Research Article

Microbial Co-Fermentation and Solvent Extraction Enhance the Phenolic Profile, Antioxidant Activity, and Antibacterial Potential of *Alpinia officinarum*

Eka Fitasari[1], Osfar Sjofjan[1*], Eko Widodo[1], Lilik Eka Radiati[1], Roy Hendroko Setyobudi[2] and Mohammad Zahoor[3]

[1]University of Brawijaya, Malang 65145, East Java, Indonesia, [2]University of Muhammadiyah Malang, Malang 65144, East Java, Indonesia, [3]University of Malakand, Chakdara, Khymber Pakhtunkhwa 18800, Pakistan.

Abstract | Synthetic antibiotics used in livestock production raise concerns related to antimicrobial resistance and chemical residues in animal-derived food products. As a natural alternative, plant-derived bioactive compounds such as *Alpinia officinarum* (red galangal) have gained increasing attention; however, their functional potential is often constrained by limited extraction efficiency and processing methods. This study aimed to evaluate the effects of microbial fermentation using *Lactobacillus plantarum* and *Rhizopus oligosporus*, individually and in combination, along with ethanol and distilled water extraction, on the phytochemical composition, antioxidant activity, and secondary metabolite profile of *A. officinarum* rhizomes. Fermented *A. officinarum* powder was extracted using a maceration method and analyzed for extraction yield, total phenolic content, total flavonoid content, and antioxidant activity using the DPPH assay (IC_{50}). Co-fermentation treatment produced the highest total phenolics (1 145.55 mg GAE 100 g^{-1}), flavonoids (85.89 mg QE 100 g^{-1}), and antioxidant activity (IC_{50} = 166.14 µg mL^{-1}). GC–MS analysis confirmed the presence of various secondary metabolites, including propionic acid, pentanoic acid 4-oxo ethyl ester (ethyl levulinate), tetradecanoic acid ethyl ester (ethyl myristate), hexadecanoic acid ethyl ester (ethyl palmitate), 2,4-di-tert-butylphenol, tetradecanoic acid (myristic acid), and n-hexadecanoic acid (palmitic acid), which have been previously reported to possess antibacterial-related properties. These findings demonstrate that dual microbial fermentation enhances the functional properties of *A. officinarum*, supporting its potential application as a natural antioxidant source rich in bioactive metabolites. This approach contributes to sustainable livestock production and aligns with Sustainable Development Goals (SDGs), particularly SDG 3 (Good Health and Well-being) and SDG 12 (Responsible Consumption and Production).

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***Correspondence** | Osfar Sjofjan, Department of Animal Nutrition and Feed, University of Brawijaya Malang 65145, East Java, Indonesia; **Email:** osfar@ub.ac.id
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Introduction

There is growing global interest in green bioprocessing technologies for the production of natural antioxidant and bioactive compounds as alternatives to synthetic chemicals (Widodo *et al*., 2019). Microorganisms play a critical role in sustainable bioprocessing of plant-derived materials by facilitating the release and transformation of bioactive compounds (Chihomvu *et al*., 2024; Qumsani, 2024). Microbial fermentation of plant substrates represents an environmentally friendly approach that can reduce reliance on synthetic antioxidants and antibiotics while addressing challenges such as antimicrobial resistance and adverse effects associated with chemical additives (Dhiman *et al*., 2025; Rachwał and Gustaw, 2025).

Alpinia officinarum Hance (red galangal) is a medicinal rhizome widely used in traditional medicine and culinary applications, known to be rich in phenolic compounds with various pharmacological effects (He *et al*., 2025). Phytochemical investigations have identified flavonoids, diarylheptanoids, phenylpropanoids, and essential oils that contribute to antioxidant activity and potential antimicrobial relevance (Aljobair, 2022; Fitasari *et al*., 2024; Lei *et al*., 2024).

Despite these promising bioactivities, a major limitation of *A. officinarum* is the low bioavailability of its phenolic compounds. Polyphenols often exhibit poor solubility, limited stability, and low intestinal absorption, resulting in reduced *in vivo* bioaccessibility (Patir *et al*., 2025).

Microbial fermentation has emerged as a promising strategy to overcome these limitations by enhancing the bioactive profile and bioavailability of plant-derived compounds. Lactic acid bacteria such as *Lactobacillus plantarum* produce enzymes including β-glucosidase, esterase, and tannase, which hydrolyze glycosidic and ester bonds in polyphenol conjugates, releasing free phenolics with higher solubility and bioactivity (Echegaray *et al*., 2023, Wahyudi *et al*., 2017). Similarly, fungal fermentation using *Rhizopus oligosporus* produces hydrolytic enzymes such as cellulases and pectinases that degrade plant structural components and release phenolic compounds.

Despite increasing interest in fermentation-based enhancement of herbal materials, studies on *A. officinarum* using dual microbial fermentation remain limited. Previous research has mainly focused on single-strain fermentation, leaving a gap in understanding the effects of fungal fermentation and bacterial–fungal co-fermentation on antioxidant activity and secondary metabolite profiles.

Therefore, this study aimed to evaluate the effects of *L. plantarum*, *R. oligosporus*, and their co-fermentation on the phytochemical composition, antioxidant activity, and secondary metabolite profile of *A. officinarum* using different extraction solvents. It was hypothesized that co-fermentation would produce synergistic effects, resulting in higher phenolic and flavonoid contents, enhanced antioxidant activity, and improved bioactive metabolite profiles associated with potential antimicrobial relevance, thereby supporting Sustainable Development Goals (SDGs), particularly SDG 3 (Good Health and Well-being) and SDG 12 (Responsible Consumption and Production).

Materials and Methods

Study location

This study was conducted in several accredited laboratories in Indonesia. Microbial culture rejuvenation was performed at the Industrial Microbiology Laboratory. Fermentation and maceration processes were carried out at the Food Engineering Laboratory, while solvent evaporation using a rotary evaporator was conducted at the Chemistry Laboratory, Universitas Tribhuwana Tunggadewi, Malang (S 7° 55' 51,74" and 112° 35' 53,54" E). Analyses of total phenolic content, total flavonoid content, antioxidant activity, and GC–MS profiling were performed at the Integrated Agricultural Laboratory, Universitas Brawijaya S 7°57'22.2552" and E 112°36'52.8948").

Preparation and standardization of microbial cultures

All culture media and glassware were sterilized using an autoclave at 121 °C for 15 min prior to use. *L. plantarum* was cultivated in de Man, Rogosa, and Sharpe (MRS) broth and agar, whereas *R. oligosporus* was cultured on Potato Dextrose Agar/Broth (PDA/PDB). The cultures were incubated at 37 °C for 24 h to 48 h. Serial dilutions ranging from 10^{-1} to 10^{-6} were subsequently prepared, and optical density was measured at 600 nm to standardize microbial populations equivalent to the 0.5 McFarland standard (1.5×10^8 CFU mL^{-1}) according to McFarland (2002).

Materials and experimental design

A. officinarum powder was obtained from Balai Materia Medica, Batu, East Java, Indonesia (S 7°51′55.386″ and E 112°31′8.238″). Proximate composition analysis of the powder revealed 92.8 % dry matter, 4.5 % crude protein, 1.9 % crude fat, 13.9 % crude fiber, 7.8 % ash, and 3617.17 kcal kg^{-1} gross energy. Pure cultures of *Lactobacillus plantarum* FNCC 0026 and *Rhizopus oligosporus* FNCC 6010 were obtained from the Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia (S 7°46′16.1″ and E 110°22′32.9″).

The experiment was arranged in a completely randomized nested design (CRD) with three replications, in which extraction solvent was nested within the fermentation treatment. The solvent extraction step was applied within each fermented substrate to characterize the extractable bioactive fraction. The primary treatment consisted of three fermentation groups: *L. plantarum* fermentation (P), *R. oligosporus* fermentation (R), and co-fermentation using both microorganisms (K). Within each fermentation treatment, extraction was performed using two solvents, namely 70 % ethanol (E) and distilled water (A). The detailed treatment combinations are presented in Table 1, and each treatment was conducted in triplicate.

Table 1: *Detail combination of the experiment*

Code	Treatments
PE	Fermented galangal *L. plantarum* extracted using ethanol
PA	Fermented galangal *L. plantarum* is extracted using distilled water
RE	Fermented galangal *R. oligosporus* extracted using ethanol
RA	Fermented galangal R. oligosporus extracted using distilled water
KE	Galangal fermentation Co-*L. plantarum* + *R. oligosporus* extracted using ethanol
KA	Galangal fermentation Co-*L. plantarum* + *R. oligosporus* extracted using distilled water

Note:
Factor A: *Type of microorganism (L. plantarum, R. oligosporus, and a combination of both at 1:1).*
Factor B: *Extraction solvent (ethanol 70 %, distilled water).*

Fermentation of A. officinarum

Fermentation was carried out following a modified method described by Lim *et al.* (2022). *A. officinarum* powder (60 mesh) was mixed with distilled water at a ratio of 1:7 (w v^{-1}) and inoculated with 10 % (v w^{-1}) microbial culture consisting of *L. plantarum*, *R. oligosporus*, or co-fermentation treatment containing 5 % *L. plantarum* culture and 5 % *R. oligosporus* culture. The samples were incubated under semi-anaerobic conditions at 35 °C ± 2 °C for 6 d. After fermentation, the samples were oven-dried at 50 °C and stored in airtight containers until further analysis.

Extraction procedure

Fermented and non-fermented powders were extracted using the maceration method following a modified procedure described by Bounyavong *et al.* (2014). Extraction was performed using 70 % ethanol or distilled water at a ratio of 1:10 (w v^{-1}) for 24 h at room temperature under dark conditions, followed by remaceration at a ratio of 1:5 (w v^{-1}) for an additional 24 h. The combined filtrates were concentrated using a rotary evaporator at 50 °C. For aqueous extracts, the concentrates were stored overnight at 5 °C, centrifuged at 5 000 rpm for 8 min – (1 rpm = 0.1047 rad/s, and subsequently reconcentrated. Extraction yield (%) was calculated using Equation (1):

$$\text{Yield (\%)} = \frac{weight\ of\ extract}{weight\ of\ sample} \text{ x } 100 \quad \text{.....(1)}$$

Determination of total phenolic content (TPC).

Total phenolic content was determined using the Folin–Ciocalteu method according to Aryal *et al.* (2019) and Damat *et al.* (2024). Briefly, 1 mL of extract solution (100 µg mL^{-1} to 500 µg mL^{-1}) was mixed with 2.5 mL of 10 % Folin–Ciocalteu reagent and incubated for 5 min. Subsequently, 2 mL of 7.5 % Na_2CO_3 solution was added, and the mixture was incubated at 50 °C for 10 min. Absorbance was measured at 765 nm using a spectrophotometer (Genesys 10S UV–Vis, Thermo Scientific, USA The results were expressed as mg gallic acid equivalent per 100 g dry weight (mg GAE100 g^{-1}).

Determination of total flavonoid content (TFC).

Total flavonoid content was determined using the aluminum chloride colorimetric method according to Aryal *et al.* (2019). The reaction mixture consisted of 1 mL extract, 0.2 mL of 10 % $AlCl_3$, 0.2 mL of 1 M CH_3COOK, and 5.6 mL distilled water. The mixture was incubated for 30 min at room temperature, and absorbance was measured at 415 nm using a spectrophotometer (Genesys 10S UV–Vis, Thermo Scientific, USA).

The results were expressed as mg quercetin equivalent per 100 g dry weight (mg QE 100 g^{-1}).

Antioxidant activity (DPPH assay)

Antioxidant activity was evaluated- using the DPPH radical scavenging method (Damat *et al.*, 2021). Equal volumes (2 mL) of 0.1 mM DPPH solution and extract (1 to 100 µg mL^{-1}) were mixed and incubated for 30 min in the dark. Absorbance was measured at 517 nm using a spectrophotometer (Genesys 10S UV–Vis, Thermo Scientific, USA). IC_{50} values were determined from linear regression. Ascorbic acid was used as a positive control.

GC–MS analysis

Secondary metabolites were identified using Gas Chromatography–Mass Spectrometry (GC–MS) (Shimadzu QP2020NX, Japan). Compound identification was carried out by comparing the obtained mass spectra with those available in the National Institute of Standards and Technology (NIST) library database.

Statistical analysis

Data were analyzed using nested analysis of variance (nested ANOVA), followed by Duncan's multiple range test at $p < 0.05$. Statistical analyses were performed using SPSS version 24.

Results and Discussion

Percentage weight yield compared to simplex weight

The percentage weight yield of fermented *A. officinarum* extract under different microbial and solvent treatments is presented in Figure 1. The average percentage weight yield ranged from 20.47 % to 32.19 %. Ethanol extraction generally produced higher yield percentages (29.37 % to 32.19 %) than distilled water extraction (20.47 % to 26.04 %). Statistical analysis showed that fermentation treatment significantly affected extraction yield ($p < 0.01$), particularly in co-fermentation treatments.

The higher extraction yield observed in ethanol extracts may be associated with improved extraction efficiency of phenolic and semi-polar compounds. Previous studies have similarly reported that microbial fermentation enhances the release of bound phenolics through enzymatic degradation of plant cell wall structures (Darina *et al.*, 2025; Yang *et al.*, 2023). Ethanol is also known to extract a broader range of phytochemicals compared with water due to its polarity characteristics.

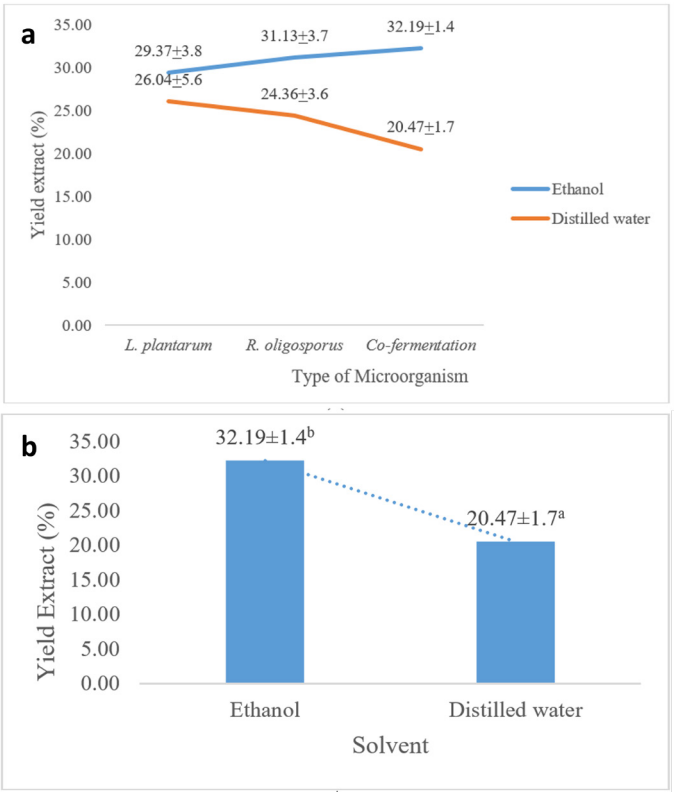


Figure 1: *Percentage weight yield of fermented A. officinarum extract*

Co-fermentation using *L. plantarum* and *R. oligosporus* likely promoted synergistic enzymatic activity, resulting in enhanced degradation of structural components and increased release of extractable bioactive compounds.

Total phenolic content of fermented A. officinarum extract

The total phenolic content of fermented *A. officinarum* extract is presented in Figure 2. Microbial treatment significantly affected total phenolic content ($p < 0.05$). Co-fermentation produced the highest phenolic content, particularly in distilled water extracts (1 145.55 mg GAE 100 g^{-1}), which exceeded the values observed in non-fermented controls.

The increase in phenolic content may be attributed to microbial enzymatic activity during fermentation. Enzymes such as cellulases, pectinases, esterases, and glycosidases facilitate degradation of plant cell wall structures and release bound phenolic compounds into more soluble forms (Darina *et al.*, 2025; Yang *et al.*, 2023). In addition, β-glucosidase activity produced by *L. plantarum* contributes to the conversion of glycosidic phenolics into more bioactive aglycone forms.

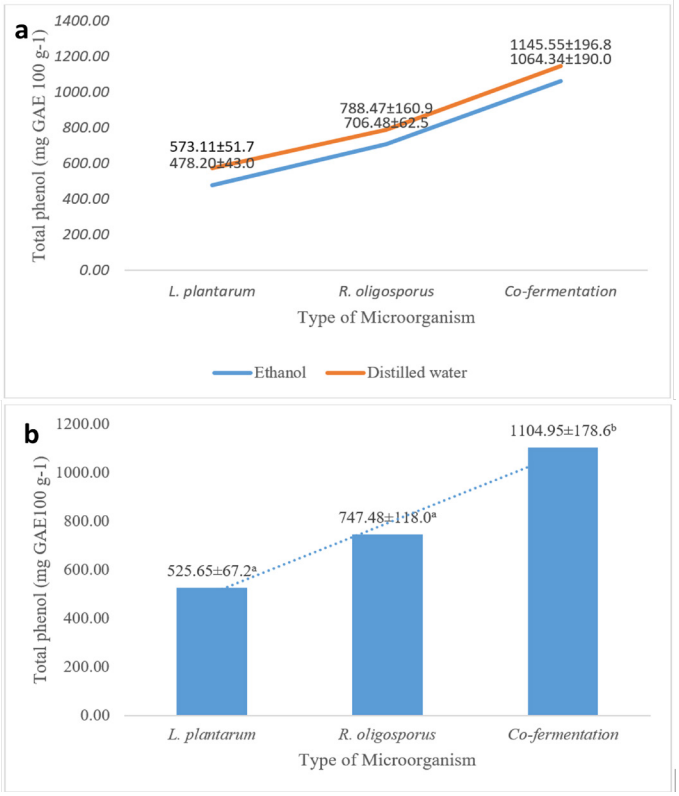


Figure 2: *Total phenolic content of fermented A. officinarum extract*

These findings are consistent with previous reports demonstrating that co-fermentation improves phenolic release and antioxidant-related phytochemical content in plant substrates (Ozturk *et al.*, 2024; Stodolak *et al.*, 2020).

Total flavonoid content of fermented A. officinarum extract
The total flavonoid content of fermented *A. officinarum* extract is presented in Figure 3. Microbial treatment significantly influenced total flavonoid content ($p < 0.05$). Co-fermentation resulted in the highest flavonoid levels, with ethanol extraction yielding 85.89 mg QE 100 g^{-1} and distilled water extraction yielding 83.99 mg QE 100 g^{-1}.

The enhancement of flavonoid content is likely related to the hydrolytic activity of enzymes such as β-glucosidase, tannase, and feruloyl esterase produced during fermentation. These enzymes facilitate the breakdown of flavonoid conjugates and release more bioactive aglycone forms.

The synergistic interaction between *R. oligosporus* and *L. plantarum* may further enhance the liberation and transformation of flavonoid compounds. Similar increases in flavonoid content following microbial fermentation have been reported in previous studies (Shen *et al.*, 2024; Stodolak *et al.*, 2020).

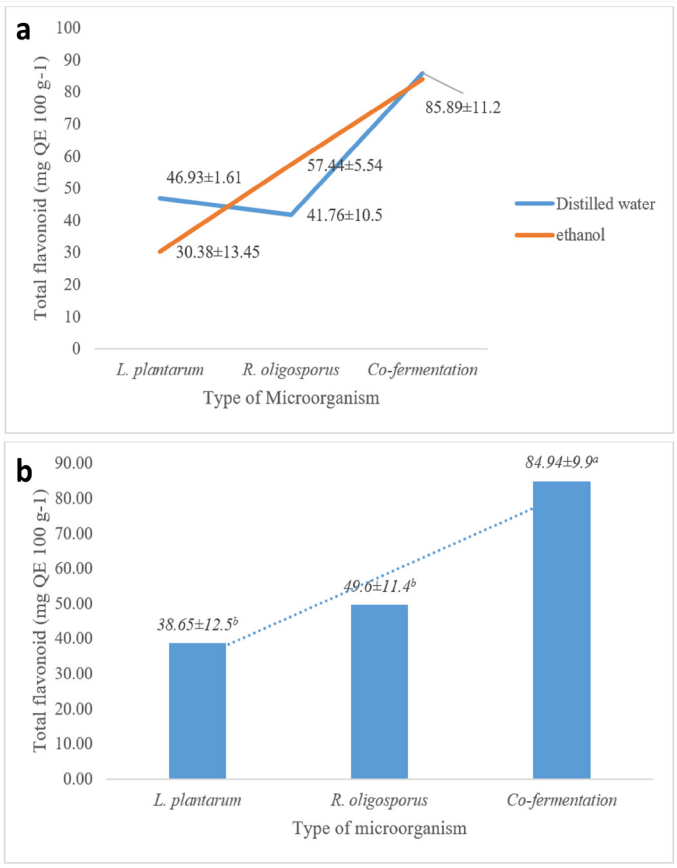


Figure 3: *Total flavonoid content of fermented A. officinarum extract*

Antioxidant activity of fermented A. officinarum extract
The antioxidant activity of fermented *A. officinarum* extract is presented in Figure 4. Microbial treatment significantly improved antioxidant activity ($p < 0.05$), as indicated by reduced IC_{50} values. The lowest IC_{50} value (166.14 µg mL^{-1}) was observed in the distilled water extract of co-fermented *A. officinarum*, indicating the strongest radical scavenging activity.

Lower IC_{50} values indicate stronger antioxidant activity because smaller concentrations are required to neutralize DPPH radicals (Gulcin and Alwasel, 2023). The improved antioxidant activity observed in co-fermented extracts is likely associated with increased phenolic and flavonoid contents.

Distilled water extracts showed antioxidant activity comparable to or slightly better than ethanol extracts, possibly due to improved extraction of polar antioxidant compounds. Similar findings have been reported in previous studies evaluating aqueous extraction of phenolic-rich materials (Chatepa *et al.*, 2024). Identification of secondary metabolites in co-fermented *A. officinarum* extract.

The identified secondary metabolites in co-fermented *A. officinarum* extract are presented in Table 2, while the GC–MS chromatogram is shown in Figure 5.

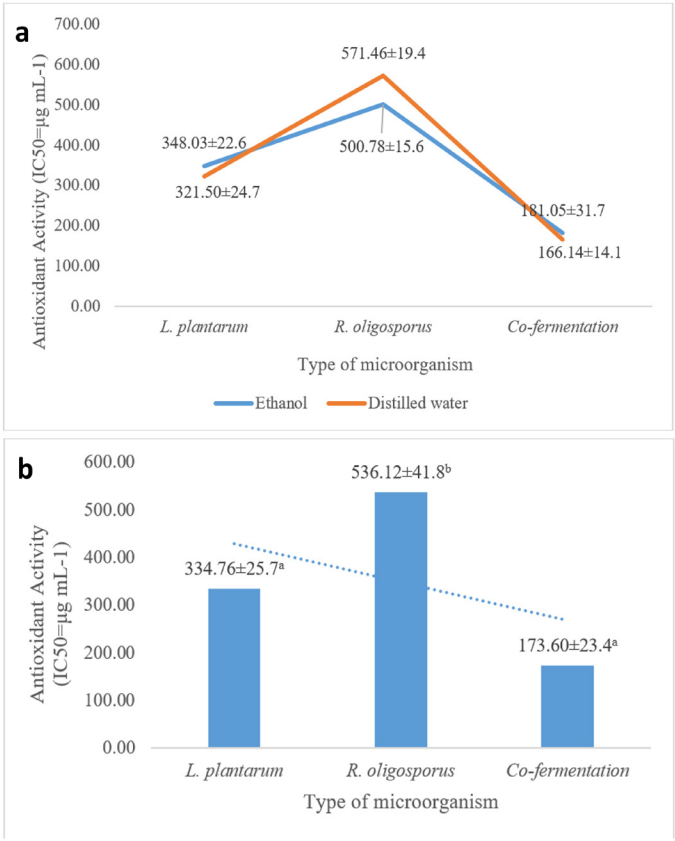


Figure 4: *Antioxidant activity (IC_{50}) of fermented A. officinarum extract*

GC–MS analysis revealed the presence of several fatty acids, esters, and phenolic-related compounds generated during microbial fermentation. Several compounds identified in this study, including propionic acid and 2,4-di-tert-butylphenol (2,4-DTBP), have been previously reported to possess antibacterial-related properties.

Long-chain fatty acids such as myristic acid and palmitic acid have also been associated with reported antibacterial effects, particularly against Gram-positive bacteria. Ethyl ester derivatives detected in the extract may result from esterification reactions during fermentation and contribute additional biological relevance.

Overall, the GC–MS results indicate that co-fermentation not only enhances the release of phenolic compounds but also contributes to the formation of new bioactive metabolites through microbial biotransformation, supporting the formation of metabolites previously associated with antimicrobial relevance in fermented *A. officinarum* extract.

Considering that *A. officinarum* is a rhizome crop cultivated in direct contact with soil environments, attention should also be given to the potential risk of microplastic contamination throughout the production chain of plant-based raw materials and fermented extracts. Previous studies have reported the occurrence of microplastics in agricultural soils and tuber crops, indicating that root- and rhizome-based commodities may act as entry points of microplastics into the feed and food chain (Setyobudi *et al.*, 2024a, 2024b, 2025).

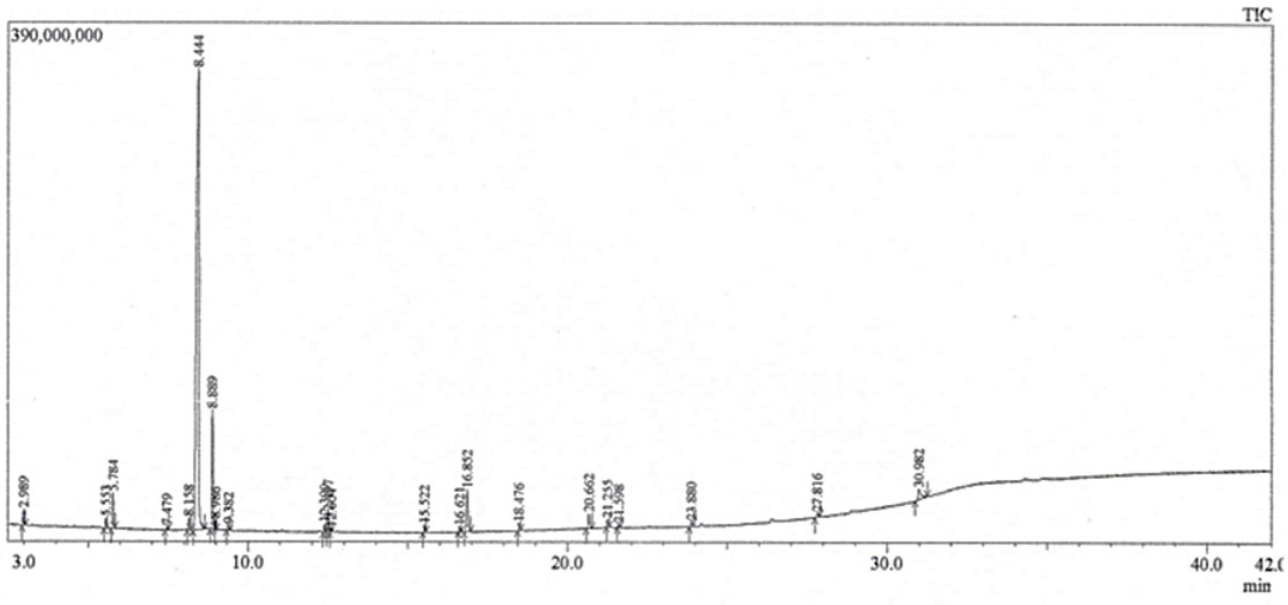


Figure 5: *GC–MS chromatogram of co-fermented A. officinarum extract*

Table 2: *Percentage weight yield of fermented A. officinarum extract under different microbial and solvent treatments*

Compound name	Compound class	Main biological activity	Reference
Diethyl sulfate	Synthetic alkylating agent (ethyl sulfate)	None – known mutagen/carcinogen (no antimicrobial use)	(National Toxicology Program, 2021)
4-Methyl-oxazole-5-carboxylic acid	Heterocyclic oxazole carboxylic acid (synthetic)	None reported; oxazole derivatives often bioactive	(Wipf *et al.*, 2005)
Cyclohexasiloxane, dodecamethyl	Cyclic siloxane (volatile silicone, synthetic)	Inert – no inherent bioactivity; used in cosmetics	(IMAP Single Assessment Report, 2016)
Cycloheptasiloxane, tetradecamethyl	Cyclic siloxane (volatile silicone, synthetic)	Inert – no inherent bioactivity; used in cosmetics	(IMAP Single Assessment Report, 2016)
Propanoic acid	Short-chain fatty acid	Broad-spectrum antimicrobial (antibacterial & antifungal); food preservative	(Antone *et al.*, 2023; Da Costa *et al.*, 2024)
Diethyl malinate (Diethyl malonate)	Diethyl ester of malonic acid (synthetic ester)	None known – used as synthetic intermediate	(Libre Texts OpenStax, 2025)
Pentanoic acid 4-oxo ethyl ester (Ethyl levulinate)	Levulinic acid ester (bio-derived keto-ester)	Broad antimicrobial (preservative in foods/cosmetics)	(Zbaqchem, 2024)
Butanedioic acid diethyl ester (Diethyl succinate)	Diethyl ester of succinic acid (ester)	None known (no reported antimicrobial activity)	-
Cyclononasiloxane, octadecamethyl	Cyclic siloxane (volatile silicone)	Inert – no antimicrobial role (GC-MS artifact)	(IMAP Single Assessment Report, 2016)
2,3-Diethoxypropionic acid ethyl ester	Ethyl diester of 2,3-dihydroxypropionic acid (synthetic)	None known (likely artifact)	-
5-Bromovaleric acid but-3-yn-2-yl ester	Halogenated unsaturated ester (synthetic)	None known (likely artifact)	-
Butanedioic acid hydroxy-diethyl ester (Diethyl malate)	Diethyl ester of malic acid (synthetic/plant)	None known – no reported antimicrobial action	-
Tetradecanoic acid ethyl ester (Ethyl myristate)	Ethyl ester of myristic acid (fatty acid ester)	Weak antibacterial (saturated fatty ester).	(Okukawa *et al.*, 2021)
Cyclooctasiloxane, hexadecamethyl	Cyclic siloxane (volatile silicone)	Inert – no antimicrobial role (cosmetic contaminant	(IMAP Single Assessment Report, 2016)
Hexadecanoic acid ethyl ester (Ethyl palmitate)	Ethyl ester of palmitic acid (fatty acid ester)	Anti-inflammatory (TNF-α, IL-6 inhibitor); no significant antibacterial	(Saeed *et al.*, 2012)
2,4-Di-tert-butylphenol	Alkylated phenolic compound	Broad antimicrobial (antibacterial, antifungal); antioxidant, antiinflamasi, sitotoksik, antivirus, insektisida, dan nematisida	(Nwobodo *et al.*, 2022; Rouvier *et al.*, 2024; Gupta *et al.*, 2023; Mishra *et al.*, 2020; Sundar *et al.*, 2025).
Tetradecanoic acid (Myristic acid)	Saturated fatty acid	Selective antibacterial (strong against Gram-positives)	(Jua´rez-Rodrı´guez *et al.*, 2021; Okukawa *et al.*, 2021)
n-Hexadecanoic acid (Palmitic acid)	Saturated fatty acid	Antibacterial and antifungal (broad-spectrum)	(Idris *et al.*, 2018; Nabi *et al.*, 2022; Ghfil *et al.*, 2025)

This issue is particularly important because the present study aims to explore the potential use of fermented *A. officinarum* as a functional feed-related ingredient. Without adequate monitoring and mitigation strategies, microplastic contamination may negatively affect livestock productivity and animal health, while also facilitating the transfer of microplastics and associated contaminants into humans through animal-derived food products (Ramachandraiah *et al.*, 2022; Setyobudi, 2026; Wahyudi *et al.*, 2025). Such conditions may ultimately threaten food safety and food security, especially in developing countries that rely heavily on sustainable livestock production systems.

Therefore, future investigations are required to evaluate the occurrence, distribution, and potential transfer of microplastics during fermentation and downstream

processing in order to ensure the safety, sustainability, and environmental integrity of fermentation-based functional products. These efforts are also aligned with Sustainable Development Goals (SDGs), particularly SDG 2 (Zero Hunger), SDG 3 (Good Health and Well-being), and SDG 12 (Responsible Consumption and Production).

Conclusions and Recommendations

This study demonstrated that co-fermentation using *L. plantarum* and *R. oligosporus* significantly enhanced extraction yield, total phenolic content, total flavonoid content, and antioxidant activity of *A. officinarum* extract compared with single-microbe fermentation and non-fermented treatments. Distilled water also showed comparable or superior performance to ethanol in several parameters, indicating its potential as an environmentally friendly extraction solvent.

GC–MS analysis confirmed that microbial fermentation promoted the formation of bioactive metabolites, including compounds previously reported to possess antimicrobial-related properties. These findings highlight the potential of co-fermentation as a sustainable strategy for developing functional *A. officinarum*-based products for food, feed, and health-related applications.

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Novelty Statement

This study presents a novel approach combining microbial co-fermentation and solvent selection to enhance the functional properties of *A. officinarum*. The findings demonstrated that co-fermentation using *L. plantarum* and *R. oligosporus*, together with distilled water extraction, effectively improved phenolic content, flavonoid levels, antioxidant activity, and bioactive metabolite formation. In addition, this study introduces a future-oriented perspective regarding potential microplastic contamination in rhizome-based raw materials intended for feed-related applications.

Author's Contribution

Eka Fitasari: Designed the study, conceptualized and elaborated the intellectual content, performed literature search, manuscript preparation, and manuscript revision.
Osfar Sjofjan: Supervisor, project administration, and manuscript review.
Eko Widodo, Lilik Eka Radiati, and Mohammad Zahoor: Manuscript review.
Roy Hendroko Setyobudi: Manuscript review, manuscript revision, template-layout, and guarantor.
All authors have read and approved the final manuscript.

Generative AI and AI-assisted technology statement
The authors declare that no generative AI was used in the generation, analysis, or interpretation of research data. AI-assisted tools were used solely for language editing and improving clarity of the manuscript.

Conflict of interest
The authors have declared no conflict of interest.

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