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Original Article

Occurrence of Multidrug Resistant *Salmonella* spp. in Poultry and Approach for Its Indigenous Bio-control

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A significant limitation to flourish poultry industry in Bangladesh is the emergence of multidrug resistance pathogenic bacteria like *Salmonella* spp. due to uncontrolled use of antibiotics for disease treatment. An alternative to antibiotics could be the application of probiotics. About 120 cloacal-swabs from poultry birds were investigated, seventy two (72) isolates of *Salmonella* spp. and twenty two (22) isolates of lactic acid bacteria (out of 50)were identified respectively by cultural, morphological and biochemical tests presumptively. Antibiotic sensitivity test of *Salmonella* spp. was performed, followed by Kirby-Bauer disc-diffusion assay with six antibiotic groups. All those isolates of *Salmonella* spp. were found to be highly resistant to β-lactam, cephalosporin, tetracycline and macrolide, highly sensitive to carbapenem and moderately sensitive to aminoglycosides. Then *Salmonella* spp. were used as a target for the prospective probiotic bacteria which were screened based on antimicrobial activity against those multidrug resistance *Salmonella* spp. In antagonism assay such as disc diffusion and one-streak method, it was revealed that five lactic acid bacteria showed antimicrobial activity against *Salmonella* spp. Further, lactic acid bacteria were characterized based on their tolerance ability to pH and NaCl, antibiotic susceptibility test. The tolerance range of Lactic acid bacteria was about pH (3.5-9.5), NaCl (4-8) % and also resistant to antibiotics groups like B lactam, aminoglycosides, and quinolone. Then 16S rDNA gene sequence analysis was performed for molecular identification of potential probiotic bacteria. One representative isolate was identified as phylogenetically closed relative to *Pediococcus acidilactici*. This study was able to demonstrate that *Pediococcus acidilactici* as anindigenous probiotic candidate to inhibit the growth of isolated multidrug resistant *Salmonella* spp. in poultry. The potent probiotic candidate *Pediococcus* sp. could be used to counter bacterial diseases in poultry, thereby it could ensure food safety in the poultry industries of Bangladesh.

Key words: Poultry, Bacterial disease, Antibiotics, Multidrug resistance, Probiotic bacteria, Antagonistic activity.

Introduction

Poultry is essential to the national economy of Bangladesh and the welfare of human beings. Commercial poultry production is ranked among the highest source of animal protein in the world[1]. Poultry production as an excellent agribusiness is flourished rapidly in Bangladesh[2]. Poultry is exposed to stressful conditionsduring large-scale rearing facilities, diseases and deterioration of environmental conditions often arise, as a consequence in serious economic losses. Among the bacterial diseases; salmonellosis is considered one of the most prevalent diseases in both humans and animals [3, 4].

In recent decades, application of veterinary medicines to control of diseases has been increased significantly. However, the rationale of using of antimicrobial agents has been questioned, because of the emergence of antimicrobial resistance among pathogenic bacteria. Moreover, the frequent and irrational uses of antibiotics have evolved antimicrobial resistance to an alarming rate[5]. In poultry, the antibiotic selection pressure for resistance in bacteria is high and as a result, a considerably huge proportion of resistant bacteria present in fecal materials[6, 7].

So, the side-effects of using antibiotics as therapeutic agents stop the likelihood of antibiotics to be used as growth stimulants for poultry, ultimately make concern in both manufacturer and consumer looking for alternatives. Probiotics are being considered to fill this gap and already some farmers are using theminstead of antibiotics[8-10]. Probiotics are live microorganisms believed to be beneficial for the host organism. According to the currently adopted definition by WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host[11, 12]. Members of the genera *Bifidobacterium* spp., *Lactobacillus* spp., *Pediococcus* spp., *Streptococcus* spp. and *Enterococcus* spp. are the most frequently used probiotics[13] Probiotics have received increasing attention as an alternative to in-feed antibiotics and for the purpose of improving productivity in the poultry industry[14].

Lactic acid bacteria (LAB), among important normal microbial population in chicken[15], produce a number of antimicrobial substances such as organic acids, free fatty acids, ammonia, reuterin, diacetyl, hydrogen peroxide and bacteriocin, which have the capacity to inhibit the growth of food spoilage and pathogenic

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organisms[16]. *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp. *and Streptococcus* spp. are referred to typical LAB genera. LAB can be tolerant to acid and bile, adhere to the intestinal epithelium of the hosts; they show an antagonistic activity against pathogenic bacteria and keep their viability during processing and storage. It is reported that LAB (lactic acid bacteria) to be used as probiotics minimally, they must have the basic properties: (a) they must be generally recognized as safe (GRAS), (b) they must have antagonistic activity against bacterial pathogenic, (c) they must be tolerant to acid and bile[17].

The current study aimed at evaluating the probiotic potential of lactic acid bacteria (LAB) isolated from poultry sample against these multidrug resistant poultry pathogens.

Materials and Methods

Sample collection

Total 120 samples of Poultry (such as cloacal sample of broiler chicken, commercial layer hen, backyard raised layer hen) were collected aseptically from some poultry farms and local market of Noakhali. Sample was collected with a sterile cotton swab and then the cotton swab was inoculated into sterile saline containing test tube. Samples were transported immediately (approximately within 1 hour) to the laboratory, the department of Microbiology, Noakhali Science and Technology University, Noakhali for microbiological analysis.

Isolation of bacterial isolates

For enrichment technique, the samples were inoculated in Tryptone Soya Broth (TSB) medium, and then incubated at least 6 hours at 37°C. It was subsequently analyzed by subculture on nutrient agar plate and respective selective agar media plates.

Target pathogenic bacteria

To identify pathogenic bacteria of poultry, Xylose Lysine Deoxycolate (XLD) media (Oxoid) and was used for isolation of the *Salmonella* spp. The sample streaked on these agar plates were incubated for 24 hours at 37°C.

Potential probiotic bacteria

To find out a control approach, an indigenous probiotic bacterium such as lactic acid bacteria was isolated using deMan, Rogosa and Sharpe (MRS agar) medium (Oxoid). After enrichment, the cloacal samples inoculated into MRS broth, then subsequently plated onto MRS agar and incubated for 24 hours to 72 hours at 37°C.

Presumptive identification of bacterial isolates

Isolated bacterial isolates were identified presumptively by microscopic and biochemical tests according to standard protocol as described in Laboratory manual for Microbiology[18].Pure culture was selected for these analyses of presumptive identification of following tests. To observe morphological characteristics, Gram staining was performed.

A series of biochemical tests were performed such as Triple Sugar Iron (TSI) agar test, Methyl Red (MR) Test, Voges-Proskauer (VP) test, Indole test. Citrate Utilization test, Urease test, oxidase test, catalase test for identification of *Salmonella* spp.

For isolated lactic acid bacteria, biochemical tests such as catalase test, oxidase test, motility test, indole test and sugar fermentation tests using different carbohydrates including Ribose, D-fructose, L-xylose, Mannitol, Raffinose, Lactose and glucose were performed.

Antimicrobial susceptibility test

The antimicrobial susceptibility test for the selected pathogenic and probiotic bacterial isolates was conducted by the Kirby Bauer method[19]. Inoculum density of bacterial isolates was adjusted to McFarland 0.5 turbidity standard (equivalent to cell density of ca: 10^8cfu/ml). A sterile cotton swab was dipped into the adjusted cell suspension and swabbed over the entire surface of Mueller-Hinton agar plate. The antimicrobial discs were dispensed onto the surface of the inoculated agar plates. The antibiotic sensitivity of the isolated *Salmonella* spp. was performed by using commercially available standardized antibiotic disks of Gentamicin (10¼g), Tetracycline (30¼g), Penicillin G (10¼g), Oxacillin (10¼g), Ampicillin (10¼g), Cefotaxime (30¼g), Ceftazidime (30¼g), Ceftriaxone (30¼g), Imipenem (10¼g), Amikacin (30¼g), Chloramphenicol (10¼g), Netilmicin (10¼g), Ciprofloxacin (10¼g).

For LAB (lactic acid bacteria) isolates, Ampicillin (10µg), Chloramphenicol (30µg), Gentamicin (10µg), Vancomycin (30µg), Streptomycin (10µg), Nalidixic acid (30µg), Ciprofloxacin (5µg), Tetracycline (30µg) and Erythromycin (15µg) antibiotic discs were employed.

The plates were incubated at 37°C for 24h and observed for the clear zone of inhibition. Susceptible and resistant isolates were defined according to the standard table given by the Clinical Laboratory and Standards Institute (CLSI, 2008)[20].

Screening of Potential probiotic

Antagonism assay

Disc Diffusion Assay

In disc diffusion method, Mueller-Hinton Agar plates were swabbed over the reference pathogenic bacterial overnight cultures. The discs were prepared by impregnating the sterile discs into the solution of different lactic acid bacteria. Finally all soaked disc were air dried for 3 hours before placing on agar plates. Within 15 min of applying the discs, the plates were inverted and incubated. Plates were incubated at 37°C for 24h. The diameters of the zones of inhibition, including the diameter of the disc were measured.

One-streak method

In this technique, cotton swab dipped into bacterial suspension (pathogen) and swabbed over the Mueller-Hinton agar plates.

Then another cotton swab or an inoculating loop inoculated into putative probiotic suspension and then streaked over the Mueller-Hinton agar plates. Plates were incubated at 37°C for 24 h. Antimicrobial activity was evaluated by measuring the inhibition zone.

Determination of pH tolerance

For the determination of pH tolerance for of the lactic acid bacterial isolates, 100¼l overnight culture of the isolates was inoculated into 5 ml MRS broth with varying pH ranging from (3.5- 9.5). The pH was adjusted with concentrated HCl or NaOH .The inoculated broths were incubated under anaerobic condition for 24 h at 37°C. Bacteria growth was measured using a spectrophotometer at 560 nm[21].

Measurement of NaCl tolerance

For the determination of NaCl tolerance, all lactic acid bacterial isolates were grown in MRS broth supplemented with different concentrations of NaCl (2,4, 6, 8, 10 and 12 percent) that were inoculated after sterilization with 1% (v/v) of overnight culture of Lactic acid bacteria and then were incubated an aerobically for 24h at 37°C. The bacterial densities were determined by visual measurement of their turbidity and were classified as Maximum growth (++), normal growth (+), and no growth (-) [21].

Molecular identification of potential probiotic

Extraction chromosomal DNA

Total DNA was extracted by boil DNA method. The method was performed according to the procedure outlined by B. Malorny (http://www.pcr.dk/DNA-purification.htm).

PCR amplification of 16S rRNA gene

Polymerase Chain Reaction (PCR) was carried out by the16S rDNA specific primers using forward 27F 5 - AGAGTTTGATCMTGGCTCAG-3"and reverse 1492R 5 - GGTTACCTTGTTACGACTT-3" primers. The PCR reaction was performed with the following program: initial denaturationat 96°C for 5 minutes, then Thirty five (35) cycles of the segments were repeated with Denaturation at 96°C for 1 minute 30 seconds, Annealing at 55°C for 1 minute, Extension at 72°C for 1 minute 30 seconds, a final extension of 10 min at 72°C. The successful amplifications of the 16S rDNA gene were examined by resolving the PCR products in 1% agarose gel, visualized under UV light and digitalized by the AlphaImager HP System Versatile Gel Imaging (USA).

Sequencing and phylogenetic analysis

After purification of the PCR products, cycle sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA) according to manufactures instruction and extension product was purified followed by capillary electrophoresis using ABI Genetic Analyzer (Applied Biosystems®, USA). Partial sequences, obtained using forward and reverse primers, were combined to full length sequences (1400 bp–1500 bp) via the Seq Man Genome Assembler (DNA star, USA). Multiple sequence alignment of the retrieved reference sequences from National Center for Biotechnology Information (NCBI) was performed with the ClustalW[22]software and was exported to the Molecular Evolutionary Genetics Analysis (MEGA) program[23]for phylogenetic tree construction using the Neighbor joining algorithm.

Results and Discussion

Occurrence of Salmonella spp. in poultry

Salmonellosis in poultry causing heavy economic loss through mortality and reduced production is a primary concern in developed, as well as developing countries[24]. Rapidly flourished poultry rearing and farming, pullorum disease and fowl typhoid have become wide spread problem in Bangladesh[25]. Therefore it is very important to address the disease causing agent, the current study was conducted in order to isolate and evaluate the status of *Salmonella* spp. in the poultry industry. *Salmonella* spp. has been isolated predominantly from poultry and is the most frequent cause of human salmonellosis. This study was designed to detect *Salmonella* spp. from poultry samples of different areas of Noakhali, Bangladesh. Out of 120 cloacal-swab of poultry samples, 72 isolates appearing black colonies on XLD agar plate (Figure 1A), were Gram negative, rod shaped with showing positive result of TSI agar, indole test, VP test, Citrate test, oxidase test, catalase test and urease test which presumptively confirmed *Salmonella* spp. This study revealed that the overall prevalence of *Salmonella* spp. was 60% (Table 1); In Mosul, Al-Hakeem et al.[26]reported that the overall prevalence of *Salmonella* spp. was 3.68%, whereas Jafari et al.[27] mentioned that the overall percentage of *Salmonella* spp. was 5.8 in south and west region of Iran. These differences in overall prevalence of *Salmonella* spp. may be related to several factors such as, environment, system of management and resistant of these chickens to be susceptible to *Salmonella* spp.[28].

Emergence of multidrug resistance Salmonella spp. in poultry

Antibiotics have been successfully used in humans and veterinary medicine as food animal growth promoting agents, prophylaxis or therapeutics. However, their indiscriminate use has created enormous pressure for selection of antimicrobial resistance among bacterial pathogens. Nowadays, there is increasing concern about the development of multidrug resistance (MDR) in bacterial species causing zoonosis and having an important animal reservoir such as *Salmonella* strains[29]. In different parts of the world, multi drug resistant strains of *Salmonella* spp. are ubiquitous in poultry and poultry environments[30]. This study attempted to reveal the multidrug resistance pattern of these isolated *Salmonella* spp. by challenging with 6 groups of antibiotics. Isolated *Salmonella* spp. exhibited100% resistance against ²-Lactam group, Cephalosporin group, Macrolides group, Tetracycline group of antibiotics. They were moderately resistant to Aminoglycosides group and sensitive to Carbapenem group of antibiotics (Figure 2) and thus considered as multidrug

Table 1: *Prevalence of Salmonella spp. and lactic acid bacteria in collected poultry samples*

Name of the places for sample collection	Presence of *Salmonella* spp.		Presence of lactic acid bacteria	
	Number of the sample	Number of *Salmonella* spp. positive sample	Number of the sample	Number of lactic acid bacteria positive sample
Bismillah Agro Farm	50	24	10	5
Sonapur Poultry Farm	30	20	10	5
Sonapurbazaar	20	16	20	7
Village home	20	12	10	5
Total	120	72	50	22

Table 2: *Observation of NaCl tolerance*

Isolates ID	Concentration of NaCl					
	2%	4%	6%	8%	10%	12%
L1	+	++	++	+	+	+
L2	++	+	+	+	-	-
L3	+	++	+	+	-	-
L4	+	++	+	+	-	-
L5	+	++	+	+	+	+

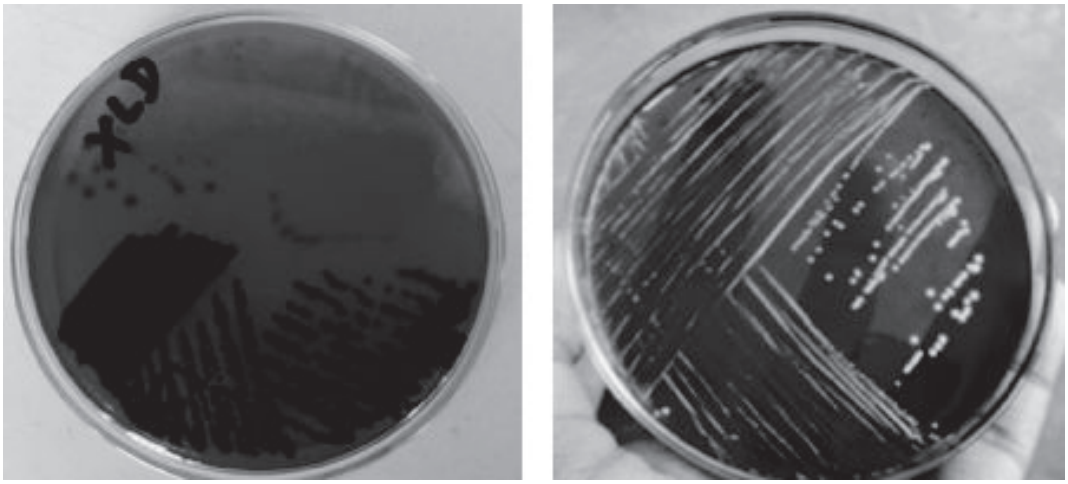

Figure 1: *Growth of Salmonella spp on XLD agar plate (A) and lactic acid bacterial isolate on MRS agar plate (B).*

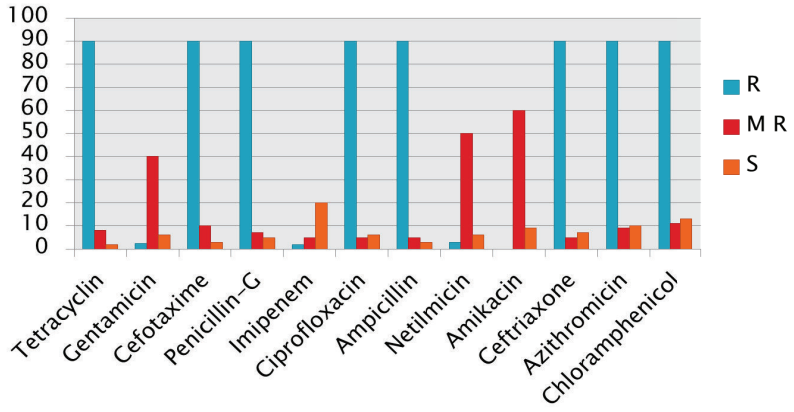

Figure 2: *Antibiotic sensitivity test of Salmonella spp. against several commercially available antibiotic commonly used in poultry industry.*

resistance. Therefore, the emergence of multidrug resistant bacteria in poultry of Bangladesh depicts the alarming situation of overuse or misuse of antibiotics. The presence of multidrug resistant *Salmonella* species in poultry of Bangladesh is also reported that poses a serious threat to public and poultry health[31].

Bioremediation approach to control multidrug resistance Salmonella spp. in poultry

This is a serious concern as far as the disease management is concerned, hence requires alternative management of control of bacterial disease. Introduction of indigenous probiotic technology could be a smart approach to combat the disease threat. The reason could be due to the failure of the foreign isolates to acclimatize with the Bangladeshi niche. This observation therefore demands to discover new probiotics from the indigenous origin to be effective in local environment. In our study, the antagonistic activity of probiotic bacteria, lactic acid bacteria isolated from poultry, against these MDR *Salmonella* spp. was analyzed. At first, cloacal-swab samples were plated onto De Man Rogosa Sharpe (MRS) medium, colonies with typical characteristics namely pure white, small (2-3 mm diameter) with entire margins (Figure 1B).The 22 isolates were gram positive, rod shaped or cocci, oxidase negative, catalase negative, non-motile, indole negative and able to ferment the disaccharides maltose, sucrose and lactose then presumptively identified as lactic acid bacteria.

In disc diffusion (Figure 3A) assay, five lactic acid bacteria isolated from animal samples were able to produce clear zones around the disc impregnated on previously swabbed with MDR *Salmonella* spp. It was also confirmed during one streak method (Figure 3B) that those five lactic acid bacterial isolates showed antibacterial activity against MDR *Salmonella* spp. isolated from poultry samples. The zones of inhibition (Figure 3) were observed. This study showed that the growth of pathogenic *Salmonella* spp. was inhibited by indigenous lactic acid bacteria which were collected from poultry sample. Kizerwetter - Swida and Binek[32] demonstrated that *L. salivarius* 3d strain reduced the number of *Salmonella enteritidis* in the group of chickens treated with *Lactobacillus* sp.

Characterization of probiotic bacteria

Many lactic acid bacteria are resistant to antibiotics. The resistance attributes are often intrinsic and non-transmissible[33]. On the other hand antibiotic resistant probiotic can benefit patients whose normal intestinal microbiota has become unbalanced or greatly reduced in number due to administration of various antimicrobial agents[34].It was found that aforementioned five lactic acid bacterial isolates were highly resistant against 6 antibiotics of standard concentration i.e. Gentamicin (10µg), Vancomycin (30µg), Streptomycin (10µg), Nalidixic acid (30µg), Ciprofloxacin (5µg), tetracycline (30µg);moderately resistant to Erythromycin (15µg) and sensitive to Chloramphenicol (30µg), Ampicillin (10µg) (Figure 4). The majority of the LAB possesses an inducible acid tolerance response (ATR) which is also known as the acid adaptive response. This property improves the survival of adapted cells upon exposure to lethal acid challenge[35]. In this study, the growth of Lactic acid bacteria in various ranges of pH (3.5-9.5) was assessed to determine the pH for optimum growth. It was observed the maximum growth; by measurement of lactic

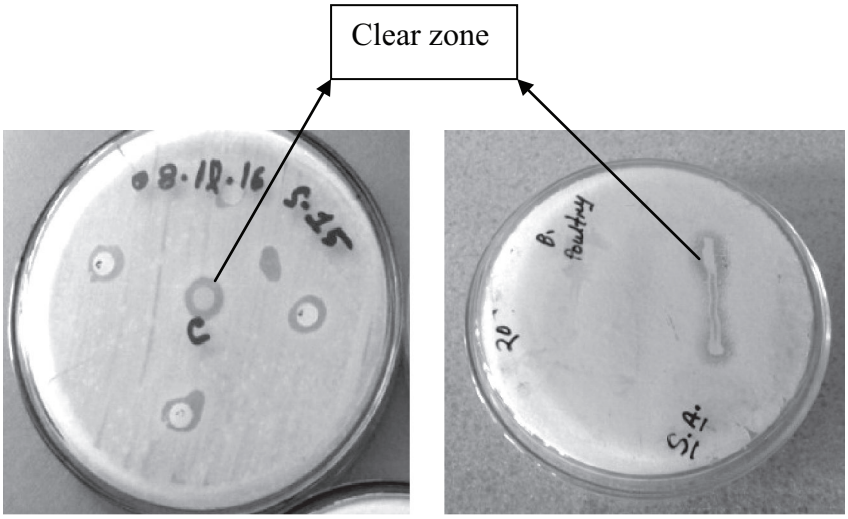

Figure 3: *The antagonistic activity of lactic acid bacteria against Salmonella spp observed both in Disk Diffusion Method (A) and One Streak Method (B). In both of the pictures, clear zone was evident that implies the antagonistic activity of lactic acid bacteria against Salmonella spp.*

acid bacterial densities to be at pH 5.5 .There was a strong correlation between the pH and the growth of the Lactic acid bacteria, the maximum growth was enhanced when the culture was controlled at pH 5.5 (Figure 5). Survival could also be observed that at acidic pH value of 3.5. In this study, it was found that the L5 had a maximum growth at pH 5.5 that exceeded the growth of other Lactic acid bacteria which had their optimal growth at different pH. NaCl may inhibit growth of certain types of bacteria. Isolated Lactic acid bacteria were able to tolerate growth 2, 4, 6, 8, 10 and 12 percent of NaCl (Table 2).in MRS broth. However, bacterial growth was correlated with various NaCl concentrations in the media with optimal growth at 4 percent NaCl while concentrations of 10% and 12% NaCl significantly inhibited the growth of lactic acid bacteria with exception of L1 and L5 that could grow at this NaCl concentration. This type of result was also found where Lactic acid bacteria isolated from gastrointestinal tract of swine that were tolerable to 4-8 % NaCl[36].

Molecular identification of potential probiotic bacteria

16S rDNA gene sequencing was performed in order to identify the potential proibiotic bacterial isolates. One representative potential probiotic isolate subject to 16S rDNA gene sequencing to interpret the close relative species after nucleotide BLAST searching in NCBI database. The isolate was phylogenetically (Figure 6) closely related to *Pediococcus acidilactici* with 98% similarity. Noohi*et al*.[37] revealed the phenotypic characteristics of lactic acid bacteria that isolated from poultry had probiotic potentiality.

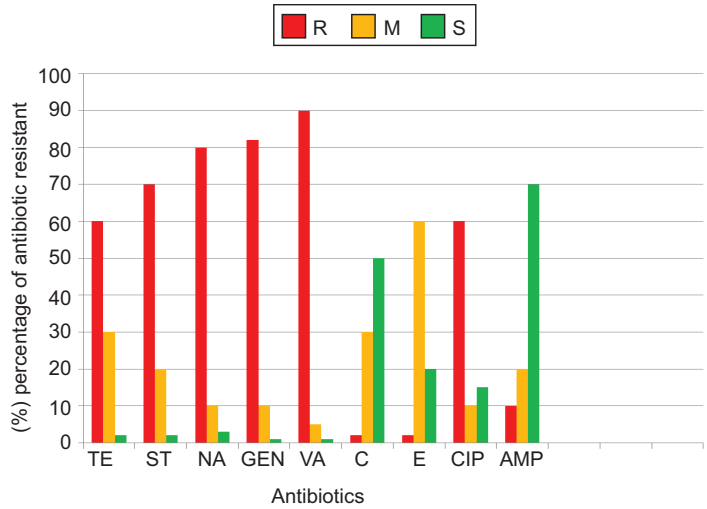

Figure 4: *Antibiotic sensitivity test of lactic acid bacteria. AMP = Ampicillin, ST = Streptomycin, CIP = Ciprofloxacin, VA = Vancomycin, C = Chloramphenicol, CN =Gentamycin, NA = Nalidixic acid, E = Erythromycin, TE = Tetracycline, FA = Fusidic acid, K = Kanamycin.* R = Resistant, S = Sensitive and M = Moderate susceptibility*

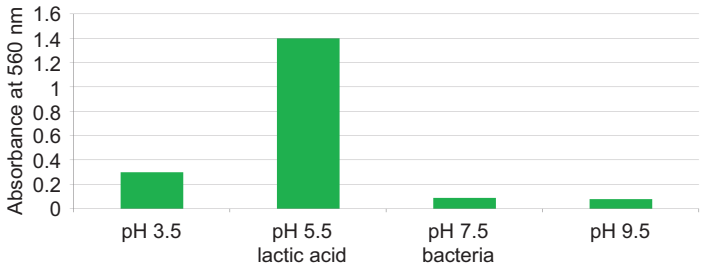

Figure 5: *pH toleranceof lactic acid bacteria.*

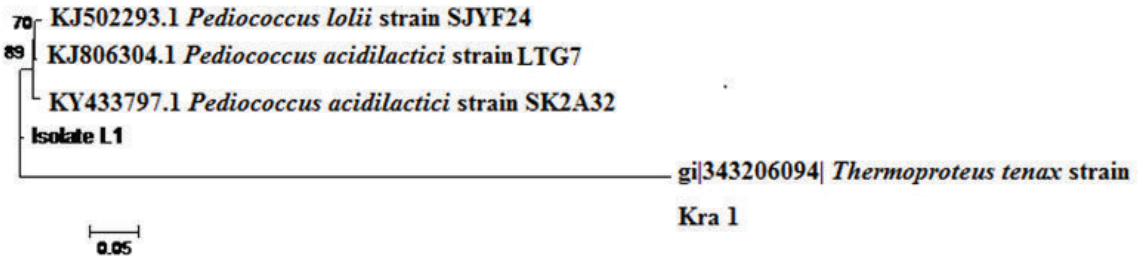

Figure 6*: Phylogenetic treeof Probiotic Bacteria. Thistree was built based on 16S rDNA gene sequence using the SeqMan Genome Assembler (Partial sequence), ClustalW (Multiple sequence), Molecular Evolutionary Genetics Analysis (MEGA) 5 program and Neighbour-Joining algorithm,*

Conclusion

The present study reveals that probiotics could be successfully used as nutritional tools in poultry feeds for promotion of growth and pathogen inhibition. The future perspective of this study is to implement our knowledge to keep the whole chain of poultry production free from multidrug resistant bacteria with an alternative use of antibiotic.

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