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Metabolite Profiling and Molecular Docking of Candidate Fungal Postbiotics Derived from *Rhizopus oligosporus*

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Abstract: Fungal postbiotics have gained increasing attention as a source of bioactive metabolites with potential applications in food preservation. Previous studies have demonstrated that candidate fungal postbiotics produced by *Rhizopus oligosporus* cultivated on edamame husks (*Glycine max* L. Merrill) contain phenolic compounds and exhibit antioxidant activity. Building on these findings, this study aimed to characterize the metabolites present in candidate postbiotics and evaluate their antibacterial potential using molecular docking. Candidate postbiotics were produced by submerged fermentation and analyzed using gas chromatography-mass spectrometry (GC-MS). The major metabolites were docked to *Staphylococcus aureus* DNA gyrase subunit B (PDB ID: 4URM). GC-MS revealed a diverse metabolite profile dominated by 2,3-butanediol, followed by glycerin, dianhydromannitol, benzoic acid, and n-hexadecanoic acid. All selected metabolites exhibited negative predicted docking score, indicating thermodynamically favorable interactions with the target protein. Among them, n-hexadecanoic acid showed the strongest predicted binding affinity, followed by benzoic acid. These findings suggest that candidate fungal postbiotics derived from *R. oligosporus*-fermented edamame husks are promising natural bioactive ingredients for food biopreservation and active food packaging. Future molecular dynamics simulations and experimental validation are needed to confirm these computational predictions.

Keywords: Active food packaging; Edamame husk; Fungal postbiotics; Fungal biotechnology; *Rhizopus oligosporus*.

Introduction

Postbiotics have become an important topic in biotics research and are defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as preparations of inanimate microorganisms and/or their components that provide health benefit to the host (Gervasoni et al., 2023). Compared with probiotics, postbiotics are more stable under different temperature and pH conditions, easier to store, and safer because they do not transfer antibiotic resistance genes to the body's natural bacteria, which is a major concern with probiotics (Isaac-Bamgboye et al., 2024; Mosallaie et al., 2025; Tong et al., 2025). Their biological activities are mainly associated with bioactive compounds such as organic acids, enzymes, exopolysaccharides, and antimicrobial peptides (Wu et al., 2025). Although bacterial postbiotics have been widely studied, filamentous fungi are receiving increasing attention because they produce a diverse range of metabolites with potential biological activities (Seidler et al., 2024).

Aligned with the principles of a circular bioeconomy, the utilization of agro-industrial waste such as edamame husk (*Glycine max* L. Merrill) provides a sustainable substrate for postbiotic production. These husks are notably

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rich in complex carbohydrates, including cellulose, hemicellulose, and pectin, which serve as an essential carbon source to support microbial growth and the formation of postbiotics (Razola-diaz et al., 2024; Wahyudi et al., 2025). One microorganism that can effectively use these compounds is *Rhizopus oligosporus*, a filamentous fungus widely used in tempeh production. This fungus produces enzymes that break down complex carbohydrates into smaller bioactive compounds during fermentation (Aktaş et al., 2025). Previous studies showed that postbiotics derived from edamame husk through *R. oligosporus* fermentation contain high level of phenolic compounds and exhibit significant antioxidant capacity (Prasetyo et al., 2025; Prasetyo & Rasmiyana, 2026). Despite this potential, the specific metabolites responsible for these biological activities have not been clearly identified because the fermented matrix contains a complex mixture of compounds (Sawant et al., 2025). Identifying these bioactive molecules is important to better understand the mechanisms behind their biological effects and to support the development of fungal postbiotic-based functional products.

To address this challenge, metabolite profiling using Gas Chromatography-Mass Spectrometry (GC-MS) can be used to identify the metabolites present in postbiotics. This technique has been widely used to detect various bioactive compounds produced during fermentation, including organic acids, alcohols, phenolic compounds, and other metabolites (Mehta et al., 2025; Sharieff et al., 2025). After the metabolites are identified, molecular docking can be used to predict their interactions with target proteins and evaluate their potential biological activities (Benzenine, Daoud, Aissaoui, Kibou, et al., 2024). Combining these approaches helps relate the chemical profile of postbiotics to their possible biological functions. Among these biological activities, antibacterial activity is particularly important because antimicrobial metabolites have potential applications in food preservation and active food packaging (Pawar et al., 2026; Thavapudalvi et al., 2024).

Therefore, this study aimed to characterize the metabolites present in candidate fungal postbiotics produced from *R. oligosporus*-fermented edamame husk using GC-MS and to evaluate their predicted interactions with *S. aureus* through molecular docking. The results are expected to improve our understanding of the bioactive compounds present in fungal postbiotics and provide a scientific basis for their future application in biopreservation and active food packaging.

Materials and Methods

Sample collection and preparation

Edamame (*Glycine max* L.) husks were collected from a local market in Jember Regency, Indonesia, and used as the fermentation substrate. *R. oligosporus* was obtained from a culture maintained at the Microbiology Laboratory, Politeknik Negeri Jember, Indonesia. The substrate was prepared following the method described by Razola-diaz et al. (2024) with minor modifications. Briefly, the edamame husks were dried in a hot-air oven at 40°C for 6 h until the moisture content was below 10%. The dried husks were then ground into powder and passed through an 80-mesh sieve to obtain a uniform particle size. A total of 3 g of edamame husks powder was suspended in 100 mL of sterile distilled water and sterilized by autoclaving at 121°C for 15 min prior to fermentation. Potato Dextrose Agar (PDA) was used for maintaining the fungal culture, and sterile distilled water was used throughout the experimental procedures.

Candidate postbiotics production

Postbiotics were produced according to the optimized protocol established in our previous study (Prasetyo et al., 2025). Briefly, each 100 ml fermentation medium containing 3 g of edamame husk powder was inoculated with 1 mL of a *R. oligosporus* spore suspension (1 x 10^7 spores/mL) and incubated at 30°C with continuous agitation at 150 rpm for five days. An uninoculated control was prepared under identical conditions without the addition of *R. oligosporus* spores. After fermentation, both the inoculated and control cultures were filtered through filter paper to separate the fermentation broth from the residual substrate. The resulting filtrate was heat-treated at 121°C for 15 min using an autoclave. The heat-treated filtrates from the inoculated cultures were stored at 4°C until further analysis.

GC-MS Analysis

The chemical profile of the postbiotics was characterized by gas chromatography-mass spectrometry (GC-MS) according to the method described by Mehta et al. (2025) with slight modifications. Prior to analysis, the samples were filtered through a 0.22 µm membrane filter and 1 µL of the filtrate was injected into GCMS-QP2020 NX system (Shimadzu, Japan) equipped with an AOC-6000 Plus autosampler. The injector temperature was maintained at 250ºC and samples were introduced in split mode. Chromatographic separation was performed using a Shimadzu SH-PolarWax-MS capillary column (30 m x 0.25 mm, 0.25 µm film thickness; polyethylene glycol stationary phase).

Helium was used as the carrier gas at a pressure of 100 kPa, with a column flow rate of 1.33 mL/min, a total flow rate of 30 mL/min, a linear velocity of 43 cm/s, and purge flow of 3.0 mL/min. The oven temperature was programmed from 100°C (held for 10 min) to 250°C at a rate of 20°C/min, followed by a 20 min hold at the final temperature. The ion source and interface temperatures of the mass spectrometer were maintained at 280°C and 250°C, respectively. Detected metabolites were identified by matching their mass spectra against the NIST17 mass spectral library. The identified metabolites were subsequently screened to select candidate bioactive compounds for further in silico analysis.

In-Silico Analysis by Molecular Docking

Molecular docking analysis was performed using the crystal structure of DNA gyrase subunit B from *Staphylococcus aureus* (PDB ID: 4URM), retrieved from the RCSB Protein Data Bank. The co-crystallized native ligand (XAM) was used to validate the docking protocol (Benzenine, Daoud, Aissaoui, Kibou, et al., 2024). Receptor preparation was performed using AutoDockTools 1.5.7. The test ligands were selected based on the highest relative abundances identified by GC-MS analysis. Molecular docking was conducted using PyRx 0.8 with the AutoDock Vina Engine. The resulting protein-ligand complexes were evaluated based on binding affinity and binding interactions, which were visualized using BIOVIA Discovery Studio Visualizer (Iqbal *et al*., 2026).

Results and Discussion

GC-MS Analysis

GC-MS analysis identified various metabolites in the candidate postbiotics produced by *R. oligosporus* grown on edamame husks. The identified compounds were classified into ten chemical classes according to their chemical structures and their relative abundance was determined based on GC-MS peak area (Fig.1). Alcohol and polyols were the predominant class, mainly represented by 2,3-butanediol, glycerin, and isosorbide. Carbohydrate derivatives were the second most abundant class, with dianhydromannitol as the major compound. Organic acids and derivatives were mainly composed of acetic acid, benzoic acid, phosphonic acid, (p-hydroxyphenyl)- and 2-propenoic acid, 3-(2-hydroxyphenyl)-, (E)-. Fatty acids and derivatives were represented primarily by n-hexadecanoic acid, tetradecanoic acid and 9,12-Octadecadienoic acid (Z, Z)-, methyl ester, while hydroquinone and phenol, 2-methoxy- were the predominant phenolic compounds.

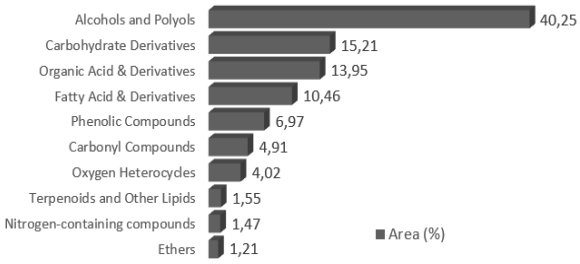


Figure 1. Distribution of chemical classes identified in candidate postbiotics produced by *R. oligosporus* grown on edamame husks based on GC-MS peak area (%).

The total ion chromatogram (TIC) obtained from GC-MS analysis showed a complex metabolite profile of the candidate postbiotics produced by *R. oligosporus* grown on edamame husks (Fig.2). A total of 52 chromatographic peaks were detected across the retention time range, indicating the presence of diverse metabolites in the postbiotic preparation. To ensure reliable compound identification, only metabolites with relatively high peak areas and spectral similarity scores of ≥ 93 were selected and are presented in Table 1. The predominant metabolites were 2,3-butanediol (19.99%), glycerin (16.81%),

dianhydromannitol (14.29%), n-hexadecanoic acid (6.45%), acetic acid (5.62%), hydroquinone (3.93%), and benzoic acid (3.05%), whereas the remaining compounds were detected at peak areas below 2%.

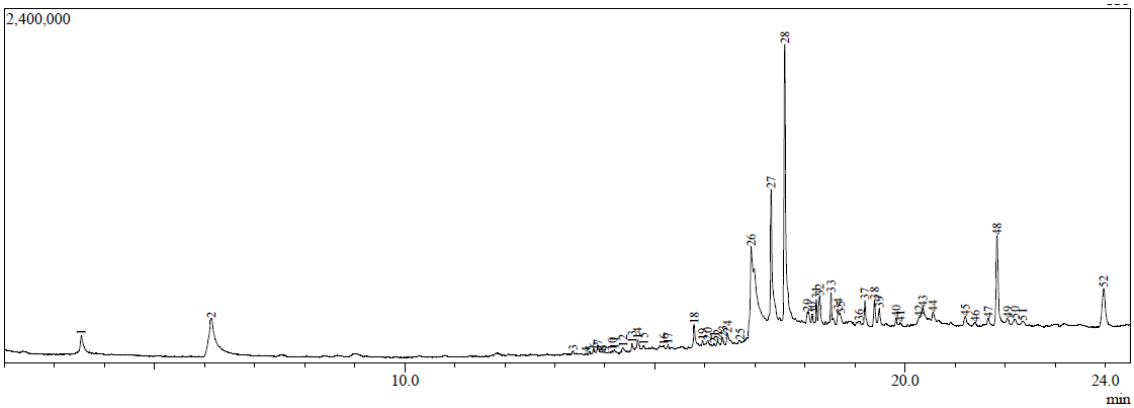


Figure 2. Total ion chromatogram (TIC) of candidate postbiotic metabolites produced by *R. oligosporus* grown on edamame husks.

Table 1. Major candidate postbiotic metabolites identified by GC-MS in the postbiotic preparation produced by *R. oligosporus* grown on edamame husks

Peak	Retention Time	Area (%)	Similarity	Name	Biological Activity
1	3.535	1.42	98	2-Propanone, 1-hydroxy-	Antibacterial (Mansur et al., 2021)
2	6.130	5.62	96	Acetic acid	Antibacterial; Antibiofilm (Harrison et al., 2023)
14	14.655	0.61	95	Phenol, 2-methoxy-	Antioxidant, Anti-inflammatory (Aqeel et al., 2023)
18	15.786	1.49	93	Phosphonic acid, (p-hydroxyphenyl)-	Anti-inflammatory (Ivanova et al., 2022)
26	16.922	19.99	94	2,3-Butanediol	Antibacterial; Antioxidant (Xu et al., 2025)
27	17.324	14.29	94	Dianhydromannitol	Antibacterial (Erdoğan Eliuz, 2022)
28	17.593	16.81	97	Glycerin	Antibacterial (Sleem et al., 2023)
32	18.289	3.05	94	Benzoic acid	Antibacterial; Antifungal (Issa & Mohammed, 2025)
33	18.521	1.74	93	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	Antibacterial (Rahman et al., 2014)
34	18.661	1.43	94	2-Propenoic acid, 3-(2-hydroxyphenyl)-, (E)-	Antibacterial (Naji et al., 2025)
37	19.197	1.55	96	Phytol	Antibacterial; Anti-inflammatory (Almeida-Bezerra et al., 2024)
38	19.389	1.53	95	Isosorbide	Bio-stimulant (Agrahari et al., 2025)
40	19.821	0.43	93	Tetradecanoic acid	Enzyme inhibitor (Lakshmanasenthil et al., 2018)
48	21.839	6.45	94	n-Hexadecanoic acid	Antibacterial; Antioxidant (Ganesan et al., 2024)
52	23.971	3.93	96	Hydroquinone	Antioxidant (Giner et al., 2022)

Note: Only the major candidate postbiotic compounds with relatively high peak areas and high spectral similarity score ≥ 93 are presented.

Molecular Docking

The molecular docking results of the selected candidate postbiotic compounds against DNA gyrase subunit B are summarized in Table 2, while the corresponding two-dimensional interaction diagrams are shown in Fig. 3. The predicted binding affinity ranged from -3.8 to -5.2 kcal/mol, whereas the native ligand (XAM) exhibited a binding affinity of -9.7 kcal/mol. Among the tested compounds, n-hexadecanoic acid showed the strongest predicted binding affinity (-5.2 kcal/mol), followed by benzoic acid, glycerin, 2,3-butanediol, and dianhydromannitol. As shown in Fig.3, n-hexadecanoic acid formed two hydrogen bonds and three hydrophobic interactions, while benzoic acid formed two hydrogen bonds and three hydrophobic interactions. Glycerin interacted predominantly through three hydrogen bonds, whereas 2,3-butanediol formed one hydrogen bond and three hydrophobic interactions. Dianhydromannitol formed two hydrogen bond interactions.

Table 2. Molecular docking results of selected candidate postbiotic metabolites against *S. aureus* (PDB ID: 4URM)

Compounds	Estimated ΔG (kcal/mol)	Residues	Category	Type of Interaction
Glycerin (PubChem CID 753)	-4.1	SER55	Hydrogen Bond	Conventional Hydrogen Bond
		ASP81	Hydrogen Bond	Conventional Hydrogen Bond
		ILE51	Hydrogen Bond	Conventional Hydrogen Bond
2,3-Butanediol (PubChem CID 262)	-4.1	SER55	Hydrogen Bond	Conventional Hydrogen Bond
		ILE86	Hydrophobic	Alkyl
		ILE175	Hydrophobic	Alkyl
		VAL79	Hydrophobic	Alkyl
Dianhydromannitol (PubChem CID 23619611)	-3.8	SER55	Hydrogen Bond	Conventional Hydrogen Bond
		ILE51	Hydrogen Bond	Carbon Hydrogen Bond
Benzoic Acid (PubChem CID 243)	-5.1	THR173	Hydrogen Bond	Conventional Hydrogen Bond
		ASP81	Hydrogen Bond	Conventional Hydrogen Bond
		ILE86	Hydrophobic	Pi-Sigma
		ASN54	Hydrophobic	Amide-Pi Stacked
		ILE175	Hydrophobic	Pi-Alkyl
n-Hexadecanoid Acid (PubChem CID 985)	-5.2	SER55	Hydrogen Bond	Conventional Hydrogen Bond
		THR173	Hydrogen Bond	Conventional Hydrogen Bond
		ILE86	Hydrophobic	Alkyl
		PRO87	Hydrophobic	Alkyl
		ILE102	Hydrophobic	Alkyl
Native Ligand (XAM)	-9.7	GLN91	Hydrogen Bond	Conventional Hydrogen Bond
		LYS93	Hydrogen Bond	Conventional Hydrogen Bond
		GLY109	Hydrogen Bond	Conventional Hydrogen Bond
		PRO87	Hydrogen Bond	Carbon Hydrogen Bond
		ALA108	Hydrogen Bond	Carbon Hydrogen Bond
		LYS111	Hydrogen Bond	Carbon Hydrogen Bond
		ILE86	Hydrophobic	Pi-Sigma
		ASN54	Hydrophobic	Amide-Pi Stacked
		ALA98	Hydrophobic	Alkyl
		ILE102	Hydrophobic	Alkyl
		ILE51	Hydrophobic	Alkyl
		ILE175	Hydrophobic	Alkyl

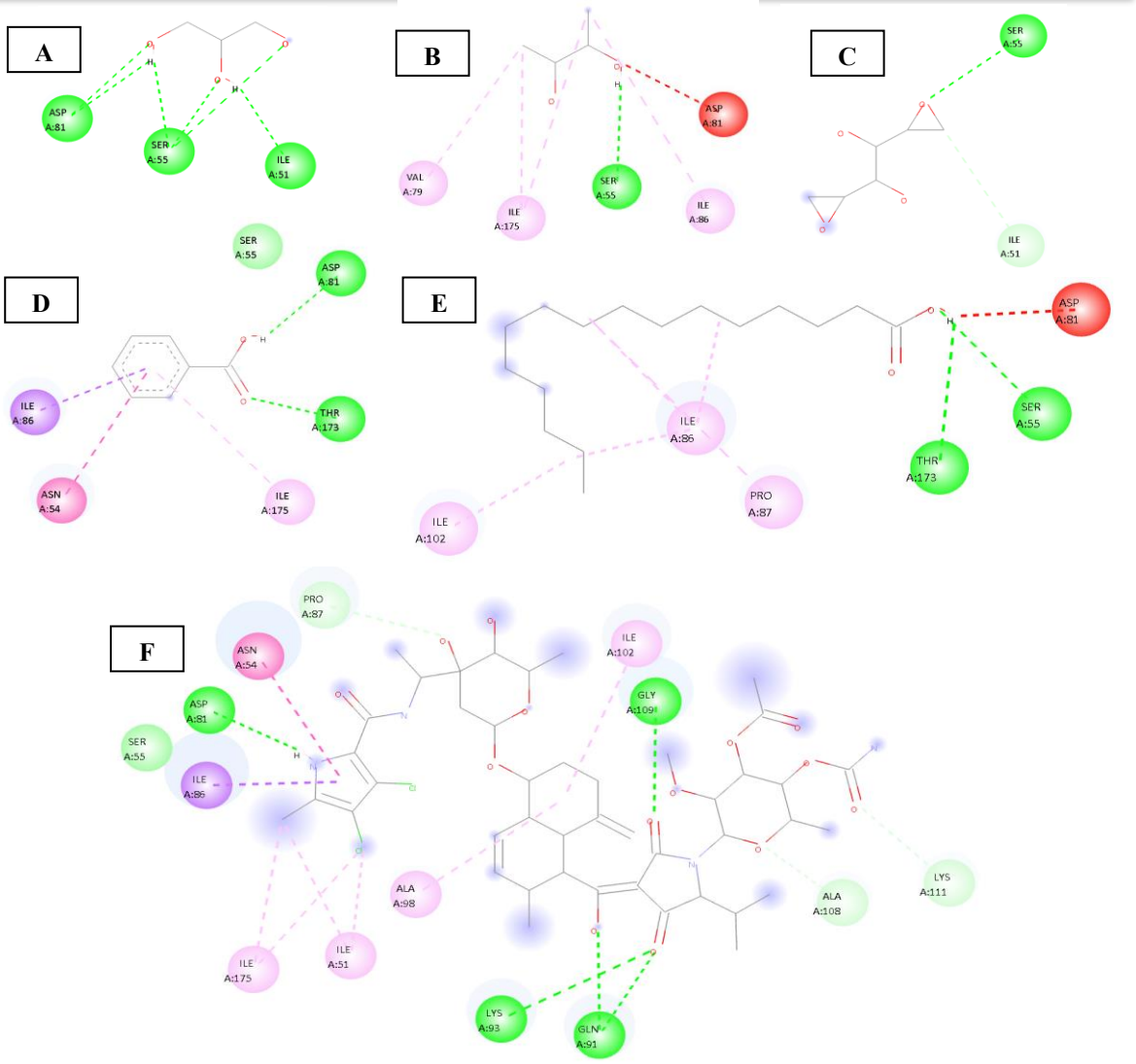


Figure 3. Two-dimensional (2D) interactions diagrams of the docked complexes between DNA gyrase subunit B from *S. aureus* (PDB ID: 4URM) and (A) Glycerin, (B) 2,3-Butanediol, (C) Dianhydromannitol, (D) Benzoic acid, (E) n-Hexadecanoic acid, and (F) XAM. Green dashed lines indicate hydrogen bonds, purple dashed lines indicate hydrophobic interactions, and red dashed lines indicate unfavorable interactions.

Discussion

GC-MS profiling of candidate fungal postbiotic metabolites

While postbiotics derived from lactic acid bacteria (LAB) have been extensively studied, research on fungal postbiotics, particularly those produced by *Rhizopus* sp. remains limited. The present study identified 2,3-butanediol as one of the major metabolites in the candidate postbiotic produced by *R. oligosporus* grown on edamame husks. The high abundance of this compound may be associated with the ability of *Rhizopus* strains to effectively utilize lignocellulosic substrates, such as edamame husks, by secreting extracellular enzymes like cellulases and hemicellulases. These enzymes break down complex plant matrices into fermentable sugars (Ho et al., 2026; Parhusip, 2023; Wang et al., 2022).

The biosynthesis of 2,3-butanediol begins with glycolysis, where fermentable sugars are converted into pyruvate. Two pyruvate molecules are then condensed to form α-acetolactate, which is subsequently converted into acetoin and finally reduced to 2,3-butanediol. This pathway helps maintain intracellular redox balance during fermentation while also producing 2,3-butanediol, a volatile metabolite known to generate buttery and

creamy aroma notes in fermented products (Huo et al., 2022; Jo et al., 2024; Ozma et al., 2023; Palaiogeorgou et al., 2023).

In addition to 2,3-butanediol, glycerin is an important metabolite produced within the same biochemical framework. During fungal fermentation, glycerin is produced to maintain intracellular redox balance by consuming excess NADH generated during sugar metabolism. Acetic acid is also commonly formed as a byproduct through an alternative pyruvate metabolic pathway (Huo et al., 2022; Jo et al., 2024). The presence of glycerin, acetic acid, and the abundant 2,3-butanediol in the present study consistent with fermentation-related metabolism, but formation specifically attributable to *R. oligosporus* requires comparison with the uninoculated control.

Dianhydromannitol is a dehydration product of mannitol that has been detected in several fungal species, suggesting that it is produced through fungal carbohydrate metabolism (Krishnaveni et al., 2026; Sutthisa et al., 2025). Although dianhydromannitol has been detected in several fungi, its occurence in fungal-derived postbiotics has rarely been reported and the metabolic pathway responsible for its formation remains unclear. Benzoic acid is another metabolite identified in the present study that can be produced during fungal fermentation through the metabolism of plant-derived aromatic compounds. Plant biomass, particularly lignin, provides a rich source of aromatic precursor that fungi can convert into valuable metabolites (Sodré & Bugg, 2024).

A. niger is well known for its ability to metabolize lignin-derived aromatic compounds, including the conversion of cinnamic acid into benzoic acid through enzymatic side-chain shortening. Benzoic acid may also be produced through the oxidation of benzaldehyde or the mandelic acid pathway (Lubbers, 2025). Since edamame husks are rich in lignocellulosic and phenolic compounds, they may provide suitable precursors for benzoic acid production during fermentation by *R. oligosporus*. Similar benzoic acid production has also been reported in *Neurospora crassa*, further supporting the ability of fungi to synthesize this metabolite from plant-derived aromatic compounds (El-Zawawy et al., 2022).

Fatty acids and derivatives represent another important group of metabolites detected in the candidate postbiotics. In the present study, n-hexadecanoic acid was identified as one of the predominant fatty acid components in the postbiotics. n-hexadecanoic acid is a saturated fatty acid that can be generated during fungal fermentation through both de novo biosynthesis and the biodegradation of lignocellulosic biomass. The fungus, *Aspergillus ochraceus* DY1 is specifically noted for its ability to efficiently degrade alkali lignin and yield n-hexadecanoic acid as a major degradation product (Nilza et al., 2024). Similar production has also been reported in *Rhizopus microsporus* and *A. oryzae,* further highlighting the capability of fungi to synthesize this fatty acid from diverse substrates (Attia et al., 2023). Within fungal cells, this metabolite is also synthesized de novo via the fatty acid synthesis pathway, in which acetyl-CoA and malonyl-CoA are sequentially condensed by the fatty acid synthase complex Fas1 and Fas2 (Gutierrez-Perez & Cramer, 2025).

Collectively, these findings indicate a diverse GC-MS profile after fermentation, while the fungal contribution to individual compounds remains to be established by comparison with the uninoculated control. These results also expand the current knowledge of the metabolite composition of *R. oligosporus*-derived postbiotics. Nevertheless, further GC-MS based metabolite profiling supported by metabolic pathway analysis is needed to identify additional metabolites and clarify the biosynthetic mechanisms underlying postbiotic production by *R. oligosporus* grown on edamame husks.

Molecular docking of candidate fungal postbiotic metabolites

To further evaluate the potential antibacterial activity of the major candidate postbiotic metabolites identified by GC-MS, molecular docking was performed against *S. aureus* DNA gyrase subunit B. This in silico approach was used to predict the binding affinity and molecular interactions of the selected metabolites with the active site of the target protein providing preliminary insights to their potential inhibitory activity (Benzenine et al., 2024).

All selected candidate postbiotic metabolites exhibited negative binding affinities toward protein target indicating spontaneous and energetically favorable ligand-protein interactions (Chibuye et al., 2026). Although their predicted binding affinities were lower than that of the native ligand, this finding is not unexpected because these

metabolites are naturally occurring small molecules rather than compounds specifically optimized to inhibit DNA gyrase. Among the tested metabolites, n-hexadecanoic acid exhibited the strongest predicted binding affinity. This may be attributed to its long hydrophobic carbon chain, which allows extensive hydrophobic contacts within the binding pocket. Its terminal carboxyl group forms stabilizing hydrogen bonds (Vishwakarma et al., 2026). Likewise, benzoic acid showed a comparable binding affinity, possibly because its aromatic ring promotes hydrophobic interactions whereas the carboxyl group contributes to hydrogen bond formation (Shi et al., 2025).

In contrast, glycerin, 2,3-butanediol, and dianhydromannitol exhibited relatively weaker binding affinities. These compounds contain multiple hydroxyl groups that readily form hydrogen bonds with amino acid residues in the binding pocket. However, their small molecular size and the absence of hydrophobic or aromatic structural features limit the number of stabilizing nonpolar interactions with the protein (Arakawa et al., 2025). These findings suggest that both hydrogen bonding and hydrophobic interactions contribute to ligand stabilization within the binding pocket of the target protein (Trezza et al., 2025).

Several metabolites also shared common interacting residues with the native ligand. Benzoic acid interacted with ASN54, ILE86, and ILE175, while n-hexadecanoic acid interacted with ILE86, PRO87, and ILE102, indicating partial overlap within the ATP-binding pocket. Similarly, 2,3-butanediol interacted with ILE86 dan ILE175, whereas glycerin and dianhydromannitol shared the ILE51 residue with the native ligand. Previous molecular docking studies of *S. aureus* DNA gyrase B (PDB ID: 4URM) identified ASN54, ILE86, and PRO87 as residues likewise involved in ligand stabilization within the ATP-binding pocket (Benzenine et al., 2024). The docking results provide preliminary hypotheses about ligand-GyrB interaction and do not independently demonstrate antibacterial activity. However, further molecular dynamics simulations and in vitro studies are necessary to validate the predicted interactions and their biological activity.

Conclusion

This study provided a preliminary GC-MS profile of candidate fungal postbiotics from *R. oligosporus*, with 2,3-butanediol identified as the predominant metabolite. Molecular docking analysis indicated that several candidate metabolites were computationally placed in the binding pocket of *S. aureus* DNA gyrase subunit B. Given the limited studies on fungal postbiotics, particularly those from *Rhizopus* species, these preliminary observations require chemical verification and experimental testing before bioactivity can be inferred. Further studies involving targeted metabolite quantification, molecular dynamics simulations, and in vitro antibacterial validation are needed to confirm their biological activity and evaluate their feasibility as functional ingredients for food biopreservation and active food packaging.

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