Project ID: 607

Competitive Research Grant (CRG)

Sub-Project Completion Report

on

Mining novel probiotics from Red Jungle fowl (*Gallus gallus*) as the alternatives to antibiotics for safe poultry production

Project Duration

May 2016 to September 2018

Department of Microbiology & Public Health
Department of Dairy & Poultry Science,
Bangabandhu Sheikh MujiburRahman Agricultural University

Submitted to
Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

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Project Implementation Unit
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Bangladesh Agricultural Research Council (BARC)
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Acknowledgement

The execution of CRG sub-project has successfully been completed by Bangabandhu Sheikh MujiburRahman Agricultural University using the research grant of USAID Trust Fund and GoB through Ministry of Agriculture. We would like to thanks to the World Bank for arranging the grant fund and supervising the CRGs by BARC. It is worthwhile to mention the cooperation and quick responses of PIU-BARC, NATP 2, in respect of field implementation of the sub-project in multiple sites. Preparing the project completion report required to contact a number of persons for collection of information and processing of research data. Without the help of those persons, the preparation of this document could not be made possible. All of them, who made it possible, deserve thanks. Our thanks are due to the Director PIU-BARC, NATP 2 and his team who have given their whole hearted support to prepare this document. We hope this publication would be helpful to the agricultural scientists of the country for designing their future research projects in order to technology generation as well as increasing production and productivity for sustainable food and nutrition security in Bangladesh. It would also assist the policy makers of the agricultural sub-sectors for setting their future research directions.

Published in: September 2018

Printed by:

Acronyms

AAg	Autoaggregation
AML	Amoxicillin
CIP	Ciprofloxacin
CN	Gentamycin
CPPS	Cumulative probiotic potential score
DXT	Doxycycline
E.	*Escherichia*
EMB	Eosin Methylene Blue
GIP	Growth inhibition percent
GIT	Gastrointestinal tract
IPPS	Individual probiotic potential score
IR	Intermediate resistance
L.	*Lactobacillus*
LAB	Lactic acid bacteria
LR	*Lactobacillus reuteri*
LS	*Lactobacillus salivarius*
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
MRS	De Man, Rogosa and Sharpe
PBS	Phosphate-buffered saline
N	Neomycin
R	Resistance
RJCe	Red Jungle fowl Cecum
RJCr	Red Jungle fowl Crop
RJF	Red Jungle fowl
RJLI	Red Jungle fowl Large Intestine
RJSI	Red Jungle fowl Small Intestine
S	Sensitive
S.	*Salmonella*
SS	Salmonella-Shigella
SHb	Surface hydrophobicity
W.	*Weissella*
WP	*Weissella paramessenteriods*
ZDI	Zone diameter inhibition

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Executive Summary

Enteric diseases are an important concern to the poultry industry because of lost productivity, increased mortality and the associated contamination of poultry products that are used for human consumption. With increasing concerns about antibiotic resistance, there is increasing interest in finding alternatives to antibiotics for poultry production. Probiotics is a unique approach that has potential to reduce enteric diseases in poultry. A huge amount of probiotic products are being imported regularly. However, probiotics imported from abroad are costly and affecting the production cost. Isolation and characterization of bacteria having probiotic properties has been reported widely. Some of them demonstrated very effective activity in poultry production. Therefore, scientists are continuously searching for noble probiotics. The state of the art of the proposed project is to search for new probiotics from Red Jungle fowl (*Gallus gallus*) in Bangladesh. Since the Jungle fowl lives in a microbes-friendly environment, it is logical to assume that their immune system is very developed and they have a very diverse intestinal ecology. This is the first report exploring the gastrointestinal digestive tract of wild Red Jungle fowl towards the development of probiotic products for the poultry industry.

A total of three Red Jungle fowls were captured from the two districts of Bangladesh, Rangamati and Bandarban. After preparing the homogenates from four different parts (crop, small intestine, large intestine and cecum) of gastrointestinal tract, a total of 39 presumptive lactic acid bacteria were isolated. Among these 39 isolates, 28% was isolated from crop, 20% from small intestine, 31% from large intestine and the remaining 21% was isolated from cecum. Molecular characterization of all the isolates targeting 16S rRNA gene revealed *Lactobacillus reuteri* as predominant isolates (71.8%) followed by *Lactobacillus salivarius* (20.5%) and *Weissella paramessenteroides* (7.7%). This is the second report so far showing the prevalence of *Weissella* Sp. in the gastrointestinal tract of chicken. The sequence analysis showed eight sequence types of *L. reuteri* representing 28 isolates, three sequence types of *L. salivarius* representing 8 isolates and three sequence types of *W. paramessenteroides* representing 3 isolates. According to the phylogenetic analysis each sequence types are different in 1 to 5 single nucleotide polymorphism which indicates that there are significant differences in polymorphic genes associated with the probiotic properties of the isolates. Upon submission of the nucleotide sequence of the 14 isolates representing each sequence type, the accession numbers have been received from GenBank.

Fourteen isolates representing each sequence types were evaluated in vitro for the eight probiotic properties such as Autoaggregation ability and Surface hydrophobicity corresponding to the adhesion/colonization; Antimicrobial activity against *E. coli* and *Salmonella* Sp. relevant to the ability to kill pathogenic microorganisms; Antibiogram profile and Hemolytic activity associated with the safety status for future use; Bile and gastrointestinal pH tolerance ability related to the survival capacity in the intestine. The selected isolates showed heterogeneous activity against the probiotic properties tested. Based on the probiotic activities the isolates were ranked by calculating cumulative probiotic potential score. The isolates having more than 50% cumulative probiotic potential score were considered as potent probiotics. Thus, the four isolates *L. reuteri*-1, *L. reuteri*-7, *L. salivarius*-3 and *W. paramesenteroides*-3 having cumulative probiotic potential score 69.5%, 57.3%, 69.5% and 63.6%, respectively were identified to be the potent probiotics for future use. However, further in vivo studies are needed to establish the isolates as potent probiotics.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. **Title of the CRG sub-project:**
   Mining novel probiotics from Red Jungle fowl (*Gallus gallus*) as the alternatives to antibiotics for the safe poultry production
2. **Implementing organization:**
   Bangabandhu Sheikh Mujibur Rahman Agricultural University
3. **Name and full address with phone, cell and E-mail of PI/Co-PI (s):**
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4. **Sub-project budget (Tk):**

4.1 Total: Tk 19,16,975
4.2 Revised (if any):

5. **Duration of the sub-project:**

5.1 Start date (based on LoA signed): 16 May 2017
5.2 End date : 30 September 2018

6. **Justification of undertaking the sub-project:**

The poultry industry is becoming a leading industry in Bangladesh. The sector has been growing at an annual rate of around 20 percent for the last two decades (Rahman et al., 2017). Nowadays, poultry is the main source of animal protein in Bangladesh. Enteric diseases are an important concern to the poultry industry because of lost productivity, increased mortality, and the associated contamination of poultry products for human consumption (human food safety). With increasing concerns about antibiotic resistance, the ban on sub-therapeutic antibiotic in many parts of the world, is increasing interest in finding alternatives to antibiotics for poultry production. Use of probiotics is a unique approach that has potential to reduce enteric diseases in poultry and subsequent contamination of poultry products (Patterson and Burkholder, 2003). Along with the other feed additives various probiotic products are being imported in Bangladesh regularly (Haque et al., 2016). However, probiotics imported from abroad are costly and affecting the production cost.

Isolation and characterization of bacteria having probiotic properties has been reported widely. Some of them demonstrated very effective activity in poultry production (Kobierecka et al., 2017; Wang et al., 2014; Blajman et al., 2015; Messaoudi et al., 2011). However, probiotics as alternatives to antibiotics in the real field are very limited. Therefore, scientists are continuously searching for noble probiotics. The state of the art of the proposed project is to searching probiotics from Red Jungle fowl (RJF; *Gallus gallus*) in Bangladesh. The RJF lives in a microbes-friendly environment. Therefore, it is logical to assume that their immune system is very developed and they have a very diverse intestinal ecology. In a recent review by Desta (2019), it has been demonstrated that the jungle fowl including RJF has non-specific immune defense mechanisms and high variability in natural immunity. They are thought to be more resilient to diseases. In addition, extensive exposure of jungle fowl to several infections in the absence of medication has enabled them to develop adaptive immunity and to accrue advantageous alleles. In Bangladesh, investigation on isolation of probiotic potent lactic acid bacteria from the gastrointestinal tract of chicken is very limited. Kabir et al. (2016), isolated 10 probiotic potent lactobacilli from broiler chickens available at selected live bird markets and identified them as *Lactobacillus crispatus*, *Lactobacillus acidophilus* and *Lactobacillus gallinarum.* According to our knowledge, no attempt has been taken to explore the gastrointestinal tract of Red Jungle fowl as a source of potent poultry probiotics. Taken together, the proposed project could be a unique approach for mining noble probiotics that could be used by the poultry industry in future.

7. Sub-project goal:

Development of probiotics for future use by the poultry industry.

8. Sub-project objective (s):

- Isolation of lactic acid bacteria (LAB) from the gastrointestinal (GI) tract of Red Jungle fowl (RJF).
- Molecular characterization of the LAB.
- In vitro evaluation of the isolated LAB for probiotic potential.

9. Implementing location (s):

Laboratories belonging to the Department of Microbiology and Public Health and Department of Dairy and Poultry Science, Faculty of Veterinary Medicine and Animal Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur - 1706, Bangladesh.

10. Methodology in brief:

10.1. Isolation of lactic acid bacteria (LAB) from the gastrointestinal (GI) tract of Red Jungle fowl (RJF)

Collection of Red Jungle fowl

A total of three Red Jungle fowls were collected. Among them two were collected from Shonaichchari mouza, Betbunia union of Rangamati district and one was collected from Baishari union, Naikhongchhari upazila of Bandarban district. The location of capturing the Red Jungle fowl has been shown in the Figure 1. The Red Jungle fowls were collected with the help of local people. The Red Jungle fowls were identified by phenotypic characteristics. After capturing, the Red Jungle fowls were immediately transported to the laboratory located at (BSMRAU), Gazipur.

Preparation of homogenates from gastrointestinal tract of Red Jungle fowl

The homogenates of the gastrointestinal tract obtained from Red Jungle fowls were prepared according to the protocol described by Musikasang et al. (2009). After sacrificing, each part of the gastrointestinal digestive tracts (crop, small intestine, large intestine and caecum) from individual Red Jungle fowl was aseptically dissected. Each of the dissected part was washed in 70% ethanol followed by washing with sterile distilled water twice. The dissected and washed gut part was then weighed and grinded with a sterilized mortar and pastel under a biosafety cabinet. After grinding the dissected part was diluted ten times with phosphate-buffered saline (PBS: 50 mM potassium di-hydrgenphosphate, 50 mM di-potassium hydrogen phosphate trihydrate, 0.85% sodium chloride, pH 7.0). This homogenate was further used for isolation of lactic acid bacteria.

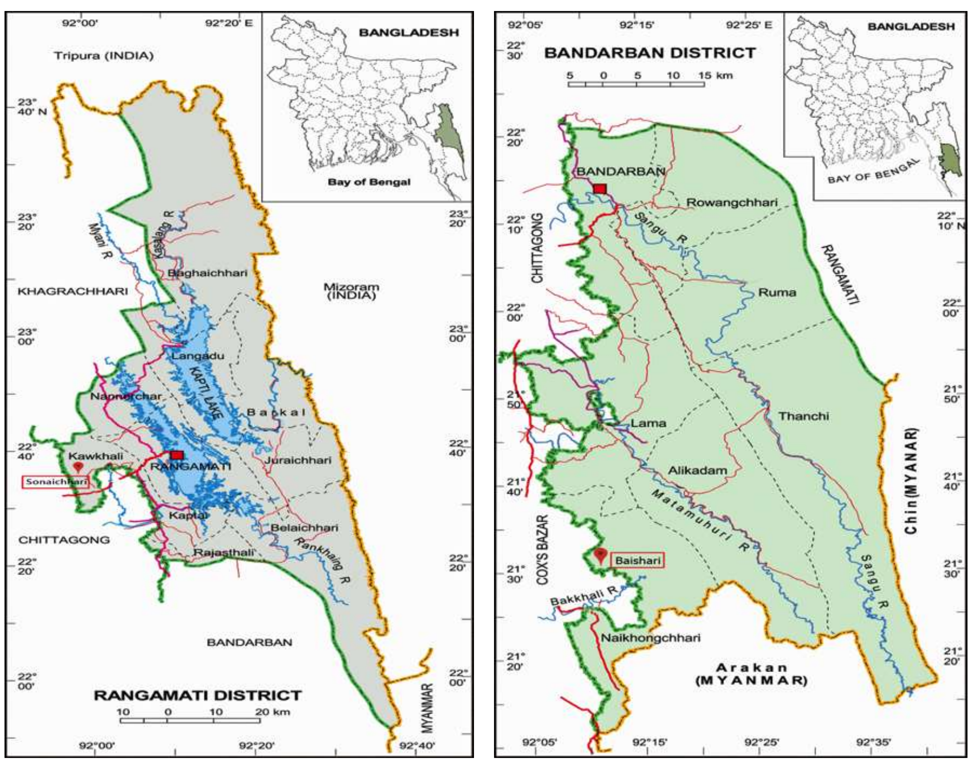

Figure 1: Locations of capturing Red Jungle fowls; indicated by the red colored box.

Isolation of lactic acid bacteria from the gastrointestinal tract of Red Jungle fowl

The lactic acid bacteria from the prepared homogenates (as mentioned in the section 10.2) were isolated according to Musikasang et al. (2009). Briefly, ten-fold serial dilution (up to 10^{-8}) of the homogenate was carried out. Each dilution was then plated onto Man, Rogosa and Sharpe (MRS) agar supplemented with 0.02% bromocresol purple and incubated anaerobically for 48h at 41°C. Colonies which exhibited a clear halo were randomly selected from the highest dilutions of each MRS agar plate. Bacterial colonies were then purified by re-streaking on MRS agar at least three times. The pure cultures were characterized using Gram stain, cell morphology and catalase reaction tests. Gram-positive and catalase-negative isolates were stored at -20°C in MRS broth supplemented with 25% (v/v) glycerol.

10.2. Molecular identification of the isolates obtained from the gastrointestinal tract of Red Jungle fowl

DNA extraction, PCR amplification and electrophoresis

Genomic DNA was extracted from each pure bacterial colony using the Bacterial Genomic DNA Isolation Kit. The extracted DNA from each colony was then amplified by PCR using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) with an expected PCR product size of around 1.5 kb (Soto et al., 2010). The PCR consisted of 35 cycles at 95°C for 30 sec, 48°C for 30 sec, and 72°C for 90 sec, with an initial denaturation (95°C for 3 min) and a final extension (72°C for 5 min). Both positive and negative controls were included in PCR to validate the results. The PCR amplified products were separated by electrophoresis on 1% agarose gel and visualized under a trans-illuminator after staining with ethidium bromide.

Nucleotide sequencing and analysis

After purification, the purified PCR products were sequenced in both directions using the representative forward and reverse primers. The nucleotide sequences and chromatograms of both forward and reverse directions were viewed using the EditSeq 5.0 and Chromas 2.4 program, respectively. The presence of double peaks at the chromatogram level was verified and the sequences were aligned and analyzed using ClustalX software (Larkin et al., 2007). Consensus sequences were then compared to similar sequences in GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/) to determine the species of the bacteria. Representative nucleotide sequences were submitted to GenBank database using Banklt, a WWW-based submission tool (https://www.ncbi.nlm.nih.gov/WebSub/tool=genbank).

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using the Molecular Evolutionary Genetics Analysis (MEGA) program version 6.06 (Tamura et al., 2007). Sequences without overlapping nucleotide positions were aligned in MEGA using a neighbor-joining analysis with representative sequences from the major bacterial species downloaded from GenBank. Bootstrap analysis was used to assess the robustness of clusters using 1,000 replicates.

Nucleotide sequence accession numbers

The representative nucleotide sequences obtained in this study were submitted to the National Center for Biotechnology Information (NCBI) and deposited in the GenBank database for the accession numbers.

10.3. In vitro evaluation of the isolated LAB for probiotic potential

Isolation of pathogenic bacteria from the gastrointestinal tract of commercial broiler chicken

One of the most important probiotic properties of a probiotic isolate is to demonstrate antibacterial activity against the pathogenic microorganisms. To carry out this test, the pathogenic microorganisms (*Escherichia coli* and *Salmonella sp.*) were isolated from the gastrointestinal tract of commercially available broiler chicken. After sacrifice, each part of the gastrointestinal digestive tract was grinded and homogenized with PBS. Serially diluted homogenate was plated on the selective media, Eosin Methylene Blue (EMB) agar media for *E. coli* and Salmonella Shigella (SS) agar media for *Salmonella* sp. The pure culture was collected by re-streaking onto the selective agar media (EMB or SS). The isolates were then stored at -20°C in nutrient broth supplemented with 25% (v/v) glycerol.

Assessment of *in vitro* probiotic potential of the isolates obtained from Red Jungle fowl

There are many methods reported to assess the *in vitro* probiotic potential of an isolate. Among them, autoaggregation and surface hydrophobicity assay (corresponding to adhesion ability), antimicrobial activity against pathogenic microorganisms, antibiotic resistance, acid and bile tolerance, hemolytic activity are important.

Autoaggregation ability

Autoaggregation ability was evaluated according to Rahman et al. (2008). Frozen stocks of the isolates were reactivated by two consecutive subcultures in MRS broth at 41°C for 24 h under anaerobic condition. After reactivating, the overnight culture of the respective isolates was inoculated at 1% (v/v) in a test tube containing 10 ml of MRS media and incubated at 41°C for 24 h under anaerobic condition. After the incubation period, the cultured media were mixed well by vortexing for 15 sec and then allowed to stand at room temperature. After 4 h interval, changes in absorbance of the top portion of the cultured media due to precipitation were monitored at 660 nm using a spectrophotometer. Autoaggregation ability was expressed as autoaggregation percentage (AAg %) and calculated using the formula:

$$\mathbf{AAg\ \% = [(A_0 - A)/A_0] \times 100;}$$

Where A_0 and A are the absorbances of cultured media at 0 h and after 4 h intervals, respectively.

From AAg%, isolates were classified into three groups: high autoaggregation (>70% AAg), medium autoaggregation (20–70% AAg), and low autoaggregation (>20% AAg). The photograph of the test tubes before and after 4 h interval was also captured for macroscopic observation. Individual probiotic potential score was given 0, 0.5 and 1 for the isolates having high, medium or low AAg ability, respectively.

Cell Surface hydrophobicity

Cell surface hydrophobicity was examined as described by Rahman et al. (2008). After growing the reactivated isolates in MRS broth with 1% (v/v) inoculum under anaerobic condition at 41°C for 24 h, bacterial cells were harvested by centrifugation at 14,000×g for 5 min, washed twice with 50 Mm K_2HPO_4 (pH 7.0) and then re-suspended in the same buffer to an absorbance of about 0.5 at 660 nm (bacterial suspension). Five milliliters of the bacterial suspension were mixed with 1 ml of xylene ($C_6H_4(CH_3)_2$) by vortexing for 120 sec and then incubated for 1 h at room temperature. Changes in absorbance on the top of the bacterial suspension due to bacterial adhesion to hydrocarbons were recorded at 660 nm using a spectrophotometer. Surface hydrophobicity was expressed as surface hydrophobicity percentage (SHb %) and calculated using the formula:

SHb % = $[(A_0 - A)/A_0] \times 100$;

Where A_0 and A are the absorbances before and after extraction with xylene.

The photograph of the test tubes before and after 4 h interval was also captured for macroscopic observation. From SHb%, isolates were classified into three groups: high SHb (>70% SHb), medium SHb (20–70% SHb), and low SHb (>20% SHb). Individual probiotic potential score was given 0, 0.5 and 1 for the isolate having high, medium or low SHb ability, respectively.

Antimicrobial activity

The antimicrobial activity of each isolate against pathogenic microorganisms was observed by applying agar well diffusion method as reported by Bian et al. (2010) with minor modifications. Mueller Hinton agar (MHA) plates were inoculated with the test organisms (*E. coli* and *Salmonella* sp. which was previously being isolated from the chicken intestine) by swab technique. Prior to swabbing, the test organisms were grown overnight in Luria-Bertani (LB) broth and the cells were re-suspended in sterilized saline solution (0.85% NaCl) equivalent to McFarland standard 0.5. After inoculation of the test organisms, four wells were created onto the surface of the agar using the backside of a sterile 1 ml micro-tip which is equivalent to 6 mm diameter. The overnight culture of the respective isolate was then centrifuged at 6,500×g for 15 min. The resulting supernatant was then sterilized using a syringe filter (0.22 µM). The remaining cell pellets were mixed with around 100 µl of fresh MRS. Freshly prepared MRS medium with the adjusted pH correspondence to the isolate cultured media was used as control. Each of the four wells was then filled with 80 µl of supernatant, cell pellets and MRS broth. All plates were incubated at 37°C for 24 h. After incubation, the inhibitory zones were measured by examination of the diameters of any clear zones around the wells using a caliper. The measured inhibitory zone was expressed as Zone Diameter Inhibition (ZDI) and considered as Highly active when ZDI ≥15; Medium active when ZDI 11-14; Less active when ZDI ≤10; and Not active in the case of absence of any inhibitory zone as described by Halder et al. (2017). Individual probiotic potential score was given 0, 0.25, 0.5 and 1 for the isolates found as Not active, less active, Medium active or high active, respectively.

Gastrointestinal pH tolerance assay

The ability of the isolates to grow at different pH was tested by performing pH tolerance assay as described by Todorov et al. (2012) with some modifications. For this study, the MRS media was adjusted at three different pH (2.5, 5.0 and 7.5). These pH values were selected considering the pH of the gastrointestinal tract of poultry that ranges from 2.5 to 8.0. Ten ml of the pH adjusted media was then placed in the test tubes. The overnight culture of each reactivated isolate was then inoculated at a level of 1%. For each isolate, control test tubes were maintained that contained media only and where the pH was not adjusted. After incubation under anaerobic condition at 41°C for 24 h, the absorbance of the cultured media was recorded at 600 nm using a spectrophotometer. The inhibition of the bacterial growth was calculated using the following formula and the result was expressed in percentage:

Growth inhibition % = $[(A_{control} - A_{at\ specific\ pH})/A_{control}] \times 100$;

Where, $A_{at\ specific\ pH}$ and $A_{control}$ are the absorbance of the cultured media with or without adjustment of pH, respectively.

According to the growth inhibition percent, the isolates were classified as high (growth inhibition <20%), medium (growth inhibition 20-70%) or low (growth inhibition >70%) acid tolerance ability. Individual probiotic potential score was given 0, 0.5 and 1 for the isolate having high, medium or low acid tolerance ability, respectively.

Bile tolerance assay

The ability of the isolates to grow in the presence bile salt was tested by performing bile salt tolerance assay as described by Todorov et al. (2012) with some modifications. For this study, the MRS media was supplemented with OX gall (Sigma) to achieve the final concentration of 0.3%. This concentration was selected considering the average bile concentration in the chicken gastrointestinal tract (0.3%). Ten ml of this media was aliquoted in each test tube. The overnight culture of each reactivated isolate was then inoculated at a level of 1%. For each isolate, control test tubes without supplementation of OX gall were also prepared. After incubation under anaerobic condition at 41°C for 24 h, the absorbance of the cultured media was recorded at 600 nm using a spectrophotometer. The inhibition of the bacterial growth was calculated using the following formula and the result was expressed in percentage:

Growth inhibition % = $[(A_{control} - A_{Ox\ gall})/A_{control}] \times 100$;

Where, $A_{Ox\ gall}$ and $A_{control}$ are the absorbance of the cultured media supplemented with or without Ox gall, respectively.

According to the growth inhibition percent, the isolates were classified as high (growth inhibition <20%), medium (growth inhibition 20-70%) or low (growth inhibition >70%) bile tolerance ability. Individual probiotic potential score was given 0, 0.5 and 1 for the isolate having high, medium and low bile tolerance ability, respectively.

Antibiogram profile

The antibiotic sensitivity of each isolate was evaluated by agar disc diffusion method according to Domingos-Lopes et al. (2017). Five different types of antibiotics (Amoxicillin, Neomycin Doxycycline, Gentamycin, Ciprofloxacin) commonly used in the poultry industry were tested. Mueller Hinton agar (MHA) plates were inoculated with the respective isolate by swab technique. Prior to swabbing, the isolate was grown anaerobically at 41°C for 24h in MRS broth and the cells were re-suspended in sterilized saline solution (0.85% NaCl) equivalent to McFarland standard 0.5. After evenly distributing the inoculum, the antibiotic discs were then placed onto the surface of the plate and incubated at 41°C. After incubation for a period of 24 h, the diameter of the inhibition zone around the disc was measured using a caliper. The measured inhibitory zone was expressed as Zone Diameter Inhibition (ZDI) and considered as Sensitive to the respective antibiotic when ZDI ≥21; Intermediate resistance/sensitive when ZDI 16-20; and Resistance when ZDI ≤15 as described by the Clinical and Laboratory Standards Institute Tables (CLSI, 2014). Individual probiotic potential score was given 0, 0.5 and 1 for the isolate found as Resistance, Intermediate resistance/sensitive or sensitive, respectively.

Hemolytic activity

The hemolytic activity was performed according to the Halder et al. (2017) with slight modification. The overnight grown MRS broth culture of all the selected 14 isolates were streaked on 5% sheep blood agar plate and incubated anaerobically at 41°C for 72 h; thereafter, the plates were observed for the formation of any clean (β-hemolysis) or greenish (α-hemolysis) hemolytic zones, or no such zone (γ-hemolysis) around the Lactobacillus colonies. Individual probiotic potential score was given 0 for α- or β-hemolysis whereas score 1 was given for the isolates demonstrated γ-hemolysis.

Determination of cumulative probiotic potential

In vitro probiotic potential of an isolate was assessed by scoring each probiotic property on a scale of 0 to 1 and termed as individual probiotic potential score (IPPS). The details score allocation for each probiotic property has been described in respective methodology section. Thus, the cumulative probiotic potential score (CPPS) was calculated using the following formula as described by Tambekar and Bhutada (2010): **CPP = Total IPPS obtained/Maximum total IPPS × 100.**

11. Results and discussion:

11.1. Isolation of lactic acid bacteria (LAB) from the gastrointestinal (GI) tract of Red Jungle fowl (RJF)

Identification of the captured bird as Red Jungle fowl

The captured bird was identified as the Red Jungle fowl by observing their phenotypic characters. As shown in the Figure 2, like the typical Red Jungle fowl the captured bird had long, golden-orange to deep-red crown and neck feathers and a dark metallic-green tail with a white tuft at the base. The captured bird also had vivid scarlet-red facial skin and dark or slate grey leg colour.

Figure 2: The Red Jungle fowl having several typical phenotypic characters

Dissection of the gastrointestinal digestive tract from the Red Jungle fowl

The dissection of the gastrointestinal digestive tract from Red Jungle fowl has been carried out at the laboratory. The representative images of the dissection process are demonstrated in Figure 3.

Figure 3: The dissection process of the gastrointestinal digestive tract

Isolation of the lactic acid bacteria (LAB)

Since the bacterial populations of gastrointestinal tract are specific and particular for different animals (Fuller, 1975), similar to other studies isolation of LAB from the dissected gastrointestinal digestive tract of Red Jungle fowl was the first step towards developing chicken probiotic. The process for dilution, plating and incubation of the sample under anaerobic condition has been shown in Figure 4. After incubation, the growth characteristics of LAB are displayed in Figure 5. The homofermentative LAB produce more yellow colour than that of heterofermentative LAB. The representative gram staining and catalase test results are also presented in Figure 5. Only gram positive and catalase negative isolates were selected for further studies. A total of 39 pure isolates were obtained following procedures mentioned above. Among these 39 pure isolates, 28% were isolated from crop, 20% from small intestine, 31% from large intestine and remaining 21% isolated from cecum, shown in Figure 5.

Figure 4: Dilution, plating and incubation of the sample under anaerobic condition

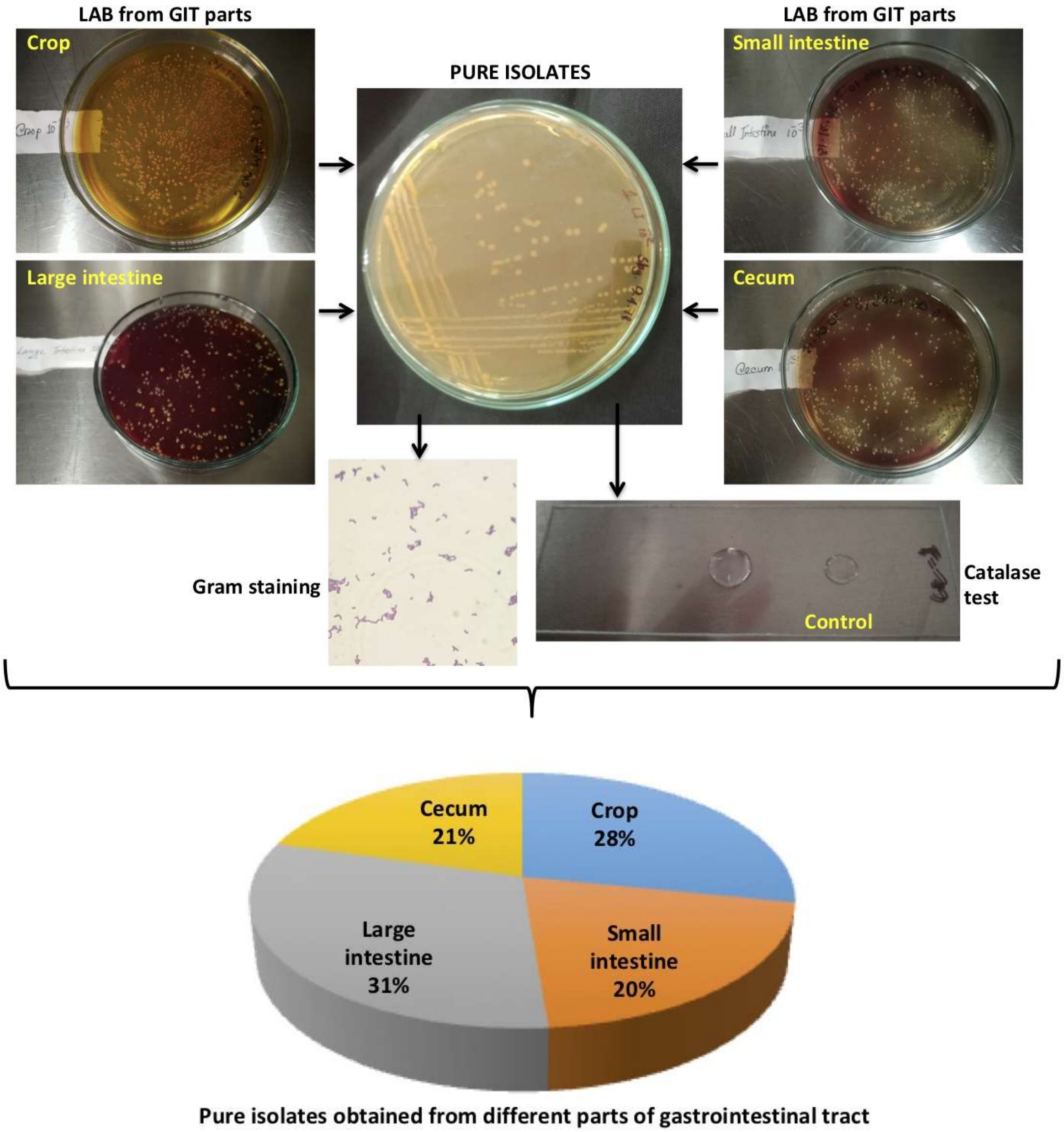

Figure 5: Isolation of pure lactic acid bacteria from the gastrointestinal homogenates of Red Jungle fowl

11.2. Molecular identification and phylogenetic analysis of the isolates

Molecular identification of all the 39 pure isolates was carried out by targeting the conserved 16S rRNA gene. The extracted bacterial DNA was amplified with universal primers 27F and 1492R which resulted amplicons of the size of around 1.5 kb. The electrophoretic patterns of the resulting amplicons are shown in Figure 6.

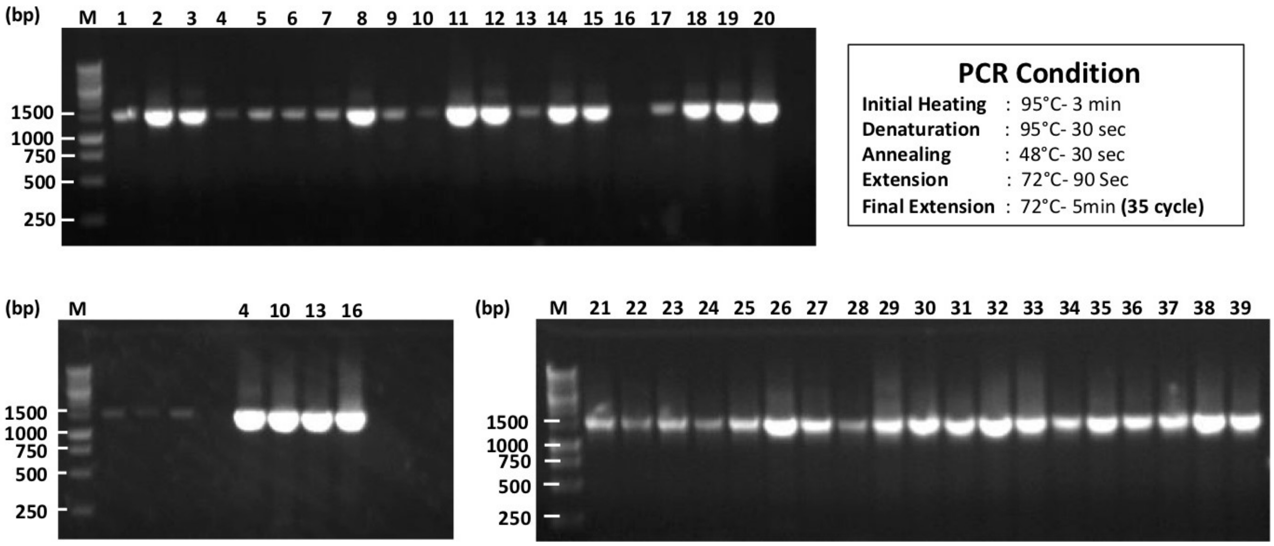

Figure 6: Agarose gel electrophoresis of the PCR amplified products from 39 isolates

The resulting amplicons were then purified and sequenced. The DNA sequence from each isolate was then analyzed by BLAST. After analyzing, 71.8% isolates were identified as *Lactobacillus reuteri*, 20.5% as *Lactobacillus salivarius*, and 7.7% as *Weissella paramessenteroides*. As demonstrated in Table 1, eight sequence types of *L. reuteri* representing 28 isolates, three sequence types of *L. salivarius* representing 8 isolates and three sequence types of *W. paramessenteroides* representing 3 isolates were detected. Figure 7 shows the distribution of the 39 isolates from different sources. Among the 28 *L. reuteri* isolates, 35.7% were isolated from large intestine followed by crop (32.1%), small intestine (21.4%) and caecum (10.7%). On the contrary, most of the *L. salivarius* were isolated from caecum (37.5%) followed by crop (25%), small intestine (12.5%) and large intestine. However, *W. paramessenteroides* were found in small intestine (33.3%) and cecum (66.7%) only. Several reports revealed *L. reuteri* or *L. salivarius* as predominant *Lactobacillus* group bacteria in chicken intestine (Kobierecka et al., 2017; Wang et al., 2014; Blajman et al. 2015; Messaoudi et al., 2011). So far our knowledge goes, only report revealed the prevalence of *Weissella* Sp. in chicken intestine, particular in the ceca is by Messaoudi et al. (2011). The main sources of *Wisselea* Sp. is known to be various fermented food products and also human (only one report). The *Wisselea* Sp. is important for their antimicrobial activity and is being considered as a promising probiotic in the future (Fusco et al., 2015). It is also interesting to mention that all the 39 isolates only belong to three bacterial species.

The phylogenetic analysis of all the sequences generated from 39 isolates showed that all the 39 isolates belong to the three bacterial species, *L. reuteri*, *L. salivarius* and *W. paramessenteroides*. The phylogenetic tree was constructed using Maximum Likelihood (ML) method in Molecular Evolutionary Genetics Analysis (MEGA) software and displayed in Figure 8. The obtained 8 sequence types of *L. reuteri*, 3 sequence types of *L. salivarius* and 3 sequence types of *W. paramessenteroides* were used in this analysis along with the related sequences retrieved from NCBI genBank. In each case, the sequence types were found to be different in 1 to 5 SNPs (single nucleotide polymorphism) that reflects in the tree showing similarity with close branching (Figure 8). Since 16S rRNA is a conserved gene and yet the sequences have certain differences, the result implies the significant differences in polymorphic genes correlating to probiotic properties of the isolates.

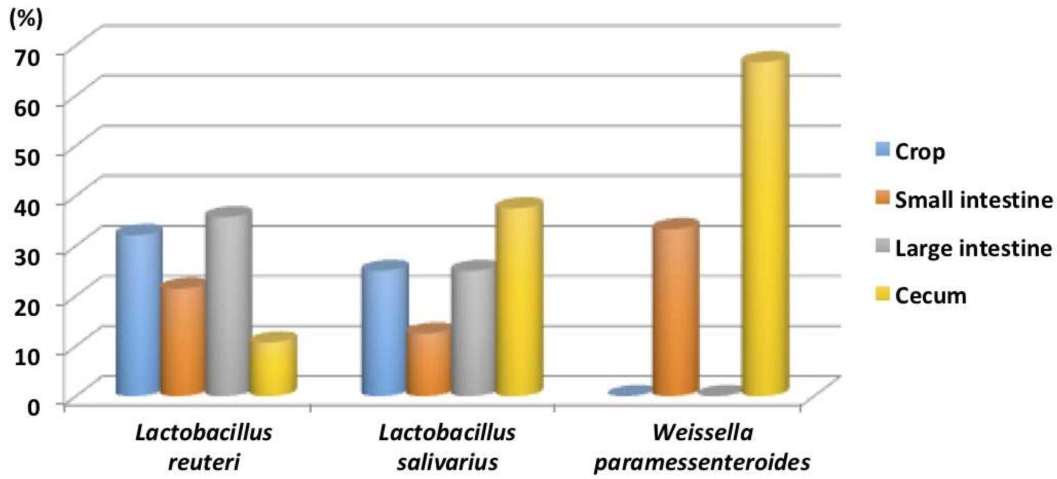

Figure 7: Distribution pattern of the identified isolates in the gastrointestinal tract of RJF

Table 1: Distribution of the isolates by sequence analysis

Species	Sequence type	Isolate identity (n)	Source
Lactobacillus reuteri	*L. reuteri*-1	RJCe-9, RJCe-34, RJCr-2, RJCr-21, RJCr-28, RJSI-20, RJLI-6, RJLI-13, RJLI-14, RJLI-37 (n = 10)	Cecum, Crop, Small intestine, Large intestine
	L. reuteri-2	RJLI-4, RJLI-36 (n = 2)	Large intestine
	L. reuteri-3	RJSI-25 (n = 1)	Small intestine
	L. reuteri-4	RJCr-23, RJCr-29, RJCr-30, RJSI-24, RJSI-32, RJLI-15 (n = 6)	Crop, Small intestine, Large intestine
	L. reuteri-5	RJCr-17, RJCr-38, RJLI-26, RJLI-33 (n = 4)	Crop, Large intestine
	L. reuteri-6	RJSI-31 (n = 1)	Small intestine
	L. reuteri-7	RJCr-7, RJLI-39 (n = 2)	Crop, Large intestine
	L. reuteri-8	RJCe-5, RJSI-19 (n = 2)	Cecum, Small intestine
Lactobacillus salivarius	*L. salivarius*-1	RJCr-1, RJLI-18 (n = 2)	Crop, Large intestine
	L. salivarius-2	RJCe-11, RJCe-35, RJCr-3, RJSI-8, RJLI-16 (n = 5)	Cecum, Crop, Small intestine, Large intestine
	L. salivarius-3	RJCe-40 (n = 1)	Cecum
Weissella paramessenteroides	*W. paramesenteroides*-1	RJCe-27 (n = 1)	Cecum
	W. paramesenteroides-2	RJSI-10 (n = 1)	Small intestine
	W. paramesenteroides-3	RJCe-12 (n = 1)	Cecum

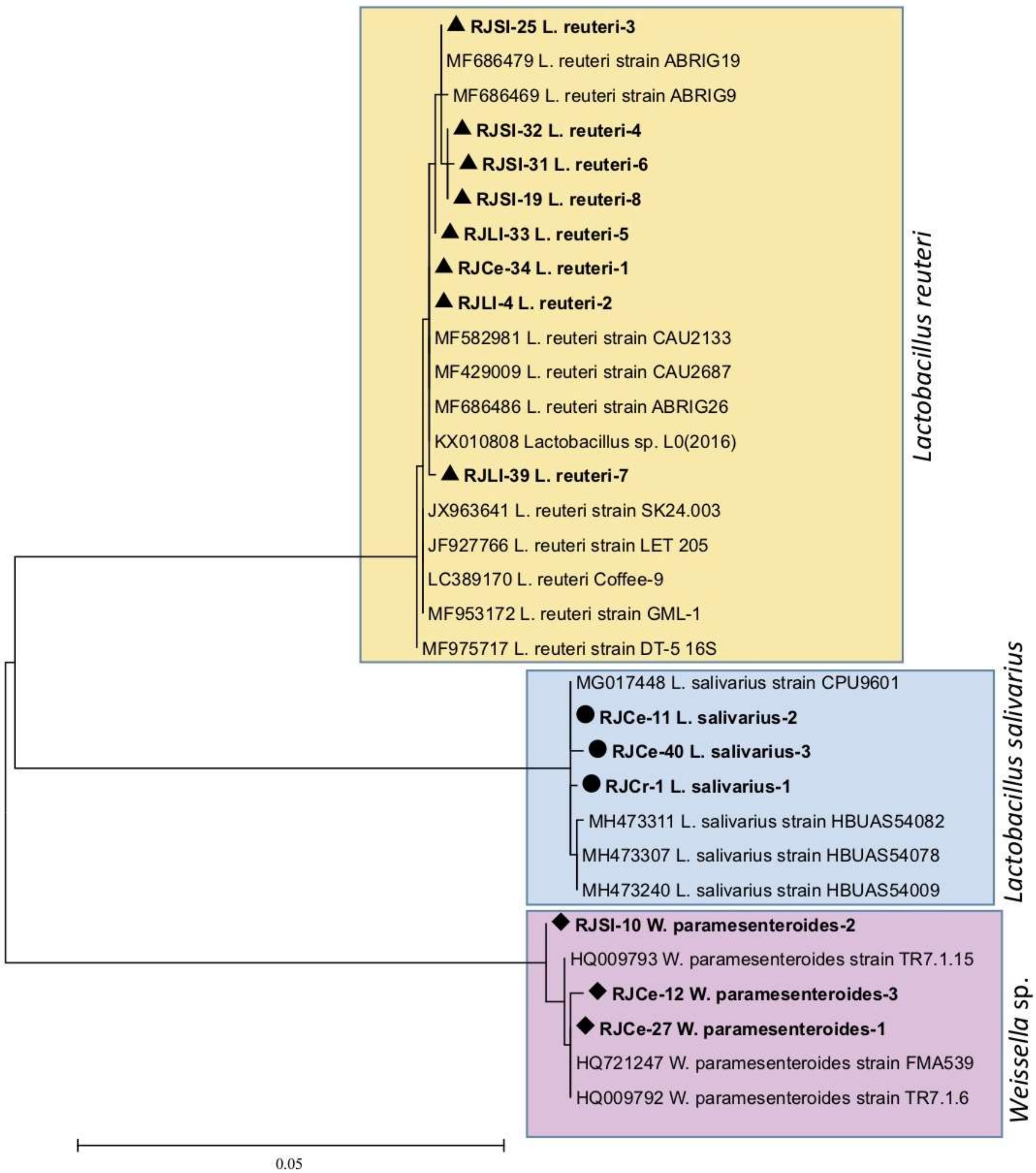

Figure 8: Phylogenetic analysis of all the sequences generated from 39 isolates

GenBank accession numbers

Upon submission of the nucleotide sequence of 14 isolates representing each sequence type, following accession numbers were received:

MK572785; MK572786; MK572787; MK572788; MK572789; MK572790; MK572791; MK572792; MK52793; MK572794; MK572795; MK572796; MK572797; MK572798

Isolation of *E. coli* and *Salmonella* Sp. from commercial broiler chicken

Figure 9 shows the pure culture of *E. coli* and *Salmonella* Sp. isolated on EMB and SS agar media, respectively. In Figure 9 (A), metallic sheen producing colonies can be seen onto the EMB agar plate which indicates the colony as *E. coli*. In Figure 9 (B), black centered translucent colonies can be seen onto the SS agar plates. As we know, *Shigella* Sp. do not ferment lactose or produce hydrogen sulfide gas, so the resulting colonies will be colourless. On the contrary, *Salmonella* Sp. do not ferment lactose, but produce hydrogen sulfide gas therefore, the resulting bacterial colonies were colourless with black centres as shown in Figure 9 (B). Based on these morphological characteristics, the isolates are considered as *E. coli* and *Salmonella* Sp.

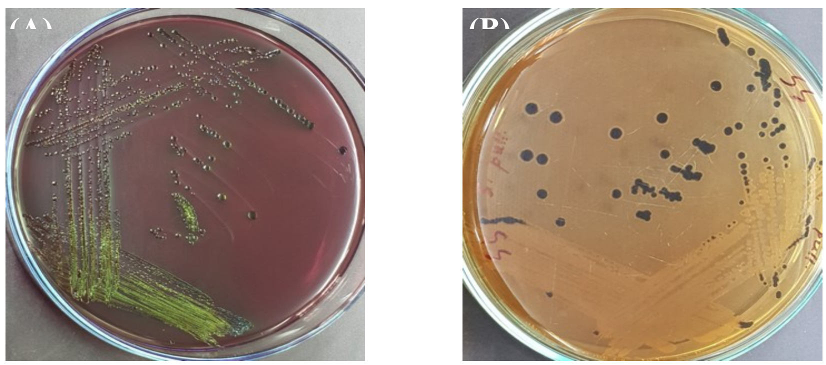

Figure 9: Representative images of isolated *E. coli* (A) and *Salmonella* Sp. (B).

11.3. Evaluation of the selected isolates for in vitro probiotic potential

A total of 14 isolates representing all the sequence types obtained from the molecular analysis of 39 isolates were selected for the evaluation of in vitro probiotic potential. Among the 14 isolates, 8 belonged to *Lactobacillus reuteri*, 3 belonged to *Lactobacillus salivarius* and the rest 3 belonged to *Wissella paramessenteriodes*. The parameters measured for the probiotic potential are Autoaggregation and surface hydrophobicity which indicates the isolates ability to colonize and adhere. The antimicrobial activity suggests the isolates ability to kill pathogenic microorganisms and also possibilities of having antimicrobial resistance genes. The acid and bile tolerance test is the indicator of a respective isolates to survive in the intestinal environment. The hemolytic activity and antibiotic resistance tests are the indicators of the safety status for future use as probiotics.

Autoaggregation and cell surface hydrophobicity

Garriga et al. (1998) and Ehrmann et al. (2002) proposed the autoaggregation and cell surface hydrophobicity test to be appropriate for the first step of screening because the tests are simple and could be applicable to a large number of test strains. The tests also show clumping of strains together and also adhesion ability to the epithelial cells indirectly but in a strong way. The autoaggregation ability and surface hydrophobicity of 14 selected isolates are shown in Table 2. As shown in the Table 2, 50% of the tested isolates were found to have high autoaggregation ability (AAg >70%) whereas 35.7% showed low autoaggregation ability (AAg <20%) and only 2 isolates (14.3%) demonstrated medium autoaggragation ability (AAg 20-70%). Analogously, 50% of the tested isolates were found to have high surface hydrophobicity (SHb >70%), 28.6% showed low surface hydrophobicity (SHb <20%) and 21.4% demonstrated medium surface hydrophobicity (SHb 20-70%). Several studies have reported that the highly autoaggregated strains usually have high surface hydrophobicity. Studies also revealed a good correlation between autoaggregation ability and surface hydrophobicity of a probiotic with the adhesion/colonization ability. The representative macroscopic images displayed in Figure 10 shows that the isolate having autoaggregation ability caused aggregation of the bacterial cells at the bottom of the test tubes whereas isolate having surface hydrophobicity resulted clear media due to the attachment of bacterial cells to the hydrocarbons.

Table 2: Autoaggregation and surface hydrophobicity ability of the selected isolates

Isolates	AAg (%)	Status	IPPS	SHb (%)	Status	IPPS
LR-1	85.7 ± 2.8	High	1	86.2 ± 1.8	High	1
LR-2	19.3 ± 1.2	Low	0	72.4 ± 1.2	High	1
LR-3	7.2 ± 0.3	Low	0	15.9 ± 0.8	Low	0
LR-4	72.1 ± 0.5	High	1	36.3 ± 1.5	Medium	0.5
LR-5	11.7 ± 1.9	Low	0	12.5 ± 1.0	Low	0
LR-6	35.7 ± 1.0	Medium	0.5	71.1 ± 1.8	High	1
LR-7	76.1 ± 1.1	High	1	75.9 ± 0.6	High	1
LR-8	78.8 ± 0.9	High	1	84.7 ± 0.9	High	1
LS-1	32.8 ± 0.9	Medium	0.5	4.1 ± 2.1	Low	0
LS-2	15.7 ± 2.1	Low	0	16.1 ± 1.1	Low	0
LS-3	81.4 ± 1.2	High	1	77.7 ± 2.2	High	1
WP-1	70.9 ± 2.3	High	1	43.3 ± 1.7	Medium	0.5
WP-2	9.9 ± 0.8	Low	0	26.6 ± 2.2	Medium	0.5
WP-3	72.3 ± 1.9	High	1	87.8 ± 1.9	High	1
Total- 14	**High- 7 (50%), Medium- 2 (14.3%), Low- 5 (35.7%)**			**High- 7 (50%), Medium- 3 (21.4%), Low- 4 (28.6%)**		

AAg: *Autoaggregation; AAg >70%:* ***High;*** *AAg 20-70%:* ***Medium****; AAg <20% :* ***Low****;*
SHb: *Surface hydrophobicity; SHb >70%:* ***High;*** *SHb 20-70%:* ***Medium****; SHb <20% :* ***Low****;*
LR: L. reuteri; LS: L. salivarius; WP: W. paramesenteroides: IPPS: Individual Probiotic Potential Score

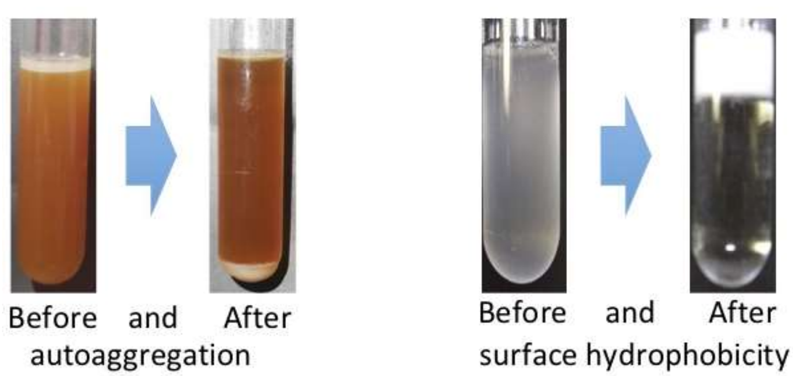

Figure 10: Representative images of autoaggregation and surface hydrophobicity assay

Antimicrobial activity against E. coli and Salmonella Sp.

The antimicrobial activity of each isolates against *E. coli* and *Salmonella* sp., isolated from the intestine of commercial broiler, were tested using the agar well diffusion assay. The representative images of the assay have been illustrated in Figure 11. The clear inhibitory zones around the well indicate the antimicrobial activity by the selected isolates. The measured inhibitory zone against *E. coli* and *Salmonella* Sp. is shown in Tables 3 and 4, respectively. As shown in Tables 3 and 4, the appreciable antimicrobial activity (ZDI ≥ 11) against *E. coli* and *Salmonella* Sp. can only be seen when cultured broth or supernatant was applied. This finding suggests the presence of antimicrobial component(s) in the cultured broth or supernatant. Among the 14 isolates tested, eight showed antimicrobial activity against *E. coli* (Table 3). On the contrary, 6 isolates showed antimicrobial activity against *Salmonella* Sp. (Table 4). These antibacterial spectra by the tested isolates are in agreement with the previous findings that lactobacilli are less active against *E. coli* and *Salmonella* sp. than the gram-positive pathogenic bacteria (Kizerwetter-Swida and Binek, 2005). In addition, it is reported that *Salmonella Pullorum* is more sensitive to the antibacterial activity of lactic acid bacteria than *Salmonella Typhimurium* and *Salmonella Enteritidis* (Jin et al., 1996). The Tables 3 and 4 also shows that the isolates were more active against *E. coli* as compared to *Salmonella* Sp. tested. Our findings are in agreement with very recent report by Shakoor et al. (2017) and Rajoka et al. (2018). The reverse results were also published by several researchers (Jin et al., 1996; Karimi Torshizi et al., 2008; Taheri et al., 2009). This kind of contradictory findings may be due to the differences in sources of tested pathogenic organisms as well as probiotics used in the studies by the respective researcher.

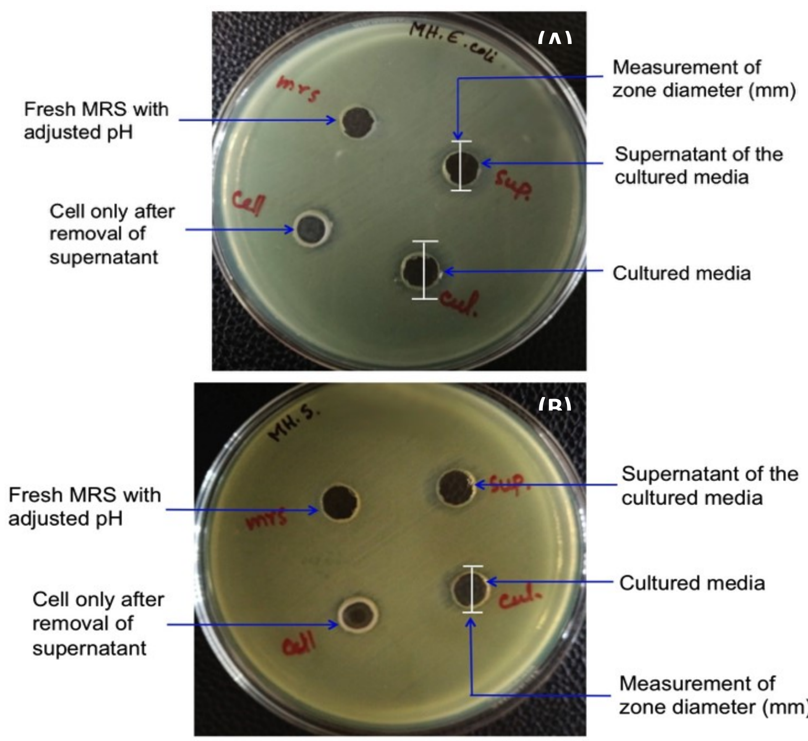

Figure 11: Representative image of antimicrobial assay against *E. coli* (A) and *Salmonella* Sp. (B)

Table 3: Antimicrobial activity (ZDI values in mm) of the selected isolates against *E. Coli*

Isolates	**Cultured broth**			**Supernatant**			**Cell only**			**Average IPPS**
	ZDI	Status	IPPS	ZDI	Status	IPPS	ZDI	Status	IPPS	
LR-1	17	High	1	21	High	1	8	Less	0.25	**0.75**
LR-2	14	Medium	0.5	16	High	1	0	NA	0	**0.50**
LR-3	0	NA	0	0	NA	0	0	NA	0	**0.00**
LR-4	0	NA	0	0	NA	0	0	NA	0	**0.00**
LR-5	0	Medium	0.5	0	NA	0	0	NA	0	**0.17**
LR-6	0	NA	0	0	NA	0	0	NA	0	**0.00**
LR-7	13	Medium	0.5	15	High	1	8	Less	0.25	**0.58**
LR-8	0	NA	0	0	NA	0	0	NA	0	**0.00**
LS-1	11	Medium	0.5	12	Medium	0.5	0	NA	0	**0.33**
LS-2	0	NA	0	0	NA	0	0	NA	0	**0.00**
LS-3	13	Medium	0.5	16	High	1	8	Less	0.25	**0.58**
WP-1	0	NA	0	0	NA	0	0	NA	0	**0.00**
WP-2	14	Medium	0.5	16	High	1	8	Less	0.25	**0.58**
WP-3	15	Medium	0.5	18	High	1	10	Less	0.25	**0.58**

ZDI: *Zone Diameter Inhibition; ZDI ≥ 15:* ***Highly active;*** *ZDI 11-14:* ***Medium active****; ZDI ≤ 10:* ***Less active****; No inhibitory zone:* ***Not active****; LR: L. reuteri; LS: L. salivarius; WP: W. paramesenteroides: IPPS: Individual Probiotic Potential Score*

Table 4: Antimicrobial activity (ZDI values in mm) of the selected isolates against *Salmonella* Sp.

Isolates	Cultured broth			Supernatant			Cell only			Average IPPS
	ZDI	Status	IPPS	ZDI	Status	IPPS	ZDI	Status	IPPS	
LR-1	14	Medium	0.5	17	High	1	8	Less	0.25	**0.58**
LR-2	0	NA	0	0	NA	0	0	NA	0	**0.00**
LR-3	0	NA	0	0	NA	0	0	NA	0	**0.00**
LR-4	0	NA	0	0	NA	0	0	NA	0	**0.00**
LR-5	0	NA	0	0	NA	0	0	NA	0	**0.00**
LR-6	0	NA	0	0	NA	0	0	NA	0	**0.00**
LR-7	11	Medium	0.5	12	Medium	0.5	0	NA	0	**0.33**
LR-8	0	NA	0	0	NA	0	0	NA	0	**0.00**
LS-1	0	NA	0	0	NA	0	0	NA	0	**0.00**
LS-2	0	NA	0	0	NA	0	0	NA	0	**0.00**
LS-3	15	High	1	15	High	1	8	Less	0.25	**0.75**
WP-1	12	Medium	0.5	11	Medium	0.5	0	NA	0	**0.33**
WP-2	14	Medium	0.5	16	High	1	0	NA	0	**0.50**
WP-3	13	Medium	0.5	15	High	1	8	Less	0.25	**0.58**

ZDI: *Zone Diameter Inhibition; ZDI ≥ 15:* ***Highly active;*** *ZDI 11-14:* ***Medium active****; ZDI ≤ 10:* ***Less active****; No inhibitory zone:* ***Not active****; LR: L. reuteri; LS: L. salivarius; WP: W. paramesenteroides: IPPS: Individual Probiotic Potential Score*

Safety status of the selected isolates

The safety status of all the 14 isolates was tested by observing their hemolytic activity and antibiogram profile against the antibiotics commonly used in the poultry industry.

Hemolytic activity

All the 14 isolates had no clear transparent or greenish zone on the blood agar plates, surrounding their colonies, and thus were found γ-hemolytic or non-hemolytic. Therefore, the individual probiotic potential score for each isolate was 1. The representative image of hemolytic assay is shown in Figure 12. The probiotic microorganisms must be safe and essentially be incompetent to cause hemolysis i.e. they would not break red blood cells. According to FAO/WHO (2002), absence of hemolytic activity is considered as safe prerequisite for the selection of probiotic strain. Hemolysis is a known virulence factor among pathogenic microorganisms. In the present study, not a single isolate caused α- or β-hemolysis, although hemolysis by the lactic acid bacteria isolated from chicken intestine is not uncommon (Powthong and Suntornthiticharoen, 2013).

Antibiogram profile

Nowadays antimicrobial resistance ability of a probiotic bacterium is being considered as negative characteristics as they may transfer their resistance genes to the pathogenic organisms when administered (European Commission, 2005). As shown in the Table 5, most of the isolates (78.65%) showed resistance (Zone diameter inhibition was ≥15 mm) against the antibiotic Ciprofloxacin followed by Neomycin (64.3%), Doxicycline (42.9%), Gentamycin (28.6%) and Amoxicillin (14.3%). However, 64.3% isolates were sensitive (Zone diameter inhibition was ≥21 mm) to the antibiotic Amoxicillin and intermediate resistance (Zone diameter inhibition was 16-20 mm) to the Gentamycin (64.3%). For better understanding, we scored each isolate for their resistance/sensitive ability against five antibiotics. The highest and lowest score out of scale 1.0 was 0.6 and 0.1, respectively. The Table 5 also shows that 4 (28.6%) of the isolates (LR 2, LR 3, LR 7 and LR 8) had an average individual probiotic potential score of 0.5 to 0.6 suggesting their possible inclusion as future probiotic isolates. Our results are partially or fully in consistent with previous findings (Messaoudi et al., 2011; Musikasang et al., 2012; Amiranashvili et al., 2016). The representative image of antibiogram profile test is shown in Figure 12.

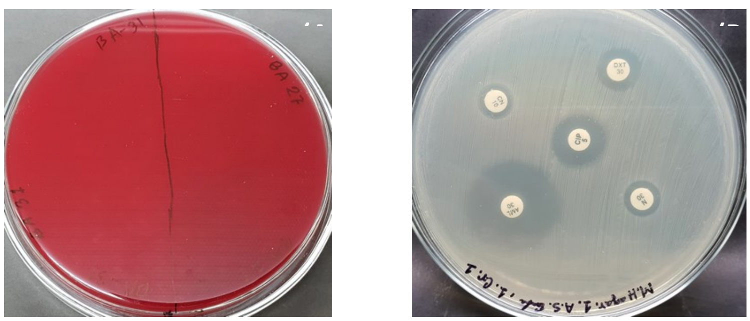

Figure 12: Representative images of hemolytic assay (A) and antibiogram profile (B).

Table 5: Antibiogram profiles (expressed as ZDI values in mm) of the selected isolates against various antibiotics commonly used in the poultry industry

Isolates name	**AML (30 µg)**			**N (30 µg)**			**DXT (30 µg)**			**CN (10 µg)**			**CIP (5 µg)**			**Average IPPS**
	ZDI	Status	IPPS	ZDI	Status	IPPS	ZDI	Status	IPPS	ZDI	Status	IPPS	ZDI	Status	IPPS	
LR-1	20	IR	0.5	13	R	0	20	IR	0.5	13	R	0	28	S	1	**0.4**
LR-2	27	S	1	17	IR	0.5	0	R	0	18	IR	0.5	16	IR	0.5	**0.5**
LR-3	26	S	1	17	IR	0.5	10	R	0	19	IR	0.5	17	IR	0.5	**0.5**
LR-4	22	S	1	15	R	0	18	IR	0.5	17	IR	0.5	15	R	0	**0.4**
LR-5	25	S	1	16	IR	0.5	0	R	0	20	IR	0.5	15	R	0	**0.4**
LR-6	18	IR	0.5	0	R	0	20	IR	0.5	19	IR	0.5	15	R	0	**0.3**
LR-7	22	S	1	16	IR	0.5	17	IR	0.5	19	IR	0.5	15	R	0	**0.5**
LR-8	25	S	1	18	IR	0.5	21	S	1	16	IR	0.5	0	R	0	**0.6**
LS-1	25	S	1	10	R	0	12	R	0	10	R	0	11	R	0	**0.2**
LS-2	21	S	1	13	R	0	8	R	0	13	R	0	13	R	0	**0.2**
LS-3	27	S	1	15	R	0	20	IR	0.5	16	IR	0.5	13	R	0	**0.4**
WP-1	0	R	0	6	R	0	19	IR	0.5	22	S	1	11	R	0	**0.3**
WP-2	16	IR	0.5	0	R	0	15	R	0	18	IR	0.5	13	R	0	**0.2**
WP-3	12	R	0	14	R	0	20	IR	0.5	15	R	0	14	R	0	**0.1**
Total- 14	Resistant= CIP- 11 (78.65%), N- 9 (64.3%), DXT- 6 (42.9%), CN- 4 (28.6%) and AML- 2 (14.3%); Sensitive= AML- 9 (64.3%); Intermediate= CN- 9 (64.3%)															

ZDI: *Zone Diameter Inhibition; ZDI ≥ 21 mm:* ***Sensitive;*** *ZDI 16-20 mm:* ***Intermediate resistance****; ZDI ≤ 15 mm:* ***Resistance****;* ***AML:*** *Amoxicillin;* ***N:*** *Neomycin;* ***DXT:*** *Doxycycline;* ***CN:*** *Gentamycin;* ***CIP:*** *Ciprofloxacin; LR: L. reuteri; LS: L. salivarius; WP: W. paramesenteroides:* ***IPPS:*** *Individual Probiotic Potential Score*

Gastrointestinal pH tolerance ability

The Table 6 shows the chicken gastrointestinal tract (GIT) pH tolerance ability of the 14 isolates. All the isolates showed less than 20 percent growth inhibition at pH 5.0 and 7.5. As we know that except proventriculus/gizzard, all the segments of the chicken GIT have pH ranging from 5.0 to 8.0 (Gauthier, 2002), therefore this result suggests the ability of these isolates to survive in these portions of the chicken GIT. However, the acid tolerance ability of all the isolates was examined by growing them at pH 2.5. As shown in the Table 6, none of the isolate had High acid tolerance ability. Only 3 isolates (4.7% of the total) showed Medium acid tolerance (20-70% growth inhibition) ability. It should be noteworthy that in the present study all the isolates were challenged for 24h at pH 2.5; however, the transit time at lower pH of the chicken GIT is around 90 min only (Gauthier, 2002). Therefore, further kinetics study is required. These findings reflected in the obtained individual probiotic potential score. Only 3 isolates (*L. reuteri*-1, *L. salivarius*-3 and *W. paramesenteroides*-3) had more than 0.8 score out of 1.0. The present findings are comparable to those reported by several researchers (Jin et al., 1998; Karimi Torshizi et al., 2008; Taheri et al., 2009; Bakari et al., 2011) that most of bacterial strains isolated from gastrointestinal tract of chickens are tolerant to pH mimicking to chicken intestine. However, most of the lactic acid bacteria showed lower to medium growth at pH 2.0 to 3.0. Blajman et al. (2015) reported analogous acid tolerance ability (at pH 2.0) by the three *L. salivarius* strains isolated from chicken intestine.

Table 6: Gastrointestinal pH tolerance ability of the selected isolates

Isolates	**pH 2.5**			**pH 5.0**			**pH 7.5**			**Average IPPS**
	GIP	Status	IPPS	GIP	Status	IPPS	GIP	Status	IPPS	
LR-1	65.4	Medium	0.5	4.51	High	1	13.8	High	1	**0.83**
LR-2	95.1	Low	0	0	High	1	6.67	High	1	**0.67**
LR-3	85.9	Low	0	0.79	High	1	8.56	High	1	**0.67**
LR-4	96.7	Low	0	0	High	1	11.2	High	1	**0.67**
LR-5	78.1	Low	0	0.24	High	1	12.0	High	1	**0.67**
LR-6	91.3	Low	0	5.94	High	1	9.98	High	1	**0.67**
LR-7	78.1	Low	0	3.86	High	1	10.8	High	1	**0.67**
LR-8	96.7	Low	0	0.97	High	1	4.58	High	1	**0.67**
LS-1	94.5	Low	0	14.7	High	1	0	High	1	**0.67**
LS-2	89.6	Low	0	0	High	1	16.2	High	1	**0.67**
LS-3	70.0	Medium	0.5	7.47	High	1	11.3	High	1	**0.83**
WP-1	86.6	Low	0	0.61	High	1	7.94	High	1	**0.67**
WP-2	93.4	Low	0	4.49	High	1	12.7	High	1	**0.67**
WP-3	69.2	Medium	0.5	9.77	High	1	13.3	High	1	**0.83**

***GIP:** Growth Inhibition percent; GIP >70%: **Low;** GIP 20-70%: **Medium**; GIP <20%: **High** acid tolerance ability; LR: L. reuteri; LS: L. salivarius; WP: W. paramesenteroides: IPPS: Individual Probiotic Potential Score*

Bile tolerance ability

Resistance to bile salts is of great importance to survival and growth of bacteria in the intestinal tract, and thus, is a prerequisite for bacteria to be used as probiotics (Havenaar et al., 1992). As shown in the Table 7, out of 14 isolates tested for their bile tolerance ability 4 were found high bile tolerance ability (<20% growth inhibition), 5 were designated as low bile tolerance ability (>70% growth inhibition) and another 5 were displayed medium ability to tolerate bile (20-70% growth inhibition) at a concentration of 0.3%. It is reported that bile resistance of some strains vary a lot among the lactic acid bacteria species and between strains themselves (Xanthopoulos et al., 1997). Several studies have shown that probiotics are usually survived well at 0.3% bile concentration (Wang et al., 2014; Kobierecka et al., 2017; Rajoka et al., 2018). It should be noted that the average bile concentration is around 0.3%, and may range up to an extreme of 2.0% during the first hour of digestion (Gotcheva et al., 2002). In the present study, appreciable number of isolates has shown their ability to survive in bile salts.

Table 7: Bile tolerance ability of the selected isolates

Isolates name	Growth inhibition (%)	Bile tolerance status	IPPS
LR-1	16.2 ± 1.6	High	1
LR-2	15.2 ± 4.4	High	1
LR-3	89.3 ± 2.1	Low	0
LR-4	53.2 ± 3.3	Medium	0.5
LR-5	85.9 ± 4.0	Low	0
LR-6	30.1 ± 2.9	Medium	0.5
LR-7	20.3 ± 4.8	Medium	0.5
LR-8	58.9 ± 3.2	Medium	0.5
LS-1	81.7 ± 2.2	Low	0
LS-2	73.9 ± 2.8	Low	0
LS-3	17.6 ± 3.1	High	1
WP-1	78.9 ± 1.6	Low	0
WP-2	42.8 ± 1.3	Medium	0.5
WP-3	19.4 ± 2.2	High	1

Growth Inhibition percent (GIP) >70%: ***Low;*** *GIP 20-70%:* ***Medium****; GIP <20%:* ***High*** *bile tolerance ability; LR: L. reuteri; LS: L. salivarius; WP: W. paramesenteroides: IPPS: Individual Probiotic Potential Score*

Cumulative probiotic potential score

The cumulative probiotic potential (CPP) score of the selected 14 isolates is shown in Table 8. Since we have evaluated eight probiotic properties (Autoaggregation ability, Surface hydrophobicity ability, Antimicrobial activity against *E. coli*, Antimicrobial activity against *Salmonella* Sp., Antibiogram profile, Hemolytic activity, Bile tolerance ability, Acid tolerance ability) and the highest score for each probiotic property was 1, therefore, the maximum possible probiotic potential score for individual isolate was 8. As shown in the Table 8, the highest CPP score was 69.5% and the lowest score was 10.9%. The highest score (69.5%) was achieved by the two isolates *L. reuteri*-1 and *L. salivarius*-3. The second highest score

(63.6%) was accomplished by the isolate *W. paramesenteroides*-3. The third highest score was 57.3% which was achieved by the isolate *L. reuteri*-7. The remaining isolates scored less than 50% CPP, therefore, were not considered as future potent probiotics. The species belonging to *L. reuteri* and *L. salivarius* have been reported to consider as probiotic and also reported to produce bacteriocin like components. Recently, *Weissella* Sp. is also being considered as promising probiotics. The *Weissella* Sp. isolated in the present study is also expected to be used as a probiotic.

Table 8: Cumulative probiotic potential (CPP) score of the selected isolates

Isolates name	Maximum possible probiotic potential score	Obtained probiotic potential score	Cumulative probiotic potential score
Lactobacillus reuteri-1	8	5.6	69.5%
Lactobacillus reuteri-2	8	3.7	45.9%
Lactobacillus reuteri-3	8	1.2	14.6%
Lactobacillus reuteri-4	8	3.1	38.4%
Lactobacillus reuteri-5	8	1.2	15.5%
Lactobacillus reuteri-6	8	2.9	37.1%
Lactobacillus reuteri-7	8	4.6	57.3%
Lactobacillus reuteri-8	8	3.8	47.1%
Lactobacillus salivarius-1	8	1.7	21.3%
Lactobacillus salivarius-2	8	0.9	10.9%
Lactobacillus salivarius-3	8	5.6	69.5%
Weissella paramesenteroides-1	8	2.8	35.0%
Weissella paramesenteroides-2	8	2.9	36.9%
Weissella paramesenteroides-3	8	5.1	63.6%

12. Research highlight/findings:

- A total of 39 isolates were purified from four different parts of the gastrointestinal digestive tracts (crop, small intestine, large intestine and cecum) of Red Jungle fowls.
- Molecular characterization of the pure isolates confirmed the predominant isolates as *Lactobacillus reuteri* (71.8%) followed by *Lactobacillus salivarius* (20.5%) and *Weissella paramessenteroides* (7.7%).
- The highest number of *L. reuteri* was found in large intestine and crop (35.7% and 32.1% respectively). On the contrary, the maximum number of *L. salivarius* (37.5%) and *W. paramessenteroides* (66.7%) was found to reside in cecum.
- Fourteen isolates representing the respective sequence types were evaluated in vitro for the probiotic properties. Based on the probiotic activities the isolates were ranked by calculating cumulative probiotic potential (CPP) score. The four isolates *L. reuteri*-1 (69.5%), *L. reuteri*-7 (57.3%), *L. salivarius*-3 (69.5%) and *W. paramesenteroides*-3 (63.6%) achieving the CPP score of more than 50% were selected as the potential probiotics for future use.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Physical (#)	Financial (Tk)	Physical (#)	Financial (Tk)	
(a) Office equipment	01	60,000	01	59,200	
(b) Lab &field equipment	02	3,50,000	02	3,50,000	
(c) Other capital items					

2. Establishment/renovation facilities: Not applicable

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	

3. Training/study tour/ seminar/workshop/conference organized: Not applicable

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	2,31,275	1,86,145	1,80,850	50,425	78.2	
B. Field research/lab expenses and supplies	11,00,900	10,88,520	11,00,900	-	100	
C. Operating expenses	96,196	96,196	96,196	-	100	
D. Vehicle hire and fuel, oil & maintenance	14,850	14,850	14,850	-	100	
E. Training/workshop/seminar etc.	-	-	-	-	-	
F. Publications and printing	55,000	23,000	19,975	35,025	36.3	
G. Miscellaneous	9,254	10,700	6,940	2,314	75	
H. Capital expenses	4,09,500	4,09,500	4,09,200	300	99.9	

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
Isolation of lactic acid bacteria (LAB) from the gastrointestinal (GI) tract of Red Jungle fowl (RJF).	RJF captured from the field; LAB isolated in the laboratory.	A total of 39 isolates were purified using various bacteriological techniques. Among them, 28% were isolated from crop, 20% from small intestine, 31% from large intestine and remaining 21% isolated from cecum.	The organisms with probiotic potential could be used in formulating a potential probiotic mix.
Molecular characterization of the LAB.	Application of molecular techniques like PCR, nucleotide sequencing, phylogenetic analysis, etc.	Molecular characterization showed the predominant isolates to be *Lactobacillus reuteri* (71.8%) followed by *Lactobacillus salivarius* (20.5%) and *Weissella paramessenteroides* (7.7%). Out of the 39 isolates, in vitro evaluation of probiotic potential of 14 isolates revealed 8 belong to *Lactobacillus reuteri*, 3 belong to *Lactobacillus salivarius* and 3 belong to *Wissella paramessenteriodes*. GenBank Accession numbers for the 14 isolates have been obtained.	
In vitro evaluation of the isolated LAB for probiotic potential.	Several laboratory assay to determine the probiotic potential	Based on the probiotic activities the isolates were ranked by calculating cumulative probiotic potential (CPP) score. The four isolates *L. reuteri*-1 (69.5%), *L. reuteri*-7 (57.3%), *L. salivarius*-3 (69.5%) and *W. paramesenteroides*-3 (63.6%) achieving the CPP score of more than 50% were selected as the potential probiotics for future use.	

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.			
Journal publication	01		
Information development			
Other publications, if any		01	Obtained 14 accession numbers from GenBank

F. Technology/Knowledge generation/Policy Support (as applied):

i. **Generation of technology (Commodity & Non-commodity):**
None.

ii. **Generation of new knowledge that help in developing more technology in future**

The four isolates *L. reuteri*-1, *L. reuteri*-7, *L. salivarius*-3 and *W. paramesenteroides*-3 from this study could be used for the development of probiotic products for the poultry industry in future.

iii. **Technology transferred that help increased agricultural productivity and farmers' income:**
None.

iv. **Policy Support:**
None.

G. Information regarding Desk and Field Monitoring

i) **Desk Monitoring**: Not done.

ii) **Field Monitoring (time& No. of visit, Team visit and output):**
03 March 2018; 01 visit; Team consisted of two honorable members from Bangladesh Agricultural Research Council (BARC). There was a threadbare discussion on research progress and future development. The monitoring team observed the research work in the laboratory. The team also observed procurement of equipment and consumables. It was advised to give special attention on molecular characterization of all the pure isolates. Proper preservation (especially store at -86°C) of all the isolates was highly recommended.

H. Lesson Learned (if any)

This type of project for mining probiotics from local resources needs more time and fund.

I. Challenges (if any)

i) Capturing Red jungle fowl was a challenge.
ii) To keep pace with the time was also challenging.

Signature of the Principal Investigator
Date …………………………
Seal

Counter signature of the Head of the organization/authorized representative
Date …………………………..
Seal

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