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Isolation, Identification and Analysis of Probiotic Properties of *Lactobacillus Spp.* From Selective Regional Yoghurts

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Abstract: Yoghurt is a potential source of probiotic lactobacilli. In the present study, *Lactobacillus* spp. were isolated from two regional yoghurts in Bangladesh, which were identified on the basis of their colony morphologies and some biochemical tests. It was observed that isolated *Lactobacillus* spp. were resistance to inhibitory substances like phenol (0.4%), NaCl (1-9%) and bile acid (0.05-0.3%). Additionally, good growths were observed in the presence of 1% NaCl and 0.3% bile acid. The isolated *Lactobacillus* spp. did show good survival abilities in acidic (pH 2.5) and alkaline (pH 8.5) conditions, while, their maximum growth was observed at pH 5.0 for lactobacilli isolated from Bogra yoghurt and at pH 6.5 for lactobacilli isolated from yoghurt of Khulna region of Bangladesh. Isolated lactobacilli were able to produce organic acid in skim milk which was determined by titrimetric method. The *Lactobacillus* spp. also did show good survival abilities in simulated gastric juice at pH 2.22 and pH 6.6 (Control). Their susceptibility to selected nine antibiotics was determined in terms of minimum inhibition concentration (MIC). The MICs results showed that, *Lactobacillus* spp. isolated from Bogra yoghurt were sensitive to amoxicillin, moderately sensitive to gentamycin, clindamycin, azithromicin and resistant to kanamycin, nalidixic acid, metronidazol, cefradine and tetracyclin. On the other hand, *Lactobacillus* spp. isolated from yoghurt of Khulna region were sensitive to gentamicin, clindamicin and resistant to amoxicillin, tetraciclin, kanamicin, nalidixic acid, metronidazol, azithromicin and cefradine. In conclusion, most of the results from the present experiments showed that, there were variations in probiotic properties of the isolated *Lactobacillus* spp. from different regions.

Key words: Regional yoghurts · *Lactobacillus* spp · Inhibitory substances · Simulated gastric juice and MIC

INTRODUCTION

The *Lactobacillus* genus consists of a genetically and physiologically diverse group of rod-shaped, Gram-positive, non-spore forming, nonpigmenetd [1], catalase negative and microaerophilic to strictly anaerobic [6] lactic acid bacteria (LAB) that have widespread use in fermented food production [2] and are considered as generally recognized as safe (GRAS) organisms and can be safely used for medical and veterinary applications [3]. In the food industry, LAB is widely used as starter cultures and has been cited to be part of human microbiota [4, 5]. In raw milk and dairy products such as cheeses, yoghurts and fermented milks, lactobacilli are naturally present or added intentionally, for technological reasons or to generate a health benefit for the consumer [6] and yogurt is one of the best-known foods that contain probiotics [7]. From the health point of view, ingestion of live cells of certain species and strains the probiotic concept of lactobacilli in adequate amounts is believed to confer several beneficial physiological effects on the host [8] such as maintaining a healthy and equilibrated intestinal microbiota and reducing incidence of intestinal infection [9]. The criteria for the *in vitro* selection of lactobacilli to be used as health-promoting, probiotic ingredients, in food and pharmaceutical preparations include antibiotic tolerance as well as the production of lactic acid that inhibits the growth of other microorganisms, which allow them to be established in the intestinal tract [10]. Bile tolerance [11] and gastric juice resistance [12] are other important characteristics of probiotic lactic acid bacteria used as adjuncts because they enable them to survive, to grow and to perform their beneficial action in the gastrointestinal tract (GIT). Although the degree of tolerance required for maximum growth in the GIT is not known, it seems reasonable that the most bile and acid-resistant species should be selected [13].

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In Bangladesh, yoghurt is perhaps the oldest fermented milk product known and consumed by large sectorsof the population as a part of their daily diet. In most of the areas of Bangladesh, different types' of traditional yogurts are found, but their probiotic role was not studied. Incorporation of probiotic microorganisms (isolated from indigenous yoghurts) in market yogurts can positively enhance health status of larger segment of communities of Bangladesh. Therefore, the present study was undertaken with the following objectives for:

- isolation and identification of *lactobacillus spp*. from yoghurts of different regions in Bangladesh.
- Determination of optimal growth and pH of isolated lactobacillus strains.
- Quantification of lactic acid production in milk.
- Determination of antibiotic and bile tolerance and.
- Determination of resistance to gastric juice.

MATERIALS AND METHODS

Collection of Samples: Yoghurt samples were collected from Grameen Dannone yoghurt, Bogra and Khulna Districts of Bangladesh due to their wide acceptance among the consumers of Bangladesh. Immediately after collection, the samples were stored aseptically in low temperature (-4°C) refrigerator to protect from contamination and deterioration. In preliminary investigations the yoghurt sample of Grameen Dannone and Barisal region were discarded due to lack of sufficient probiotic properties.

Media: The bacteria *Lactobacillus* spp. were isolated from yoghurt samples by using modified MRS broth and MRS agar media [14]. Additionally, 0.05% cysteine was added to MRS to improve the specificity of this medium for isolation of lactobacillus [15]. The pH of the media was adjusted to 6.5.

Isolation of Bacteria: Lactobacillus was isolated from yoghurts by using MRS medium. One gram of each sample was dissolved into 100 ml of MRS broth at pH 6.5. After dissolving into MRS broth they were shaken homogeneously and were incubated at 37°C for 24 h in aerobic condition.. The cultures were subjected to five subculture at 37°C under low pH (pH 4.5) and anaerobic condition in the presence of 10% CO_2 to remove unwanted bacteria. After seven subcultures, the bacterial culture was streak onto MRS agar media at pH 4.8. Finally, the single colony of lactobacillus was isolated by observing their colony morphology and some biochemical tests (Gram staining, catalase, endospore and motility test) and the culture were maintained in MRS broth at pH 5.5.

Identification: The isolated bacteria were identified as *Lactobacillus* spp. by observing their morphological characteristics and by means Gram staining, motility test, catalase test, endospore test, milk coagulation activities, 0.4% bacteriostactic phenol tolerance test and 1-10% NaCl tolerance test. MRS broth containing inhibitory substances such as 0.4% phenol and 1-10% NaCl were inoculated with 1% (v/v) 24 h active culture of lactobacillus and incubated (anaerobically) for 24 h at 37°C in the presence of 10% CO_2.

Determination of Optimal Growth and pH: For the determination of optimal growth and pH of lactobacillus, 1% (v/v) fresh over night culture of lactobacillus were inoculated into MRS broth with varying pH ranging from 2.5-8.5. The pH were adjusted with concentrated acetic acid (99%) and 5 N NaOH. The inoculated broths were incubated in anaerobic condition 24 h at 37°C in the presence of 10% CO_2. After 24 h of incubation growth of the bacteria were measured using a spectrophometer, reading the optical density at 560 nm (OD_{560}) against the uninoculated broth.

Assay for NaCl Tolerance: For the determination of NaCl tolerance of isolated lactobacillus 10 test tube containing MRS broth were adjusted with different concentration (1-10%) of NaCl. After sterilization, each test tube was inoculated with 1% (v/v) fresh over night culture of lactobacillus and incubated at 37°C for 24 h. After 24 h of incubation their growth were determined by observing their turbidity. Maximum growth were indicated as double positive sign (+ +), normal growth as single positive sign (+) and no growth were indicated as negative sign (-).

Quantification of Organic Acid and Determination of pH Value: One percent (v/v) 24 h active culture of lactobacillus was used to inoculate 10% sterilized skim milk obtained from Milk Vita Co-operative Bangladesh Ltd. and initial pH (6.68) was determined by a digital electrode pH meter (Hanna, Model No. 211). The inoculated skim milk was incubated at 37°C for 72 h and samples were collected in every 24 h, 48 h and 72 h and liquids of coagulated milk were separated by filtration. pH of the separated liquid was recorded using a digital electrode pH meter and quantification of organic acid was performed through titration with 0.1 N NaOH.

Assay for Bile Tolerance: Bile tolerances of isolated lactobacillus from yoghurts were determined using the protocol described by Graciela and Maria [13].

Assay for Gastric Juice Resistance: Gastric juice resistances of isolated lactobacillus from yoghurts were assayed using the protocol described by Graciela and Maria [13].

Assay for Sensitivity to Antibiotic: Sensitivity of lactobacillus to different antibiotics was determined by the minimum inhibitory concentration (MIC) assay. The assay was done using the method described by Andrews [16]. According to this method, stock solutions 128 µg/ml of 9 different selected antibiotics such as amoxicillin, gentamicin, tetracyclines, clindamicins, kanamycin, nalidixic acid, metronidazole, azithromicin and cefradine were prepared. All antibiotics except Kanamycin were obtained from Square Pharmaceuticals Ltd. Bangladesh. Stock solutions of selected antibiotics were two-fold diluted in the range 0.25 µg/ml to 1024 µg/ml in MRS broths. For preparation of the test inoculums the 16 h active cultures of lactobacillus were adjusted to 0.5 McFarland standards (10^7 to 10^8 cfu/ml of bacteria) by adding sterile distilled water. 50 µl of test inoculums were inoculated into each dilution and incubation was done for 24 h at 37°C in the presence of 10% CO_2 in a CO_2 incubator.

RESULTS

Identification: Bacteria isolated from different yoghurts were identified as *Lactobacillus* spp. by observing their colony morphology, physiological and as well as some biochemical characteristics. Microscopically they were Gram-positive (Figure 1), rod shaped (Figure 2), non-motile, catalase negative and absence of Endospore (Figure 3). The isolates have the abilities to coagulate milk and were able to tolerate inhibitory substances such as 0.4% bacteriostatic phenol and showed growth in MRS broth containing 1-9% NaCl. Their characteristics have shown in Table 1.

Optimal Growth and pH: Maximum growth (OD= 2.054) of isolated lactobacilli from Bogra yoghurts was observed at pH 5.0 and maximum growth of lactobacilli isolated from Khulna yoghurt (OD= 1.93) was observed at pH 6.5. The OD reading was the average value of two readings, where control OD was 0.152. The results are shown in Figure 4.

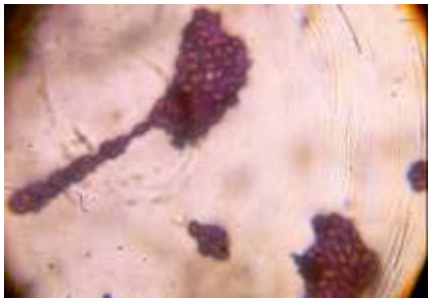

Fig. 1: Gram-positive *Lactobacillus* spp.

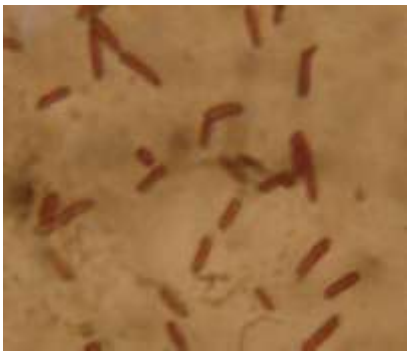

Fig. 2: Isolated rod-shaped *Lactobacillus* spp.

Fig. 3: Non-spore forming *Lactobacillus* spp.

Tolerance to NACL: The isolated lactobacilli from yoghurts were able to tolerate 1-9% NaCl. The results are shown in Table 2.

Quantification of Organic Acid and Determination of Ph Value: The results have shown in Table 3.

Bile Tolerance: Isolated *Lactobacillus* spp. was able to survive in 0.05, 0.1, 0.15 and 0.3% bile acid. The isolated *Lactobacillus* spp. was also able to multiply in above mentioned concentrations of bile acid. In the Figure 5 the optical density values against incubation time are shown.

Gastric Juice Resistance: Isolated *Lactobacillus* sp. (Isolate-2) was able to survive in gastric environment at low pH (2.2) but they were not able to multiply. In per ml artificial gastric juice (pH 2.2) the colony forming unit at

Table 1: Morphological, physiological and biochemical characteristics of isolated *Lactobacilli*

Biochemical and physiological characteristics	Isolate-1	Isolate-2	Isolate-3	Isolate-4
Colonies morphology	1.0 mm, white, rough, irregular and round	small 0.1-0.5 mm, rough, dull and brownish	white, shiny, smooth and 1.0 mm diameter	Small circular, white-creamy in colors and disc like
Gram stain	+	+	+	+
Motility test	Non motile	Non motile	Non motile	Non motile
Catalase test	-	-	-	-
Endospore test	-	-	-	-
0.4% phenol	+	+	+	+
Milk coagulation	+	+	+	+
NaCl tolerance	+	+	+	+

+: positive, -: negative

Table 2: Tolerance to NaCl of isolated *Lactobacilli*

Concentration of NaCl (%)	*Lactobacilli* isolated from yoghurt of Khulna region	*Lactobacilli* isolated from yoghurt of Bogra Region
1	++	++
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	+	+
9	+	+
10	-	-

Legend: ++, good growth, +, visible growth, -, no growth

Table 3: Organic acids (%) and pH in skim milk produced by isolated *Lactobacillus* spp

Sources of Bacteria	Name of the bacteria	Incubation time (Hour)	Incubation temp. (°C)	Organic acid (%)	pH
Bogra yoghurt	*Lactobacillus* spp.	24	37	2.290	5.09
		48	37	6.615	3.73
		72	37	6.530	3.65
Khulna yoghurt	*Lactobacillus* spp.	24	37	2.130	5.13
		48	37	3.920	4.50
		72	37	6.260	3.88

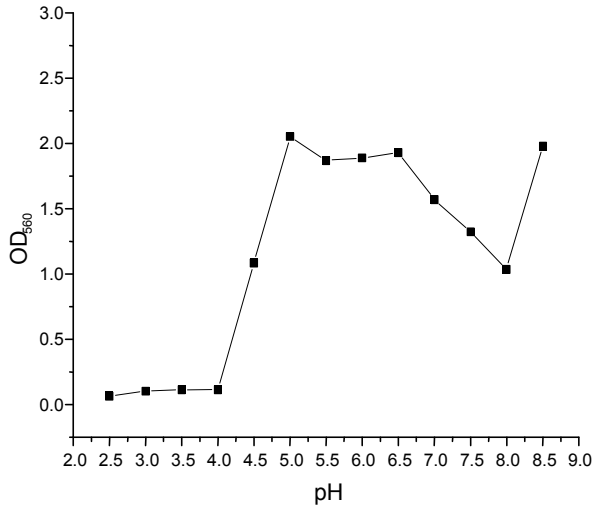

Fig. 4: Optimal growth and pH of isolated *Lactobacillus* sp. (Isolate-2) from Bogra yoghurt sample

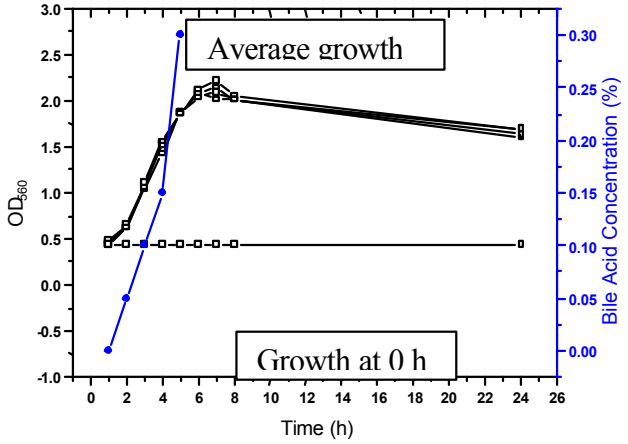

Fig. 5: Bile acid tolerance of *Lactobacillus* sp. (Isolate-2) isolated from Bogra yoghurt.

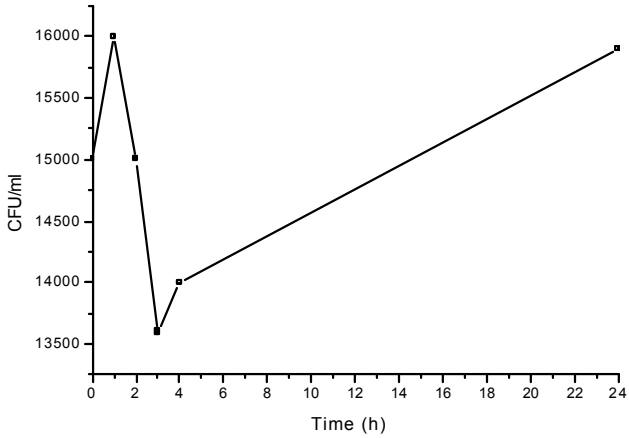

Fig. 6: Survival abilities of *Lactobacillus* sp. (Isolate-2) in simulated gastric juice at pH 2.2.

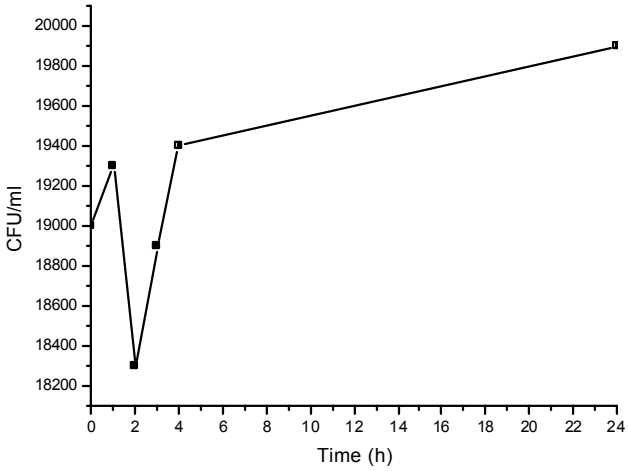

Fig. 7: Survival and partial multiplication abilities of isolated *Lactobacillus* sp. (Isolate-2) in simulated gastric juice at pH 6.6.

Table 4: Minimal Inhibitory Concentrations (MIC) of selected antibiotics for isolated *Lactobacillus* spp. from different yoghurts

		MIC (µg/ml)								
Sources of bacteria	Species	Amoxi	Genta	Tetra.	Clinda	Kana	Nalid	Metro	Azit	Cefra
Bogra Yoghurt	*Lactobacillus* spp.	2	8	32	8	256	>1024	>1024	8	128
Yoghurt Khulna	*Lactobacillus* spp.	256	16	128	8	>1024	>1024	>1024	>1024	>1024

Legend: Amoxi – amoxicillin, Genta – gentamycin, Tetra – tetracyclin, Clinda – clindamycin, Kana – kanamycin, Nalid – nalidixic acid, Metro – metronidazol, Azit – Azithromicin, Cefra – Cefradine

0 h, 1 h, 2 h, 3 h, 4 h and 24 h were 1.5×10^4 (CFU/ml), 1.6×10^4 (CFU/ml), 1.5×10^4 (CFU/ml), 1.36×10^4 (CFU/ml), 1.4×10^4 (CFU/ml) and 1.59×10^4 (CFU/ml) respectively. On the other hand, they were able to survive and had partial multiplication abilities at pH 6.60. In per ml gastric juice (pH 6.60) the colony forming unit at 0 h, 1 h, 2 h, 3 h, 4 h and 24 h were 1.9×10^4 (CFU/ml), 1.93×10^4 (CFU/ml), 1.83×10^4 (CFU/ml), 1.89×10^4 (CFU/ml), 1.94×10^4 (CFU/ml) and 1.99×10^4 (CFU/ml) respectively. Results are shown in Fig. 6 and Fig. 7 as colony forming unit (CFU/ml) after 0,1,2,3, 4 h and after 24 h incubations.

Sensitivity to Antibiotic: Sensitivity of *Lactobacillus* spp. and to different antibiotics was determined in term of minimum inhibitory concentration (MIC). The results are shown in the Table 4.

DISCUSSION

The isolated Lactobacilli were identified as Isolate-1 (1.0 mm, white, rough, irregular, round and white colony), Isolate-2 (small 0.1-0.5 mm, rough, dull and brownish colony), Isolate-3 (white, shiny, smooth and 1.0 mm diameter) and Isolate-4 (2.0-2.5 mm, white, shiny, smooth and disc like colony) which were comparable with the study of Tharmaraj and Shah [17].

pH is an important factor which can dramatically affect bacterial growth. In our experimental design we have observed the growth of our isolated *Lactobacillus* spp. in various pH values ranges from 2.5 to 8.5. The reason for choosing this pH range was to determine whether LAB species can grow in acidic and alkaline conditions and also to predict the optimum pH value for good growth. From the experimental results, it was found that the isolated *Lactobacillus* spp. from yoghurts is able to survive in extreme acidic pH (pH 2.5 to 3.5) and basic pH (pH 7.5 to 8.5). Maximum growth (OD= 2.054) of isolated lactobacilli from Bogra yoghurts was observed at pH 5.0 and for lactobacilli isolated from Khulna yoghurt maximum growth (OD= 1.93) was observed at pH 6.5. From our results we also found a variation of optimum growth pH.

NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria. the current results showed that *Lactobacillus* spp. isolated from yoghurts were able to tolerate 1-9% of NaCl and good growth was observed at 1% NaCl. Our experimental results have the similarities with the findings of Elezete and Carlos [18], incase of lactobacilli isolated from gastrointestinal tract of swine that were tolerable to 4-8% NaCl.

The present experiment indicates that organic acid production was increased with the incubation time. On the other hand, pH of the media decreased with the increasing acid production. Highest acidity (6.53%) and lowest pH (3.65) was observed after 72 h incubation at 37°C for probiotic LAB isolated from Bogra yoghurt. On the other hand, probiotic bacteria isolated from yoghurt of Khulna region of Bangladesh showed the acid (6.26%) and low pH (3.88) value after 72 h incubation. This investigation indicates that, there is a minor variation in organic acid production by Lactobacilli due to their regional variation. This finding has the connection with the findings of Haddadin *et al*. [19] who stated that, a strain of *Lactobacillus acidophilus* isolated in North America may well be genetically different from a species recovered in Europe or the Middle East.

In this experimental design, 0.05-0.3% of bile concentration were used, as it corresponded to that found in the human intestinal tract and 0.3% bile is the maximum concentration that is present in healthy men [13]. Therefore, before selection of probiotic bacteria for human consumption it must be endurable to 0.3% bile concentration [20]. Our isolated *Lactobacillus* spp. from both yoghurts (Bogra and Khulna regions of Bangladesh) are able to tolerate up to 0.3% of bile concentrations.

The viable cell count of *Lactobacillus* spp. on MRS agar media were 1.5×10^4 (CFU/ml) at 0 h and 1.59×10^4 (CFU/ml) at 24^{th} h (cell plated from artificial gastric juice at pH 2.2). These results indicate that there were no losses of viability of cell in simulated gastrointestinal condition. On the other hand, cell plated from artificial gastric juice with pH 6.6 the viable cell counts were 1.9×10^4 (CFU/ml) at 0 h and 1.99×10^4 (CFU/ml) at 24^{th} h. These results indicate that cells were able to survive and may have little multiplication abilities in artificial gastric juice at pH 6.6.

From this experiment the results indicate that in GIT environment the gastric juice will have minor or possibly no adverse effect on our isolated probiotic bacteria. Bacterial culture plated on MRS agar medium from both simulated gastric juices (pH 2.2) and 6.6) showed four morphologically different colonies at 0 h and 24 h.

Lactic acid bacteria (LAB) from fermented products may act as a reservoir of antimicrobial-resistance genes [21]. From the MIC values of 9 tested antibiotics it was found that, *Lactobacillus* spp. isolated from Bogra yoghurt of Bangladesh sensitive to amoxicillin (MIC = 2 μg/ml), moderately sensitive to gentamycin (MIC = 8 μg/ml), clindamycin (MIC = 8 μg/ml), azithromicin (MIC = 8 μg/ml) and resistant to kanamycin (MIC = 256 μg/ml), nalidixic acid (MIC > 1024 μg/ml), metronidazol (MIC > 1024 μg/ml), cefradine (MIC = 128 μg/ml) and tetracyclin (MIC = 32 μg/ml). On the other hand, *Lactobacillus* spp. isolated from yoghurt of Khulna region of Bangladesh were sensitive to gentamicin (MIC = 16 μg/ml), clindamycin (MIC = 8 μg/ml) and resistant to amoxicillin (MIC = 256 μg/ml), tetracyclin (MIC = 128 μg/ml), kanamycin (MIC > 1024 μg/ml), nalidixic acid (MIC > 1024), metronidazol (MIC > 1024 μg/ml), azithromicin (MIC > 1024 μg/ml) and cefradine (MIC > 1024 μg/ml). This investigation revealed that, some antibiotics such as amoxicillin, gentamycin, clindamycin and azithromicin intake can drastically drop the *Lactobacillus* spp. from intestinal microflora, on the other hand five antibiotics viz. tetracyclin, kanamycin, nalidixic acid, metronidazol and cefradine will not influence the growth of lactobacilli population. From our experiments we did find that *Lactobacillus* spp. isolated from yoghurt of Khulna region have shown broad range of resistances to most of the antibiotics including amoxicillin. This was possibly due to wide use of antibiotics in veterinary medicine and agriculture which could be contributing to the dissemination of resistances into yoghurt related ecological niches and is supported by the opinion of Flórez *et al*. [21]. In Bangladesh, a wide ranges of non-specific antibiotics treatment is a common practice, as a result many pathogenic bacteria in the geographical location of Bangladesh has gained resistance to several groups of antibiotics including amoxicillin.

In conclusion, the experimental results showed that isolated *Lactobacillus* spp. are able to tolerate inhibitory substances such as 0.05-0.3% bile acid, 0.4% phenol, 1-8% NaCl and also able to survive in simulated gastric (pH 2.2) condition, as well as in alkaline (pH 8.5) condition. The isolated lactobacilli were able to produce organic acid in milk. From the MIC results, We have found that the *Lactobacillus* spp. are sensitive to amoxicillin, gentamycin, clindamycin, azithromycin resistant to kanamycin, nalidixic acid, metronidazol, cefradine and tetracyclin which are most frequently used antibiotics in Bangladesh regarding treatments of human and animals.

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