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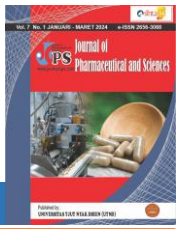

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# Plant-Based Matrix-Derived Lactic Acid Bacteria Strain SBM10 from Fermented Red Rice Syrup: In Vitro Safety Assessment and Characterization

# Strain Bakteri Asam Laktat SBM10 dari Matriks Berbasis Nabati Berupa Fermentasi Sirup Beras Merah: Evaluasi Keamanan In Vitro dan Karakterisasi

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## Abstract

Plant-based fermentation offers a route to develop nondairy probiotic candidates, yet syrup-like matrices remain underexplored compared with solid or beverage systems. This study investigated fermented red rice syrup (prepared from red rice and barley malt powder) as a source of lactic acid bacteria (LAB) and advanced one isolate, SBM10. Cultivation on MRS supplemented with $CaCO_3$ yielded acidogenic colonies. A stable morphotype (SBM10) was purified and exhibited a LAB consistent profile, Gram-positive rods, catalase negative, TSIA K/A without $H_2S$, Simmons citrate negative, gelatinase negative, $CO_2$ positive in Durham and γ-hemolytic on 5% sheep blood agar. Under gastrointestinal stress models (4 h, 37 °C), tolerance relative to controls was 27.14% at pH 3.0 and 35.58% in 0.3% oxgall. In disk diffusion assays, the cell-free supernatant (CFS) showed no inhibition of *Escherichia coli* or *Staphylococcus aureus*. Thus, no antibacterial activity was detected by disk diffusion under the conditions tested, whereas amoxicillin produced zones of 13.95 mm and 12.43 mm, respectively. SBM10 presents a safety-supportive, LAB-typical profile with partial tolerance to gastric-like acidity and bile. Although no disk diffusion antagonism was detected for the CFS, the results motivate taxonomic confirmation and process/formulation optimization, such as improving acid/bile robustness and re-evaluating antimicrobial potential with complementary assays, to clarify the suitability of SBM10 for plant-based functional applications.

***Keywords*: *Fermented red rice syrup, Lactic acid bacteria, Probiotic safety evaluation, Acid and bile tolerance, Antibacterial activity.***

## Abstrak

Fermentasi berbasis bahan nabati menawarkan jalur pengembangan kandidat probiotik non susu, namun matriks yang menyerupai sirup masih kurang dieksplorasi dibandingkan sistem berbentuk padat atau minuman. Studi ini mengevaluasi sirup beras merah fermentasi (disiapkan dari beras merah dan bubuk barley malt) sebagai sumber bakteri asam laktat (BAL) dan memilih satu isolat, SBM10, untuk karakterisasi lanjutan. Kultur pada MRS yang disuplementasi $CaCO_3$ menghasilkan koloni asidogenik. Morfotipe stabil (SBM10) dimurnikan dan menunjukkan profil konsisten BAL, batang Gram positif, katalase negatif, TSIA K/A tanpa $H_2S$, Simmons sitrat negatif, gelatinase negatif, $CO_2$ positif pada tabung Durham, serta γ-hemolitik pada agar darah dengan darah domba 5%. Pada model uji toleransi saluran cerna (4 jam, 37 °C), pertumbuhan relatif terhadap kontrol mencapai 27,14% pada pH 3,0 dan 35,58% pada 0,3% oxgall. Dalam uji difusi cakram, supernatan bebas sel tidak menunjukkan penghambatan terhadap *Escherichia coli* maupun *Staphylococcus aureus*. Dengan demikian, tidak terdeteksi aktivitas antibakteri melalui metode difusi cakram pada kondisi uji kami, sedangkan amoksisilin menghasilkan zona hambat masing-masing 13,95 mm dan 12,43 mm. Secara keseluruhan, SBM10 memperlihatkan profil khas BAL yang mendukung aspek keamanan dengan toleransi parsial terhadap keasaman menyerupai lambung dan garam empedu. Meskipun tidak terdeteksi antagonisme pada metode difusi cakram untuk CFS, temuan ini mendorong konfirmasi taksonomi serta optimasi proses/formulasi misalnya peningkatan ketahanan terhadap asam/empedu dan penilaian ulang potensi

antimikroba dengan uji pelengkap untuk memperjelas kesesuaian SBM10 bagi aplikasi fungsional berbasis nabati.

***Kata Kunci: Fermentasi sirup beras merah, bakteri asam laktat, evaluasi keamanan probiotik, toleransi asam dan empedu, aktivitas antibakteri.***



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## Introduction

The global rise of fermentation-based functional foods has coincided with increasing consumer interest in plant-based probiotic formats, driven by health, lactose intolerance, and sustainability considerations. [1]. The urgency for safe, effective microbial interventions is underscored by the substantial worldwide burden of bacterial antimicrobial resistance, estimated at 4.95 million associated deaths in 2019 [2]. In parallel, modern dietary patterns characterized by low fiber intake have been linked to gut microbiota disruption and elevated disease risk, highlighting the need for strategies that support intestinal homeostasis [3]. Within this context, nondairy probiotic products have expanded across diverse plant matrices and emphasize rigorous in vitro evaluation to ensure viable, functional strains for food applications. [4]. Complementarily, recent reviews indicate that plant-based matrices can successfully host probiotics while aligning with evolving consumer preferences [5].

Red rice is a promising substrate owing to its bioactive profile. Comprehensive chemical characterization has shown that red rice is rich in phenolic constituents, predominantly flavan-3-ols, supporting its antioxidant potential. [6]. In pigmented rice, these phenolics concentrate in the bran layer, such as anthocyanins, proanthocyanidins, flavonoids, and related compounds, which materially contribute to antioxidant capacity [7]. Barley malt complements this matrix, malting yields flours rich in soluble carbohydrates, free phenolic acids, and free amino acids (including GABA), with higher antioxidant properties than unmalted barley, supporting use as a bio-functional base [8]. Consistent with this, controlled malting of barley significantly increases total polyphenol content and radical scavenging activity compared with unmalted grain and derived cereal products, strengthening the rationale for incorporating malt into functional formulations [9]. Together, these characteristics provide a coherent biochemical rationale for using cooked red rice supplemented with barley malt powder as a plant-based fermentation medium for LAB.

Red rice syrup fermentation was prepared by washing and cooking red rice at medium-high heat, adding water and barley malt powder to the cooked rice, and allowing the mixture to ferment [10]. Beyond process feasibility, rice-derived syrups have shown physiological effects in animal models, including improvements in triglyceride values over 28 days, with lower values relative to baseline [11]. Complementing these findings, a separate study reported that rice syrup lowered serum uric acid, urea, and creatinine concentrations and was associated with improved renal histopathology compared with the negative control. [12]. These findings suggest that rice-based syrup systems can serve not only as vehicles for microbial growth but also as carriers with potential functional benefits.

Fermentation can modulate phenolic profiles and enhance antioxidant capacity, thereby elevating the functional value of plant foods [13]. In rice systems, LAB fermentation of pigmented rice bran has been reported to increase the levels of bioactive compounds and antioxidant properties, indicating the suitability of rice substrates for functional fermentation [14]. Rice-based beverages have also been shown to serve as viable nondairy carriers for LAB, supporting live populations and producing expected physicochemical changes [15]. Clinically, a randomized, double blind, placebo-controlled trial found that a rice drink



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fermented with *Lactiplantibacillus plantarum* JSA22 reduced abdominal distension in individuals with irritable bowel syndrome, underscoring its clinical relevance as a rice-based probiotic platform [16].

Mechanistically, LAB contribute through both cell viability and the production of metabolites, including organic acids, bacteriocins, exopolysaccharides, and vitamins that can inhibit foodborne pathogens and support gastrointestinal health [17]. Contemporary syntheses further highlight LAB bacteriocins as promising agents for promoting gut health and bioprotection in foods [18]. Standard screening of candidate probiotic strains typically focuses on foundational safety and robustness attributes, such as absence of hemolysis and tolerance to acidic conditions and bile salts, as demonstrated in recent phenotypic assessments of Lactobacillus salivarius CGMCC20700 [19].

Despite advances in rice-based fermentations, most studies have concentrated on pigmented rice bran or on rice beverages as nondairy probiotic carriers, leaving syrup matrices comparatively underexplored. In particular, the isolation and phenotypic safety or robustness characterization of LAB recovered specifically from fermented red rice syrup prepared with barley malt powder remain unexplored, representing the key novelty addressed here. This study examines fermented red rice syrup as a source of LAB and characterizes isolate SBM10, with emphasis on safety, supportive, and function-relevant properties to inform plant-based functional applications.

## Experimental Section

### Production of Red Rice Syrup Fermentate

Red rice (200 g) was rinsed with distilled water in a sterile 1.0 L beaker, combined with 500 mL of distilled water, and cooked until the grains softened. The cooked rice was then cooled to approximately 50 °C, after which diastatic barley malt powder (60 g) was incorporated and mixed thoroughly to obtain a uniform mash. This mash was maintained at 50 °C for 60 min to facilitate enzymatic hydrolysis of starch into simpler sugars, then cooled to approximately 37 °C and transferred to a sterile 1.0 L Erlenmeyer flask covered with aluminum foil. Fermentation was subsequently conducted in a shaking incubator set to 37 °C and 300 rpm for 120 h.

### Isolation of SBM10 from Red Rice Syrup

Red rice syrup fermentate was first enriched by inoculating 1 mL of the sample into 9 mL sterile MRS broth and incubating at 37 °C for 24 h to favor the outgrowth of presumptive LAB. After enrichment, tenfold serial dilutions ($10^{-1}$-$10^{-5}$) were prepared, and 100 µL aliquots from each dilution were spread onto MRS agar containing 1% (w/v) $CaCO_3$ and incubated at 37 °C for 48 h. Dissolution of $CaCO_3$ by organic acids produced optically clear halos that served as a primary indicator of acidogenic colonies; such colonies were picked as LAB candidates and purified by repeated streaking on MRS agar until single, morphologically uniform colonies were obtained. Purity was confirmed by four-quadrant re-streaking [20].

### Characterization of SBM10

Purified LAB underwent phenotypic profiling by Gram staining, catalase test, Triple Sugar Iron Agar (TSIA) test, Simmons Citrate utilization, gelatinase activity, and evaluation of gas production from glucose. Gram staining was performed on heat-fixed smears following the canonical four-step protocol: crystal violet, Gram's iodine, ethanol decolorization, and safranin counterstain, as previously described by Ruby Khan et al. [21]. Catalase activity was assessed on a glass slide smear by applying a drop of 3% $H_2O_2$, with bubble or froth formation indicating a positive result [22]. TSIA slants were inoculated by stabbing through the center to the tube bottom, streaking the slant, and incubating at 37 °C for 24 h before recording slant/butt reactions. LAB isolates ferment glucose, sucrose, and lactose, as indicated by the yellowing of the medium [23]. Citrate utilization was assessed on Simmons Citrate Agar slants streaked and incubated at 37 °C for 24-48 h, a green to blue shift denoted alkalinization and a positive reaction, whereas persistence of green indicated non-utilization (negative) [24]. Gelatinase activity was determined in nutrient gelatin tubes incubated at 30 °C for 7–14 days. Retained liquefaction after chilling the tubes at 4 °C for one hour was considered a positive result [25]. Gas production from glucose was evaluated by inoculating isolates into MRS broth containing an inverted Durham tube and incubating at 30 °C. The presence of gas trapped in the Durham tube was recorded as a positive result [26].



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**In Vitro Safety Characterization of SBM10**

In vitro safety characterization of LAB isolates involved assessing hemolytic activity and determining tolerance to acidic pH and bile salts. Hemolytic activity was evaluated by streaking isolates onto blood agar plates containing 5% sheep blood and incubating at 37 °C for 24-48 h, with β-hemolysis defined as a clear zone around colonies, α-hemolysis as a greenish discoloration, and γ-hemolysis as the absence of hemolysis [27].

Acid tolerance was evaluated as described by Xiaoxue Kong et al. [28], with minor modifications. In which isolates were first incubated at 37 °C for 24 h, then inoculated into MRS broths adjusted to defined test conditions adjusted with 1 M HCl and control pH values, pH 3.0 (simulated gastric-like condition) and pH 6.5 (control). Inoculated tubes were incubated at 37 °C for four hours, after which tolerance was quantified by measuring absorbance at 600 nm ($OD_{600}$). In this assay, $OD_{600}$ served as a turbidity-based proxy for tolerance over the four-hour exposure. OD-based readings do not discriminate viable from non-viable cells and therefore approximate total biomass rather than actual viability. [29]. All tolerance assay was performed as a single exploratory run, and no inferential statistics were performed. The acid tolerance was expressed as:

$$\text{Growth/Survival Percentage (\%)} = \frac{OD_{600}\ \text{pH 3.0}}{OD_{600}\ \text{pH 6.5}} \times 100\%$$

Bile salt tolerance was evaluated as described by Abdel Tawab et al. [30], with minor modifications. LAB cultures grown in MRS at 37 °C were standardized, then inoculated into sterile MRS supplemented with 0.3% (w/v) oxgall, with bile-free MRS as the control. All tubes were incubated at 37 °C for four hours, and $OD_{600}$ was recorded at four hours. As for the acid assay, $OD_{600}$ was used here as a practical proxy for tolerance, with the same limitation that turbidity cannot distinguish live from dead cells. The bile salt tolerance was defined as:

$$\text{Growth/Survival Percentage (\%)} = \frac{OD_{600}\ (\text{MRS} + \text{bile})}{OD_{600}\ (\text{MRS control})} \times 100\%$$

**Antibacterial activity of SBM10**

Antibacterial activity was determined by a disk diffusion assay using cell-free supernatant (CFS) from strain SBM10 to assess the isolates' antibacterial effects. Assays were performed in duplicate on the isolates. Test pathogens (Escherichia coli and Staphylococcus aureus) were grown and their suspensions standardized to an optical density of 0.10 at 600 nm using a UV–Vis spectrophotometer. The standardized suspensions were then evenly spread over Nutrient Agar (NA) plates with sterile cotton swabs to generate confluent lawns. CFS was obtained from SBM10 cultures by centrifugation, then sterile-filtered through a 0.22 μm filter. Sterile 6 mm paper disks were impregnated with the CFS and placed onto the inoculated NA surface together with an amoxicillin disk (positive control) and a sterile distilled water disk (negative control). Plates were incubated at 37 °C for 24 h, after which inhibition zones were measured in millimetres using a caliper [31]. Disk diffusion testing was performed as a single exploratory run; inhibition zone diameters are reported as single-run point values without mean ± SD, and no inferential statistics were performed.

## Results and Discussion

**Isolation of SBM10 from Red Rice Syrup**

Following inoculation onto $CaCO_3$-supplemented MRS and 48 h incubation at 37 °C, evident colony growth was observed from the sample. A consistent morphotype was recovered on MRS agar and designated SBM10; colonies were circular with smooth, entire margins, convex in elevation, and white to creamy in appearance, as shown in Figure 1.

These macroscopic attributes align with recent descriptions of LAB colony phenotypes on MRS, where isolates from Indonesian fermented foods frequently exhibited creamy white pigmentation with circular form and convex elevation, reinforcing their preliminary assignment as LAB candidates [32]. In parallel, a study by Olorunshola et al. reported that presumptive LAB typically formed cream colored, circular, convex colonies with entire margins, further supporting the morphological consistency observed for SBM10 [33].



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**Characterization of SBM10**

Before downstream evaluations, isolate SBM10 was first profiled morphologically and biochemically, including Gram staining and cell shape assessment, the catalase assay, TSIA, Simmons citrate, gelatin liquefaction, and $CO_2$ production in Durham tubes. The characteristics of isolate SBM10 are shown in Table 1.

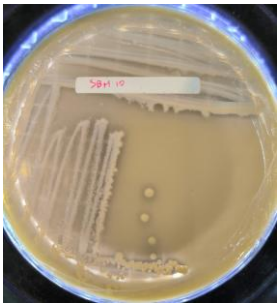

**Figure 1.** Colony morphology of isolate SBM10 on MRS with $CaCO_3$

**Table 1.** Characterization of SBM10 isolate

| Isolate | Characteristics | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Gram staining | Morphology | Catalase | TSIA | $H_2S$ Production | Gelatinase Test | Simmons Citrate Test | Gas Production |
| SBM10 | + | Bacilli | - | K/A | - | - | - | + |

Isolate SBM10 exhibited a Gram-positive reaction with rod-shaped (bacillary) morphology on microscopy, as shown in Figure 2, and a negative catalase test. These traits are consistent with the canonical descriptors of lactic acid bacteria (LAB), which are typically Gram-positive, non-spore-forming rods or cocci, and generally catalase-negative; these phenotypes were likewise used as first-pass selection criteria in recent LAB screening studies. [34].

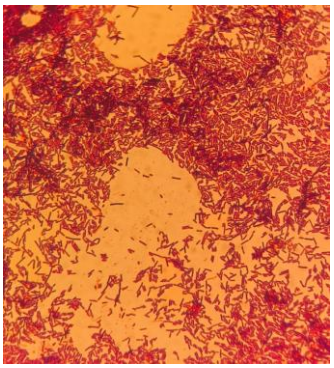

**Figure 2.** Gram-stained micrograph of isolate SBM10

The Triple Sugar Iron Agar (TSIA) test, SBM10, exhibited an alkaline slant/acid butt (K/A) with no $H_2S$ shown in Figure 3, indicating glucose fermentation in the butt without lactose/sucrose fermentation on the aerobic slant. Recent work isolating LAB from fermented milk applied the TSIA system to track sugar fermentation and $H_2S$ formation, and likewise noted $H_2S$ negativity among LAB candidates, aligning with the absence of $H_2S$ observed for SBM10. [35]. For gelatin hydrolysis, SBM10 was gelatinase negative, a desirable safety trait in probiotic selection. A recent survey of LAB from the Ethiopian fermented beverage Cheka reported no gelatinase activity among candidate isolates used for probiotic development, consistent with our observation for SBM10 [36]. Beyond simple phenotype, gelatinase (GelE) is recognized as a virulence-associated factor in certain bacteria because of its roles in proteolysis and biofilm biology. Thus, the absence of gelatinase activity supports the safety profile of SBM10 [37].

SBM10 was Simmons citrate negative; it did not utilize citrate as the sole carbon source under the test conditions. This outcome is consistent with the contemporary understanding of citrate metabolism in LAB, which requires specific uptake and catabolic machinery and is restricted to particular taxa. In contrast, many foodborne LAB do not express this trait broadly. Hence, a negative Simmons citrate test is not unusual in LAB



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screenings [38]. Finally, gas production from glucose was positive in Durham tubes for SBM10, implicating a heterofermentative route that yields $CO_2$ alongside acids/ethanol. Comparative LAB screens frequently record acid without gas in Durham tubes for many probiotic candidates, indicative of homofermentative metabolism, highlighting that SBM10's gas-positive pattern sits within the recognized metabolic diversity of LAB [39]. In terms of interpretation, Durham tube $CO_2$ formation is a standard criterion for assigning heterofermentative behavior in LAB, with the presence of bubbles signifying gas generation from glucose [40]. In beverage/syrup formats, ongoing $CO_2$ generation may cause package swelling, foaming, or texture changes, which can be undesirable for shelf-stable products. Conversely, a slight, controlled carbonation could be acceptable or even desirable in some drinks if headspace, residual sugars, temperature, and inoculum are managed. In short, packaging design and sugar control need to be aligned with the intended use, minimize post-production metabolism for shelf-stable syrups, or allow controlled activity for fresh/chilled products.

**Figure 3.** Triple Sugar Iron Agar (TSIA) profile of SBM10

**In Vitro Safety Characterization of SBM10**

On 5% sheep blood agar, isolate SBM10 exhibited γ-hemolysis (no hemolysis), growth without clearing or greenish discoloration around colonies, indicating an absence of detectable hemolytic activity under the assay conditions. Consistent with probiotic selection criteria that preclude hemolytic activity as a recognized virulence factor, recent work on food-derived lactic acid bacteria reported γ-hemolysis on sheep blood agar with no α- or β-hemolysis, paralleling the SBM10 phenotype and aligning with prior reports [41]. Interpreting this outcome within standard blood-agar taxonomy, β-hemolysis is read as a clear zone (complete lysis), α-hemolysis as a green halo (partial lysis), and γ-hemolysis as the absence of a visible zone [42]. From a safety standpoint, contemporary probiotic assessments explicitly include hemolytic activity as a required criterion, with non-hemolytic behavior considered supportive of strain safety for food applications [43].

In the acid tolerance test, SBM10 cultured in MRS adjusted to pH 3.0 showed an $OD_{600}$ of 0.241 relative to the control $OD_{600}$ of 0.888, corresponding to a Growth/Survival Percentage of 27.14 %. When contrasted with recent LAB screens that quantified survival at pH 3.0, several food-derived isolates retained markedly higher viability (89–97 % after three h), underscoring that SBM10's tolerance under strong acid is comparatively low within the range reported for candidate probiotics. [44]. In terms of performance thresholds, contemporary work has considered strains with >50 % survival at pH 3.0 (≈3 h) as "acid-tolerant," indicating that SBM10's 27.14 % represents limited survival under the present assay conditions. [45]. Mechanistically, acid stress in bacteria is mitigated by $F_1F_0$-ATPase-mediated proton efflux that helps maintain cytoplasmic pH. Reduced growth at pH 3.0 is consistent with the substantial energetic burden required for pH homeostasis at low external pH [46].

As determined by the bile salt tolerance test, SBM10 attained an $OD_{600}$ of 0.316 in 0.3% (w/v) oxgall-supplemented MRS (4 h, 37 °C) compared with 0.888 in the bile-free control, equating to a Growth/Survival Percentage of 35.58 %. Comparable studies report isolated dependent survival around 60-66 % at 0.3% bile after four h, highlighting wide strain variability and situating SBM10 toward the lower end of bile resilience under matched exposure times [47]. Regarding adequacy, probiotic selection studies frequently treat 0.3 % bile as a physiologically relevant screening level and often apply a >50 % survival benchmark. SBM10's 35.58 % reflects modest tolerance that may limit persistence through small-intestinal bile exposure [48]. At the cellular level, one established mechanism of bile adaptation in LAB is bile salt hydrolase (BSH) mediated deconjugation of bile acids, which reduces their detergent toxicity toward bacterial membranes. The extent of such activity contributes to inter-strain differences in bile survival [49]. Representative $OD_{600}$ readings and Growth/Survival Percentage values under acidic (pH 3.0) and bile (0.3% oxgall) conditions are summarized in Table 2.



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**Table 2.** $OD_{600}$ and Growth/Survival Percentage profiles of isolate SBM10 in acidic and bile media

| Isolates SBM10 | $OD_{600}$ | Growth/Survival Percentage (%) |
|---|---|---|
| Control | 0.888 | - |
| Acid Condition | 0.241 | 27.14 |
| Bile Condition | 0.316 | 35.58 |

Relative to commonly applied screening benchmarks at pH 3.0 and 0.3% bile (>50% survival), SBM10's 27.14% and 35.58% indicate partial resilience under the present conditions. A plausible explanation is ecological adaptation to a sugar-rich syrup matrix with limited acid/bile selective pressure, yielding lower baseline robustness than strains selected from harsher niches. SBM10 originated from a sugar-rich syrup niche that imposes limited acid and bile selection pressures, favoring fast growth in benign conditions rather than survival traits for gastric/intestinal transit. This origin plausibly yields lower basal expression of acid homeostasis systems and bile-resistance under our assay conditions, consistent with the present partial tolerance profile.

While plant-derived LAB can exhibit heterogeneous gastrointestinal tolerance, lower baseline resilience is not unusual when the source environment does not chronically impose intense acid/bile stress. Given this partial tolerance, delivery format matters; functional use will likely require protective strategies, for example, microencapsulation and/or co-formulation with buffering matrices and potentially gradual acid/bile pre-adaptation during propagation to enhance robustness before raising survival to the intestine. Such approaches are routinely used to help candidate LAB traverse gastric acidity and early bile exposure.

Our acid and bile results were based on $OD_{600}$ at four hours, which does not differentiate viable from non-viable cells. Decreases in OD can also reflect cell lysis or flocculation, not solely growth inhibition. Accordingly, these readouts should be interpreted as preliminary indicators. Interpretation is limited by the lack of biological replication and the use of $OD_{600}$-only proxies, precluding statistical generalization. Future work will corroborate these findings with post-treatment viable counts (CFU/mL) to quantify survival more accurately, along with biological replicates ($n \geq 3$) with mean ± SD and appropriate statistical testing (e.g., t-tests/ANOVA).

**Antibacterial activity of SBM10**

The antibacterial assay of the SBM10 isolate, performed in a disk diffusion method with cell-free supernatant (CFS), showed no detectable inhibition zones against Escherichia coli or Staphylococcus aureus under the conditions tested. In contrast, the amoxicillin-positive control produced clear zones (13.95 mm for E. coli and 12.43 mm for S. aureus), and the distilled water-negative control produced none. Representative assay images are provided in Figure 4, and the corresponding inhibition data are summarized in Table 3.

**Table 3.** Disk diffusion measurements of isolate SBM10 and controls on E. coli and S. aureus.

| Isolate/Control | Inhibition zone (mm) | |
|---|---|---|
| | *E. coli* | *S. aureus* |
| SBM10 | - | - |
| Amoxicillin (+) | 13,95 | 12.43 |
| Distilled water (–) | - | - |

In contrast, recent in vitro studies have reported measurable antagonism of LAB preparations against E. coli and S. aureus in plate-based assays, demonstrating that inhibitory outcomes are strain- and condition-dependent across LAB [50]. Methodologically, a lack of zone in disk or sound diffusion does not by itself exclude antimicrobial potential, because agar diffusion tests are sensitive to compound diffusivity, matrix effects, and assay setup. Contemporary evaluations emphasize complementing diffusion assays with quantitative endpoints, such as MIC, to avoid false negative interpretations [51]. Moreover, the antibacterial activity of many LAB supernatants is pH-dependent. When the supernatant is closer to neutrality, inhibitory activity often diminishes relative to acidic preparations, reflecting the contribution of organic acids to growth suppression [52]. To directly test this, future work will include MIC assays performed with pH-adjusted CFS (e.g., acidified to pH 4.5 versus neutral) alongside neutralization controls.


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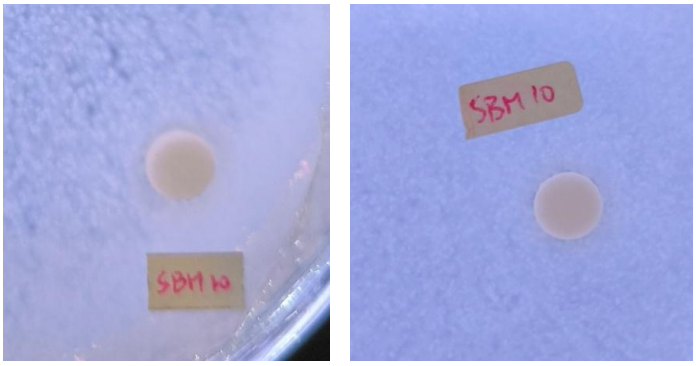


(a) (b)

**Figure 4.** Antibacterial activity of SBM10 CFS in disk diffusion. (a) isolates against *E. coli*; (b) isolates tested against *S. aureus*

At the mechanistic level, the principal antimicrobials implicated for LAB include organic acids (lowering extracellular pH and collapsing proton motive force) and bacteriocins that disrupt cell membranes and essential cellular processes [53]. Beyond bacteriocins, LAB also deploy non-bacteriocin effectors, notably hydrogen peroxide ($H_2O_2$), diacetyl, and reuterin to antagonize Gram-positive and Gram-negative bacteria via bacteriostatic and bactericidal effects [54]. $H_2O_2$ may be poorly captured in diffusion-based formats; targeted follow-up (e.g., catalase challenge or $H_2O_2$ quantification) can be performed.

## Conclusions

This study provides a preliminary assessment of a presumptive lactic acid bacterium (SBM10) isolated from fermented red rice syrup, through baseline phenotypic and safety evaluation. SBM10 showed a profile consistent with LAB, Gram-positive bacilli, catalase-negative, TSIA K/A with no $H_2S$, gelatinase-negative, Simmons citrate-negative, $CO_2$-positive in Durham, and non-hemolytic ($\gamma$-hemolysis), supporting its preliminary in vitro safety. In gastrointestinal stress models, SBM10 retained 27.14% tolerance at pH 3.0 and 35.58% tolerance in 0.3% oxgall (4 h, 37 °C), indicating partial resilience that may benefit from process or formulation optimization for functional applications. Examples include microencapsulation to enhance gastric juice resistance, co-formulation with buffering matrices, and gradual acid/bile pre-adaptation during propagation. In disk diffusion assays using cell-free supernatant, no inhibition of *E. coli* or *S. aureus* was observed under the conditions tested, underscoring the strain- and method-dependent nature of antagonism. Given the absence of detectable disk diffusion antagonism and the partial acid/bile tolerance, the present probiotic value of SBM10 lies primarily in its safety-supportive profile and the uniqueness of its plant-syrup origin, rather than in superior functional performance at this stage. We acknowledge two principal limitations of this study: reliance on four h $OD_{600}$ readouts for acid and bile tolerance without post-exposure viable counts, and the use of a disk diffusion method for antibacterial activity, both of which constrain interpretation and will be addressed in future work. Accordingly, SBM10 should be regarded as a potential candidate for further development, contingent on (i) molecular identification (16S rRNA and/or WGS) for accurate genus/species assignment and (ii) replicated functional testing with biological replicates ($n \geq 3$), mean ± SD, CFU-based survival, and MIC assays with pH-adjusted CFS. In sum, molecular identification and further replicated functional testing are prerequisites for any probiotic claims and must precede product-oriented assertions.

## Conflict of Interest

The authors declare no conflicts of interest.

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