformation

Check for updates

Academic Editor: Shun Yao

Received: 4 September 2026
Revised: 29 September 2026
Accepted: 29 September 2026
Published: 1 October 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

1. Introduction

Optimization of bioprocesses such as fermentation often requires careful coordination of multiple variables, including temperature, pH, and reaction time. These factors interact in a complex, nonlinear manner that can significantly influence the outcome of biochemical transformations. Traditional "one-factor-at-a-time" approaches are insufficient for identifying optimal conditions in such multivariable systems, leading to inefficient experimental

designs and suboptimal results. For example, optimizing fermentation conditions using the OFAT method can miss synergistic interactions between variables such as temperature and pH, which are captured more effectively by multivariate approaches like response surface methodology (RSM). By contrast, response surface methodology (RSM) provides a robust statistical framework for modeling and optimization of processes affected by multiple parameters. By using techniques such as central composite design (CCD) and Box–Behnken design, RSM allows for the development of predictive mathematical models and efficient exploration of factor interactions [1–4]. RSM has been widely adopted in food science and biotechnology for improving extraction efficiency, fermentation yield, and functional compound production [2–4]. This approach not only enables process optimization but also reduces the number of experimental trials, conserving time, cost, and resources [1,3]. Furthermore, RSM's applicability to both empirical and mechanistic modeling makes it particularly valuable for developing scalable and reproducible fermentation processes [2,4]. RSM has also been applied recently to optimize lactic acid bacteria fermentation of plant materials to enhance bioactive compound yields and antioxidant activity [5,6].

Fermentation, and particularly microbial fermentation using lactic acid bacteria (LAB), has emerged as a promising method for enhancing the nutritional and functional value of plant-derived materials. LAB can produce diverse enzymes such as tannase and β-glucosidase, which can catalyze the hydrolysis of high-molecular-weight polyphenols into more bioavailable forms [7–10]. Recent studies have shown that extracellular tannase-producing *Lactiplantibacillus plantarum* strains hydrolyze galloylated ellagitannins from fruit extracts [11], and that *Weissella* species are attracting increasing attention for their biotechnological and probiotic potential [12]. LAB, including genera such as *Weissella*, *Lactobacillus*, and *Bifidobacterium*, have been recognized for their Generally Recognized As Safe (GRAS) status and functional benefits in food systems [9,10,13]. Additionally, the antioxidant properties of plant-based foods have been shown to improve after fermentation with LAB, making this approach particularly attractive for the development of functional food ingredients [10,13].

Juglans mandshurica, commonly known as the Manchurian walnut, is a deciduous tree native to East Asia and has been traditionally used for both medicinal and nutritional purposes [14]. The fruit of *J. mandshurica* is particularly rich in ellagitannins, a class of high-molecular-weight polyphenols, which are known for their antioxidant, anti-inflammatory, and anticancer properties [15]. However, owing to their polymeric structure, ellagitannins have poor bioavailability and require hydrolysis to release ellagic acid, which is the major bioactive metabolite responsible for most of their biological effects [15,16]. Conventional hydrolysis methods such as acid or thermal treatment are often harsh and may degrade sensitive compounds and generate undesirable byproducts [16]. Therefore, microbial fermentation has been proposed as a mild and eco-friendly strategy to achieve the bioconversion of ellagitannins into ellagic acid [7,17].

Previous studies have demonstrated the bioavailability of ellagic acid following the consumption of ellagitannin-rich foods, including pomegranate juice [18]. Additionally, the metabolism of ellagic acid by gut microbiota has been linked to the formation of urolithins, which show health benefits such as cancer cell proliferation inhibition [19,20]. Lactic acid bacteria have also been shown to transform punicalagin from a pomegranate extract into ellagic acid and urolithins [7], and solid-state fermentation of walnut press cake with *Aspergillus oryzae* and *Rhizopus oligosporus* has been reported to liberate ellagic acid from walnut ellagitannins [21], although a later study using a different batch of press cake observed no increase [22]. These findings highlight the importance of the development of efficient strategies for the conversion of ellagitannins into bioavailable forms such as ellagic acid in food and nutraceutical applications [15–17,19,20].

Based on the above considerations, this study aims to optimize the fermentation process of *J. mandshurica* fruit extract using *Weissella confusa*, a LAB strain known for its enzymatic activity in polyphenol-rich substrates. By applying RSM within a CCD framework, we sought to determine the optimal fermentation conditions, namely temperature, pH, and incubation time, that maximize ellagic acid yield. Furthermore, antioxidant activity and total polyphenol content of the fermented extract were evaluated to assess its functional potential [8–10,13].

The novelty of this study lies in the strain–substrate combination: *W. confusa*, which is well documented for lactic acid and exopolysaccharide production but not for ellagitannin bioconversion, was applied for the first time, to our knowledge, to *J. mandshurica* fruit, an underused regional resource, for ellagic acid accumulation. The process was optimized and experimentally validated using RSM within a CCD framework (relative error 0.016%).

2. Materials and Methods

Materials and reagents. Ellagic acid hydrate (technical grade, Cat. No. 372749), gallic acid, DPPH, potassium persulfate, Folin–Ciocalteu phenol reagent, sodium carbonate, dimethyl sulfoxide (DMSO), butylated hydroxyanisole (BHA), and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). ABTS was obtained from Wako Pure Chemical Industries (Osaka, Japan). MRS broth was purchased from BD Difco (Sparks, MD, USA). Ethanol (95% HPLC grade for extraction and 99% for sample dilution), hydrochloric acid, and sodium hydroxide were purchased from Duksan Pure Chemical (Ansan, Republic of Korea), and acetonitrile and acetic acid (HPLC grade) were purchased from Merck (Darmstadt, Germany). PVDF syringe filters (0.45 μm) were purchased from Whatman (Maidstone, UK). *W. confusa* CBF20-N-001 (KCTC18992P) was deposited at the Korean Collection for Type Cultures (KCTC; Jeongeup, Republic of Korea). *J. mandshurica* fruits were harvested in Gangwon-do, Republic of Korea, in July 2024. The HPLC system (Shimadzu, Kyoto, Japan) was equipped with a ProntoSIL C18-ace-EPS column (Bischoff Chromatography, Leonberg, Germany), and absorbance was measured using a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA, USA).

2.1. Sample Preparation

Juglans mandshurica fruits were harvested from Gangwon-do, Republic of Korea. After removing the outer shells, approximately 100 g of kernels were obtained and used for a single extraction experiment (n = 3). The fruits were sliced and extracted once with 95% HPLC-grade ethanol diluted to 20% (v/v) at a 1:7 (w/v) ratio at 25 °C for 6 h under constant shaking at 110 rpm. Aqueous ethanol has been used to extract phenolic compounds from walnut (*Juglans*) green husk [23]. The extract was filtered through a 0.45 μm membrane, concentrated under reduced pressure using a rotary evaporator (40 °C, 100 mbar, 5 h), and freeze-dried (−80 °C, 0.1 mbar, 2 days) to obtain a powder form. The dried extract was stored at 4 °C in an airtight desiccator until use in fermentation experiments. *Weissella confusa* CBF20-N-001 was isolated from human feces and identified as *W. confusa* based on 16S rRNA gene sequence analysis. The strain has been deposited at the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology, under accession number KCTC18992P (deposit date: 7 April 2022).

2.2. RSM Design

2.2.1. Experimental Design

To optimize the fermentation conditions for ellagic acid accumulation, CCD, which is a type of RSM, was carried out using Minitab 16 software (Minitab Inc., State College, PA, USA). In our preliminary experiments, *W. confusa* was cultured alone under different

temperature, pH, and incubation time conditions. The results showed that the highest bacterial growth rate was observed at 35 °C, pH 7, and 24 h. Based on the preliminary studies, three independent variables were selected: fermentation temperature (X_1, 30–40 °C), pH (X_2, 4–7), and fermentation time (X_3, 8–20 h). Each variable was encoded at three levels (−1, 0, 1), and a total of 20 experimental runs were conducted (Table 1). The fermentation experiments were performed according to the randomized order suggested by the design matrix. The actual values of the variables (X_1, X_2, and X_3) were converted to coded values (T, p, and t) using Equation (1):

$$T = \frac{X_1 - 35}{5}, p = \frac{X_2 - 5.5}{1.5}, t = \frac{X_3 - 14}{6} \quad (1)$$

where X_1, X_2, and X_3 are the actual values of temperature (°C), pH, and time (h), respectively.

Table 1. Independent variables for fermentation of 20% ethanol extract of *J. mandshurica* fruit by *W. confusa* and their investigated levels in coded and actual form.

Independent Variable	Units	Levels				
		−1.682	**−1**	**0**	**+1**	**+1.682**
Temperature	°C	26.59	30	35	40	43.41
Initial pH	pH	2.98	4	5.5	7	8.02
Fermentation Time	h	3.91	8	14	20	24.09

Central composite design.

For inoculum preparation, the *W. confusa* stock, stored at −80 °C in an ultra-low temperature freezer, was inoculated into MRS broth [24] at a 10:1 (MRS broth:stock, *v*/*v*) ratio and pre-cultured at 38 °C for 24 h with shaking under aerobic conditions. The cells were then harvested by centrifugation and resuspended in fresh MRS broth, and the cell density was adjusted to OD_{600} = 0.01 with MRS broth prior to inoculation. For fermentation, 1 g of *J. mandshurica* extract was mixed with the inoculated MRS broth to a final volume of 15 mL in a 50 mL tube closed with an air-permeable rubber stopper, and the cultures were incubated with shaking at 150 rpm under aerobic conditions at the temperature and for the time specified by the experimental design (26.59–43.41 °C and 3.91–24.09 h; Table 2). The initial pH of the fermentation medium was adjusted to the value required by the experimental design (pH 2.98–8.02) using 1 N HCl or 1 N NaOH before inoculation. The pH was not controlled during fermentation; therefore, the pH values in this study refer to the initial pH. Fermentation was carried out under different conditions of temperature, pH, and time according to the experimental design. Samples were collected at designated time points, and ellagic acid content was analyzed to evaluate ellagic acid accumulation.

Table 2. Experimental variables for optimization of ellagic acid content of 20% ethanol extract from *J. mandshurica* fruit after fermentation by *W. confusa* based on CCD using RSM.

Exp. No.	Run Order	Fermentation Parameters			Ellagic Acid
		Temperature, °C	**Initial pH**	**Time, h**	**μg/mL**
1	16	26.59	5.5	14	35.51
2	13	30	4	8	36.59
3	17	30	4	20	43.48
4	1	30	7	8	35.45
5	10	30	7	20	54.39
6	18	35	2.98	14	30.06
7	14	35	5.5	3.91	33.09
8	3	35	5.5	14	47.28

Table 2. *Cont.*

Exp. No.	Run Order	Fermentation Parameters			Ellagic Acid
		Temperature, °C	Initial pH	Time, h	µg/mL
9	4	35	5.5	14	45.24
10	5	35	5.5	14	45.21
11	8	35	5.5	14	46.29
12	12	35	5.5	14	47.00
13	15	35	5.5	14	47.13
14	7	35	5.5	24.09	59.25
15	6	35	8.02	14	31.47
16	20	40	4	8	33.56
17	19	40	4	20	42.17
18	9	40	7	8	33.66
19	2	40	7	20	55.67
20	11	43.41	5.5	14	38.92

2.2.2. Results Analysis

The concentration of ellagic acid (µg/mL) was used as the response variable and was subjected to multiple regression analysis to derive the prediction equation. Analysis of variance (ANOVA) was conducted to evaluate the significance and fitness of the model. The resulting regression equation was evaluated using the coefficient of determination (R^2) to assess the agreement between the predicted and actual values. To visualize the effects of variable interactions, three-dimensional response surface plots were generated by fixing one factor at its central value (temperature = 35 °C, pH = 5.5, time = 14 h), while varying the other two factors (Table 2).

2.3. HPLC Analysis

2.3.1. Chromatographic Conditions

HPLC (Shimadzu, Kyoto, Japan) was performed to analyze the main compounds in *J. mandshurica* walnut ethanol extract. Analysis of ellagic acid was carried out using a ProntoSIL C18-ace-EPS HPLC column (4.6 × 250 mm, 5 µm; Bischoff Chromatography, Leonberg, Germany) at 40 °C. The solvent A and solvent B used as mobile phases were 0.5% acetic acid in deionized water and 0.5% acetic acid in acetonitrile, respectively, with a solvent A to solvent B ratio of 80:20 (isocratic). Acetonitrile and acetic acid were HPLC grade. The HPLC was operated at a flow rate of 1.0 mL/min for 20 min, and the injection volume was 10 µL. Similar conditions have been used for the HPLC-UV determination of ellagic acid [25]. Ellagic acid was quantified using the UV detector of the HPLC system operated at 254 nm. For peak identification, UV spectra (190–370 nm) were subsequently recorded with a photodiode array (PDA) detector (SPD-M20A, Shimadzu, Kyoto, Japan) (Section 2.3.4).

2.3.2. Standard Solutions and Calibration

Ellagic acid hydrate (technical grade, Sigma-Aldrich, St. Louis, MO, USA, Cat. No. 372749) was used as the reference standard. A stock solution (1000 µg/mL) was prepared by dissolving 1 mg of the standard in 1 mL of ethanol, and working standards (500, 250, 100, and 10 µg/mL) were prepared by serial dilution for construction of the calibration curve. The calibration equation is given in Equation (2):

$$y = 4.25048 \times 10^{-5}x + 0.8439 \quad (2)$$

where y is the ellagic acid concentration (μg/mL) and x is the peak area (R^2 = 0.999). Linearity and range of the calibration curve were evaluated according to ICH Q2(R2) [26]. Ellagic acid in the samples was quantified from this external calibration curve.

2.3.3. Sample Preparation for HPLC Analysis

After fermentation, the culture broth was centrifuged at 4000× *g* for 10 min, and the supernatant was filtered through a 0.45 μm PVDF filter. The filtrate was diluted 5-fold with 99% ethanol prior to HPLC injection. Each sample was injected in triplicate.

2.3.4. Identification and Method Validation

Peak identity was confirmed by a standard addition test, in which a known amount of authentic ellagic acid standard was added to the sample. The corresponding peak increased proportionally in area, without any shift in retention time or the appearance of a shoulder or split peak, confirming co-elution with the standard. The method was validated in accordance with ICH Q2(R2) [26] for specificity, linearity, range, and precision (Table S1). Peak identity was further confirmed by comparing the UV absorption spectrum (190–370 nm) of the sample peak with that of the authentic ellagic acid standard, recorded subsequently with the PDA detector (Figure S1, Supplementary Materials); both showed absorption maxima at approximately 254 and 360 nm. Specificity was assessed by analyzing non-inoculated MRS medium and the non-fermented extract under the same conditions. Repeatability was assessed by six replicate analyses at 100 μg/mL, and intermediate precision by analysis on 3 different days.

2.4. Data Modeling and Statistical Validation

2.4.1. Data Analysis

Experimental design, data fitting, and regression modeling were conducted using the Minitab software (see Section 2.2.1). A second-order polynomial model was fitted to the data, and ANOVA was performed to evaluate the significance of the model and its variables [4]. The regression coefficients were used to derive predictive equations with the fermentation time, temperature, and pH as the input variables. The coefficient of determination (R^2) was used to assess the model fitness, and F-values were applied to evaluate statistical significance.

2.4.2. Prediction and Verification

The optimal fermentation conditions were predicted by identifying the overlapping range among the response variables. A representative central point was selected within the predicted range, and estimated values were calculated using the regression equations. To verify the model accuracy, experimental trials were conducted under the predicted conditions, and actual values were compared with the predicted values. Relative error (%) was used to assess the predictive validity of the model.

2.4.3. Statistical Processing

All experiments were performed in triplicate. Data were expressed as mean ± standard deviation (SD), and statistical significance was evaluated by one-way ANOVA followed by Tukey's HSD post hoc test ($p < 0.05$), using the Minitab 16 software.

2.5. Total Polyphenol Content Assays and Antioxidant Activity

2.5.1. Total Polyphenol Content Assay

The total polyphenol content (TPC) of optimized fermented *J. mandshurica* extract (OFJME) was determined using the Folin–Ciocalteu method [27] with slight modifications for a microplate format [28]. OFJME and Folin–Ciocalteu phenol reagent were mixed in a

1:1 ratio and reacted for 3 min. Then, 10% Na_2CO_3 was added to the mixture, which was then incubated at 25 °C for 1 h. The absorbance was measured at 760 nm using a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA, USA). The TPC of OFJME was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract. All analyses were performed in triplicate. Prior to the TPC assay, freeze-dried OFJME powder was dissolved in 10% DMSO at a concentration of 10 mg/mL. As the whole fermentation product, including residual MRS medium constituents and microbial biomass, was freeze-dried without a prior separation step, the reported TPC values (mg GAE/g) reflect the total freeze-dried fermentate rather than the plant-derived phenolics alone; this should be taken into account when interpreting the TPC increase relative to the non-fermented JME control.

2.5.2. DPPH and ABTS Radical Scavenging Assays

The antioxidant activity of OFJME was evaluated through DPPH and ABTS radical scavenging assays.

The DPPH assay was performed based on the method by Blois (1958) [29]. A 1:2 reaction mixture of OFJME and 0.2 mM DPPH solution was prepared and incubated at 25 °C for 10 min. The absorbance was then measured at 517 nm using a SpectraMax M5 microplate reader. Each sample was analyzed in triplicate.

For the ABTS assay, 2.45 mM potassium persulfate and 7 mM ABTS were mixed and left to react in the dark at 25 °C for 24 h to form ABTS radical cations. OFJME and the ABTS working solution were mixed and incubated at room temperature for 10 min. The absorbance was measured at 734 nm [30].

The radical scavenging activity was calculated using Equation (3):

$$\text{Scavenging effect } (\%) = \left[1 - \frac{A_S - A_{SB}}{A_C}\right] \times 100 \quad (3)$$

S, Sample; SB, Sample black; C, Control.

3. Results

3.1. Optimization of Ellagic Acid Accumulation Through Fermentation

Ellagic acid concentration, quantified by HPLC as described in Section 2.3 and used as the main response variable in this study, is presented for each experimental run in Table 2.

3.1.1. CCD: Effects of Independent Variables and Model Correlation for Fermented *J. mandshurica* Extract

To determine the optimal fermentation conditions for ellagic acid accumulation from *J. mandshurica* extract, CCD was conducted. Three independent variables, namely the coded values of fermentation temperature (T), pH (p), and time (t), were tested to build a second-order regression model.

The regression coefficients obtained from the analysis are presented in Table S2 (Supplementary Materials). The model equation was obtained as shown in Equation (4):

$$\begin{aligned} Y = 46.216 + 0.065T + 1.888p + 7.355t - 2.318T^2 - 4.606p^2 + 0.849t^2 \\ +0.479Tp + 0.600Tt + 3.181pt \end{aligned} \quad (4)$$

where Y is the predicted ellagic acid concentration (μg/mL); T, p, and t are the coded values of fermentation temperature, pH, and time, respectively, calculated from the actual values using Equation (1); and the numerical values are the regression coefficients (Table S2 (Supplementary Materials)).

As shown in Table S2 (Supplementary Materials), the linear terms of pH and time were statistically significant (p = 0.048 and $p < 0.001$, respectively), while the temperature

term was not significant ($p = 0.940$). However, the pH × time interaction term ($p = 0.016$) and the quadratic pH term ($p < 0.001$) were highly significant, indicating that interaction and quadratic effects both play a substantial role in ellagic acid accumulation. The model had a coefficient of determination (R^2) of 0.929 and an adjusted R^2 of 0.866, indicating a good fit within the tested design space. The predicted R^2 was 0.49, and the lack of fit was statistically significant (Table 3); nonetheless, the model's optimum was closely confirmed by experimental validation (predicted 64.05 μg/mL vs. observed 64.04 ± 0.95 μg/mL), supporting its practical usefulness for identifying the best fermentation condition within the tested design space [31]. Experimental validation was limited to this single condition within the design space; validating additional points, particularly away from the optimum, would be needed to more fully characterize the model's predictive capability across the design space.

Table 3. ANOVA results for the response of dependent variables.

Source	DF	Seq SS	Adj SS	Adj MS	F	*p*
Regression	9	1260.37	1260.37	140.041	14.66	0.000 *
Linear	3	787.38	787.38	262.460	27.47	0.000 *
Temperature	1	0.06	0.06	0.057	0.01	0.940
pH	1	48.60	48.60	48.603	5.09	0.048 **
Time	1	738.72	738.72	738.721	77.33	0.000 *
Square	3	387.34	387.34	129.115	13.52	0.001 *
Temperature * Temperature	1	57.60	77.59	77.587	8.12	0.017 **
pH * pH	1	319.41	305.01	305.014	31.93	0.000 *
Time * Time	1	10.33	10.33	10.331	1.08	0.323
Interaction	3	85.64	85.64	28.547	2.99	0.082
Temperature * pH	1	1.84	1.84	1.836	0.19	0.670
Temperature * Time	1	2.88	2.88	2.880	0.30	0.595
pH * Time	1	80.92	80.92	80.925	8.47	0.016 **
Residual Error	10	95.53	95.53	9.553		
Lack-of-Fit	5	91.13	91.13	18.226	20.71	0.003
Pure Error	5	4.40	4.40	0.880		
Total	19	1355.90				

Coefficient of determination (R^2) = 92.9%, adjusted (R^2) = 86.6%. * $p < 0.01$ indicates that model terms are significant at the 99% level. ** $p < 0.05$ indicates that model terms are significant at the 95% level.

Table 3 provides the ANOVA results for model significance. The regression model was statistically significant ($p < 0.001$). Among the ANOVA components, the linear term for time and the quadratic term of pH showed the highest F-values (77.33 and 31.93, respectively), highlighting their strong impact on ellagic acid accumulation. As noted above, the significant lack-of-fit ($p = 0.003$) indicates that the second-order model does not fully account for all sources of variation within the experimental domain, even though its optimum was confirmed by experimental validation.

Taken together, the results presented in Table S2 (Supplementary Materials) and Table 3 confirm that fermentation time and pH are key variables for maximizing ellagic acid accumulation. Although temperature alone did not show a significant effect, its interaction and quadratic terms suggest that it contributes in conjunction with the other parameters.

3.1.2. Response Surface Analysis of Optimized Fermented *J. mandshurica* Extract

Based on the second-order regression model, response surface and contour plots were constructed to visualize the influence of temperature, pH, and time on ellagic acid accumulation from *J. mandshurica* extract fermented by *W. confusa*. Among the experimental

runs, the highest observed yield was 59.25 µg/mL at 35 °C, pH 5.5, and 24.09 h (Exp. No. 14, Table 2), while the model-predicted optimum is presented in Section 3.1.3.

Figure 1A shows the ellagic acid content as a function of temperature and pH, with time fixed at 14 h. The highest ellagic acid yield was observed at approximately 32–38 °C and pH 4.5–7, with the maximum near 35 °C and pH 5.5–6, suggesting that near-neutral to mildly acidic conditions and mid-range temperatures favor ellagic acid accumulation. Outside of this range, a gradual decrease in the yield was observed, indicating either potential thermal degradation or suboptimal enzyme activity under less favorable conditions [32].

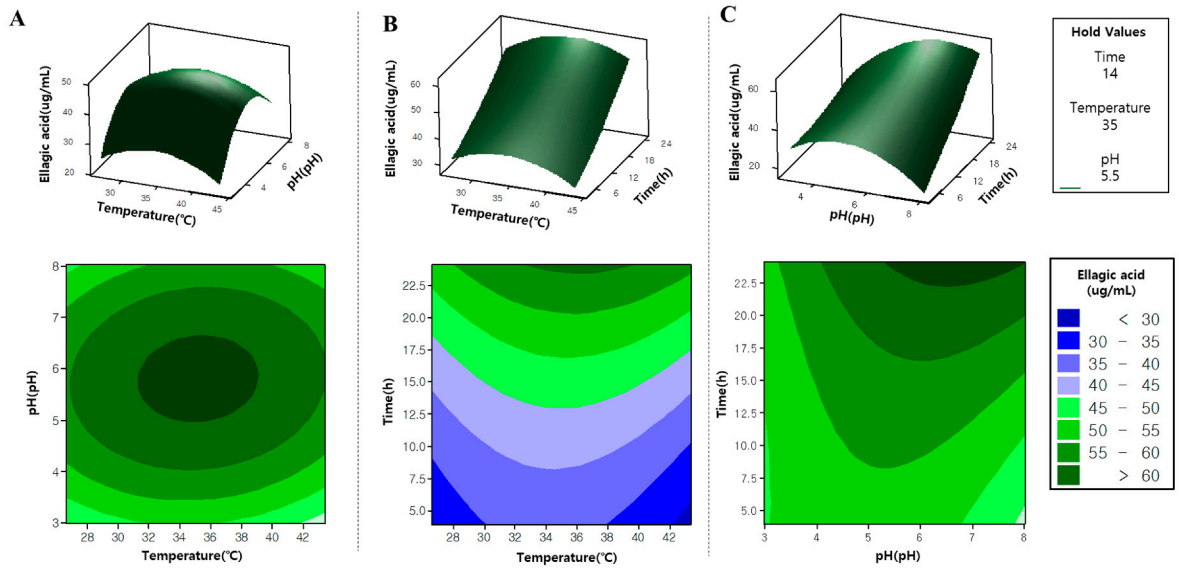


Figure 1. Response surface plots of ellagic acid content from fermentation of *J. mandshurica* extract by *W. confusa*: (**A**) as a function of temperature and pH, with time fixed at 14 h; (**B**) as a function of temperature and time, with pH fixed at 5.5; (**C**) as a function of pH and time, with temperature fixed at 35 °C. Fermentation conditions: inoculum density, OD_{600} = 0.01; substrate concentration, 1 g of extract per 15 mL (approximately 6.7%, *w*/*v*); shaking speed, 150 rpm; aerobic conditions with an air-permeable stopper.

Figure 1B shows the response surface of ellagic acid content relative to temperature and time, with pH held constant at 5.5. The maximum yield is obtained for the temperature range of 34–38 °C and time range of 20–24 h. Within the tested range, the yield increased with time up to the upper limit of the design (24.09 h); the response at longer times was not evaluated.

Figure 1C presents ellagic acid content as a function of pH and time, with the temperature fixed at 35 °C. The yield increased steadily with pH until approximately 6.5–7.0 and with fermentation time until 20–24 h, indicating that optimal conditions are achieved under slightly acidic to neutral pH and sufficient incubation time. The nearly concentric contour plot suggests a well-defined maximum response zone.

These results confirm that ellagic acid accumulation from *J. mandshurica* via microbial fermentation is significantly influenced by the interplay of temperature, pH, and time. Accurate optimization of these parameters is essential for cost-effective and scalable maximization of bioactive compound recovery.

3.1.3. Optimization of Ellagic Acid Fermentation Process

The optimal fermentation conditions for maximizing ellagic acid accumulation from *J. mandshurica* extract fermented by *W. confusa* were determined using RSM. As shown in Figure 2, the predicted optimal values were a temperature of 36.61 °C, initial pH of 6.70, and

fermentation time of 24.09 h. These values lie within the axial range of the central composite design (Table 1), with the optimal fermentation time corresponding to the +1.682 axial point, and therefore do not constitute extrapolation beyond the experimental domain. Under these conditions, the ellagic acid content was predicted to reach 64.05 μg/mL.

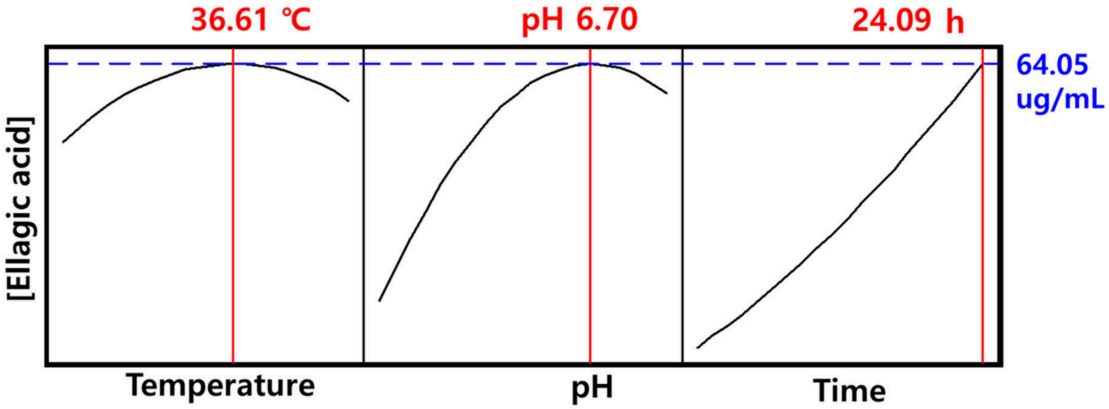


Figure 2. Optimization graph of the response surface for ellagic acid content in *J. mandshurica* extract fermented by *W. confusa*. Fermentation conditions: inoculum density, OD_{600} = 0.01; substrate concentration, 1 g of extract per 15 mL (approximately 6.7%, *w*/*v*); shaking speed, 150 rpm; aerobic conditions with an air-permeable stopper.

The optimization graph (Figure 2) clearly demonstrates that the maximum ellagic acid concentration was achieved near the apex of each response curve for the individual variables. The ellagic acid content increased with temperature and pH up to the optimal point, after which it declined slightly, indicating a classic quadratic response pattern. This behavior suggests that while moderate increases in temperature and pH enhance microbial enzymatic activity and ellagitannin hydrolysis, excessive values may denature enzymes or destabilize ellagic acid, resulting in decreased yield [32]. By contrast, ellagic acid concentration increased consistently with time within the tested range, suggesting a continued accumulation of the compound during fermentation up to the optimal time point. Because the optimal time coincides with the upper limit of the experimental domain and the yield increased with time throughout the tested range, the optimum with respect to time should be regarded as the best condition within the tested range, and longer fermentation times should be examined to determine whether the yield can be increased further.

These results validate the predictive capability of the quadratic regression model used in this study. The consistency between the model's predictions and the peak values observed in the individual variable plots demonstrates the reliability of RSM for fermentation optimization. Moreover, the use of *W. confusa* appears to support efficient ellagic acid accumulation within a relatively short fermentation duration at mild acidic and sub-physiological temperature conditions.

To validate the predicted model, a verification experiment was performed in triplicate under the optimized conditions (36.61 °C, pH 6.70, and 24.09 h). The experimental ellagic acid yields were 63.12, 63.98, and 65.01 μg/mL, with an average of 64.04 ± 0.95 μg/mL. This result is in excellent agreement with the predicted value (64.05 μg/mL), with a relative error of only 0.016%, confirming the reliability and accuracy of the RSM model.

Overall, this RSM-based optimization model provides a valuable guideline for maximizing ellagic acid accumulation under cost-effective and biologically feasible fermentation settings.

3.2. Antioxidant Activity and HPLC Analysis of OFJME

The antioxidant activity of OFJME was assessed using TPC, DPPH, and ABTS radical scavenging assays (Table 4). The TPC of OFJME (141.41 ± 2.32 mg GAE/g) was significantly higher ($p < 0.05$) than that of the unfermented JME (98.17 ± 0.89 mg GAE/g), indicating a high abundance of phenolic compounds released during fermentation.

In the DPPH assay, OFJME exhibited a dose-dependent radical scavenging effect, ranging from 46.78 ± 3.58% at 50 μg/mL to 85.47 ± 0.85% at 400 μg/mL. The ABTS assay showed similar trends, with activity increasing from 56.73 ± 2.01% to ≥99.99%, which was comparable to butylated hydroxy anisole (BHA) and ascorbic acid (AA) used as the positive controls. These results indicate that OFJME possesses strong antioxidant potential.

Additionally, Figure 3 shows the HPLC chromatographic profiles of the non-inoculated control, OFJME, and standard ellagic acid. The chromatograms of the control (Figure 3A) and OFJME (Figure 3B) were compared with the ellagic acid standard (Figure 3C) to confirm the presence of ellagic acid in the extract.

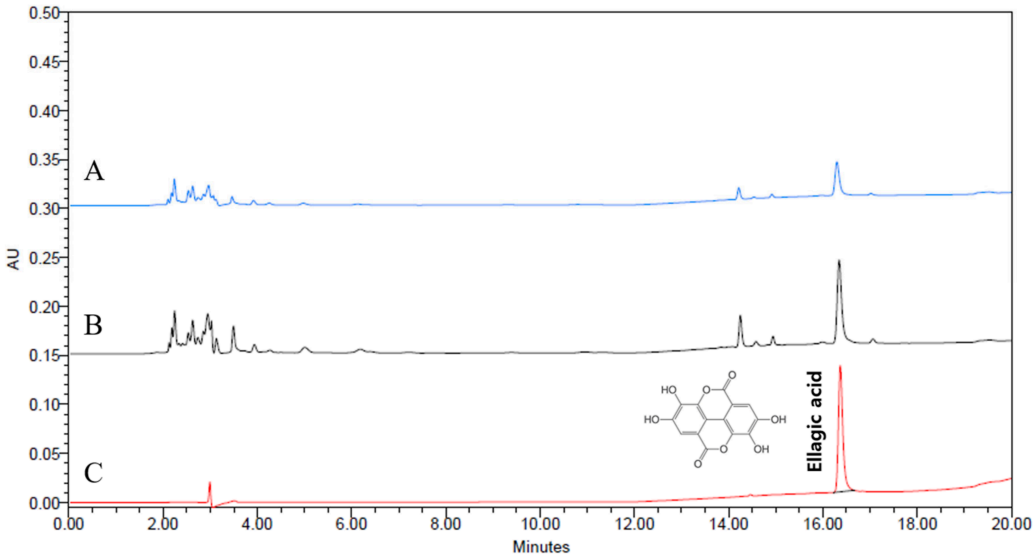


Figure 3. Representative HPLC chromatograms of (**A**) the non-inoculated control (*J. mandshurica* extract in MRS broth incubated without *W. confusa* under the same conditions as B), (**B**) the *J. mandshurica* extract fermented by *W. confusa* under the optimized conditions (36.61 °C, initial pH 6.70, 24.09 h), and (**C**) the ellagic acid standard. Detection was performed at 254 nm. The ellagic acid peak (retention time ≈ 16.3 min) is indicated. Fermentation conditions: inoculum density, OD_{600} = 0.01 (B only); substrate concentration, 1 g of extract per 15 mL (approximately 6.7%, *w*/*v*); shaking speed, 150 rpm; aerobic conditions with an air-permeable stopper.

Table 4. Antioxidant activities of 20% ethanol extract from *J. mandshurica* fruit fermented by *W. confusa* using CCD.

	TPC [1] (mg GAE/g)	DPPH Radical Scavenging Activity (%) Concentration (μg/mL)				ABTS Radical Scavenging Activity (%) Concentration (μg/mL)			
		50	100	200	400	50	100	200	400
JME [3]	98.2 ± 0.9 [b]	12.3 ± 2.8 [d]	20.9 ± 2.6 [d]	23.7 ± 4.4 [d]	41.9 ± 2.7 [c]	24.8 ± 1.2 [d]	40.8 ± 0.5 [d]	75.8 ± 2.2 [c]	96.9 ± 0.5 [b]
OFJME [4]	141.4 ± 2.3 [a]	46.8 ± 3.6 [b]	65.1 ± 1.1 [b]	82.0 ± 1.5 [b]	85.5 ± 0.8[b]	56.7 ± 2.0 [c]	88.0 ± 1.7 [c]	97.9 ± 0.8 [b]	≥99.99 [a]
BHA [2]		34.9 ± 0.2 [c]	58.3 ± 2.0 [c]	75.4 ± 1.8 [c]	83.6 ± 0.2[b]	82.2 ± 1.8 [b]	90.5 ± 0.2 [b]	93.5 ± 0.5 [b]	96.8 ± 1.6 [b]
AA [2]		76.4 ± 3.8 [a]	88.1 ± 2.7 [a]	≥99.99 [a]	≥99.99 [a]	88.1 ± 0.3 [a]	94.8 ± 1.0 [a]	≥99.99 [a]	≥99.99 [a]

[1] TPC: total phenolic content was expressed in terms of mg gallic acid equivalents. [2] Positive controls used were butylated hydroxy anisole (BHA) and ascorbic acid (AA), respectively. [3] *J. mandshurica* extract. [4] Optimized fermented *J. mandshurica* extract. Data represent the means ± SD in triplicate. Different superscript letters within the same column indicate significant differences at $p < 0.05$ (one-way ANOVA followed by Tukey's HSD).

4. Discussion

This study successfully established an optimized microbial fermentation strategy to maximize the ellagic acid content and antioxidant potential of *J. mandshurica* fruit extract by utilizing *W. confusa* [10]. By applying RSM with CCD, the optimal fermentation conditions were identified as 36.61 °C, pH 6.70, and 24.09 h. Under these predicted conditions, the validated ellagic acid yield reached 64.04 ± 0.95 μg/mL, as confirmed by HPLC analysis [1]. The good coefficient of determination of the quadratic regression model (R^2 = 0.929), together with the close agreement between predicted and validated yields, supports the suitability of RSM for modeling and optimization of ellagic acid accumulation from plant-based polyphenols under mild fermentation conditions [4]. It should be noted that this study measured the increase in ellagic acid content but did not directly quantify ellagitannin depletion; therefore, the results demonstrate enhanced ellagic acid accumulation rather than direct evidence of ellagitannin-to-ellagic acid conversion.

Direct comparison with previous fermentation-based ellagic acid studies is complicated by differences in substrates, microorganisms, and reporting bases (Table 5). Most studies used ellagitannin-rich pomegranate materials. Lactic acid bacteria (*Lactiplantibacillus plantarum* and *Streptococcus thermophilus*) fermenting pomegranate peel with *Schisandra chinensis* under RSM-optimized conditions yielded 99.49 ± 0.47 mg/g [5], and *Aspergillus niger* GH1 grown on punicalagin purified from pomegranate peel reached 42.02 mg/g [33]. Thirty lactic acid bacteria converted punicalagin from a pomegranate extract into ellagic acid without forming urolithins [7], and solid-state fermentation of walnut press cake with *Aspergillus oryzae* or *Rhizopus oligosporus* liberated ellagic acid, with a maximum at 48 h [21]. The concentration obtained in the present study (64.04 μg/mL of fermentation broth, corresponding to approximately 0.96 mg per gram of the dilute ethanol extract used as substrate) is substantially lower than the peel-based yields. This likely reflects the fact that those studies used ellagitannin-rich peel material or purified punicalagin, whereas the present study used a dilute ethanol extract of *J. mandshurica* fruit. Future studies using a more concentrated substrate or raw *J. mandshurica* fruit material would allow a more direct, mass-normalized comparison.

Table 5. Comparison of the present study with previous studies on the fermentative production or release of ellagic acid.

Substrate	Microorganism	Process/Optimization	Key Conditions	Ellagic Acid (as Reported)	Ref.
J. mandshurica fruit extract (20% ethanol; 1 g in 15 mL MRS)	*W. confusa* CBF20-N-001 (lactic acid bacterium)	Submerged; CCD-RSM (temperature, initial pH, time)	36.61 °C, initial pH 6.70, 24.09 h	64.04 ± 0.95 μg/mL broth (≈0.96 mg/g extract)	This study
Pomegranate peel + *Schisandra chinensis*	*L. plantarum* + *S. thermophilus* (lactic acid bacteria)	RSM (Box–Behnken)	37 °C, 33 h	99.49 ± 0.47 mg/g	[5]
Punicalagin purified from pomegranate peel	*A. niger* GH1 (fungus)	Solid-state culture on polyurethane foam	36 h	42.02 mg/g	[33]
Punicalagin-rich pomegranate extract (30 μg/mL punicalagin in MRS)	30 lactic acid bacteria strains (screening)	Submerged; screening with proteomics	37 °C, 5% CO_2, 24 h	≈1.0–1.4 μg/mL (≈20-fold vs. extract without bacteria); no urolithins	[7]
Walnut (*J. regia*) press cake	*A. oryzae*, *R. oligosporus* (fungi)	Solid-state; optimization of hydration, acetic acid, and NaCl	25 °C; maximum at 48 h	Liberation reported; degradation dominated at 72 h	[21]

As shown in Table 5, the present study differs from previous studies in substrate, microorganism, and process. Previous processes mostly used ellagitannin-rich pomegranate materials and/or filamentous fungi in solid-state culture, whereas this study used a dilute ethanol extract of *J. mandshurica* fruit fermented by the food-grade lactic acid bacterium

W. confusa in a submerged process. The advantages of the present work are (i) a new substrate–strain combination, which to our knowledge has not been reported previously; (ii) mild conditions and a relatively short fermentation time (36.61 °C, 24.09 h) without solid supports or fungal cultivation; and (iii) statistical optimization of three factors by CCD-RSM with experimental validation. However, the absolute ellagic acid concentration is lower than those reported for peel-based systems, and direct comparison is limited by differences in substrates, reporting bases, and analytical methods.

In addition to enhanced ellagic acid accumulation, the optimized fermented extract (OFJME) displayed substantially improved antioxidant activity, as demonstrated by DPPH and ABTS radical scavenging rates exceeding 85% and 99%, respectively [34]. These results suggest that microbial fermentation with *W. confusa* not only enriches bioactive compound concentrations, particularly ellagic acid, but also modifies their chemical profiles to boost their functional properties, as illustrated by the overlapped chromatograms in Figure 3 [35]. The significant increase in the TPC of OFJME compared to non-fermented controls is compatible with a fermentation-mediated release of bound phenolics, potentially via tannase- and β-glucosidase-type activities that have been reported in other LAB species to hydrolyze ellagitannins into bioavailable ellagic acid [15]. As tannase/β-glucosidase activity and ellagitannin depletion were not directly measured for *W. confusa* in the present study, this remains a literature-based interpretation rather than a demonstrated mechanism, and direct enzymatic characterization is warranted in future work. This interpretation is in line with previous studies on the bioconversion of tannin-rich plant materials by LAB, supporting the role of microbial fermentation as an eco-friendly alternative to conventional chemical hydrolysis approaches [36]. Furthermore, strain-specific metabolic capabilities are increasingly recognized as critical determinants of microbial biotransformation efficiency. Previous research has shown that different bacterial strains possess distinct enzymatic repertoires that directly influence their ability to modulate bioactive compounds [37]. In this context, the remarkable performance of *W. confusa* highlights the importance of careful strain selection for maximizing functional outcomes in fermentation-based strategies.

The industrial relevance of using *W. confusa* is also noteworthy [38]. Its GRAS status and its demonstrated ability to function effectively under mild fermentation conditions suggest potential for functional ingredient development [39], although this remains to be evaluated beyond the single-strain, laboratory-scale system used here. Compared to acid or thermal hydrolysis, microbial fermentation provides a sustainable, lower-energy, and consumer-acceptable alternative, contributing to the ongoing shift toward greener processing technologies in the food and nutraceutical sectors [40]. Such strategies resonate strongly with consumer preferences for "clean-label" products and minimal processing, adding further value to the proposed OFJME production protocol [30].

From a technological viewpoint, the ability of *W. confusa* to carry out biotransformation at relatively mild temperatures and near-neutral pH demonstrates its robustness and process compatibility [41]. This is highly advantageous in industrial settings where energy consumption and product safety are major concerns [42]. Furthermore, the enhanced antioxidant properties and increased phenolic content of OFJME make this extract a promising functional food ingredient for health applications beyond antioxidation due to the potential anti-inflammatory, anti-aging, and anti-carcinogenic effects reported for ellagic acid and its derivatives [43].

However, it is important to acknowledge some limitations. This study focused on three independent variables (temperature, pH, and time), yet other parameters such as inoculum density, agitation rate, dissolved oxygen, and substrate concentration can also influence the bioconversion efficiency and should be included in future optimization studies [44]. Moreover, while the in vitro antioxidant activity results are encouraging, translating these

findings into in vivo efficacy requires further studies on the bioavailability, absorption, metabolism, and excretion of ellagic acid from OFJME [45]. Animal studies and clinical trials will ultimately be needed to confirm the safety and efficacy of these compounds in humans [31]. In addition, although this study referred to the enzymatic hydrolysis of ellagitannins, we did not experimentally confirm enzyme activities or intermediate metabolites. In addition, the ellagitannin content of the extract before and after fermentation was not measured, so the relative contributions of ellagitannin hydrolysis and the release of ellagic acid already present in the extract could not be distinguished. A non-inoculated extract/MRS control, analyzed under the optimal conditions and shown in Figure 3A, exhibited a detectable ellagic acid peak, indicating some contribution of heat and pH to ellagitannin hydrolysis; testing this control across the full range of temperature and pH conditions would further help quantify this contribution. This limitation will be addressed in future studies using metabolomics or multi-omics approaches. Because DPPH and ABTS activities were measured for the whole freeze-dried fermentate, they reflect the combined contribution of ellagic acid, other phenolics, and medium- or biomass-derived components; resolving these contributions individually would be a useful next step.

Beyond these limitations, future work could further characterize the OFJME metabolome and evaluate the scalability of this process for food, nutraceutical, and cosmeceutical applications.

5. Conclusions

This study successfully optimized the fermentation conditions for ellagic acid accumulation from *Juglans mandshurica* fruit extract using *Weissella confusa* through response surface methodology (RSM). The optimal parameters were identified as 36.61 °C, pH 6.70, and 24.09 h, resulting in a predicted ellagic acid yield of 64.05 μg/mL. This prediction was experimentally verified at the predicted optimum condition using three independent replicate fermentations (n = 3), resulting in an actual yield of 64.04 ± 0.95 μg/mL (relative error 0.016%), confirming good agreement between the predicted and experimental yields at this single validated condition. In addition, the fermented extract demonstrated significantly enhanced antioxidant activities, including DPPH radical scavenging activity of 85.47% and ABTS activity exceeding 99.99%, along with an increased total phenolic content of 141.41 mg GAE/g. These results confirm that microbial fermentation using *W. confusa* effectively enhances the bioactivity and polyphenol content of *J. mandshurica* extract.

The application of RSM provided a statistically validated framework for optimizing this laboratory-scale bioprocess. These findings suggest the potential of combining microbial biotransformation with statistical modeling to valorize underutilized polyphenol-rich resources, although this was demonstrated with a single strain and substrate under laboratory conditions. Further research on in vivo efficacy, toxicology, stability, economic feasibility, and process scale-up would be needed to evaluate the public health and commercial potential of this approach.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations13100285/s1, Table S1: Validation results of the HPLC method for ellagic acid; Table S2: Estimated regression coefficients for the formula of ellagic acid accumulation as a function of temperature, time and pH; Figure S1: Overlaid UV absorption spectra (190–370 nm) of the ellagic acid peak in the fermented sample (red) and the authentic ellagic acid standard (black), recorded with a PDA detector. Both spectra show absorption maxima at approximately 254 nm and a secondary band near 360 nm.

Author Contributions: Conceptualization, T.-H.K.; methodology, M.H. and J.-H.K.; software, M.H.; validation, M.H., J.-H.K. and D.-H.C.; formal analysis, M.H. and D.-H.C.; investigation, M.H., J.-H.K., D.-H.C. and J.-H.H. (Joon-Hee Han); resources, J.-H.H. (Jin-Hee Han) and T.-H.K.; data curation, M.H.; writing—original draft preparation, M.H.; writing—review and editing, T.-H.K., J.-H.H. (Jin-Hee Han) and J.-H.H. (Joon-Hee Han); visualization, M.H.; supervision, T.-H.K.; project administration, T.-H.K.; funding acquisition, T.-H.K. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by two funding sources: (1) the ANCHOR program through the Gangwon ANCHOR Center, funded by the Ministry of Education (MOE) and the Gangwon State (G.S.), Republic of Korea (2026-ANCHOR-10-002); and (2) a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health and Welfare, Republic of Korea (grant number: RS-2025-24535069).

Institutional Review Board Statement: Not applicable. This study did not involve human participants; the bacterial strain used had been previously isolated and deposited at the KCTC (KCTC18992P).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are contained within the article or Supplementary Materials.

Conflicts of Interest: The authors received research funding from the Gangwon ANCHOR Center (2026-ANCHOR-10-002) and the Korea Health Industry Development Institute (KHIDI) (RS-2025-24535069). The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results. The authors declare no other conflicts of interest.

References

1. Myers, R.H.; Montgomery, D.C.; Anderson-Cook, C.M. *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*, 4th ed; John Wiley & Sons: Hoboken, NJ, USA, 2016.
2. Kim, Y.J. Response surface approach to integrated optimization modeling for parameter and tolerance design. *J. Korean Soc. Qual. Manag.* **2002**, *30*, 58–67.
3. Sim, C.H. Application of response surface methodology for the optimization of process in food technology. *Food Eng. Prog.* **2011**, *15*, 97–115. [CrossRef]
4. Baş, D.; Boyacı, İ.H. Modeling and optimization I: Usability of response surface methodology. *J. Food Eng.* **2007**, *78*, 836–845. [CrossRef]
5. Liu, H.-M.; Xu, P.-F.; Cheng, M.-Y.; Lei, S.-N.; Liu, Q.-L.; Wang, W. Optimization of fermentation process of pomegranate (*Punica granatum* L.) peel and *Schisandra chinensis* (Turcz.) Baill and the biological activities of fermentation broth: Antioxidant activity and protective effect against H_2O_2-induced oxidative damage in HaCaT cells. *Molecules* **2021**, *26*, 3432. [CrossRef]
6. Muzykiewicz-Szymańska, A.; Kucharska, E.; Kucharski, Ł.; Pełech, R.; Nowak, A. Application of response surface methodology for obtaining fermented extract of *Sanguisorba officinalis* L. herb with high antioxidant activity, polyphenols, and lactic acid content: Analysis of the content and skin permeation of selected phenolic acids. *RSC Adv.* **2025**, *15*, 44597–44610. [CrossRef]
7. Caballero, V.; Estévez, M.; Tomás-Barberán, F.A.; Morcuende, D.; Martín, I.; Delgado, J. Biodegradation of Punicalagin into Ellagic Acid by Selected Probiotic Bacteria: A Study of the Underlying Mechanisms by MS-Based Proteomics. *J. Agric. Food Chem.* **2022**, *70*, 16273–16285. [CrossRef]
8. Vaquero, I.; Alberto, M.R.; de Nadra, M.C. Effects of phenolic compounds from wine on the growth of lactic acid bacteria and oenological yeasts. *Food Control* **2007**, *18*, 840–846.
9. Parvez, S.; Malik, K.A.; Ah Kang, S.A.; Kim, H.Y. Probiotics and their fermented food products are beneficial for health. *J. Appl. Microbiol.* **2006**, *100*, 1171–1185. [CrossRef]
10. Filannino, P.; Di Cagno, R.; Gobbetti, M. Metabolic and functional paths of lactic acid bacteria in plant foods: Get out of the labyrinth. *Curr. Opin. Biotechnol.* **2018**, *49*, 64–72. [CrossRef]
11. Pulido-Mateos, E.C.; Lessard-Lord, J.; Desjardins, Y.; Roy, D. Biotransformation of camu–camu galloylated ellagitannins by *Lactiplantibacillus plantarum* with extracellular tannase activity. *Food Funct.* **2024**, *15*, 7189–7199. [CrossRef]
12. Fusco, V.; Chieffi, D.; Fanelli, F.; Montemurro, M.; Rizzello, C.G.; Franz, C.M.A.P. The *Weissella* and *Periweissella* genera: Up-to-date taxonomy, ecology, safety, biotechnological, and probiotic potential. *Front. Microbiol.* **2023**, *14*, 1289937. [CrossRef]

13. Tamang, J.P.; Watanabe, K.; Holzapfel, W.H. Review: Diversity of microorganisms in global fermented foods and beverages. *Front. Microbiol.* **2016**, *7*, 377. [CrossRef]
14. Luan, F.; Wang, Z.; Yang, Y.; Ji, Y.; Lv, H.; Han, K.; Liu, D.; Shang, X.; He, X.; Zeng, N. *Juglans mandshurica* Maxim.: A review of its traditional usages, phytochemical constituents, and pharmacological properties. *Front. Pharmacol.* **2021**, *11*, 569800. [CrossRef]
15. Landete, J.M. Ellagitannins, ellagic acid and their derived metabolites: A review about source, metabolism, functions and health. *Food Res. Int.* **2011**, *44*, 1150–1160. [CrossRef]
16. Pinto, D.; Santos, J.; Caleja, C.; Ferreira, I.C.; Amaral, J.S. Optimization of hydrolysis conditions to recover ellagic acid from Rubus ulmifolius Schott leaves using response surface methodology. *J. Appl. Res. Med. Aromat. Plan.* **2017**, *6*, 1–8.
17. Tomás-Barberán, F.A.; Selma, M.V.; Espín, J.C. Interactions of gut microbiota with dietary polyphenols and consequences to human health. *Curr. Opin. Clin. Nutr. Metab. Care* **2016**, *19*, 471–476. [CrossRef]
18. Seeram, N.P.; Lee, R.; Heber, D. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. *Clin. Chim. Acta* **2004**, *348*, 63–68. [CrossRef]
19. Espín, J.C.; Larrosa, M.; García-Conesa, M.T.; Tomás-Barberán, F.A. Biological significance of urolithins, the gut microbial ellagic acid–derived metabolites: The evidence so far. *Evid. Based Complement Altern. Med.* **2013**, *2013*, 270418. [CrossRef]
20. González-Sarrías, A.; Tomás-Barberán, F.A.; García-Villalba, R.; Núñez-Sánchez, M.Á.; García-Conesa, M.T.; Espín, J.C. The gut microbial ellagic acid–derived metabolite urolithin A decreases prostate cancer cell proliferation and expression of the prostate-specific antigen (PSA) in vitro. *Eur. J. Nutr.* **2015**, *54*, 1059–1068.
21. Brück, W.M.; Díaz Escobar, V.D.; Droz-dit-Busset, L.; Baudin, M.; Nicolet, N.; Andlauer, W. Fermentative liberation of ellagic acid from walnut press cake ellagitannins. *Foods* **2022**, *11*, 3102. [CrossRef]
22. Brück, W.M.; Erismann, Y.; Andlauer, W. Fermentative liberation and transformation of bioactive compounds: Ellagic acid from nut press cake ellagitannins. *Appl. Sci.* **2024**, *14*, 1141. [CrossRef]
23. Fernández-Agulló, A.; Pereira, E.; Freire, M.S.; Valentão, P.; Andrade, P.B.; González-Álvarez, J.; Pereira, J.A. Influence of solvent on the antioxidant and antimicrobial properties of walnut (*Juglans regia* L.) green husk extracts. *Ind. Crops Prod.* **2013**, *42*, 126–132. [CrossRef]
24. De Man, J.C.; Rogosa, M.; Sharpe, M.E. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **1960**, *23*, 130–135. [CrossRef]
25. Assunção, P.I.D.; da Conceição, E.C.; Borges, L.L.; de Paula, J.A.M. Development and validation of a HPLC-UV method for the evaluation of ellagic acid in liquid extracts of *Eugenia uniflora* L. (*Myrtaceae*) leaves and its ultrasound-assisted extraction optimization. *Evid. Based Complement. Altern. Med.* **2017**, *2017*, 1501038. [CrossRef]
26. ICH Harmonised Guideline, Validation of Analytical Procedures Q2(R2), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, Geneva. 2023. Available online: https://database.ich.org/sites/default/files/ICH_Q2(R2)_Guideline_2023_1130.pdf (accessed on 3 September 2026).
27. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152–178. [CrossRef]
28. Ainsworth, E.A.; Gillespie, K.M. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. *Nat. Protoc.* **2007**, *2*, 875–877. [CrossRef]
29. Blois, M.S. Antioxidant determinations by the use of a stable free radical. *Nature* **1958**, *181*, 1199–1200. [CrossRef]
30. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef]
31. Bezerra, M.A.; Santelli, R.E.; Oliveira, E.P.; Villar, L.S.; Escaleira, L.A. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta* **2008**, *76*, 965–977. [CrossRef]
32. Senavirathna, T.; Shafaei, A.; Lareu, R.; Balmer, L. Unlocking the Therapeutic Potential of Ellagic Acid for Non-Alcoholic Fatty Liver Disease and Non-Alcoholic Steatohepatitis. *Antioxidants* **2024**, *13*, 485. [CrossRef]
33. Ascacio-Valdés, J.A.; Buenrostro, J.J.; De la Cruz, R.; Sepúlveda, L.; Aguilera, A.F.; Prado, A.; Contreras, J.C.; Rodríguez, R.; Aguilar, C.N. Fungal biodegradation of pomegranate ellagitannins. *J. Basic Microbiol.* **2014**, *54*, 28–34. [CrossRef]
34. Hur, S.J.; Lee, S.Y.; Kim, Y.-C.; Choi, I.; Kim, G.-B. Effect of fermentation on the antioxidant activity in plant-based foods. *Food Chem.* **2014**, *160*, 346–356. [CrossRef]
35. Rezac, S.; Kok, C.R.; Heermann, M.; Hutkins, R. Fermented foods as a dietary source of live organisms. *Front. Microbiol.* **2018**, *9*, 1785. [CrossRef]
36. Pontonio, E.; Di Cagno, R.; Tarricone, S.; Giuliani, G.; Roano, R.; Centomani, I.; Gobbetti, M.; Rizzello, C.G. Lactic acid fermentation of pomegranate juice as a tool to improve antioxidant activity. *Front. Microbiol.* **2019**, *10*, 1550. [CrossRef]
37. Hussein, Z.A.; Talib, Z.A. Study The Bacterial Resistance of Antibiotic From Opportunistic Bacteria. *SHIFAA* **2024**, *2024*, 63–79. [CrossRef]
38. Granato, D.; Barba, F.J.; Bursać Kovačević, D.B.; Lorenzo, J.M.; Cruz, A.G.; Putnik, P. Functional foods: Product development, technological trends, efficacy testing, and safety. *Annu. Rev. Food Sci. Technol.* **2020**, *11*, 93–118. [CrossRef]

39. Marco, M.L.; Tachon, S. Environmental factors influencing the efficacy of probiotic bacteria. *Curr. Opin. Biotechnol.* **2013**, *24*, 207–213. [CrossRef]
40. Chemat, F.; Rombaut, N.; Sicaire, A.G.; Meullemiestre, A.; Fabiano-Tixier, A.S.; Abert-Vian, M. Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrason. Sonochem.* **2017**, *34*, 540–560. [CrossRef]
41. Montgomery, D.C. *Design and Analysis of Experiments*, 8th ed.; John Wiley & Sons: Hoboken, NJ, USA, 2013.
42. Granato, D.; Nunes, D.S.; Barba, F.J. An integrated strategy between food chemistry, biology, nutrition, pharmacology, and statistics in the development of functional foods: A proposal. *Trends Food Sci. Technol.* **2017**, *62*, 13–22. [CrossRef]
43. Tomás-Barberán, F.A.; Espín, J.C. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *J. Sci. Food Agric.* **2001**, *81*, 853–876. [CrossRef]
44. Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Rémésy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242S. [CrossRef]
45. Wishart, D.S. Metabolomics for investigating physiological and pathophysiological processes. *Physiol. Rev.* **2019**, *99*, 1819–1875. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.