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Screening, isolation and evaluation of probiotic potential *Lactobacillus acidophilus* strains from available sources in Bangladesh

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Abstract

Probiotics, live microorganisms that promote health by balancing the gut microbiota, have gained popularity in food and supplements. This study aimed to identify potential probiotic strains of *Lactobacillus acidophilus* isolated from available yoghurt/fermented food sources in Bangladesh. The research addressed the need for indigenous strains to cater to the local population's health requirements in the face of imported probiotic products dominating the market. Eight yoghurt samples from Bogra District were collected and cultured using Man Rogosa and Sharp (MRS) broth and agar. The isolated lactobacilli were further characterised through sequencing, and the *Lactobacillus acidophilus* LA-5 strain was identified in all isolates. *Lactobacillus acidophilus* LA-5 is a probiotic strain that has been employed in food and dietary supplements. Additionally, a stock contamination test was conducted to ensure sample purity. *In vitro* tests were performed to assess the probiotic potential, including acid tolerance, bile salt tolerance, antibiotic sensitivity, and storage ability in order to mimic the gut environment and industrial processing. The LA-5 strain exhibited sensitivity to amikacin, gentamicin, and levofloxacin and the ability to grow after long-term storage at -80°C. These findings highlight the promising probiotic potential of indigenous strains in Bangladesh, specifically *Lactobacillus acidophilus* LA-5. The results can guide further research and contribute to the development of locally sourced probiotics tailored to the Bangladeshi population, which have been used by the local population for many years in fermented food.

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Introduction

According to the Food and Agriculture Organization, "probiotics" are living microorganisms that, when consumed in sufficient quantities, enhance the health of the host [1]. The therapeutic effects of probiotics are mostly due to the presence of good bacteria, which can compete with and limit the growth of bad bacteria. In this way, probiotics support the maintenance of a balanced gut flora. Research into the makeup of probiotic bacteria, their potential benefits, and their effects on human health has exploded in recent years. As a result of this increased understanding, more people are now using probiotics. Growing knowledge of gut health and an increased focus on individual wellbeing can be related to the increase in demand for probiotic-functional foods, drinks, and dietary supplements. Researchers are motivated to keep working on creating new and better probiotic formulations because of the rising demand. *Lactobacillus acidophilus* LA-05 is a probiotic bacteria strain that has been studied for its capacity to stimulate the immune system, improve digestive health, and increase overall well-being. It is notable for its ability to cling to intestinal epithelial cells while surviving the stomach's acidic environment. Research has shown that it can alleviate symptoms linked to some gastrointestinal issues, prevent or reduce the risk of certain infections, and affect the immune system. It is generally regarded as safe to ingest and has been found in a wide range of foods and dietary supplements [2].

Recent studies have looked into how probiotics could be utilized to treat or prevent diseases, keep individuals healthy, and reduce the risk of future illness. By boosting the proportion of beneficial bacteria and reducing the quantity of dangerous ones, consuming probiotics helps the host's gut maintain a healthy microbial balance and reduces the risk of stomach and bowel diseases [3-5]. Probiotics have been demonstrated to aid those who struggle to digest lactose, facilitate nutrient absorption, and lessen or prevent allergies in those who are predisposed to them [3, 6]. They can also help to prevent cancer, control blood pressure, inhibit mutations, prevent bone loss, lower cholesterol, and alter the immune system [3]. Probiotics have also been proven to help alleviate the symptoms of alcoholic liver disease, colitis, constipation, inflammatory bowel disease, and irritable bowel syndrome, as well as reduce the risk of breast, colon, and liver cancer [7].

Fermented food products are a subset of food products that break down carbohydrates in different ways when probiotic bacteria are present [8]. They are now a source of nutrition as well as functional and probiotic foods that are good for health or protect from diseases that are spread through food.

Probiotics and other tools from many domains must be used to combat the multifaceted problem of food-borne diseases (FBDs) [9]. Along with several strains of *Streptococcus*, *Bifidobacterium,* and *Lactobacillus*, probiotic bacteria also include *Lactococcus lactis* and a few *Enterococcus* species [10]. Probiotic lactic acid bacteria (LAB) are important in food fermentation because they limit the growth of spoilage or pathogenic bacteria while also improving the flavor, fragrance, and texture of fermented foods. They are gram-positive, acid-tolerant, nonsporulating, nonrespiring rod or cocci microorganisms found all throughout nature that can be used in the food industry [11, 12].

Lactobacillus bacteria (LAB) can be found in a variety of environments, including milk, fermented foods, animal intestines, freshwater fish, soil samples, sugar cane plants, and poultry farms [13]. LABs are useful in treating a range of disorders caused by drug-resistant pathogenic bacteria because they may produce enzymes, reduce infections, enhance immunological responses, and provide nutrients [14]. In this study, we sought to identify native *Lactobacillus acidophilus* strains from sources in Bangladesh and test their potential for use as industrially processed probiotics. We also sought to identify *L. acidophilus* strains with good probiotic characteristics, such as resistance to gastric acidity and bile salts, eradication of pathogenic bacteria, and production of beneficial metabolites.

We are confident that the results of this study will contribute to the creation of probiotic products made from traditional fermented foods that will improve Bangladeshi citizens' health. This finding can potentially be utilized as a starting point for future research into locating and testing different probiotic strains from local sources.

Materials and methods

Sampling

Eight yoghurt samples were gathered in Bangladesh's Bogra District. To prevent degradation and contamination after collection, the samples were kept aseptically at +4°C in a refrigerator until additional analysis was performed.

Isolation and Purification of Lactobacillus spp from Yoghurt

Lactobacillus spp. was isolated from regional yoghurt by using de Man, Rogosa, and Sharpe (MRS) agar (Hi-Media, India) medium at a specific pH of 6.5 [15]. The material was diluted up to ten logarithmic (10^{-10}) fold by dissolving one gram in nine milliliters of a 0.15% buffered peptone water solution. After ensuring a specified pH level, the diluted sample was inoculated onto the MRS agar plate and incubated under anaerobic conditions using an anaerobic jar (BBL, Gas Pak Anaerobic Systems) at 37°C for 48 hours [16]. Following the incubation period, colonies were chosen at random from the plates for additional purification. They were then subcultured three times on new MRS agar plates, evaluated for gram-staining, and their cell shape was assessed. For further characterization, the cultures were maintained at −80°C C in MRS broth that included 20% (v/v) glycerol [17].

Bacterial characterization

Colony morphology

Repeated streaking on MRS agar media was used to further purify the bacteria on the plates. Colony morphologies, including color, shape, and size, were then inspected with the naked eye, although sometimes microscopic scrutiny was required to distinguish one colony from another.

Gram staining

Gram staining was performed in accordance with a previously established methodology, with some changes [18]. On MRS agar, bacterial cultures were cultivated anaerobically for 48 hours at 37°C. A single colony was removed aseptically and smeared on a clean, dry slide before being heat-fixed. After 30 seconds in a crystal violet solution, the heat-fixed smear underwent a 5-second water rinse. Following a minute-long application of gram iodine solution, the slide was washed with tap water for 5 seconds. The slide was then rinsed for 5 seconds after being decolored with 95% ethanol for 15 to 30 seconds. Finally, a counterstain of safranin was applied for 60–80 seconds and then washed with water. The gram-positive bacteria were then identified using a light microscope because they stained blue to purple, whereas the gram-negative bacteria stained pink to red.

Microearophilic test

An organism known as a microaerophile needs surroundings with lower oxygen concentrations than those found in the atmosphere to exist (i.e., <21% O_2; typically 2-10% O_2). Microaerophilic *Lactobacillus spp*. was cultivated on selective agar media plates by fixing the two selective media like a sandwich and spreading them on a selective agar media plate under incubation at 37°C overnight.

Stock contamination test

A small amount (10 g) of the yoghurt from stock was taken in 1 L of autoclaved, distilled water and mixed well in an autoclaved Duran bottle. Then the solution was allowed to settle with the solid particles on the bottom of the Duran bottle. The upper, clear water was then filtered, and the filtered paper was placed on commercially available Compact Dry EC plates. Before placing the filter paper, 800 µL distilled water was added to each plate, and the plate was set for 15 minutes. After that, filter paper was placed on the plate for 5 minutes and then removed by autoclaved forceps. The plate was incubated overnight at 37°C. On the next day, the blue and purple colonies were evaluated to check for contamination.

Acid tolerance test

Following Conway *et al.*'s recommendations [19], the acid-salt tolerance test was carried out. The cultures were grown overnight in MRS broth at 37 °C, subcultured into 10 mL of fresh MRS broth, and then incubated for an additional 24 hours. The cultures were centrifuged at 2000 rcf for 10 minutes at 4 °C, and the pellets were then cleaned twice in PBS (Sigma) before being re-suspended in 1 mL of PBS. In a subsequent step, 0.1 mL of culture suspension was introduced to a set of tubes containing 2 mL of sterile PBS with varying pH levels. The evaluated pH levels were 2, 3, 4, 5, and 7. The pH of the PBS was altered using hydrochloric acid (2M). For one and two hours, the tubes were incubated. The optical density at 590 nm was assessed following the incubation period.

Bile salt tolerance test

The Gilliland *et al.* technique was used to see how effectively lactic acid bacteria from some Bogra District yoghurts handled bile salts [20]. *Lactobacillus* strains were cultured in MRS broth overnight, and 0.1 mL of the culture suspension was injected into tubes containing 10 mL of MRS broth with 0.3% chicken bile (Sigma) or without bile (as controls). At 37°C, the infected tubes were incubated. Each *Lactobacillus* strain in each treatment was subjected to three tests, each with a duplicate. Increased absorbance at 600 nm was measured hourly for 6 hours using a spectrophotometer (DU-65, Bechman, Fullerton, USA). For both the control and bile cultures, growth curves were constructed, and the periods required for turbidity to achieve an absorbance of 0.3 were determined. The time difference between control and bile cultures was interpreted as a growth delay caused by bile inhibition [21]. This experiment was also carried out using MRS agar plates.

Molecular characterization

Polymerase chain reaction (PCR) and sequencing were employed to identify the isolates' species.

Genomic DNA extraction and sequencing

Genomic DNA was extracted from samples of bacteria using a genomic DNA isolation kit (Promega, USA) and adhering to the manufacturer's instructions. In the following step, a DNA purification kit (Promega, USA) was used to clean the DNA samples. Genomic DNA concentrations were balanced using agarose gel recording and UV spectrophotometry. Gel filtration was used to remove the salt from the genomic DNA that had been purified using Sephacryl S-400 (GE Healthcare, USA).

The isolated DNA was subjected to PCR using a universal 16S rRNA-specific primer pair (forward primer: 27F5'-AGAGTTTGATCCTGGCTCAG-3', and reverse primer: 1492R5'–TACGGHTACCTTGTTACGACTT-3'). In a 25 µL reaction mixture, 1 µL forward primer, 1 µL reverse primer, 1.5 µL dNTPs, 5 µL buffer (5X), 2.5 µL $MgCl_2$, 0.25 µL Taq DNA polymerase, and 13.75 µL nuclease-free water were added. Enzyme activation at 95 °C for 10 minutes initiated the PCR reaction.

The process was then repeated 35 times, with denaturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds, and elongation at 72°C for 1 minute. After that, the last extension was carried out for 20.0 minutes at 72°C. The gel electrophoresis was observed using the amplified PCR product.

This PCR product was sent to Invent Technologies Ltd., Dhaka, Bangladesh, to perform sequencing. After its species confirmation, further experiments were performed using only this sample.

Assay for sensitivity to antibiotic

The minimum inhibitory concentration (MIC) assay determines sensitivity to various antibiotics. Andrews (2001) described the procedure for performing the assay. A total of 19 different antibiotics were used in this assay: Amikacin, Amoxyclav, Ampicillin, Ampicillin + Sulbactam, Azithromycin, Aztreonam, Bacitracin, Carbenicillin, Cefepime + Tazobactam, Cefuroxime, Cephotaxime, Cephradine, Chloramphenicol, Imipenem, Doripenem, Gentamicin, Levofloxacin, Neomycin, and Rifampicin.

Storage ability test

A previous study [22] was utilized to determine how well the experimental samples could be preserved. The samples were grown in MRS broth media at 37°C and subcultured for 24 hours on a shaker in 10 mL of MRS broth. Following a centrifugation of the culture at 2000 rcf for 10 minutes at 4°C, the supernatant was removed, and skim milk containing pellets was added. Then freeze-drying was done between -50 °C and -80 °C at around 200 mT. And the freeze-dried samples were stored at -80 °C. After six months, the sample was again cultured using the MRS broth and agar media to check its ability to grow again.

Results

Phenotypic and genotypic Identification of LAB from yoghurt sample

All of the samples' bacterial colonies were found to be regular, rod-shaped, smooth, greyish, and non-spore-forming. Due to their violet-blue colour, all of the presumedly identified bacteria were discovered to be Gram-positive. In the area between two agar plates with a lack of oxygen and hydrogen, Lactobacillus species can flourish. Rummaging the microaerophilic trait in *Lactobacillus spp*. suggests it can survive at lower amounts of oxygen or in an anaerobic condition, whereas microaerophilic demands conditions containing lower quantities of oxygen or even none than are present in the atmosphere. All isolates in this research were identified by the sequencing method, and the isolates were characterised as the *Lactobacillus acidophilus* LA-5 strain.

Stock contamination test

The stock contamination test was done to identify whether there were any pathogenic-containing microbes in yoghurt with *Lactobacillus spp*. Samples were grown on selective media for *Lactobacillus spp*. identification. Among them, samples 4 and 7 were contaminated by pathogenic *Salmonella spp*. and *Shigella spp*. (Figure 1).

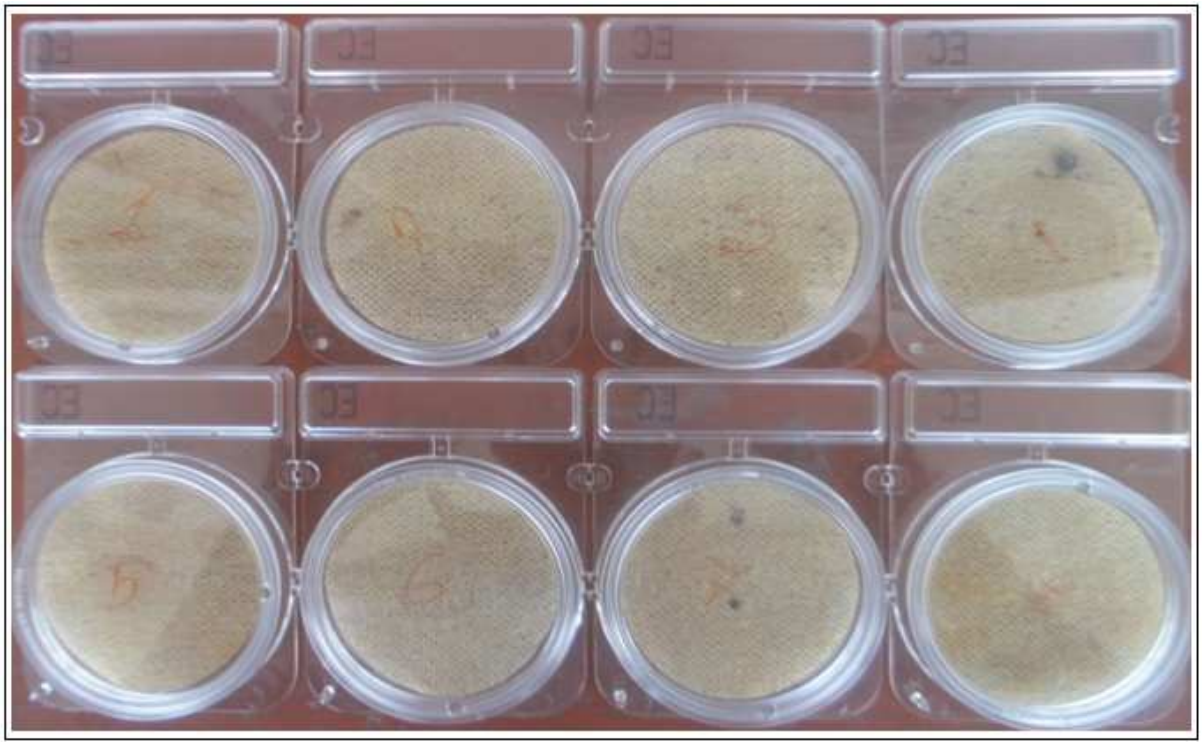

Fig. 1. Stock contamination test.

Acid tolerant test

An acid tolerance test was conducted to assess the viability of probiotic cultures in the stomach and intestines. The test involved measuring the optical density to determine the growth rate of bacterial cultures in broths with varying pH levels. While there

was no clear pattern observed in the change in optical density as pH values decreased, the *L. acidophilus* LA-5 strain demonstrated the ability to survive across all tested pH levels. Figure 2 presents the results, depicting the optical densities at 590 nm plotted against time for different pH levels in a line diagram representing the acid tolerance test. The data shown in the figure represent the average value of a single isolate at different pH levels (7, 5, 4, 3, and 2) after 6 hours of incubation at 37°C.

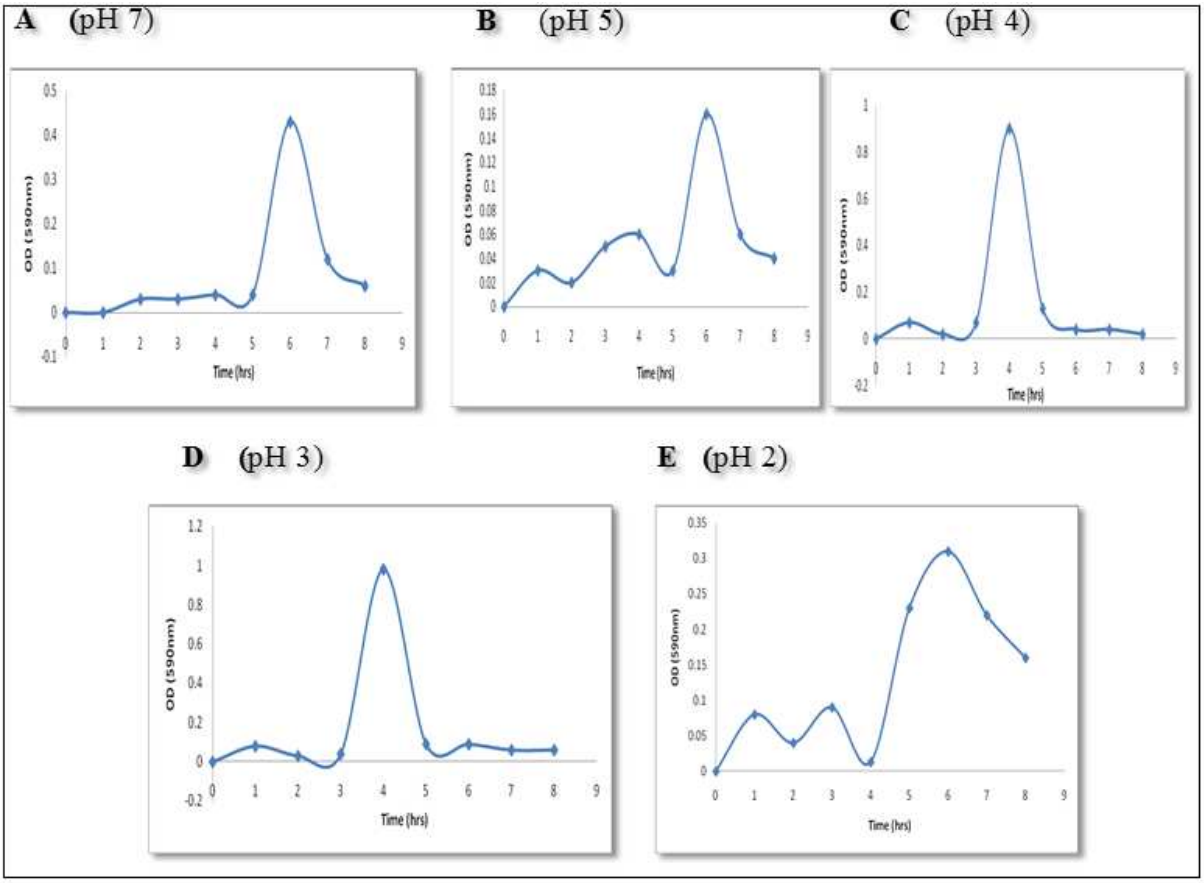

Fig. 2. Shows the growth curve of isolates at pH 7, pH 5, pH 4, pH 3, and pH 2. As it is seen from the above figure (A = pH 7), the optical density varies with time, reaches a maximum at 6 hours, and then decreases gradually over time. The OD takes the lowest value, which is zero at one hour. (B = pH 5), the optical density varies with time, reaches the maximum point at 6 hours, and then decreases gradually over time. The OD takes its lowest value at 2 hours and then again increases over the next 3 hours. (C = pH 4), the optical density varies with time, shows a peak point at 4 hours, and decreases gradually over a period. (D = pH 3). There is a resemblance between two curves at pH 4 and pH 3, with only a time lag. As can be seen from the above figure, the optical density varies with time, reaches a maximum at 4 hours, and then decreases gradually over time. The OD reaches its lowest value at hour 2. (E = pH 2): This curve is more scattered than the previous ones. As can be observed from the above figure, the optical density fluctuates significantly with time. It reaches its peak value at 6 hours and decreases sharply over time. In the initial period, OD fluctuates between one and zero.

Bile salt tolerance test

A bile salt tolerance test was performed to determine whether probiotic cultures could survive in the stomach and intestines. The degree of cloudiness in the broth served as a gauge of the amount of bacterial growth at different bile salt concentrations. The turbidity of the culture goes down as the amount of bile salts in the culture broth goes up, and it goes up as the time of incubation goes up. The isolates of probiotic bacteria were able to live in bile salt, which is 0.3% inhibitory. They were also able to multiply at the above-mentioned concentration of bile acid after

6 hours of incubation at 37°C. The optical density (OD) values at 600 nm against time and concentration shown in figure 3 represent tolerance to artificial bile salt. 100 µL of bacterial culture containing approximately 10^7 isolated bacteria along with MRS culture medium containing 0.3% inhibitory substance bile were poured into a single test tube, and the same procedure was followed for all the isolates. Data were expressed as the average value of eight isolates with a concentration of 0.3% after 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, and 6 hours of incubation at 37°C.

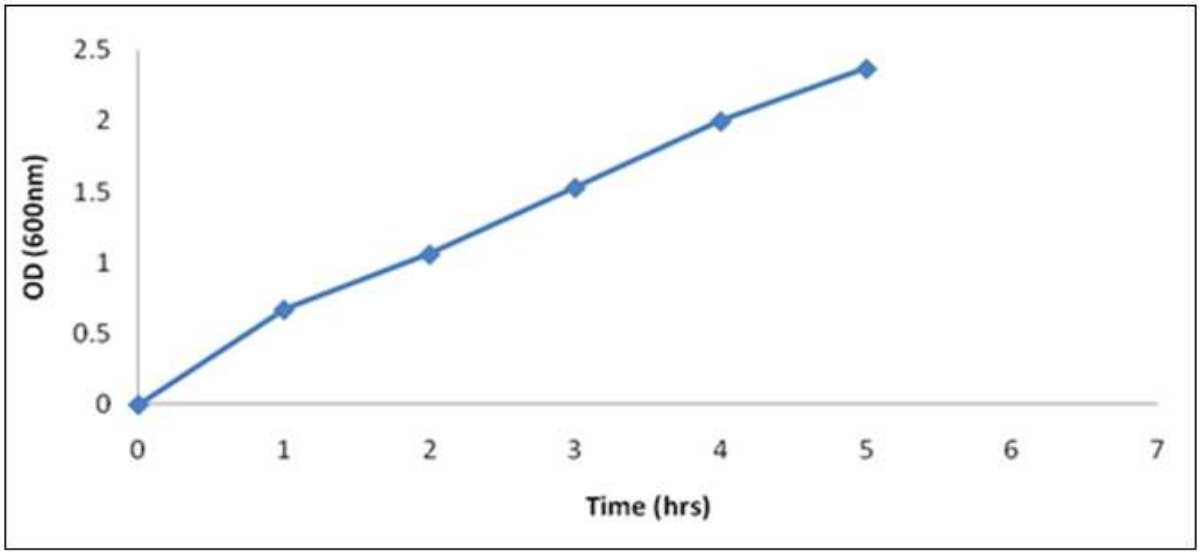

Fig. 3. The bile salt tolerance of the detected probiotic bacteria from a sample among eight of the OD was found in yoghurt at 0.3% inhibitory substance bile concentrations after 0, 1, 2, 3, 4, 5 and 6 hours of incubation at 37°C.

Plate assay for bile salt tolerance test

LAB (lactic acid bacteria) producing bile salt hydrolases were streaked onto MRS agar plates containing different concentrations of bile salt (0.1%, 0.3%, and 0.5%). This resulted in the deconjugation of bile salts, causing isolated colonies to exhibit characteristics such as an opaque, granular white appearance or the formation of halos around the colonies. These observations indicated the activation of bile salt hydrolase by LAB, as shown in figure 4. To assess the bile salt hydrolase activity of each isolate, a sensitive radiochemical assay was employed. Among the strains that produced precipitated halos, tauro (carbonyl-14C) cholic acid (TCA) yielded the most clearly defined halos. No deoxycholic acid was detected on the agar plates of both the uninoculated bile salt plates and the corresponding control plates.

Fig. 4. Manifestation of bile salt hydrolase activity through plate assay using a bile salt plate on solid MRS medium containing 0.1%, 0.3% and 0.5% bile salt.

Assay for sensitivity to antibiotic

The *L. acidophilus* LA-5 strain was sensitive to Amikacin, Gentamicin, and Levofloxacin. On the other hand, it was resistant to Amoxyclav, Ampicillin, Ampicillin + Sulbactam, Azithromycin, Aztreonam, Bacitracin, Carbenicillin, Cefepime + Tazobactam, Cefuroxime, Cephotaxime, Cephradine, Chloramphenicol, Imipenem, Doripenem, Neomycin, and Rifampicin shows in figure 5.

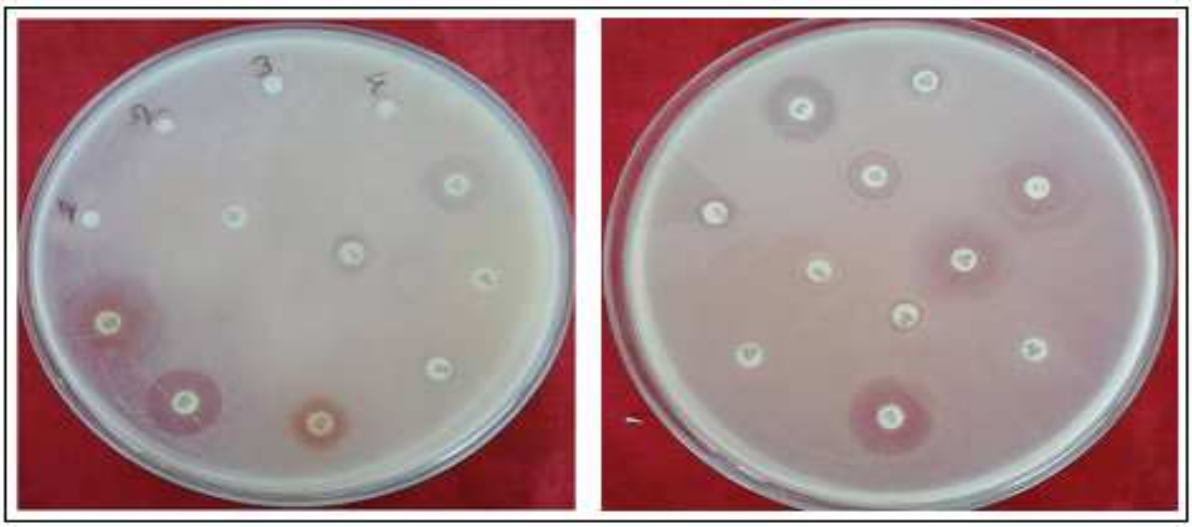

Fig. 5. Culture sensitivity test of the isolates with different antibiotic discs, including control (marked as 1, 2, 3 and 4 on the left plate).

Storage ability test

Industrial processing that is the lyophilization process of the probiotic strains remained a challenge. Therefore, we lyophilized the strain kept in the shelf as well in -80 °C to check revival rate in CFU. After 6 months of storage, it was found that the isolated *L. acidophilus* LA-5 strain was able to grow efficiently both on selective MRS agar plates and in selective MRS broth media. After 10 times dilution the achievable picture is shown in Figure 6. However, initial CFU was 1.5 X 10^{10} which later reduced to 1 x 10^{10} CFU. This implies that the strain is capable of tolerating the lyophilizing process.

Discussion

Indigenous probiotics, also known as probiotic strains particular to a geographic location or people, are very important since they have the ability to offer specific health advantages and support a healthy microbiome. Actually, native probiotics are ideally matched to the local environmental circumstances because they have evolved organically to flourish in particular geographical areas. This modification increases their effectiveness in providing health advantages to people in those specific locations.

The goal of this study was to isolate, screen, and evaluate Lactobacillus acidophilus strains from indigenous sources in Bangladesh, with a focus on yoghurt from the Bogra district. Lactobacilli are widely used in the creation of conventionally fermented dairy products because they can increase the shelf life of food goods while bringing about desired changes in taste, flavor, and texture. These cultures are regarded as safe and valuable for commercial and scientific purposes [23]. Probiotic LABs are frequently administered through fermented dairy products like yoghurt, cheese, and other fermented milk products due to their distinct presence in the intestinal epithelium and human gastrointestinal tract and their traditional use in these foods and dairy products without major issues [24].

MRS agar was used to selectively grow *Lactobacillus spp*. based on what was learned in earlier investigations [25, 26]. The characteristics of colonies

in typical culture environments are able to identify *Lactobacillus spp*. [27, 28]. In accordance with earlier findings [29, 30], gram staining examination revealed that all bacterial samples were stained blue, indicating that they were gram-positive. The results of the microaerophilicity test indicated that Lactobacillus spp. may flourish in anaerobic environments. Since Lactobacillus acidophilus is a microaerophilic strain, oxygen concentration is important for its growth because even modest oxygen concentrations can cause a reduction in lactate production [31, 32].

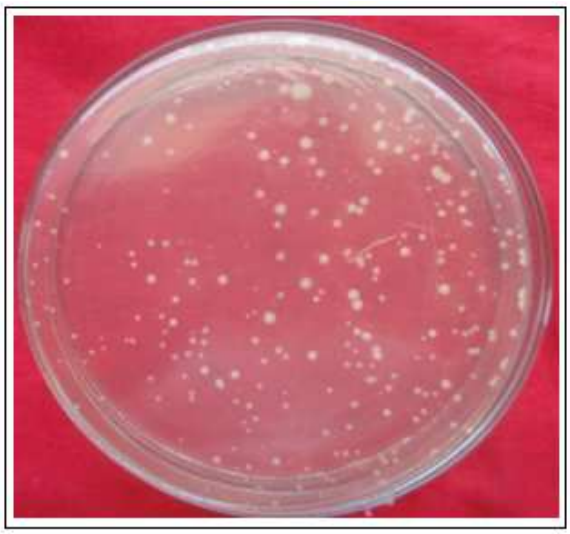

Fig. 6. *L. acidophilus* grow on selective MRS agar media after freeze drying.

The study also revealed that two yoghurt samples contained the potentially hazardous bacteria *Shigella spp*. and Salmonella spp. Because of this, it's crucial to conduct a stock contamination test to prevent unintended infections from harmful organisms. After 6 hours of incubation, all of the LAB strains tested in this investigation were able to tolerate 0.3% bile salt in MRS broth media, exhibiting a noticeably faster rate of growth [33-35]. For potential probiotic bacteria to survive and develop in the GIT, they must be able to tolerate or endure intestinal bile salt. As a result, it is crucial for probiotic selection [36]. The study also demonstrated that Lactobacillus species have the ability to produce bile salt hydrolase (BSH), an enzyme that catalyzes the deconjugation of bile salts. BSH is an intracellular enzyme that is often found in intestinal bacterial species such as *Lactobacillus* [37], *Clostridium* [38], *Bacteroides* [39] and *Bifidobacterium* [40]. BSH's biological function in these bacteria is unknown. However, BSH has recently received attention because of its potential therapeutic benefits for reducing serum cholesterol [41, 42]. Radiochemical tests using tauro (carbonyl-14C) cholic acid as a substrate were used to measure BSH activity [43]. This discovery is especially significant since it suggests that probiotic LAB strains may be able to influence bile acid metabolism in the human gastrointestinal tract.

The acid tolerance test was used to determine whether or not probiotic microorganisms could pass through the stomach and intestines. Acidity is a significant detriment to the viability of lactobacilli [44]. Singh *et al*. discovered that the pH of the intestine's gastric juice is 2.0–3.0, making it difficult for ingested bacteria to survive [45]. As a result, the goal of this study was to find probiotics that can resist acidic conditions (pH 2.0) in the presence of 0.3% bile salts. The *L. acidophilus* LA-5 strain grew at all pH levels examined (7, 5, 4, 3, and 2). At pH 2.0, the optical density changed dramatically over time but peaked at 6 hours. This was congruent with the findings of Wang *et al*. and Tulumoglu *et al*., who found great survival in Lactobacillus bacteria isolated from the faces of breastfed infants and children [46, 47].

Probiotics' ability to manage antibiotics, especially when administered after antibiotic therapy, is one of their most crucial qualities. The susceptibility to antimicrobials was evaluated using the disc diffusion method. Amikacin, Gentamicin, and Levofloxacin all caused adverse reactions in the L. acidophilus LA-5 strain. Contrarily, it was impervious to Amoxycillin, Ampicillin, Ampicillin + Sulbactam, Azithromycin, Aztreonam, Bacitracin, Carbenicillin, Cefepime + Tazobactam, Cefuroxime, Cephotaxime, Cephradine, Chloramphenicol, Doripenem, Imipenem, Neomycin, and Rifampicin. The resilience of bacteria was influenced by their genus, species, strain, and logarithmic growth phase. Antibiotic therapy may change the composition of the intestinal microbiota,

upsetting the intestinal tract's homeostasis. Lactobacilli species are thought to be helpful in preventing these issues [48], but they shouldn't have genes that can transfer to other bacterial populations [49].

Freeze-drying, also called lyophilization or cryodesiccation, is a way to remove water from a material to make it last longer or make it easier to transport. Due to the widespread use of freeze-drying in the pharmaceutical industry to preserve products, numerous research studies have examined how various cryoprotectants affect probiotics. The stability of several *Lactobacillus* strains following freeze-drying is unknown in great detail [22]. In this study, the isolated *L. acidophilus* LA-5 strain's capacity to develop after an extended period of storage at -80 °C was investigated. It may still successfully grow on selective MRS agar plates and selective MRS broth media after six months of storage. This characteristic makes the probiotic candidate more durable during storage.

The present study isolated, screened, and evaluated *Lactobacillus acidophilus* strains from available sources in Bangladesh, with a focus on yoghurt produced in Bogra district. The study discovered strains with potential as probiotics because they could thrive in anaerobic environments and tolerate intestinal bile salt. The findings of this study contribute to the growing body of research on probiotics and their potential applications in the food and health industries, keeping in mind that indigenous probiotics have co-evolved alongside human hosts within a particular population. This co-evolution facilitates a more harmonious interaction between the probiotics and the host's immune system, potentially enhancing their ability to confer health benefits.

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Conflict of Interests

The authors state that they have no potential conflicts of interest.

Author contributions

The study procedure was conceptualized and designed.: Ariful Haque; Carried out experiments: Ariful haque, Zohorul Islam Moon, Dipa Roy and Saiful Haq; Analysis and interpretation of data: Dipa Roy, Zohorul Islam Moon and Ariful Haque; Manuscript preparation: Dipa Roy; Finalizing the manuscript: Ariful Haque; Critical review of the manuscript for significant intellectual substance : All authors; Funding was obtained: Institutional Internal Funding, University of Rajshahi; Technical, material, or administrative assistance: Institute of Biological Sciencces, Rajshahi, Bangladesh; Supervision: Ariful Haque; Data visualization: Ariful Haque and Dipa Roy.

Ethical approval and consent to participate

The Institutional Animal, Medical Ethics, Biosafety, and Biosecurity Committee (IAMEBBC) of the Institute of Biological Sciences, University of Rajshahi, provided its ethical approval. 37 (21)/320/IAMEBBC/IBSc Memo

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