



Molecular Identification of *Lactobacillus* from Austagram Cheese

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ABSTRACT

Background: The most significant and frequently employed bacteria in the dairy food industry are lactic acid bacteria. To produce cheese commercially, carefully chosen starting cultures are needed. These starter cultures are mainly composed of lactic acid bacteria (LAB).

Methods: To isolate and identify *Lactobacillus* from Austagram cheese, biochemical tests were used and for molecular characterization, a PCR test was used.

Result: A total of 112 raw cheese samples from different producers were analyzed for detection of *Lactobacillus*, of which 97 samples were found positive in primary isolation using culture media and the overall prevalence of *Lactobacillus* was 86.60%. In the case of a biochemical test, the overall prevalence was 81.25% and in the case of a PCR test, the overall prevalence was 73.21%. In the case of gram staining, the bacteria were stained blue and purple and rod-like bacilli or sphere-shaped cocci without spores were visible under the microscope. A total of 97 gram-positive microorganisms were identified as *Lactobacillus*, of which 61 were rod-shaped bacilli and the remainder, 36, were cocci. Molecular characterization of LAB strains found in traditional cheeses will improve our understanding of traditional food microbiota and help us find probiotic bacteria with significant benefits for industry and human health.

Key words: Austagram cheese, Bacilli, Cocci, Gram staining, Lactic acid bacteria (LAB), Polymerase chain reaction (PCR).

INTRODUCTION

The present evidences emphasize the health benefits of some microorganisms. In this regard, probiotics are currently among the most studied beneficial microorganisms (Cassani *et al.*, 2020). Lactic acid bacteria (LAB) are a diverse group of microorganisms, having the ability to produce lactic acid as the main product, which prevents the proliferation of food spoilage bacteria and pathogens. They are gram-positive, nonspore forming, cocci or rods, with the ability to secrete a variety of antimicrobial compounds, for example, organic acids, bacteriocins, *etc.* (Quigley *et al.*, 2013). LAB are normal inhabitants of the healthy gut microbiota in animals and can be found in the milk and dairy products such as cheese (Bihola *et al.*, 2025).

The term "probiotic" is derived from the combination of two Greek words "pro" and "bios," which together mean "for life". Noble prize winner Metchnikoff first discovered the favorable influence of bacteria on gut flora, is thought to have suggested the first concept of probiotics in 1907. Ferdinand Vergin most likely invented the word "probiotic"; he compared advantageous effect ("probiotika") of some specific bacteria with the harmful effects of antimicrobial agents including antibiotics on the gut microbiome in his 1954 paper "Anti- und Probiotika". Probiotics are defined by the FAO and WHO as "beneficial live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill *et al.*, 2014). About 60-70% of the functional food industry is anticipated to be probiotic foods (Kołożyn-Krajewska and Dolatowski, 2012; Bakhti *et al.*, 2025). Lactic acid bacteria are considered one of the most important probiotic organisms that are usually used in cultured dairy products (Dahou *et al.*, 2024).

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Nowadays, there is a global interest to isolate LAB from food commodities for application in functional food and dietary supplement (Chiang and Pan, 2012). LAB isolated from different food sources drawn a lot of attention in combating food-associated pathogens and spoilers, biodegradation of chemical contaminants and development of unique food with special interests for the consumers (Hajigholizadeh *et al.*, 2020). Antimicrobial peptides and volatile organic acids produced by Lactobacilli make them one of the most significant and versatile probiotic bacteria (Moradi *et al.*, 2020).

In recent years, there has been a surge of interest in the identification of LAB to know their effect on starter culture

as well as the bacteriocin activity of LAB. From a microbiological perspective, there is little information available regarding the diversity of LAB in cheese in Bangladesh. If identified, LAB present in cheese can be evaluated for their potential ability to improve product quality. In Dhaka, Chattogram and Jashore districts of Bangladesh, researchers characterized and evaluated lactic acid bacteria from dahi, milk, cheese and yogurt for potential probiotic features (Afrin *et al.*, 2021; Reuben *et al.*, 2020). In Bangladesh, molecular detection of *Lactobacillus* or probiotics present in cheese and their antimicrobial performance has not been well studied. This is consistent with the theory that the efficiency of probiotic cultures is reliant on the former host from which they are derived, that they function most effectively in a host that is similar to the host from which they were originally isolated and that they are affected by the geographical location from where the isolates are originated. Therefore, considering the above facts, the study was conducted for the following objectives:

Objectives

- To isolate and identify *Lactobacillus* from austagram cheese.
- To molecular characterization of *Lactobacillus* from austagram cheese by PCR test.

MATERIALS AND METHODS

Study area and sample collection

The study was carried out in different parts of Austagram upazila in Bangladesh. It is wedged with regard to latitudes 24°13' and 24°27' north and 90°59' and 90°15' east. From November 2021 to April 2022, a total of six months were devoted to the execution of the study. A total of 112 cheese samples from different locations were collected randomly at different cheese manufacturers in Austagram upazila and placed into airtight plastic bags. All samples were sent to the Dairy Science Laboratory, Sylhet Agricultural University in ice packs containing a cooler and stored at 4°C until tested.

Media preparation

For media preparation manufacturer's instructions were followed in order to prepare each of the media including MRS Broth, MRS Agar, Nutrient Agar, BHI Broth, TSI Agar, Simmons Citrate Agar, MR-VP Broth (Company: Himedia, India) that were utilized in this experiment.

Isolation of bacteria

In a test tube, 1 g of cheese was taken and 9 ml of water (containing NaCl) were thoroughly mixed. In a test tube, 9 ml of MRS (De Man, Rogosa and Sharpe) broth was taken and 1 ml of cheese sample was added, which was then incubated at 37°C for 24 hours to observe the bacterial culture. All primary culture colonies were subcultured on MRS agar and incubated at 37°C for 24 hours under a strict microaerophilic environment. From MRS agar, large white colonies were subcultured into MRS agar. The cultures were streaked in MRS Agar twice to obtain the pure culture. Finally, from the

pure culture, desired bacterial colonies were collected for morphological characterization, biochemical tests and DNA extraction. The remaining colonies were stored in BHI and 15% glycerin for further use.

Gram's staining procedure

A smear was prepared on a clean glass slide from the desired bacterial colony and air-dried the smear. 5 drops of crystal violet were added and then rinsed away with water after one minute. After adding 5 drops of iodine solution and rinsing briefly with water for 30 seconds, excess water was eliminated. The slide was tilted, decolorized with alcohol and rinsed with water within 5 seconds. 5 drops of safranin were added and allowed to remain for one minute before being washed briefly with water and surplus water was removed. Examined at 100X magnification with oil immersion under microscope.

Biochemical and physiological profiling of isolate

Catalase test, Carbohydrate fermentation test by TSI Agar, Citrate utilization test by Simmons Citrate Agar, Methyl-Red Test were performed according to the method outlined by Mac Faddin and Jean F (2000).

Molecular detection of *Lactobacillus* from Austagram cheese

DNA extraction

Lactobacillus culture was collected in a microcentrifuge tube containing 1 ml of nuclease-free water. 200 µl of cell culture was harvested by centrifugation at 13,000 rpm for 30 seconds and the supernatant was discarded. Then 20 µl volume of Proteinase K solution with a concentration of 20 mg/ml was put into a 1.5 ml microcentrifuge tube at the start of the experiment. After that, 200 µl of Lysis Solution was added into the 1.5 ml micro-centrifuge tube that contained the Proteinase K solution (Table 1). In the event that the volume of the sample is less than 200 µl, the necessary volume of PBS was added. After adding the Binding Solution in an amount of 200 µl to the sample tube, it was well mixed by pulse-vortexing for a period of 15 seconds. After that, it was left to incubate at 56°C for ten minutes. After that, 200 µl of absolute ethanol was added and thoroughly mixed using pulse-vortexing for fifteen seconds. Following this procedure, a quick spin down was carried out in order to obtain the drips that were sticking under the lid. Without wetting the tube's rim, the lysate was transferred with extreme caution

Table 1: Reagents used in DNA extraction.

Reagents	Amounts
Lysis solution	200 µl
Proteinase K solution (20 mg/ml)	20 µl
Binding solution	200 µl
Absolute ethanol	200 µl
Washing solution 1	500 µl
Washing solution 2	500 µl
Elution solution	30 µl

Table 2: Primers used for molecular identification of genus *Lactobacillus*.

Target genes	Primers sequences (5'-3')	Amplicon size	Reference
16S rRNA	F: AGAGTTTGATCCTGGCTCAG R: GGTACCTTGTACGACTT	1350 bp	(Saeed <i>et al.</i> , 2020)

from the lower reservoir of the spin column into the top reservoir of the spin column. After one minute centrifugation at a speed of 13000 rpm was carried out. The flow-through was poured out and the spin column was assembled using the 2.0 ml collecting tube. Following the addition of 500 µl of Washing 1 Solution, the spin column containing the collecting tube was spun at a speed of 13000 rpm for one minute. The flow through was poured off and the spin column was assembled using the 2.0 ml collecting tube. Once again, 500 µl of Washing 2 Solution was added to the spin column with collection tube and the apparatus was centrifuged at a speed of 13000 revolutions per minute for one minute. In order to get rid of any remaining ethanol in the spin column, additional centrifugation at a speed of 13000 rpm for one minute was performed. The spin column was moved into a fresh micro-centrifuge tube that had a capacity of 1.5 ml. At last, 30 µl of the elution solution was poured into the spin column with the microcentrifuge tube and it was allowed to stand for at least one minute. The genomic DNA was extracted by centrifuging the sample at 13000 revolutions per minute for one minute. The eluted DNA samples were kept at a temperature of - 20°C until further examination.

PCR protocol for genus *Lactobacillus*

Chemicals and reagents needed: Primer-1 set, Nuclease Free water, 2X PCR Master Mix (GoTaq Promega Green Master Mix). The universal gene for *Lactobacillus* was targeted for molecular confirmation of suspected colonies. Polymerase chain reaction using the primers indicated in Table 2 amplified the target gene from samples. The PCR reaction was conducted in 20 µl reaction mixtures containing 1 µl (10 pmol/µl) of each forward and reverse primer, 5 µl of ready-to-use master mix and 8 µl of nuclease-free water. In the final step, 5 µl of DNA template was added to each reaction tube (Table 3). This mixture was mixed very well by repeated pipetting in a PCR tube.

Gel electrophoresis of amplified PCR products

For separating and analyzing the PCR products 1.5% agarose gel was used. 0.9g of agarose powder was added in 60 ml of TAE and swirled to mix into a 250 ml conical flask (Table 5). The flask was kept in a micro-oven for 30 seconds to dissolve the agarose and then cooled down at 60-70°C. 5 µl of safe gel stain dye was added into it and mixed well. Then the gel was poured slowly into the gel plate. Gel comb having 13 well was placed into plate before pouring the gel. Gel plate was left for 15-30 minutes for solidification. Comb was removed from gel carefully after solidification and the gel along with the gel plate was placed into the electrophoresis tank. Then TAE buffer was poured into gel electrophoresis tank and the gel submerged to 2-5 mm depth. After that 4 µl of 100-bp DNA Marker was placed in

Table 3: Composition of reaction mixture for the amplification of 16S rRNA gene.

Components	Amount
Master Mix (2x conc.)	5 µl
Forward primer	1 µl
Reverse Primer	1 µl
Nuclease free water	8 µl
Extracted DNA	5 µl
Total volume	20 µl

Table 4: Thermal cycle for genus *Lactobacillus*.

Steps	Temperature	Time	Cycle
Initial denaturation	94°C	3 min	1
Denaturation	94°C	45 sec	35
Annealing	56°C	45 sec	
Extension	72°C	45 sec	
Final extension	72°C	7 min	1

Table 5: Reagents for 1.5% agarose gel preparation.

Components	Amount
Agarose Le	0.9 g
Safe Gel Stain	5 µl
TAE buffer (TRIS-Acetate- EDTA)	60 ml

the first well and a negative control was maintained in the second well. Then 5 µl of PCR products were loaded from the third well onward. After loading all the samples, the gel tank was covered and the gel was run at 100 volts for 30 minutes (Table 4). The gel run was stopped when the DNA Marker had run half the length of the gel. The gel was taken very carefully from the gel tank after unplugging it. Then the gel was observed under a UV trans-illuminator.

Determination of *Lactobacillus* prevalence

Prevalence of *Lactobacillus* =

$$\frac{\text{Total number of } Lactobacillus \text{ positive samples}}{\text{Total number of cheese samples}} \times 100$$

RESULTS AND DISCUSSION

Primary isolation of positive samples by culture media

A total of 112 raw cheese samples from different producers were analyzed for detection of *Lactobacillus* where 97 samples were found positive in primary isolation using culture media. The overall prevalence of *Lactobacillus* was found 86.60% (Table 6). *Lactobacillus* on MRS agar produced white or creamy yellow single colonies with round edges and smooth surfaces, with diameters ranging from 0.5 to 3 mm (Fig 1).

Table 6: Results of different biochemical tests of *Lactobacillus* isolates from Austagram Cheese samples.

Name of tests	Result	Interpretation
Catalase test	No bubbles	Catalase (-)
Carbohydrate fermentation test by TSI agar	Yellow color slant and butt	TSI (+)
Citrate utilization test by Simmons citrate agar	No change in the color	Citrate (-)
Methyl red test	Red or pink color on the surface medium	MR (+)

TSI = Triple Sugar Iron agar, MR = Methyl red.

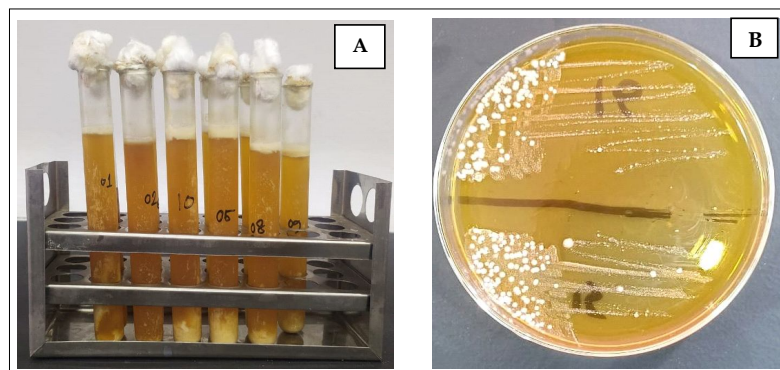


Fig 1: Growth of *Lactobacillus* in MRS broth (A). Creamy yellow single colonies of *Lactobacillus* with round edges on MRS agar (B).

Morphological examination

The bacteria were stained blue and purple and rod-like bacilli or sphere-shaped cocci without spores were visible under the microscope (Fig 2). Total 97 gram-positive microorganisms were presumptively identified as *Lactobacillus*. The majority of tested isolates were rod-shaped bacilli that were 61 isolates and the remainder 36 isolates were cocci.

Biochemical tests

From 97 isolates we found 91 isolates positive in different biochemical tests for *Lactobacillus*. The overall prevalence was 81.25% in biochemical tests.

1) Catalase test: The absence of bubble indicated that the isolated bacteria lack catalase and consequently incapable of mediating the decomposition of hydrogen peroxide into oxygen (Fig 3). The catalase test is used to assess whether or not bacteria have the enzyme catalase, which is involved in the conversion of hydrogen peroxide into water and oxygen.

2) Carbohydrate fermentation test: All of the isolates produced acid by fermenting the three basic sugars (Lactose, Glucose and Sucrose). The change in color from reddish to yellow in both slant and butt indicated a drop in the pH because of acid production without the formation of gas or hydrogen sulfide.

3) Citrate utilization test: The isolates were unable to utilize citrate as a solitary source of carbon and were not able to generate sodium bicarbonate or ammonia. Thus, there was no alteration in the color of media (Fig 3). All of the isolates were tested negative for citrate.

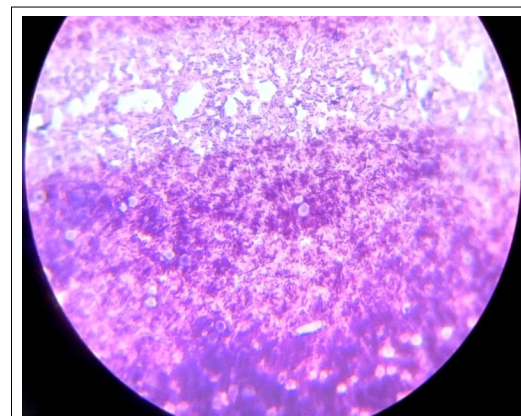


Fig 2: Rod-like bacilli of *Lactobacillus* under microscope after Gram's staining.

4) Methyl-Red test: The appearance of red or pink color on the surface medium after adding 5 drops of methyl red suggested acidity and indicated that the isolates were MR test positive (Fig 3).

Overall prevalence of *Lactobacillus* in Austagram cheese

In this study, a total of 82 isolates were positive in PCR test with specific band. So, overall prevalence of *Lactobacillus* was 73.21% in this study. The overall prevalence of *Lactobacillus* in Austagram cheese is presented in Table 7. In agarose gel electrophoresis we found specific band on 1350 bp for *Lactobacillus* positive isolates (Fig 4).

According to the results of this study, *Lactobacillus* isolates from Austagram cheese produced 0.5-2 mm

diameter sized, white to yellowish white color colonies with round edge on MRS agar when incubated at 37°C. This is similar to the findings of Jose *et al.*, (2015) who also found milky white round colony on MRS agar surface (Jose *et al.*, 2015). *Lactobacillus* from Yoghurt samples was detected by Hoque *et al.*, (2010) by observing their morphology and several biochemical and physiological properties. Gram positive rods or sphere shaped bacteria was observed under the microscope. The isolated bacteria were catalase negative, citrate negative and MR positive. *Lactobacillus* isolates were found to be capable of fermenting lactose, glucose and sucrose. As a result, the current study's findings

were found to be similar to those of Hoque *et al.*, (2010). Similar morphological and biochemical results of lactic acid bacteria isolated dahi samples were also found in the study of Harun-Ur- Rashid *et al.*, (2007). Because other species cannot grow in this medium, the MRS medium was selected for initial identification of the genus *Lactobacillus* and other lactic acid bacteria in this research (De MAN *et al.*, 1960). The same results were observed by Lopez-Diaz (2000) suggesting that the genus *Lactobacillus* was prominent in MRS. All 97 isolates were classified as probiotic bacteria based on their colony morphology on MRS agar, physiological properties like salt tolerance and biochemical

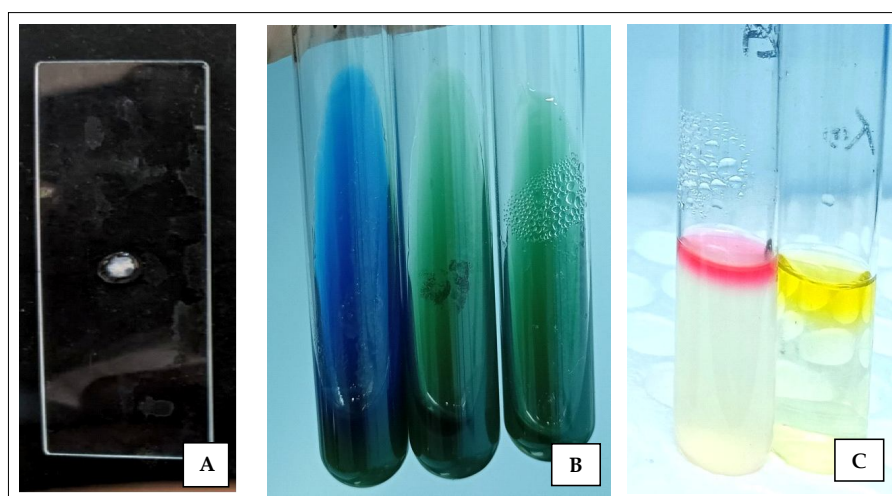
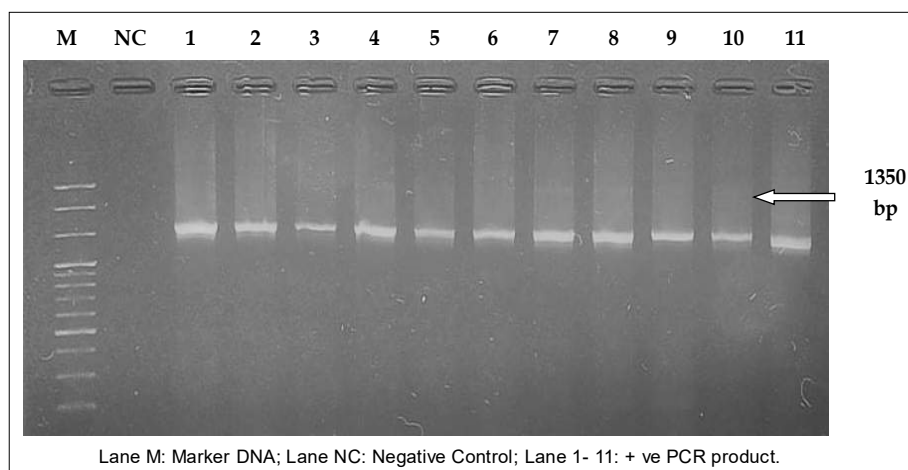


Fig 3: Biochemical characteristics of *Lactobacillus*: A) In Catalase test *Lactobacillus* produced no bubble; B) In citrate utilization test by Simmons citrate agar *Lactobacillus* did not utilize citrate; C) In MR test *Lactobacillus* produced stable red color.



Lane M: Marker DNA; Lane NC: Negative Control; Lane 1- 11: + ve PCR product.

Fig 4: Specific gene amplification by PCR and *Lactobacillus* genus detected by 1.5% agarose gel electrophoresis.

Table 7: Prevalence of *Lactobacillus* in Austagram cheese.

Genus	Total sample	Cultural positive	Prevalence (%)	Biochemical test positive	Prevalence (%)	PCR positive	Prevalence (%)
<i>Lactobacillus</i>	112	97	86.60	91	81.25	82	73.21

characteristics like carbohydrate fermentation pattern, absence of catalase enzyme, unable to utilize citrate as a sole source of carbon.

The biochemical prevalence of *Lactobacillus* isolates from Austagram cheese was determined 81.25% in the current study. Based on PCR, the prevalence of *Lactobacillus* isolates from Austagram Cheese was 73.21%. This is comparable to Abdullah and Osman's findings, in which the *Lactobacillus* genus was found to be 69.23% in white cheese analyzed by cultural, physiological and biochemical tests (Abdullah and Osman, 2010). Saeed *et al.*, (2020) showed the prevalence of *Lactobacillus* isolated from goat raw milk was 15% based on PCR. That's why it may be stated that cheese contains more probiotic organism like *Lactobacillus* than other milk based products.

CONCLUSION

Lactobacillus is a kind of probiotic that has numerous uses for the health of humans as well as animals. In this study, the prevalence of *Lactobacillus* in Austagram Cheese was found 73.21% by PCR assay. Because of their unique ability to grow under the highly selective conditions of ripening cheese, lactobacilli typically are the dominant microflora of Austagram cheese beyond different age. Their capacity to sustain themselves by consumption of compounds other than lactose, such as lactate, citrate, glycerol, N-acetylamino sugars, amino acids and other metabolites or autolysis products of starter bacteria, would partially explain their competitive advantage over other bacterial genera. Lactobacilli gain access to the cheese-making process through pre- or post-pasteurization contamination, most entering via the latter mechanism. Species of lactobacilli most often associated with Austagram cheese. In addition to possessing antibacterial characteristics, certain criteria must be met when selecting *Lactobacillus* isolates due to the fact that *Lactobacillus* may transmit antibiotic resistance genes to other organisms. Also, new strains of *Lactobacillus* species that may be good for human and animal health must be identified and characterized through more research. *Lactobacillus* and bacteriocin molecules might play even more exciting roles in the near future, including quorum sensing and site- targeted medicine delivery.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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