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Isolation of Probiotic Bacteria from Rice, Molecular Identification and Preparation of Probiotic Based Rice Milk -Shelf Life and Nutritional Analysis

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ABSTRACT
Probiotic bacteria are beneficial organisms present in our small intestine, to regulate important functions in our body. Poor eating habits, consumption of chlorinated drinking water, stress and certain disease conditions, consumption of alcohol and the use of antibiotics are known to alter the composition and activities of gut flora. Probiotics might help our body's normal microbiota return to a healthy condition after being disturbed. A healthy gut microbiome can boost our overall immunity. This study focuses on the isolation and characterization of probiotic bacteria from rice (Oryzasativa) and preparation of probiotic based rice milk. The probiotic bacterium was isolated from matta rice. Characters like NaCl tolerance, bile salt tolerance test were performed. The probiotic strain was identified to be Weissellacibaria by 16srRNA sequencing. Thin layer chromatography, Bio autography, UV-Visible spectrophotometry, Fourier transform infrared spectroscopy was carried out to identify the antimicrobial compounds in the sample. Antibacterial study and Antifungal study were performed by agar well diffusion method to evaluate the bacterial inhibition efficacy. The nutrient analysis of probiotic based rice milk were estimated by protein estimation, carbohydrate test, total phenolic content, pH, moisture and ash content estimation. Results of the test were graphically represented. Microbial load of the samples were also studied during different stages of shelf life. The study shows overall improvement in quality of rice milk in its nutritional functionality.Antimicrobial efficiency of the probiotic bacteria was also indicated. The results from the study suggested that Weissellacibaria has exemplary technological properties that can be implemented in the pharmaceutical and food industry.

KEYWORDS: *Matta rice, Probiotics, rice milk, shelf life, microbial load.*

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I. INTRODUCTION

The FAO/WHO established live microorganism definitions as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" with growing interest among scientists since their discovery thirty years ago [1]. Strains within *Lactobacillus*, *Bifidobacterium* and *Weissella* genera among others benefit gastrointestinal health through their action of managing gut microbes while helping immune functions and restricting harmful organisms while sustaining intestinal barrier stability [2]. Host metabolism and vitamin synthesis and protection against pathogens remain essential functions of the human gut microbiome which also supports digestion and metabolic processes. The fine equilibrium among gut microbes breaks down frequently because antibiotic usage and inadequate eating patterns and stress elements combined with alcohol consumption and exposure to chemically treated waters [3]. The consumption of probiotic-rich foods presents an appealing method to restore microbial balance while improving human wellness during times of microbial imbalance.

The increasing numbers of lactose-intolerant individuals and milk-allergic people along with the growing vegan population led to the development of non-dairy probiotic delivery products [4]. The increased demand for plant-based probiotic beverages stems from their appeal to both health and cultural preferences among people who use soy, oats, legumes and cereals as fermentation sources. Rice stands as the most popular cereal choice in Asia and other areas because it remains hypoallergenic so researchers use it as a prime substance to create fermented products. Rice milk that includes probiotics functions as a therapeutic beverage

because it unites the nutritional attributes of rice with the beneficial effects of probiotics.South Indian communities widely consume matta rice as a parboiled red rice that contains dietary fibre along with phenolic compounds and minerals and resistant starches which support microbial fermentation [5]. The bioactive components of these rice products exhibit antioxidant and anti-inflammatory benefits which strengthen the health benefits consumers can get from rice-based beverages. Probiotic strain fermentation of rice milk improves the nutritional accessibility and tasteful properties while enhancing functional features [6]. Through fermentation with probiotics manufacturers can produce antimicrobial agents including organic acids and hydrogen peroxide and bacteriocins which prevent pathogenic microbial growth [7].

The shelf life investigation plays a vital role during commercial development procedures for probiotic products. The market acceptability and functional performance of the beverage depend directly on how well the probiotic cells survive throughout storage. The experimental analysis of microbial counts occurred throughout various storage intervals to measure both product stability and product safety conditions. Weissellacibaria-fermented rice milk maintained both its probiotic viability and its nutritional values across storage periods indicating its role as an environmentally friendly beverage with health benefits. This research demonstrates that traditional food products including matta rice contain useful probiotic microorganisms which make it possible to manufacture functional probiotic drinks from non-dairy sources. *Weissellacibaria* shows potential in the development of plant-based foods through rice milk production by demonstrating its ability to boost food nutrients while fighting against harmful microorganisms. These technological advancements help both functional food science research and consumer needs for sustainable food choices that align with culture and promote health benefits.

II. MATERIALS AND METHODS

Collection of sample and isolation of *Lactobacillus sp.*

The whole rice samples were procured from local store in Karukachal. Two varieties of rice were collected, Matta &Navara. Then, these samples were placed in paper bags and labelled with name of rice, date of collection, and other details. Collected samples were brought to laboratory for further studies.0.1g of rice powder was transferred to the MRS broth and was incubated at 37 0 C for 24-48hrs for the isolation of Probiotic strain.

Isolation of pure culture

The 48hour old culture suspension of isolated bacteria was used for the isolation of pure culture. MRS agar plate was prepared and a loopful of Lactobacillus culture was streaked on it and incubated at 37^0 C for 24 hours. The obtained pure culture was used for further study.

Biochemical Characterization of the Isolate

NaCl Tolerance Test

The isolates were tested for NaCl tolerance at different concentrations of NaCl (0%, 1%, and 5%) in MRS broth. The isolated lactic acid bacteria were inoculated into MRS broth with NaCl and incubated anaerobically at 37°C for 24hours. Growth of bacteria was observed by measuring the absorbance at 600 nm [8]. The isolates were inoculated into MRS broth without NaCl served as control.

Bile Salt Tolerance Test

The lactic acid bacterial isolates were tested for bile salt tolerance by using Bile Salt at 0.3% in MRS broth. The culture was inoculated into MRS broth with bile and incubated at 37ºC for 24 hrs. The growth was detected by absorbance at 600 nm [9]. The bacterial culture inoculated into MRS broth without bile served as control.

Confirmation of the bacteria using molecular techniques

DNA isolation by Phenol-Chloroform-Isoamyl alcohol Method

For DNA isolation, pure culture was inoculated onto autoclave sterilized Luria Bertani Broth and incubated at 37ºC for 24 hours. After incubation, it was centrifuged at 5000 rpm for 5 minutes and the pellet was collected. Along with the pellet, 500µl of saline EDTA and 20µl of lysozyme was added and mixed well and incubated for 30 minutes at 37°C. After 30 minutes, 150µl of 10% SDS was added and incubated for 15 min. 180µl of phenol, 160µl of chloroform and10µl of iso-amyl alcohol were added and incubated at 65ºC for 15-30 minutes and centrifuged. The aqueous phase was collected and the DNA was precipitated with half the volume of sodium acetate and 0.2 volume of isopropanol. The tube was mixed and centrifuged for 10 minutes at 10000 rpm. The

DNA pellet was collected and washed with 70% and 100% ethanol. The pellet was air dried and re-suspended in 40µl of 1X TE buffer [10].

PCR amplification and sequencing

The amplification of DNA was carried out in a reaction mixture with a final volume of 20µl containing 2µl of template DNA, 2µl of each 16SrRNA forward (5'- AGAGTTTGATCCTGGCTCAG-3') and16SrRNA reverse (5'ACGGCTACCTTGTTACGACTT-3'), 8µl of PCR master mix, 4µl of distilled water. A gradient PCR was followed in the experiment. The PCR reaction condition were as follows: 94ºC for 3 minutes, followed by 20 cycles of denaturation at 94ºC for 15s, annealing at 53ºC, 55 º C for 15 s at 30°C and extension at 72ºC for 2 minutes, and a final extension at 72ºC for 15 s. The PCR product was analysed using 1.5% agarose gel. Later the sample was used for the sequencing study and the obtained data was submitted to NCBI gene bank for the accession number [10].

Preparation of production media and extraction of antibacterial compound

Under aseptic condition, 100µl of culture was transferred to autoclave sterilized MRS broth and incubated .The grown culture was initially allowed for the centrifugation for 10 minutes at 12000 rpm and separated the supernatant and pellet. To this ethyl acetate was added (60%) and allowed for stirring for 1hour and later incubated in cooling condition for overnight. The aqueous layer was separated from the mixture and this was used for further study [11].

Characterization of the antibacterial compounds

Thin Layer Chromatography

For the extracted compounds, a thin layer chromatography (TLC) assay was carried out using silica gel sheets (Silica gel 60 F 254 20 x 20 cm gel thickness: 0.25mm, Merck) with a mobile phase made up of Methanol: Acetic acid: Formic acid in the ratio of 4:2:3. The compound was applied to the silica plate using a tooth pick until it becomes thickens. Then it was placed in chromatographic chamber for the development of the spot. After the development of the mobile phase, the spot was visualized using 0.2% ninhydrin in acetone and the Retention factor of the separated compound was calculated [12].

Bioautography

Bio autography of the identified compound was done on nutrient agar. To the sterilized solidified agar media 70µl of the *E coli* culture was swabbed using cotton swab and the visible spot developed on TLC sheet was placed and incubated at 37ºC for 24 hours. The zone of inhibition was noted in millimetres[13]

UV-Visible spectrophotometric analysis

UV-visible spectrophotometric analysis was studied on the sample using a UV-visible spectrophotometer (Labtronics LT-291). The sample was examined under visible and UV light in the wavelength ranging from 200-600nm for the detection of peaks [12]

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared (FTIR) was used to identify the characteristic functional groups in the sample to identify the antibacterial compound. It provides the information about the structure of a molecule could frequently be obtained from its absorption spectrum. Prior to spectrum analysis, the dried sample was prepared by KBr pellet and stabilized. The IR spectrum was obtained using Shimadzu infrared spectrometer; The FT-IR analysis was carried out within a range of 4000–400 cm^{-1} [12]

Antibacterial study by Agar well diffusion method

Nutrient Broth medium was used to subculture the bacteria and was incubated at 37 °C for 24 hrs. 70µl cultures of *E coli, S aureus, and K pneumoniae,* were taken and spread on the autoclave sterilized Mueller Hinton agar media using cotton swabs. Wells were made with cork borer and the extracted antibacterial compound were added to the respective wells along with negative (DMSO) and positive control (Chloramphenicol- C 30mcg) and incubated at 37ºC for 24 hours. In order to evaluate the antibacterial activity of the samples, the diameter of the zone was measured in millimetres [14]

Antifungal study by Agar well diffusion method

80 µl fungal spore culture of *Aspergillusflavus*and*Fusariumoxysporum* was swabbed sterilized on malt extract agar using sterilized cottion swabs. Wells were made with cork borer and samples were added to the respective wells along with 5µl of Fluconazole as a positive control (standard antibiotic-10mg/ml), and incubated for 3days at room temperature. After incubation, zone of inhibition was measured in millimeters [14]

Preparation of rice milk and fortification of Lactobacillus

10g of rice powder was taken for the preparation of rice milk and then heated at 90^0 C for 15-20 minutes followed by cooling. After the cooling process of rice powder, it was added with 5% of honey. Then 5g of rice sample was taken carefully in two containers and incubated for 2 hours in a shaking incubator. After the preparation of rice milk, the isolated probiotic pellet (1ml pellet) was added and incubated at room temperature for 10 to 15 days. The rice milk without probiotic pellet serves as control [15].

Nutrient Analysis of the fortified rice milk

Carbohydrate estimation by Anthrone Method

0.5ml of sample was mixed with 2.5ml of the anthrone reagent and allowed to stand in boiling water bath for 10 minutes. The sample was cooled to room temperature and colour developed was read at 620 nm in a UV spectrophotometer. A standard graph was drawn by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis. From the graph calculated the amount of mg/g of carbohydrate present in the sample [16]

Protein estimation by Lowry's method

Pipetted out 0.5 ml of the sample extract in a test tube and added 2.5 ml of Solution C prepared by the combination of Solution A-(2% Na_2CO_3 in 0.1 N NaOH) and Solution B (0.5 % $CuSO_4$ solution) at a ratio of 50:1 ratio. After mixing the tube was allowed to stand for 10 minutes, and added 0.2 ml of Folin- Ciocalteau reagent, mixed well and incubated at room temperature in the dark condition for 30 minutes. After incubation the OD was read at 660 nm using spectrophotometer. Concentration of protein was estimated using a standard graph of BSA and the amount of protein in the sample can be calculated in mg/ ml. [17]

Moisture and Ash content of the drug

About 3 to 5 g sample were added to the dried and pre weighed crucible and spread uniformly. This was dried and heated at 105^0 C for 3h to get the moisture content. The sample was again incubated in the same condition until constant weight to get the ash content of the sample and the percentage of the moisture and ash was calculated [18]

Total phenolic content

To the 0.2 ml of extract, 0.2ml of 10% Folin–Ciocalteau's reagent was added, mixed by gentle shaking, and kept for 5 minutes in room temperature, followed by adding 1 ml of Na_2CO_3 (7%, w/v) and the reaction mixture was made up to 3 ml with distilled water and incubated at 30^0 C for 30 min. The absorbance of the sample was recorded at765 nm using a UV-Vis spectrophotometer (Labtronics LT291, Microprocessor). The phenolic content of the extract was estimated from the standard curve of Gallic acid and the results were expressed in Gallic acid equivalent (GAE)/g of extract[19]

pH Analysis

The pH of the fermented material was measured by a glass probe digital pH meter (ELICO, India.

Microbial load

Microbial load was tested with nutrient broth.100 µl of the rice milk sample was added to the broth then this was incubated at 37^OC for 24 hours. After incubation the microbial load was tested by taking the absorbance at 600nm using spectrophotometer. Pour plate method also done for the confirmation of the bacterial contamination in the rice milk. To the sterilized petri plate 10 µl of the sample (overnight incubated broth with rice milk) was added and mixed with molten sterilized nutrient agar. After solidification, the plate was incubated and number of bacterial growth was checked and recorded as CFU/ml [20]

III. RESULT AND DISCUSSION

The market interest in functional health drinks without dairy content promotes the development of plant-based beverages which include probiotics in rice milk. These drinks provide gut health support through beneficial microorganisms and present additional therapeutic values plus nutritional improvements. Rice milk which contains the probiotic strains *Lactobacillus* undergoes improved shelf life preservation together with enhanced antimicrobial capability and nutritional benefits.

Isolation of Probiotic bacteria from rice varieties

Two traditional rice varieties Matta and Navara were sourced from a local store in Karukachal for probiotic bacterial isolation during the current study. The Matta rice sample showed the most visible turbidity when cultured in de Man, Rogosa, and Sharpe (MRS) broth at 37 °C for 24–48 hours. The significant turbidity in the solution indicated higher bacterial numbers implying viable lactic acid bacteria found in carbohydrate-rich environments such as fermented rice [6].A small amount of the cultured sample was transferred onto Bifidobacterium agar plates to acquire single bacterial isolates. Incubated colonies displayed white features with small size, convex shape, and smooth edges along with non-pigmented appearance. Scientific studies on similar cereal-based fermentation processes have demonstrated the same morphological markers as those found in *Lactobacillus spp*. [21]. Selective agar helped minimize contaminated bacteria while enabling the targeted recovery of select probiotic strains.Lactic acid bacteria proliferate inside rice substrates because the natural carbohydrates enable microbial acidity required for bacterial colonization and probiotic functionality [22]. The rice variety Matta demonstrated superior performance as a growth medium for probiotics and thus showed promise for developing probiotic beverages.Preliminary tests show that rice offers a feasible option for both providing probiotic culture and serving as their growth substrate while continuing the increasing popularity of non-dairy probiotic food products.

Biochemical Characterization of the isolate

The biochemical evaluation of the isolated probiotic strain analysed its NaCl tolerance alongside bile salt resistance because these parameters show how well the strain survives within the gastrointestinal (GI) environment and food applications. The MRS broth with 0%, 1% and 5% NaCl and 0.3% bile salt concentrations was used to evaluate the isolate's osmotic stress resistance and its ability to tolerate bile salts.The bacterial isolate achieved its most vigorous growth at the 1% NaCl condition (OD: 0.40) after which it demonstrated lower growth rates at control tube (0.20) and 5% NaCl (0.16). Optimal salt tolerance exists in this isolate which makes it suitable for application in fermented foods since they commonly contain diverse salt quantities. The maintenance of cell viability while processing and storing depends on high NaCl tolerance [23]. Microorganisms that fail to survive saline environments typically become ineffective before getting consumed.

The isolate managed well in MRS broth containing 0.3% bile salt by achieving an OD of 0.38. The present bacteria displayed active growth as demonstrated by turbidity in the provided test tubes. The 0.3% bile salt concentrations commonly found in small human intestines do not appear harmful to this strain due to its bile salt resistance [24]. Resistance to bile salts presents as an important advantage because most bacteria suffer damage from these substances when the membranes break down and enzymatic functions degrade.The isolated lactic acid bacterium demonstrates promising potential to be used as a probiotic agent within fortified rice milk because it successfully tolerates important physiological stress factors. This dual tolerance against NaCl salt as well as bile salts fulfils the necessary FAO/WHO requirements for identifying potential probiotic strains [1].

Confirmation of the bacteria using molecular techniques

Taxonomic identification of the isolated bacterium was established through molecular methods to surpass traditional morphological and biochemical techniques. Organisms liberated high-yield genomic DNA by applying the Phenol-Chloroform-Isoamyl Alcohol (PCI) method which remains the standard operational technique for bacterial culture DNA extraction. The bacterial cell destruction required lysozyme enzymatic activity to break membranes before the addition of SDS for additional cell membrane breakdown. A combination of phenol and chloroform organic solvents in conjunction with isoamyl alcohol functioned to purify nucleic acids after protein and other impurity removal. Further analysis required suspending the DNA pellet in TE buffer after washing it with 70% ethanol followed by 100% ethanol and air-drying [25].The analysis using agarose gel electrophoresis (AGE) revealed orange-colored DNA bands that appeared under the ultraviolet light after ethidium bromide staining confirmed the successful extraction of DNA. A standard PCR reaction amplified the 16S rRNA gene by using universal primers which had sequences as 5’-AGAGTTTGATCCTGGCTCAG-3’ (forward) and 5’-ACGGCTACCTTGTTACGACTT-3’ (reverse). The

PCR amplification showed successful results when the machine used a gradient method at 53–55°C for annealing temperatures and the 1.5% agarose gel analysis confirmed the correct band size.

The product from amplification proceeded to partial sequencing of 16S rRNA genes before researchers used the BLAST tool from the NCBI database for sequence analysis. Analysis of the aligned sequence showed high similarity to *Weissellacibaria* which proves to be a lactic acid bacterium with both probiotic value and beneficial functions for food fermentations [26]. *Weissellacibaria* exists within the Leuconostocaceae family where it frequently appears during isolation of fermented plant-based sources. *Weissellacibaria* possesses three significant characteristics that include exopolysaccharide production and tolerance to acidic pH and bile salts while producing bacteriocins and organic acids to inhibit pathogens [27].The results from this study show that *Weissellacibaria* was present just as previous reports determined its presence in fermented foods made with rice and cereal. The strain offers great potential for probiotic use because it survives gastrointestinal conditions effectively and provides gut health benefits. A unique accession number was obtained from the NCBI GenBank for the 16S rRNA gene sequence which will allow referencing this strain for comparison and future investigations.

Characterization of the antibacterial Compound

TLC stands as a beneficial technique for breaking down and recognizing bioactive compounds found in crude extracts. TLC analysis served as the method for examining the bacterial isolate's metabolic composition during this research. TLC analysis showed that the chromatogram displayed visible spots representing both the crude and ethyl acetate extract samples which confirmed the presence of different compounds. Separation occurred through differences in polarity because ethyl acetate extract demonstrated defined and clear bands which proved this solvent was effective in extracting bioactive metabolites [28].

The assessment of antimicrobial activity in the separated compounds continued through bioautography evaluation procedures. The TLC plates need to be directly added to agar plates containing indicator microorganisms for the bioautography method. Researchers observed clear inhibition zones developing from two parts of the agar surface that contained the chromatogram culture after it was placed on the agar. The antimicrobial active components concentrated themselves in specific regions of the TLC plate so areas showing clear zones matched these locations.Bioautographic analysis uses TLC methods to both detect and identify potential antimicrobial agents in microbial extract samples efficiently. Secondary metabolite production by the isolated strain *Weissellacibaria* results in measurable antimicrobial areas detected through the inhibitory zones on agar surfaces. Numerous studies have validated that *Weissella* species create bacteriocins and antimicrobial substances [29].

FTIR spectroscopy as a Fourier-transform technique analyzed the functional group distributions in bioactive compounds extracted from the probiotic microorganism. Various distinctive absorption peaks appeared in the FTIR spectrum. O-H stretching vibrations produce a wide peak at 3270.95 cm^{-1} thus identifying the hydroxyl groups commonly detected in phenolic and alcohol compounds [30]. The C-H stretching peak of alkanes shows its occurrence at 2932.73 cm^{-1}. A strong peak at 1634.84 cm^{-1} represents C=C or C=O stretching thus indicating alkenes or carbonyl groups are present as these compounds commonly exhibit antimicrobial properties. The overlapping 1410.46 cm^{-1} and 1236.50 cm^{-1} peaks may indicate C–H bending and C–O stretching vibrations which point to aromatic rings and ether linkages present in the substance. At 1000–1100 cm^{-1} wavelength a set of absorption peaks indicates the existence of structures which resemble carbohydrates and polysaccharides. The experimental data verifies that alkenes and carboxylic acids and alcohols exist in the probiotic indicating their potential role in antimicrobial activity as described by [31].

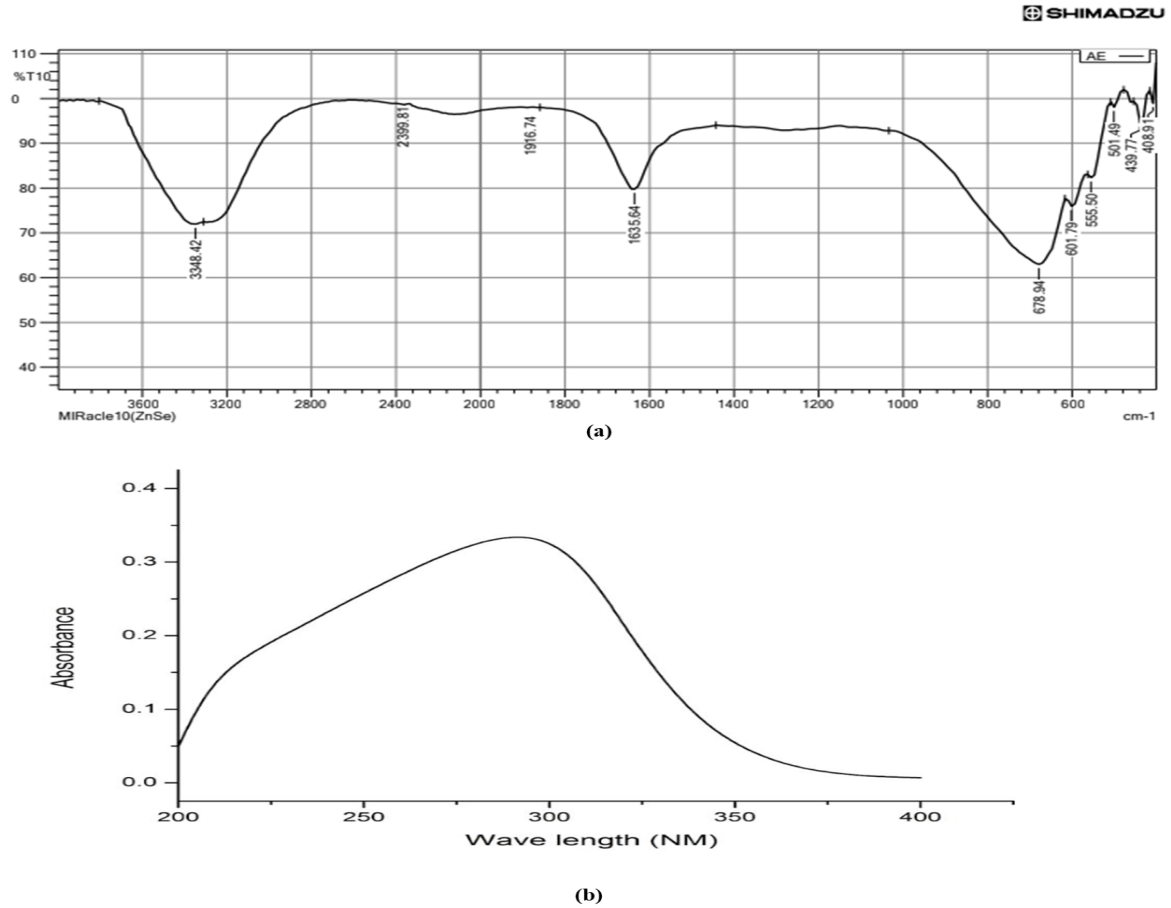

Figure 1: (a) FTIR Spectrum and (b) UV spectrum of the extracted compound

The ethyl acetate extract displayed its main absorbance peak at wavelength 295 nm which indicates the presence of conjugated π-electron systems like aromatic compounds or unsaturated ketones. The UV region absorbance indicates electronic transitions (π–π*) that are common in phenolic compounds and flavonoids which represent bioactive components [32]. UV absorbance shows an initial steep rise from 200–300 nm and then decreasing patterns in the wavelengths above 350 nm that indicates natural bioactive molecules with aromatic structures. The analysis data supports FTIR results indicating that the isolate creates secondary metabolites that demonstrate antimicrobial as well as antioxidant capabilities.

Antibacterial Activity

The antibacterial evaluations of crude and purified extracts focused on testing three major clinical pathogens which included *Klebsiellapneumoniae, Staphylococcus aureus* and *Escherichia coli* using the agar well diffusion method as an assessment procedure. The studied organisms showed different levels of sensitivity to both crude and purified samples based on the measured inhibitory effects.The crude extract demonstrated the maximum zone size (20 mm) against *Klebsiellapneumoniae* and exhibited a similar inhibition area (19 mm) compared to the purified extract. The antibiotic disc chloramphenicol produced an inhibition zone of 17 mm as a positive control but DMSO as solvent control showed no inhibition activity which demonstrates that tested bioactive compounds within the samples caused antimicrobial effects. The study demonstrates that both unprocessed and refined extract fractions harbour major bacterial inhibiting substances likely including bacteriocins together with organic acids which effectively fight Gram-negative bacterial strains [33].

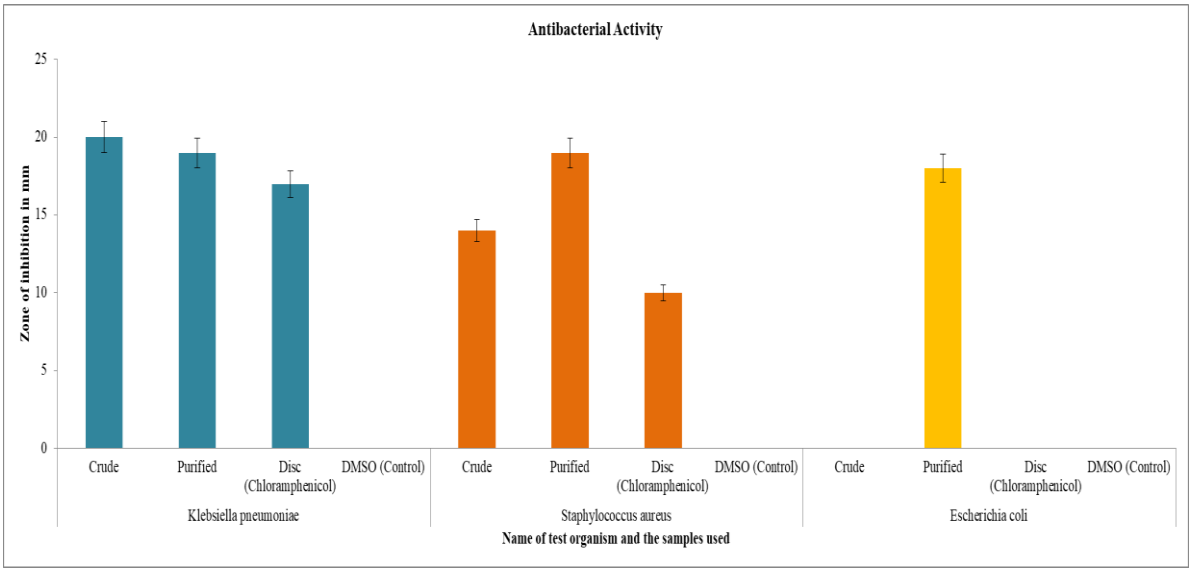

Figure 2: Graph showing the Antibacterial Activity produced by the crude and purified samples against 3 different pathogens

Both the crude extract (14 mm) and purified extract (19 mm) inhibited *Staphylococcus aureus* bacteria whereas chloramphenicol (10 mm) proved less effective. The purification process led to higher levels of active metabolites in the final product which may have occurred due to the removal of contaminants or specific fraction concentration. Multiple research studies confirm that purification techniques boost antimicrobial effectiveness of probiotic metabolites [34].The crude extract from *Escherichia coli* produced no inhibitory effects which demonstrate poor availability of inhibitory compounds or blocking substances within the extract. The purified metabolite extract exhibited an 18 mm inhibitory zone against *E. coli* cells. Bioactivity enhancement and specific targets become possible after purification because of different activity levels detected between unstable components and purified products. The tests in pure DMSO wells proved there was no interplay from the solution due to the total lack of inhibitor effects.

Antifungal Activity

The agar well diffusion method assessed the antifungal properties of crude extract together with purified extract against *Fusariumoxysporum*and*Aspergillusflavus*. The sample tests showed no signs of antifungal effects since they produced no inhibitory zones. The purified extracts showed strong antifungal behaviour yielding inhibition zones which reached 10 mm and 13 mm when tested against *F. oxysporum* and *A. flavus*.Crude extracts lacked detectable antifungal effects because they contained inactive substances or substances that affected bioactive compound detections across the well plate tests. Purification procedures probably separated these compounds from interfering substances thus improving both their antifungal effectiveness along with their concentration levels. Purified antifungal metabolites produced by lactic acid bacteria such as phenyl lactic acid and cyclic dipeptides remain undetected when present in crude extracts according to [35] yet demonstrate potent effect after their purification process.Tests with fluconazole did not display inhibitory effects against fungal species due to these fungi's resistance to antifungal drug treatment. The fungi specifically including *Aspergillusflavus* and *Fusariumoxysporum* have built-in protection against azole compounds causing them to become resistant or highly resistant to these compounds. According to [36] azole-resistant *Aspergillus* strains are appearing more frequently in clinical environments to create major difficulties for healthcare services. *Fusarium* species maintain intrinsic resistance toward antifungal drugs encompassing azoles and echinocandins along with expelling various substances through unique cell wall construction and pumping mechanisms [37].

The antifungal effect detected in the purified extract might stem from natural substances produced by probiotic bacteria *Lactobacillus gasseri*. Metabolic metabolites demonstrate their antifungal activity through disruption of cell membranes and blocking spore germination in addition to affecting essential metabolic enzymes.The active substance in the purified extract originates from bioactive compounds produced by probiotic bacteria. Multiple antifungal compounds found in the extract include organic acids (lactic acid and phenyllactic acid) along with hydrogen peroxide and proteinaceous substances such as bacteriocins as reported in [38]. These metabolites probably attack cell membranes while affecting metabolic enzymes and blocking spore germination processes.

Preparation of Rice milk and Fortification of Probiotics

A rice milk solution containing 10g rice powder and 5% honey received the isolated probiotic culture and showed beneficial results after being incubated at room conditions for 10 to 15 days. The treated sample demonstrated stability through visual examination because it displayed no signs of contamination including fungal growth or discoloration or off-odour's or gas production. The absence of turbidity indicators and sedimentation particles contributed to the effective microbiological stability of the formulation.A temperature of 90°C applied for 15–20 minutes dismantled most of the native microbial life in the rice substrate which reduced the possibility of microbial contamination in the fermentation process. Several research studies demonstrated that thermal treatment maintains hygienic standards in plant-based substrates which serve as components for producing probiotic beverages [39]. The incorporation of honey played a dual function in this mixture by improving both nutritional and sensory aspects of rice milk as well as showing antimicrobial effects from hydrogen peroxide release and acidic conditions. The bacterial growth environment inside rice milk worked well for the isolated probiotic strain since fermentation resulted in positive outcomes with no spoilage detected. The carbohydrates along with micronutrients present in rice-based substrates provide suitable conditions for probiotic survival due to their supportive properties as reported by [40]. The shaking incubation of samples for two hours prior to probiotic addition helped both to distribute nutrients evenly and to create a homogeneous mixture which benefits bacterial adaptation and colonization processes.

Nutrient Analysis of Fortified Rice milk

The carbohydrate and protein measurement of rice milk demonstrated significant variations between untreated and treated samples. The rice milk fermentation with the isolated probiotic strain produced treated samples with anthrone concentration of 140.53 mg/ml at 620 nm in the Anthrone test for carbohydrates whereas the control sample showed concentration of 96.94 mg/ml at this wavelength. The higher optical density reading at 620 nm confirms that the treated rice milk contained more carbohydrate substances. The observed carbohydrate increase supports enzymatic activity of the probiotic since it breaks down complex polysaccharides into simpler and more soluble sugars. The enzyme production capability of lactic acid bacteria is standard because they synthesize amylolytic enzymes to enhance the availability of carbohydrates [41].

The protein content measured in the treated specimen increased according to the Lowry method where it achieved protein concentration of 77.79mg/ml at 660 nm compared to 54.53mg/ml found in the control sample. The elevated protein concentration in fermented products correlates with bacterial multiplication processes and activities during fermentation together with probiotic-induced protein formation by biomass production and rice protein hydrolysis into peptides and free amino acids. Biological availability of protein increases when protein fragments break down into smaller components that improve overall nutritional value [42].The incubation period without contamination proves that fermentation succeeded while the microbial purity of the treated sample remained intact. Rice milk processed by probiotic fermentation shows an improved nutritional value through enhanced biochemical traits which include elevated protein and carbohydrate concentrations. The research confirms previous studies which demonstrate fermentation successfully enhances the nutritional content and digestibility of plant substances.

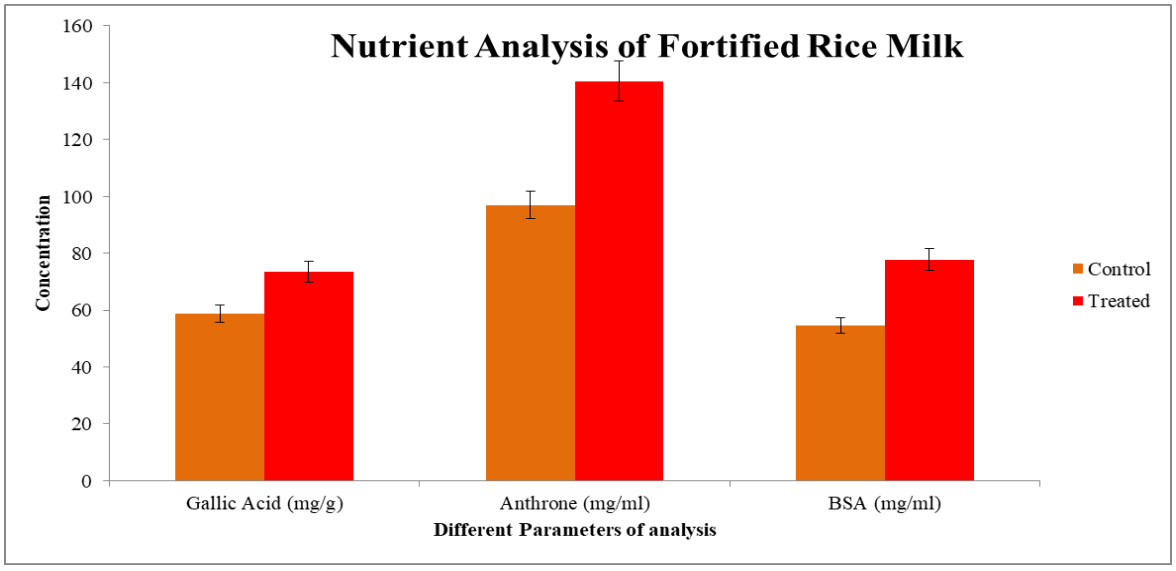

Figure 3: Graph Showing the Different nutrient analysis result of Fortified Rice Milk

The phenolic compound measurements indicate that treated rice milk showed higher levels when compared to its untreated counterpart. The total phenolic content reached 73.44 mg/g GAE in the treated rice milk whereas the control maintained 58.76 mg/g GAE. During fermentation probiotic bacterial enzymatic activity led to an increase in the phenolic compounds likely because of their enzymatic properties. The study employed *Weissellacibaria* LAB species as well as other members of its group which demonstrate β-glucosidase hydrolytic enzymes that break down phenolic compounds present in complex plant structures.The enzymes active during fermentation break down glycosidic bonds found in phenolic conjugates which frees the trapped phenolic acids and thus increases the overall total phenolic content. The process of phenolic acid release creates both antioxidant power improvements along with functional value enhancement in the product because phenolic compounds demonstrate different biological activities including anti-inflammatory, anti-carcinogenic and cardioprotective properties [43].Results show that fermented rice milk gained additional phenolic content through microbial bioactive compound modification aligning with previous research about fermentation techniques for cereal-based beverages [44]. Probiotic fermentation enhances the bioavailability of health-promoting phytochemicals in rice milk as well as maintaining their preserved state.

Proximate Analysis of the Fortified Rice milk

The proximate analysis of rice milk samples with and without probiotic treatment showed modifications in moisture, ash content and pH after fermentation. The treated rice milk obtained 3.6% moisture while the control remained at 1.9% moisture. The metabolic process of probiotic strains caused both intracellular water release and additional water retention through polysaccharide breakdown and hydrophilic molecule increase during fermentation. The research indicates that fermented plant-based matrices show enhanced water retention after microbial fermentation.Hence the total mineral content increased in the treated sample by nearly 1.6% when compared to the control sample which had 2.3% ash content. The microbial decomposition of rice material components creates conditions for mineral solubilization when minerals become available during microbial fermentation.Fermentation succeeds in improving mineral availability since it degrades anti-nutritional phytates which are common mineral binding factors [45]. The probiotic processing of rice milk demonstrates better mineral nutritional value than standard rice milk according to these findings.

The treated rice milk displayed a decreased pH value with 7.0 after fermentation while the control remained at 7.5. The decline of pH represents a typical pattern in probiotic fermentation because organic acids including lactic acid create acidification of fermentation media (Klayraung et al., 2009). The limited pH decrease indicates healthy probiotic metabolic activity to prevent spoilage microorganisms while increasing shelf stability which represents an essential quality factor for probiotic drink products.The results establish the biochemical changes resulting from probiotic intervention. The elevated moisture content and increased ash values and minimal acidification show that the rice milk contains better nutritional characteristics along with increased microbial stability which makes it a potentially beneficial plant-based food product.

Microbial Load Analysis

The study evaluated microbial loads in control and treated rice milk samples through OD measurement at 600 nm wavelength after 15, 30 and 45 days. The control rice milk bulk showed rising microbial growth from Day 15 to Day 45 according to measurements that recorded 0.087 OD initially then 0.192 and later 0.385 OD. The rising microbial growth of rice milk without treatment indicates normal microbial activity because probiotic cultures or antimicrobial agents were not applied. The microbial growth in rice milk as a carbohydrate-based food depends on treatment or preservation methods according to [47].The treated rice milk maintained lower optical density readings at each measurement date starting from 0.005 on Day 15 up to 0.018 on Day 45. The treatment process including probiotics addition with honey application successfully prevented microorganisms from growing. Research proves that honey exhibits antimicrobial effects primarily from its high osmotic power, acidic nature, and its hydrogen peroxide component. The production of organic acids by *L. gasseri* includes bacteriocins alongside lactic acid since these acids help both lower bacterial pH levels and inhibit the growth of spoilage or pathogenic bacteria. The testing results indicated that the treated rice milk retained microbial stability throughout the 45-day storage period. The preservation impact was sustained because microbial growth increased very little (0.005 units reached 0.018 units of OD). The control sample showed abnormal microbial activity because its readings at OD increased by more than four times over the observation period. The research proves that combining probiotic bacteria with honey as natural antimicrobials helps increase the storage durability of plant-based milk alternatives. A promising approach for enhancing functional food product microbial safety emerges from the combined ability of probiotic metabolites alongside honey.

V. CONCLUSION

This study established a probiotic-enriched formulation process for rice milk that incorporated a specific Lactobacillus strain. The combination of heating rice powder and supplementing it with honey followed by probiotic fermentation led to the development of a product featuring enriched nutritional and functional properties. Total carbohydrate and protein levels increased alongside phenolic content following microbial analyses of the treated sample when compared to the control, which demonstrated successful microbial metabolism throughout enrichment. The fermentation process led to elevated carbohydrate and protein concentrations because of microbial breakdown and biosynthesis activities with additional phenolic compounds indicating antioxidant enhancement.The microbial load analysis proved that probiotic protection in the treated sample achieved sustained low optical density values during 45 days of testing which validated the hygienic formulation preparation method. The treated rice milk sustained an acidic pH value which favoured the survival of probiotics. The physical characteristics of rice milk experienced modifications because the microbial fermentation process raised moisture and ash content levels.The antibacterial and antifungal activity tests performed on fermented products delivered positive results. The analytical extract showed remarkable inhibition zones which exceeded findings from standardized antibiotics for particular pathogens thus exhibiting potential therapeutic benefits of formulated probiotics.This study shows that rice milk fortified with probiotics delivers both nutritional value and microbial safety benefits that rival traditional dairy products. The nutritional product shows promise for functional food applications because it has enhanced biochemical composition together with superior antimicrobial activity. Relevant development studies must focus on product appraisal through sensory testing along with evaluations of in-vivo efficacy and prolonged product stability which will facilitate commercial viability and wider nutritional utilization.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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