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Fermented Rice Water Derived Probiotics: A Novel Approach to Scalp Health

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ABSTRACT

The increasing demand for natural and sustainable solutions in personal care has led to growing interest in the use of probiotics for scalp health. This study focuses on fermented rice water as a novel, bioactive-rich medium for the development of probiotic formulations aimed at improving scalp conditions. Fermented rice water, traditionally used for skin and hair care, serves as a natural reservoir of lactic acid bacteria and other beneficial microbes. These microbes produce enzymes, organic acids, and antimicrobial compounds that can help restore scalp microbiota balance, reduce dandruff and support hair follicle health. The research includes microbial profiling, probiotic potential assessment, and preliminary application testing. The findings suggest that fermented rice water-derived probiotics offer a promising, non-toxic alternative to chemical-based scalp treatments, aligning with the trend toward microbiome-friendly and eco-conscious hair care solutions.

Key words: Probiotics, Natural source of probiotics, Antifungal activity

1. INTRODUCTION

Probiotics are live microbial supplements that provide health benefits by improving the balance of the gut microbiota. Their beneficial effects have been recognized since the early 20th century when it was suggested that consuming fermented milk could counteract the adverse effects of gut bacteria. Probiotics primarily consist of lactic acid bacteria such as Lactobacillus acidophilus, Bifidobacterium bifidum, and Enterococcus faecium, which play a key role in maintaining intestinal health. Probiotics work through several mechanisms, including producing antimicrobial substances, competing for adhesion sites on the gut lining, competing for nutrients, and stimulating the immune system. One well-established benefit of probiotics is their ability to alleviate conditions such as antibiotic-associated diarrhea and pseudomembranous colitis caused by Clostridium difficile. They also improve lactose digestion, with studies showing that lactase-deficient individuals tolerate yogurt better than milk due to the presence of probiotic bacteria. However, probiotics require continuous ingestion to maintain their beneficial effects, as they do not permanently colonize the gut **(Fuller, R., 1991).** Probiotics play a beneficial role in scalp health by promoting hair growth and controlling dandruff. They achieve this by modulating the immune system and gut-hair axis, influencing pathways such as Wnt/β-catenin, IGF-1, and VEGF. Meta-analysis

of clinical and preclinical studies showed that probiotics significantly increased hair follicle count, skin thickness, and VEGF levels. For dandruff control, probiotics showed a non-significant effect on adherent dandruff but significantly reduced free dandruff. Additionally, probiotics improve scalp microbiota by inhibiting dandruff-causing fungi like Malassezia and modulating inflammatory responses. While promising, more research is needed to establish standardized probiotic formulations and their mechanisms for hair and scalp health **(Yin, Chang-Shik *et al*., 2024).** Probiotics play a crucial role in maintaining scalp health by balancing the microbiome, reducing inflammation, and supporting hair growth. The scalp microbiota, similar to skin microbiota but with unique characteristics, is influenced by environmental factors like moisture, pH, and sebum content. Disruptions in this balance can lead to conditions such as dandruff and hair loss. Androgenetic alopecia (AGA) and dandruff are associated with microbial dysbiosis, increased inflammation, and oxidative stress. Research has shown that probiotic-derived postbiotics, such as bioactive peptides and short-chain fatty acids, can directly interact with scalp cells, enhancing hair follicle health and preventing hair thinning and greying. Postbiotics, derived from Lactobacillus paracasei, have demonstrated efficacy in reducing inflammatory markers (IL-6, IL-8, MCP-1) and oxidative stress while inhibiting Staphylococcus aureus biofilm formation. These properties help in maintaining a healthy scalp environment, promoting hair growth, and preventing premature hair loss and aging **(Bifulco, Guglielmo *et al.*, 2022).**

Fermented rice water as probiotic source

Fermented rice water is a traditional byproduct of rice fermentation that has gained attention for its potential benefits for scalp and hair health. It contains bioactive compounds like organic acids, phenolic compounds, vitamins, and peptides, along with beneficial microbial strains that contribute to its antibacterial and antifungal properties. Research has shown that fermented rice water can be effective against scalp fungal infections caused by Malassezia and Trichophyton rubrum, which are responsible for dandruff and seborrheic dermatitis. The study also explores its incorporation into a shampoo formulation, offering a natural and sustainable alternative to conventional scalp treatments while promoting overall scalp health **(Ayyadurai, Gowri, *et al*., 2022).** Fermented rice water is a natural probiotic rich in beneficial microorganisms like lactic acid bacteria, which improve nutrient bioavailability and gut health by breaking down complex carbohydrates and proteins. Fermentation increases resistant starch, dietary fibre, protein content, and essential nutrients, making it a cost-effective, nutritious, health-promoting beverage **(Rajendran *et al*., 2024).** The study highlights the potential of probiotics as a safe and effective alternative to traditional antifungal drugs for managing fungal infections. Probiotics, like Lactobacillus and Bifidobacterium, demonstrate significant antifungal activity by inhibiting fungal biofilm formation, preventing mycelial growth, and blocking fungal adhesion to host cells. Various hypotheses have been suggested to explain their antifungal properties. The ability of probiotics to suppress pathogenic fungi is likely influenced by the interactions between the pathogens and the probiotics. When probiotics are cultured alongside a pathogen, they may compete for receptor and binding sites, nutrients, and growth factors. The study advocates further exploration into the molecular mechanisms underlying probiotic antifungal activity and suggests incorporating probiotics with traditional antifungal therapies to address resistance limitations **(Wu *et al*., 2022).**

1 MATERIALS AND METHODS

1.1 Sample collection

White rice samples were purchased from the local market in Chennai.

1.2 Preparation of fermented rice water

For the fermentation process, each 5 g portion of rice was cooked in water at a ratio of three times its volume. The rice was cooked until fully softened, ensuring thorough hydration. Following cooking, the rice samples were then soaked in water at a ratio of three times their volume and left to ferment naturally for 12 h at room temperature (approximately 28–30•C). The fermentation relied on naturally occurring microorganisms present in the environment, eliminating the need for inoculation with specific strains.

1.3 Culture medium

MRS (De Man, Rogosa, and Sharpe) agar is a selective medium used for the isolation and growth of lactic acid bacteria, particularly Lactobacillus species. MRS medium is used to support the growth of different probiotic strains present in fermented rice water. After inoculation, the plates are incubated anaerobically at 37°C for 24–48 hours to allow optimal growth of lactic acid bacteria.

1.4 VIABILITY OF PROBIOTIC STRAINS IN FERMENTED RICE WATER USING PREBIOTICS (INULIN)

The rice extract was dispensed into containers and added with different concentrations (1%, 2%, and 3% w/v) of inulin.The fermentations were performed in Duran screw-capped glass bottles at 37 C for 24 h.

The viable cell count, lactic acid content, reducing sugar content, and free amino nitrogen contents of the fermented rice extracts were determined.

1.4.1 Viable Cell Count

Viable cell counts were determined using the standard plate count method with MRS medium at 37 C for 24 hrs. Briefly, 1 mL of each sample was vortexed aseptically with 9 mL of sterile physiological saline (0.85% NaCl) to make a series of dilutions. The plates were incubated at 37 C for 24 hours. The viable cell counts were expressed as $\log^{10}$ values per milliliter.

1.4.2 Titratable Acidity Analysis

Titratable acidity expressed as the percentage of lactic acid was determined using the titration method. The sample (2 mL) was mixed with distilled water (18 mL) and titrated with 0.1 N NaOH. Phenolphthalein (1 mL) was used as an indicator. Each milliliter of 1 N NaOH is equivalent to 90.08 mg of lactic acid.

1.4.3 Reducing Sugar Analysis

Reducing sugars were determined using the 3, 5-dinitrosalicylic acid, with glucose as the standard.

1.4.4 Free Amino Nitrogen Analysis

The concentration of free amino nitrogen (FAN) was estimated using ninhydrin with glycine solution as the control.

1.5 PHYTOCHEMICAL ANALYSIS OF FERMENTED RICE WATER

Qualitative phytochemical studies were conducted to identify the presence of bioactive compounds such as Alkaloids, flavonoids, glycosides, steroids, phenols and proteins.

1.5.1 Test for Alkaloids (Wagner's reagents)

To 1ml of plant extract, 1.5 ml of 1% hydrochloric acid (HCI) was added. 2 drops of Wagner's reagent were added. The presence of alkaloids showed the formation of an orange precipitate.

1.5.2 Test for Flavonoid

To 1ml of plant extract, A few drops of ferric chloride hexahydrate ($FeCl3.6H_2O$) solution was added. The formation of an intense green colour indicates the presence of flavonoid.

1.5.3 Test for Phenols

To 1 ml of plant extract, a few drops of 5% FeCl3-6H2O solution were added. A deep blue-black colour indicated the presence of tannins.

1.5.4 Test for Cardiac glycosides

To 1 ml of plant extract, 3ml of glacial acetic acid (CH3COOH) was added. 1 drop of 5% FeCl36H2O. 0.5 ml of concentrated sulphuric acid (H2SO4) was added carefully along the sides of the test tube. The blue colour indicates the presence of Cardiac glycosides.

1.5.5 Test for Steroids

To 1ml of plant extract, 5ml of chloroform (CHCl3) and 2ml acetic anhydride ((CH3CO)2O) were added. then 2ml of concentrated H2SO4 was added. The red dish-brown coloration at the interface shows the presence of steroids.

1.5.6 Test for Proteins (Biuret test)

To 1 mL of plant extract, 1 mL of 1% sodium hydroxide (NaOH) was added, followed by 2 drops of 1% copper sulphate ($CuSO_4$) solution. The presence of proteins was indicated by the formation of a violet or purple color.

1.5.7 Total Phenolic Content- Quantitative Assay

The Folin-ciocalteu spectrophotometric method was used to determine the total phenolic content in the fermented rice water sample.

1.6 ISOLATION OF TEST ORGANISM

1.6.1Culture Media

Sabouraud dextrose agar

Sabouraud Dextrose Agar (SDA) is a selective fungal growth medium commonly used for the isolation and identification of dermatophytic fungi, including Trichophyton rubrum. For the isolation of T. rubrum, SDA is supplemented with the antibiotic chloramphenicol.

1.7ANTIFUNGAL ACTIVITY BY AGAR WELL DIFFUSION METHOD

1.7.1 Inoculum preparation

Stock inoculums of T. rubrum was prepared from 10-day cultures in SDA at 28 °C to induce sporulation. Fungal colonies were covered with 5 ml of sterile saline solution (NaCl 0.85 % w/v), the surface gently scraped with a sterile loop, and the resultant mixture of fungal units was then transferred to a sterile tube. The turbidity of the final inoculum was standardized according to a McFarland scale 0.5 tube and adjusted to a fungal population of 10^6 colony former units (CFU). Inoculum quantification was confirmed by plating 0.01 ml of inoculum suspension in Sabouraud dextrose agar (SDA). The dishes were incubated at 28 °C and examined daily for the presence of fungal colonies which were counted as soon as growth became visible.

1.7.2 Agar-well diffusion method

The assay was conducted by the agar well diffusion method. About 15 to 20 ml of potato dextrose agar medium was poured in the sterilized petri dishes and allowed to solidify. A fungal lawn was prepared using 5-day-old culture strains. The fungal strains were suspended in an SDA broth and adjusted to a turbidity of 0.5 Mac Farland standards (10^6 CFU/ml). 1 ml of fungal strain was spread over the medium using a sterilized swab. Using a flamed sterile borer, wells of 4 mm diameter were punctured in the culture medium and required concentrations of serially diluted extract in each well. The plates thus prepared were left for diffusion of extracts into media for one hour in the refrigerator and then incubated at 30ºC. After incubation for 48h, the plates were observed for the zone of inhibition. The diameter zone of inhibition was measured and expressed in millimeters.

1.8ANTI-INFLAMMATORY ACTIVITY

The in vitro anti-inflammatory activity of fermented rice water was evaluated using the egg albumin denaturation assay. 0.2 mL of 1-2% fresh egg albumin solution was collected and mixed with 2 ml of different concentrations of fermented rice water (FRW) and 2.8 mL of phosphate-buffered saline (pH 7.4) were mixed to form a reaction mixture of a total volume of 5 ml, to assess its ability to inhibit protein denaturation. Diclofenac sodium was used as a positive control for comparison. The reaction mixture, consisting of egg albumin, phosphate-buffered saline (PBS), and the test sample, was incubated at 37°C for 15-30 minutes. After incubation, heat-induced denaturation was performed by exposing the mixture to 70°C for 10 minutes. The absorbance of the solution was then measured at 660 nm using a UV-Vis spectrophotometer. The percentage inhibition of protein denaturation was calculated.

1.9 DOCKING STUDIES

Docking study provides insights into the molecular interactions between target protein and small molecule such as drug. This helps researchers understand how these molecules bind and interact, which is crucial for drug discovery and development. It also aids in the virtual screening of compounds to identify potential drug candidates, which helps in saving time and resources by prioritizing the most promising compounds for further experimental testing. This process involves preparing the protein and ligand structure, performing docking simulations, analysing the results, and validating the predictions through experiments.

1.9.1 AlphaFold Protein Structure Database

The AlphaFold Protein Structure Database (AlphaFold DB) is a public repository that provides predicted 3D protein structures generated by AlphaFold. This database offers structural models for a vast range of proteins, including those from humans, pathogens, and model organisms. In molecular docking studies, AlphaFold DB is invaluable for obtaining predicted protein structures when experimental data is unavailable. Carboxypeptidase 2 (AF-A6XHF7-F1-v4), derived from Trichophyton rubrum, has been used for docking studies. Carboxypeptidases are enzymes involved in proteolysis, playing a role in fungal metabolism and virulence. The structure of AF-A6XHF7-F1-v4, retrieved from AlphaFold DB, provides a model for studying ligand interactions, including potential antifungal agents.

1.9.2 PUBCHEM

PubChem is a comprehensive online resource that provides valuable information on various chemical compounds. It serves as a vast library for chemistry, offering researchers, students, and enthusiasts a wealth of data. By utilizing PubChem, users can access detailed insights into specific compounds, including their chemical structures, properties, and even biological activities. This invaluable tool plays a crucial role in advancing scientific knowledge and facilitating research in the field of chemistry. The ligand chosen for this study is 12-Hydroxydodecanoic Acid, a bioactive compound derived from fermented rice water, which is being explored for its antifungal properties. Its hydroxyl and carboxyl functional groups may contribute to interactions with fungal enzymes or membranes, potentially disrupting fungal growth. Molecular docking studies can help identify its binding affinity to fungal targets, aiding in the development of antifungal treatments.

1.9.3 Molegro Molecular Viewer (MMV)

It is a robust visualization and analysis tool used in computational chemistry and molecular docking studies. It provides an intuitive interface for examining molecular interactions, protein-ligand docking results, and structural conformations. MMV enables researchers to visualize hydrogen bonding, hydrophobic interactions, electrostatic forces, and other critical molecular interactions that influence binding affinity. The software supports various molecular file formats, allowing seamless integration with docking tools like AutoDock. Additionally, its interactive 3D visualization capabilities enhance the interpretation of docking results, aiding in the refinement of drug candidates. In this study, MMV was employed to analyze the docking interactions between the target protein and ligand, facilitating a comprehensive understanding of their binding mechanisms.

1.9.4 AutoDock

AutoDock is a powerful molecular docking software widely used in computational chemistry for predicting the binding interactions between small molecules and target proteins. It employs advanced algorithms to estimate binding affinity, orientation, and molecular interactions, making it an essential tool in drug discovery and design. With its efficient docking simulations and user-friendly interface, AutoDock enables researchers to explore ligand-receptor interactions, accelerating the identification of potential therapeutic candidates. In this study, AutoDock was utilized to investigate the binding interactions between the target protein and the ligand.

2 RESULTS

2.1 PREPARATION OF FERMENTED RICE WATER

White rice samples purchased from the local market in Chennai were allowed for the fermentation process. The rice was cooked until fully softened, ensuring thorough hydration. Following cooking, the rice samples were soaked in water and left to ferment naturally for 48-72 hrs at room temperature (approximately 28–30 ◦ C). Bubbles were visible indicating the fermentation process. **(Figure 1 & 2)**

2.2 ISOLATION AND CHARACTERIZATION OF LACTOBACILLUS SPP. FROM FERMENTED RICE WATER

The isolation and characterization of Lactobacillus spp. from fermented rice water were conducted using morphological and biochemical approaches. After 24–48 hours of incubation on MRS agar the following characteristics were observed.

2.2.1 Macroscopic morphology

On MRS agar, Lactobacillus colonies typically appear as small to medium-sized (1–3 mm) circular or irregular colonies with a raised or convex elevation. They have a smooth, creamy, or mucoid texture with entire or slightly irregular margins. The colonies are usually white, off-white, or pale yellow, with an opaque to translucent appearance and a shiny or glossy surface. These morphological features help in the preliminary identification of Lactobacillus species. **(Figure 3)**

2.2.2Microscopic morphology

Under the microscope, Lactobacillus grown on MRS medium appears as gram-positive, non-spore-forming, rod-shaped bacteria arranged singly. **(Figure 4)** They are non-motile and do not form endospores. Gram staining reveals a purple coloration, confirming their gram-positive nature. These characteristics help differentiate Lactobacillus from other bacteria. Gram staining confirmed Gram-positive, rod-shaped bacteria, and the catalase test was negative. Biochemical tests revealed glucose, lactose, maltose, and sucrose fermentation with acid production **(Figure 7)**. The isolates were tested negative for oxidase and indole and Methyl Red (MR) positive, Voges-Proskauer (VP) negative. **(Figure 5) (Table 1)**

2.3VIABILITY OF PROBIOTIC STRAINS IN FERMENTED RICE WATER USING PREBIOTICS (INULIN)

2.3.1 Viable count

The viability of probiotic strains in fermented rice water supplemented with prebiotics was assessed by measuring the viable cell count. **(Figure 8)** The results showed that the addition of inulin supported probiotic survival, with a viable count of approximately **2.33 X 10^6 CFU/ml**. This indicates that inulin provided a favourable environment for probiotic growth, likely by serving as a fermentable substrate that enhanced bacterial viability. **(Figure 9) (Table 2).** The findings suggest that incorporating prebiotics such as inulin in fermented rice water can improve the stability and survival of probiotic strains, with enhanced probiotic benefits.

2.3.2 Titratable Acidity Analysis

The titratable acidity (TA) analysis of fermented rice water samples showed a progressive increase in acidity with the addition of inulin. The TA of the control sample (T0) without inulin was 0.54%, while the rice water supplemented with 1% inulin (T1), 2% inulin (T2), and 3% inulin (T3) exhibited TA values of **0.90%, 1.26%,** and **1.44%,** respectively. **(Figure 10) (Table 3)**

Titratable acidity % was calculated using the formula:

$$\text{TA (\% Lactic acid)} = \left(\frac{V \times N \times 90.08}{Sample\ volume}\right) \times 100$$

This increase in acidity correlates with higher lactic acid production, suggesting that inulin enhances the growth and metabolic activity of lactic acid bacteria (LAB) during fermentation. The results indicate that inulin acts as a prebiotic, promoting microbial fermentation and acidification of the rice water. However, while increased acidity may contribute to improved preservation and potential probiotic benefits, excessive acidification could influence the sensory acceptability of the final product.

2.3.3 Reducing Sugar Analysis

The reducing sugar content in fermented rice water was quantified using the 3,5-dinitrosalicylic acid (DNS) method, with glucose as the standard. The optical density (OD) values for the standards and test samples were recorded at 540 nm. The standard curve was generated using glucose concentrations for each standard. **(Figure 11)**

The optical density (OD) values of glucose standards ranged from **0.048 to 0.265**. Test samples of fermented rice water showed increasing OD values with inulin addition up to 2%, followed by a slight decrease at 3%. Corresponding reducing sugar concentrations, derived from the standard curve, showed a similar trend: the highest sugar content was observed in the 2% inulin sample **(1.202 mg/mL**), while the 3% inulin sample had a lower concentration **(0.941 mg/mL)** than the 1% and 2% inulin samples. The control (no inulin) had the lowest sugar content (0.717 mg/mL). **(Table 4)**

This suggests that inulin enhances sugar metabolism during fermentation, likely promoting microbial activity that contributes to sugar breakdown. However, at higher concentrations (3% inulin), the metabolic balance may shift, leading to sugar consumption or utilization by fermenting microbes.

2.3.4 Free Amino Nitrogen Analysis

The Free Amino Nitrogen (FAN) content of fermented rice water was determined using the ninhydrin method, with glycine as a standard for calibration. The standard curve, constructed using glycine as a reference, established a linear relationship between OD_{570} absorbance and FAN concentration (mg/L), validating the accuracy of the ninhydrin method for FAN estimation. **(Figure 12)** The fermented rice water **without inulin (T0) exhibited the lowest FAN concentration (2.91 mg/L)**, suggesting minimal amino acid release in the absence of a prebiotic. When **inulin was introduced at 1% and 2% (T1, T2)**, FAN levels increased slightly, peaking at **4.37 mg/L in T2 (2% inulin)**, indicating an optimal balance between protein hydrolysis and microbial activity. However, at **3% inulin (T3), FAN decreased to 3.30 mg/L**, likely due to a metabolic shift towards carbohydrate fermentation, reduced protease activity, and possible acidification inhibiting protein breakdown**. (Table 5)** These findings suggest that **2% inulin**

supplementation enhances FAN production, making it the most suitable concentration for promoting amino acid availability in fermented rice water. The standard curve and sample values were plotted to visualize the trend**.**

2.4 PHYTOCHEMICAL ANALYSIS OF FERMENTED RICE WATER

The phytochemical analysis of fermented rice water was performed. **Table 6** represents the results of the Phytochemical analysis with the extract, which shows the presence of flavonoids, phenols, and proteins.

2.4.1 Total Phenolic Content- Quantitative Assay

The **Total Phenolic Content (TPC)** of the fermented rice water sample was determined using the **Folin-Ciocalteu method**, with **gallic acid** as the standard. A standard curve was generated using known concentrations of gallic acid ranging from **0.0100 to 0.0600 mg/mL**, with corresponding absorbance values at **765 nm**. **(Figure 13)** The absorbance of the fermented rice water sample was measured as **1.75**, and using the standard curve equation, the TPC was calculated as **0.0473 mg GAE/mL**. **(Table 7)** This result suggests that fermented rice water contains a significant amount of phenolic compounds, which may contribute to its potential **antioxidant and anti-inflammatory properties**. The standard curve and sample value were plotted to visualize the trend.

2.5 ISOLATION OF TEST ORGANISM

Morphology of fungi (Figures 14 & 15)

S.NO	Identification	Macroscopic morphology	Microscopic morphology
1	*Trichophyton rubrum*	Colonies were flat to slightly raised, white to cream to downy, with either no reverse pigment or a yellow-brown to wine-red reverse.	Isolate showing slender clavate microconidia and cigar-shaped macroconidia, some with terminal appendages.

2.6 ANTIFUNGAL ACTIVITY BY AGAR WELLDIFFUSION METHOD

The antifungal activity of fermented rice water (FRW) against Trichophyton rubrum was evaluated using the agar well diffusion assay. The results showed that FRW exhibited a zone of inhibition of 16 mm at and 14 mm at a concentration of 200 ug/ml and 150 ug/ml, respectively. The positive control (Fluconazole 1

mg/mL) produced a zone of 20 mm, while the negative control (sterile water) showed no inhibition. **(Figure 16)**

2.7 ANTI-INFLAMMATORY ACTIVITY

The anti-inflammatory potential of fermented rice water was evaluated through its ability to inhibit protein denaturation, a key factor in inflammation. In this study, albumin protein was selected as the target, and its denaturation was induced using heat treatment. Various concentrations of fermented rice water extract were tested to determine their efficacy in preventing the denaturation process. The results demonstrated that the extract exhibited significant anti-inflammatory activity. The percentage inhibition of protein denaturation was calculated by comparing the extent to which the extracts prevented albumin denaturation. As presented in **Table 9**, the extracts displayed notable inhibition of protein denaturation, suggesting their effectiveness. The inhibition levels observed with fermented rice water were comparable to those of diclofenac sodium, a well-known standard anti-inflammatory drug**. (Graph 4) (Figure 17)**

The percentage inhibition of protein denaturation was calculated using the formula:

$$\text{Inhibition (\%)} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}}\right) \text{x } 100$$

These findings highlight the anti-inflammatory potential of fermented rice water and suggest its possible application as an alternative or complementary treatment to conventional anti-inflammatory medications

2.8 DOCKING STUDIES

The molecular docking study of Carboxypeptidase 2 (**Figure 18**) and 12-Hydroxydodecanoic Acid (**Figure 19 & 20**) was conducted using AutoDock 4.2.6. The docking results revealed a binding energy score of -4.99 kcal/mol (**Figure 21**), indicating a moderate interaction between the ligand and the active site of the protein. Visualization of the docked complex using Molegro Molecular Viewer confirmed that 12-Hydroxydodecanoic Acid aligns well within the catalytic pocket of Carboxypeptidase 2 (**Figure 22 & 23**). The presence of 12-Hydroxydodecanoic Acid as a key antifungal compound further supports its potential role in controlling fungal infections, making it a promising natural ingredient for scalp care formulations.

LIST OF FIGURES

Figure 1: Fermented rice water

Figure 2: Soaking rice

Figure 3: Lactobacillus spp on MRS Plate

Figure 4: Catalase negative

Figure 5: MR Positive, VP Negative with control

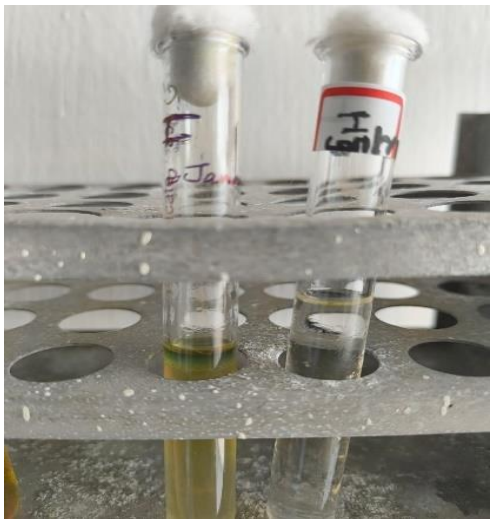


Figure 6: Indole negative with control

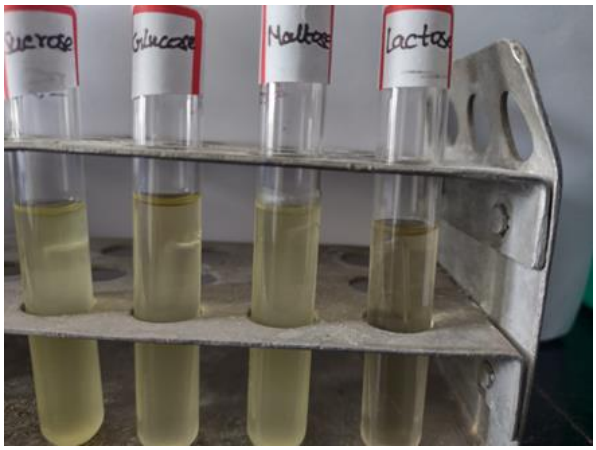


Figure 7: Sugar fermentation- Sucrose, glucose, Maltose, and lactose acid production

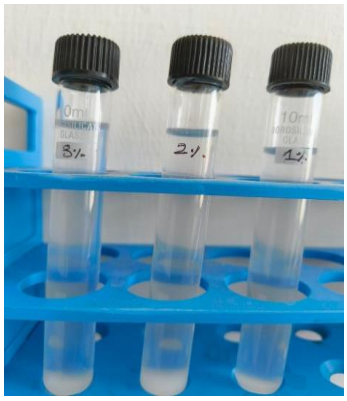


Figure 8: Inulin with different concentrations 1%, 2% and 3% in fermented rice water

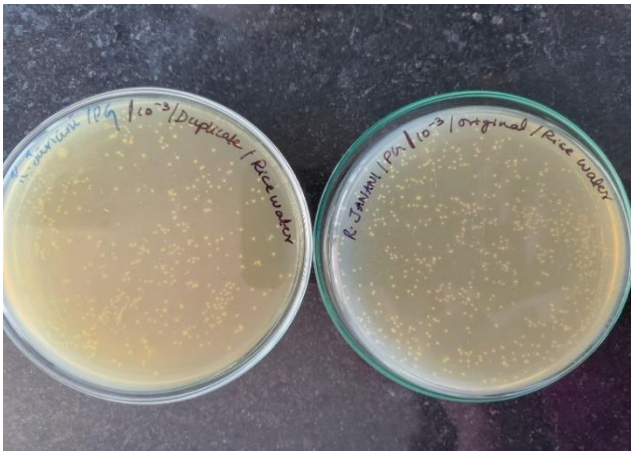


Figure 9: Viable count- Isolated colonies of Lactobacillus spp on MRS Medium

Figure 10: Titratable acidity analysis- Development of pink colour – Endpoint

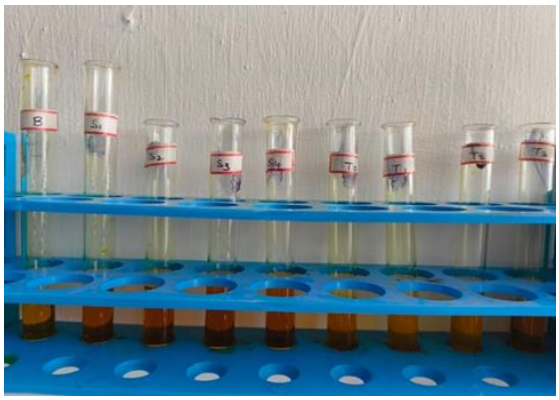


Figure 11: Reducing sugar analysis

Figure 12: Free amino nitrogen analysis

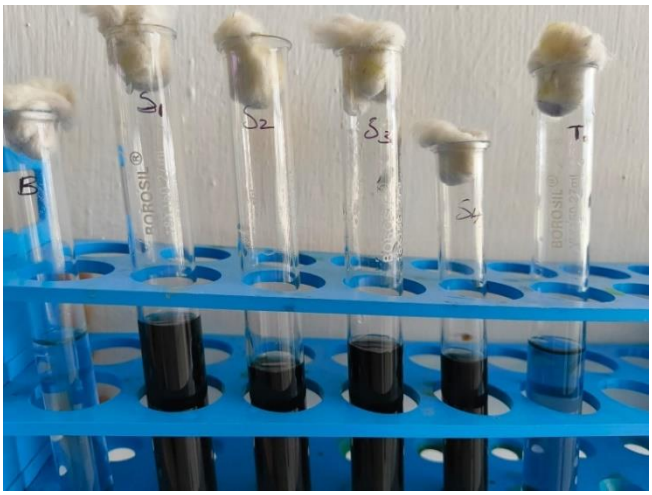


Figure 13: Total phenolic content

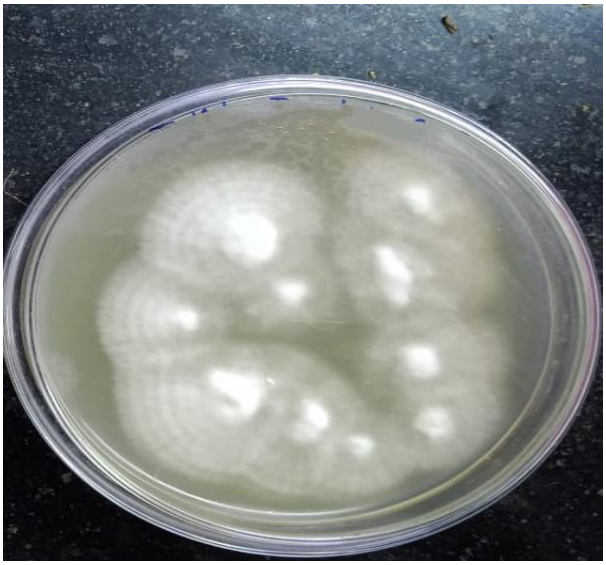


Figure 14: Macroscopic morphology

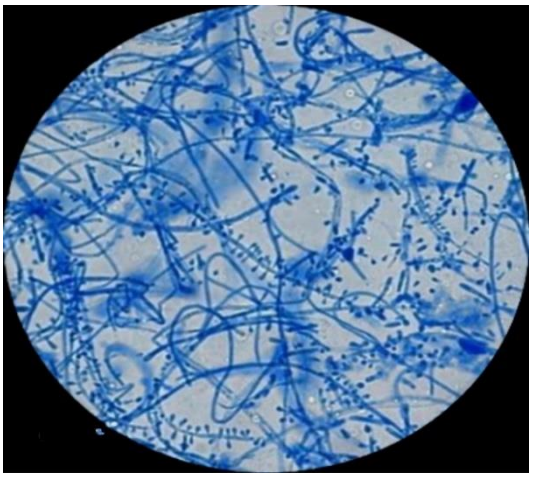


Figure 15: Microscopic morphology

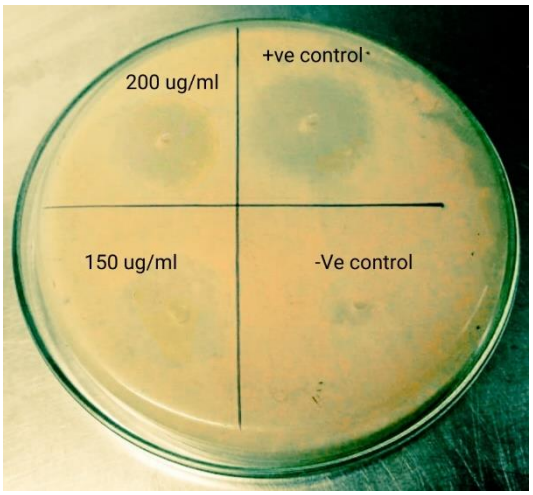


Figure 16: Antifungal activity by agar well diffusion

Figure 17: Anti-inflammatory activity

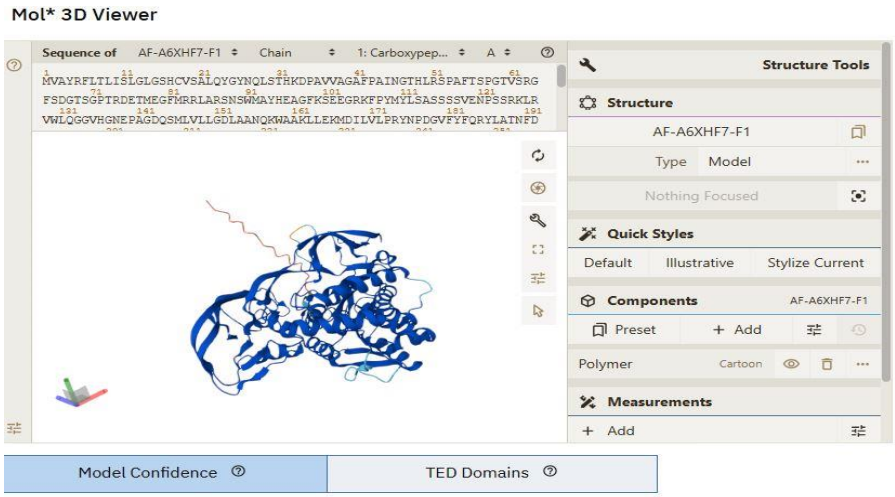


Figure 18: Protein structure retrieved from AlphaFold DB

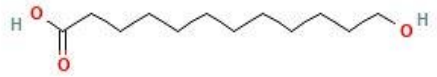


Figure 19: 2-D Structure of ligand

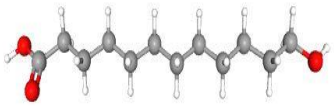


Figure 20: 3-D Structure of ligand

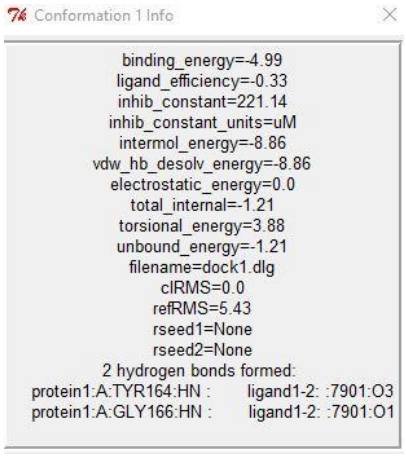


Figure 21: Binding energy (-4.99 Kcal/mol)

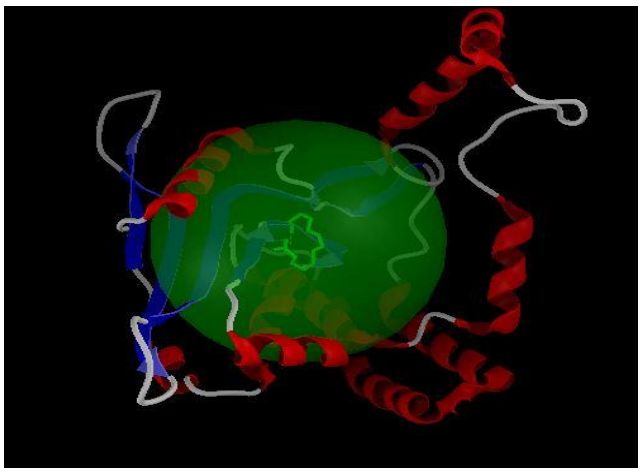


Figure 22: Final conformation

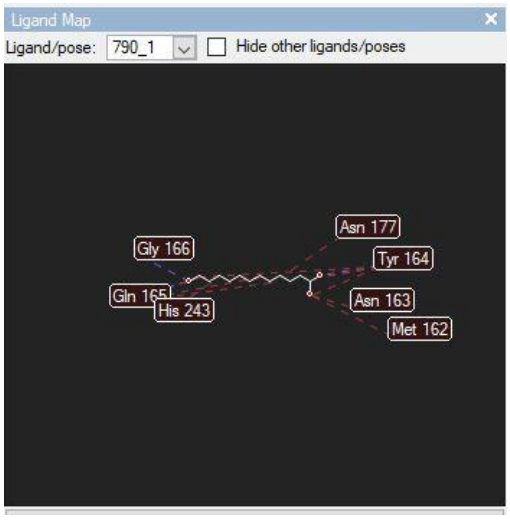


Figure 23: Ligand map

LIST OF TABLES

Table 1: Preliminary and Biochemical tests

Test	**Result**
Gram Staining	**Gram-positive rods**
Motility	**Non-motile**
Catalase Test	**Negative (-)**
Oxidase Test	**Negative (-)**
Sugar Fermentation	
Glucose	**Positive (+), Acid production**
Lactose	**Positive (+), Acid production**
Sucrose	**Positive (+), Acid production**
Maltose	**Positive (+), Acid production**
Indole Test	**Negative (-)**
Methyl Red Test	**Positive (+)**
Voges-Proskauer Test	**Negative (-)**

Table 2: Viable count

S.No	**Dilution**	**Colony count (CFU/ml)**		**Average**
1.	**10^{-1}**	**TNTC**	**TNTC**	**-**

2.	**10^{-2}**	**TNTC**	**TNTC**	**-**
3.	**10^{-3}**	**230**	**235**	**232.5**

Table 3: Titratable acidity analysis

Sample	NaOH Volume (mL)	Titratable Acidity (% Lactic Acid)
T0 (No Inulin)	**0.6 ml**	**0.54%**
T1 (1% Inulin)	**1.0 mL**	**0.90%**
T2 (2% Inulin)	**1.4 mL**	**1.26%**
T3 (3% Inulin)	**1.6 mL**	**1.44%**

Table 4: Reducing sugar analysis

Sample	OD at 540 nm	Glucose Concentration (mg/mL)
Blank	**0.000**	**-**
S1 (Standard)	**0.045**	**0.200**
S2 (Standard)	**0.054**	**0.400**
S3 (Standard)	**0.066**	**0.600**
S4 (Standard)	**0.082**	**0.800**
Test 0 (No Inulin)	**0.080**	**0.797**
Test 1 (1% Inulin)	**0.105**	**1.203**
Test 2 (2% Inulin)	**0.119**	**1.431**
Test 3 (3% Inulin)	**0.098**	**1.089**

Table 5: Free nitrogen amino analysis

Sample	OD at 570 nm	FAN Concentration (mg/L)
Blank	**0.000**	**-**
S1 (Standard)	**0.500**	**50**
S2 (Standard)	**1.020**	**100**
S3 (Standard)	**1.550**	**150**

S4 (Standard)	**2.040**	**200**
Test 0 (0% Inulin)	**0.020**	**2.91**
Test 1 (1% Inulin)	**0.028**	**3.69**
Test 2 (2% Inulin)	**0.035**	**4.37**
Test 3 (3% Inulin)	**0.024**	**3.30**

Table 6: Phytochemical analysis of fermented rice water

S. No	**PHYTOCOMPOUNDS**	**SAMPLE**
1	**Alkaloids**	-
2	**Flavonoids**	+
3	**Glycosides**	-
4	**Steroids**	-
5	**Phenols**	+
6	**Proteins**	+

Table 7: Total phenolic content

S. No	**OD Value at 765 nm**	**Concentration of Gallic acid (mg/mL)**
Blank	**0.00**	**0.0000**
S1 (Standard)	**0.50**	**0.0100**
S2 (Standard)	**0.86**	**0.0200**
S3 (Standard)	**1.54**	**0.0400**
S4 (Standard)	**2.15**	**0.0600**
Sample	**1.75**	**0.0473**

Table 8: Antifungal activity assay

Sample	**Concentration**	**Zone of Inhibition (mm)**
Fermented Rice Water	**200 ug/ml**	**16 mm**
Fermented Rice Water	**150 ug/ml**	**14 mm**

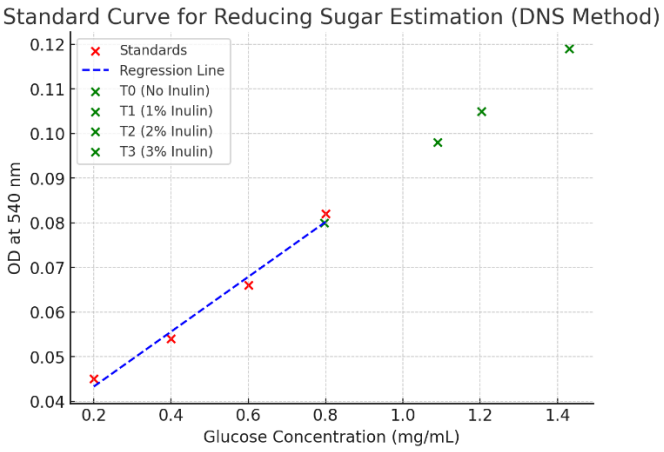
Fluconazole (Positive Control)	**1 mg/mL**	**20 mm**
Sterile Distilled Water (Negative Control)	**-**	**0 mm**

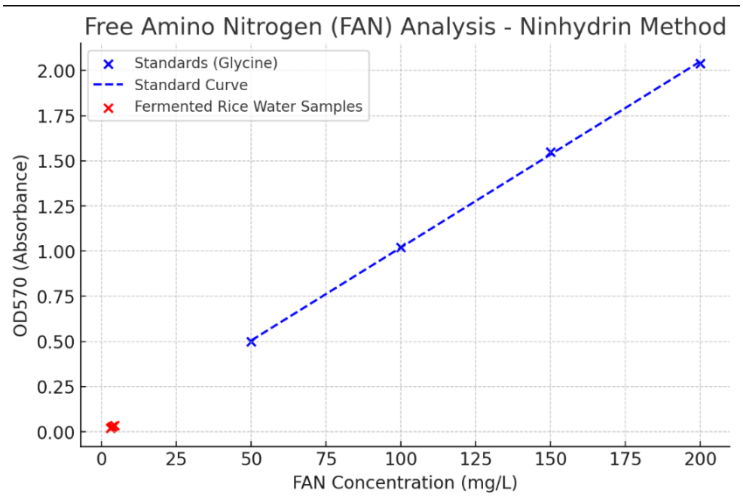
Table 9: Anti-inflammatory activity

Sample	**Mean Absorbance (660 nm)**	**Inhibition (%)**
Control	**0.806**	**0.00%**
Diclofenac (Standard Drug)	**0.298**	**63.02%**
FRW (100 µL)	**0.544**	**32.50%**
FRW (250 µL)	**0.435**	**46.02%**
FRW (1000 µL)	**0.337**	**58.19%**

LIST OF GRAPHS:

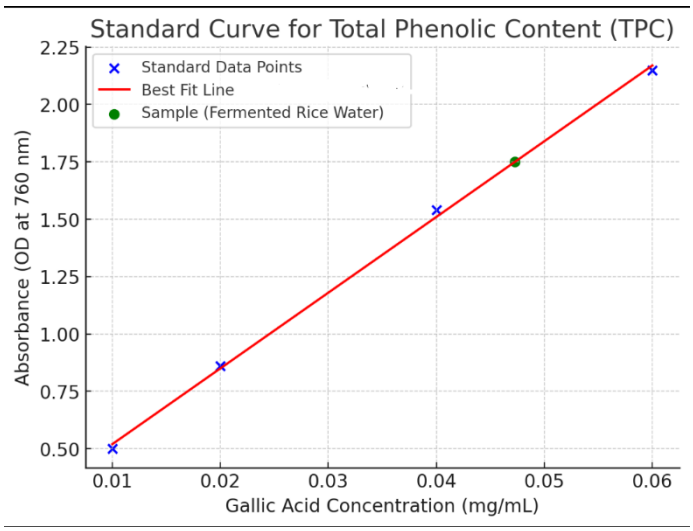
Graph 1: Standard curve for Reducing sugars

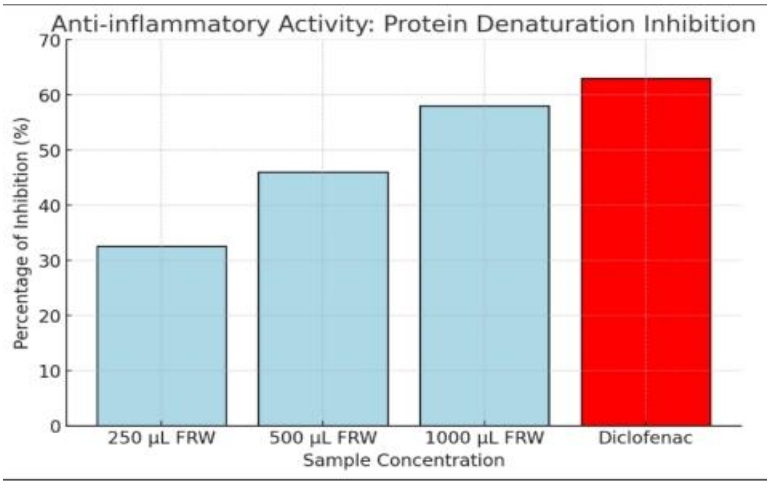




Graph 2: Standard curve for Free Amino Nitrogen analysis

Graph 3: Standard curve for Total Phenolic content





Graph 4: Anti-inflammatory activity assay

3 DISCUSSION

The preparation of fermented rice water in this study involved cooking, soaking, and natural fermentation, influencing its microbial composition, biochemical properties, and potential probiotic benefits. The method used in this study aligns with traditional fermentation practices. **Balakrishnaraja *et al*., (2023)** reported that fermented rice water can be prepared using either boiling or soaking methods, both of which enhance its nutrient composition and bioactivity. In their study, whole grains of Oryza sativa were boiled in deionized water (1 L) for 30 minutes, followed by cooling to 30°C and filtration through cotton gauze. This process facilitated the extraction of essential nutrients, including starches, amino acids, vitamins, and antioxidants, making *the* rice water nutritionally rich. Fermentation occurs when the rice water is left at room temperature for 24–48 hours, allowing the proliferation of naturally occurring lactic acid bacteria and yeast. This fermentation process significantly enhances the bioactive compound content, leading to improved antioxidant, antimicrobial, and hair-care properties. Additionally, pH analysis of the fermented rice water revealed a mild acidity (pH ~6.5), indicating moderate fermentation, which contributes to its beneficial effects.

In this study, Lactobacillus species were successfully isolated from fermented rice water, confirming their identity through morphological and biochemical tests. The isolates were Gram-positive, rod-shaped, and non-motile, with acid production observed during glucose, lactose, and sucrose fermentation. These findings align with previous research that identifies Lactobacillus spp. as beneficial microorganisms capable of producing antimicrobial compounds. Similar studies have reported the presence of Lactobacillus plantarum and Lactobacillus rhamnosus in fermented foods, demonstrating their antifungal properties through lactic acid and bacteriocin production **(Abdel-Nasser et al., 2023).** The current study reinforces this by highlighting the inhibitory effects of Lactobacillus isolates against T. rubrum.

In this study different inulin concentrations (1%, 2%, and 3%) were used to assess their effect on probiotic viability. The results indicated that 3% inulin provided optimal growth conditions, with a viable cell count of approximately 2.33×10^6 CFU/mL. Inulin acted as a fermentable prebiotic, enhancing bacterial metabolism and leading to increased titratable acidity and sugar utilization. The highest titratable acidity (1.44% lactic acid) was observed at 3% inulin concentration, which may have improved the antimicrobial properties of the fermented rice water. **Su, Henriksson, and Mitchell (2007**) investigated the effects of prebiotics—specifically soybean oligosaccharide (SOS), fructooligosaccharide (FOS), and inulin—on the survival and retention of probiotic strains (*Lactobacillus acidophilus* LAFTI L10, Bifidobacterium lactis LAFTI B94, and Lactobacillus casei LAFTI L26) in mice. The findings demonstrated that these prebiotics significantly enhanced the survival and prolonged the retention period of the probiotic strains in vivo. **Savedboworn, Wanticha, *et al*., 2017** investigated that fermentation in rice extract resulted in a peak viable cell count of 8.42 log CFU/mL after 24 hours, with 2% inulin supplementation yielding the highest growth rate (0.157/hr). Inulin significantly enhanced Lactobacillus viability, with 1–3% inulin supplementation increasing CFU counts, particularly at 2%, which resulted in the highest viable cell numbers (8.90 log CFU/mL). Inulin also influenced fermentation by increasing lactic acid production and lowering pH (3.69–3.83) compared to the control (pH 4.04). Additionally, inulin promoted greater sugar consumption and FAN reduction, indicating enhanced probiotic metabolism. Storage stability tests showed that 2% inulin provided the best probiotic survival, with 30.16% viability at 4°C after 52 days and 7.84% at 30°C after 31 days, suggesting its protective role in maintaining probiotic integrity. These results

confirm that inulin enhances the growth, metabolism, and storage stability of L. plantarum in fermented rice extract, highlighting its potential as a functional prebiotic in probiotic-rich foods.

The antifungal potential of fermented rice water was evaluated against Trichophyton spp., a common fungal pathogen responsible for scalp infections such as tinea capitis. The study demonstrated that fermented rice water exhibited a clear zone of inhibition in the agar well diffusion assay. The results highlight the potential use of fermented rice water as a natural alternative for controlling fungal infections of the scalp. Since scalp fungi contribute to dandruff, itching, and hair loss, incorporating fermented rice water into scalp sprays, shampoos, or hair treatments may offer a sustainable and chemical-free solution for maintaining scalp health.

Danial, A. M., Medina, A., & Magan, N. (2021) investigated the antifungal properties of *Lactobacillus plantarum* strain HT-W104-B1, isolated from Malaysian fermented foods, against *Trichophyton rubrum*, a dermatophytic fungus responsible for skin infections. A total of 66 lactic acid bacteria (LAB) strains were screened, and four (L. plantarum HT-W104-B1, L. plantarum MCC 2156, Pediococcus acidilactici 1498, and Pediococcus pentosaceus 1426) demonstrated significant antifungal activity. Dual-culture assays showed that these LAB strains inhibited T. rubrum growth but had no effect on T. interdigitale, indicating strain-specific antifungal action. Further analysis revealed that the cell-free supernatant of L. plantarum HT-W104-B1 was primarily responsible for fungal inhibition, with minimum inhibitory concentration (MIC), IC50, and minimum fungicidal concentration (MFC) values of 20 mg/mL, 14 mg/mL, and 30 mg/mL, respectively. The study identified six bioactive compounds in the LAB supernatant, with L-lactic acid (19.1 mg/g CDW) and acetic acid (2.2 mg/g CDW) being the dominant antifungal metabolites. A comparative study on keratin agar media demonstrated that the natural mixture of lactic and acetic acids was significantly more effective in controlling T. rubrum than the individual compounds alone. The LAB supernatant also inhibited fungal biofilm formation, a crucial factor in T. rubrum's resistance to conventional antifungal treatments. The findings suggest that *L. plantarum* HT-W104-B1 produces organic acids and antifungal metabolites that effectively inhibit *T. rubrum*, offering a natural, probiotic-based alternative for treating dermatophytic infections**.**

(Kwon, Hyuk-Ju, *et al.*, 2022) The study evaluated the anti-inflammatory effects of bioconversioned rice extract (RE) on Caco-2 cells, which are human intestinal epithelial cells. To investigate its anti-inflammatory potential, bioconversioned RE was tested for its ability to inhibit S. aureus-induced IL-8 expression, a key marker of inflammation. The results demonstrated that while untreated RE had a moderate inhibitory effect, LP-RE and LR-RE significantly reduced IL-8 expression, indicating stronger anti-inflammatory activity.

In this study, the anti-inflammatory property of fermented rice water was tested by performing an albumin denaturation assay. The extracts were subjected to inhibit albumin denaturation, and the percentage of inhibition was calculated using the formula employed in the study of **HDT, Madhuranga (2023)**. Diclofenac sodium was used as a reference drug. The results obtained were comparable to that of diclofenac sodium. Thus, fermented rice water can potentially be used as an anti-inflammatory agent

(Zaugg, Christophe, *et al.*, 2008) As key virulence factors in Trichophyton rubrum, Carboxypeptidases play a crucial role in degrading keratinized tissues and facilitating nutrient assimilation, making them promising targets for antifungal studies. Since these enzymes contribute to fungal survival by breaking down large peptides into assimilable amino acids, inhibiting their activity could disrupt fungal growth and

infection persistence. In this study, Molecular docking studies against carboxypeptidases have helped evaluate their inhibitory potential. Identifying strong interactions between fermented rice water metabolites and carboxypeptidases has provided insights into novel antifungal mechanisms, particularly for scalp health applications. Bioactive compounds may reduce fungal colonization on the scalp by targeting these enzymes, alleviating conditions such as dandruff and scalp infections. This research could lead to the development of natural, probiotic-based scalp treatments, enhancing both antifungal efficacy and scalp microbiome balance.

4 SUMMARY

The study explores the antifungal potential of fermented rice water (FRW) against Trichophyton rubrum, a scalp pathogen. FRW, prepared by fermenting white rice for 48–72 hours, promotes the growth of beneficial Lactobacillus spp., as confirmed through biochemical tests. The viable cell count, lactic acid content, reducing sugar content, and free amino nitrogen content of the fermented rice extracts were determined, showing enhanced probiotic viability. Probiotic viability improved with 2% inulin, enhancing metabolic activity. Phytochemical analysis revealed flavonoids, phenols, and proteins, contributing to antioxidant and antimicrobial properties. Antifungal activity, assessed via the agar well diffusion method, showed FRW inhibiting T. rubrum, comparable to standard drugs. Increased lactic acid production created an acidic environment unfavourable for fungal growth. FRW also exhibited anti-inflammatory effects, inhibiting protein denaturation similarly to diclofenac sodium. Molecular docking of 12-Hydroxydodecanoic Acid with carboxypeptidase 2 (binding energy -4.99 kcal/mol) supported its antifungal potential. The study highlights FRW as a natural alternative to chemical antifungals, minimizing resistance and side effects.

5 CONCLUSION

This study highlights the potential of fermented rice water (FRW) as a natural antifungal agent against Trichophyton rubrum, a major scalp pathogen. The antifungal activity of FRW is attributed to its rich composition of organic acids, probiotics, and bioactive metabolites, which synergistically inhibit fungal growth. The progressive decrease in pH and increase in lactic acid content during fermentation contributed significantly to its inhibitory effects. Additionally, inulin supplementation (2%) enhanced probiotic viability and activity, reinforcing its role in scalp health. Scalp fungal infections caused by Trichophyton, Microsporum, and Malassezia species are often accompanied by inflammation. FRW's ability to inhibit protein denaturation further supports its potential as an alternative to conventional antifungal and anti-inflammatory treatments. Further research is recommended to validate its effectiveness through in vivo studies, formulation optimization, and clinical trials. Future research should focus on developing FRW-based probiotic formulations like shampoos and sprays for scalp health. Personalized treatments using scalp microbiome analysis could enhance effectiveness against fungal infections.

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APPENDIX

MEDIA:

MRS MEDIUM

COMPOSITION	GRAMS/LITER
Proteose peptone	10.000
HM Peptone B	10.000
Yeast extract	5.000
Dextrose (Glucose)	20.000
Tween 80 (Polysorbate 80)	1.000
Ammonium citrate	2.000
Sodium acetate	5.000
Magnesium sulphate	0.100

Manganese sulphate	0.050
Dipotassium hydrogen phosphate	2.000
Agar	12.000

Final pH (at 25°C) 6.5±0.2

SABOURAUD DEXTROSE AGAR

COMPOSITION	**GRAMS/LITER**
Dextrose (Glucose)	40.000
Mixture of Peptone and Tryptone	10.000
Agar	15.000

pH after sterilization (at 25°C) 5.6±0.2

SABOURAUD DEXTROSE BROTH

COMPOSITION	**GRAMS/LITER**
Dextrose (Glucose)	20.000
Mixture of Peptone and Tryptone	10.000

pH after sterilization (at 25°C) 5.6±0.2

REAGENTS:

0.5 Mc FARLAND STANDARD:

1% Barium chloride - 0.05 ml

1% Sulphuric acid - 9.95 ml

DNS REAGENT:

Dinitrosalicylic acid (DNS) – 10 g

300 g of sodium potassium tartrate (Rochelle salt) – 300 g

0.5 N NaOH – 800 ml

Distilled water – Made up to 1 litre

PHENOLPHTHALEIN INDICATOR SOLUTION:

Phenolphthalein powder - 0.5 g

95% Ethanol - 50 mL

Distilled water – Make up to 100 ml

NINHYDRIN REAGENT:

Ninhydrin - 1.5 g

Ethanol - 50 mL

Citrate buffer (pH 5.5–6.0) - 25 mL

Distilled water - Make up to 100 mL

FOLIN-CIOCALTEU REAGENT:

Sodium tungstate - 85 g

Sodium molybdate - 25 g

Concentrated hydrochloric acid - 70 mL

Concentrated phosphoric acid - 50 mL

Distilled water - 1500 mL

Lithium sulphate - 100 g

PHOSPHATE BUFFER SALINE:

Sodium chloride - 8.000 g

Potassium chloride - 0.200 g

Disodium hydrogen phosphate - 0.240 g

Potassium dihydrogen phosphate - 0.140 g

CITRATE BUFFER (PH 5.5–6.0):

Citric acid – 2.1 g

Sodium citrate – 2.94 g

Distilled water – 100 ml