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Assessment of probiotic application of lactic acid bacteria (LAB) isolated from different food items

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Lactic acid bacteria (LAB) are regarded as effective probiotic organisms and used with a view of augmenting the safety of the food. In the present study, five food items (meat, fish, apple, milk and carrot) were selected having high nutritive and economic value, assumptive of harboring lactic acid bacteria. A total of 29 LABs were isolated from 5 different samples after 2 batches of fermentation. All of the isolates were then tested against 4 most frequently encountered pathogens, *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium,* and *Vibrio cholerae*. Among all these isolates, those from meat and fish samples showed positive average inhibitory coefficient (AIC) against two target pathogens, while those from milk and carrot showed positive AIC against three pathogens each, and isolates from apple revealed positive AIC against all four pathogens used. More than 50% of the isolates were found to inhibit or mask the pathogens when allowed to grow along with the individual pathogen on the each tested food item. Out of 29 isolates, 17 were found to successfully inhibit *Escherichia coli,* 11 worked against *S. aureus,* 11 against *S. typhimurium,* and 13 showed significant effect against *V. cholerae*. Among these isolates ML4, ML8, AP5 and CR3 most notably showed the potential to inhibit or mask at least three of the target pathogenic strains.

Key words: Lactic acid bacteria; probiotics; competitive inhibition

A large proportion of diseases originate from food rendered as unsafe due to bacterial growth or toxin production, hence everyone is at risk from food-borne illness (1). Thus bacteria could pose a great threat to food safety and security. On the other hand, bacteria have been used in ensuring food safety and food preservation for over 6,000 years (2, 3). It is an inexpensive and manageable tool, which imparts some extrinsic defenses to the food while leaving the intrinsic defense factors of the food unchanged (4). All these factors have cumulative or individual effects antagonistic to pathogenic or toxigenic microorganisms that render the food safer (5, 6). On top of that, nutritional value of food fermented with bacteria when compared to non fermented one is higher regarding protein, vitamin and mineral contents (7).

Fermentation processes enhance food safety by reducing toxic compounds such as aflatoxins and cyanogens, and producing antimicrobial factors such lactic acid, bacteriocins, carbon dioxide, hydrogen peroxide and ethanol which facilitate inhibition or elimination of food-borne pathogens (8-10**).** Therapeutic properties of fermented foods have also been reported (11). In addition to its nutritive, safety and preservative effects, fermentation process imparts a diversity of flavors, textures and aromas (12).

The most common probiotic candidate within the human digestive tract is the *Lactobacillus* spp. (13, 14). Some strains of *Lactobacillus acidophilus* have natural antibiotic producing and cancer fighting properties (15). These strains are particularly beneficial against infectious bacteria such as *Streptococcus*, *Staphylococcus*, *Salmonella*, *Clostridium botulinum*, and *E. coli* (16-19). Some strains of *L. acidophilus* have even shown impressive effects against viral infections including polio, HIV, and herpes (20, 21), and can also produce hydrogen peroxide which has the potential to kill undesirable *Candida* yeast and prevent its overgrowth (19).

Lactic acid bacteria are known to release various enzymes and vitamins into the intestinal lumen (22). These exert synergistic effects on digestion, alleviating symptoms of intestinal malabsorption (13). Bacterial enzymatic hydrolysis may enhance the bioavailability of protein and fat (23) and increase the production of free amino acids, short chain fatty acids (SCFA), lactic acid, propionic acid and butyric acid (24). When absorbed, these SCFAs contribute to the available energy pool of the host (25, 26) and may protect against pathological changes in the colonic mucosa (27, 28).

The present study was undertaken to isolate lactic acid bacteria (LAB) from some foods known to support LAB growth with a view of using the isolates in the long run to ferment those foods, and determination of their interactions with some food-borne pathogenic bacteria

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frequently encountered in Bangladesh (29). As there is no published report on assessing the efficacy of LABs against pathogens in Bangladesh, this study was carried out to shed some light on that.

MATERIALS AND METHODS

Samples chosen for this study were selected on the basis of higher possibility of harboring naturally occurring LAB (30) as well as having higher promise in terms of economic and nutritional value. Samples included meat (beef), cow-milk, fish (*Pangasius bocourti*), apple and carrot.

Sample processing. Two batches of samples were collected from Karwan Bazar and Moghbazar markets in Dhaka city. About 100g of sample for each item was collected aseptically using sterile container. Approximately 20g of each sample (except milk) were mixed with 80 ml of sterile water and then homogenized with stomacher for 5-10 minutes and then 10 fold dilution was performed once. pH was recorded for each homogenized sample before adjusting it to 5.5 to give selective advantage to the LAB.

Isolation of LABs. Processed samples were kept at room temperature inside paper boxes for 3 days for natural fermentation to occur. Then, dilution up to 10^{-6} was performed for each fermented sample. 0.1ml from each sample was spread on Rogosa SL Agar and incubated at 37 °C for 48 hours under limited oxygen concentration by sealing the agar plates with parafilm.

Pathogenic microrganisms. Four clinical isolates including *Staphylococcus aureus, E. coli, S. typhimurium,* and *V. cholerae* were collected from Microbiology Laboratory of Stamford University Bangladesh. These pathogens were selected due to their involvement in food-borne gastrointestinal illnesses.

Competitive inhibition assay (CIA). Four sets of test tubes (each for a target pathogen) were filled with 5 ml of sterile homogenized food sample. In each set, one test tube was assigned for each bacterium isolated from the test samples. In addition, 5 test tubes were taken as negative control. Medium used for each of the isolates was the original food type from which the LABs were isolated, i.e. the isolates from meat samples were tested for interaction with the target pathogens in autoclaved homogenized meat, milk-isolates were tested against target pathogens in milk, and so on for the others. All the test tubes (except the negative controls) were inoculated with 0.1 ml of each of the test organisms suspended in normal saline and kept for fermentation at 37 °C. After 48 hours, 4 sets were inoculated with 4 target organisms respectively and were further incubated for 24 hours. 100-fold dilution was performed for each of the 4 sets of samples. Mannitol salt agar, MacConkey agar, *Salmonella-Shigella* agar, and thiosulfate-citrate-bile salt-sucrose agar were used for enumeration of *S. aureus, E. coli, S. typhimurium,* and *V. cholerae*, consecutively. Average Inhibition Coefficient (AIC) for each group of LAB isolated from a particular type of food against each of the four target pathogens was determined by using the following formula-

Average Inhibition Coefficient (AIC) = [{Total count in negative control – Average count for each group of isolates} / Total count in negative control].

RESULTS

Physical parameters. After initial processing, the pH of all the samples were measured and were found to range from 5.4 for apple to 7.1 for fish samples. The pH for all the samples was then adjusted to 5.5 and then, after fermentation, the pH was again measured for all the samples and found to be in range from 2.0 for carrot sample to 7.2 for meat sample (Fig. 1).

Frequency of LAB. After two batches of fermentation, a total of 29 bacteria were isolated. Among these 29 isolates, 3 (MT1-3) were found from meat samples, one (FS1) from fish samples, 9 (CR1-9) from carrot samples, 9 (ML1-9) from milk samples, and 7 (AP1-7) isolates were found from apple samples. All the isolates were presumptively confirmed to be LAB by Gram staining and subsequent observation under microscope for their characteristic morphology.

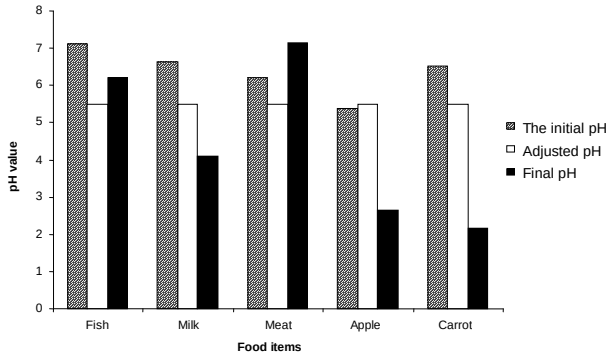

FIG. 1. Trend of pH change before and after fermentation. The bar chart indicates pH drops for milk, apple, and carrot samples while increases in case of fish and meat samples after fermentation.

Determination of AIC. After competitive inhibition assay (CIA), 3 meat isolates (MT1, MT2 and MT3) showed varied results (Table 1). MT1 showed positive result against *E. coli*, MT2 against all stains but *S. aureus*, and MT3 were positive against 2 of the target strains, namely *E. coli* and *S. typhimurium*. Overall, *E. coli* was inhibited with an average inhibition coefficient (AIC) of 0.95 while *S. typhimurium* was also moderately inhibited (Fig. 2a).

TABLE 1. Count (10^3 cfu/ml) of target pathogens after competition with meat isolates

Isolate	*Staphylocuccus aureus* (cfu/ml)	*Escherichia coli* (cfu/ml)	*Salmonella typhimurium* (cfu/ml)	*Vibrio cholerae* (cfu/ml)
MT1	280	5	172	70
MT2	415	10	121	10
MT3	370	4	2	125
N.C.	270	130	165	60

N.C. – Negative Control

TABLE 2. Number (10^3 cfu/ml) of target pathogens after competition with fish isolates

Isolate	*Staphylocuccus aureus* (cfu/ml)	*Escherichia coli* (cfu/ml)	*Salmonella typhimurium* (cfu/ml)	*Vibrio cholerae* (cfu/ml)
FS1	355	8	14	270
N.C.	159	225	46	170

N.C. – Negative Control

The fish isolate showed positive result against *S. typhimurium* and *E. coli*, but was ineffective against *S. aureus* and *V. cholerae* (Table 2). AIC for fish isolates was found to be 0.96 and thus very promising against *E. coli*, and fairly promising against *S. typhimurium*, while no such effect was observed against *S. aureus* or *V. cholerae* (Fig. 2b).

Milk isolates showed mixed result in CIA. Among 9

isolates, *S. aureus* was inhibited by ML1, ML3, ML4, ML6, and ML8. *E. coli* was inhibited by ML1, ML4, ML5, ML7, and ML9. *S. typhimurium* was inhibited by ML1, ML 4, and ML8. *V. cholerae* was inhibited by ML1, ML 4, and ML8. ML4, ML5, and ML8. Among these isolates ML4 was found to have inhibited all four target strains (Table 3). Milk isolates showed fairly promising AIC against *S. aureus* (0.14) and *E. coli* (0.36) but ineffective against *S. typhimurium* and *V. cholerae* (Fig. 2c).

Table 4 shows that *S. aureus* was inhibited by 4 (AP2, AP4, AP5, AP7), *E. coli* was inhibited by 3 (AP1, AP4, AP6), *S. typhimurium* was inhibited by 2 (AP 2, AP5) and *V. cholerae* was inhibited by 3 (AP3, AP5, AP7) of the apple isolates. Overall, the apple isolates showed positive effect against all four target strains with AIC 0.33 against *S. aureus*, 0.23 against *E. coli*, 0.10 against *S. typhimurium*, and 0.26 against *V. cholerae* (Fig. 2d).

Table 5 shows that *S. aureus* was inhibited by 3 (CR1, CR2, CR7), *E. coli* was inhibited by 4 (CR3, CR5, CR8, CR9), *S. typhimurium* was inhibited by 3 (CR3, CR6,

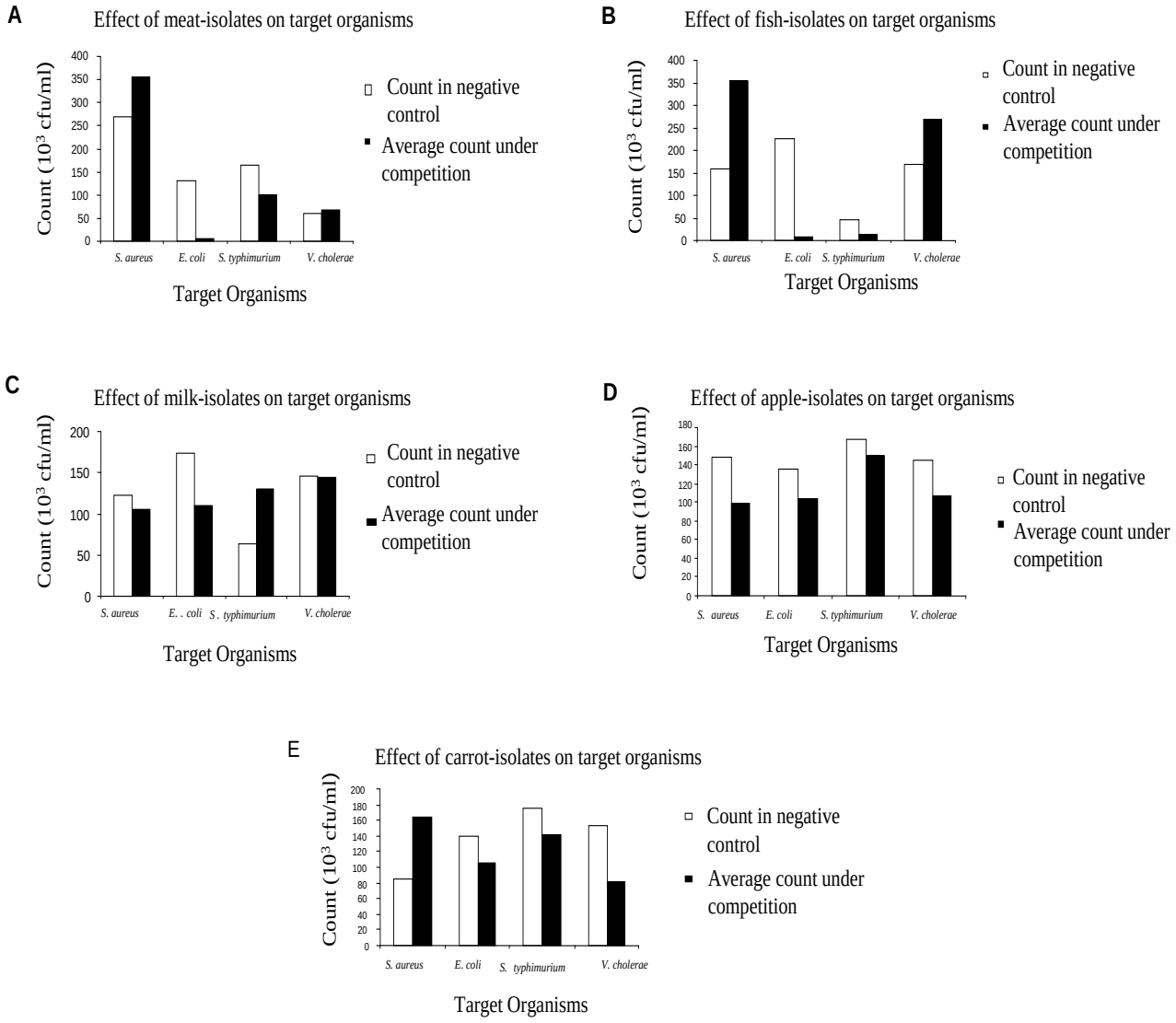

FIG. 2. Competative inhibition of the target pathogens by the LAB isolates. (A) isolates from meat and (B) fish showed average positive result against *E. coli* and *S. typhimurium*. Milk isolates (C) on an average were fairly inhibitive against *E. coli* only. Apple isolates (D) were moderatelyinhibitive against all four target pathogens and carrot isolates (E) on average were inhibitory against all but *S. aureus*.

TABLE 3. Count (10^3 cfu//ml) of target pathogens after competition with milk isolates

Isolate	*Staphylococcus aureus* (cfu/ml)	*Escherichia coli* (cfu/ml)	*Salmonella typhimurium* (cfu/ml)	*Vibrio cholerae* (cfu/ml)
ML1	5	28	71	183
ML2	126	175	121	175
ML3	4	227	141	271
ML4	9	3	7	3
ML5	274	14	174	1
ML6	9	183	186	182
ML7	172	4	227	155
ML8	18	176	73	15
ML9	204	14	115	173
N.C.	122	173	63	145

N.C. – Negative Control

TABLE 4. Number (10^3 cfu/ml) of target pathogens after competition with apple isolates

Isolate	*Staphylococcus aureus* (cfu/ml)	*Escherichia coli* (cfu/ml)	*Salmonella typhimurium* (cfu/ml)	*Vibrio cholerae* (cfu/ml)
AP1	252	7	184	192
AP2	10	149	5	170
AP3	160	155	270	3
AP4	4	4	216	162
AP5	109	166	3	19
AP6	153	5	195	184
AP7	5	234	177	20
N.C.	148	135	167	145

N.C. – Negative Control

CR8), and *V. cholerae* was found to be inhibited by 5 (CR2, CR3, CR5, CR7, CR9) of the carrot isolates. On an average, carrot isolates did not have any inhibitory role on *S. aureus*, moderately effective against *E. coli* and *S. typhimurium*, and fairly effective against *V. cholerae* with an AIC of 0.47 (Fig. 2e).

DISCUSSION

During the screening of LAB strains, it was very important to ensure ease and credibility. Various methods for this purpose were already reported by many researchers. Among those, spot-on-lawn assay, microtiter plate assay (31), agar well diffusion assay (32), multi-well plate assay (33), etc. were most notable. All of these techniques have few limitations. Moreover, as the goal of our experiment was to assess

TABLE 5. Number (10^3 cfu/ml) of target pathogens after competition with carrot isolates

Isolate	*Staphylococcus aureus* (cfu/ml)	*Escherichia coli* (cfu/ml)	*Salmonella typhimurium* (cfu/ml)	*Vibrio cholerae* (cfu/ml)
CR1	2	197	110	191
CR2	9	174	192	1
CR3	350	4	0	20
CR4	360	182	223	142
CR5	220	9	221	11
CR6	174	158	5	179
CR7	5	150	238	2
CR8	154	30	9	172
CR9	203	55	276	5
N.C.	85	140	175	152

N.C. – Negative Control

the inhibitory effect of fermented foods, the experiment was designed accordingly. Competitive inhibition assay (CIA) was designed, keeping in mind the ultimate goal of fermentation. It was anticipated that the process of assessing the capacity of LABs to enhance microbiological safety of food by inhibiting pathogenic strains in food could effectively be carried out though leaving them to compete in similar environments. The intrinsic capacity of foods fermented with LABs to ward off pathogenic contaminants was put to test. In the end, the method was proven effective as a screening technique for identifying the promising strains as far as food borne bacterial illness is concerned. It ended up with unveiling 11 isolates to be effective against *S. aureus*, 17 against *E. coli*, 11 against *S. typhimurium* and 13 against *V. cholerae*.

In our study, average inhibition coefficient (AIC) was calculated for each food type against each of the target strains. It can be used as a gross parameter of the candidate LABs from each sample type. Isolates from apple origin showed most uniform index of AIC ranging from 0.10 to 0.33 which were found to be positive against all four target strains. Isolates from meat and fish could effectively suppress two of the target strains each; particularly, both groups were highly efficient against *E. coli* with AIC of 0.95 in fermented meat and 0.96 in fermented fish. Milk and carrot isolates revealed fairly positive AIC indicating the positive result.

One very important consideration is that the changes caused by fermentation can sometimes be disadvantageous. However, fermentation provides beneficial results if controlled carefully. It can therefore be a highly appropriate technique for use in developing countries and remote areas where access to sophisticated

equipments is limited. If we can tackle the associated problems, lactic acid producing bacteria will not only serve as probiotic agents, but the microbiological food safety can also be ensured with considerable health and economic impact.

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