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Fermented Black Rice Extract (*Oryza sativa*) Induces Morphological Changes in Pathogenic Microorganisms

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Abstract. Black rice (*Oryza sativa* L.) contains nutrients and bioactive compounds with potential application in health, including antimicrobial properties. To date, the application of fermentation to black rice to enhance its bioactivity, particularly as an antimicrobial, remains unclear. This study aims to evaluate the antimicrobial activity of solid fermented black rice against Gram-positive bacteria, Gram-negative bacteria, and fungi. Black rice was fermented using *Saccharomyces cerevisiae* under solid-state conditions. Extraction was carried out by maceration using 96% ethanol. Antimicrobial activity was assessed using the well diffusion test method, agar dilution, and scanning electron microscope (SEM). Fermented black rice extract showed antimicrobial activity against all tested microbes. The minimum inhibitory value obtained was 20% against all microbes, but the minimum bactericidal/fungicidal concentration (MBC/MFC) could not be determined. The SEM analysis showed morphological changes in microbes exposed to fermented black rice extract, including cell shrinkage, elongation, and lysis in *Bacillus cereus*, fragmentation and irregular cell shape in *Escherichia coli*, cell size reduction in *Staphylococcus aureus*, and bleb formation in *Candida albicans*. These changes indicate the mechanism of microbial growth inhibition. We found that fermented black rice (EFBR) extracts produce different effects from unfermented black rice extracts (EUBR), which tend to induce biofilm formation. We propose that EFBR functions as an antimicrobial by rupturing cell walls, preventing cell division and DNA synthesis. This research provides new insights into natural antimicrobial mechanisms at the cellular level and offers a potential alternative for addressing antimicrobial resistance. Solid fermented black rice may serve as a valuable source of antimicrobial compounds for developing health products or natural preservatives.

Keywords: antimicrobial, black rice, scanning electron microscope, solid fermentation.

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INTRODUCTION

Black rice (*Oryza sativa* L.) contains various bioactive compounds such as flavonoids, anthocyanins, phytic acid, proanthocyanidins, tocopherols, tocotrienols, gamma oryzanol, and phenolic compounds (Sanghamitra et al., 2017). Previous research showed that ethanol extracts of several varieties of Javanese black rice contained leucoanthocyanidins, phenolics, proteins, tannins, flavonoids, quinones, anthraquinones, and glycosides (Fatchiyah et al., 2020). Black rice is traditionally known as a food ingredient, but its bioactive compounds hold opportunities for applications in the health and medicine industries (Seechamnanturakit et al., 2018).

Black rice's health potential is mainly attributed to its phenolic content. Among different rice bran colors, black rice has the highest total phenolic content (Wijayanti et al., 2023). However, the bioavailability and solubility of these phenolic compounds are relatively low (Thilagavathi et al., 2019). Fermentation has been shown to enhance the bioavailability of insoluble-bound phenolic compounds in rice (Prabhu et al., 2014; Wijayanti et al., 2017).

Microbial fermentation of plant materials increases the production of active ingredients, improves bioavailability of phytochemicals and nutrients, and enhances antioxidant and

antibacterial activities (Lee et al., 2019; Sinaga et al., 2022). Fermentation of white rice bran has been reported to increase levels of phenolic compounds and the digestibility of proximate nutrients (Jannah et al., 2020). Several microorganisms have been used in the rice fermentation process, including *Rhizopus oligosporus* (Budijanto et al., 2022), *Rhizopus oryzae* (Jannah et al., 2020), *Lactobacillus casei* (Lee et al., 2019), and *Saccharomyces cerevisiae*, which is recognised for its effectiveness in solid-state fermentation (Prabhu et al., 2014).

A notable benefit of fermentation is its ability to increase phytochemical content and produce antimicrobial compounds (Murugan et al., 2018; Sutthanut et al., 2022). These antimicrobial compounds are useful to address the growing problem of antibiotic resistance, which occurs when antibiotics are overused or prescribed incorrectly (Missa et al., 2024). Black rice contains bioactive compounds that may inhibit the growth of pathogenic microorganisms, however, research on the antimicrobial potential of fermented black rice remains limited.

Antimicrobials have different effects on Gram-positive and negative bacteria due to variations in cell wall composition (Epand et al., 2016; Wu et al., 2016). Additionally, fungal cells exhibit distinct responses to antimicrobial compounds (Lyu et al., 2016). In this study, the antimicrobial activity of fermented black rice extract against six pathogenic microorganisms known to cause infection problems and exhibit resistance to antimicrobials was examined. The pathogenic microorganisms were selected to represent a diverse spectrum of pathogens, including rod-shape Gram-positive bacteria (*Bacillus cereus* and *Propionibacterium acnes)*, rod-shape Gram-negative bacteria (*Escherichia coli)*, cocci-shaped Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus. mutans)* and fungi (*Candida albicans)*, all of which have been documented to exert resistance to antimicrobials and antifungal, respectively (Dessinioti & Katsambas, 2016; Uddin et al., 2021; Wijayanti et al., 2024).

To date, no prior studies have been reported on the antimicrobial effect of black rice extract, especially fermented, on the six selected microorganisms. Therefore, this study investigates the antimicrobial activity of fermented black rice and its impact on microbial morphology. Preliminary detection of antimicrobial activity was performed by the agar-well diffusion method. Subsequently, minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC) were determined by the agar dilution method. To further understand the mechanism of action of antimicrobial compounds produced from fermented black rice, the Scanning Electron Microscope (SEM) technique was used to visualise morphological changes in microorganism cells after exposure to antimicrobial compounds (Lyu et al., 2016; Wijayanti et al., 2021).

Through this approach, we hope to obtain more in-depth information on how fermented black rice affects microbial structures at the microscopic level. This study compares the antimicrobial effect of fermented black rice extract (EFBR) with unfermented black rice extract (EUBR). The exploration of fermented black rice as an antimicrobial compound may provide an effective solution for infection caused by bacteria and fungi while providing an understanding of its mechanism of action. Furthermore, this research supports the use of fermented black rice as a sustainable alternative for infection treatment that is affordable for the community. It may also serve as the basis for developing fermented black rice-based health and pharmaceutical products.

METHODS

Fermentation of Black Rice

Black rice was purchased from the local store in Malang, East Java, Indonesia. The rice was ground into powder and then sieved to obtain fine powder. The rice powder was sterilized in an autoclave at 121°C, 1 atm, for 15 minutes. Next, 1% (w/w) instant yeast starter (*S. cerevisiae*), was dissolved in distilled water and added to the sterile black rice powder. As a control, an identical batch of sterilized black rice powder was prepared but without the addition of a starter. Fermentation was carried out for 20 hours at room temperature (Mirsalami & Mirsalami, 2024).

Extraction of Fermented Black Rice

Fermented black rice powder was extracted with 96% ethanol solvent in a ratio of 1:10. The extraction was carried out using the maceration method for 3x24 hours. The macerate was filtered with a Buchner vacuum funnel, evaporated with a rotary evaporator and continued in an oven until a concentrated extract was obtained. The same extraction process was also applied to unfermented black rice to allow a direct comparison of antimicrobial activity (Rahayu et

al., 2023).

Antimicrobial Assay

Preparation of Microbial Suspensions

Microbial cultures were obtained from CV. Wiyasa Mandiri, Malang, East Java, Indonesia, including *B. cereus, E. coli, P. acnes, S. aureus, S. mutans,* and *C. albicans*. The microbial culture medium used were Bacillus Agar Base (Himedia) for *B. cereus*, Eosin Methylene Blue Agar (EMBA, Oxoid) for *E. coli*, Blood agar (Himedia) for *P. acnes* and *S. mutans*, Mannitol Salt Agar (MSA, Himedia) for *S. aureus*, and Saboraud's Dextrose Agar (SDA, Himedia) for *C. albicans*. Each 24-hour microbial culture was inoculated on Nutrient Broth (NB, Himedia) for bacteria and Saboraud's Dextrose Broth (SDB, Himedia) for fungi and incubated for 24 hours at 37°C. The suspension was analysed for turbidity using a UV-Vis spectrophotometer at 625 nm until a suspension with a % transmittance 25 was obtained. For fungi, the % transmittance was set to 90 at 530 nm (Sanuddin et al., 2024).

Agar-Well Diffusion Method

Each bacterium was inoculated into Mueller Hinton Agar (MHA, Himedia) media, while the fungi were inoculated on SDA media using the pour-plate technique. After the media had solidified, a well was made with a 7 mm cork-borer. EFBR dissolved in 10% dimethyl sulfoxide (DMSO) was put into the well and then incubated at 37°C for 24-48 hours. The diameter of the inhibition zone was measured using a caliper. The same procedure was also applied using EUBR (Murugan et al., 2018).

Agar Dilution Method

Microbial cultures were inoculated on MHA (for bacteria) and SDA (for fungi) media, followed by the addition of varying concentrations (0-50%) of EFBR/EUBR in 10% DMSO using the pour-plate technique. Incubation was carried out at 37°C for 24 hours. The growing colonies were counted with a colony counter (Schumacher et al., 2018).

SEM Analysis

Microbes were inoculated on liquid media, NB for bacteria and SDB for *C. albicans*. Each culture was treated with EUBR and EFBR, respectively. As a control, cultures without the addition of extracts were used. Incubation was carried out at 37°C for 18 hours. The SEM analysis was carried out following the reported method at the Institute of Biosciences, Brawijaya University (Wijayanti et al., 2021).

Data Analysis

All antimicrobial assays were conducted in triplicate. The diameter of the inhibition zone of EFBR was compared with EUBR using a T-test. MIC determination was based on the concentration of extracts that resulted in a significant decrease in the number of colonies analysed by ANOVA followed by Tukey's HSD test. All statistical analyses were performed with GraphPad Prism 8 software. Microbial cell morphology observed using SEM was analysed qualitatively.

RESULTS AND DISCUSSION

Black rice was fermented using *S. cerevisiae* to increase its antimicrobial activity. As a comparison, black rice without fermentation was also used in this study. Several indicator microbes were used in the antimicrobial assay, which represents Gram-positive and Gram-negative bacteria, rod, and coccus forms, and fungi, including *B. cereus, E. coli, P. acnes, S. aureus, S. mutans,* and *C. albicans*. Preliminary screening was conducted to detect the antimicrobial activity of black rice extract using the well-diffusion method. The presence of antimicrobial activity is indicated by the formation of an inhibition zone around the well after incubation for a certain time (Araújo et al., 2024), where a larger inhibition zone suggests greater antimicrobial activity (Bubonja-šonje et al., 2020).

EFBR showed antimicrobial activity against all test microbes, as indicated by the presence of inhibition zones (Figure 1). The inhibition zone diameter produced by EFBR was significantly larger than that of EUBR against *B. cereus, E. coli,* and *S. mutans*. The zone of inhibition of EFBR against *C. albicans* was also greater than that of EUBR but the difference was not significant. However, EFBR showed a smaller inhibition zone diameter against *S. aureus* compared to EUBR, while no significant difference was observed on *P. acnes*.

Based on the inhibition zone diameter, EFBR demonstrated medium antimicrobial activity (6-10 mm) against *B. cereus, P. acnes*, and *S. mutans*, while its activity is weak (<6 mm) against *E. coli, S. aureus*, and *C. albicans* (Missa et al., 2024). In contrast, EUBR shows medium antimicrobial activity against *P. acnes* and *S. aureus*, but weak activity against the remaining tested microbes.

The observed variation in microbial sensitivity may be due to differences in cell membrane composition (Abu-Zaid et al., 2022).

Considering the antimicrobial activity of EFBR, a further test was conducted to determine the MIC and MBC/MFC values using the agar dilution method. The extracts were prepared at varying concentrations of 0-50% and then inoculated on the media with the addition of each microbial indicator. After the incubation process, the number of colonies was counted. The MIC values were defined as the lowest concentration of extract that causes a significant decrease in the number of colonies, while the MBC/MFC value was determined as the concentration at which no growth of microbial colonies was observed (Schumacher et al., 2018).

Figure 2 shows the inhibition of microbial growth at varying concentrations of EFBR/EUBR. The higher the concentration of the extract, the fewer the number of microorganisms grow. Interestingly, all microbes showed the same growth inhibition curve, where at 20% concentration there was a significant decrease in the number of colonies (ANOVA, $P<0.05$), leading to their MIC values. The MBC/MFC value was not obtained, since the microbial growth was still observed at the highest concentration. Future studies may evaluate higher extract concentration to determine the MBC/MFC values.

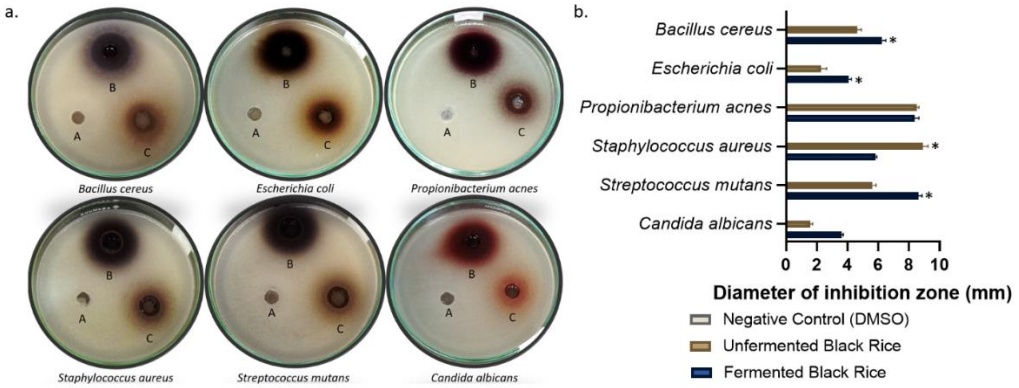
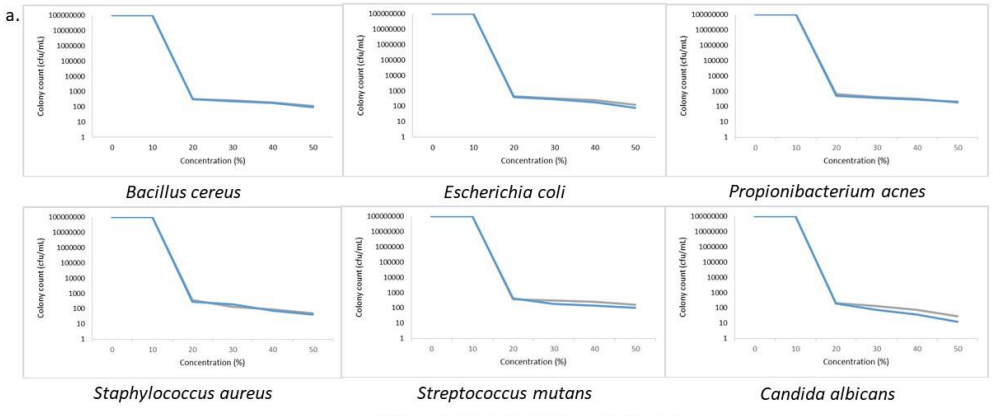


Figure 1. Diameter of zone inhibition of black rice extract. a. Test results using agar well diffusion, A: negative control (DMSO), B: extract of unfermented black rice (EUBR), and C: extract of fermented black rice (EFBR); b. Summary diagram of inhibition zone diameter. An asterisk notation indicates a significant difference between EFBR and EUBR in each microbe (T-test, $P<0.05$).



b.

	B. cereus		*E.coli*		*P. acnes*		*S. aureus*		*S. mutans*		*C. albicans*	
	EUBR	EFBR	EUBR	EFBR	EUBR	EFBR	EUBR	EFBR	EUBR	EFBR	EUBR	EFBR
MIC (%)	20	20	20	20	20	20	20	20	20	20	20	20
MBC/MFC (%)	-	-	-	-	-	-	-	-	-	-	-	-

Figure 2. Microbial growth inhibition by black rice extract at 0-50% concentration. a. growth inhibition curve of each microbe; b. MIC and MBC/MFC value of black rice extract. EUBR: extract of unfermented black rice, EFBR: extract of fermented black rice.

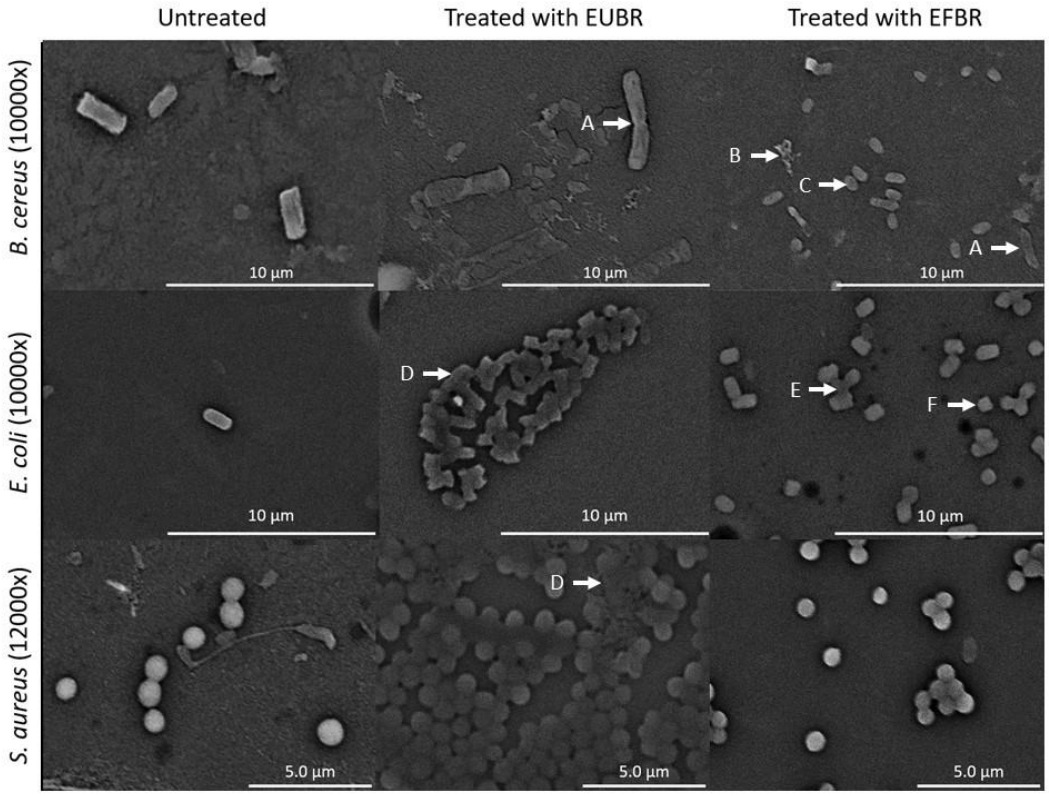


Figure 3. SEM analysis of an extract of fermented black rice effect on bacteria. EUBR: extract of unfermented black rice, EFBR: extract of fermented black rice. The white arrow shows an elongated cell (A), cell debris (B), shrinking cell (C), biofilm formation (D), irregularly shaped cells (E), and fragmented cells (F).

To investigate the effect of exposure to black rice extract on microbial cell morphology, SEM analysis was performed. In this analysis, four microbes representing each group were selected, including *B. cereus* representing Gram-positive rod bacteria, *E. coli* representing Gram-negative rod bacteria, *S. aureus* representing Gram-positive coccus bacteria, and *C. albicans* from the fungi group.

SEM analysis in Figure 3 shows that *B. cereus* cells exposed to EFBR are smaller (0.69-1.37 µm) than the untreated cells (1.68-2.58 µm), indicating that they cannot grow optimally. The cell shape is also different from the untreated cells. Among the shrunken cells, elongated cells were observed (Figure 3(A)). Moreover, there is the presence of cell debris, indicating cell lysis (Figure 3(B)). In contrast, EUBR-treated *B. cereus* cells did not shrink but instead, became longer (4.54-4.89 µm), which appears as cells that have failed to divide. Cell elongation is a known response to DNA synthesis inhibition, while changes in cell size indicate abnormal cell division (Cushnie et al., 2016).

The EFBR effect in *E. coli* was similar to that of EUBR, where treated cells are smaller (0.63-1.13 µm) than untreated cells (1.49-1.99 µm) and exhibit irregularly, fragmented shapes. Additionally, *E. coli* cells exposed to EUBR showed biofilm formation. In *S. aureus*, both EFBR and EUBR caused cell shrinkage while maintaining a normal cocci shape (0.59-0.99 µm). However, *S. aureus* cells exposed to EUBR appeared to form biofilms. The normal cell size was 0.86-1.15 µm. The reduced cell size and other morphological changes in the cell indicate that the extract might have an impact on the bacterial cell membrane (Musini & Giri, 2019).

Similar antimicrobial exposure effects have been reported previously using purple rice ferulic acid extract against *Salmonella typhimurium* and *Listeria monocytogenes*. Exposure to *S. Typhimurium* caused cell shrinkage, biofilm formation, and cell lysis, while exposure to *L. monocytogenes* caused cell damage and lysis (Wijayanti et al., 2021). The cell elongation observed in *B. cereus* upon exposure to EUBR is similar to the filamentation effect of peptoids on *E. coli*, where cells continuously elongate but do not undergoing cell division. This phenomenon is a response to stress due to antimicrobial exposure (Mojsoska et al., 2017). However, cell shrinkage

in *B. cereus* differs from the previous study using diallyl sulfide, which resulted in bubble formation on the *B. cereus* cell surface (Manjhi et al., 2024). The antimicrobial effect of black rice extract is different from those of other antimicrobial agents. For example, antimicrobial peptides have been shown to cause changes in the cell surface of *S. aureus* (Chaparro et al., 2018), while bacteriocins induce irregular cell shape (Kranjec et al., 2020).

Both *E. coli* and *S. aureus* are known to produce biofilm bacteria (Ball et al., 2022; Kranjec et al., 2020). Biofilm formation is a well-known bacterial self-defense mechanism in response to stress by antimicrobial exposure (Kaplan, 2011). Interestingly, while EUBR tends to cause the formation of biofilms, no biofilms were observed in the cell treated with EFBR. This suggests that EUBR may trigger a cell defense response, while EFBR effectively inhibits biofilm formation.

In *C. albicans*, the cells that were exposed to EFBR (2.12-2.94 µm) and EUBR (2.66-3.72 µm) showed a smaller size than the untreated cells (3.28-4.60 µm) (Figure 4). The cell surface looked uneven, with noticeable bleb formation. In addition, in cells exposed to EFBR, there is cell debris, indicating cell lysis. Similar morphological changes have been observed in *C. albicans* cells treated with antimicrobial peptides, where bleb formation occurred, indicating cell membrane damage. This effect supports that the antimicrobial effect can damage the cell membrane and subsequently kill the microbes (Lyu et al., 2016).

Exposure to EFBR caused distinct effects on the cell morphology in the indicator microbes. The effect of EFBR is also different from the effect produced by EUBR. This indicates that microbial species exhibit different morphological changes in response to antimicrobials and that each antimicrobial substance also has varying effects. Changes in microbial cell morphology can be affected by multiple factors, including the type of antimicrobial material, antimicrobial concentration, duration of antimicrobial exposure, and incubation conditions. In addition, types of indicator microorganisms may also show unique responses to alterations in cell morphology (Cushnie et al., 2016).

A summary illustration of the effects of EFBR on microbial cells is presented in Figure 5. The EFBR causes cell shrinkage, elongation, lysis, fragmentation, and also bleb formation. Based on the resulting microbial cell morphological changes, we propose that EFBR exerts its antimicrobial action by disrupting cell walls, inhibiting cell division, and inhibiting DNA synthesis.

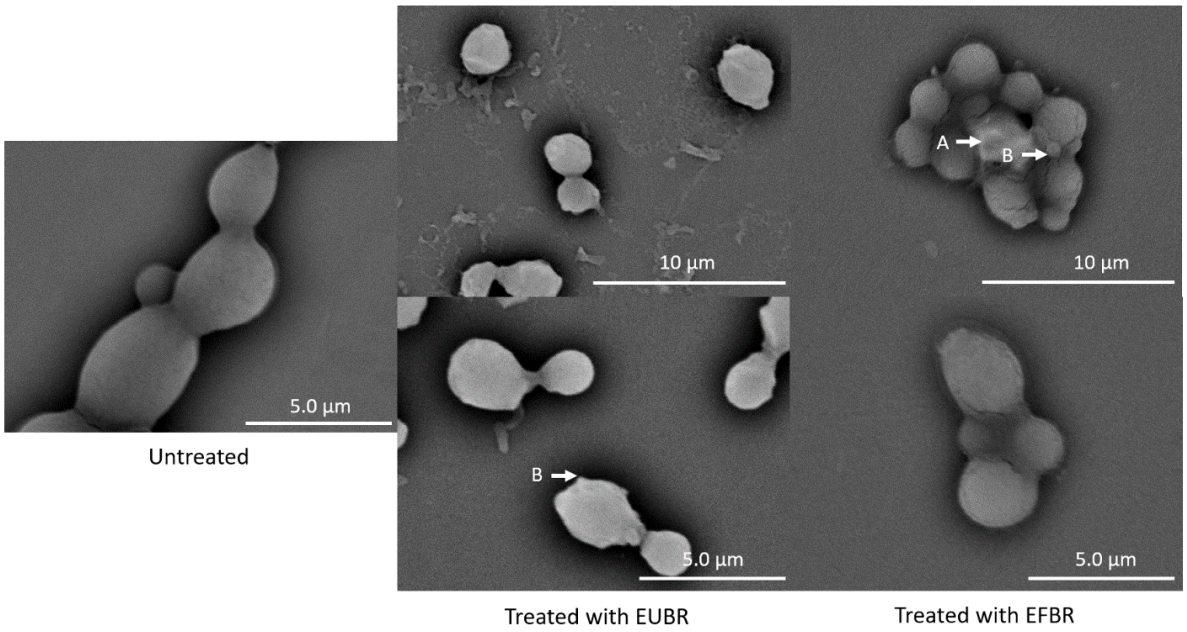


Figure 4. SEM analysis of an extract of fermented black rice effect on *C. albicans* at 8000 and 12000 magnifications. EUBR: extract of unfermented black rice, EFBR: extract of fermented black rice. The white arrow shows cell debris (A) and bleb formation (B).

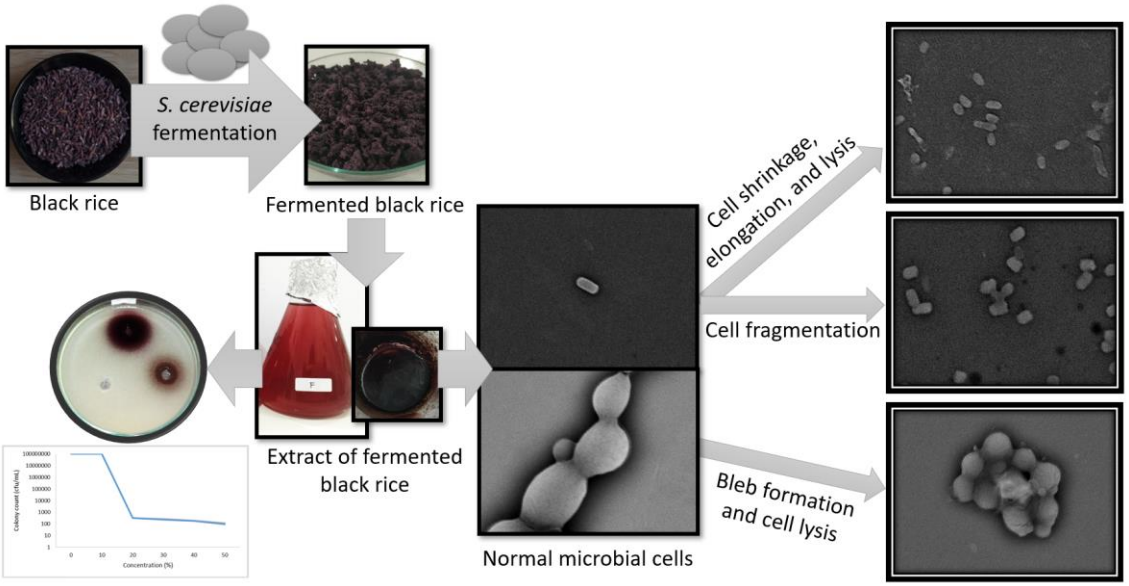


Figure 5. Summary illustration of antimicrobial activity of fermented black rice extract inducing changes in microbial cell morphology.

The current study highlights the potential of fermented black rice to be utilized as an antimicrobial material. These results provide an understanding of its mechanism of action as an antimicrobial, demonstrating that different microbes respond differently to EFBR treatment. The same microbe may respond differently to different antimicrobial exposures. The fermentation process changes the chemical components of the black rice, thus resulting in different antimicrobial mechanisms. Unlike EUBR, EFBR produces an inhibitory effect without allowing microbial cells to defend themselves through biofilm production. This indicates that the fermentation process enhances the antimicrobial activity of black rice.

Overall, this study contributes significantly to scientific advancement, especially in microbiology, pharmacy, and biotechnology, by providing insights into the cellular mechanisms through which fermented black rice extracts induce microbial morphological changes. It highlights the potential of fermented black rice extract as an effective antimicrobial agent and a promising alternative for treating microbial infections. In addition, it initiates further research into the bioactive compounds contained in fermented black rice and how they can be utilized in medicine or the pharmaceutical industry. It also promotes the identification of specific bioactive components and assesses their effectiveness against antibiotic-resistant microbes. This offers a solution to the growing problem of antibiotic resistance. For the community, this research introduces a more affordable, effective, and natural infection treatment solution to reduce reliance on chemical antibiotics through the utilization of local natural resources. Moreover, it opens opportunities for industries to develop black rice-based products that are beneficial for health and food while encouraging the development of standardized fermentation techniques to ensure products with high quality.

CONCLUSION

EFBR exhibits antimicrobial activity against all tested microbes and causes morphological changes in microbial cells. The exposure of EFBR generally leads to cell shrinkage, cell elongation, cell fragmentation, bleb formation, and cell lysis. The antimicrobial effect of EFBR is also different from EUBR, where it tends to promote the formation of biofilms as a microbial defense mechanism. The mechanism of action of EFBR is possibly mediated through cell wall destruction, inhibition of DNA synthesis, and inhibition of cell division. However, other mechanisms may also be involved and should be further investigated through membrane permeability assays, protein and nucleic acid synthesis inhibition assays, or protein leakage assays. Further research is also suggested to analyze the effects of EFBR on other pathogens, identify active compounds responsible for its antimicrobial activity, and evaluate the antimicrobial activity and toxicity *in vivo*. The current study suggests that EFBR is a promising antimicrobial agent with potential applications in

the development of more effective antimicrobial drugs, for overcoming antimicrobial resistance, and in other innovations within the pharmaceutical, healthcare, and food industries.

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