

## Research Article

### Screening of potential *Lactobacillus* species from buffalo milk and evaluation of their antimicrobial activity

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**Abstract:** *Lactobacillus*, widely studied lactic acid bacteria (LAB), are very common inhabitants of milk from different sources such as cow milk, goat milk, buffalo milk, etc. Our target was to isolate some *Lactobacillus spp.* from buffalo milk and evaluate their antimicrobial activity. We selected buffalo milk as our sample with an expectation that it may contain *Lactobacillus spp.* with some better qualities because of its high protein and mineral levels and low cholesterol contents. Ten (10) buffalo raw milk samples, collected from different regions of Chittagong district of Bangladesh, were tested for isolation and identification of *Lactobacillus spp.* Isolates showing antimicrobial activity against any two of the eight selected Gram positive and Gram negative pathogenic bacteria were considered as potential. Two potential *Lactobacillus spp.* from 22 primary isolates were selected on the basis of their antimicrobial activity by cross streak method. The selected potential isolates were identified as *Lactobacillus brevis*(B3<sub>4</sub>) and *Lactobacillus fermentum* (B7<sub>3</sub>). *Lactobacillus brevis* showed activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* where as *Lactobacillus fermentum* against *Shigella dysenteriae* and *Bacillus subtilis*. It was also observed that both identified *Lactobacillus spp.* show their best antimicrobial activity against these pathogenic bacteria after incubation at 37°C temperature for 24 hours. Both the species were tested for their bacteriocin activity against the respective pathogenic bacteria. From our present study, it is revealed that *Lactobacillus spp.* from buffalo milk has some probiotic properties and can be considered for use against various human pathogens.

**Keywords:** Lactic acid bacteria, *Lactobacillus spp.*, Buffalo milk, Bacteriocin, Antimicrobial activity, human pathogens

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#### INTRODUCTION

Milk is valued as nearly balanced diet and complete food, because all the essential components including proteins and minerals are available [1]. Also, milk is a proper medium for microorganisms because all the parameters for microbial growth e.g., pH, temperature, nutritional contents, water activity etc. are in optimal [2]. Various microorganisms including yeasts, molds and bacteria are present in raw milk but only the lactic acid bacteria (LAB) produce lactic acid by fermenting milk sugars. The term lactic acid is derived from lactique which was named by Lavoisier after discovery by Scheela. US FDA approved lactic acid as GRAS (Generally Recognized as Safe) for consumption as food additives. A Nobel laureate eminent scientist named Elie Metchnikoff stated that lactic acid bacteria are beneficial for health and help in promoting long life [3]. Lactic acid bacteria are the most studied microorganisms for beneficiary to human health and research on LAB are diversified for significant advancement [4].

Thus lactic acid bacteria predominate in raw milk. *Lactobacillus spp.* are widely known as lactic acid

producing bacteria and they are gram positive, non spore forming and coccobacilli or rod shaped and they secrete a bioactive protein named bacteriocin that produces antagonistic activity against many pathogens [5-6]. Bacteriocin cause death to pathogen by interfering cell wall synthesis or by causing pore formation and thus lactic acid bacteria determine therapeutic properties of milk [7]. Buffalo milk is rich in nutrition and is preferred by consumer [8]. Buffalo is generally stronger than cow or goat and produce fatter milk. The aim of our present study is to isolate potential *Lactobacillus spp.* from buffalo milk and to elucidate their antimicrobial activity. Buffalo milk containing potential *Lactobacillus spp.* will help in combating against some common pathogenic bacteria that are responsible for causing various gastrointestinal disorders in human. Thus our study will reveal a potential significance of local buffalo milk for beneficiary use against various gastrointestinal diseases.

#### MATERIALS AND METHODS

##### *Collection of sample*

A total of 10 raw milk samples of buffalo were collected from different regions in Chittagong district of

Bangladesh. Keeping in an ice box (4°C), the samples were transported immediately to the microbiology laboratory, University of Chittagong, Bangladesh, and analyzed for isolation and identification of *Lactobacillus spp.*

#### *Growing of lactic acid bacteria*

After enriching the milk samples in MRS broth [9] for 48 hours, one (1) ml of milk sample was mixed with pre sterilized 9 ml of saline water to make dilution of the sample to  $10^{-1}$ . Then, serial dilution procedure was followed and each sample was diluted up to  $10^{-7}$ . For isolation  $10^{-3}$  to  $10^{-7}$  dilutions were preferred and 0.1 ml from each dilutions were plated on MRS agar and incubated at 37°C for 24 hours (h). After incubation well isolated colony grown on MRS agar plates were randomly picked and morphologically distinct colonies were coded individually, sub cultured on MRS agar slants and preserved in freezer at 4°C for further study [10].

#### *Detection of potential lactic acid bacteria*

The isolated lactic acid bacteria were screened for their potentiality to show antagonistic activity against some selected gram negative and gram positive pathogenic bacteria. The test pathogens include: Gram positive bacteria- *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus* and Gram negative bacteria- *Escherichia coli*, *Shigella dysenteriae*, *Salmonella typhimurium*, *Vibrio cholerae*, *Pseudomonas aeruginosa*. The test pathogens were obtained from freeze-dried stock culture collection of Department of Microbiology, University of Chittagong, Bangladesh. The antagonism among the test pathogens and isolated LAB was detected by cross streak method [11]. Briefly, each pure isolated LAB culture was streaked individually on different nutrient agar (NA) plates in a single line. The plates were then incubated at 37 °C for 3 days to allow the isolates to secrete antimetabolite(s) into the medium. After the incubation period, the test pathogens were diluted and were cross-streaked along the line of fully grown isolates. Each streaking was started near the edge of the plates and streaked toward the growth line of the isolated LAB. The plates were then incubated for 24 hours at 37°C. The ability of any isolated LAB to inhibit growth of the test pathogen as indicated by a zone of inhibition along its growth line. The isolates showing growth inhibition against at least two test pathogens were considered as potential antagonistic LAB and further characterized, while the isolated LAB failed to show antagonism against at least two test pathogens were excluded from further characterization in our study.

#### *Identification of Lactobacillus spp.*

For identification, the selected LAB isolates were examined for their morphological characteristics, e.g., size, shape, cell arrangement and staining properties e.g., gram staining, spore staining, acid fast

staining. Cultural properties including form, colour, elevation, margin, surface of colonies on MRS agar plate and slant were also recorded. Physiological and biochemical characteristics of the isolates were evaluated by Voges–proskauer, methyl red, indole, catalase, oxidase, urease, citrate utilization, nitrate reduction, gelatin liquefaction and H<sub>2</sub>S production tests. The ability of the organisms in fermenting a number of carbohydrates including glucose, xylose, arabinose, lactose, inulin, glycerol, starch, and manitol were also performed. The isolates were identified up to species based on comparative analysis of the observed characteristics with the standard description of bacterial strains in Bergey's Manual of Determinative Bacteriology, 8<sup>th</sup> edition [12].

#### *Screening of bacteriocin activity*

The selected LAB isolates were grown at optimized conditions in MRS broth and the culture filtrates were obtained by centrifugation at 5000 rpm for 15 minutes. The culture filtrates were neutralized to pH 7 by 0.1N NaOH. The bacteriocin was assayed by agar well diffusion method [13].

#### *Antimicrobial activity at different incubation temperature*

The pathogens which showed susceptibility to the isolates in the previous cross streak method for detection of potential isolates were selected in this test. The isolates were grown in MRS broth medium for 48 hours at 10°C, 27°C, 37°C and 45°C. After incubation the culture broth were filtered through sterilized 0.2 µm pore size whatman filter paper (Whatman International Ltd., Maidstone, England). Then antimicrobial activity of the culture filtrates was assayed against the respective pathogenic bacteria by agar well diffusion method. Hundred (100) micro litre (µl) of culture filtrate was poured in each hole and after incubation the zone of inhibition was measured in milimetre (mm) scale.

#### *Antimicrobial activity at different incubation period*

To test antimicrobial activity at different incubation period, the isolates were grown in MRS broth medium at 37°C for 18, 24, 48, 72, 96 and 120 hours. Then antimicrobial activity of the culture filtrate was assayed in the same manner that was followed for different incubation temperature.

## **RESULT AND DISCUSSION**

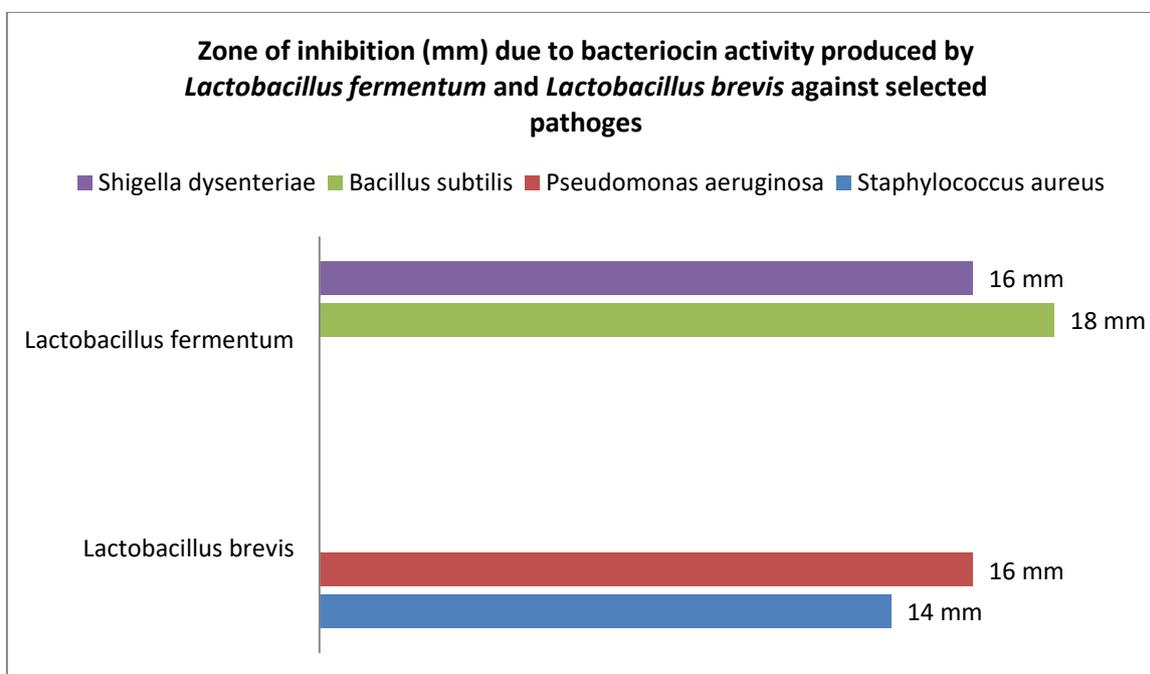
After pour plating of the buffalo raw milk samples on MRS agar media, isolates were then selected on the basis of colony characteristics. A total of 22 isolates were isolated from the milk samples, transferred to MRS agar slants and preserved.

*Screening of potential isolates and identification of lactic acid bacteria*

The isolates were examined for inhibitory activity against pathogenic bacteria and those isolates were considered as potential which inhibit at least two pathogenic bacterial growth. On the basis of inhibiting at least 2 pathogenic bacteria, 2 from the 22 isolates were found as potential. The coded B3<sub>4</sub> isolate found to inhibit the growth of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and B7<sub>3</sub> were found inhibitory against *Staphylococcus aureus*, *Shigella dysenteriae*. The two selected isolates were then assayed for cultural characteristics, morphology, staining properties, biochemical properties and fermentation to different carbohydrates. Scrutinizing the properties with that described in Bergey's Manual, the isolates were identified as *Lactobacillus brevis* (B3<sub>4</sub>) and *Lactobacillus fermentum* (B7<sub>3</sub>).

*Screening for bacteriocin activity*

Acidic nature of culture filtrate can inhibit the pathogenic bacteria. So, after neutralization of the culture filtrate with 0.1N NaOH the remained metabolites may be bacteriocin which is responsible for inhibiting growth of pathogenic bacteria [14-15]. The test was done using agar well diffusion method and the zone of inhibition representing in figure 1, produced by culture filtrate of the isolates was measured in millimeter (mm) scale. Though all the selected pathogenic bacteria were assayed for inhibitory activity against the isolates but similarity was found with the antagonistic activity analysis for each isolate by cross streak method. Bacteriocins from *Lactobacillus spp.* are proteinaceous exhibiting bactericidal mode of action, synthesized ribosomally and are effective against both gram positive and gram negative bacteria [16-17].



**Fig-1: Zone of inhibition (millimeter scale) showing bacteriocin activity against the selected pathogens by the isolates. Zone of inhibition by *Lactobacillus fermentum* against *Shigella dysenteriae* is 16 mm and *Bacillus subtilis* is 18 mm, and by *Lactobacillus brevis* against *Pseudomonas aeruginosa* is 16 mm and *Staphylococcus aureus* is 14 mm.**

*Antimicrobial activity at different incubation temperature*

The zone of inhibition produced against the selected pathogenic bacteria by the culture filtrate of the isolates after incubating them at 10°C, 27°C, 37°C and 45°C was considered for antimicrobial activity. In the result it was observed that identified LAB *Lactobacillus brevis* produced zone of inhibition against *Staphylococcus aureus* and *Pseudomonas aeruginosa* and the maximum inhibition zone diameter was 17 mm (millimeter) and 23 mm respectively for these two pathogens. Another identified LAB *Lactobacillus fermentum* was found to show maximum zone of

inhibition of 18.5 mm and 16 mm against *Bacillus subtilis* and *Shigella dysenteriae* respectively. From the figure 2 and figure 3, it is clear that both the LAB isolates revealed their maximum antimicrobial activity at incubation temperature of 37°C. Temperature is an important factor for proper growth of bacteria, and in that proper temperature bacteria show their best metabolic activity as well as release of high amount of metabolic bi-products like bacteriocin. So, to use *Lactobacillus brevis* and *Lactobacillus fermentum* as antagonistic to pathogenic bacteria they need to grow at 37°C temperature.

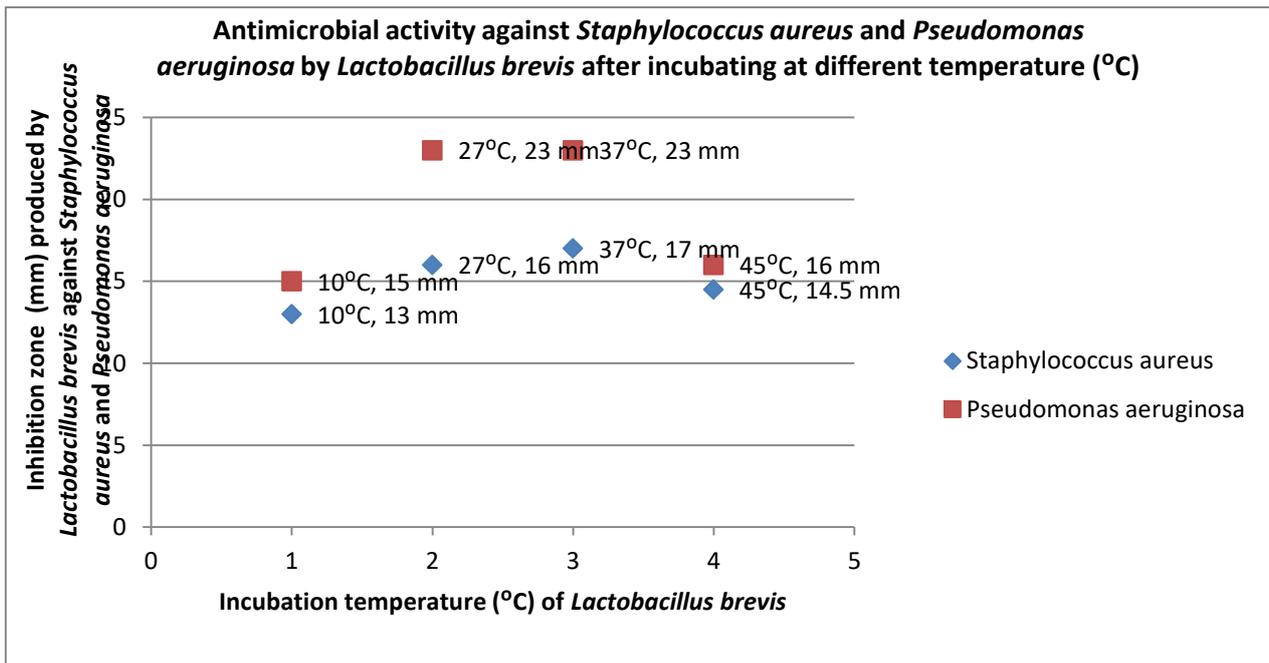


Fig-2: After incubating at different temperatures (degree centigrade), the isolates showing zone of inhibition against selected pathogens. Culture filtrates of *Lactobacillus brevis*, after growing it at different temperature produced zone of inhibition against *Staphylococcus aureus* (10°C- 13mm, 27°C- 16mm, 37°C- 17mm, 45°C- 14.5mm) and *Pseudomonas aeruginosa* (10°C- 15mm, 27°C- 23mm, 37°C- 23mm, 45°C- 16mm).

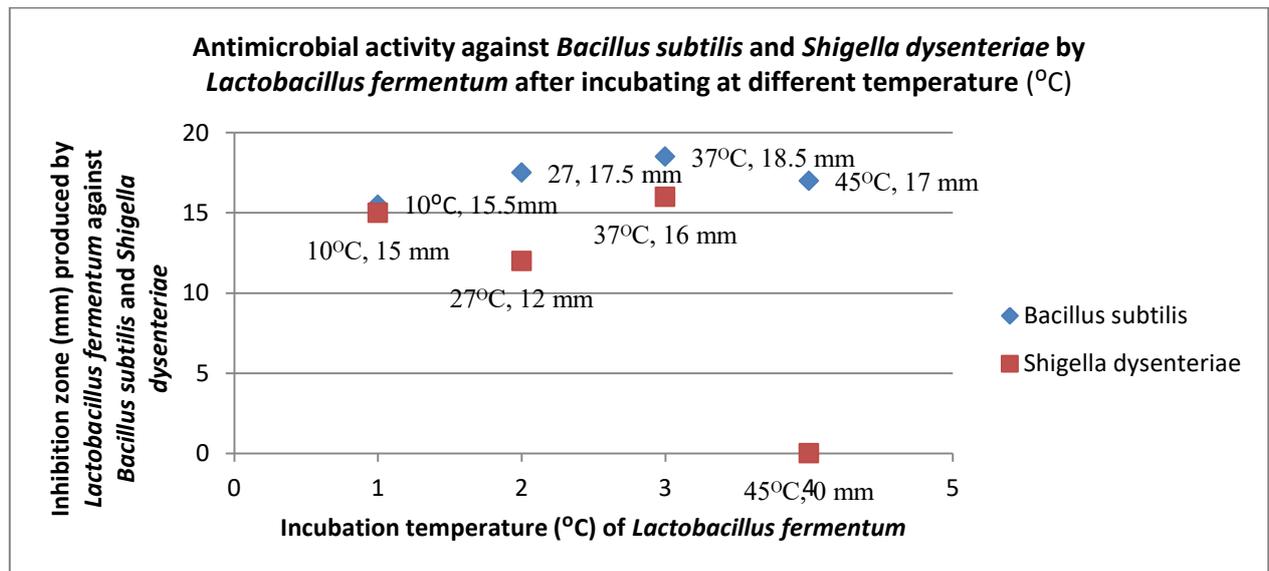


Fig-3: After incubating at different temperatures (degree centigrade), the isolates showing zone of inhibition against selected pathogens. Culture filtrates of *Lactobacillus fermentum*, after growing it at different temperature produced zone of inhibition against *Bacillus subtilis* (10°C- 15.5mm, 27°C- 17.5mm, 37°C- 18.5mm, 45°C- 17mm) and *Shigella dysenteriae* (10°C- 15mm, 27°C- 12mm, 37°C- 16mm, 45°C- 0mm).

*Antimicrobial activity at different incubation period*

MRS broth culture filtrates of the two isolates after growing for different incubation period *i.e.*, 18, 24, 48, 72, 96 and 120 hours at 37°C, analyzed against pathogenic bacteria, and it was observed that *Lactobacillus brevis* showed inhibitory activity against *Staphylococcus aureus* up to 96 hours of incubation and against *Pseudomonas aeruginosa* at 48 hours of

incubation. *Lactobacillus fermentum* showed inhibitory activity against *Shigella dysenteriae* up to 72 hours of incubation and against *Bacillus subtilis* at 48 hours of incubation. From the figure 4 and figure 5 it is lucid that the two LAB isolates are best antagonistic to pathogens at 24 hours of incubation. Our study shows similarity with findings of Fazeli *et al.* [18] where they observed best antagonistic effects of *Lactobacillus lactis* against

*Salmonella typhimuriam* after 24 hours of incubation. Antimicrobial activity is important criteria for *Lactobacillus spp.* to use against various diseases caused by pathogens. Our study reveals that the

identified *Lactobacillus brevis* can be used against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and *Lactobacillus fermentum* against *Bacillus subtilis* and *Shigella dysenteriae*.

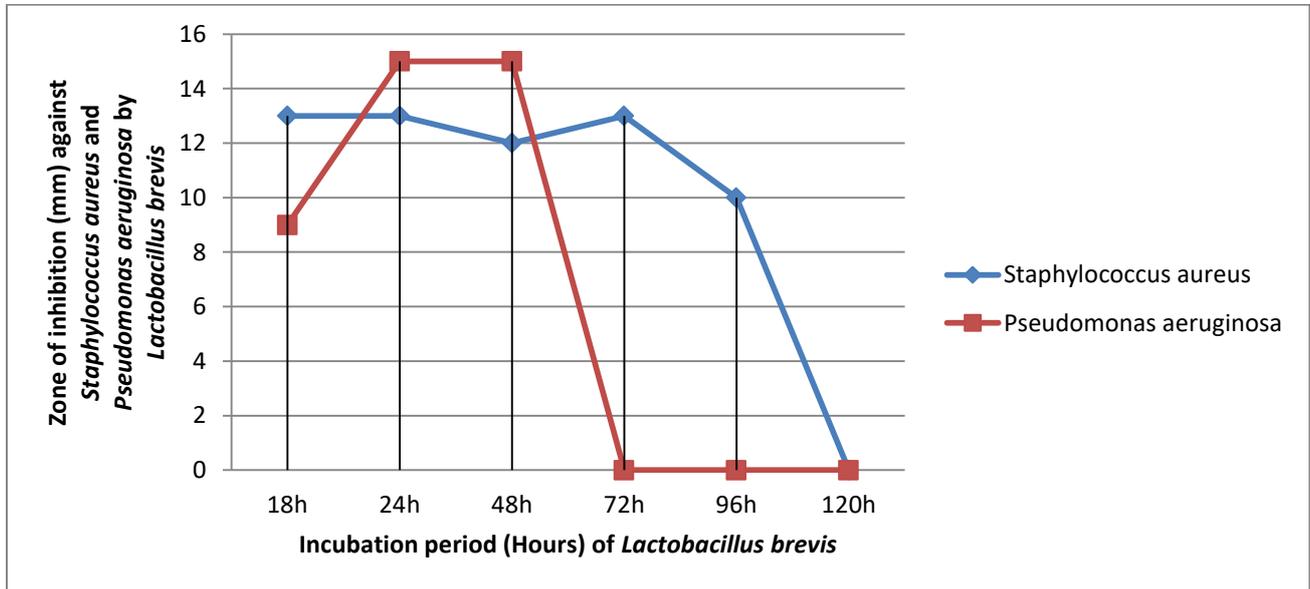


Fig- 4: Zone of inhibition against *Staphylococcus aureus* (18 h- 13mm, 24h- 13mm, 48h- 12mm, 72h- 13mm, 96h- 10mm, 120h- 0mm) and *Pseudomonas aeruginosa* (18h- 9mm, 24h- 15mm, 48h- 15mm, 72h- 0mm, 96h- 0mm, 120h- 0mm) due to treatment of culture filtrates of *Lactobacillus brevis* after growing it for different incubation period (Hours)

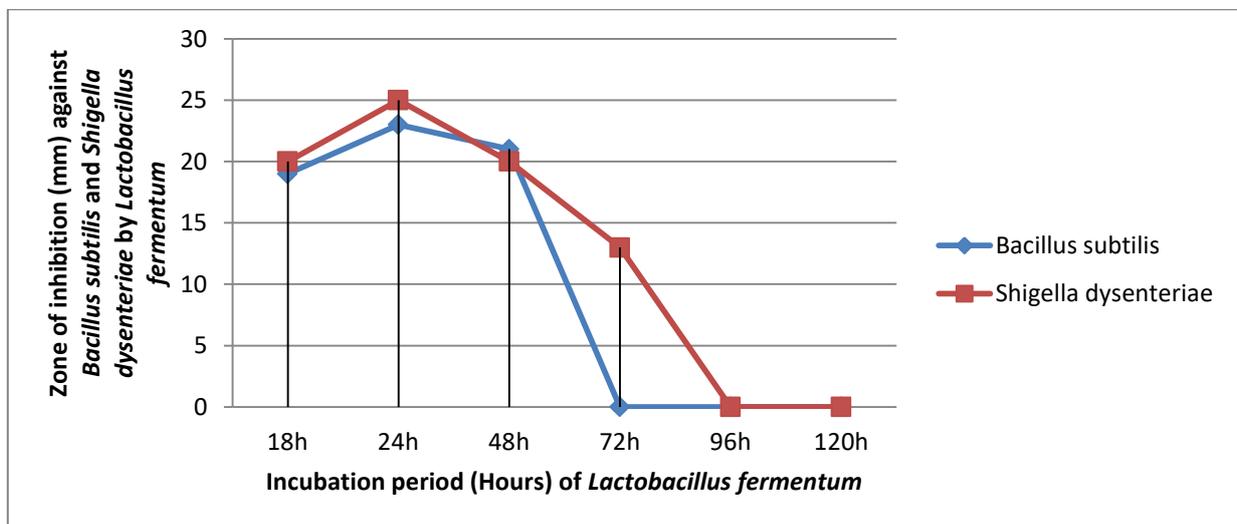


Fig-5: Zone of inhibition against *Bacillus subtilis* (18 h- 19mm, 24h- 23mm, 48h- 21mm, 72h- 0mm, 96h- 0mm, 120h- 0mm) and *Shigella dysenteriae* (18h- 20mm, 24h- 25mm, 48h- 20mm, 72h- 13mm, 96h- 0mm, 120h- 0mm) due to treatment of culture filtrates of *Lactobacillus fermentum* after growing it for different incubation period (Hours)

**CONCLUSION**

There are available researches on *Lactobacillus* species from different types of milk samples, but very few information are there on Chittagong, Bangladesh based study for *Lactobacillus* species from buffalo milk. We tried to identify only the potential *Lactobacillus spp* from the sample, and we got only 2 potential isolates from 10 buffalo milk samples.

Our target was to find out optimum incubation temperatures and incubation period for the isolates to show best antimicrobial activity against selected pathogens. The selected pathogens are very common causing different types of diseases mainly gastrointestinal disorders in human. Best outcome of antimicrobial activity against selected pathogens assure the isolates for beneficiary use in human. So, selecting

the optimum incubation period and incubation temperature, are important, because these parameters are important for formulating the isolates and for beneficiary use in human. Our result shows that identified *Lactobacillus fermentum* and *Lactobacillus brevis* can be used against common pathogenic bacteria, and for best beneficiary outcome they need to incubate at 37°C for 24 hours. The study was done targeting some parameters but for final conclusion more *in vitro* tests have to be carried out with pragmatic usage.

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