Al-Muthanna J. For Agric Sci Online ISSN:2572-5149
Print ISSN: 2226-4086
Vol. 13 , Issue 1. 2026

https://muthjas.mu.edu.iq/ http://doi.org/10.52113/mjas04/13.1/17

Enhancing Phenolic Content and Antioxidant Activity during Fermentation using a Selected Lactic Acid Bacteria Isolate from a Local Milk

Maher Rasheed Salman[1] , Amna Ahmed Abbas[1], Fouad Razzaq Al-Burki[1], Yahya A. Al-Ethari[2]

[`1] Jabir Ibn Hayyan University for Medical and Pharmaceutical Sciences - Faculty of Pharmacy

[2] Directorate of Najaf Agriculture, Iraq

Abstract

Lactic acid bacteria (LAB) are widely used in fermented foods due to their safety as bio-preservatives. In this study, several strains of organic acid-producing lactic acid bacteria were isolated from locally produced milk to investigate their potential use in fermentation and select the most efficient isolate, and evaluate its effect on improving the physicochemical and functional properties during fermentation. Three strains of lactic acid bacteria were isolated, all Gram-positive and catalase-negative. Three strains of lactic acid bacteria were isolated, all of which were Gram-positive and catalase-negative. The LAB1 isolate was chosen for fermentation due to its high growth rate and superior stability. The fermentation process lasted 48 hours. During that time, we monitored several key factors: pH, the amount of acid present, the concentration of phenols, and the effectiveness of the antioxidant in combating oxidation. After those 48 hours, the pH decreased from 6.64 to 4.23, and the acid levels rose from 0.19% to 0.95%. The results show that fermentation with lactic acid bacteria made more phenolic compounds available, and the antioxidant activity went up. So, using the

extract and microbial fermentation together could improve the food product. This study suggests that local lactic acid bacteria isolates may be beneficial in enhancing the nutritional value of fermented foods.
Key words: Fermentation, Lactic Acid, TTA, TPC, DPPH

I. Introduction

Given the vital role they play in fermentation processes and improving the chemical and physical properties of food, lactic acid bacteria (LAB), belonging to genera such as Leuconostoc, Lactobacillus, and Streptococcus, are among the most important microbes used in traditional and modern food industries (Sharma et al., 2020), due to their ability to produce lactic acid, which leads to a decrease in pH and thus improves microbial safety and extends shelf life (Admassi, 2018).

Microbial fermentation using lactic acid bacteria (LAB) contributes to enhancing the total phenolic content (TPC) and antioxidant activity of fermented foods (Li *et al*., 2022; Al-Burki *et al*., 2022).

Multiple studies have shown that fermentation can increase phenolic content by 30–70% compared to unfermented raw materials, due to the release of bound phenolic compounds or their conversion into more bioavailable forms (Nisa *et al*., 2019; Anumudu *et al*., 2024).

Lactic acid bacteria make stuff that stops foodborne pathogens from growing in food and keeps it from going bad too fast. These things are like natural defenders against spoilage. Some of these things have been tied to biosubstituted compounds(Garcia *et al*., 2019). When using the DPPH test, the free radical scavenging capacity is usually noticeably improved, with some fermented food products recording increases in inhibition exceeding 80–100% after 48 hours of fermentation compared to time zero, which is attributed to the increase in phenolic compounds and antioxidant metabolites resulting from LAB (Septembre-Zhao *et al*., 2021). (Rizzello *et al*., 2017) found that when testing 27 strains of lactic acid bacteria for fermenting quinoa flour, the highest activity in removing DPPH

free radicals (more than 80%) was observed when fermenting using Lb. plantarum strains T6B4, T6C16 and T0A10, while only Lb. The bacterial strains plantarum T1B6, T6B4 and T0A10 exhibited antioxidant activity against ABTS free radicals.

The effectiveness of antioxidants in phenolic compounds lies in their ideal chemical structure, which facilitates the transfer of electrons or hydrogen from the hydroxyl groups located along the aromatic ring, giving them the ability to eliminate free radicals and form bonds with metals (Shi *et al*., 2022).

Our study aimed to isolate and characterize lactic acid bacteria from a traditional food product, and then select the most effective strain to evaluate its effect on fermentation by monitoring changes in pH and total acidity, as well as studying the development of total phenolic content and antioxidant activity.

II. Materials and methods

Collection of samples

Samples were collected from local markets and included locally produced milk. The samples were transported in sterile containers. To keep them safe and avoid microbial changes before analysis, they were stored at 4°C until analysis took place.

Isolation of Lactic Acid Bacteria

To prepare sequential decimal dilutions of the samples, a sterile saline solution with a concentration of 0.85% sodium chloride was used. We cultured 0.1 mL of these dilutions on MRS agar using the surface diffusion method. The plates incubated at 37°C for 48 hours under semi-anaerobic conditions.

Colonies that showed typical features of lactic acid bacteria, like a white or creamy color, round shape, and smooth surface, were selected and purified through re-culture. We performed Gram staining and catalase tests. The isolates that were Gram-positive and catalase-negative were considered potential lactic acid bacteria, and they were then stored on MRS agar at 4°C until use (Cappuccino and Welsh, 2017).

Morphological Characterization of Isolates

The selected isolates were characterized morphologically using a light microscope at ×1000 magnification (with immersion oil) to determine cell shape (rodicular

or spherical) and arrangement (pairs or chains). The morphological characteristics of colonies growing on MRS medium, including colony size, color, height, edge shape, and surface texture, were also recorded according to standard bacteriological methods (Cappuccino & Welsh, 2017).

Preparation of the Bacterial Inoculum

At a temperature of 37°C for 24 hours, the selected isolate LAB1 was cultured in MRS broth. Then, we tuned the bacterial density to around 10^7–10^8 CFU/mL. We did this by checking the optical density at 600 nm (OD_{600}) with a UV-Vis spectrophotometer. This gave us a standard batch to work with for all our tests (Madigan *et al*., 2018).

Fermentation Process

A 2% (v/v) bacterial inoculum was added to pasteurized milk as a fermentation medium, and the samples were incubated at 37°C for 24–48 hours. Fermentation samples were collected at 0, 24, and 48 hours for all targeted analyses, in accordance with established principles for lactic acid bacteria fermentation (Leroy and De Vuyst, 2004).

pH Measurement

The pH of the samples was measured using a pre-calibrated digital pH meter at 25°C. All measurements were performed in triplicate, and the results were expressed as mean ± standard deviation (AOAC, 2019).

Estimation of total acidity

10 mL of the sample were taken for titration of total acidity (TTA), a few drops of phenolphthalein reagent were added, and then titrated with 0.1 M sodium hydroxide solution until a pink color appeared for 30 seconds. The results were expressed as a percentage of lactic acid based on the standard equation (Nielsen, 2017).

Estimation of total phenolic content

Using the Folin-Ciocalteu reagent, the total phenolic content (TPC) was estimated. 0.5 mL of the sample was mixed with 2.5 mL of the diluted reagent, and 2 mL of 7.5% sodium carbonate solution was added. The samples were incubated for 30 minutes in the dark, and the absorbance was measured at a wavelength of 765 nm. The results were expressed in mg of gallic acid equivalent/100 mL (Singleton et al., 1999).

Antioxidant Activity Test (DPPH)

Using the DPPH free radical assay, the antioxidant activity was estimated, according to the steps described by Brand Williams et al. (1995), the sample was left in the dark for 30 minutes to incubate. Then, the amount of something that was absorbed was measured, and the wavelength was measured at 517 nm, and from that, % free radical inhibition was figured out. The standard equation:

%Inhibition= sample*A* control – *A*\ control – A ×100

Statistical Analysis

All experiments were performed with three replicates, and results are expressed as mean ± standard deviation.

T-test or ANOVA was used at a significance level of $p < 0.05$ (Montgomery, 2017).

II. Results and discussion

Results of Isolation and Morphological Characterization

The results of table (1) indicates the morphological and biochemical characteristics of three bacterial isolates obtained from locally produced milk. The studied isolates showed smooth, circular colonies and were Gram-positive and catalase-negative, confirming their classification as lactic acid bacteria. Isolate LAB1 was selected for fermentation due to its higher growth and greater stability during the isolation and purification stages, and this reflects its metabolic efficiency and its ability to adapt to the fermentation medium, and this is consistent with what (Hussain, 2022) found.

The data in Table (1) illustrate also the morphological and biochemical characteristics of three isolates from lactic acid bacteria obtained from locally produced milk. Due to its high growth rate and superior stability, LAB1 isolate was selected for the fermentation process. Figure (1) shows a microscopic image of the selected isolate, which displays short, Gram-positive rod-shaped cells. LAB1 isolate was chosen for fermentation because it showed higher growth and better stability.

.

TABLE 4. Morphological Characteristics of Lactic Acid Bacteria Isolates

Isolation	Colony Shape	Color	Gram test	Catalase	Cell Shape
LAB1	Round, smooth, slightly raised	Creamy white	Positive	Negative	Short rods
LAB2	Round, convex	white	positive	negative	spheroids in chains
LAB3	Round, smooth	yellowish-white	positive	negative	long rods

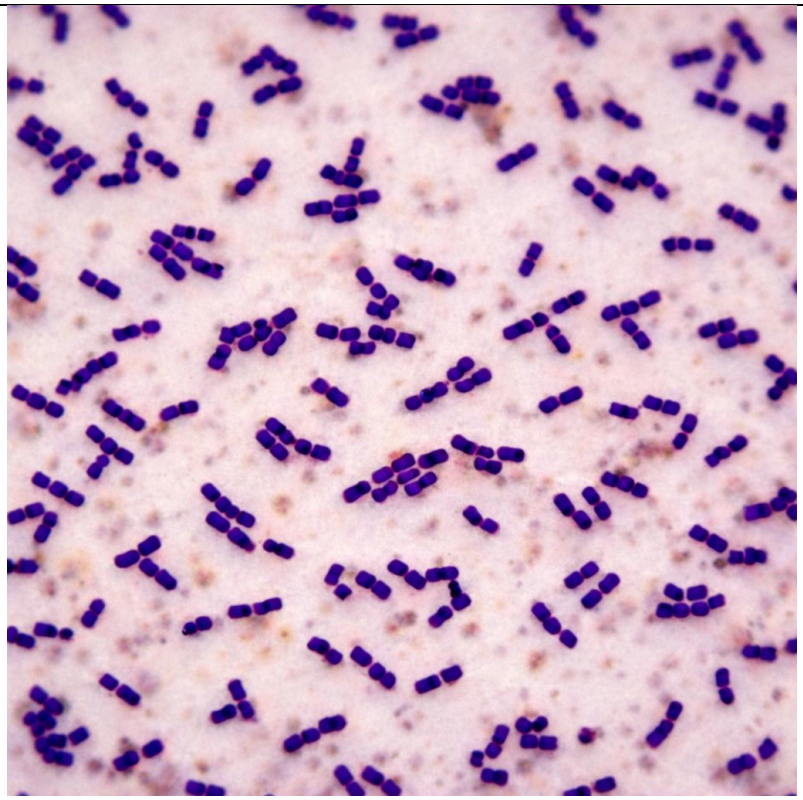

Fig. 1. Light microscopic image of the selected LAB1 isolate showing Gram-positive short rod-shaped cells (1000× magnification).

Changes in pH during fermentation

The results of Table 2 indicate a significant decrease in pH during fermentation using the LAB1 isolate. The pH decreased from 6.64 ± 0.04 at time zero to 4.88 ± 0.06 after 24 hours, and then to 4.23 ± 0.03 after 48 hours of fermentation, with significant differences ($p < 0.05$). This gradual decrease reflects the high

metabolic activity of the selected isolate and its ability to produce organic acids, particularly lactic acid, during fermentation. These results are consistent with what has been described in the literature, where a decrease in pH to values below 4.5 is considered an indicator of the efficiency of lactic acid bacteria in fermentation and an improvement in the microbial safety of fermented food products (Anumudu *et al*., 2024).

The pH decreased significantly during the fermentation period (Table 2), which is also evident in Figure 2, showing the downward trend in pH values over time.

TABLE 2. pH change during the fermentation period

Time (hours)	**pH (average ± SD)**
0	6.64 ± 0.04
24	4.88 ± 0.06
48	4.23 ± 0.03

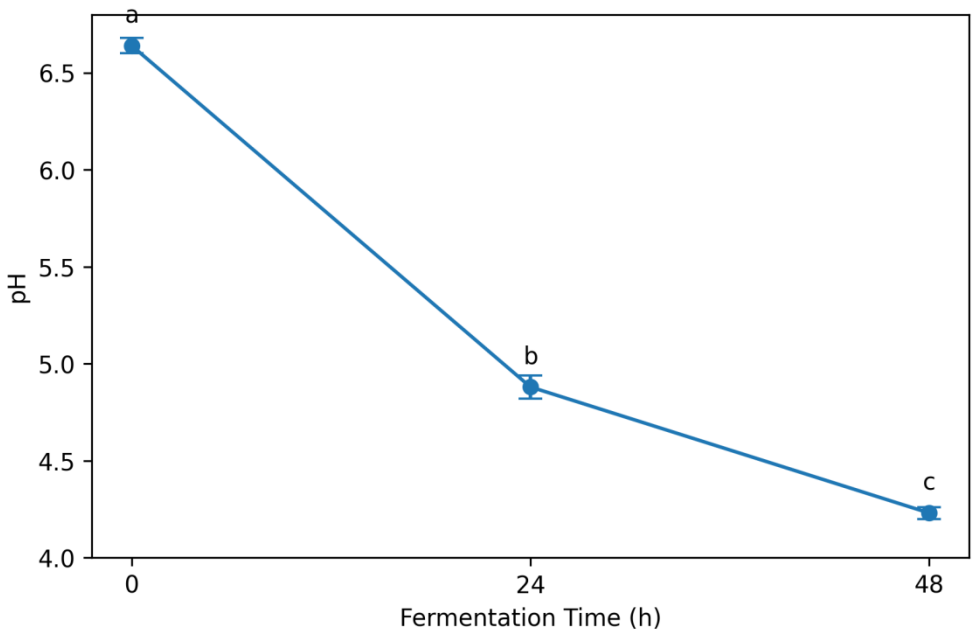

Fig. 2. Changes in pH values during fermentation by the selected lactic acid bacteria isolate (LAB1). Values represent mean ± SD (n = 3). Different letters indicate significant differences among fermentation times ($p < 0.05$).

Development of Titratable Acidity

The results presented indicate that during the fermentation period and using LAB1 isolate, a significant increase in total titrated acidity was observed (Table 3). At time zero, the acidity was recorded at 0.19 ± 0.03%, then it rose to 0.74 ± 0.05% after 24 hours of fermentation, and then to 0.95 ± 0.04% after 48 hours. This gradual increase shows a clear inverse relationship with the decrease in pH value, confirming the continuous production of organic acids during the fermentation process and the fermentation efficiency of the selected isolate and its ability to enhance the chemical properties of the culture medium and ss shown in figure (3), These results are consistent with what was stated by (Okoye *et al*., 2024), namely that strains of lactic acid bacteria have shown exceptional potential as biopreservatives.

TABLE 3. Total Titration Acidity

Time (hours)	**PH % (± SD)**
0	0.19 ± 0.03
24	0.74 ± 0.05
48	0.95 ± 0.04

Significant gradual increase over time ($p < 0.05$)

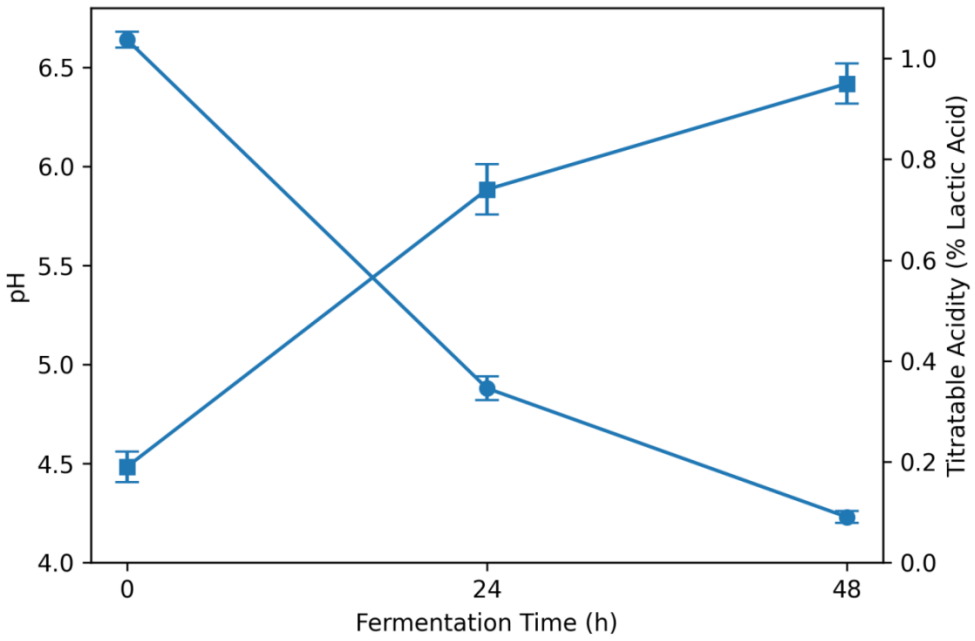

Fig. 3. Dual-axis representation of the inverse relationship between pH decrease and titratable acidity (TTA) increase during fermentation by LAB1. Data are expressed as mean ± SD (n = 3).

Changes in Total Phenolic Content (TPC) During Fermentation

Table 4 shows that the total phenolic content went up steadily as fermentation went on with the LAB1 isolate. At the start, it was 41.3 ± 2.2 mg GAE/100 mL. After a day, it got to 58.5 ± 2.7 mg GAE/100 mL, and after two days, it hit 67.3 ± 2.8 mg GAE/100 mL. This shows that fermentation helps free up phenolic compounds because the lactic acid bacteria are breaking them down. It also demonstrates the positive effect of fermentation on improving the functional properties of local milk, which is consistent with the findings of (Admassie, 2018). Furthermore, this increase in phenolic content coincides with an improvement in DPPH activity, as clearly illustrated in Figure (4).

TABLE 4. Change in Phenolic Content

Time (hours)	**TPC (mg GAE/100 mL ± SD)**
0	41.3 ± 2.2
24	58.5 ± 2.7

Time (hours)	TPC (mg GAE/100 mL ± SD)
48	67.3 ± 2.8

DPPH % inhibition

The results shown in Table (5) indicate a significant increase in free radical removal activity during fermentation using LAB1 isolate. The inhibition percentage at time zero increased from 32.4 ± 1.6% to 54.6 ± 2.4% after 24 hours of fermentation, and after 48 hours it rose to 64.5 ± 2.4%, with significant differences ($p < 0.05$). This gradual improvement in free radical scavenging capacity reflects the positive effect of fermentation in enhancing antioxidant activity, which is consistent with the increase recorded in total phenolic content during the same period, indicating a functional relationship between these two variables (Anumudu *et al*., 2024).

TABLE 5. DPPH Activity

Time (hours)	% inhibition (± SD)
0	32.4 ± 1.6
24	54.6 ± 2.4
48	64.5 ± 2.4

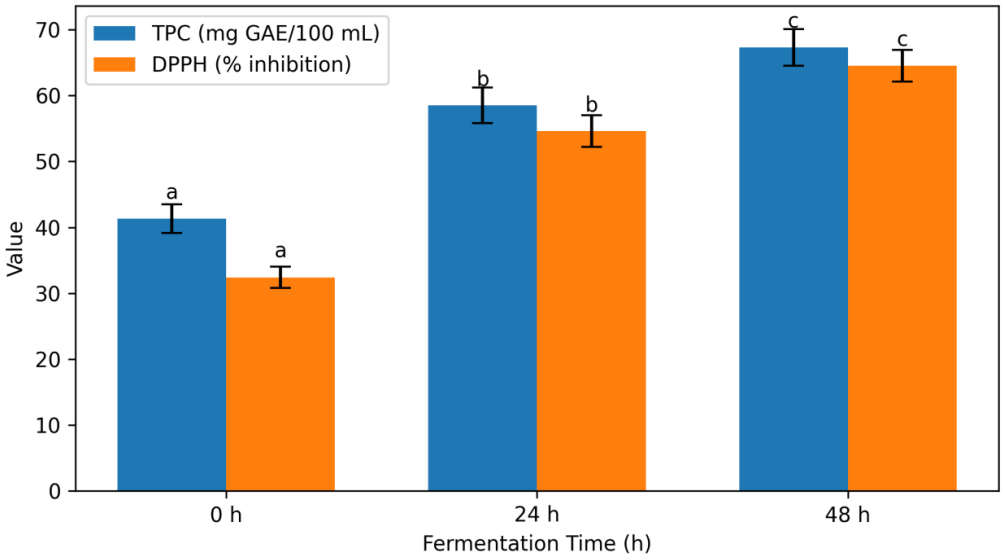

Fig. 4. Changes in total phenolic content (TPC) and DPPH radical scavenging activity during fermentation by LAB1. Values are expressed as mean ± SD (n = 3). Different letters indicate significant differences among fermentation times ($p < 0.05$).

Overall Percentage Improvement After 48 h of Fermentation

The data of table (6) shows the overall effect of fermentation using LAB1 isolate after 48 hours compared to time zero, through a 35% decrease in pH value, accompanied by a significant increase in total titrated acidity of approximately 400%, reflecting the efficiency of the selected isolate's fermentation activity. A clear increase of 65% in total phenolic content was also recorded, along with a 99% improvement in antioxidant activity, as measured by the DPPH method. These results, taken together, reflect the vital role of selected bacteria in improving the physical, chemical, and functional properties of locally fermented milk (Sharma *et al*., 2020).

TABLE 6. Percentage change after 48 hours compared to time zero

variable	**% change**
pH	↓ 35
Total acidity	↑ 400≈%
Phenolic content	↑ 65%

variable	% change
DPPH activity	↑ 99%

VI. References

Al-Burki, F. R., Ismail, E. N., Majeed, D. M., & Hasan, S. F. (2022). Determination of genetic diversity in selected salt tolerance and sensitive wheat by random amplified polymorphic cDNA. Iraqi Journal of Science, 63(12), 5157–5165.

Admassie, M. (2018). A review on food fermentation and the biotechnology of lactic acid bacteria. *World Journal of Food Science and Technology*, *2*(1), 19-24.

Anumudu, C.K., Miri, T., Onyeaka, H. (2024). Multifunctional applications of lactic acid bacteria: Enhancing safety, quality, and nutritional value in foods and fermented beverages. *Foods, 13(23), 3714.*

Brand-Williams, W., Cuvelier, M. E., Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology, 28(1), 25–30.*

Cappuccino, J.G., Welsh, C.T. (2017). Microbiology: A laboratory manual (11th ed.). Pearson.

De Man, J.C., Rogosa, M., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology, 23(1), 130–135.*

Filannino, P., Di Cagno, R., Gobbetti, M., & Rizzello, C.G. (2018). Metabolic and functional paths of lactic acid bacteria in plant foods: Get out of the labyrinth. *Current Opinion in Biotechnology, 49, 64–72.*

García, C., Rendueles, M., & Díaz, M. (2019). Liquid-phase food fermentations with microbial consortia involving lactic acid bacteria: A review. *Food Research International*, *119, 207-220.*

Hossain, T. J. (2022). Functional genomics of the lactic acid bacterium Limosilactobacillus fermentum LAB-1: metabolic,

probiotic and biotechnological perspectives. *Heliyon, 8(11).*

Hur, S.J., Lee, S.Y., Kim, Y.C., Choi, I., & Kim, G.B. (2014). Effect of fermentation on the antioxidant activity in plant-based foods. *Food Chemistry, 160, 346–356*.

Hutkins, R.W. (2018). Microbiology and technology of fermented foods (2nd ed.). Wiley-Blackwell.

ISO. (2017). Microbiology of the food chain—Preparation of test samples (ISO 6887-1). International Organization for Standardization.

Leroy, F., De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology, 15(2), 67–78.*

Li, X., He, T., Mao, J., Sha, R. (2022). Effects of lactic acid bacteria fermentation on physicochemical properties, functional compounds and antioxidant activity of edible grass. *Fermentation, 8(11): 647.*

Madigan, M. T., Bender, K. S., Buckley, D. H., Sattley, W. M., & Stahl, D. A. (2018). Brock biology of microorganisms (15th ed.). Pearson.

Montgomery, D. C. (2017). Design and analysis of experiments (9th ed.). Wiley.

Nielsen, S.S. (2017). Food analysis (5th ed.). Springer.

Nisa, K., Rosyida, V. T., Nurhayati, S., Indrianingsih, A. W., Darsih, C., & Apriyana, W. (2019). Total phenolic contents and antioxidant activity of rice bran fermented with lactic acid bacteria. In *IOP Conference Series: Earth and Environmental Science* . 251: 1, p. 012020. IOP Publishing.

Okoye, C.O., Gao, L., Wu, Y., Li, X., Wang, Y., & Jiang, J. (2024). Identification, characterization and optimization of culture medium conditions for organic acid-producing lactic acid bacteria strains from Chinese fermented vegetables. *Preparative Biochemistry & Biotechnology, 54(1), 49-60.*

Rizzello, C.G.; Lorusso, A.; Russo, V.; Pinto, D.; Marzani, B.; Gobbetti, M. Improving the

antioxidant properties of quinoa flour through fermentation with selected autochthonous lactic acid bacteria. Int. J. *Food Microbiol. 2017, 241, 252–261.*

Septembre-Malaterre, A., Remize, F., & Poucheret, P. (2018). Fruits and vegetables, as a source of nutritional compounds and phytochemicals: Changes in bioactive compounds during lactic fermentation. *Food Research International, 104, 86–99.*

Shahidi, F.; Zhong, Y. Measurement of antioxidant activity. J. Funct. *Foods 2015, 18, 757–781.*

Sharma, R., Garg, P., Kumar, P., Bhatia, S. K., & Kulshrestha, S. (2020). Microbial fermentation and its role in quality improvement of fermented foods. *Fermentation, 6(4), 106.*

Shi, L., Zhao, W., Yang, Z., Subbiah, V., & Suleria, H. A. R. (2022). Extraction and characterization of phenolic compounds and their potential antioxidant activities. *Environmental Science and Pollution Research, 29(54), 81112-81129.*

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates by means of Folin–Ciocalteu reagent. *Methods in Enzymology, 299, 152–178.*

Tamang, J. P., Watanabe, K., & Holzapfel, W. H. (2016). Review: Diversity of microorganisms in global fermented foods and beverages. *Frontiers in Microbiology, 7, 377.*

Tamime, A. Y., & Robinson, R. K. (2007). Yoghurt: Science and technology (3rd ed.). Woodhead Publishing.

Zhao, Y. S., Eweys, A. S., Zhang, J. Y., Zhu, Y., Bai, J., Darwesh, O. M., & Xiao, X. (2021). Fermentation affects the antioxidant activity of plant-based food material through the release and production of bioactive components. *Antioxidants, 10(12), 2004.*