

## Assessment of Potential Probiotic *Lactobacillus* Strains Isolated from Goat Milk

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**Abstract:** Probiotics are a group of microbes that may help directly in enhancing resistance against human intestinal pathogens and in the prevention of gastrointestinal disorders. This study aimed to isolate *Lactobacillus* spp. from various raw goat milk samples for their probiotic properties. A total of 16 isolates were primarily screened from the collected goat milk samples. Only 3 isolates were found to exhibit remarkable inhibitory activity against multiple pathogenic bacteria. These probiotic strains were analyzed to elucidate their cultural, morphological and biochemical features, and were identified as *Lactobacillus bulgaricus*, *Lactobacillus casei* subsp. *casei*, and *Lactobacillus helveticus*. The strains were found to exhibit the maximum growth at pH 7 and 37°C temperature for 18 hours of incubation. In addition, they were found to produce antimicrobial metabolites that are inhibitory to pathogens, able to tolerate bile salt at concentration upto 3%, but their inhibitory activity was hampered with trypsin treatment at concentration of 0.5 mg/ml. The strains were found susceptible to erythromycin, chloramphenicol, gentamicin and ciprofloxacin, but resistant against penicillin-G, ampicillin and amoxicillin. The present study thus elucidates that goat milk naturally inhabits *Lactobacillus* spp. which can be potential candidates in treatment of various infectious diseases in human mainly related with gastrointestinal system.

**Keyword:** Antimicrobial activity, Bacteriocin, Goat milk, *Lactobacillus* spp., Probiotics

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### I. Introduction

Lactic acid bacteria (LAB) occur naturally as indigenous microflora in raw milk. Goat milk is the second variety of milk mostly produced in the world, and it could be an alternative to substitute the consumption of cow milk due to evidence that it does not induce allergies, present high digestibility, and also possess high nutritional quality [1].

Study on lactic acid bacteria specially *Lactobacillus* spp. are drawing interests significantly in worldwide due to its applied beneficiary effects in the prevention, control and treatment of diseases and health maintenance [2]. Now, *Lactobacillus* spp. are widely used as probiotics which play a key role in enhancing resistance to colonization by exogenous, potentially, pathogenic organisms in the intestinal tract. They produce a variety of substances e.g., bacteriocin, nisin, lactacin etc. that are effective against different types of enteric pathogens like *E. coli*, *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Bacillus* spp., *Klebsiella* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Proteus* spp. etc. These effects can be described as the improvement of lactose digestion and the treatment of diarrheal disorders [3]. Moreover, *Lactobacillus* spp. are considered as alternative to antibiotics due to emergence of antibiotic resistance pathogens [4].

Raw milk represents a source of new strains of LAB with the potential to inhibit undesirable microflora [5]. Our present study was designed to characterize some *Lactobacillus* spp. from various goat milk samples collected from different regions of Chittagong district to investigate their optimum growth conditions, and some probiotic properties like sensitivity to antibiotics, antagonistic activity to pathogenic bacteria, bile salt tolerance, and sensitivity to proteolytic enzymes. *Lactobacillus* strains having probiotic properties are effective to treat various gastrointestinal disorders in human. So, the outcome of this study may reveal the quality of local goat milk of Chittagong district for inhabiting potential probiotic *Lactobacillus* strains.

### II. Materials And Method

#### 2.1 Sample

Three raw goat milk samples were collected from three different rural areas mainly Raozan, Rangunia and Hathazari of Chittagong district. Milk samples were collected aseptically in sterile glass bottles, and packed in sterile polythene bags. After packing, the milk samples were carried to microbiology laboratory, university of Chittagong within four hours, via sampling carrier with maintaining the temperature at 4°C.

## 2.2 Isolation of *Lactobacillus* spp.

Colonies were grown on nutrient agar (NA) medium, and colonial growths with different cultural appearances were streaked on individual de-Mann, Rogosa and Sharpe (MRS) agar plates. MRS agar media is a selective media for isolation of *Lactobacillus* spp. [6]. After incubating the plates at 37 °C, well isolated colonies were purified by repeated plating. Pure culture of obtained *Lactobacillus* spp. were maintained in MRS agar slant for subsequent analysis.

## 2.3 Detection of antagonistic activity of the isolated *Lactobacillus* spp.

Some commonly encountered pathogenic bacteria were considered to demonstrate the antagonistic activity of isolated *Lactobacillus* spp. The 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 pathogens were obtained from freeze-dried stock culture collection of Department of Microbiology, University of Chittagong. The antagonism among pathogens and the isolated *Lactobacillus* spp. were detected by cross streak method [7]. Briefly, each pure isolated *Lactobacillus* culture was streaked individually on different 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 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 *Lactobacillus* spp. The plates were then incubated for 24 hours at 37°C. The ability of any isolated *Lactobacillus* to inhibit growth of the pathogens as indicated by a zone of inhibition along its growth line. The *Lactobacillus* spp. showing growth inhibition against at least two pathogens were considered as potential antagonistic and further characterized; while the isolated *Lactobacillus* spp. failed to show antagonism against at least two test pathogens were excluded from further characterization in our study.

## 2.4 Identification of isolated *Lactobacillus* spp.

The selected isolates were examined for their morphological properties, such as size, shape, cell arrangement and staining properties. 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 isolates in fermenting a number of sugars 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].

## 2.5 Optimization of growth parameters

Optimum temperature, pH and incubation period were determined. Optimum growth temperature was determined by growing the selected isolates in MRS broth and incubating at different temperatures (10, 27, 37 and 45°C). MRS broth of different pH (pH 2, 3, 5, 7 and 9) were inoculated and incubated to determine the optimum pH. The selected isolates were incubated for different duration (18, 24, 48, 72, 96 and 120 hours) to determine the optimum incubation period. The optimum parameters for highest growth of the identified *Lactobacillus* spp. were determined by measuring and comparing the optical density (OD) at 600 nm (OD<sub>600</sub>).

## 2.6 Assay of antibacterial activity by agar well diffusion method

Antimicrobial activity of *Lactobacillus* strain is mainly due to secretion of bacteriocin and other antimicrobial metabolites. To qualify screening procedure, antimicrobial activity test was retested by agar well diffusion method. The isolates were incubated on MRS broth at 37 °C for 24 hours. After incubation the culture broth were filtered with the help of whatman filter paper (Whatman International Ltd., Maidstone, England), and the culture filtrate was assayed against all the pathogenic bacteria. Antibacterial activity of the *Lactobacillus* strain was determined by measuring the “zone of inhibition” expressed in millimeter (mm) in diameter.

## 2.7 Bile salt tolerance of the isolates

10 ml MRS broth was prepared with varied concentration (i.e.; 0.1, 0.5, 1.0, 2.0 and 3.0% [w/v]) of bile salt. After inoculation with 0.5ml inoculums of each selected *Lactobacillus* spp., the tubes were then incubated at 37°C for 24 hours. After incubation, 0.1 ml of culture from each tube was pour plated on NA media and incubated at optimized growth conditions. The degree of tolerance was evaluated by comparing the number of colony forming unit (cfu) produced on the plates [9].

## 2.8 Assay of antibiotic sensitivity pattern

To assess the antibiotic sensitivity pattern, disk diffusion method was followed. Culture inoculums of the isolates grown in MRS broth was taken as amount of 0.5 ml, and was mixed in 5 ml of the same medium containing 0.5% agar, and aseptically poured into glass Petri dishes containing MRS agar medium. The antibiotic disks (Penicillin G, Ampicillin, Amoxicillin, Ciprofloxacin, Gentamicin, Chloramphenicol, and Erythromycin; manufacturer-oxid) were placed on the surface of the plate at equidistance. The plates were then kept at 4°C for 1 hour for proper diffusion of antibiotics. The plates were incubated at 37 °C for 24 hours. The antibiotic sensitivity or resistance was determined by observing the presence of zone of inhibition. The zone of inhibition was measured by a millimeter scale [10] [11].

## 2.9 Sensitivity to proteolytic enzymes

The isolated *Lactobacillus* spp. were grown in MRS broth, incubated at optimized conditions and centrifuged to collect cell free supernatants. The supernatants were treated with trypsin (0.5 mg/mL) for 1 hour at 30 °C, respectively. The treated supernatants were heated at 100 °C for 20 minutes. The resultant supernatants were used for assessment of antimicrobial activity as described by Nowroozi *et al* [12].

# III. Results

## 3.1 Isolation of antagonistic organisms

It was a primary screening of isolating a few potential *Lactobacillus* spp. from large number of unidentified *Lactobacillus* isolates. Bacterial colonies from NA medium and, repeated streaking them on MRS agar plates; 16 heterotrophic bacterial isolates were isolated. The isolates were tested for antagonistic activity against selected test pathogens and the isolate which inhibit at least two pathogenic bacterial growth were considered as potential. On the basis of better growth inhibition of test pathogens by the isolates, three isolates coded as R<sub>8a</sub>, R<sub>10b</sub>, R<sub>12a</sub> from sixteen isolates (R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub>, R<sub>5</sub>, R<sub>6</sub>, R<sub>7</sub>, R<sub>8a</sub>, R<sub>8b</sub>, R<sub>8c</sub>, R<sub>9</sub>, R<sub>10a</sub>, R<sub>10b</sub>, R<sub>11</sub>, R<sub>12a</sub>, R<sub>12b</sub>) were selected for subsequent studies.

## 3.2 Identification of *Lactobacillus* spp.

The three selected potential isolates grown on MRS agar plates were investigated for their morphological, cultural, physiological, and biochemical features. After scrutinizing the properties with that described in Bergey's Manual, and the isolates R<sub>8a</sub>, R<sub>10b</sub>, R<sub>12a</sub> were identified as *Lactobacillus bulgaricus*, *Lactobacillus casei* subsp. *casei* and *Lactobacillus helveticus* respectively.

## 3.3 Optimum Growth parameters for the selected *Lactobacillus* spp.

To investigate optimum growth parameters of the selected isolates, they were incubated at different pH of the media, at different incubation temperature for different incubation period. The growth of the selected isolates at different pH of the medium (pH 2, 3, 5, 7, 9) were observed. The three isolates were observed best growth at neutral pH (Fig. 1). Decreasing of pH or increasing of pH from neutral value is less growth yielding of the isolates. Similarly, it was also found that the isolates showed best growth at temperature 37°C (Fig. 2) and at incubation period of 18 hours (Fig. 3).

## 3.4 Assay of antibacterial activity by agar well diffusion method

Culture filtration excludes cell debris and extraneous materials. So, after filtration the culture filtrate contains only a mixture bacteriocin and other antimicrobial metabolites. The zone of inhibition produced (Table-1) by the isolates against target pathogens indicates it's potentiality of antimicrobial properties. Identified Isolate *Lactobacillus bulgaricus* showed inhibitory properties against *Bacillus subtilis* and *Pseudomonas aeruginosa*, *Lactobacillus casei* subsp. *casei* against *Bacillus cereus*, *Staphylococcus aureus*, *Vibrio cholerae*, and *Lactobacillus helveticus* against *Bacillus subtilis* and *Bacillus cereus*. These isolates were tested against all the selected pathogenic bacteria but the outcome was similar to the result obtained during antagonistic assessment for each isolates by cross streak method.

## 3.5 Bile salt tolerance of the isolates

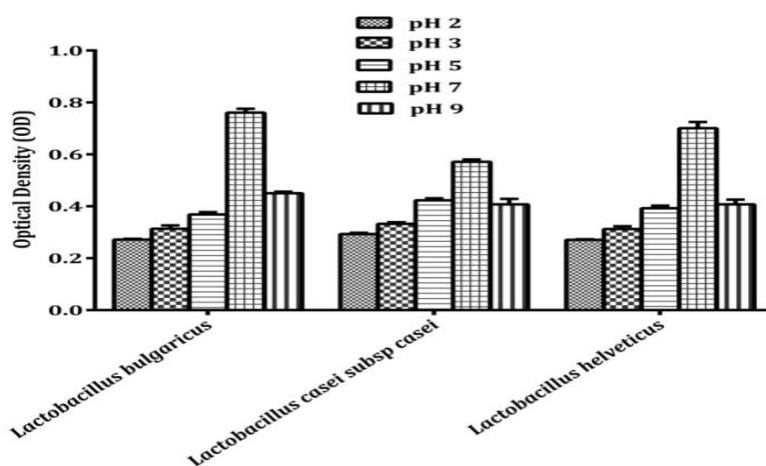
Probiotic organisms have to tolerate bile salt concentration in the intestinal tract or stomach for their survival. The three isolates were assayed for their sensitivity to bile salt concentration (i.e.; 0.1, 0.5, 1.0, 2.0 and 3.0% [w/v]). It was observed that growth of the selected isolates decreases with the increasing of bile salt concentration in the medium and our study shows that the isolates can tolerate bile salt having concentration upto 3% (Fig. 4).

### 3.6 Assay of antibiotic sensitivity pattern

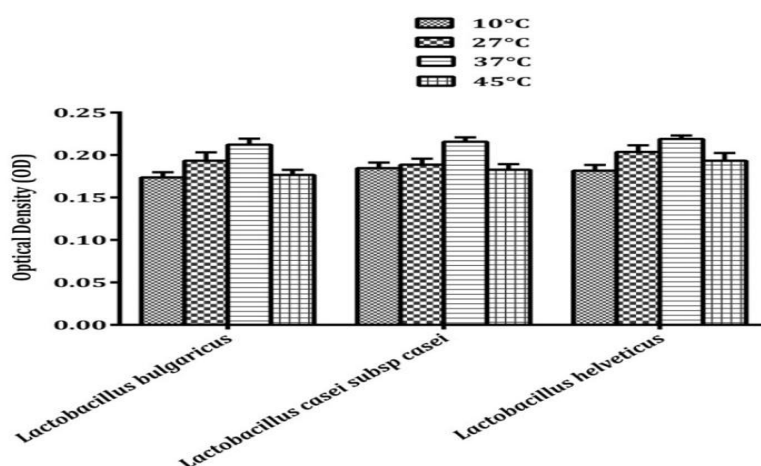
Knowing the isolates either susceptible or resistant to antibiotics is important because therapeutic activity of the isolates may be influenced when a combination is occurred with antibiotics. The result showed that all the three isolates were susceptible to erythromycin, chloramphenicol, gentamicin and ciprofloxacin but resistant to penicillin-G, ampicillin and amoxicillin (Table- 1).

### 3.7 Sensitivity to proteolytic enzymes

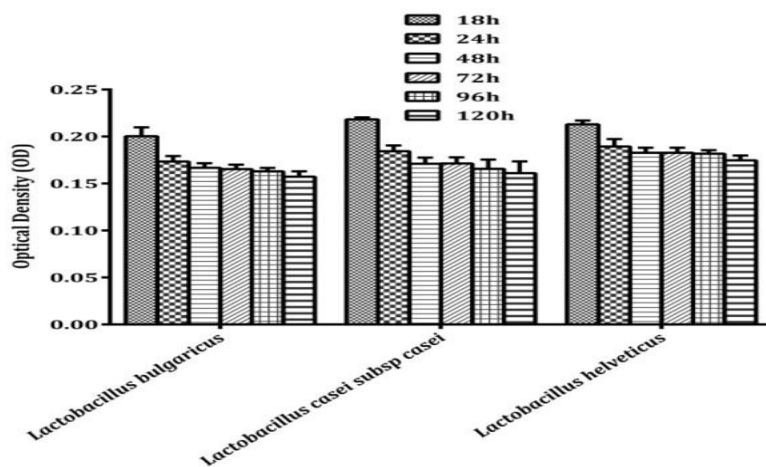
Sometimes proteolytic enzyme may inactivate the proteinaceous antimicrobial substances. To observe the influence of proteolytic enzymes on bacteriocin and other antimicrobial metabolites, the culture supernatant was treated with a proteolytic enzyme trypsin. The Table-1 showed that the antibacterial activity of bacteriocin and other antimicrobial metabolites were destroyed by trypsin treatment (0.5 mg/ml), as the respective pathogenic bacteria showed resistance against the isolates.



**Fig. 1:** *Lactobacillus bulgaricus* (R8a), *Lactobacillus casei subsp. casei* (R10b), and *Lactobacillus helveticus* (R12a) were grown at different pH (2, 3, 5, 7, and 9) in MRS broth medium for 24 hours. The optimum pH for highest growth of the identified *Lactobacillus spp.* was determined measuring and comparing the optical density (OD) at 600 nm. OD was measured by using Spectrophotometer T60 UV-Visible Spectrophotometer (PG INSTRUMENTS, UK). The data is representative of three independent experiments.

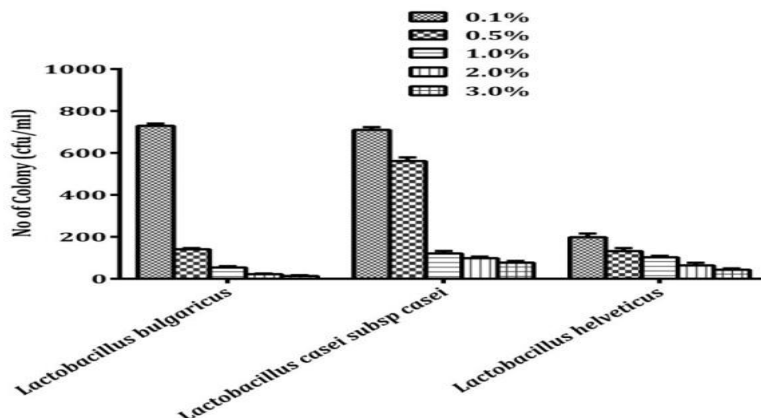


**Fig. 2:** *Lactobacillus bulgaricus* (R8a), *Lactobacillus casei subsp. casei* (R10b), and *Lactobacillus helveticus* (R12a) were grown in MRS broth medium for different incubation temperatures i.e, 10 °C, 27 °C, 37 °C, 45 °C. The optimum temperature for highest growth of the identified *Lactobacillus spp.* was determined measuring and comparing the optical density (OD) at 600 nm. OD was measured by using Spectrophotometer T60 UV-Visible Spectrophotometer (PG INSTRUMENTS, UK). The data is representative of three independent experiments.



**Fig. 3:** *Lactobacillus bulgaricus* (R8a), *Lactobacillus casei subsp. casei* (R10b), and *Lactobacillus helveticus* (R12a) were grown in MRS broth medium for different incubation periods i.e, 18h, 24h, 48h, 72h, 96h, 120h .

The optimum incubation period for highest growth of the identified *Lactobacillus spp.* was determined measuring and comparing the optical density (OD) at 600 nm. OD was measured by using Spectrophotometer T60 UV-Visible Spectrophotometer (PG INSTRUMENTS, UK). The data is representative of three independent experiments.



**Fig. 4:** *Lactobacillus bulgaricus* (R8a), *Lactobacillus casei subsp. casei* (R10b), and *Lactobacillus helveticus* (R12a) were grown in MRS broth medium containing different concentrations of bile salt i.e., 0.5%, 1.0%, 1.5%, 2.0%, 3.0% for 18 h at 37 °C. After incubation, pour plate method was used to assess sensitivity of the isolates to bile salt tolerance. The data is representative of three independent experiments.

**Table-1: Antibiotic sensitivity pattern, inhibition activity of the isolates, and assessment of trypsin effects on inhibitory activity of the isolates**

Isolates	Sensitivity to different antibiotics							Pathogenic bacteria	Zone of inhibition due to secretion of bacteriocin and other antimicrobial metabolites by the isolates against pathogenic bacteria	Inhibitory activity of bacteriocin and other antimicrobial metabolites of the isolates against pathogenic bacteria after treatment with trypsin (0.5 mg/ml)
	Penicillin-G	Ampicillin	Erythromycin	Chloramphenicol	Amoxicillin	Gentamicin	Ciprofloxacin			
<i>Lactobacillus bulgaricus</i>	-	-	32 mm	21 mm	-	12 mm	30 mm	<i>Bacillus subtilis</i>	18.5 mm	-
								<i>Pseudomonas aeruginosa</i>	17.5 mm	-
<i>Lactobacillus casei subsp casei</i>	-	-	15 mm	34.5 mm	-	16 mm	33 mm	<i>Bacillus cereus</i>	19 mm	-
								<i>Staphylococcus aureus</i>	17 mm	-
								<i>Vibrio cholerae</i>	17 mm	-
<i>Lactobacillus helveticus</i>	-	-	35 mm	25 mm	-	14 mm	26 mm	<i>Bacillus cereus</i>	18 mm	-
								<i>Bacillus subtilis.</i>	21 mm	-

Note: “mm” indicates the measurement of zone of inhibition by the isolates against the pathogenic bacteria, “-” indicates resistance to the isolates or incapability to inhibit the pathogenic bacteria by the isolates.

#### IV. Discussion

The study was designed to identify some lactic acid bacteria (LAB) especially *Lactobacillus spp.* from goat milk samples collecting from different rural areas of Chittagong, Bangladesh. Lactic acid bacteria has beneficial effects on human gastrointestinal tract like correction of lactose malabsorption, alleviation of viral and drug induced diarrhea, inflammatory bowel syndrome, absorption of fatty acids through intestine etc. LAB produce these beneficial effects by restoration of normal intestinal flora, elimination of intestinal pathogens, reinforcement of intestinal barrier capacity to foreign antigens, stimulation of nonspecific immunity such as phagocytosis, stimulation of humoral immunity and production of anti-inflammatory products [13].

MRS agar media is used for cultivation of *Lactobacillus spp* [14]. So, after growing the isolates on MRS agar media they were assayed for antagonistic properties in NA medium. Three isolates were selected for further research from the antagonistic result of the isolates against minimum two pathogenic bacteria. The *Lactobacillus spp* can produce acetate, ethanol, carbon di-oxide (CO<sub>2</sub>), hydrogen peroxide, di-acetyl and bacteriocins which have inhibitory effects towards other bacteria especially against pathogen bacteria like *E. coli*, *Pseudomonas aeruginosa* [15].

Growth conditions influence on the growth of the bacteria. Our study was to identify optimum growth parameters for *Lactobacillus bulgaricus*, *Lactobacillus casei subsp. casei* and *Lactobacillus helveticus* because bacteriocin production from *Lactobacillus spp.* is also dependent on incubation parameters [16] [17]. *Lactobacillus salivarius* can show highest cell growth at pH 6.5 of MRS broth medium and at 37°C incubation temperature [18]. *Lactobacillus spp.* can grow after treatment of their growing media with 2% or 3% concentration of bile salt [10] [19]. In our experiment we found that our isolated *Lactobacillus spp.* are able to grow at 3% bile salt concentration treated medium. Resistance to bile salt toxicity is one of the criteria used to select probiotic strains that would potentially be capable of performing effectively in human gastrointestinal tract [20]. Colonization in gastrointestinal tract is an important criterion for LAB and ability to adhere to the intestinal epithelium is regarded as a prerequisite to colonize the human gastrointestinal tract (GIT) for exerting beneficial effects like exclusion of enteropathogenic bacteria [21].

LAB are generally susceptible to antibiotics due to inhibition in their protein synthesis [21]. Resistance to antibiotics indicates that the isolated *Lactobacillus spp.* if introduced in patients treated with antibiotic therapy may be helpful in controlling intestinal disorders. On the other hand, sensitivity indicates that patients taking these antibiotics will not be appropriate for probiotic treatment. So, our isolated strain will not be used along with the erythromycin, chloramphenicol, gentamicin and ciprofloxacin.

Bacteriocin and other antimicrobial metabolites produced from *Lactobacillus spp.* are inhibitory to various pathogenic bacteria like *Bacillus subtilis*, *Bacillus Megaterium*, *Stapylococcus aureus*, *E. Coli* etc. [22] [23]. Our present study showed that bacteriocin or bacteriocin like metabolites secreted from the *Lactobacillus bulgaricus*, *Lactobacillus casei subsp casei* and *Lactobacillus helveticus* are also inhibitory against the selected pathogens. Bacteriocin forms the pores in the membrane of sensitive cells and depletes the transmembrane potential and /or the pH gradient resulting in the leakage of cellular materials [24]. Human gastro-intestinal tract can be considered as enzymatic system. Trypsin is secreted from pancrease which is also called an endo-enzyme hydrolyses the internal peptide bounds of the outer layers of bacterial cells [25]. So, trypsin can inhibit activity of isolated *Lactobacillus spp.* in gastrointestinal tract. In our study, trypsin concentration of 0.50 mg/ml affects on selected *Lactobacillus spp.* because the isolates treated with that concentration inhibited antagonistic properties (Table- 1) against selected pathogenic bacteria. A similar study was also observed from Nowroozi et al [12].

#### V. Conclusion

Goat milk is an alternative choice of cow milk, and people in the rural areas are used to goat farming or cow farming. As our study suggests that goat milk is a proper source of potential probiotic properties containing *lactobacillus spp.*, so the people can choose goat milk considering not only the nutritious quality but also it's preventive efficiency to various disease caused by enteropathogenic bacteria.

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