Antagonism of Lactic Acid Bacteria Against Selected Pathogenic Bacteria and Spoilage Fungi


1Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia 7003, Bangladesh
2Department of Biochemistry and Molecular Biology, Jahangirnagar University, Savar, Dhaka 1342, Bangladesh

Abstract: Lactic acid bacteria (LAB) have been studied for many years to be used as biopreservative due to production of various antimicrobial compounds. This study was carried out for the determination of natural antimicrobial activity of LAB isolated from curd samples. LAB were screened for antagonistic activity against Gram-negative Escherichia coli and Gram-positive Staphylococcus aureus and Bacillus subtilis. The inhibitory activity was determined by a disc diffusion method. Strong (+++) and moderate (++) inhibition were found for E. coli and weak (+) inhibition was found for S. aureus. No zone of inhibition was found for B. subtilis. Studies were made to compare the production of antibiotic components by LAB in different nutrient media (LAPT broth with different supplements). The media LAPT with riboflavin (1.5 mg/l) and LAPT with vitamin B mixture (1.5 mg/l) showed the positive result. LAB were also screened for antagonism against spoilage fungi isolated from potato samples and showed antifungal activity against Aspergillus niger. This study led to suggest that food-derived LAB strains could be selected for biotechnological application to control pathogenic bacteria and spoilage fungi.

Key words: Biopreservative • Antagonism • Lactic Acid Bacteria • Pathogenic Bacteria • Spoilage Fungi

INTRODUCTION

The search for alternative bioproducts to replace chemicals and toxic pesticides, has found growing interest in recent times because the extensive use of synthetic chemicals and pesticides in food and agriculture may pose a health risk for human and animals and affect the ecological equilibrium of the environment [1]. For this purpose, using bacteria or natural compounds which exhibit the same inhibitory effect on pathogenic and spoilage microbes was not only shown to be efficient in storage life extension and nutritive and safety value retention, of food products but also the environment safeguarding [2, 3]. Such bacteria are known as “biological control agents” [4].

Lactic acid bacteria (LAB) are a group of Gram-positive, nonspore forming, immobile and catalase negative bacteria, which excrete lactic acid as major end product and generally recognized as safe (GRAS) organisms [5]. They are also selected as probiotic, which are able to promote health and prevent infections against enteropathogenic bacteria [6, 7]. Different Lactobacillus spp. isolated from yoghurt samples collected from different regions of Bangladesh showed variation in probiotic properties [8]. LAB usually harbor in carbohydrate-rich environments and are found in various food products such as milk, plant, meat, intestinal mucosa of human and animals [6, 9] but especially proliferate in different fermented foods [10]. Owing to particular physiological and biochemical traits,
such as exopolysaccharide production, organic acids, aromatic compounds, tolerance to low water activity and antimicrobial production \[3, 11, 12\], LAB found different industrial applications, either by their biopreservatives or techno-functional properties \[13, 14\].

LAB have been studied for their inhibitory action against different microbes but not well established yet. Antagonistic activity of LAB is connected with the inhibitory compounds they produce. Metabolites with inhibitory properties include lactic acid, hydrogen peroxide and bacteriocins \[15\]. Growing attention has been devoted to bacteriocins, which play a considerable role in regulation of pathogenic microorganisms in the food and environment. Bacteriocins are ribosomally synthesized antimicrobial peptides that are active against other bacteria, either of the same species (narrow spectrum), or across genera (broad spectrum). These are typically plasmid borne, heat resistant and typically bactericidal in action \[16, 17\].

LAB have been reported to show antagonistic properties against pathogenic microorganisms. Therefore it is possible to use LAB as biopreservative. In this study, pathogenic bacteria were collected and spoilage fungi were isolated from potato samples and LAB were isolated from curd samples. Then antimicrobial activity of LAB against pathogenic bacteria (Escherichia coli, Staphylococcus aureus and Bacillus subtilis) and spoilage fungi (Aspergillus niger) were investigated to enumerate their antagonistic properties.

**MATERIALS AND METHODS**

**Collection of Bacterial Isolates:** Bacterial isolates namely Escherichia coli, Staphylococcus aureus, Bacillus subtilis were collected from stock culture of food microbiology section, IFST, BCSIR, Dhaka, Bangladesh. Pure cultures of bacteria were maintained at 4°C on nutrient agar slants. Morphological characteristics such as shape, size, form, opacity and pigment production turbidity of the 24 hours bacterial cultures were observed. The identity of these bacterial isolates was confirmed through conventional biochemical tests \[18\].

**Isolation and Identification of Fungi (Aspergillus Niger):** The Aspergillus niger was isolated from potato (Solanum tuberosum) samples collected from Hatirpul maket of Dhaka city. Sample of 20 gm was transferred aseptically into sterile conical flask jar and 180 ml Ringer solution was added. The sample was homogenized with a blander (electric) at 6000 RPM for 5-10 minutes and diluted as needed. About 0.2 ml of the diluted homogenate was spread onto each Petri dish containing potato dextrose agar (PDA). The sample was homogenously distributed on the plate using a glass spreader in a backward and forward movement while rotating the plate. Then the plates were covered leaving them for 1-2 hours to dry before inverting and inoculation. The prepared dishes were incubated at room temperature for 48-72 hours. The plates were screened for the presence of discrete colonies after the incubation period. All colonies on the dishes were counted using a colony counter (Yc-2A, pram optical works Ltd Tokyo) and following back calculation, the actual numbers as colony forming unit (CFU/gm or ml) and the results per dilution were recorded.

Aspergillus isolates were identified in the level of genus following standard method \[19\] on Sabouraud Glucose Agar 4% (SGA4%). To improve the sensitivity and specificity of routine culture approach for identification of Aspergilli in the level of species, four differential media were used including czapek dox agar (CZ), czapek yeast agar (CYA), malt extract agar (MEA) and czapek yeast 20% sucrose agar. Morphological features of Aspergillus cultures were studied, the major and remarkable macroscopic features in species identification were the colony diameter, color (conidia and reverse), exudates and colony texture. Riddle’s classic slide culture method \[20\] was used for microscopic study of standard strains and most of our isolates. Microscopic characteristics for the identification were conidial heads, stipes, color and length vesicles shape and seriation, metula covering, conidia size, shape and roughness also colony features including diameter after 7 days, color of conidia, mycelia, exudates and reverse, colony texture and shape. Fungi was picked up carefully by using sterile loop and transferred into PDA slant and then incubated at room temperature.

**Isolation of LAB:** LAB were isolated from curd sample collected form Hatirpul market of Dhaka city by the accumulation method as described by Chen et al. \[21\], with some modifications. Samples of 1 g were aseptically transferred into 15 mL tubes containing 5 mL of Mann Rogasa Sharpe (MRS) broth and incubated anaerobically at 30°C for 3 days. After incubation, samples were serially diluted in 0.75% NaCl solution. Fractions of 0.1mL of the dilutions ranging between 10^-2 and 10^-8 were plated in duplicate on the surface of MRS agar supplemented with 0.0025% of bromocresol green and 0.01% cycloheximide to inhibit fungal growth. The plates were incubated in the same conditions. The different colonies of acid-producing
bacteria, determined by a yellow zone in the media around each colony, were picked and purified on MRS agar. Overnight incubated cultures of isolated were Gram-stained and examined microscopically for morphology and phenotype. Catalase test was performed by adding few drops of 3% hydrogen peroxide in a tube containing 24 h old culture of each isolate. Gram-positive and catalase-negative isolates were selected and maintained in broth with 25% glycerol at -80°C for further identification. The isolates were also tested for gas production from D-glucose (using inverted Durham tubes in MRS broth), growth at different temperatures (10 and 45°C), different pH (4.0 and 9.6) and different concentration of NaCl (3, 6.5, 8 and 9%) in MRS broth.

**Antagonistic Activity of LAB Against Pathogenic Bacteria:** Isolated LAB (isolates were designated as L1, L2 and L3 that grew at 37°C, 23°C and 45°C respectively) were maintained in sterile skim milk. The cultures were growing in sterile skim milk for hours at 37°C, 23°C and 45°C respectively.

The strains of LAB were screened for their ability to inhibit the growth of *E. coli*, *S. aureus* and *B. subtilis*. The antimicrobial activity was determined by disc diffusion assay. Melted nutrient agar was inoculated with 1% of an 18-24 hour old broth culture of the test organism. Eight milliliter of this seeded agar were poured into sterile Petri dishes and allowed to solidify. A sterile filter paper disc of 6.25 mm diameter was dipped into the LAB culture and disc placed on the seeded agar surface. Several discs could be placed on one plate. Each test culture was assayed in duplicate. The plates were incubated at 5°C for 2 hours to allow the test material to diffuse into the agar and then incubated at 37°C, 23°C and 45°C according to the type of LAB for 16-18 hours. After incubation the plates were examined for zone of inhibition around the various discs. Very strong inhibition (+++++) was assigned to zones of 20 to 25 mm diameter, correspondingly, strong inhibition (+++) 15 to 18 mm diameter, moderate inhibition (+) 12 to 14 mm diameter, weak inhibition (+) 9 to 10 mm diameter, no inhibition (-) and doubtful (±) cases were also recorded.

Next studies were made to compare the production of the antibiotic component(s) by LAB in different media containing LAPT (including yeast extract, peptone, tryptone, glucose and Tween) broth with different supplements. The following media were used: LAPT+ casein (1%), LAPT+ skim milk (1%), LAPT+ Riboflavin (1.5 mg/l), LAPT+ vitamin-B mixture (1.5 mg/l).

**Antagonistic Activity of Lab Against Isolated Fungi:**

Isolated LAB were sub-cultured on MRS agar slant and streaked out in a line on MRS agar plate. Then the fungi *A. niger* was isolated from PDA slant and streaked in line beside the LAB. MRS agar plates were then incubated at 35-36°C for 48 hours and observed.

**RESULTS**

**Identification of Bacterial Isolates by Conventional Biochemical Tests:** Identities of the test bacterial isolates were confirmed through biochemical characterization for catalase, indole production, Voges-Proskaur, methyl red, citrate utilization, H$_2$S production, oxidase, urease, phenylalanine deamination, triple sugar iron agar, ornithine decarboxylase and lysine decarboxylase (Table 1).

**Morphological Identification of Isolated Fungal Species:**
The morphological method with four different media was used for identification of *A. niger*. The microscopic features used for identification of *A. niger* was as follows:

- **size:** 400-3000, stripes color: slightly brown, surface: smooth walled, vesicle serration: biseriate large size, metula covering: entirely, shape: globule, conidia surface: very rough irregular.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Cat</th>
<th>IP</th>
<th>VP</th>
<th>MR</th>
<th>CU</th>
<th>HSP</th>
<th>OX</th>
<th>Urease</th>
<th>PD</th>
<th>OD</th>
<th>LD</th>
<th>Butt</th>
<th>Slant</th>
<th>Gas</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>A</td>
<td>K</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>A</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>N</td>
<td>N</td>
<td>N</td>
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</tbody>
</table>

Cat = Catalase, IP= Indole Production, VP = Voges-Proskaur, MR = Methyl Red, CU = Citrate Utilization, HSP = H$_2$S production, OX = Oxidase, PD = Phenylalanine Deaminase, OD = Ornithine Decarboxylase, LD = Lysine decarboxylase, TSI = Triple sugar Iron Agar test, A = Acidic, K = Alkaline, N = Not done, + sign denotes positive and - sign denotes negative.
Table 2: Antimicrobial activity of lactic acid bacteria (LAB) against pathogenic bacteria

<table>
<thead>
<tr>
<th>Test organism</th>
<th>LAB strain</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>+++ (15-16 mm)</td>
<td>+ (10 mm)</td>
<td>++ (12-14 mm)</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>+(12-14 mm)</td>
<td>-</td>
<td>+ (8-10 mm)</td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Sign (++++)+, (+++), (++), (+), (-) and (±) refers to strong inhibition of test cultures (zone of inhibition: 20-25 mm), strong inhibition (zone of inhibition: 12-18 mm), moderate inhibition (zone of inhibition: 12-14 mm), weak inhibition (zone of inhibition: 9-10 mm), no inhibition and doubtful respectively; L1, L2 and L3 were designated as isolated LAB grew at 37°C, 23°C and 45°C respectively.

Table 3: Inhibitory activity of lactic acid bacteria (LAB) on different nutrient media.

<table>
<thead>
<tr>
<th>Nutrient media</th>
<th>Test organism</th>
<th>LATP + casein (1%)</th>
<th>LATP + skim milk (1%)</th>
<th>LATP + riboflavin (1.5 mg/l)</th>
<th>LATP + vitamin-B mixture (1.5 mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Escherichia coli</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Staphylococcus aureus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Bacillus subtilis</td>
<td>-</td>
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</tr>
</tbody>
</table>

LAPT is a broth including yeast extract, peptone, tryptone, glucose and Tween.

Antagonistic Activity of LAB Against Pathogenic Bacteria: The antimicrobial activity of isolated LAB against E. coli, S. aureus and B. subtilis was observed by disc diffusion method. It was observed that all of the LAB isolates possessed significant antagonistic activity against E. coli and did not possess any inhibitory activity against B. subtilis. Against S. aureus, L1 and L3 isolates showed weak inhibition but L2 did not exhibit any inhibitory action (Table 2).

Comparative Inhibitory Activity of LAB on Different Nutrient Media: The productions of the antibiotic component(s) by LAB in different media were observed. The following media were used: LAPT + casein (1%), LAPT + skim milk (1%), LAPT + riboflavin (1.5 mg/l) and LAPT + vitamin-B mixture (1.5 mg/l). The results are shown in Table 3. LAB showed inhibitory activity against test organisms in medium containing LATP + vitamin-B mixture (1.5 mg/l).

Antagonistic Activity of Lab Against Isolated Spoilage Fungi A. Niger: The fungi A. niger was isolated from different potato samples and identified. LAB inhibited the growth of fungi on PDA as shown by the Fig. 1.

DISCUSSION

Fermentation of various foods by LAB is one of the oldest forms of biopreservation practiced by mankind. Bacterial antagonism has been recognized for over a century, but in recent years this phenomenon has received more scientific attention, particularly the use of various strains of LAB. LAB have a broad spectrum of antagonistic activity against both Gram-(+)ve and Gram-(−)ve microorganisms, mainly through production of metabolites such as lactic acid, hydrogen peroxide and bacteriocins [22-24].

The aim of the study was to isolate LAB from curd samples and screen their antagonistic activity against selected pathogenic bacteria and spoilage fungi. The bacterial isolates were collected from referred...
laboratory and the fungi were isolated from potato samples. The isolated LAB strains showed significant inhibitory action against *E. coli* while moderate and no inhibition against *S. aureus* and *B. subtilis* respectively (Table 2). The high inhibitory potential of isolated LAB against *E. coli* highlights the biotechnological potential to control these pathogenic bacteria which cause many human diseases. Further studies should be conducted to elucidate the nature of the antibacterial metabolites produced by selected LAB. The isolates also exhibited antagonistic potential against the isolated fungi *A. niger* from potato samples (Figure 1). There is another interesting finding that LAB exhibited better antibacterial properties in the media LAPT with riboflavin (1.5 mg/l) and LAPT with vitamin-B mixture (1.5 mg/l) (Table 3). The findings from the study were in line with the findings from similar studies conducted [25-28], where LAB strains isolated from different samples showed significant antimicrobial activities against different bacterial isolates.

The present study showed that selected environmental LAB could offer an excellent source for active metabolite to control different pathogenic bacteria and spoilage fungi. The different substances such as organic acids, hydrogen peroxide, cyclic dipeptides and phenolic and proteinaceous compounds produced by LAB could be responsible for the detected antibacterial and antifungal activity. Further large scale study should be conducted to enumerate the mechanism of this antagonistic activity exerted by LAB against bacteria and fungi.

**CONCLUSIONS**

In this study, the results showed a high rate of antimicrobial activity among the isolates indicating that curd may be a common source for the selection of LAB with important technological potential, which are useful for the biocontrol of pathogenic bacteria and spoilage fungi. Further investigations to elucidate the nature of inhibiting compounds should be considered and to establish LAB as bio-control agent.

**ACKNOWLEDGEMENTS**

Authors would like to greatly acknowledge the support provided by the food microbiology section of Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh.

**Conflict of Interests:** There is no conflict of interests.

**REFERENCES**


