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Antibacterial Test of Bacterial Isolates from Red Rice Syrup

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ABSTRACT

Red rice syrup, as a fermentation product, contains bioactive components with antimicrobial potential, thereby requiring exploration of the associated microbes and their activities. This study aims to isolate bacteria from fermented red rice syrup, characterize their phenotypic properties, and assess the antibacterial activity of the supernatant against *Escherichia coli* and *Staphylococcus aureus*. Isolation was carried out on MRS agar (37 °C, 48 hours), followed by Gram staining, catalase testing, and antibacterial activity testing using the disc diffusion method. Five isolates (SBM1–SBM5) were obtained, all of which were Gram-positive with coccobacillus morphology and catalase-positive. In the antibacterial assay, the supernatant from the five isolates did not produce inhibition zones against either indicator bacterium, whereas the amoxicillin control produced inhibition zones of 13.95 mm (*E. coli*) and 12.43 mm (*S. aureus*), and the negative control showed no inhibition. In summary, the isolates from red rice syrup exhibited consistent phenotypic profiles; however, under the test conditions applied, no antibacterial activity was detected against *E. coli* or *S. aureus*. These findings provide a foundation for further studies to clarify taxonomic identity and to optimize more sensitive assays for assessing biological activity.

Keywords: red rice syrup, lactic acid bacteria, Gram-positive, coccobacillus, antibacterial test

INTRODUCTION

Diseases caused by pathogenic bacteria remain a serious public health issue worldwide. The increasing prevalence of antibiotic resistance has further worsened the situation, thereby encouraging research into antibacterial alternatives derived from natural sources that are safer and more effective [1].

The antimicrobial resistance (AMR) crisis has been recognized as a global threat to the effectiveness of infection treatment, the safety of medical procedures, and food security; therefore, research and innovation efforts to discover safe and effective alternative antimicrobial agents have become a priority at the international level [2].

One food commodity that has been widely studied is red rice. This type of rice is known to contain various bioactive compounds, including polyphenols, flavonoids, and other phenolic components. These substances not only function as antioxidants but also have been reported to inhibit microbial growth in various laboratory assays. Studies on rice components, such as husk, bran, and endosperm, have demonstrated antibacterial activity against both Gram-positive and Gram-negative bacteria using diffusion methods and solution-based tests [3].

Processing methods can also affect rice's antibacterial properties. Fermentation and enzymatic hydrolysis treatments, for example, can modify the metabolite composition, thereby enhancing inhibitory activity against bacteria. Findings from studies on fermented rice bran and hydrolyzed proteins derived from rice seeds have shown increased antibacterial activity after these treatments, thereby reinforcing the understanding that rice-derived products hold substantial potential as sources of antimicrobial agents [4].

As a promising solution, the exploration of processed rice products, including modified bran or fermented products, can reveal new sources of antimicrobial metabolites. Recent studies indicate that fermentation treatments and enhanced extraction techniques applied to rice bran or rice syrup can release or increase the levels of phenolic compounds and other metabolites with potential antimicrobial properties. In addition, associative microbes (e.g., lactic acid bacteria) are known to produce secondary metabolites, such as bacteriocins, organic acids, and small peptides, that exhibit antimicrobial activity. Therefore, investigating both the chemical components of rice and the metabolite-producing microbes in processed products represents a rational strategy for discovering new antibacterial agents [5].

Red rice syrup is a product of rice starch hydrolysis, widely used as a sweetener. Although scientific evidence regarding its antibacterial effects remains limited, minor non-carbohydrate components, such as phenolics or small peptides, are suspected to provide biological effects if successfully extracted and

identified. Therefore, it is important to conduct a study to determine whether isolates from red rice syrup possess antibacterial activity [6].

The novelty of this study lies in its emphasis on microbial isolates from red rice syrup rather than solely on testing plant extracts, and in its focus on screening for antibacterial activity using isolate supernatants. By assessing the supernatant of the isolates (secondary metabolites released into the medium) using the disc diffusion assay, this study aims to detect antibacterial agents produced by microbes during growth in the red rice syrup matrix, an approach rarely reported in the literature on processed rice products. This approach is relevant given the widely documented role of microbial metabolites (e.g., bacteriocins, organic acids, and peptides) as potential antimicrobial agents derived from fermented foods [7].

Recent findings indicate that variations in rice varieties, differences in fractions (husk, bran, endosperm), and extraction or processing methods strongly determine the resulting bioactive profiles. This fact highlights the need for targeted studies evaluating isolates from specific processed products, including red rice syrup, to assess their effectiveness against target bacteria in laboratory settings [8].

Based on the limited scientific data available regarding the antibacterial activity of isolates from red rice syrup, this study was conducted to isolate bioactive compounds from the product and to test their inhibitory effects against the test bacteria. The results of the study are expected to enrich the literature on the use of rice-based products as natural antibacterial sources, while also opening opportunities for the development of functional food products and alternative materials for microbial control [9].

This study aims to isolate bacteria from red rice syrup and to assess the in vitro antibacterial potential of the isolated supernatants using the disc diffusion assay. The screening results are expected to identify isolates that produce metabolites with inhibitory activity against the test bacteria, thereby serving as candidates for further metabolite characterization studies.

RESEARCH METHODS

1. Preparation of Red Rice Syrup Fermentation

Preparation of red rice syrup began with thoroughly washing the red rice to remove any adhering impurities. A total of 500 grams of red rice was then cooked in 2 liters of water at 95–100 °C for approximately 40–60 minutes, until fully cooked and the starch began to be released, after which the solution was cooled to approximately 50 °C. To this solution, sufficient water was added along with 50 grams of barley malt powder for every 500 grams of red rice, stirred until homogeneous, and incubated at 50 °C for 1–2 hours to allow the amylase enzymes in the malt to break down the starch into simple sugars. The hydrolysate solution was then filtered using muslin cloth or a fine sieve to separate the liquid from the residue, cooled to approximately 37 °C, and transferred into a sterile container. The fermentation stage was carried out by incubating the liquid at 37 °C for 48 hours in a sterile closed container [10].

2. Isolation of Bacteria from Red Rice Syrup

The bacterial isolation process was conducted based on a modified method [11]. A total of 10 mL of red rice syrup sample was added to 90 mL of de Man, Rogosa, and Sharpe (MRS) broth, and the mixture was incubated at 37 °C for 24 hours to obtain an initial culture suspension. From this suspension, 1 mL of the supernatant was collected and serially diluted using sterile buffer through a serial dilution method (10^{-1} to 10^{-5}). Sterile Petri dishes containing MRS agar (which had been sterilized using an autoclave) were prepared, then 0.1 mL of sample from each dilution level was dropped onto the agar surface and evenly spread using a sterile spreader to ensure homogeneous distribution. All plates were incubated at 37 °C for 48 hours under anaerobic conditions, after which colony growth was directly observed on the plates (24–48 hours). Colonies with characteristics typical of lactic acid bacteria, including coccoid or bacillary shape, white to cream coloration, and small to medium size, were selected and collected using a sterile inoculating needle, then regrown on fresh MRS agar through purification subculturing. The purification process was repeated until a single pure colony was obtained, after which the purified lactic acid bacteria (LAB) isolates were stored in 25% (v/v) glycerol solution at −20 °C for subsequent characterization.

3. Characterization of Bacterial Isolates

The pure bacterial isolates were characterized through a series of tests, namely Gram staining and the catalase test. Gram staining was used to identify the Gram characteristics and cell morphology of the lactic acid bacteria (LAB). The examination began with preparing a smear of the LAB colony on a sterilized glass slide. The colony was collected using a sterile inoculating needle, placed on the slide, then one drop of sterile distilled water was added and spread evenly to form a thin layer. After the smear dried, fixation was carried out by passing the glass slide over a Bunsen flame two to three times. The staining process was then performed: the sample was first treated with crystal violet solution for 1 minute, then rinsed with water. After that, iodine solution was applied for one minute and rinsed again. The decolorization step was conducted using 95% alcohol for 15–30 seconds until the color stopped dissolving, after which the slide was immediately rinsed with water. As a counterstain, safranin solution was applied for 1 minute, then rinsed again. After air-drying, the preparation was observed under a microscope at 1000x magnification with immersion oil [12].

The catalase test was conducted to determine the presence of the catalase enzyme in the bacterial isolates. A pure bacterial colony was collected using a sterile inoculating needle and placed on a sterilized glass slide. Subsequently, 1–2 drops of 3% hydrogen peroxide (H_2O_2) solution were added directly to the

colony surface, and the reaction was observed for 10–30 seconds. Lactic acid bacteria, which belong to the facultative anaerobes, generally show negative results in the catalase test, as indicated by the absence of gas bubble formation after the addition of H_2O_2 [13].

4. Antibacterial Activity Test

The antibacterial activity assay was conducted in two repetitions using the disc diffusion method against *Escherichia coli* and *Staphylococcus aureus*. Before testing, the bacterial isolates and the test bacteria were grown separately in MRS broth and Nutrient Agar (NA) at 37 °C for 24 hours. After incubation, a small portion of culture from each bacterium was aseptically transferred into sterile physiological saline, and the cell density was adjusted to an OD600 of 0.10 using a UV–Vis spectrophotometer [14].

The pathogenic suspension was then evenly spread on the surface of the NA using a sterile cotton swab to produce a homogeneous growth layer. Paper discs with a diameter of 6 mm were subsequently immersed in the bacterial culture supernatant, briefly drained, and then placed on the surface of the NA inoculated with the pathogen. As a comparison, discs containing amoxicillin were used as the positive control, and discs containing distilled water were used as the negative control, both placed using sterile forceps. All plates were incubated in an inverted position at 37 °C for 24 hours. Antibacterial activity was determined by measuring the diameter of the inhibition zone (in mm) around each disc, and the results from the two repetitions were averaged [14].

RESULTS AND DISCUSSION

1. Isolation of Bacteria from Red Rice Syrup

Lactic acid bacteria (LAB) were isolated from the fermented red rice syrup (SBM) sample using the selective medium MRS agar. After incubation at 37 °C for 48 hours, colony growth was observed on the medium surface. The colonies that appeared were generally circular, had convex elevation, smooth surfaces, and white to cream coloration, consistent with the morphological characteristics of LAB.

From the initial isolation, five candidate colonies (SBM1–SBM5) were obtained with relatively similar morphology, indicating the possibility of phenotypic homogeneity among the isolates. Each colony was then subcultured by repeated streak plate techniques on MRS agar to obtain pure cultures and ensure consistent morphology. The pure isolates were prepared for short-term storage using MRS broth supplemented with 25% (v/v) glycerol and stored at −20 °C to maintain viability until subsequent characterization stages (microscopic morphology, biochemical tests such as Gram staining and catalase, and antibacterial activity assays). Visual documentation of the isolation results for colonies SBM1–SBM5 is presented sequentially in Figure 1.

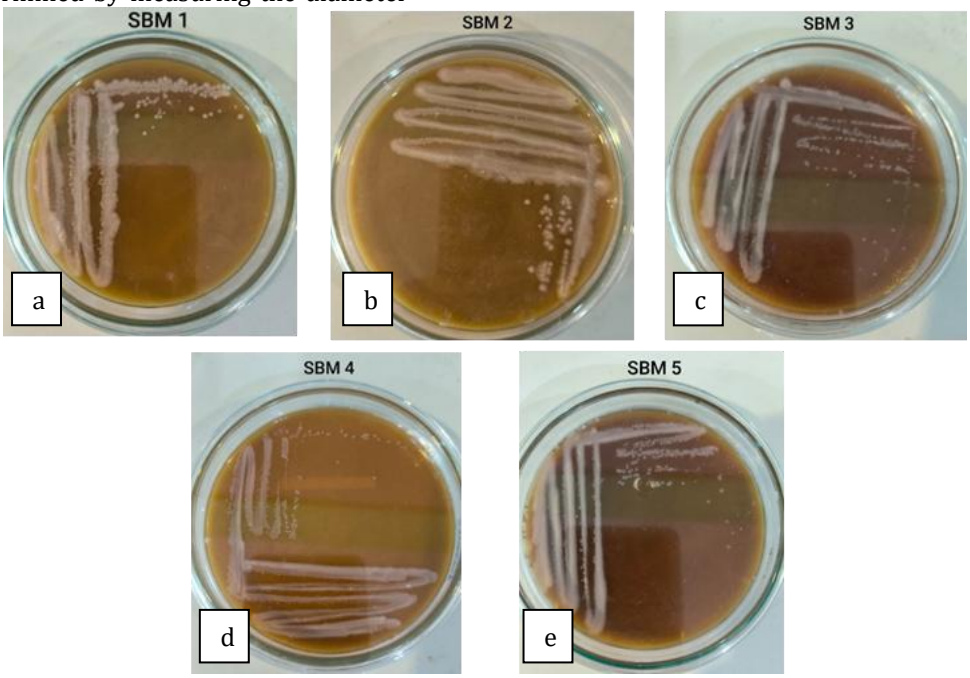

Figure 1. Bacterial isolate colonies on MRS agar. (a) isolate SBM1; (b) isolate SBM2; (c) isolate SBM3; (d) isolate SBM4; (e) isolate SBM5.

The isolation of five candidate colonies (SBM1–SBM5) from fermented red rice syrup on MRS agar medium with 48 hours of incubation at 37 °C indicates that the syrup fermentation environment provides highly conducive conditions for the growth of lactic acid bacteria (LAB). The observed colony morphology, including white to cream coloration, circular shape, and convex elevation, is highly consistent with the classical characteristics of LAB on MRS agar, supporting the interpretation that these isolates are indeed dominant LAB [15]. This success appears to result in a combination of the fermentation pH, the readily available carbohydrate substrate (resulting from

starch hydrolysis in the syrup), and the selective MRS medium, which strongly supports LAB growth. These factors simultaneously produced a relatively homogeneous LAB population. The incubation conditions also have an important role, as the temperature and incubation duration allowed LAB to dominate without substantial competition from other non-LAB microbes, making the obtained isolates easy to purify using serial streak-plate techniques [16].

The main findings of this study are the presence of phenotypically homogeneous LAB isolates from fermented red rice syrup and the ability to store the cultures in the short term, maintaining viability when cryoprotected with glycerol and stored at −20 °C. Furthermore, this study has prepared isolate supernatants (secondary metabolites) for antibacterial testing using the disc diffusion assay, providing preliminary evidence that the metabolites released by the LAB isolates can be explored as potential antimicrobial agents. This represents an important starting point because many similar studies only evaluate LAB cells directly or perform basic biochemical tests, without an explicit focus on the supernatant as a source of bioactive metabolites.

Factors highly likely to explain the results include the quality of the fermented red rice syrup matrix, which provides a source of hydrolyzed sugars highly favorable to LAB; the strongly selective nature of MRS medium; and optimal purification conditions. The red rice syrup matrix not only supplies simple sugars but also other growth factors, such as peptides or ions, that may support LAB growth more effectively than that of other microbes. Meanwhile, MRS is the standard medium for LAB isolation because it provides optimal nutrients, dextrose, and pH conditions, thereby effectively suppressing the growth of non-target microbes. Repeated streak-plate purification ensures that the phenotype obtained is a pure culture, reducing the likelihood of contamination and heterogeneity [17].

The morphological results and the isolation approach reported here are consistent with studies on other fermented rice-based products that have identified dominant LAB with similar colony morphology and successfully isolated them using MRS agar. These studies also confirm that rice-based fermentation provides a practical source of LAB with biotechnological potential and preliminary antimicrobial activity [18].

The strength of this study lies in its selective and application-relevant approach: by targeting the supernatant of LAB isolates, this study opens a pathway for discovering natural antimicrobial metabolites that may be safer and more suitable for functional food or biopreservation applications. In addition, isolates purified using the streak-plate method and cryopreserved strengthen the potential reproducibility of the isolates for further studies. However, this study also has important limitations: the use of only the disc diffusion assay for the supernatant yields qualitative results and does not provide quantitative data, such as the minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC). In addition, molecular identification (for example, 16S rRNA sequencing) has not yet been performed on isolates SBM1–SBM5; therefore, the specific taxonomy and phylogenetic relationships with other LAB isolates remain unclear.

2. Characterization of Bacterial Isolates

Gram staining of the five isolates obtained from fermented red rice syrup (SBM1–SBM5) showed that all were Gram-positive, indicated by the purple coloration (retention of crystal violet–iodine). Morphologically, the bacterial cells appeared coccobacillary, with short length-to-width ratios, and were generally arranged solitarily or in pairs in the visual field. Variability among the isolates was relatively small; therefore, the microscopic morphological profile can be considered homogeneous across SBM1–SBM5. Documentation of the microscopic observations is presented sequentially in Figure 2 (SBM1–SBM5).

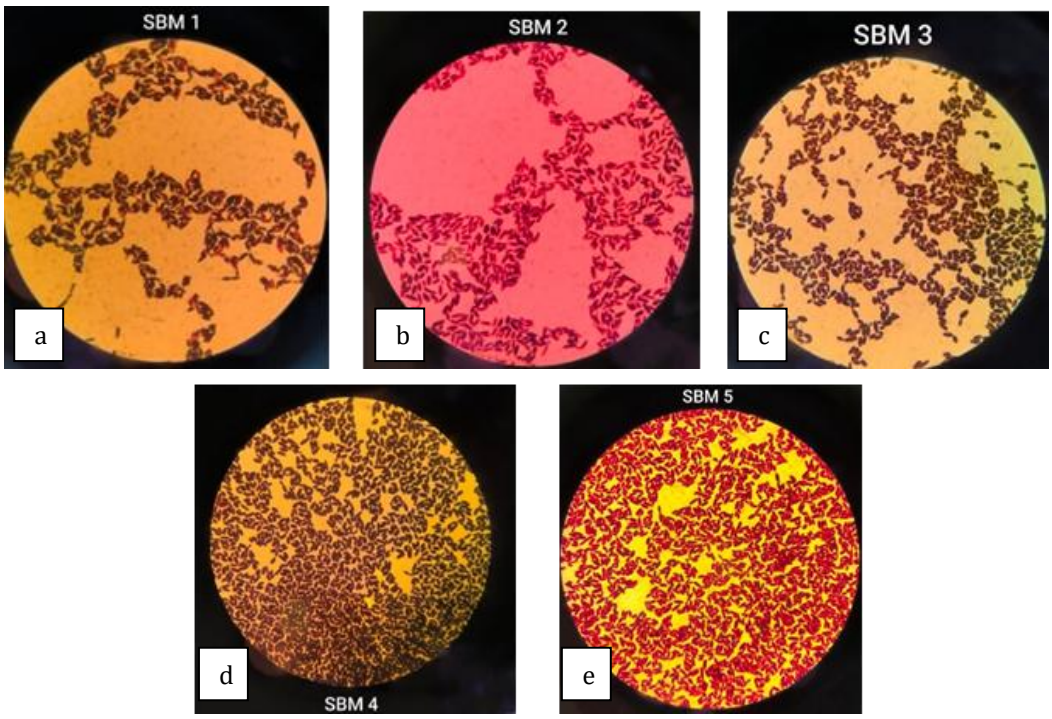

Figure 2. Gram staining results. (a) isolate SBM1; (b) isolate SBM2; (c) isolate SBM3; (d) isolate SBM4; (e) isolate SBM5.

Microscopically, all isolates showed Gram-positive characteristics with a homogeneous coccobacillary morphology, a profile consistent with the general description of LAB (a group of acid-tolerant, non-spore-forming, aerotolerant or facultative anaerobic bacteria) that may morphologically appear as cocci, short bacilli, or coccobacilli [19]. The alignment between the colony characteristics on MRS and the Gram-positive coccobacillary morphology strengthens the interpretation that the obtained isolates represent the LAB group typically expected in cereal- or rice-based fermentation [20].

The limited number of colonies (SBM1–SBM5) and the phenotypic homogeneity observed are likely to reflect the dominance of one or several LAB strains that are most adaptive to the fermentation ecology of red rice syrup, for example, due to the carbohydrate composition, acidity, and the characteristic phytochemical compounds of red rice that selectively shape specific microbial communities. The pattern of "a few similar isolates" is consistent with reports on rice fermentation, in which Gram-positive LAB with coccobacillary or short coccoid-bacillary morphology frequently become the dominant populations recovered through culture-based isolation from cereal matrices. These findings support the interpretation that the applied isolation procedure successfully captured the ecologically relevant LAB fraction in the red rice substrate, so the relatively uniform profile of SBM1–SBM5 can be viewed as a reflection of community dominance during fermentation [17].

Enzymatic testing using the catalase assay showed positive results for all isolates. After dropping a 3% H_2O_2 solution onto the fresh colonies, gas bubbles were observed within the 10–30 second observation interval, indicating H_2O_2 decomposition activity. This result was consistent across repeated tests among the isolates; therefore, it can be summarized that the preliminary characterization profile of the five isolates is Gram-positive, coccobacillary morphology, and catalase-positive. A summary of the characterization results is presented in Table 1.

Table 1. Summary of the characterization of isolates SBM1–SBM5

Isolate	Characteristics		
	Gram Staining	Morphology	Catalase
SBM1	+	Coccobacilli	+
SBM2	+	Coccobacilli	+
SBM3	+	Coccobacilli	+
SBM4	+	Coccobacilli	+
SBM5	+	Coccobacilli	+

In the catalase assay, all isolates (SBM1–SBM5) exhibited positive catalase results (the appearance of gas bubbles within 10–30 seconds after the application of 3% H_2O_2). The catalase-positive phenotype in LAB can be explained by the presence of heme-dependent catalase in certain species, particularly within the Lactobacillus/Latilactobacillus sakei group. Recent studies have shown that L. sakei possesses the katA gene (heme-dependent catalase) and a heme uptake

system (an ECF-like transporter), and catalase activity is expressed when heme is available in the environment. Thus, biologically, a catalase-positive result does not necessarily exclude isolates from the LAB group but may instead reflect an oxidative-stress tolerance strategy under specific conditions [21].

The manganese-dependent "pseudocatalase" mechanism (Mn-catalase) has also been shown in *Lactiplantibacillus (L.) plantarum*. A 2021 study confirmed that Mn-catalase is crucial for aerobic growth and resistance to H_2O_2 in *L. plantarum*, indicating that some LAB can degrade H_2O_2 without heme, leading the catalase test to appear positive. In the context of plant-based fermentation, such as red rice syrup, this finding provides a reasonable explanation for the positive catalase results observed in the SBM isolates, even though the standard medium (MRS) is not enriched with heme [22]. Taxonomically, LAB are classically described as catalase-negative, yet several strains are recognized to produce "pseudocatalase". This consensus has been summarized in recent comprehensive reviews of fermented foods. Therefore, the positive catalase results in SBM1–SBM5 remain consistent with the LAB identity indicated by Gram staining and coccobacillary morphology [23].

The main finding of this study is that all isolates obtained from red rice syrup fermentation showed a consistent profile of lactic acid bacteria (LAB), characterized by Gram-positive traits, homogeneous coccobacillary morphology, and positive catalase activity, which can be explained by heme-dependent catalase mechanisms and manganese-based pseudocatalase. This consistency in characteristics indicates that red rice fermentation tends to select for specific LAB communities that are competitive and stable, a phenomenon also reported in other cereal-based fermentations. The phenomenon of isolate homogeneity observed in this study is in line with the findings of Yumnam et al. (2025), who reported the dominance of short Gram-positive LAB in rice-based fermentations [19]. It is also consistent with the report by Abidin (2024), which demonstrated that the fermentation of rice-washing water produced LAB isolates that were relatively uniform phenotypically [24]. The positive catalase activity observed is also consistent with the findings of Peacock and Hassan (2021) on *Lactiplantibacillus plantarum* and Verplaetse et al. (2020) on *L. sakei*, both of which showed that certain LAB strains can demonstrate catalase activity under specific conditions via pseudocatalase mechanisms or heme-dependent catalase. The alignment of these findings strengthens the interpretation that our results are not an anomaly but instead reflect the adaptive capacity of LAB within plant-based fermentation environments [25] [26].

The factor most likely to cause the uniform phenotype and the positive catalase activity is the fermentation conditions of the red rice syrup, ranging from the availability of simple carbohydrates, the phenolic content of red rice, the progressively decreasing pH during fermentation, to the possible presence of micronutrient catalysts such as Mn^{2+}, which selectively support the growth of certain LAB strains. The strength of this study lies in the consistency of the isolation process (producing five stable isolates) and the focus on cell-free supernatant as the basis for antibacterial testing, thereby providing an initial overview of the potential of extracellular LAB metabolites from the red rice substrate as antimicrobial agents [25].

3. Antibacterial Activity Test

Antibacterial activity testing against *Escherichia coli* and *Staphylococcus aureus* was performed for each isolate (SBM1–SBM5) in duplicate. Based on observations after 24 hours of incubation at 37 °C, none of the SBM1–SBM5 isolates formed inhibition zones around the discs for either test bacterium. This pattern of absence of inhibition zones was consistent across both repetitions, indicating that, under the applied test conditions, the culture supernatants of the isolates did not exhibit detectable antibacterial activity against *E. coli* or *S. aureus* using the disc diffusion method. Visualization of the results for the isolates against *E. coli* is presented in Figure 3, and against *S. aureus* in Figure 4.

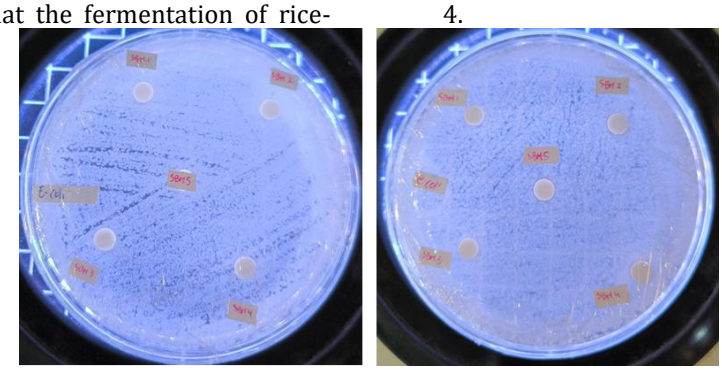

Figure 3. Disc diffusion assay results of isolates SBM1–SBM5 against *E. coli*

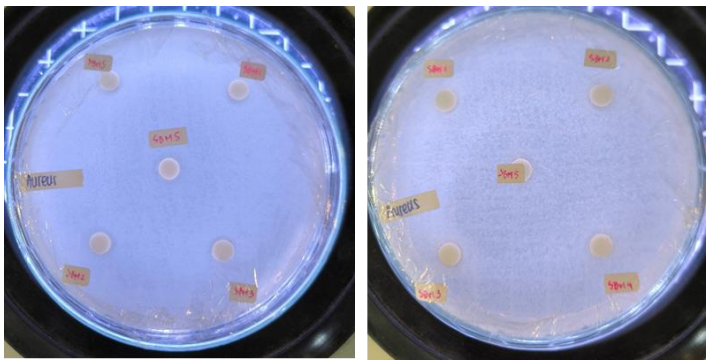

Figure 4. Disc diffusion test results of isolates SBM1–SBM5 against *S. aureus*

As a comparison, the positive control (amoxicillin) produced an average inhibition zone of 13.95 mm against *E. coli* and 12.43 mm against *S. aureus*, confirming the validity and sensitivity of the test method used. Meanwhile, the negative control (sterile distilled water) did not produce an inhibition zone for either test bacterium. Visual documentation for the controls against *E. coli* and *S. aureus* is presented in Figure 5.

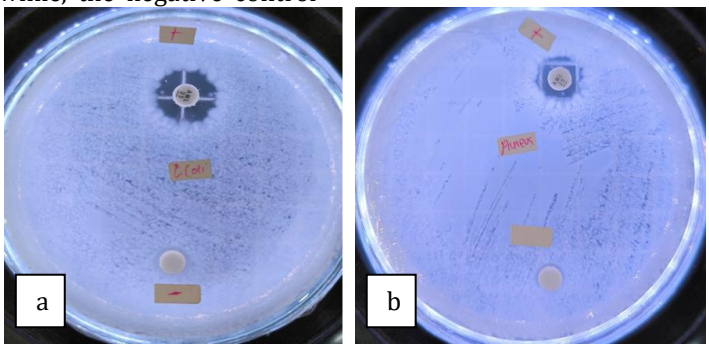

Figure 5. Disc diffusion test results for the amoxicillin and distilled water controls. ((a) Test control on *E. coli*; (b) Test control on *S. aureus*.

Overall, these results indicate that under the applied test conditions (NA medium, 37 °C, 24 hours, disc diffusion method, culture supernatant), the supernatants of isolates SBM1–SBM5 did not exhibit inhibitory activity against the indicator bacteria, either the Gram-negative *E. coli* or the Gram-positive *S. aureus*. The antibacterial test results are summarized in Table 2.

Table 2. Antibacterial activity of bacterial isolates against *E. coli* and *S. aureus*

Isolate/Control	Inhibition Zone Diameter (mm)	
	E. coli	*S. aureus*
SBM1	-	-
SBM2	-	-
SBM3	-	-
SBM4	-	-
SBM5	-	-
Amoxicillin (+)	13.95	12.43
Aquadest (–)	-	-

In this study, the supernatants of all isolates (SBM1–SBM5) did not form inhibition zones against *Escherichia coli* or *Staphylococcus aureus* after 24 hours of incubation at 37 °C, whereas the positive control (amoxicillin) produced average inhibition zones of 13.95 mm (*E. coli*) and 12.43 mm (*S. aureus*), indicating that the assay was valid; the negative control showed no inhibition. The finding of "no detectable zones" under these test conditions can be interpreted as reflecting the limitations of the disc diffusion method in terms of sensitivity and diffusion for certain bioactive compounds (e.g., peptides or bacteriocins), which can result in undetected activity. Recent methodological references recommend quantitative testing (MIC) or alternative approaches (e.g., agar well diffusion) to overcome diffusion limitations and provide a more accurate representation of activity [21].

In the disc diffusion assay conducted, none of the LAB isolates (SBM1–SBM5) produced inhibition zones against *Escherichia coli* ATCC 25922 or *Staphylococcus aureus* ATCC 25923, whereas the positive control in the form of amoxicillin produced average inhibition diameters of 13.95 mm and 12.43 mm, respectively. This indicates that the test method was capable of detecting antimicrobial activity in the positive control but not in the LAB preparations tested. Similar negative results have also been reported previously; *Lactobacillus acidophilus* ATCC 4356 was reported to inhibit MRSA and MSSA only through the soft agar overlay method, but did not show antimicrobial effects when tested using agar well diffusion, punch hole, or paper disc methods,

which reinforces that certain assay formats indeed fail to display specific antimicrobial activities [28].

A recent study by Amirloreport et al. also reported the absence of antimicrobial activity using the disc diffusion method and agar well diffusion when testing bacteriocin extracts against various *E. coli* strains, showing that certain Gram-negative bacteria can yield zero-zone outcomes under standard agar diffusion conditions [29]. The sensitivity of the method is also affected by the type of preparation tested; in a study on LAB from Sumba horse milk, the non-filtrate fraction (containing cells) did not produce inhibition zones against several pathogens using the disc diffusion method, whereas the cell-free preparation generated measurable inhibition zones, indicating that the matrix and assay format affect detection outcomes [30]. In addition, pH neutralization of LAB supernatant can eliminate clear zones in *Lactobacillus casei* ATCC 39392 and *L. rhamnosus* ATCC 7469; neutralized CFS did not produce inhibition zones, whereas acidic CFS generated halos of 9–9.4 mm. These findings indicate that acidity is often the primary factor contributing to the inhibitory effects observed in agar-based assays [31].

From the perspective of LAB metabolite biology, recent literature indicates that the antimicrobial activity of CFS (cell-free supernatant) is strongly influenced by the growth phase and metabolite composition, particularly the accumulation of organic acids. In *Pediococcus acidilactici*, activity begins to be detected at approximately 12 hours and increases until the stationary phase (around 48 hours). This increase does not always correlate with total protein content, indicating a strong involvement of diffusible soluble components, such as lactic acid. Thus, the absence of zones in the present assay most likely reflects an insufficient concentration or composition of active metabolites, or a lack of effective diffusion into the agar under the disc diffusion setting, rather than eliminating the antimicrobial potential of the isolates [32].

In addition, differences in the resistance of the target cell wall also determine the outcome: LAB bacteriocins or biopeptides are generally more effective against Gram-positive bacteria, whereas Gram-negative bacteria such as *E. coli* possess an outer membrane rich in lipopolysaccharides that limits the penetration of many antimicrobial molecules, resulting in lower inhibitory effects or requiring supportive conditions. Therefore, the absence of zones in *E. coli* is consistent with the outer membrane barrier mechanism, while the lack of zones in *S. aureus* under this setting further points to diffusion or concentration limitations rather than a complete absence of activity. Accordingly, more sensitive follow-up assays (pH neutralization, CFS concentration, well diffusion, MIC) are worth considering in the next stage of the research [33].

Overall, the finding that all supernatants of isolates SBM1–SBM5 did not produce inhibition zones against *Escherichia coli* or *Staphylococcus aureus* under the disc diffusion test conditions (NA, 37 °C, 24 hours, two repetitions) should be interpreted as an empirical result that is valid yet contextual: valid because the positive control (amoxicillin) and negative control functioned as expected, but contextual because the disc diffusion method has well-known limitations when applied to cell-free supernatant (CFS) containing large molecules, peptides, or components with low diffusibility [34]. The methodological literature states that disc diffusion is most sensitive to small compounds that readily diffuse into agar, whereas peptides, bacteriocins, and several secondary metabolites are often poorly differentiated by this method, which may result in false negatives even when activity is present at sufficiently high concentrations or under other conditions. Therefore, the absence of inhibition zones in this study is more likely to reflect issues of diffusion, concentration, or metabolite production phase rather than absolute evidence of a lack of antibacterial potential in the isolates [35].

A logical biological explanation for this pattern is the combination of (a) the growth phase and the timing of supernatant harvesting, which determines metabolite composition. Several studies show that the accumulation of metabolites such as organic acids or bacteriocins often reaches its peak in the late-log to stationary phase; thus, harvesting the supernatant at an inappropriate time can result in a low concentration of active components; (b) the chemical nature of the dominant metabolites (for example, organic acids that diffuse easily versus peptides or bacteriocins that are relatively large), which affects their ability to penetrate the agar matrix; and (c) the biological characteristics of the target bacteria: Gram-negative bacteria are often less susceptible to many bacteriocins because of the outer LPS membrane that blocks penetration; thus, it is not unexpected that *E. coli* is more resistant in the same assay, whereas several bacteriocins show clearer activity against Gram-positive bacteria. Therefore, the negative results in the disc diffusion assay do not eliminate the possibility that the supernatant contains bioactive metabolites; rather, they emphasize the need for more sensitive and quantitative follow-up steps such as concentration of the CFS, pH neutralization to reveal non-acid effects, proteolytic treatment to confirm peptide nature, agar well diffusion assays that are more suitable for larger molecules, and MIC/MBC determination using dilution methods (broth microdilution). These recommendations are consistent with recent guidelines and methodological studies that discuss the limitations of disc diffusion for natural products and outline alternative evaluation strategies for CFS and bacteriocins [36] [37].

Comparison with the literature on cereal fermentation products shows partial consistency: several studies isolating LAB from rice fermentation or cereal-based products have reported clear antibacterial activity from CFS or bacteriocins after concentration and/or purification steps (for example, protein fractionation and protease assays), whereas other studies that relied solely on disc diffusion also reported similar negative results until the samples were enriched or concentrated. Thus, our results, which were initially negative in the disc diffusion assay, align with several methodological reports and differ from studies that performed concentration or purification before testing; these differences are more related to testing procedures and sampling phases rather than biological contradictions among studies. The practical implication is that this study provides preliminary evidence that can guide subsequent efforts, namely molecular identification of the isolates (16S rRNA) to place them within taxonomic context and existing literature, optimization of supernatant harvesting time (monitoring pH or OD for the production phase), concentration of the CFS (for example, lyophilization or ultrafiltration), pH-neutralization testing and protease treatment to detect peptide characteristics, as well as MIC/MBC assays for activity quantification. These steps will strengthen claims of antibacterial activity or demonstrate the absence of such activity under more sensitive conditions.

CONCLUSION

This study successfully isolated five bacterial isolates (SBM1–SBM5) from fermented red rice syrup through cultivation on MRS agar and incubation at 37 °C for 48 hours. The resulting colonies exhibited white–cream coloration, circular shape, and convex elevation, and were subsequently purified for further characterization. Microscopically, all isolates displayed Gram-positive characteristics and a relatively homogeneous coccobacillary morphology, indicating initial phenotypic uniformity and the consistency of characteristics expected for bacteria obtained under those fermentation conditions. The catalase assay for all five isolates showed positive results, indicated by the appearance of gas bubbles within 10–30 seconds after the application of 3% H_2O_2. In the antibacterial activity test using the disc diffusion method against *Escherichia coli* and *Staphylococcus aureus*, the culture supernatants of SBM1–SBM5 did not produce inhibition zones in both repetitions, whereas the positive control (amoxicillin) exhibited average inhibition zones of 13.95 mm for *E. coli* and 12.43 mm for *S. aureus*, and the negative control (aquadest) showed no inhibition. Based on the findings, it is recommended that this isolation stage be followed by a more precise determination of taxonomic identity (e.g., 16S rRNA) to definitively associate the observed phenotypic profile with the corresponding species or strain. In addition, there is a need for follow-up assay designs that are more sensitive to the diffusibility of active compounds, such as pH neutralization of the supernatant before testing, supernatant concentration, the use of well diffusion and/or MIC determination based on broth microdilution, as well as the alignment of test media with disc diffusion standards.

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