

ANTIBACTERIAL EFFECT OF FERMENTED COOKED BLACK RICE WATER AGAINST GASTROENTERITIS CAUSING BACTERIA

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Science degree in Biotechnology

by

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**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Mr. Viswaganesh.V (40770057)** who carried out the project entitled "**ANTIBACTERIAL EFFECT OF FERMENTED COOKED BLACK RICE WATER AGAINST GASTROENTERITIS CAUSING BACTERIA**" under my supervision from September 2022 to March 2023

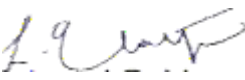

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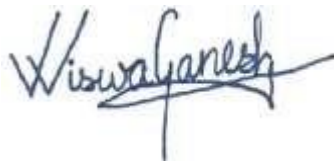
DECLARATION

I **Viswaganesh.V** hereby declare that the Project Report entitled **Antibacterial Effect of Fermented Cooked Black Rice Water Against Gastroenteritis Causing Bacteria** done by me under the guidance of **Dr. S.Gracy Jenifer** at Genoseq Diagnostics, Plot No.3, Loganathan Nagar, Near Vandalur Bypass Road, Chennai – 600048. Tamil Nadu is submitted in partial fulfilment of the requirements for the award of Bachelor of Science degree in Biotechnology.

DATE :

12/05/23

PLACE : Chennai

A handwritten signature in blue ink, appearing to read 'Viswaganesh.V', with a stylized flourish at the end.

SIGNATURE OF THE CANDIDATE

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ABSTRACT

The current project work was carried out using black rice. Black rice was cooked and soaked in water to ferment for 12 and 24 hrs in mud pot and conical flask. The bacterial species was isolated from the fermented cooked black rice water, and it was identified as *Lactobacillus spp.* using gram staining and biochemical tests. The growth of bacteria was better in mud pot when compared to the conical flask. The growth of *Lactobacillus* in 24 hrs was more compared to 12 hrs. The study of antibacterial activity using disc diffusion method was carried out against three common gastroenteritis causing bacteria viz., *Salmonella typhimurium*, *Staphylococcus aureus* and *Escherichia coli*. The study results showed that, the fermented cooked black rice water has antibacterial effect against *Salmonella typhimurium* with 10 mm zone of inhibition and *Staphylococcus aureus* with 12 mm zone of inhibition.

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1. INTRODUCTION

India, our country is an agriculture country. Agriculture plays a vital role in India's economy. More than 54.6% of the total workforce is engaged in agriculture and allied sector activities. Agriculture added 18.8% (First Advance Estimates) of country's Gross Value Added (GVA) for the year 2021-22 [7]. Economic Survey notes that 65% (2021 data) of the country's population lives in the rural areas and 47% of the population is dependent on agriculture for livelihood [22]. In the 2021-22 statistics released by Ministry of Agriculture and farmers shows that Rice is still our major source of food and India produced 127.93 million tonnes of Rice which stands first among other food grains [23].

Traditionally in India, we follow different ways to preserve and use rice to satisfy our daily food needs. One of the traditionally followed ways is to use the left-over rice of the day. The leftover cooked rice of the day is soaked in water and left it without disturbing overnight [24]. The next morning, the rice-soaked water is consumed. Our ancestors knew about fermentation and the importance. The tradition of consuming fermented cooked rice water in India has been passed down through generations [20]. This Fermented water is believed to have numerous health benefits due to its rich nutrient content [31]. This water is a probiotic drink that can help to balance the gut microbiome and promote the growth of good bacteria, can improve gut health and boost the immune system [9]. It is also a good source of vitamins and minerals, including B vitamins and iron, which are important for overall health and wellbeing [31].

The fermented cooked rice water contains many types of probiotic bacteria that are beneficial for our gut health. The lactic acid bacteria (LAB) that are commonly found in fermented foods are also found in fermented cooked rice water. They include species such as *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei* [16]. These probiotic microbes aid in the process of fermentation and produce lactic acid, which provide the drink its characteristic sour taste. The process of

fermentation also creates a wide range of beneficial compounds such as vitamins, enzymes and amino acids [31]. Probiotic bacteria help to improve digestive health, boosts the immune system and reduces inflammation.

Black rice or purple rice is a type of rice of the *Oryza sativa species*. It has a deep black colour and turns deep purple upon cooking. It gets its black colour from a type of pigment present in it called anthocyanins. The high anthocyanin content in it makes it rich in antioxidants and is believed to be anti-inflammatory [29]. A very important health benefit of black rice is its high fibre content. In addition to its high fibre content, black rice is also a good source of iron, which is essential for healthy blood and oxygen transport throughout the body. It also contains other important nutrients such as vitamin E, zinc, and magnesium. Black rice has a slightly nutty and earthy flavour and a chewy texture that sets it apart from other rice varieties. Black rice has been scientifically proven to have antioxidant activity [19], antitumor [29], anti-inflammatory [29], anti-proliferative [10], anticancer [10], anti-diabetes and hyperlipidaemia [37] and anti-atherosclerosis activity [36]. The anthocyanin derivatives cyanidin-3-O-glucoside also has beneficiary effects [21].

Bacterial gastroenteritis is a type of gastroenteritis that is caused by bacterial infections in the gastrointestinal tract. The condition is characterized by inflammation and irritation of the stomach and intestines, which can lead to symptoms such as diarrhoea, abdominal pain, cramping, nausea, and vomiting [26]. Bacterial gastroenteritis is typically caused by the ingestion of contaminated food or water that contains harmful bacteria. The most common bacteria that cause gastroenteritis include *Salmonella*, *Escherichia coli*, *Shigella*, *Campylobacter* and *Staphylococcus* [12,15].

Symptoms of bacterial gastroenteritis can vary depending on the severity of the infection and the type of bacteria involved. In some cases, symptoms may be mild and self-limited, while in other cases they may be severe and require medical attention. Severe cases of bacterial gastroenteritis can lead to dehydration,

electrolyte imbalances, and other complications [15]. Treatment for bacterial gastroenteritis typically involves antibiotics to target the specific bacteria causing the infection. In some cases, supportive care such as fluid and electrolyte replacement may also be necessary to prevent dehydration and other complications [6].

Prevention of bacterial gastroenteritis involves proper food handling and preparation, including cooking meat and poultry to appropriate temperatures, washing hands and surfaces that come into contact with raw meat, and avoiding cross-contamination of food items. It is also important to drink clean, safe water and practice good hygiene, such as washing hands regularly with soap and water [6].

This project aims to test the ability of fermented cooked black rice water to fight against common gastroenteritis causing bacteria (*Escherichia Coli*, *Staphylococcus aureus*, *Salmonella typhi*).

2. LITERATURE SURVEY

2.1. Health benefits of black rice - a review

Balasubramaniam Jaya Prasad et al., 2019, has done a review on the health benefits of black rice. He has stated “The main theme of this research article is to define the scientifically proven medicinal properties of black rice. The health properties of black rice are experimentally renowned, and gathered empirical data regarding the physiological and pharmacological activity of black rice remarkably supports the use of black rice in nutritional therapy.” Pigmented rice varieties contain more of micronutrients and macronutrients. Compared to ordinary rice, black rice contains superior protein and essential amino acid content present in it. Anthocyanin is the major pigment present in the black rice [31]. Black rice has been scientifically proven to have antioxidant activity [19], antitumor [29], anti-inflammatory [29], anti-proliferative [10], anticancer [10], anti-diabetes and hyperlipidaemia [37] and anti-atherosclerosis activity [36]. The anthocyanin derivatives cyanidin-3-O-glucoside also has beneficiary effects [21].

2.2. Study on isolation of potentially probiotic *Lactobacillus* species from fermented rice

N. Jeygowri et al., 2015 has done a study on isolation of potentially probiotic lactobacillus species from fermented rice. Probiotics are microbes that are helpful for the helpful for the host by improving the intestinal microbial balance of the host [8]. Commonly used probiotics are *Bifidobacteria*, *Lactobacilli* and *certain yeasts*. Adding probiotic to food provides many health benefits like increasing the frequency of bowel movements [4] and stimulate cell mediated immunity [30]. They have used deMan Rogosa and Sharpe agar with 0.1-0.2% sorbitol which acts as a selective media for *Lactobacillus* spp. MRS, MRS-sorbitol (0.2%) and MRS-L-cysteine (0.25%) media were used in order to improve the specificity of the medium for the isolation of different species of *Lactobacillus*. The pH of the MRS, MRS sorbitol (0.2%) and MRS L cysteine (0.25%) media were 6.2, 6 and, 5.8 respectively. They have used red raw and white rice and cooked it with water. Fermentation was carried out by soaking the cooked rice in sterile distilled water overnight in clay pots at ambient temperature (27 °C). Fermented rice steep for the fermented rice was

collected and vortexed and diluted in sterile saline solution to obtain concentrations 10^{-0} , 10^{-1} , 10^{-2} and 10^{-3} dilutions. The MRS plates were inoculated and kept in the incubator. The identification of the bacterial colony was done by observing the colony morphology and using biochemical tests. Gram staining, Catalase test, Spore staining and Motility test were performed. The predominant genus isolated was found to be *Lactobacillus spp.*

2.3. Probiotic and anticancer activity of fermented rice water

Thilagavathi P, Rekha A and Soundhari C, have tested the probiotic and anticancer activity of fermented water. The lactic acid bacteria present in fermented rice breakdown the anti-nutritional factors in the rice resulting in an improved bioavailability of micronutrients and minerals such as iron, potassium, and calcium. These activities are enhanced by the phenolic compounds that are p-hydroxybenzoic acid derivative and syringic acids and hydroxycinnamic derivatives present in fermented rice [27]. They have used raw white rice to cook with water. Overnight fermentation was done. They have isolated lactobacillus on MRS agar medium plate by quadrant streaking. The bacterial colony was identified by microscopic examination and biochemical tests such as catalase test, endospore staining and motility test.

2.4. Isolation and characterization of probiotic from fermented rice, idly and dosa batter and screening of antimicrobial activity

Ajay Kumar Sahu, Rahul Nemani, Rupali Sinha, Pratima Pradhan, have isolated probiotic microbes from Fermented rice, idly and dosa batter and screened it for antimicrobial activity. The samples were collected, and serial dilution method was done to obtain stepwise dilution of the samples. The samples were inoculated onto nutrient media plates and incubated for 24 hrs at 37 °C. The Colonies observed were identified by using biochemical tests and microscopic examination. They were tested for antimicrobial susceptibility. They have done this by using the disk diffusion susceptibility method. They have used Mueller-Hinton agar plates and commercially prepared fixed concentration paper antibiotic disk.

2.5. Isolation and characterization of bacteria from fermented cooked-rice

Mst. Kusum Kaly & Muhammad Nurul Matin & Khondokar Nasirujjaman & Md. Motiur Rahman, have isolated Bacteria from Rice samples collected from 3 different districts of Bangladesh. The rice was soaked in water and left overnight. A small amount of aqueous sample was taken for the isolation. Spreading and streaking techniques were used on LB agar (Luria Bertani agar) media to isolate bacteria with an incubation period of 16 hrs at 37 °C. A series of biochemical tests were carried out to identify the isolated bacterial colony such as MacConky agar test, Mannitol Salt agar test, EMB Agar test, TSI test, Simmons Citrate test, Urea Hydrolysis test, BSA test, Catalase test and Starch Hydrolysis test. The antibiotic sensitivity test was done using the disc diffusion method. The isolated strains were grown in LB liquid media and incubated at 37 °C for 24 hrs. 50 ul of the sample was taken using pipette and dispensed on the plates and spread using L-rod. The antibiotic discs were placed. The plates were incubated at 37 °C for 24 hrs.

2.6. Nutritional analysis and antimicrobial activity of fermented rice water

T. Kumaran et al., have done the study on fermented white rice water. Raw white rice was cooked with water for 30 minutes. The rice was soaked in water overnight. The fermented water sample was collected at 0 hrs and 24 hrs. Nutritional analysis was done using 50 ml of the sample. 2 ml was taken for isolation of microorganism isolation. They have studied various characteristics of the rice water such as physical characteristics, nutritional factors, micronutrients, macronutrients and minerals.

3. AIM AND SCOPE

3.1. AIM

To isolate and identify the bacteria present in fermented cooked black rice water.

To check the antibacterial activity of fermented cooked black rice water against common gastroenteritis causing bacteria.

3.2. SCOPE

The probiotic nature of the Fermented black rice water can be confirmed by isolating and identifying the bacteria present in the fermented black rice water.

By checking the antibacterial activity of the fermented black rice water against common gastroenteritis causing bacteria, the use of the fermented black rice water against controlling the gastroenteritis can be confirmed.

4. MATERIALS AND METHODS

4.1. MATERIALS

The project work was carried out using the below mentioned materials such as black rice, pressure cooker, RO water, sterile distilled water, conical flask, mud pot, sterile cotton swabs, cotton plugs, autoclave, petri plates, inoculation loop, test tubes, Eppendorf tubes, micropipettes. And Bacterial culture media such as Nutrient Agar powder, Mueller Hinton Agar powder, Nutrient Broth, TSI slant, Citrate Agar slant, Indole test reagent and Urease Agar slant. And common laboratory instruments such as Vortex mixer, Incubator, Laminar Air Flow Hood, Refrigerator, Microscope were used.

2. METHODS

1. *Cooking Of Rice*

Good quality commercially available black rice is selected for this study 200 gm of black Rice is taken in a container and washed 3 times properly and the water is drained out fully. The washed black rice is transferred to a 2-litre pressure cooker. 400 ml of RO water is added to black rice in the pressure cooker. The pressure cooker is closed with the lid and weight provided and kept on the gas stove. The rice is cooked for 15 minutes. The Pressure cooker maintains 15 lbs pressure (121 °C) for 15 minutes. The cooker is allowed to cool to room temperature without opening the lid of the Cooker. The Cooked and cooled black rice is Transferred to sterile conical flask and plug it with a cotton plug and kept it for further processing. (Fig.4.1)



Fig.4.1: Cooked Black Rice – Ready to be Transferred for Fermentation

4.2.2. Fermentation Of Cooked Black Rice

Three numbers of 500ml conical flask with cotton plug were taken and sterilized using autoclaving at 15 lbs pressure (121°C) for 15 minutes [34]. 1 litre of RO water is taken in a 1 litre conical flask and sterilised by autoclaving at 15 lbs pressure (121 °C) for 15 minutes [34]. 100 grams of cooked black rice prepared as per the 4.2.1 protocol is transferred to a 500ml sterile conical flask and 200 ml of sterile RO Water is added and it is cotton plugged. This conical flask is named as “CFS-12”. Similarly, one more conical flask was prepared and named as “CFS-24”. Procedure control was prepared by adding 200 ml of sterile RO water without cooked black rice in the sterile conical flask and it is cotton plugged. This conical flask is named as “CF-C”. The whole procedure was carried out under Laminar flow.

Similarly, three numbers of mud pots of 500 ml are taken and sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes [34]. 100 grams of cooked black rice prepared as per the 4.2.1 protocol is transferred to one of the sterile Mud pots and 200 ml of sterile RO Water is added and it is covered properly with the

sterile paper. This Mud pot is named as “MP-S-12”. Similarly, one more mud pot was prepared and named as MP-S-24. Procedure control was prepared by adding 200 ml of sterile RO water without cooked black rice in the other sterile mud pot and it is covered with sterile paper. This mud pot is named as “MPC”. The whole procedure was carried out under Laminar flow. Both the above-mentioned conical flask of CFS-12, CFS-24 & CFC and the mud pot of MPS-12, MPS-24 & MPC were kept in room temperature without disturbance. (Fig.4.2)



Fig.4.2: Cooked Black Rice Transferred as Per The Experimental Setup

4.2.3. Preparation Of Nutrient Agar Media Petri Plate

Nutrient agar PH 6.8 of HiMedia (Annexure 1) is used for the Nutrient agar media preparation. 23.00 grams of Nutrient agar powder is suspended in 1000 ml distilled water taken in a 1000ml conical flask. Nutrient agar powder is mixed by Heating it to boiling to dissolve the medium completely with intermittent stirring to avoid agar clumps and sticking to the bottom of the conical flask. The dissolved Nutrient Agar conical flask is Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. And the flask is Cooled down to 45-50 °C, ear bearing hot. Pour the molten sterilised nutrient agar mixture into sterile petri dishes to a depth of about 4-5mm inside the Laminar flow under aseptic condition. Allow the agar to solidify by leaving the petri

dishes on a level surface until the agar has cooled and hardened. Store the petri dishes upside down in a refrigerator until use.

4. Isolation Of Bacteria

1. Isolation of Bacteria from Conical Flask Fermented Rice Water

The Conical flask kept for fermentation as per procedure 4.2.2 was taken for isolation of bacteria as per the procedure given below. After 12 hrs of incubation, 10ul of fermented water was taken using a sterile nichrome loop from CFS-12 was taken and streaked on the nutrient Agar petri plate prepared as per the procedure 4.2.3. This Streaked plate was labelled as CFS-12. This plate was incubated at 37Deg C in an incubator [25] for 12 hrs.

Similarly, after 24 hours of incubation 10ul of fermented water was taken from CFS-24 flask and streaked on Nutrient agar plate and incubated at 37deg c. for 12 hours.

Similarly, 10ul of fermented water was taken from CFS-C flask and streaked on Nutrient agar plate and incubated at 37deg c for 12 hours.

After 12 hours of incubation, the CFS-12 plate was taken out from the incubator and observed for bacterial growth. The Colony morphology was noted down meticulously. Isolation of pure colony was performed by taking a single colony from the CFS-12 plate and further streaked on to a fresh Nutrient Agar plate. This plate is incubated at 37° C for 12 hours. After 12 hours the plate was taken and observed the colony morphology and make sure it is pure without any other contamination. This isolated pure culture was taken for further identification.

Similarly, after 24 hours of incubation, the CFS-24 plate was taken out from the incubator and observed for bacterial growth. The Colony morphology was noted down meticulously. Isolation of pure colony was performed by taking a single colony from the CFS-24 plate and further streaked on to a fresh Nutrient Agar plate. This plate is incubated at 37° C for 12 hours. After 12 hours the plate was taken and observed the colony morphology and make sure it is pure without any other contamination. This isolated pure culture was taken for further identification.

After 12 hours of incubation, the CFC plate was taken out from the incubator and observed for bacterial growth.

4.2.4.2. Isolation of Bacteria from Mud Pot Fermented Rice Water

The mud pots kept for fermentation as per procedure 4.2.2 was taken for isolation of bacteria as per the procedure given below. After 12 hrs of incubation, 10ul of fermented water was taken using a sterile nichrome loop from MPS-12 was taken and streaked on the nutrient Agar petri plate prepared as per the procedure 4.2.3. This Streaked plate was labelled as MPS-12. This plate was incubated at 37Deg C in an incubator [34] for 12 hrs.

Similarly, after 24 hours of incubation 10ul of fermented water was taken from MPS-24 flask and streaked on Nutrient agar plate and incubated at 37deg c. for 12 hours.

Similarly, 10ul of fermented water was taken from MPC flask and streaked on Nutrient agar plate and incubated at 37deg c for 12 hours.

After 12 hours of incubation, the MPS-12 plate was taken out from the incubator and observed for bacterial growth. The Colony morphology was noted down meticulously. Isolation of pure colony was performed by taking a single colony from the MPS-12 plate and further streaked on to a fresh Nutrient Agar plate. This plate is incubated at 37° C for 12 hours. After 12 hours the plate was taken and observed the colony morphology and make sure it is pure without any other contamination. This isolated pure culture was taken for further identification.

Similarly, after 24 hours of incubation, the MPS-24 plate was taken out from the incubator and observed for bacterial growth. The Colony morphology was noted down meticulously. Isolation of pure colony was performed by taking a single colony from the MPS-24 plate and further streaked on to a fresh Nutrient Agar plate. This plate is incubated at 37° C for 12 hours. After 12 hours the plate was taken and observed the colony morphology and make sure it is pure without any other contamination. This isolated pure culture was taken for further identification. (Fig.4.3)

After 12 hours of incubation, the MPC plate was taken out from the incubator and observed for bacterial growth.



Fig.4.3: Isolation of Bacteria from Fermented Cooked Black Rice water by Streaking on Nutrient Agar Media Plate.

4.2.5. Identification Of Bacteria

The Isolated pure colony from CFS-12, CFS-24, MP-12 and MP-24 was taken further for identification as per the procedure given below.

The Colony morphology such as colour, shape, periphery, surface, and nature of each plate was observed and noted down meticulously. (Fig.4.4)



Fig.4.4: Isolated Bacterial Colony from Fermented Black Rice water

4.2.5.1. Gram Staining

The individual Isolated colony was taken for gram staining. Clean and grease free microscopic slide was taken, and one small drop of sterile saline water is kept on the centre on the slide. Using the sterile inoculation loop individual pure isolated colony was touched and the cell mass was transferred to the drop of the sterile saline water kept on the microscopic slide. Thin bacterial smear was prepared by evenly missing the bacterial cell mass mixed in the saline water on the centre of the slide using the loop. Then the slide was kept to air dry. The bacterial cells were heat fixed to the slide by passing the slide through the flame of a Bunsen burner by gently heating the slide. Once the bacterial cells are heat fixed, crystal violet stain was added on to the slide to cover the slide surface fully and leave the slide without disturbing for 1 minute. The Slide was rinsed with distilled water to remove the excess crystal violet stain. Then Iodine solution was added onto the slide covering the slide surface fully and left it for 1 minute without disturbing. Then the slide was rinsed with distilled water to remove any excess iodine. Then small amount of Absolute ethanol was added on to the slide covering the entire surface to decolorize and immediately the slide was rinsed with distilled water. The decolourization step was done as quick as possible to avoid false positive. Then small amount of safranin solution was added on to the slide covering the entire surface and left for 1 minute to counter stain. The Slide was rinsed with distilled water to remove any excess safranin. Then the slide was air dried. Once the slide is fully dried, the slides were taken for microscopic examination [33].

4.2.5.2. Microscopic observation

The gram-stained slide as per the above procedure 4.2.5.1 was taken and the slide was kept under the microscope slide holder position. First the slide was observed under with 100x oil immersion objective lens with 10X eye piece lens to get total 1000x magnification to observe the bacterial cells and determine their gram status. Under the 1000x magnification microscopic field Gram-positive bacteria will appear purple or blue, while gram-negative bacteria will appear red or pink [14]. This gram nature of the bacteria was observed and noted down individually for every isolate, isolated from CFS-12, CFS-24, MP-12, MP-24.

4.2.5.3. Biochemical Test

Once the Gram nature of the bacteria was identified as Gram positive the biochemical tests required for the identification of Gram positive bacteria were selected and done as per the procedure given below.

4.2.5.3.1. Indole Test

One loopful of pure culture is Transferred sterile glass slide and a thin smear is prepared using a sterile loop. One drop of Kovac's reagent is added over the bacterial smear without disturbing the smear. The Slide was observed a red colour change at the surface of the reagent. The presence of a red colour change indicates the presence of indole, and this is reported as Indole positive [5].

4.2.5.3.2. Citrate Test

Premade Simmons Citrate agar slant of HiMedia was used for this analysis. Loopful of pure isolate colony culture was streaked using a sterile inoculation loop onto the Simmons citrate agar slant surface under aseptic condition. The slant was Incubated at 37° C in an incubator [2]. The slant was observed for growth and colour change for 24 to 48 hours. If the bacterial species is able to utilize citrate, it will grow on the slant and produce an alkaline by product, which will cause the pH indicator in the

medium to change from green to blue. This colour change was observed for each isolate and noted down.

4.2.5.3.3. TSI Test (Triple Sugar Iron):

Premade TSI Agar Slant of HiMedia was used for this analysis. Loopful of pure isolate colony culture was taken and inoculated onto the TSI agar slant by stabbing into the centre of the TSI agar slant under aseptic condition using a sterile inoculation loop. The Inoculated TSI slant was Incubate at 37° C in an incubator for 18 to 24 hours. The slant was observed for growth and colour change and gas production. The TSI agar contains lactose, sucrose, and glucose, as well as the pH indicator phenol red. If the bacterial species is able to ferment any of the sugars, it will produce acid, which will cause the pH indicator to turn yellow. If the bacterial species produces gas during fermentation, it will appear as cracks or bubbles in the agar [5]. These changes were observed for each isolated and noted down.

4.2.5.3.4. Urease Test

Premade Urea Agar Slant of HiMedia was used for this analysis. Loopful of pure isolate colony culture was taken and inoculated onto the Urea agar slant using a sterile inoculation loop under aseptic condition. The Inoculated Urea slant was Incubate at 37° C in an incubator for 12 to 24 hours. The slant was observed for growth and colour change [13]. The urea agar contains the pH indicator phenol red, which turns pink or red in an alkaline environment. If the slant turns pink or red, it indicates the presence of urease activity and thus a positive for Urease activity. This was observed and noted for each isolate.

4.2.6. Preparation of Mueller-Hinton Agar Plates

Mueller Hinton agar powder of HiMedia (Annexure 2) was used for the preparation of Mueller- Hinton Agar plate. 38.00 grams of Mueller- Hinton Agar powder is suspended in 1000 ml distilled water taken in a 1000ml conical flask. The Mueller-Hinton agar power is mixed in the water by Heating it to boiling to dissolve the media

completely with intermittent stirring to avoid agar clumps and sticking to the bottom of the conical flask. The dissolved Mueller-Hinton Agar conical flask is Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. After autoclave, the flask is Cooled down to 45 to 50 °C, (ear bearing hot). Pour the molten sterilised Mueller-Hinton agar mixture into sterile petri plates to a depth of about 4-5mm inside the Laminar flow under aseptic condition. Allow the agar to solidify by leaving the petri dishes on a level surface until the agar has cooled and hardened. Store the petri dishes upside down in a refrigerator until use.

7. *Testing of Antibacterial Activity of Fermented Black Rice water*

1. *Fermented Cooked Black Rice water Disc Preparation*

Sterile disc of 6mm diameter of HiMedia was used for this protocol. The sterile discs were taken on a sterile petri plate and each disc was kept isolated inside the plate base. 500ul of fermented Black rice water prepared as per the protocol 4.2.2 is poured on to each disc under aseptic condition inside the laminar flow. The Disc was allowed to air dry under aseptic condition and once again 500ul of fermented black rice water is added to each disc, allowed the disc to dry and the step was repeated one more time. The dried disc was stored aseptically inside the sterile petri plate sealed with sterile parafilm.

4.2.7.2. *Pure Culture Procurement and Subculture*

The pure cultures Viz. Salmonella typhimurium (MTCC No.98), Staphylococcus aureus (MTCC No.96), Escherichia coli (MTCC No.443) were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The lyophilised cultures were revived following the methods given by MTCC as per the protocol given below. The ampules tip was broken carefully with cotton wrapped around. Then the cotton plug which was kept inside was removed and 500ul of Nutrient Broth was added to make suspension of the culture. Then loopful of suspension was streaked on Nutrient Agar plate. This whole procedure was carried out under

aseptic condition inside a Laminar Air flow hood. The plates were incubated at 37 °C for 24 hours. After 24 hours the grown colonies were subcultured and used for further studies. (Fig.4.5 & 4.6)



Fig 4.5: MTCC Pure culture ampules



Fig 4.6: MTCC Pure culture - Subculture

4.2.7.3. Antimicrobial Activity Testing by Disc Diffusion method

The pure culture, procured from MTCC, which is subcultured as per the protocol 4.2.6.2 was taken for the antimicrobial activity testing of Fermented cooked black Rice water by disc diffusion method [1]. Loopful of bacterial culture was taken into a sterile Eppendorf tube containing 100ul of nutrient broth (HiMedia). the bacterial mass was suspended into the Nutrient broth by vortexing. A sterile cotton swab was taken and immersed into the Nutrient broth containing the bacterial suspension.

Then a uniform smear was applied gently onto the Mueller-Hinton agar plate prepared as per the protocol 4.2.6 using the dipped cotton swab which contain the bacterial mass. The Mueller-Hinton agar plate was turned to 60° and smeared again with the same dipped cotton swab. This procedure was repeated to cover the entire surface of the agar plate with bacterial cell mass. One number of Fermented Black rice water disc prepared by the procedure 4.2.61 was taken and placed onto the centre of the bacteria smeared Mueller- Hinton Agar plate.

This procedure was done for all the 3 cultures procured for the test individually.

One control plate was prepared without a bacterial smear and one fermented black rice water disc was place in the centre. The plates were incubated at 37 °C in incubator for 12 to18 hours.

The plates were observed for the presence of a zone of inhibition around the antibiotic disks. The diameter of the zone of clearance (inhibition) is measured and noted down against each bacterium individually.

5. RESULT AND DISCUSSION

1. RESULT

1. *Isolation of Bacteria from Fermented Cooked Black Rice water:*

The bacterial isolates, isolated from the fermented rice water kept in Conical Flask (CFS-12 & CFS-24) and Mud Pot (MPS-12 & MPS-24) were taken for identification. Morphologically single type of colonies was observed. The morphological features of the colony are given below in the Table 5.1. (Fig.5.1, Fig.5.2)



Fig.5.1: Bacterial Growth after 12 hours & 24 hours of Fermentation of Cooked Black Rice.



Fig.5.2: NO Bacterial Growth in Control plate after 12 hours of Fermentation of Cooked Black Rice

Table 5.1: Bacterial Colony Morphology

FEATURE	OBSERVATION
Colour	White/ Off white
Shape	Circular
Periphery	Irregular
Surface	Smooth and convex
Nature	Translucent

Further the gram stain and biochemical test were performed, and the results are given below in Table 5.2. Under microscope the bacterial cells were observed as Gram positive short rod shape with rounded ends, arranged in pairs and short & long chains (Fig.5.3)

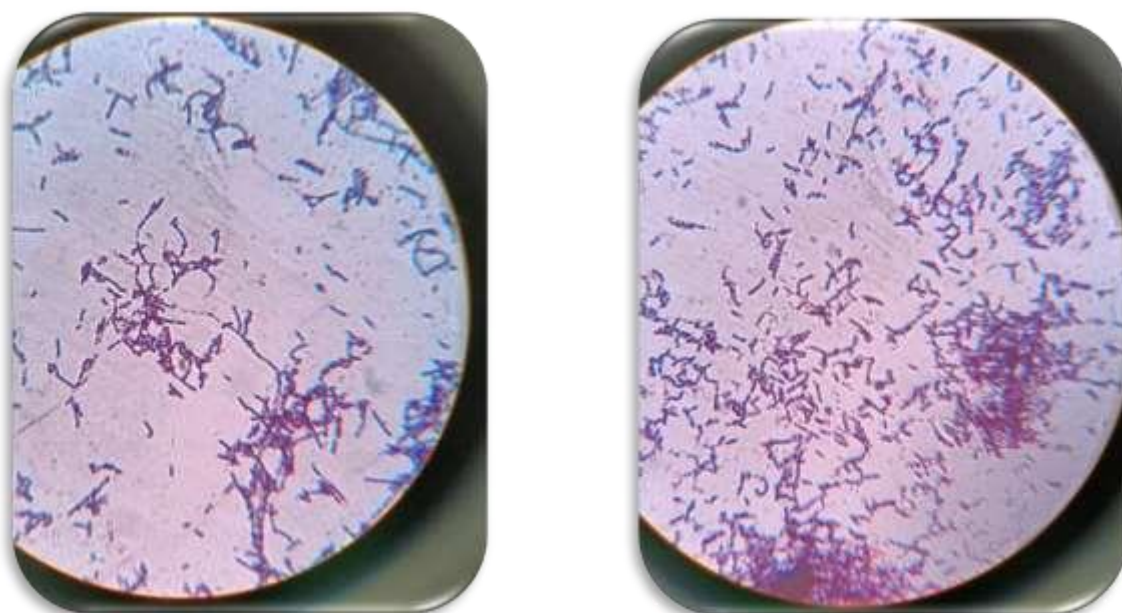


Fig.5.3: Microscopic Photo of Gram Stain of the Isolate.

Table 5.2 : Gram stain & Biochemical Test Result

SAMPLE Name	GRAM STAIN	INDOLE TEST	CITRATE TEST	UREASE TEST	TSI TEST
CFS-12	Positive	Negative	Negative	Negative	Negative
CFS-24	Positive	Negative	Negative	Negative	Negative
MPS-12	Positive	Negative	Negative	Negative	Negative
MPS-24	Positive	Negative	Negative	Negative	Negative

The biochemical tests showed that Indole test- negative; citrate test- Negative; Urease test- negative and TSI test-Negative (Fig.5.4). With these test results along with Gram stain and the Bacterial morphological features results which showed that it is a Gram-positive rod shape bacteria were collectively correlated, and the Bacteria is identified. Based on the collective results the bacteria are identified as *Lactobacillus species*. Also, it was noted that the isolates, isolated from the fermented black rice water kept in Conical flask and Mud pod was same and it is *Lactobacillus species*.



Fig.5.4. Biochemical Test Results of the Isolate

5.1.2. Disc Diffusion Method

The zone of inhibition for the three gastroenteritis-causing bacteria was checked after 24 hrs. of incubation. *Salmonella typhimurium* showed 10mm zone of inhibition. *Escherichia coli* showed no zone of inhibition (Fig.5.5, Fig.5.6, Fig.5.7). *Staphylococcus aureus* showed 12mm zone of inhibition (Table 5.3). The control plates with plain sterile discs showed no zone of inhibition for all three bacteria. (Fig.5.8, Fig.5.9, Fig.5.10)



**Fig.5.5. Zone of Inhibition
*Staphylococcus aureus***



**Fig.5.6. Zone of Inhibition
*Salmonella typhimurium***



**Fig.5.7. Zone of Inhibition
*Escherichia coli***



**Fig.5.8. Control
*Staphylococcus aureus***



**Fig.5.9. Control
*Salmonella typhimurium***



**Fig.5.10. Control
*Escherichia coli***

Table 5.3: Zone of Inhibition Measured in mm

Bacteria	Zone of inhibition (mm)
<i>Salmonella typhimurium</i>	10
<i>Escherichia coli</i>	Nil
<i>Staphylococcus aureus</i>	12

5.2 DISCUSSION

The rice variety selected was unique, which is considered as one of the rare and highly nutritious rice variety which is used by royal families [18]. Even now, this rice variety is used in rare and very important occasions such as Marriages by some ethnic groups of India such as “Nagarathar”, so called “Karaikudi Chetiyar”. The other name of this rice is also known as “Forbidden Rice”, Emperor’s Rice, “Kavuni Rice”. There are several similar research works done with other varieties of rice. Thus, in this study it is of interest to select the Black Rice for the project work.

In this current study, fermentation of cooked Black rice was carried out in mud pot and conical flask. The reason of selecting two different containers including mud pot is to mimic the actual traditional method of fermenting cooked rice. Generally, fermentation of cooked rice was carried out in mud pots. This is the reason the mud pot was selected for the current study. To compare the traditional with the laboratory method, conical flask also was used for fermenting cooked black rice.

The current study result of more bacterial growth in the mud pot fermented cooked black rice water clearly exhibit better fermentation in mud pot compared to conical flask. The reason may be because, the tiny pores in mud pot acts as a natural air filter which provides aeration. This aeration may reduce the fermentation efficiency, in turn the bacterial growth is better. Whereas, in case of conical flask, the fermentation occurs continuously which may increase the pH of the medium. Thus, the growth of bacteria is low. This comparison was evident why mud pot was used for fermentation of cooked rice traditionally.

For the current study, two time periods of fermentation, 12 hours and 24 hours were selected. The reason for selecting two time periods is to compare the effect of time period on fermentation. This comparative study result (5.1.1 and fig.5.1) showed increased bacterial growth in 24 hrs fermentation over 12 hrs fermentation. Even though there are by-products of fermentation which may inhibit the growth of bacteria, in this study it was evident that 24 hrs of fermentation gives better bacterial growth compared to 12 hrs of fermentation. Considering the probiotic nature of fermented rice water, this study showed drinking 24 hrs fermented rice water may be more beneficial than drinking 12 hrs fermented rice water. On the other hand,

further study has to be done to prove the threshold level of probiotic bacteria present in the fermented rice water when consumed.

In this study, *Lactobacillus spp.* was isolated and identified from both mud pot and conical flask fermented cooked black rice water. In this current study we have used nutrient agar plate to isolate any possible bacteria present in the fermented cooked black rice water. There are earlier studies [28,32], which used selective agar media for the specific isolation of *Lactobacillus*. Though nutrient agar media was used in the current study, only *Lactobacillus spp.* was isolated. Thus, it is evident that fermented cooked rice water has *Lactobacillus* as the major or the only bacteria present.

The isolation of *Lactobacillus* in the current study matches with studies carried out by other researchers [16]. Even though the rice variety is unique in terms of nutritional value, when it is compared for its probiotic nature, it is similar with other rice varieties since the bacteria isolated, *Lactobacillus spp.* is similar with other rice varieties. In this study the species-specific identification was not carried out. Thus, it is not possible to conclude that the species of *Lactobacillus* present in the fermented cooked black rice water is similar with the species of *Lactobacillus* present in other fermented cooked rice water. Hence, further study has to be carried out for species specific identification of the isolate.

It is common nowadays to get frequent gastric problems due to the current food habits. Gastroenteritis causing common bacteria such as *Salmonella spp.*, *Staphylococcus spp.*, *Escherichia spp.*, affects the native gut microbial flora. This enables the faster growth gastritis causing bacteria [17]. The gut microbial flora has a very good symbiotic and probiotic relationships among themselves. When this relationship is affected, it is advised to take probiotic externally to compensate the loss of the gut microbial flora so that it naturally evades the gastritis causing bacteria [11].

In this study, *Lactobacillus spp.* that was isolated from the fermented cooked black rice act as probiotic as well as symbiotic relation with gut microbial flora. The study done by [35] showed that *Lactobacillus* increases the symbiotic and probiotic relation with gut microflora. So, the current study also proves that consuming

probiotic rich (*Lactobacillus*) fermented cooked black rice water prevents the unwanted gastritis.

In the current study, antibacterial effect of fermented cooked black rice water was carried out against three common gastroenteritis causing bacteria viz., *Salmonella typhimurium*, *Staphylococcus aureus* and *Escherichia coli*. The study results show that the fermented cooked black rice water has antibacterial effect against *Staphylococcus aureus* and *Salmonella typhimurium*. The zone of inhibition for *Staphylococcus aureus* is 12 mm and the zone inhibition for *Salmonella typhimurium* is 10 mm. The study done by [32,3], also showed zone of inhibition *Staphylococcus aureus* is 15 mm and the inhibition for *Salmonella typhimurium* is 8.57 mm. In the current study, there was no zone of inhibition showed for *Escherichia coli*.

6. SUMMARY AND CONCLUSION

6.1. SUMMARY

The current project work was carried out using black rice. Black rice was cooked and soaked in water to ferment for 12 and 24 hrs in mud pot and conical flask. The bacterial species was isolated from the fermented cooked black rice water, and it was identified as *Lactobacillus spp.* using gram staining and biochemical tests. The study of antibacterial activity using disc diffusion method was carried out against three common gastroenteritis causing bacteria viz., *Salmonella typhimurium*, *Staphylococcus aureus* and *Escherichia coli*. The study results show that, the fermented cooked black rice water has antibacterial effect against *Salmonella typhimurium* and *Staphylococcus aureus*.

6.2 CONCLUSION

The project work proves, the presence of *Lactobacillus spp.* in fermented cooked black rice water. Considering the earlier works done on the probiotic nature of *Lactobacillus spp.*, the current study also proves that consuming the fermented cooked black rice water increases probiotic and symbiotic relation in gut microflora, which prevents the growth of unwanted or pathogenic organisms. Current study also proves the fermented cooked black rice water has antibacterial effect against gastroenteritis causing bacteria, which correlates with the earlier studies. Thus, it is evident that consuming fermented cooked black rice water inhibits the growth of gastroenteritis causing bacteria.

Considering the current study along with other earlier related studies carried out on fermented rice water, proves following traditional method of food preparation and following the traditional eating habits is always beneficial even in current scenario.

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Nutrient Agar pH 6.8

M561

Intended use

Nutrient Agar is used for the cultivation of bacteria and for the enumeration of organisms in water, sewage, faeces and other materials.

Composition**

Ingredients	Gms / Litre
Peptone	5.000
HM Peptone B #	3.000
Agar	15.000
Final pH (at 25°C)	6.8±0.2

**Formula adjusted, standardized to suit performance parameters

Equivalent to Beef extract

Directions

Suspend 23.00 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. If desired, the medium can be enriched with 5 - 10% v/v sterile defibrinated bloods. Mix well and pour into sterile Petri plates

Principle And Interpretation

Nutrient Agar is a basic culture medium used for maintenance or to check purity of subcultures prior to biochemical or serological tests from water (1) and Dairy (2). This medium may be used as slants or plates for routine work with non-fastidious organisms. Nutrient Agar, pH 6.8 has relatively simple formulation which provides the necessary nutrients for the growth of many microorganisms which are not very fastidious. Many bacteria have the optimum pH growth range of 6.6 to 7.0. Wetmore and Gochenour (3) maintained cultures of *Malleomyces* and *Pseudomonas* on Nutrient Agar to which glycerol was added. Greenberg and Cooper (4) employed this medium in cultivation of Staphylococci for the preparation of vaccines and antigens. Nutrient Agar has relatively simple formulation which provides the necessary nutrients for the growth of many microorganisms which are not very fastidious.

HM Peptone B contains vitamins, organic nitrogen compounds, salts and little carbohydrates (5). Peptone provide amino acids and long chain peptides for the organisms.

Type of specimen

Clinical samples - Blood ; Food and dairy samples; Water samples

Specimen Collection and Handling

For clinical samples follow appropriate techniques for handling specimens as per established guidelines (8,9).

For food and dairy samples, follow appropriate techniques for sample collection and processing as per guidelines (2,6,7).

For water samples, follow appropriate techniques for sample collection, processing as per guidelines and local standards (1).

After use, contaminated materials must be sterilized by autoclaving before discarding.

Warning and Precautions :

In Vitro diagnostic Use only. Read the label before opening the container. Wear protective gloves/protective clothing/eye protection/ face protection. Follow good microbiological lab practices while handling specimens and culture. Standard precautions as per established guidelines should be followed while handling clinical specimens. Safety guidelines may be referred in individual safety data sheets

Limitations :

This medium is general purpose medium and may not support the growth of fastidious organisms.

Performance and Evaluation

Performance of the medium is expected when used as per the direction on the label within the expiry period when stored at recommended temperature.

Please refer disclaimer Overleaf.



Mueller Hinton Agar

M173

Intended Use:

Recommended for determination of susceptibility of microorganisms to antimicrobial agents isolated from clinical samples.

Composition**

Ingredients	Gms / Litre
HM infusion B from #	300.000
Acicase ##	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.3±0.1

**Formula adjusted, standardized to suit performance parameters

- Equivalent to Beef infusion from

- Equivalent to Casein acid hydrolysate

Directions

Suspend 38.0 grams in 1000 ml purified/ distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates. Note: The performance of this batch has been tested and standardised as per the current CLSI (formerly, NCCLS) document M6-protocols for Evaluating Dehydrated Mueller Hinton Agar.

Principle And Interpretation

The Mueller Hinton formulation was originally developed as a simple, transparent agar medium for the cultivation of pathogenic *Neisseria* species (1). Other media were subsequently developed that replaced the use of Mueller Hinton Agar for the cultivation of pathogenic *Neisseria* species, but it became widely used in the determination of sulfonamide resistance of gonococci and other organisms. Mueller Hinton Agar is now used as a test medium for antimicrobial susceptibility testing (2). Mueller Hinton Agar is recommended for the diffusion of antimicrobial agents impregnated on paper disc through an agar gel as described in CLSI Approved Standard (3). Mueller Hinton Agar has been selected by the CLSI for several reasons:

- It demonstrates good batch-to-batch reproducibility for susceptible testing.
- It is low in sulfonamide, trimethoprim and tetracycline inhibitors.
- It supports the growth of most non-fastidious bacterial pathogens and
- Many data and much experience regarding its performance have been recorded (4).

Kirby-Bauer et al recommended this medium for performing antibiotic susceptibility tests using a single disc of high concentration (5). WHO Committee on Standardization of Susceptibility Testing has accepted Mueller Hinton Agar for determining the susceptibility of microorganisms because of its reproducibility (6). Mueller Hinton Agar with 5% sheep blood and Mueller Hinton Agar with Hemoglobin have been recommended for antimicrobial susceptibility testing of *Streptococcus pneumoniae* and *Haemophilus influenzae*.

HM infusion B from and acicase provide nitrogenous compounds, carbon, sulphur and other essential nutrients. Starch acts as a protective colloid against toxic substances present in the medium. Starch hydrolysis yields dextrose, which serves as a source of energy. These ingredients are selected for low thymine and thymidine content as determined by MIC values for

Enterococcus faecalis with sulfamethoxazole trimethoprim (SXT).

The Kirby-Bauer procedure is based on agar diffusion of antimicrobial substances impregnated on paper discs. This method employs disc with a single concentration of antimicrobial agent and the zone diameters observed are correlated with minimum inhibitory concentration (MIC) values (7,1,2). A standardized suspension of the organism is swabbed over the entire surface of the medium.

Please refer disclaimer Overleaf.