Asian Australas. J. Biosci. Biotechnol. 2016, 1 (1), 54-65

Asian-Australasian Journal of Bioscience and Biotechnology
ISSN 2414-1283 (Print) 2414-6293 (Online)
www.ebupress.com/journal/aajbb

Article

Isolation, identification, molecular characterization and screening of probiotic activities of *Lactobacillus* species from poultry sources at live bird markets in Mymensingh, Bangladesh

S. M. Lutful Kabir[1a], S. M. Mushfiqur Rahman[1a], Sucharit Basu Neogi[2], Mohummad Muklesur Rahman[3] and M. Shahidur Rahman Khan[1]

[1]Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh, Bangladesh
[2]Centre for Food and Waterborne Diseases, International Centre for Diarrheal Disease Research, Mohakhali, Dhaka, Bangladesh
[3]Veterinary Surgeon, District Veterinary Hospital, Gazipur, Bangladesh

[a]S. M. Lutful Kabir and S. M. Mushfiqur Rahman contributed equally to this work

*Corresponding author: Professor Dr. S. M. Lutful Kabir, Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh. Tel.: +88-091-67401-6/Ext. 2394; Fax: +88-091-61510; E-mail: lkabir79@gmail.com

Received: 10 March 2016/Accepted: 03 April 2016/ Published: 30 April 2016

Abstract: A study was conducted to isolate and characterize *Lactobacilli* from the gastrointestinal (GI) tract of broiler chickens in different markets along with evaluation of probiotic ability and antibiotic sensitivity of *Lactobacillus* isolates during the period of July, 2014 to December, 2014. The caecum and cloacal contents of 100 broiler chickens from different markets were collected and cultured on Man Rogosa and Sharp (MRS) broth and agar. *Lactobacilli* were isolated and characterized by using phenotypic (cell morphology, Gram's staining, physiological and biochemical tests which are specific for *Lactobacillus* genus) and genotypic methods (PCR and RAPD). The identified *Lactobacilli* species were screened for probiotic properties by *in vitro* tests like acid tolerance and bile tolerance. Total eighty two isolates were identified as *Lactobacillus* based on morphological, physiological and biochemical tests which are specific for *Lactobacillus* genus. All of the *Lactobacillus* isolates were further identified at species level as *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus galinarum* and other unclassified *Lactobacillus* species based on sugar fermentation tests. The prevalence of *Lactobacillus crispatus, Lactobacillus acidophilus* and *Lactobacillus gallinarum* species found in the chickens were 21.95%, 18.29% and 17.07% respectively. A total of 10 *Lactobacillus* isolates were amplified by using 16S rRNA gene-based universal primers. Furthermore, out of 10 *Lactobacillus* isolates 4 molecular patterns were detected and 6 *Lactobacillus* isolates were clonal by randomly amplified ploymorphic DNA (RAPD) method in this study. Out of 15 *Lactobacillus* isolates, 10 isolates demonstrated probiotic ability as determined by bile tolerance test and acid tolerance test. Moreover, the results of antibiotic sensitivity showed that all of the isolates tested were sensitive to tetracycline and gentamicin. However, most of the isolates tested were resistant to ampicillin and cefradine. Potential probiotic bacterial strains from poultry sources were identified as well as characterized in this study.

Keywords: poultry; *Lactobacillus* species; PCR; RAPD; probiotic activities

1. Introduction

The genus *Lactobacillus* is one of the major members of the lactic acid bacteria, a definition which groups Gram positive, catalase-negative bacterial species able to produce lactic acid as a main end-product of the fermentation of carbohydrates (Felis and Dellaglio, 2007). These bacteria colonize in the small intestine and

caeca of chickens, a week after hatch (Mead, 1997). They help maintain the natural balance of organisms (microflora) in the intestines and help support a healthy digestive system (Dunne *et al*., 1999).The genus *Lactobacillus* has been isolated from dairy products, meat products, sewage, plants, and animal intestines (Kandler and Weiss, 1986) and currently, this genus contains more than 130 species assigned to twelve *Lactobacillus* clades (Neville and O'Toole, 2010). At present, they are widely used as probiotics in efforts to reduce the numbers of pathogens residing in the intestinal tract and to maintain a balanced microbiota (Tannock *et al.*, 2000; Apa´s *et al.*, 2010; Grimoud *et al*., 2010).

The poultry industry is now facing a ban for the use of antibiotic feed additives for disease prevention and growth enhancing supplements. Probiotics were used to overcome this problem (Kabir, 2009). In Bangladesh, the use of probiotics in poultry is gradually being increased. But surprisingly, there is no local probiotic for the huge poultry industry in Bangladesh. The probiotic market in Bangladesh is completely dependent on the importation of probiotic materials and thus, every year the country counts a handsome amount of money for importation of such materials. The reason of the dependency to the imported probiotics may be that there is no probiotic bacteria isolated yet in Bangladesh even though the existence of potential sources. Therefore, in this study, appropriate strategies was used for the isolation, identification and characterization of potential probiotic *Lactobacillus* strains from poultry sources, in order to evaluate their suitability for commercial use in poultry industry.

2. Materials and Methods

2.1. Collection of sample for isolation of bacteria

The present research was conducted during the period from July, 2014 to December, 2014 in the Bacteriology Laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh. A total of 100 samples were collected from ceacum and cloacal region of broiler chickens from two different places at Kamal-Ronjit (KR) market and SES MOR in Mymensingh district. Sterile cotton sticks with sterile normal saline was inserted to collect the cloacal contents of chickens and ceacum content was collected after postmortem of chickens and placed in sterile vials contained MRS broth. All the samples were carried to the laboratory immediately in an insulating foam box with ice.

2.2. Isolation of bacteria in pure culture

Samples were enriched in MRS broth at 37°C for 24 hours. The overnight bacterial broths were streaked onto MRS agar and incubated at 37°C for 24 hours for the growth of *Lactobacilli.* Purification was done by streak plate method as described by Awan and Rahman (2002). Morphologically distinct and well isolated colonies were picked with a sterilized loop. Colonies were streaked onto a new MRS agar plate by four-phase streaking pattern. Loop was again sterilized and touched with other colony. Streaking was done on other new MRS agar plate. In a similar way, all the distinct colonies were transferred onto MRS agar plates by streaking method. All the streaked plates were placed in incubator at 37^{o}C an aerobically for 24 hours for growth. Again, morphologically distinct colonies were shifted to other new MRS agar plates until pure colonies were obtained. Then pure colonies were isolated and grown in MRS broth at 37^{o}C an aerobically for 24 hours.

2.3. Identification of cultural isolates

Lactobacilli were presumptively identified following the methods described in Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986). Identification of bacteria was performed on the basis of colony morphology, Gram′s staining reaction (cell morphology was examined after Gram's staining and spore staining), motility test and biochemical tests like glucose fermentation, lactose fermentation, indole production, Voges-Proskauer, methyl red and citrate utilization tests were performed.

2.4. Confirmation of *Lactobacillus* isolates by molecular method

For the identification of *Lactobacillus* genus, DNA templates prepared from the *Lactobacillus* isolates were amplified by 16s rRNA universal primers. PCR was performed to amplify 16S rRNA of *Lactobacillus* spp. using previously published primers (Magne *et al*., 2006). After PCR reaction, 10 µl of the amplified product was mixed with 1µl of 6x modified loading buffer and used for gel electrophoresis. Molecular weight marker (Invitrogen, USA) was loaded in each of the gel for comparison of the size of the amplified products as well as gel image normalization. Electrophoresis was performed at 70 V for 4 hr and 30 min using New Horizon, Life technologies electrophoresis system (electrode distance 30 cm) with 11 cm x 24 cm tray. Tris-Borate with EDTA (TBE: 89 mM Tris-Borate, I mM EDTA) was used as the running buffer for the electrophoresis. After

electrophoresis, the gel was stained in ethidium bromide solution (5µg/ml) for 15 min and de-stained in tap water for 10 min with shaking.

2.5. Molecular typing of *Lactobacillus* strains based on randomly amplified polymorphic DNA (RAPD) fingerprint analysis

Genomic DNA from the bacterial isolates was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA) and eluted in 200 µl elution buffer AE (10 mM Tris-HCI P^H 9.0, 0.5 mM EDTA). DNA concentrations were determined spectrophotometrically by absorbance at 260 nm (A260). The DNA were diluted to 20 ng/µl by TE buffer and stored at -20^0C until analyzed. The amplification reactions were carried out in 50 µl volumes using Taq DNA polymerase and buffer system (Invitrogen, USA). Twenty-five nano grams of purified total DNA was mixed with a 25 µl reaction mixture containing 2.5 µl of 10X PCR buffer, 1.5 µl of MgCl2 (50 mM), 1.0 µl of deoxy nucleotide triphosphates (10 mM each of dATP, dCTP, dGTP, dTTP), 2.0 µl primer (10 µM), 2.5 units Ampli Taq DNA polymerase (Invitrogen, Life Technologies, USA) and an appropriate volume of sterile Milli Q water. A 10-mer oligonucleotide RAPD primer, i.e., 5'-CAG GCG CACA-3' was used in the PCR assay (Wong *et al*., 2003). PCR was performed using a thermal cycler (MJ Research, Cetus, USA). After PCR reaction, 10 µl of the amplified product was mixed with 1µl of 6x modified loading buffer and used for gel electrophoresis. Molecular weight marker (Invitrogen, UA) was loaded in each of the gel for comparison of the size of the amplified products as well as gel image normalization. Agarose gel, 1.8% wt/vol, was opted as the appropriate concentration for electrophoresis separation of the long-range RAPD product. Electrophoresis was performed at 70 V for 4 hr and 30 min using New Horizon, Life technologies electrophoresis system (electrode distance 30 cm) with 11 cm x 24 cm tray. Tris-Borate with EDTA (TBE: 89 mM Tris-Borate, I mM EDTA) was used as the running buffer for the electrophoresis. After electrophoresis, the gel was stained in ethidium bromide solution (5 µg/ml) for 15 min and de-stained in tap water for 10 min with shaking. The fingerprint pattern in the gel was analyzed by careful visual scrutinization by comparing the number, size and intensity of the amplified products of each of the *Lactobacillus* strains, which can be used as probiotic. The fingerprint patterns were subjected to typing based on banding similarity and dissimilarity.

2.6. Maintenance of stock culture

These isolated species were preserved in MRS broth medium containing 20% (v/v) glycerol as frozen stocks at -80 °C. The glycerol stocks of samples were prepared by mixing 0.5 ml of active cultures and 0.5 ml MRS medium including 40% sterile glycerol.

2.7. *Lactobacillus* species for probiotic properties tests

2.7.1. Bile salt resistance test

The ability of isolated species to grow in the presence of bile salts was determined in MRS broth as described by Dunne *et al.* (2001). MRS broth (100 ml) mixed with 0.3% (w/v) of bile salt (Sigma) was prepared and autoclaved at 121^{o}C for 15 minutes. All the test tubes were filled with sterilized MRS broth (10 ml in each tube). The tubes were inoculated with each culture suspension of *Lactobacillus* spp. and incubated at 37^{o}C. The growth was checked after 24 hours of incubation and again the culture was streaking in the MRS agar plate and incubated overnight for growth.

2.7.2. Tolerance to acidic pH

Tolerance of isolated *Lactobacilli* to acidic p^H was determined by growing bacteria in acidic MRS broth. Test was replicated twice. A total of 100 ml MRS broth was prepared and sterilized in autoclave. Separately, p^H 4.0 and p^H 3.0 were adjusted with 1 M HCl and 0.5 M NaOH in flask 1 and 2 respectively. In each set, test tubes were labeled as 4.0 and 3.0 p^H. Five milliliter MRS broth was measured in each test tube with the help of sterilized pipette. Culture of each isolated species of *Lactobacilli* was poured in each broth tube. All the tubes were incubated at 37^{o}C anaerobically and survival was evaluated by spreading in the agar plate again for the organisms growth.

2.7.3. Antibiotics sensitivity tests

Antibiotic resistance of isolated species of *Lactobacilli* was determined to 6 commonly used antibiotics by disc diffusion method on solid Mueller- Hinton (MH) agar plates as MH agar is recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for antibiotic sensitivity test.

3. Results

3.1. Isolation of *Lactobacillus* spp. from cloaca and ceacum

Lactobacillus spp. were procured from intestinal contents of chicken after culturing on MRS broth and agar. The results of morphology and colony characteristics of *Lactobacillus* spp. are presented in Table 1.

3.2. Prevalence of *Lactobacillu*s spp. in chicken

Lactobacillus spp. was detected in 82 out of 100 samples. Of them 37 were detected positive from cloacal swab and 45 from caecum. However, a total of 82 isolates were identified as *Lactobacillus* spp. on the basis of morphologically, staining characteristics, growth and colony characteristics in MRS agar. The prevalence of *Lactobacillus* spp. was recorded as 74% in cloaca, 90% in ceacum of broiler chickens. Over all prevalence of *Lactobacillus* spp. in chicken gastrointestinat tract was 82% (Table 2). Biochemical and sugar fermentation test were performed to determine the various species of *Lactobacillus.* The prevalence of *Lactobacillus* in the gastrointestainal tract were *Lactobacillus crispatus (*21.95%), *Lactobacillus acidophilus* (18.29%), *Lactobacillus gallinarum* (17.07%) and 42.69% were unclassified (Table 3).

Table 1. Morphology, staining characteristics of isolated bacteria.

Name of isolates	Color and shape of colonies	Shape	Arrangement	Gram staining reaction	Spore arrangement
Lactobacillus acidophillus	regular, smooth, entire margin, grayish	rods	singly and tapering ends	G +ve	non-spore forming
Lactobacillus crispatus	regular, small, translucent, grayish colonies	rods	Singly and short chains	G +ve	non-spore forming
Lactobacillus gallinarum	Small white colonies	rods	Singly and short chains	G +ve	non-spore forming

Table 2. Isolation of *Lactobacillus* species from poultry sources in Mymensingh.

Origin of sample	No. of sample	No. of *Lactobacillus* spp.
Cloacal swab	50	37 (74%)
Caeca	50	45 (90%)
Total	100	82 (82%)

Table 3. Results of percentages (%) of *Lactobacillus* spp. available in poultry sources in Mymensingh.

Name of isolates	No. of isolates (n=82)	% of the isolates recovered from poultry sources
Lactobacillus crispatus	18	21.95%
Lactobacillus acidophilus	15	18.29%
Lactobacillus gallinarum	14	17.07%
Others Lactobacillus spp.	35	42.69%
Total	82	100

3.3. Molecular detection of *Lactobacillus* spp. by PCR

DNA extracted from ten *Lactobacillus* isolates were used in the polymerase chain reaction (PCR) assay in the investigation Centre for Food and Waterborne Diseases Laboratory, ICCRD'B, Dhaka. Universal 16S rRNA PCR primers targeting 16S rRNA of *Lactobacilli* amplified 472 bp fragments of DNA confirmed the identity of *Lactobacillus* spp. (Figure 1).

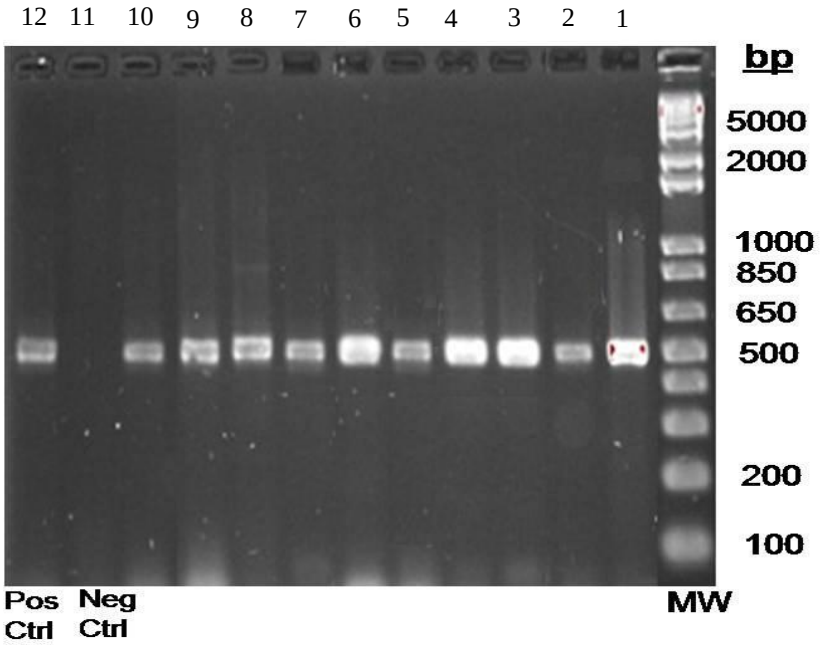

Figure 1. Universal 16S rRNA PCR for *Lactobacillus* strains. Lane MW, 1 Kb Plus DNA Ladder marker (Life Tech). Lanes: 1, isolate 2; 2, isolate 7; 3, isolate 8; 4, isolate 9; 5, isolate 10; 6, isolate 11; 7, isolate 12; 8, isolate 13; 9, isolate 17; 10, isolate 20; 11, negative control; 12, positive control.

3.4. Molecular characterization of *Lactobacillus* spp. by RAPD

The RAPD fingerprinting assay showed excellent reproducibility and efficient discriminative power to check clonal relationship among the tested *Lactobacillus* strains. One of the major aspects of the present study was to determine clonal identity of representative *Lactobacillus* strains. A total of ten isolates of *Lactobacilli* were analyzed by this RAPD method in the investigation Centre for Food and Waterborne Diseases Laboratory, ICCRD'B, Dhaka. In this study, four patterns were detected and six strains were clonal by RAPD method.

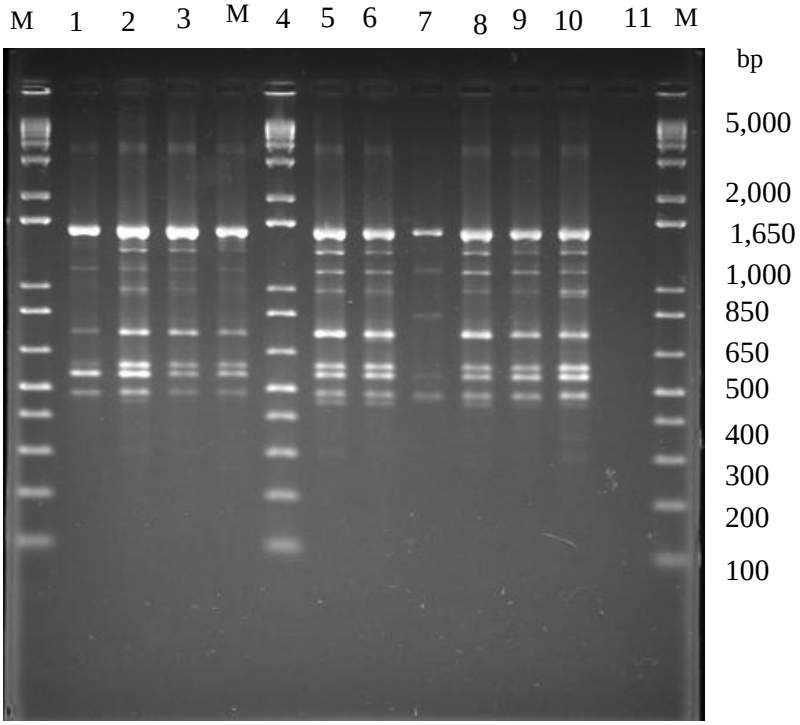

Figure 2. Amplified DNA polymorphisms of *Lactobacillus* isolates. Lane M, 1 Kb Plus DNA Ladder marker (Life Tech). Lanes: 1, isolate 2 (pattern A); 2, isolate 7 (pattern B); 3, isolate 8 (pattern C); 4, isolate 9 (pattern C); 5, isolate 10 (pattern B); 6, isolate 11 (pattern B); 7, isolate 12 (pattern D); 8, isolate 13 (pattern B); 9, isolate 17 (pattern B); 10, isolate 20 (pattern B); 11, negative control.

3.5. Results of probiotic properties tests

3.5.1. Result bile salt resistance test

The results of bile salt resistance test were presented in Table 4. Tolerance level was found variable among all test species of *Lactobacillus* spp. All the tested cultures showed variable resistance against 0.3% concentrations of bile salt. Out of fifteen inoculated test isolates ten *Lactobacillus* isolates were showed positive growth and color change in the test tubes which indicated that *Lactobacillus* spp. were grown in the bile containing medium. This positive culture was inoculated in the MRS agar medium for further growth for the final confirmation of *Lactobacillus* spp. After 36 hours incubation the growth colonies were creamy white and some of them were grayish-white.

3.5.2. Result of tolerance to acidic pH

The results of bile salt resistance test were presented in Table 4. Tolerance to acidic environment by all isolated species of *Lactobacilli* were tested. The survival of *Lactobacillus* species at p^H 3 and p^H 4 was observed after 24 hrs of incubation. All *Lactobacillus* spp. showed consistency in terms of tolerance to p^H 4. Survival at p^H 3 was promising for all isolates. Tolerance level of all species was found significantly variable. Out of fifteen test *Lactobacillus* isolates, there were 13 (87%) test isolates showed positive growth in MRS broth after 24 hours of incubation which p^H 4 was maintained. On the other hand, 11 (73%) test isolates (out of fifteen test isolates) showed positive growth of *Lactobacillus* at p^H 3. It was confirmed that the isolated *Lactobacillus* bacteria showed probiotics characteristics.

3.5.3. Results of antibiotics resistance tests

Antibiotic resistance and susceptibility of isolated *Lactobacilli* species was determined against commonly used antibiotics discs of gentamicin, tetracycline, ampicillin, bacitracin, cefuroxime, cefradine by standard disc diffusion method. The zone of inhibition was measured by calipers in millimeters. The results were expressed as sensitive or resistant (NCCLS, 1997). A total ten isolates which showed the probiotic ability were used for this test. Result showed that *Lactobacillus* spp. were more sensitive against tetracycline and gentamicin. Only one isolates (out of ten isolates) were resistant against gentamicin. In our result, about 10% of *Lactobacillus* spp. were resistant against gentamicin. On the other hand, small zone of inhibition was observed against ampicillin and cefradine. It was showed that seven (out of ten isolates) isolates were resistant against cefradine and six isolates were resistant against ampicillin. So the resistance percentage of *Lactobacillus* spp. against ampicillin and cefradine were 60% and 70% respectively. *Lactobacillus* species were also sensitive against bacitracin and cefuroxime. Slight zone of inhibition was observed against bacitracin and cefuroxime. Three isolates were resistant against bacitracin and four isolates were resistant against cefuroxime. The resistance percentage of *Lactobacillus* spp. against bacitracin and cefuroxime were 30% and 40% respectively. Result was shown in Table 5.

Table 4. Results of tolerance to acidic p^H and bile salt test.

Range of p^H and bile	Total sample incubated	Positive growth	Negative growth	percentage
p^H 4	15	13	2	87%
p^H 3	15	11	4	73%
Bile salt	15	10	5	67%

Table 5. Results of antimicrobial sensitivity test.

Antibiotic name / Isolates no.	Diameter of zone of inhibition (mm)					
	Gentamicin (10µg/disc)	Tetracycline (30µg/disc)	Ampicillin (25µg/disc)	Bacitracin (10µg/disc)	Cefuroxime (30µg/disc)	Cefradine (25µg/disc)
1	25	24	No zone	12	14	8
2	12	22	7	13	11	9
3	16	19	12	No zone	16	8
4	27	30	7	16	20	10
5	22	20	No zone	10	12	13
6	15	19	No zone	12	15	16
7	12	22	12	16	18	8
8	20	26	18	17	12	12
9	23	18	13	12	10	10
10	22	24	No zone	No zone	8	No zone
No(%) of resistance	1(10%)	0(00%)	6(60%)	3(30%)	4(40%)	7(70%)

Resistant zone was considered: Less than 13 mm.
Sensative zone was considered: More than 13 mm.

4. Discussion

The present work was an important endeavor for *Lactobacillus* species research in Bangladesh. The colonies having typical cultural characteristics were selective as presumptive *Lactobacillus* species. Man's Rogosa and Sharps (MRS) agar, selective cultural media for *Lactobacillus* species, were used to culture the organism. The media used in this study were selected on the basis of experience of the past researchers (Behira *et al.,* 2009; Gunasekaran *et al.,* 2012*).*

In this study, apparently healthy chickens (broiler) were used for sample collection. Total 100 samples, 50 caecum contents and 50 cloacal samples, were cultivated on MRS agar for the isolation of *Lactobacillus* species. Total 82 isolates were identified as *Lactobacillus* spp. observing the colony characteristics. Colony characteristics of *Lactobacillus* species observed on MRS agar were similar to the findings of Veera Jothi *et al.* (2012). In Gram's staining, the morphological characteristics of isolated *Lactobacillus* species exhibited Gram-positives, rods, arranged in single or chain or in palisade shaped, non-motile, non-spore forming bacteria under microscope which was supported by other researchers (Felies *et al*., 2007; Behira *et al.,* 2009; Hee-Jin Kim *et al*., 2011; Veera Jothi *et al.,* 2012*).*

The identity of *Lactobacillus* species was confirmed by sugar fermentation and biochemical tests. *Lactobacillus crispatus, Lactobacillus acidophilus* and *Lactobacillus gallinarum* were isolated and identified by sugar fermentation tests as per the procedure followed by Lan (2003). Khalil *et al.* (2007) identified *Lactobacillus* genus based on morphological and biochemical tests. The isolates identified in this study were in agreement with some researchers (Kandler *et al*., 1986; Soumaya *et al.,* 2011; Shruthy *et al*., 2011; Zhang *et al*., 2011).

In this study PCR assay using universal 16S rRNA PCR primers targeting 16S rRNA of *Lactobacilli* amplified 472 bp fragments of DNA confirmed the identity of *Lactobacillus* spp. Total 10 pure colonies were subjected for confirmation of *Lactobacillus* isolates by PCR methods and all of the isolates confirmed as *Lactobacillus* spp. In conventional studies, PCR-based methods have been performed to detect single or a few species of *Lactobacillus*. Similar results were also observed by some researchers (Hyuk-sang *et al.,* 2004; Prashant *et al.,* 2009; Hurtado *et al*., 2011).

Partial 16S rRNA PCR primers were used to identify the isolates to the species level but randomly amplified polymorphic DNA (RAPD) profiles were used to differentiate *Lactobacillus* isolates to the strain level. Based on this studies, four strains were analyzed out of ten isolates by RAPD method. This method was also used by some researchers (Martin *et al.,* 2009; Klose *et al*., 2010; Morandi *et al*., 2011). RAPD fingerprinting has been successfully used to resolve clonal lineages of bacterial strains of a particular target species. Wong *et al.* (1999) developed a RAPD protocol with lower amplification stringency and higher percent agarose gel (1.8% agarose). The improvisation of the PCR reagents in the recent years facilitated the development of long-range RAPD assay by utilizing the advantage of the commercially available long-range Taq polymerase and buffer system. The higher fidelity of the polymerase provides reliable and longer amplification fragments. The long-range RAPD PCR proved useful and indispensable in many areas of research including bacterial molecular typing with a better resolution of the genomic polymorphism of different strains. In this study, the representative *Lactobacillus* strains were subjected to RAPD fingerprinting following Wong *et al*. (1999) with improved reagents of the long range PCR system. RAPD method utilizes single primer complementary to specific repeat sequences in the bacterial genome. One of the major aspects of the present study was to determine clonal identity of representative *Lactobacillus* strains, which showed their potential to be considered as useful probiotic. In this study, four patterns were detected and six strains were clonal by RAPD method. A previously optimized protocol (Wong *et al.,* 1999) for the interpretation of fingerprint patterns of *V. parahaemolyticus*, an enteric bacterial pathogen causing diarrhea, was verified and applied. The RAPD fingerprinting assay showed excellent reproducibility and effecient discriminative power to check clonal relationship among the tested *Lactobacillus* strains.

The overall prevalence of *Lactobacillus* spp. in healthy chickens was recorded as 82% in Mymensingh, Bangladesh. Dubos *et al. (*2011) reported 75% prevalence in apparently healthy chickens, which was slightly lower than this study, whereas Naser-Tajabadi *et al.* (2011) and Soumaya *et al.* (2011) observed the prevalence of various *Lactobacillus* spp. in healthy chickens. It was noticed that there was a little variation in the overall prevalence of *Lactobacillus* spp. in healthy chickens in this study compared to other studies. Several factors might be responsible for such variations such as sanitary condition of farms, unrestricted movements of individuals, vehicles and wild life (Matin, 2014), ventilation system of the farms etc.

The flora of digestive tract of birds was studied extensively and it was found different from the flora of mammals (Smith, 1965; Perez de Rozas *et al.,* 2004). Bacterial population begins to colonize from the first day of life and it spreads throughout gastrointestinal tract and typical microbiota is settled as soon as feed is given. Among important bacteria of microbiota, lactic acid bacteria occupy a dominant place and contribute a lot in

balancing ecology of gastrointestinal tract (Apajalahti *et al*., 2004). Among lactic acid bacteria, *Lactobacilli* has dominance in the anterior part of gut i.e. crop, duodenum and jejunum. Most of the studies revealed that *Lactobacilli* were mostly inhabited in crop and caecum region (Sarra *et al.,* 1992). *Lactobacilli* efficiently colonize the stratified squamous epithelium lining of the crop, which functions as a food storage pouch. In the crop, carbohydrate digestion begans with moistening and microbial fermentation (Hammes and Vogel, 1995). In crop and caecum of chicken, acids were produced by *Lactobacilli* through fermentation process that decrease the p^H of crop and in turn this improves the absorption of feed nutrients (Sarra *et al.,* 1992; Boling *et al*., 2001). However, bacteriostatic property of *Lactobacilli* in crop and ceacum was due to low p^H, but the bactericidal activity could not be accounted for by the p^H alone (Fuller, 1973).

In chicken, mainly bacterial population constituted crop and caeca and, to a lesser extent, the small intestine. From crop to terminal ileum, Gram positive, particularly facultative anaerobes are dominant while caeca is mainly composed of strict anaerobes. The crop and caecum was largely consisted of *Lactobacilli*, enterococci and yeast (Fuller, 1984; Gong *et al*., 2002). Many *Lactobacillus* strains, isolated from various sources, were being used as probiotic agents and it was unlikely that each species/strain possesses all of the characters that will make it a suitable probiotic. The exposure of organisms to stressful conditions, usually encountered in the gastrointestinal tract as well as antimicrobial substances might affect their probiotic properties (Salminen *et al*., 1998).

Bacteria must tolerate bile salts concentration for their metabolic activity as well as to colonize in the gastrointestinal tract (Havenaar *et al*., 1992). Therefore, it was necessary to evaluate the resistance ability of bacteria to bile acids before using them as probiotics (Lee and Salminen, 1995). In this study, *Lactobacillus* species which were isolated from intestinal contents of chicken were found tolerant to high concentration (1.0%) of bile salt. The results of resistance against bile salt are supported by the findings of Gilliland (1979) who reported that *Lactobacilli* which were isolated from animal intestines showed high tolerance to biliary salts than species isolated from milk products. Similar results were found in another study conducted by Patel *et al.* (2004). In this study, *Lactobacillus reutrei* was isolated from human intestines and found highly resistant to 2.0% bile salt. The normal level of bile salt in intestines is nearly 0.3% whereas 1.0% concentration of bile salt was used in this studies. The resistance ability is variable among *Lactobacillus* species as well as among different strains (Xanthopoulos, 1997) because resistance to bile salt is due to the presence of bile salt hydrolase (BSH), an enzyme that reduces toxic effects by conjugating bile (Du Toit *et al*., 1998). The absence of activity of BSH exposes bacterial species to be sensitive to bile salts that has been reported to affect glycolipids and phospholipids of bacterial cell membranes. So cell membrane permeability will be modified by bile salts (Taranto *et al*., 2003). BSH activity is mostly found in the species of *Lactobacillus* which are isolated from faeces or intestines of animals (Bateup *et al*., 1995; Tanaka *et al.,* 1999). This is in accordance with our results as isolated from chickens, showed high resistance to bile salts.

As acidic conditions are prevailing in the individual segments of gastrointestinal tract of chicken due to the production of hydrochloric acid and enzymes like pepsin (Pepsin, p^H=3.0). In caeca region, p^H ranges from 5.60 to 5.83 and 6.08 to 6.58 in the colon (Gabriel *et al*., 2006). So bacterial species must be acid tolerant and pass through gastric transit to be used as probiotics. In this study, tolerance to acidic environment of isolated *Lactobacillus* species was determined after culturing in acidic medium. All the species showed different but acceptable survival at acidic p^H. Our results showed that isolated bacterial species can survive in acidic p^H. Thus acid tolerant strains have advantage to survive in the gastrointestinal tract.

The development and use of antibiotic has been one of the most important steps towards controlling of infectious bacterial diseases in 21st century. However, the subsequent appearance and spread of antibiotic resistance in pathogenic organisms have made many currently available antibiotics ineffective (Khan *et al.,* 2007; Khan, 1992). Antibiotics has been used broadly in human, veterinary medicine, agriculture and aquatic practices and it has progressively in increased particularly in the developing countries. Currently, antimicrobial resistance is a rising public health threat and has been designed by the WHO a rising public health trouble (Sharma and Malik, 2012).

One of the most important characteristics of probiotics was their resistance against antibiotics particularly when these are used after antibiotic treatment. We used the disk diffusion method to determine the antimicrobial susceptibility and resistance of 10 representative *Lactobacillus* species to six antibiotics. The antibiotics used for the tests included ampicillin, gentamicin, tetracycline, bacitracin, cefuroxime, cefradine. All 10 *Lactobacillus* isolates were resistant to ampicillin, bacitracin, cefuroxime and cefradine. But all *Lactobacillus* isolates were sensitive to gentamicin and tetracycline. Among the selected ten isolates 00, 10, 30, 40, 60 and 70% were found to be exhibiting a significant degree of resistance to tetracycline, gentamicin, bacitracin, cefuroxime, ampicillin and cefradine respectively. As far as antibiotic susceptibility of isolated *Lactobacilli* is concerned, results

indicated that almost all the species were found resistant to commonly used antibiotics except tetracycline and gentamicin. These results are supported by many reports indicating that lactic acid bacteria are resistant to commonly used principal antibiotics (Halami *et al*., 2000; Nawaz *et al*., 2011; Bobcek *et al*., 2011; Coppola *et al*., 2005). Nemcova *et al*. (1997) isolated 13 strains of *Lactobacilli* from suckling piglets and conducted their antibiotic susceptibility test and found they were susceptible to many common feed additive antibiotics. The resistance of bacteria depended upon their genus, species, strain and logarithmic growth phase. Antibiotic therapy may cause changes in the composition of intestinal microflora and as result there is disturbance in intestinal homeostasis. *Lactobacilli* species are regarded as effective in preventing such kinds of complications (McFarland and Elmer, 1997). However, one of the important feature of resistant probiotic bacteria is that they should not have the genes which can be transferred to other bacterial population (Zhau *et al.*, 2011).

5. Conclusions

Potential probiotic bacterial strains from poultry sources were identified as well as characterized in this study. It is recommended that these locally isolated *Lactobacilli* may be used as probiotics after species/strain identification through molecular methods and extensive field trials.

Acknowledgements

This study was supported by a financial grant (Project no. 2013/117/MOST) from the Ministry of Science and Technology, Government of the People's Republic of Bangladesh, Bangladesh Secretariat, Dhaka–1000, Bangladesh.

Conflict of interest

None to declare.

References

Apa AL, J Dupraz, R Ross, SN Gonzalez and ME Arena, 2010. Probiotic administration effect on fecal mutagenicity and microflora in the goat's gut. Journal of Bioscience and Bioengeneering,110: 537–540.

Apajalahti JH, A Kettunen and H Graham, 2004. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. J. Worlds Poult. Sci., 60: 223–232.

Awan JA and SU Rahman, 2002. Microbiology Manual.Unitech Communications, Faisalabad–Pakistan.

Bateup J, MA McConnell, HF Jenkinson and G Tannock, 1995. Comparison of *(Lactobacillus)* strains with respect to bile salt hydrolase activity, colonization of the gastrointestinal tract and growth rate of the murine host. Appl. Environ. Microbiol., 61: 1147-1149.

Behira B, M Moulay and M Kihal, 2009. Probiotic potential of thermotolerants*Lactobacilli* isolated from chicken gastrointestinal digestive and their use as poultry feed. World Appl. Sci. J., 7: 951-957.

Bobcek B, L Gajdosova, M Kacaniova, J Lejkova and L Hleba, 2011. Antibacterial susceptibility of *Lactobacilli* isolated from pork meat of conventional breeding. Biotech. Anim. Husbandry, 27: 799-807.

Boling SD, JL Snow, CM Parsons and DH Baker, 2001. The effect of citric acidon the calcium and phosphorus requirements of chicks fed cornsoyabean meal diets. Poult. Sci., 80:783–788.

Coppola R, M Succi, P Tremonte, A Reale, G Salzano and E Sorrentino, 2005. Antibiotic susceptibility of *L. rhamnosus* strains isolated from Parmigiano Reggiano cheese. Le Lait, 85: 193-204.

du Toit M, CM Franz, LM Dicks, U Schillinger, P Haberer, B Warlies, F Ahrens and WH Holzapfel, 1998.Characterization and selection of probiotic *Lactobacilli* for a preliminary minipig feeding trail and their effect on serum cholesterol level, faeces moisture contents. Int. J. Food Microbiol., 40: 93-104.

Dubosn C, N Vega, C Carvallo, P Navarrete, C Cerda, O Brunser and M Gotteland, 2011. Identification of *Lactobacillus* spp. in colostrum from Chilean mothers. Archivos Latinoamericanos de Nutricion, 61: 66-68.

Dunne C, L Murphy, S Flynn, L O'mahony, S O'halloran, M Feeney, D Morrissey, G Thornton, G Fitzgerald, C Daly, B Kiely, EM Quigley, GC O'sullivan, F Shanahan and JK Collins,1999. Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. Antonie Van Leeuwenhoek,76: 279-292.

Dunne C, L O'Mahony, L Murphy, G Thornton, D Morrissey, S O'Halloran, M Feeney, S Flynn, G Fitzgerald, C Daly, B Kiely, GC O'Sullivan, F Shanahan and JK Collins, 2001. In-vitro selection criteria for probiotic bacteria of human origin: corrlation with in vivo findings. Am. J. Clin. Nutr.,73: 386-392.

Felis GE and F Dellaglio, 2007.Taxonomy of Lactobacilli and Bifidobacteria. Curr. Issues Intestinal Microbiol., 8: 44–61.

Fuller R, 1973. Ecological studies on the *Lactobacillus* flora associated with the crop epithelium of the fowl. J. Appl. Bacteriol., 36:131–139.

Fuller R, 1984. Microbial activity in the alimentary tract of birds. Proceedings of the Nutrition Society, 43: 55-61.

Gabriel I, M Lessire, S Mallet and JF Guillot, 2006. Microflora of the digestive tract: critical factors and consequences for poultry. World's Poul. Sci. J., 62: 499-511.

Gilliland SE, 1979. Beneficial interrelationships between certain microorganisms and human: candidate microorganisms for use as dietary adjuncts. J. Food Prot., 42: 164-167.

Gong J, RJ Forster, H Yu, JR Chambers, R Wheatcroft, PM Sabour and S Chen, 2002. Molecular analysis of bacterial populations in the ileum of broiler chickens and comparison with bacteria in the cecum. FEMS Microbiol. Ecol., 41: 171-179.

Gong J, W Si, RJ Forster, R Huang, H Yu, Y Yin, C Yang and Y Han, 2007. 16S rRNA gene-based analysis of mucosa associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crop to ceca. FEMS Microbiol. Ecol., 59:147–157.

Grimoud J, H Durand, C Courtin, P Monsan, F Ouarne, V Theodorou and C Roques, 2010. In vitro screening of probiotic lactic acid bacteria and prebiotic glucooligosaccharides to select effective synbiotics. Anaerobe,16: 493-500.

Gunasekaran S, R Karunakaran, V Balakrishnanand and M Parthiban, 2012. Identification and in vitro evaluation of species specific probiotic for feeding broiler chicken using probiotic scores. International Journal of Veterinary Science, 1: 64-68.

Halami PM, A Chandrashekar and K Nand, 2000. *Lactobacillus farciminis* MD, a newer strain with potential for bacteriocin and antibiotic assay. Lett. Appl. Microbiol., 30: 197–202.

Hammes WP and RF Vogel, 1995.The genus *Lactobacillus*. In: B. J. B. Wood and W. H. Holzapfel (ed.), The lactic acid bacteria, Vol. 2. The genera of lactic acid bacteria. Blackie Academic, London, United Kingdom. pp. 19–54.

Havenaar R, B Ten Brink and J Huisin't Veld, 1992. Selection of strains forProbiotic use. In: *Probiotics.* The Scientific Basis, R. Fuller, (Ed).Chapman and Hall, London), pp. 209–221.

Hee-Jin K, E Seok-Jin, P Sung-Joon, P Chang-Jun and K Geun-Bae, 2011. *Lactobacillus alvi* spp. nov., isolated from the intestinal tract of chicken. FEMS Microbiol. Lett., 323: 83–87.

Hurtado A, NB Othman, N Chammem, M Hamdi, S Ferrer, C Reguant, A Bordons and N Rozes, 2011. Characterization of *Lactobacillus* isolates from fermented olives and their bacteriocin gene profiles. Food Microbiol., 28:1514-1518.

Kabir SML, 2009.The role of probiotics in the poultry industry. Int. J. Mol. Sci.,10: 3531-3546.

Kabir SML, MM Rahman, MB Rahman, MM Rahman and SU Ahmed, 2004.The dynamics of probiotics on growth performance and immune response in broilers. Int. J. Poult. Sci., 3: 361-364.

Kabir SML, MM Rahman, MB Rahman, MZ Hosain, MSI Akand and SK Das, 2005. Viability of probiotics in balancing intestinal flora and effecting histological changes of crop and caecal tissues of broilers. Biotechnology, 4: 325-330.

Kandler O and N Weiss, 1986. Genus *Lactobacillus*. Bergey's Manual of Systematic Bacteriology. Baltimore, USA. pp. 1209–1234.

Khalil R, R Mahrous, K Halafawy, K Kamaly, J Frank and M Soda, 2007. Evaluation of the probiotic potential of lactic acid bacteria isolated from faeces of breast-fed infants in Egypt. Afr. J. Biotechnol., 6: 939-949.

Khan M, D Raoult, H Richet, H Lepidi and B La Scola, 2007. Growth-promoting effects of single-dose intragastrically administered probiotics in chickens. Br. Poult. Sci., 48: 732-735.

Khan ML, I Ullah and MT Javed,1992. Comparative study of probiotic, TM-50, Biovin 40 and Albac on the performance of broiler chicks. Pak. Vet. J., 12: 145-157.

Klose V, R Bruckbeck, S Henikl, G Schatzmayr and AP Loibner, 2010. Identification and antimicrobial susceptibility of porcine bacteria that inhibit the growth of Brachyspirahyodysenteriae in vitro. J. Appl. Microbiol., 108: 1271-1280.

Kwon HS, EH Yang, SW Yeon, BH Kang and TY Kim, 2004. Rapid identification of probiotic *Lactobacillus* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. FEMS Microbiol. Lett., 239: 267–275.

Lan PT, TL Binh and Y Benno, 2003. Impact of two probiotics *Lactobacillus* strains feeding on fecal *Lactobaclli*and weight gains in chickens. J. Gen. Appl. Microbiol., 49: 29-36.

Lee YK and S Salminen, 1995.The coming age of probiotics. Trends in Food Sciences and Technology, 6: 241–245.

Magne F, M Abely, F Boyer, P Morville, P Pochart and A Suau, 2006. Low species diversity and high interindividual variability in faeces of preterm infants as revealed by sequences of 16S rRNA genes and PCR-temporal temperature gradient gel electrophoresis profiles. FEMS Microbiol. Ecol., 57:128-138.

Martin R, M Olivares, M Perez, J Xaus, C Torre, L Fernandez and JM Rodriguez, 2010. Identification and evaluation of the probiotic potential of Lactobacilli isolated from canine milk. Veterinary Journal, 185: 193-198.

Martin R, S Delgado, A Maldonado, E Jimenez, M Olivares, L Fernandez, OJ Sobrino and JM Rodriguez, 2009. Isolation of Lactobacilli from sow milk and evaluation of their probiotic potential. J. Dairy Res., 76: 418-425.

McFarland LV and GW Elmer, 1997. Pharmaceutical probiotics for the treatment of anaerobic and other infection. Anaerobe, 3: 73-78.

Mead GC, 1997. Bacteria in the gastrointestinal tract of birds. Gastrointestinal Microbiology, Chapman and Hall, New York. pp. 216-240.

Nawaz M, J Wang, A Zhou, C Ma, X Wu, JE Moore, BC Millar and J Xu, 2011.Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Curr. Microbiol., 62: 1081-1089.

NCCLS, 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed., vol. 17, no. 2. National Committee for Clinical Laboratory Standards, Wayne, PA.

Nemcova R, A Laukova, S Gancarcikova and R Kastel, 1997. In vitro studies of porcine *Lactobacilli* for possible probiotic use. Appl. Environ. Microbiol., 110:413-417.

Neville BA and PW O'Toole, 2010. Probiotic properties of *Lactobacillus salivarius* and closely related *Lactobacillus* species. Future Microbiol., 5: 759–774.

Patel HM, SS Pandiella, RH Wang and C Webb, 2004. Influence of malt, wheat, and barley extracts on the bile tolerance of selected strains of Lactobacilli. Food Microbiol., 21: 83-89.

Perez de Rozas, AM Roca, R Carabano, C de Blas, M Francesch, J Brufau, SM Marin-Orue, J Gasa, S Campoy, J Barbe and I Badiola, 2004. A comparative study of intestinal microbial diversity from birds, pigs and rabbits by restriction fragment length polymorphism analysis. Reproduction Nutrition Development, 44: 4.

Prashant, SK Tomar, S Rameshwar, SC Gupta, DK Arora, BK Joshi and K Dinesh, 2009. Phenotypic and genotypic characterization of *Lactobacilli* from Churpi cheese. Dairy Science and Technology, 89: 531-540.

Salminen S, A Wrigh, L Morelli, P Marteau, D Brassart, WM de Vos, R Fonden, M Saxelin, K Collins, G Mogensen, SE Birkeland and T Mattila-Sandholm,1998. Demonstration of safety of probiotics, a review. Int. J. Food Microbiol., 44:93–106.

Sarra PG, L Morelli and V Bottazzi, 1992. The lactic microflora of fowl. In: B. J. B. Wood (Ed.), The lactic acid bacteria in health and disease, Vol. 1. Elsevier Applied Science, London, U.K. pp. 3-19.

Sharma D and A Malik, 2012. Incidence and prevalence of antimicrobial resistant *Vibrio cholerae* from dairy farms. Afr. J. Microbiol. Res., 25: 5331-5334.

Shruthy V, M Pavithra, S Gowri and RG Asit, 2011. Probiotic potentials among lactic acid bacteria isolated from curd. International Journal of Research in Ayurveda and Pharmacy, 2: 602-609.

Smith HW, 1965. Observations on the flora of the alimentary tract of animals and factors affecting its composition. J. Path. Bacteriol., 89: 95-122.

Soumaya M, K Gilles, R Albert, F Mounir, P Hervé, D Djamel, M Mohamed and D Xavier, 2011. Identification of Lactobacilli residing in chicken ceca with antagonism against *Campylobacter*. International Microbiology, 14: 103-110.

Tajabadi N, M Mardan, MYA Manap, M Shuhaimi, A Meimandipour and L Nateghi, 2011. Detection and identification of *Lactobacillus* bacteria found in the honey stomach of the giant honeybee Apisdorsata. Apidologie, 42 642-649.

Tanaka H, K Doesburg, T Iwasaki and I Mireau, 1999. Screening of lactic acid bacteria for bile salt hydrolase activity. J. Dairy Sci., 82: 2530-2335.

Tannock GW, K Munro, HJM Harmsen, GW Welling, J Smart and PK Gopal, 2000. Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. Appl. Environ. Microbiol., 66: 2578–2588.

Taranto MP, ML Fèrnandez Murga, G Lorca and GF de Valdez, 2003. Bile salts and cholesterol induce changes in the lipid cell membrane of *Lactobacillus reutrei*. J. Appl. Microbiol., 95:86-91.

Veera VJ, KTK Anandapandian and S Shankar, 2012. Bacteriocin production by probiotic bacteria from curd and its field application to poultry. Archives of Applied Science Research, 4: 336-347.

Wong H, C Liu, T Pan, T Wang, C Lee and DY Shih, 1999. Molecular typing of *Vibrio parahaemolyticus* isolates, obtained from patients involved in food poisoning outbreaks in Taiwan, by random amplified polymorphic DNA analysis. J. Clin. Microbiol., 37: 1809–1812.

Xanthopoulos V, E Litopoulou-Tzanetaki and N Tzanetakis, 1997. *In vitro* study of *Lactobacillus* species strains on bile tolerance and cholesterol removal. In: Lactic Acid Bacteria – Caen, University Press, Caen.

Zhi-dan Z, G Xiao-hua and X Hai-rong, 2011. Screening of *Lactobacillus* with amylolytic activity and its identification.Chinese Journal of Microecology, 23: 769-779.

Zulkifli I, N Abdullah and NM Azrin, 2000. Growth performance and immune response of two commercial broiler strains fed diets containing *Lactobacillus* cultures and oxytetracycline under heat stress conditions. Br. Poult. Sci., 41: 593-597.