

*Article*

## **Isolation, identification and molecular detection of selected probiotic bacteria from broiler chickens and their related environment**

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**Abstract:** This study was aimed to isolate and identify the potential probiotic bacteria from broiler chickens and their related environment of the two districts in Bangladesh by using cultural and biochemical techniques and detection of the selected isolated probiotic potential bacterial species by using polymerase chain reaction (PCR). In this context five probiotic bacteria species of five bacteria genera were screened out from 750 samples in where there were 186 *Lactobacillus* genus isolates, 102 *Bacillus* genus isolates, 112 *Streptococcus* genus isolates, 220 *Enterococcus* genus isolates and 33 *Bifidobacterium* genus isolates on the basis of biochemical properties like 10% solution of total 22 different sugars. Other biochemical tests like MR-VP, Oxidase, Catalase, Citrate utilization, MIU and Indole test were major. Genus was detected through specific genus target gene and 16S r DNA based PCR. Selected probiotic bacteria species were detected through species specific target gene PCR. We confirmed 23 *Lactobacillus acidophilus* species isolates, 14 *Bacillus subtilis* isolates, 19 *Streptococcus thermophilus* isolates, 31 *Enterococcus faecium* isolates and 09 *Bifidobacterium bifidum* isolates at species level with *ddl* gene, *apr-E* gene, *16S-23S ITS* gene, *BIBI* gene, *Stp-TH* gene primers. The novelty of this study is at first time in Bangladesh, we isolated and identified five selected potential probiotic species from the gastro-intestinal tract of broiler chickens and their related environmental sources by not only using traditional cultural, biochemical techniques but also genus and species specific target gene based PCR.

**Keywords:** *Lactobacillus*; *Bacillus*; *Streptococcus*; *Bifidobacterium*; *Enterococcus*; biochemical; PCR; probiotic; broiler chickens

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### **1. Introduction**

The widespread use of antibiotics all around the world not only as therapeutic and prophylactic agents but also as growth promoters in poultry industry has led to a wide increase in antibiotic resistance and the emergence of untreatable multidrug resistant strains of bacteria and other microbes (Kabir, 2009). However, the growing concern of the international community on the use of antibiotics in animal production has led to an increase of the so called antibiotic free production (Cristiano *et al.*, 2019). But what should be the alternative for this and what was the background of this study? European Union banned the utilization of antibiotics as growth promoters in all of edible animal production in 2006. Now for what we isolated and identified common probiotic bacterial species belongs to *Lactobacillus*, *Bacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus* genus. Probiotic is derived from Greek word “Probios” and it means for life. According to FAO and WHO the term “Probiotic” refers to “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO, 2002; WHO, 2002). Another way, it is defined as a “live microbial product” which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989). Probiotic species belonging to *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*,

*Candida*, and *Saccharomyces* have a beneficial effect on broiler performance, pathogen inhibition and immunomodulation (Kabir, 2009). The ultimate target of this research was to develop a cost effective and potent probiotic for the poultry industry by isolation, identification and molecular detection of selected potential bacterial species from the gastro-intestinal tract of locally rearing broiler chickens and their related environmental sources like litter, feeder, water drinkers with cultural, biochemical and molecular techniques. *Lactobacillus*, *Bacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus* genus were chosen as probiotic for the poultry industry because they are beneficial commensals of chickens and have proven as potential probiotic through probiotic ability tests. Moreover, these probiotic bacteria strongly aggregate with other pathogenic bacteria, compete for adhesion to epithelial receptors of host, modify the structure and function of intestinal epithelium of host, modify total microbiota of host intestine, take apart for competition for nutrients of host body and produce some specific substances like organic acids, bacteriocine, dipicolinic acids (Peera and James, 2013). In Bangladesh, there are many foreign commercial probiotics are available in market and their indiscriminate use without local reliable experimental support can't be justified. These products are also expensive for the poultry rearer as well. That's why we attempted to isolate, identify and molecular detection of all above mentioned probiotic bacteria. Yet, the proper chain handle of these live bacteria containing imported product preparation hasn't maintained neither at seller nor farmer level. Indeed, it's a great problem here. Therefore, this unique research was undertaken for isolation, identification and detection of selected multi bacterial species from totally indigenous sources at the first time in Bangladesh.

## 2. Materials and Methods

### 2.1. Study area

A total of 750 samples were collected from 10 farms of two districts in Bangladesh. The lab work and other accessories work were performed mainly in Department of Microbiology and Hygiene, Department of Physiology, Central Laboratory of Bangladesh Agricultural University, Bangladesh.

### 2.2. Collection of samples

Total samples were collected from gastro-intestinal tracts of locally rearing broiler chickens like 300 caecum, 300 cloacal swab samples and their related environment which includes the samples from 50 water drinkers, 50 feeder and 50 floor litter samples. After collection the samples were preserved at 0.1% peptone water and cool ice box until reached to laboratory.

### 2.3. Isolation and morphological characteristics of probiotic bacteria

The total samples were processed and somewhere it was needed to grind with mortar and pestle at the very beginning of processing. The sample aliquots were inoculated in Nutrient broth, MRS broth and BSM broth then incubated at 37<sup>o</sup> C for 24 hrs to 36 hrs. After then, we have gone for primary culture in specific agar media and then secondary culture for isolation of selected bacteria in selective enrich agar media. Sub cultured into specific selective media until obtaining single pure colonies of five bacterial species of five genera. Tentative pure colonies were isolated through morphological characterization, Gram's staining and culture characteristics. In where, another technique was biochemical characterization by fermentation of 10% solution of different 22 sugars. Other biochemical properties were characterized by the MR-VP Test, Oxidase, Catalase Test, Indole Test, Citrate utilization Test, MIU (Motility) Test. Somewhere we used zigzag streaking method or straight streaking method and finally we found pure colonies of *Lactobacillus* on MRS Agar<sup>TM</sup> which were distinct white or whitish yellow and Gram staining properties included positive, rod or ovoid shaped with tapering end (Kamrun *et al.*, 2016). Gram staining positive, rod with square ends, large rod shaped organisms, arranged in chain are characteristics of *Bacillus subtilis* (Jones *et al.*, 1987). The surface was finely granular and slightly sticky, large, sporulated, rod-shaped bacteria (Merchant and Packer, 1967). Maximum of all these species are sub terminal, endospore formed, motile and on Bacillus Differentiation agar (Himedia, India) we found large ellipsoidal, slightly sticky, blue pure colonies. Members of the genus *Enterococcus* include gram positive, facultatively anaerobic cocci that are ovoid in form and can occur in singlet, pairs or short chains (Facklam, 2002). On Enterococcus Faecium agar base (Himedia, India) we found luxuriant green colored pure colonies. *Bifidobacterium bifidum* colonies were convex, creamy, white or grayish, glossy, smooth, neat-edged, soft, sticky on BSM agar media and TOS-Propionate Agar media. The colonies were dew drop like small flat distinct brown colored on MRS agar media. In Nutrient Agar medium, normally produced small grayish round colonies. In Streptococcus Thermophilus Isolation Agar Medium<sup>TM</sup>, the colonies were round, flat, large grayish sticky.

For cultural morphological characterization, we used MRS agar (Himedia, India), Hicrome Bacillus agar (Himedia, India), Bacillus Differentiation agar (Himedia, India), Streptococcus Thermophilus isolation agar (Himedia, India), Enterococcus Faecium agar base (Himedia, India), Enterococcus Differential Agar Base (Himedia, India), TOS-Propionate Agar acc. (Sigma -Aldrich, India).

#### 2.4. Identification of probiotic bacteria by using biochemical tests

Biochemical characterization was performed by fermentation of 10% solution of different 22 sugars like D-Galactose, D-Fructose, L-Arabinose, Maltose, L-Rhamnose, D-Mannitol, D-Sorbitol, Methyl-alpha-D-Glucopyranoside, Amygdaline, D-Ribose, Melebiose, Lactose, Raffinose, Sucrose, Melizitose, Xylose, Trehalose, Arginine, Salicine, NAG amine, Glucose, Dextrose, L-Rhamnose. Other biochemical properties were characterized by the MR-VP Test, Oxidase, Catalase Test, Indole Test, Citrate utilization Test, MIU (Motility) Test. Finally, we confirmed about five specific probiotic bacterial species of five genera through molecular detection (PCR).

#### 2.5. Molecular detection of probiotic bacteria

Finally, we confirmed about five specific probiotic bacterial species of five genera through molecular detection (PCR). Genus was detected through specific genus target gene and 16S r DNA based PCR. Selected probiotic bacteria species were detected through species specific target gene PCR. We confirmed *Lactobacillus acidophilus* species isolates, *Bacillus subtilis* isolates, *Streptococcus thermophilus* isolates, *Enterococcus faecium* isolates and *Bifidobacterium bifidum* isolates at species level with *ddl* gene, *apr-E* gene, *16S-23S ITS* gene, *BIBI* gene, *Stp-TH* gene primers accordingly.

##### 2.5.1. DNA extraction

DNA was extracted from pure colonies of five selected probiotic bacteria samples as follows / regards: harvested up to  $2 \times 10^9$  bacterial cells in a 1.5 ml micro centrifuge tube centrifugation for 10 minutes at 5000xg. Then discarded the supernatant and resuspended the pellets in 180  $\mu$ l of Gram positive bacteria lysis buffer (20 mM Tris-HCl, Ph 8.0, 02 mM EDTA, 1.2% Triton x 100, add lysozyme to 20 mg/ml immediately before use). Incubated for 30 minutes for 37°C, then we added 200  $\mu$ l of lysed sample solution and then added 20  $\mu$ l proteinase-k. Mixed properly by vortexing and pipetting to obtain a uniform suspension. Incubated the sample at 56°C while shaking water bath until the cells were completely lysed (30 minutes), then added 20  $\mu$ l of RNase-A solution (Thermo-Fisher Scientific, Lithuania), mixed by vortexing and kept the mixture for 10 minutes at room temperature. Then 400  $\mu$ l of 50% Ethanol was added and properly mixed by pipetting. Transferred the prepared lysate solution to a Gene JET Genomic DNA purification column and inserted in a collection tube and centrifuged the column for 01 minute at 6000xg. Discarded the collection tube containing the flow-through solution. The Gene JET Genomic DNA purification column was placed into a new 02 ml collection tube. 500  $\mu$ l of wash Buffer-I with ethanol was added. Then centrifuged at 8000xg for 01 minute. Discarded the flow-through and placed the purification column back into collection tube. 500  $\mu$ l of wash Buffer-II with ethanol was added into the Gene JET Genomic DNA purification column. Centrifuged at  $\geq 12000$ xg speed for 03 minutes, respined the column for 01 minute at maximum speed. Discarded the collection tube containing the flow-through solution and the Gene JET Genomic DNA purification column was transferred to a sterile 1.5 ml eppendorf tube. Finally, 200  $\mu$ l of Elution Buffer was added to the centre of Gen JET Genomic DNA purification column membrane to elute genomic DNA. Incubated for 02 minutes at room temperature and centrifuged at 8000xg for 01 minute. Discarded the purification column tube, then used the purified DNA immediately in downstream applications and remaining was stored at -20°C.

##### 2.5.2. PCR analysis

###### 2.5.2.1. *Lactobacillus* and *Lactobacillus acidophilus* PCR based detection

Identification of the *Lactobacillus acidophilus* isolates with genus specific primers designed by Magne *et al.*, 2006. Target 472 bp 16S rRNA gene fragment from all suspected *Lactobacillus* isolates were amplified by PCR using the primers 16S rRNA with FW: 5'-CTC CTA CGG GAG GCA GCA GT-3' and REV: 5'-GGACTACCAGGGTATCTA A-3'; inwhere, PCR condition for each suspected *Lactobacillus* genus isolate was as follows: 25  $\mu$ l of PCR mixture contained with 12.5  $\mu$ l master mix (GoTaq<sup>®</sup> G2 Green Master Mix, 2X: GoTaq<sup>®</sup> G2 DNA Polymerase was supplied in GoTaq<sup>®</sup> G2 reaction buffer with Ph 8.5, 400  $\mu$ M d-ATP, 400  $\mu$ M d-GTP, 400  $\mu$ M d-CTP, 400  $\mu$ M d-TTP and 3 mM MgCl<sub>2</sub>, Madison, WI USA), FW: 1  $\mu$ l (10 pmol/ $\mu$ l), REV: 1

μl (10 pmol/μl), Nuclease Free Water: 5.5 μl, DNA Template: 5 μl, initial denature temperature at 94°C for 5 minutes, 30 cycles of 94°C for 60 seconds at annealing at 55°C for 60 seconds, extension at 72°C for 90 seconds & final 72°C for 10 minutes. Final PCR product was loaded in 1.5% agarose gels with Bench Top 100 bp DNA Ladder (consists of 11 double-stranded DNA fragments with sizes from 100-1500 bp, Promega, Madison, WI USA) and ran at 80 Volt for 25 minutes. Finally, identification of *Lactobacillus acidophilus* isolates with species specific primers according to Song *et al.*, 2000. A 210 bp target 16S-23S rDNA *ISR* (Intergenic Spacer Region) Laci-I: FW:5-TGCAAAGTGGTAGCGTAAGC-3, 23–10C: REV:5-CCTTTCCC TCACGGTACTG-3; in where, 12.5 μl master mix, FW: 1μl (10 pmol/μl), REV: 1 μl (10 pmol/μl), Nuclease Free Water: 5.5 μl, DNA Template: 5 μl and total volume of PCR mixture was 25 μl, initial denaturation temperature was 95°C for 03 minutes, annealing at 68°C (60 seconds), 72°C for 1 minute, concluded with each reaction a 10 minutes final extension at 72°C.

#### 2.5.2.2. *Bacillus* and *Bacillus subtilis* PCR based detection

Detection of *Bacillus* isolates with genus specific primers have described by Kim *et al.*, 2010. In where, target PCR band product size was 1.5 kb (1500 kb) 16S rRNA gene from all suspected *Bacillus* isolates were amplified by PCR using forward and reverse primers sequence with 27F: 5'-AGAGTTTGATCCTGGCTCAG-3', 1492R: 5'-GGCTACCTTGTTACGACTT-3'. Each PCR product was amplified according to the conditions as follows: initial denaturation temperature was 30 cycles, at 95°C for 1 minute, annealing at 45°C for 1 minute, extension at 72°C for 90 s and 10 minutes final incubation at 72°C. Isolates of *Bacillus subtilis* detection at species level was carried out according to Alireza Sadeghi *et al.*, 2012. Target 744 bp *apr-E* genes with FW and REV primers sequence with *apr-E*: FW: TTTACGATGGCGTTCAGCAAC, *apr-E*: REV: GGAAGTGCCTTCA TTTCCGGCT. Total volume of PCR product was 25 μl; initial denaturation temperature was 94°C for 4 minutes followed by 35 cycles, annealing at 57°C for 40 seconds, extension 72°C for 1 minutes, concluded with Each reaction a 05-minutes final extension at 72°C, primers concentration of 2 μL of each primer with 0.5 mmol L<sup>-1</sup>. Final PCR product was loaded in 1.5% agarose gels with BenchTop 100 bp DNA Ladder (consists of 11 double-stranded DNA fragments with sizes from 100-1500 bp, Promega, Madison, WI USA) and ran at 80 Volt for 25 minutes.

#### 2.5.2.3. *Bifidobacterium* and *Bifidobacterium bifidum* PCR based detection

Detection of *Bifidobacterium* isolates with genus specific primers have described by Langendijk *et al.* (1995). In where, target PCR band product size was 510 bp 16S rRNA gene from all suspected *Bifidobacterium* isolates were amplified by PCR using forward and reverse primers sequence with Bif164-f: 5'-GGGTGGTAATGCCGGATG-3', Bif662-r: 5'-CCACCGTTACACC GGGAA-3'. Each PCR product was amplified according to the conditions as follows: initial denaturation temperature was 1st cycle of 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, annealing at 60°C for 1 minutes, extension at 68°C for 2 minutes and 68°C for 7 minutes. Isolates of *Bifidobacterium bifidum* detection at species level was carried out according to Matsuki *et al.*, 1998. Target 278 bp *BIBI* gene with FW and REV primers sequence with FW: 5-CCACATGATCGCATGTGATTG-3, REV: 5-CCG AAG GCT TGC TCCCAA-3. Total volume of PCR product was 25 μl; initial denaturation temperature was one cycle of 94°C for 5 minutes followed by 35 cycles of 94°C for 20 seconds, annealing at 55°C for 20 seconds, extension at 72°C for 30 seconds, concluded with final reaction cycle at a 72°C for 5 minutes, primers concentration of 25 μM of each primer. Final PCR product was loaded in 2% agarose gels with BenchTop 100 bp DNA Ladder (consists of 11 double-stranded DNA fragments with sizes from 100-1500 bp, Promega, Madison, WI USA) and ran at 80 Volt for 25 minutes.

#### 2.5.2.4. *Streptococcus* and *Streptococcus thermophilus* PCR based detection

Molecular detection of *Streptococcus* genus using PCR, primers pair specific to 16S rDNA of *Streptococcus* was performed according to Sato *et al.*, 2003. The sequence of 16S rDNA was amplified by using total PCR mixture of 25ul volume for each suspected isolates. The target amplicon size was 1505 bp with 8UA-FW and 1492-REV primers sequence of 8UA-FW: 5'-AGAGTTTGATCMTGGCTCAG-3', 1492-RW: 5'-TACGGYTACCTGTTACGACTT-3', 15 minutes at 95°C for initial heat activation and denaturation with 35 cycles, 1 minute at 55°C for annealing, extension at 1.5 minutes at 72°C & a final elongation of 72°C for 10 minutes and primer concen. 1 μl of each primer (5 μM).

(\*Reaction conditions for *S. thermophilus* PCR were 0.2 μg/μl DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, a 200 μM concentration of each dATP, dCTP, dGTP, and dTTP, 1 μM Stp Th-F, 1 μM Stp Th-R, 2.5

units Applied Biosystems Ampli Taq DNA polymerase. Amplification conditions were 95°C for 3 minutes; 35 cycles. Reaction conditions varied regarding MgCl<sub>2</sub> concentration (2.5 mM) as well as Taq polymerase (2.5 units Applied Biosystems Ampli Taq DNA polymerase described by Jeanne *et al.* (2005). For all isolates of suspected *S. thermophilus*, 968 bp of *Stp-TH-FW* and *Stp-TH-REV* fragment was detected confirming the species to the genus *Streptococcus*. *Streptococcus thermophilus* species were isolated from the all samples according to Lick *et al.*, 2001. Applied Biosystems AmpliTaq DNA polymerase with *Stp-TH-FW*: 5-CAC TAT GCT CAG AAT ACA-3, *Stp-TH-REV*: 5-CGA ACA GCA TTG ATG TTA-3, in where, PCR condition for each suspected *Streptococcus thermophilus* isolate was as follows: 25 µl of PCR mixture contained with 12.5 µl master mix, Forward primer :1 µM, Reverse primer:1 µM, Nuclease Free Water: 5.5 µl, DNA Template: 5 µl, initial (denaturation at 94°C for 20 seconds, annealing at 58°C for 30 seconds, extension at 68°C for 30 seconds; 68°C for 5 minutes of final extension. Final PCR product was loaded in 1.5% agarose gels with BenchTop 100 bp DNA Ladder (consists of 11 double-stranded DNA fragments with sizes from 100-1500 bp, Promega, Madison, WI USA) and ran at 80 Volt for 25 minutes.

### 2.5.2.5. *Enterococcus* and *Enterococcus faecium* PCR based detection

A systematic molecular approach, followed by the targeting genus and species specific gene based PCR. The amplicon size of genus of *Enterococcus* was 112 bp with Ent-1 FW, Ent-2 Rev primers targeting the 16S-23S ITS rDNA according to Ke *et al.*, 1999. In where, sequence of Ent-1: FW: 5-TACTGACAAACCATTTCATGATG-3, Ent-2: Rev: 5-AACTT CGTCA CCAACGCGAAC-3, in where, PCR condition for each suspected *Enterococcus* isolate was as follows: 25 µl of PCR mixture contained with 12.5 µl master mix (GoTaq<sup>®</sup> G2 Green Master Mix, 2X: GoTaq<sup>®</sup> G2 DNA Polymerase was supplied in GoTaq<sup>®</sup> G2 reaction buffer with Ph 8.5, 400 µM d-ATP, 400 µM d-GTP, 400 µM d-CTP, 400 µM d-TTP and 3 mM MgCl<sub>2</sub>, Madison, WI, USA), Forward primer: 0.2 µM/µL, Reverse primer: 0.2 µM/µL, Nuclease Free Water: 5.5 µl, DNA Template: 5 µl, initial denature temperature at 3 minutes at 95°C and then followed by 35 cycles of 30 seconds, at 95°C, annealing at 30 seconds, at 55°C & extension at 1 minutes at 72°C, with a 7 minutes final 72°C. Molecular detection of *Enterococcus faecium* from suspected isolates were performed according to Dukta-Malen *et al.*, 1995, targeting a 550bp *ddl* gene based species specific PCR. In where, PCR using forward and reverse primers sequence with *ddl* FW: 5'-TAGAGACATTGAATATGCC-3', *ddl* Rev: 5'-CTAACATCGTGTAAAGCT-3'. Each PCR product was amplified according to the conditions as follows: initial denaturation temperature was 94 °C for 2 minutes for the 1st cycle and 30 cycles of 94 °C for 01 minute, annealing at 54°C for 01 minute, extension at 72°C for 01 minute and 72°C for 10 minutes for the last cycle. Final PCR product was loaded in 1.5% agarose gels with BenchTop 100 bp DNA Ladder (consists of 11 double-stranded DNA fragments with sizes from 100-1500 bp, Promega, Madison, WI USA) and ran at 80 Volt for 25 minutes.

## 2.6. Ethical approval

The protocol was approved by the Animal Welfare and Experimentation Ethics Committee of the Bangladesh Agricultural University and approval no: AWEEC/BAU/2019(44).

## 3. Results

### 3.1. Isolation and preliminary identification of probiotic bacteria

#### 3.1.1. *Lactobacillus* and *Lactobacillus acidophilus*

On this study we observed that colonies on Lactobacillus MRS Agar<sup>™</sup> (Himedia, India) were distinct white or whitish yellow, smooth round looking form. Gram's staining properties included positive, rod or ovoid shaped with tapering end. *Lactobacillus* species which was primarily confirmed as *Lactobacillus acidophilus* though performed negative result reaction in case of 10 % carbohydrate (sugar) fermentation of D-Galactose, D-Sorbitol, Melebiose, Melizitose and MR, Indole, Oxidase, Citrate test but showed positive reaction in case of D-Fructose, L-Arabinose, Maltose, L-Rhamnose, D-Mannitol, D-Sorbitol, Methyl-alpha-D-Gluco-pyranosid, Amygdaline, D-Ribose, Lactose, Raffinose, Sucrose, Xylose, Trehalose, Arginine, Salicine, NAG amine and VP test (Tables 1 and 2). Carbohydrate fermentation tests were performed with the basal medium phenol red broth (ADSA, Barcelona, Spain).

### 3.1.2. *Bacillus* and *Bacillus subtilis*

The surface was finely granular and slightly sticky, large, sporulated, rod-shaped bacteria (Merchant and Packer, 1967). Maximum of all these species as ellipsoidal, sub terminal endospore formed motile and on Bacillus Differentiation agar (Himedia, India) we found large ellipsoidal, slightly sticky, blue pure colonies. On this research study, primarily suspected *Bacillus subtilis* species performed negative reaction in case of D-Galactose, D-Fructose, L-Rhamnose, Arginine, NAG amine, Melizitose 10% carbohydrate (sugar) fermentation and MR, Indole, Catalase, Oxidase, but showed positive results in 10% sugar solution of L-Arabinose, Maltose, D-Mannitol, D-Sorbitol, Amygdaline, Dextrose, D-Ribose, Melebiose, Sucrose, Lactose, Xylose, Dextrose, Raffinose, Trehalose, Salicine fermentation and VP, Citrate, ONPG, Gram's staining (Tables 1 and 2).

### 3.1.3. *Streptococcus* and *Streptococcus thermophilus*

In Nutrient Agar medium, normally produced small grayish round colonies. In Streptococcus Thermophilus Isolation Agar Medium™, the colonies were round, flat, large grayish sticky in our study. On Blood Agar, it was non-haemolytic responded. Primarily suspected *Streptococcus thermophilus* species performed negative reaction in case of Maltose, L-Rhamnose, D-Mannitol, Amygdaline, Arginine, NAG amine, 10% carbohydrate (sugar) fermentation and MR, VP, Indole, Oxidase, Citrate utilization, but showed positive results in 10% sugar solution of D-Galactose, D-Fructose, L-Arabinose, D-Sorbitol, Dextrose, D-Ribose, Melebiose, Sucrose, Melizitose, Xylose, Trehalose, Salicine, Raffinose fermentation, Catalase reaction and Gram staining (Tables 1 and 2). Gram's staining properties also included the cocci, non sporeforming, chain formed, but also in pair or single form microorganisms.

### 3.1.4. *Enterococcus* and *Enterococcus faecium*

On Enterococcus Faecium agar base (Himedia, India) we found luxuriant green colored pure colonies. On this research study, primarily suspected *Enterococcus faecium* species D-Galactose, D-Fructose, L-Arabinose, Amygdaline, Dextrose, D-Ribose, Sucrose, Melizitose, Arginine, Trehalose, Salicine, Raffinose showed positive results in 10% carbohydrate (sugar) fermentation, Oxidase reaction and Gram's staining but performed negative result in 10% sugar fermentation of Maltose, L-Rhamnose, D-Mannitol, D-Sorbitol and MR, VP, Indole, Catalase, Citrate, ONPG (Tables 1 and 2). Biochemical tests, such as sugar fermentation, coagulase, catalase, MR, VP, and indole tests, were performed as per the standard methods of Cheesbrough (1985).

### 3.1.5. *Bifidobacterium* and *Bifidobacterium bifidum*

On used BSM broth and then referred to selective TOS-Propionate Agar acc™ Medium acc. (Sigma-Aldrich, St. Louis, USA), the colonies were convex, creamy, white or grayish, glossy, smooth, neat-edged, soft, sticky. On Lactobacillus MRS Agar™ (Himedia, India), the colonies were dew drop like small flat, distinct brown colored. The findings in this study, primarily suspected as *Bifidobacterium bifidum* fermented D-Galactose, D-Fructose, Amygdaline, D-Ribose, Sucrose, NAG amine, Melebiose but did not ferment L-Arabinose, Maltose, L-Rhamnose, D-Mannitol, D-Sorbitol, Dextrose, Arginine, Melizitose, Xylose, Trehalose, Salicine, Raffinose. We pointed out that MR, VP, Catalase, Citrate reaction, Gram staining performed positive but Oxidase and Indole reactions were negative (Tables 1 and 2).

## 3.2. Molecular detection of probiotic bacteria

We found 186 *Lactobacillus* genus isolates, 102 *Bacillus* genus isolates, 112 *Streptococcus* genus isolates, 220 *Enterococcus* genus isolates and 33 *Bifidobacterium* genus on the basis of biochemical properties like 10% solution of total 22 different sugars. Other biochemical tests like MR-VP, Oxidase, Catalase, Citrate utilization, MIU and Indole test were major. Genus was detected through specific genus target gene and 16S r DNA based PCR. Selected probiotic bacteria species were detected though species specific target gene PCR. We confirmed 23 *L. acidophilus* species isolates, 14 *B. subtilis* isolates, 19 *S. thermophilus* isolates, 31 *E. faecium* isolates and 09 *B. bifidum* isolates at species level.

### 3.2.1. *Lactobacillus* and *Lactobacillus acidophilus*

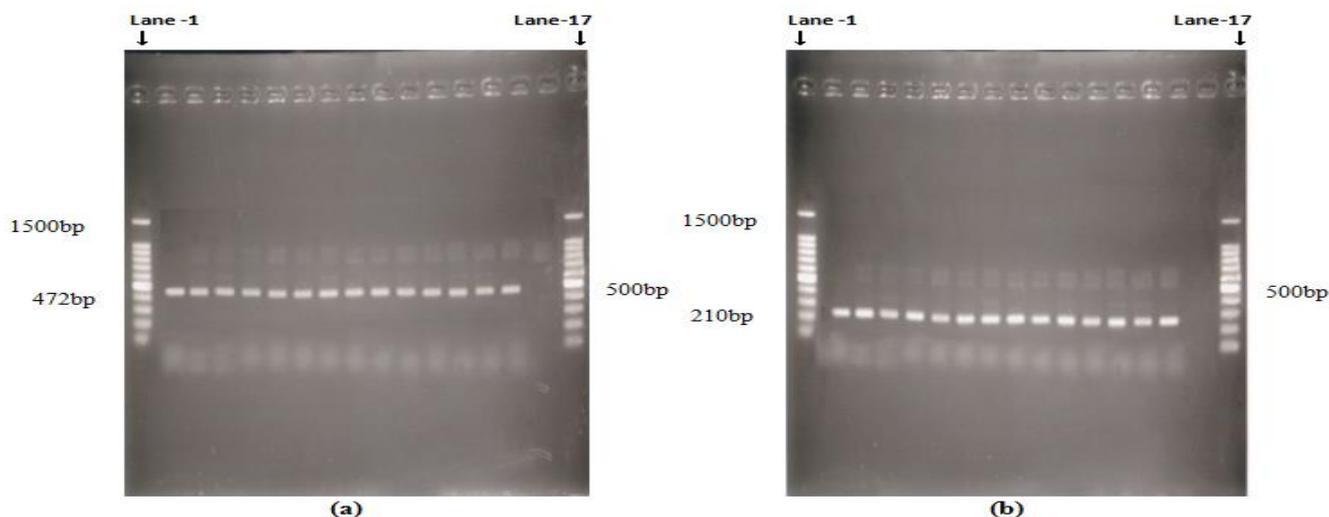
Target 472 bp 16S rRNA gene fragment from all suspected *Lactobacillus* isolates were amplified by PCR, using the primers 16S rRNA (Figure 1a). *Lactobacillus acidophilus* isolates with species specific primers according to Song *et al.*, 2000. A 210 bp target 16S-23S rDNA ISR gene was used to confirm *L. acidophilus* species (Figure 1b).

**Table 1. Results of biochemical tests.**

<i>Lactobacillus</i> spp.	<i>Bacillus</i> spp.	<i>Streptococcus</i> spp.	<i>Bifidobacterium</i> spp.	<i>Enterococcus</i> spp.
D-Galactose (-ve) = 186/750	D-Galactose (-ve) = 102/750	D-Galactose(+ve)= 112/750	D-Galactose(+ve)= 33/750	D-Galactose (+ve) = 220/750
D-Fructose (+ve) = 186/750	D-Fructose (-ve) = 102/750	D-Fructose (+ve) = 112/750	D-Fructose (+ve) = 33/750	D-Fructose (+ve) = 220/750
L-Arabinose (+ve) = 186/750	L-Arabinose (+ve) = 102/750	L-Arabinose (+ve) = 112/750	L-Arabinose(-ve)= 33/750	L-Arabinose (+ve) = 220/750
Maltose (+ve) = 186/750	Maltose (+ve) = 102/750	Maltose (-ve) = 112/750	Maltose (-ve) = 33/750	Maltose (-ve) = 220/750
L-Rhamnose (+ve) = 186/750	L-Rhamnose (-ve) = 102/750	L-Rhamnose (-ve) = 112/750	L-Rhamnose(-ve)=33/750	L-Rhamnose (-ve) = 220/750
D-Mannitol (+ve) = 186/750	D-Mannitol (+ve) = 102/750	D-Mannitol (-ve) = 112/750	D-Mannitol(-ve) = 33/750	D-Mannitol (-ve) = 220/750
D-Sorbitol (-ve) = 186/750	D-Sorbitol (+ve) = 102/750	D-Sorbitol (+ve) = 112/750	D-Sorbitol (-ve) = 33/750	D-Sorbitol (-ve) = 220/750
Methyl-alpha-D-Gluco pyranoside (+ve) = 186/750	Amygdaline (+ve) = 102/750	Amygdaline (-ve) = 112/750	Amygdaline(+ve)=33/750	Amygdaline (+ve) = 220/750
Amygdaline (+ve) = 186/750	Dextrose (+ve) = 102/750	Dextrose (+ve) = 112/750	Dextrose (-ve) = 33/750	Dextrose (+ve) = 220/750
D-Ribose (+ve) = 186/750	D-Ribose (+ve) = 102/750	D-Ribose (+ve) = 112/750	D-Ribose (+ve) = 33/750	D-Ribose (+ve) = 220/750
Melebiose (-ve) = 186/750	Melebiose (+ve) = 102/750	Melebiose (+ve) = 112/750	Melebiose (+ve) = 33/750	Melebiose (+ve) = 220/750
Lactose (+ve) = 186/750	Arginine (-ve) = 102/750	Arginine (-ve) = 112/750	Arginine (-ve) = 33/750	Xylose (+ve) = 220/750
Raffinose (+ve) = 186/750	NAG amine (-ve) = 102/750	NAG amine (-ve) = 112/750	NAG amine(+ve) = 33/750	NAG amine (+ve)= 220/750
Sucrose (+ve) = 186/750	Sucrose (+ve) = 102/750	Sucrose (+ve) = 112/750	Sucrose (+ve) = 33/750	Sucrose (+ve) = 220/750
Melzitose (-ve) = 186/750	Melzitose (-ve) = 186/750	Melzitose (+ve) = 112/750	Melzitose (-ve) = 33/750	Melzitose (+ve) = 220/750
Xylose, Trehalose, Arginine, Salicine, NAG amine, Raffinose (+ve) = 186/750	Lactose, Xylose, Dextrose, Treha lose, Salicine, Raffinose (+ve) = 102/750	Xylose, Trehalose, Salicine, Raffinose (+ve) = 112/750	Xylose, Trehalose, Salicin, Raffinose (-ve) = 33/750	Arginine, Trehalose, Salicine, Raffinose (+ve) = 220/750
Primarily, confirmed as <i>Lacto bacillus acidophilus</i>	Primarily, confirmed as <i>Bacillus subtilis</i>	Primarily, confirmed as <i>Streptococcus thermophilus</i>	Primarily, confirmed as <i>Bifidobacterium bifidum</i>	Primarily, confirmed as <i>Enterococcus faecium</i>

**Table 2. Identification of selected probiotic bacterial species by biochemical properties.**

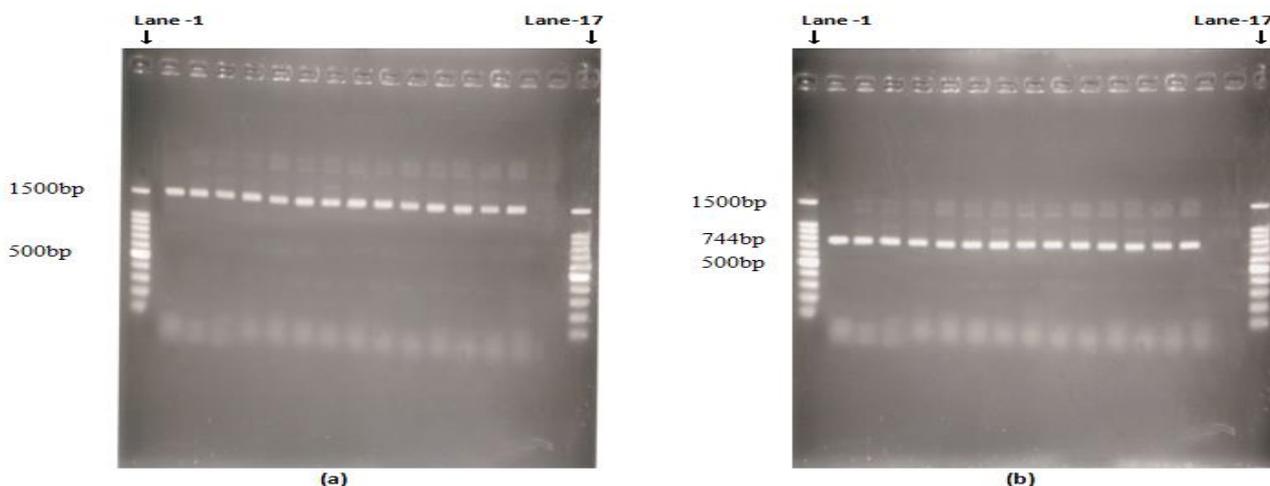
Name of Bacterial species	Voges-Proskauer (VP)	Methyl-Red (MR)	Indole test	Motility Indole Urea (MIU)	Citrate utilization test	Catalase test	Oxidase test
<i>Lactobacillus</i> species (186/750)	+ve	+ve	-ve	-ve	-ve	-ve	-ve
<i>Bacillus</i> species (102/750)	-ve	+ve	-ve	+ve	+ve	+ve	+ve
<i>Streptococcus</i> species (112/750)	-ve	-ve	-ve	-ve	-ve	+ve	-ve
<i>Bifidobacterium</i> species (33/750)	+ve	+ve	-ve	-ve	+ve	+ve	-ve
<i>Enterococcus</i> species (220/750)	-ve	-ve	-ve	-ve	-ve	-ve	+ve



**Figure 1. (a)** Detection of *Lactobacillus* genus by 16S r RNA gene based PCR (Lane-1 & 17= 100bp Ladder DNA and Lane 2 - 15= positive samples & Lane 16= Negative). **(b)** Detection of *Lactobacillus acidophilus* by 16S-23S *ISR* gene based PCR (Lane-1 & 17= 100bp Ladder DNA and Lane 2 - 15= positive samples & Lane 16= Negative).

**3.2.2. Bacillus and Bacillus subtilis**

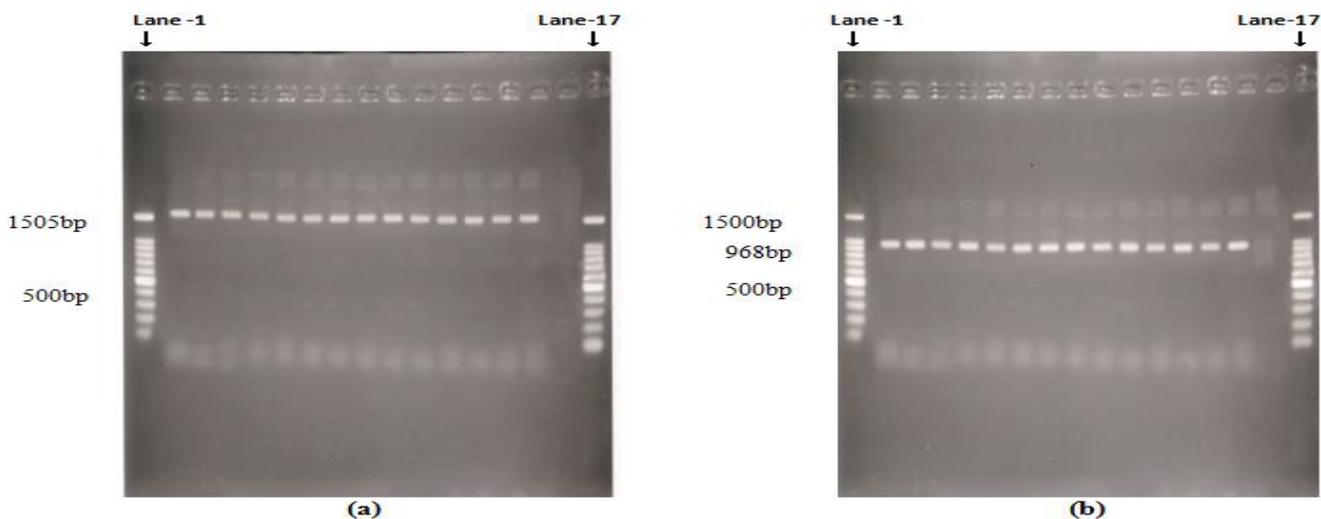
Target PCR band product size was 1.5 kb (1500 kb) in where, 16S rRNA gene from all suspected *Bacillus* isolates were amplified by PCR using 27F forward and 1492R reverse primers sequence (Figure 2a). Target 744 bp *apr-E* genes with FW and REV primers sequence for *B. subtilis* (Figure 2b).



**Figure 2. (a)** Detection of *Bacillus* genus by 16S r RNA gene based PCR (Lane-1 & 17= 100bp Ladder DNA and Lane 2- 15= positive samples & Lane 16= Negative). **(b)** Detection of *Bacillus subtilis* by *apr-E* gene based PCR (Lane-1 & 17= 100bp Ladder DNA and Lane 2- 15= positive samples & Lane 16= Negative).

**3.2.3. Streptococcus and Streptococcus thermophilus**

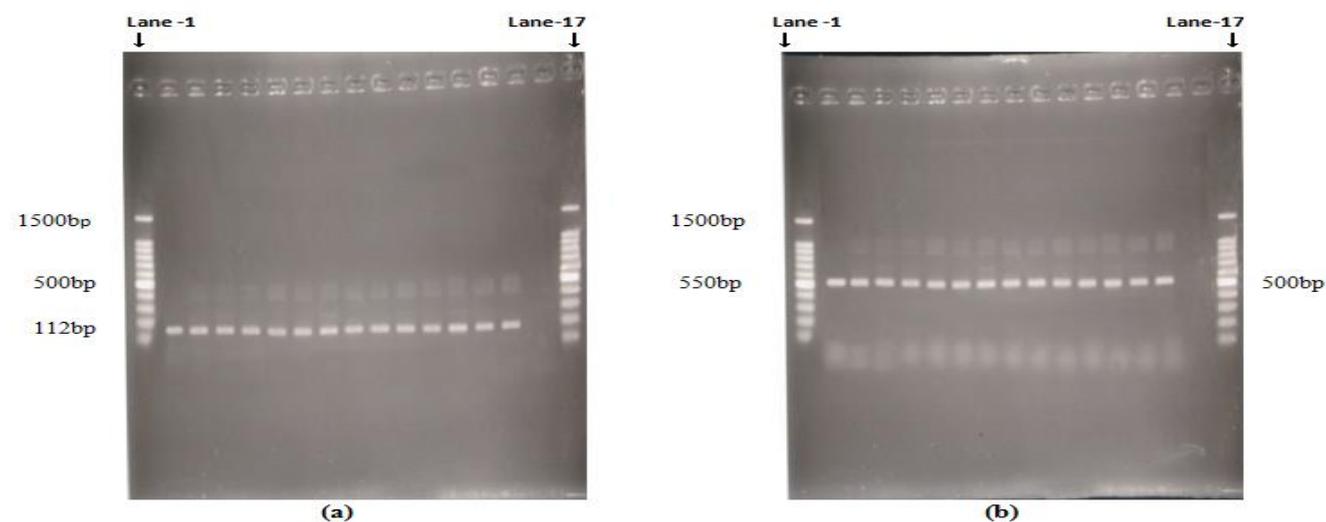
The target amplicon size was 1505 bp with 8UA-FW and 1492-REV primers sequence (Figure 3a) by using primer pair specific to 16s rDNA of the genus *Streptococci*. All isolates of suspected *S. thermophilus*, a fragment 968 bp of Stp-TH-FW and Stp-TH-REV was detected confirming the correction assignment of the species to the genus *Streptococcus* (Figure 3b).



**Figure 3.** (a) Detection of *Streptococcus* genus by 16S r RNA gene based PCR (Lane-1 & 17= 100bp Ladder DNA and Lane 2- 15= positive samples & Lane 16= Negative. (b) Detection of *Streptococcus thermophilus* by *Stp-TH* gene based PCR (Lane-1 & 17= 100bp Ladder DNA and Lane 2-15= positive samples& Lane 16= Negative.

**3.2.4. Enterococcus and Enterococcus faecium**

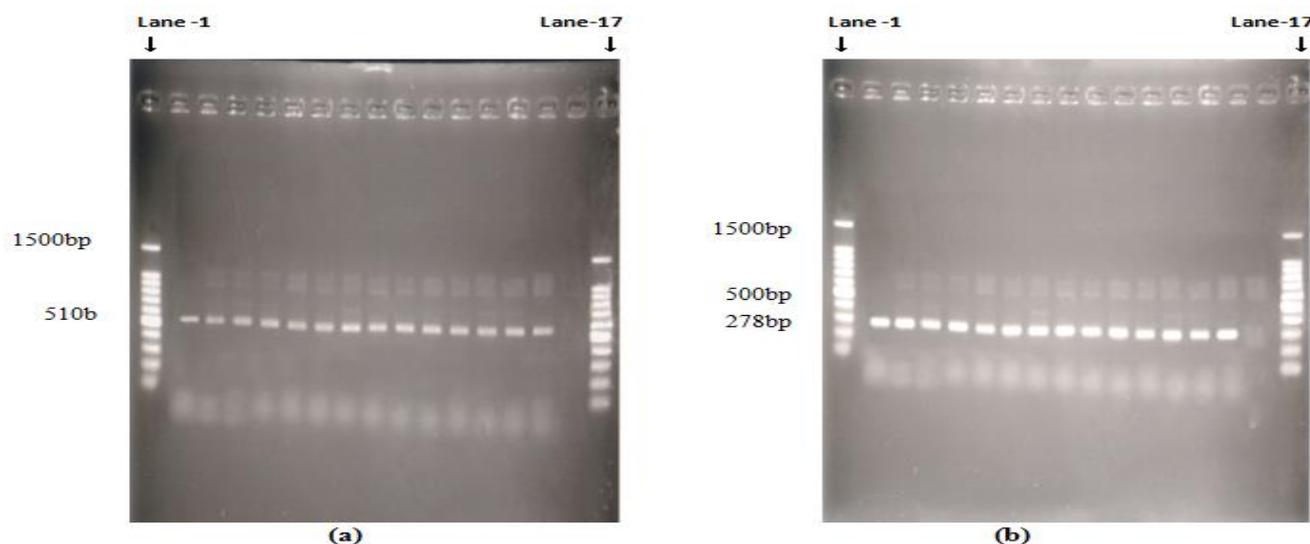
The amplicon size of genus *Enterococcus* was 112 bp with Ent-1 FW, Ent-2 Rev primers targeting the 16S-23S *ISR* rDNA (Figure 4a). Molecular detection of *E. faecium* from suspected isolates was performed targeting a 550 bp *ddl* gene based species specific PCR (Figure 4b).



**Figure 4.** (a) Detection of *Enterococcus* genus by 16S-23S r DNA *ISR* gene based PCR (Lane-1 & 17= 100bp Ladder DNA and Lane 2 - 15= positive samples & Lane 16= Negative. (b) Detection of *Enterococcus faecium* by *ddl* gene based PCR (Lane-1 & 17= 100bp Ladder DNA and Lane 2 - 15= positive samples & Lane 16= Negative.

**3.2.5. Bifidobacterium and Bifidobacterium bifidum**

In where, target PCR band product size was 510 bp 16S rRNA gene from all suspected *Bifidobacterium* isolates and was amplified by using Bif164- forward and Bif662- reverse primers with sequence ( Figure 5a). We targeted a segment of 278 bp *BIBI* gene with FW and REV primers with sequence for detection of *B. bifidum* (Figure 5b).



**Figure 5. (a)** Detection of *Bifidobacterium* genus by 16S r RNA gene based PCR (Lane-1 & 17= 100bp Ladder DNA and Lane 2-15 = positive samples & Lane 16= Negative. **(b)** Detection of *Bifidobacterium bifidum* by BIBI gene based PCR (Lane-1 & 17= 100bp Ladder DNA and Lane 2 – 15 = positive samples & Lane 16= Negative.

#### 4. Discussion

Carbohydrate tests produced a yellow color for a positive result and a red or red-orange color for a negative result agreeing to Albert and Anicet (1999). All these results were found relevant to the findings of Kamrun *et al.*, 2016. On this study, we also observed that *Lactobacillus* species especially in case of MR, Indole, Oxidase, Citrate test *Lactobacillus acidophilus* species showed negative result. But positive reaction in case of VP test which is very similar to other research reports of many research workers on *Lactobacillus acidophilus* species. According to Parvaneh *et al.* (2016) D-Fructose, D-Galactose, L-Rhamnose, D-Mannitol, D-Sorbose, D-Ribose, Lactose, Raffinose, VP, Catalase showed negative result in case of *L. acidophilus* species which was not completely match with our study result. We had established our accuracy through PCR techniques at genus and species level which they did not do it, they only perform biochemical profiles. According to many researchers, this difference may due to variation in source of origin. Then all isolates were examined under bright field microscope to observe their microscopic features. These isolates were found gram positive, short and medium rod shaped, non-spore forming bacterium which indicates them to be member of *Lactobacillus* species (Thamaraj and Shah, 2003). The isolates were found catalase and oxidase negative and in IMViC (indole, methyl-red, voges proskauer, citrate utilization) tests all isolates were also found negative, thereby these might confirm the isolates were *Lactobacillus* species (Dhanasekaran *et al.*, 2010). It differed from our study result. According to Narwade *et al.* (2015) *Lactobacillus acidophilus* species are non motile, indole test negative, methyl red test negative, voges proskauer positive, citrate negative, catalase negative, yellow colored colonies, size < 1mm, round shape, Gram's staining positive. Finally, our study results were compared with Bergey's manual of Bacteriology & isolates were characterized as *Lactobacillus* species and specifically as *L. acidophilus* species. Some biochemical tests such as catalase test, oxidase test, indole test, Methyl Red (MR) test, Voges Proskauer (VP) test, citrate utilization test and carbohydrate fermentation patterns were performed as delineated by Bergey's manual systematic bacteriology to identify the *Lactobacillus* species (Hensyl, 1994). Biochemical tests, such as sugar fermentation, coagulase, catalase, MR, VP, and indole tests, were performed as per the standard methods (Cheesbrough, 1985). Gram's staining properties included the cylindrical rods, straight / slightly curved with rounded ends in case of long chain, presented in single or in chain. Spores are arranged in pair. Colony morphology on Nutrient Agar showed small, grayish and amoeboid with crenate margin and wool like edge. The surface was finely granular and somewhat dry with a ground glass appearance. The growth was membranous and slightly sticky. On Bacillus Medium™ and Bacillus Differentiation Agar™ (Himedia, India), *Bacillus subtilis* formed large, flat distinct, blue colonies. On later, from others biochemical tests and PCR technique, we were confirmed about those blue colored colonies which was *Bacillus subtilis*. Merchant and Packer (1967) reported that *Bacillus* species were Gram positive, cylindrical rods, straight or somewhat curved with rounded ends, present in singly or in chain. They stated that *Bacillus* species were on any nutrient agar medium was small, grayish with wool like edge. The surface was finely granular and slightly sticky, large, sporulated, rod-shaped bacteria which indicated very close to our observation. This study report is very similar

to O'Donnell *et al.* (1980) though in case of Rhamnose, Galactose, N-Acetyl-Glucose-Amine, VP we observed negative result but he found variable. Granum (2001) reported that *Bacillus* species can ferment dextrose, maltose, lactose, sucrose and mannitol with production of acid. They are positive for Voges-Proskauer and methyl red test. On Nutrient Agar, colonies were thick, grayish-white or cream colored. Gram's staining showed positive, rod with square ends, large rod shaped organisms, arranged in chain and morphology and staining characteristics of *B. subtilis* recorded in this study are in agreement with the finding of Jones *et al.* (1987). According to Honda *et al.* (1982) all the isolates of *B. subtilis* fermented dextrose, sucrose, lactose, maltose but did not ferment mannitol with the production of acid within 24 h-48 h of incubation and reported positive reaction in VP test, negative reaction in MR and Indole test which are dissimilar to our study report. We also found maximum of all these species as ellipsoidal, sub terminal, endospore formed, motile and in case of other results not only MR, Catalase, Oxidase, Citrate, ONPG, VP, Indole but also maximum 10% carbohydrate sugar fermentation results are supported by Logan and Berkeley (1984) in where exceptions occurred in Fructose, Raffinose. Our study observation is also supported by Svetlana and Jain (2010). Bacterial identification was conducted based on morphological, physiological and biochemical tests and the results were compared with Bergey's Manual of Determinative Bacteriology, 8th edition (Buchanan and Gibbons, 1974) and The Genus Bacillus: Agriculture Handbook No. 427. The findings in this study were in accordance to the study of Devriese *et al.* (1987) where in L-Arabinose showed negative result and Raffinose, L-Rhamnose, Melizitose, Xylose showed variable positive and negative result though which is dissimilar to our study reports but the other results including these species as non-motile are same to our study reports. In Blood Agar, the colonies were small translucent with no haemolysis. In Hicrome™ Enterococcus Faecium Agar Base (Himedia, India), the colonies were luxuriant, distinct, green-colored in case of *E. faecium*. According to Alber and Anicet (1999) D-Galactose, D-Fructose, Arginine, L-Arabinose, Amygdaline, Dextrose, D-Ribose, Melebiose, Xylose, NAG amine, Sucrose, Melizitose, Trehalose, Salicine, Raffinose and Oxidase reaction showed positive results in all of these sugar fermentation with a report of non-motile but performed negative results not only in case of Rhamnose, Sorbitol, Melizitose but also in case of MR, VP, Indole, Catalase, Citrate, ONPG reaction which are similar to our study results. But those of the fermentation results of Maltose, Raffinose are not same to our results. Rhamnose, Raffinose and Xylose fermentation results were variable (i.e, both positive and negative), reported by Schleifer *et al.* (1984) and Collins *et al.* (1986) which are not alike with our study results. Members of the genus *Enterococcus* include gram positive, facultatively anaerobic cocci that are ovoid in form and can occur in singlet, pairs or short chains (Facklam, 2002) which were accordance to our study results. *Enterococcus* species are homo fermentative lactic acid bacteria that lack cytochrome enzymes. In biochemical screens, *Enterococcus* species normally exhibit catalase-negative properties; yet some strains produce pseudocatalase and can appear to be weakly catalase positive (Murray, 1990). Other studies from many researchers pointed out that *E. faecium* from faecal samples and other sources exhibited similar results in biochemical reactions like acid production from arabinose, lactose, adonitol, sucrose and arginine dihydrolase and minor difference in the biochemical tests like mannitol, sorbitol and raffinose. According to Pelinescu *et al.* (2009), L-Arabinose, Starch, Glycogen showed positive result but Sorbitol and Raffinose showed negative result. Bridge and Sneath (1983) mentioned variable positive and negative result in case of Raffinose, D-Ribose, Melizitose, Maltose, Sorbitol, Mannitol, Galactose, Glycerol, Trehalose, Xylose, Arginine fermentation, VP but stated positive result in Lactose, Fructose, Starch fermentation, Catalase test and negative result in Rhamnose, Arabinose, Salicin, Amygdaline, NAG amine fermentation, MR, Indole, Oxidase, Motility Indole Urea test, Citrate test. In Blood Agar, Gamma haemolysis was variable responded in case of *S. thermophilus*. Holt *et al.* (1994) pointed out that Arabinose, Salicin, Maltose, Mannitol, Glycerol, Xylose fermentation were negative and Sucrose, Lactose showed positive result but not detected in Raffinose, Trehalose fermentation, VP reaction. Wasilewska *et al.* (2003) concluded that among 100 samples 90 samples found positive to Galactose, Lactose, Fructose, 50 samples found positive to Melibiose, Starch, Sucrose and every 10 samples found positive to Arabinose, Ribose, Maltose, Mannitol, Sorbitol, Salicine, Xylose, Lactose, Trehalose, Starch, Melizitose, Raffinose. They also concluded that though MR, VP, Catalase, Citrate reaction were positive but Indole, Oxidase reaction were negative. A conclusion from Iwana *et al.* (1993) prevailed that acid production by carbohydrate fermentation was detected by a change of the color of the phenol red indicator from red to yellow on the 4<sup>th</sup> day and 10<sup>th</sup> day of incubation. They also distinguished clearly that *B. animalis* and *B. longum* showed positive in case of Arabinose, xylose, Raffinose but *B. bifidum* showed negative result in our study. *B. animalis* and *B. longum* showed negative reaction in case of Ribose fermentation but we found it as a positive one. Fatima *et al.* (2013) marked that Maltose, Lactose, Galactose, Fructose, Melibiose, Sucrose, Salicine, Glucose fermentation, Citrate reaction was positive but Melizitose, Raffinose, Glycerol, Mannose, Rhamnose, Arabinose, Xylose, Trehalose, Ribose, Mannitol fermentation, Catalase, Oxidase, Indole reaction were negative. They commented colony

morphology as white, cream spatulate extremities, rod cell morphology. Gram's reaction was positive but non spore formed. In original description of Bifidobacteria, Tissier (1889) described curved rods and rods with ends split to give the characteristic "Y" shape which led to the designation of "bifid." In addition, bizarre small branches and bulbous or swollen ends were seen less frequently. Scardovi (1986) commented these bacteria are gram-positive pleomorphic, non-spore-forming, and non-motile, anaerobic bacteria with spatulate extremities (Cronin *et al.*, 2011). Colonies are Gram positive, characterized by various forms, but often "bifid" forms (Scardovi, 1986) that are typical of Bifidobacteria. All the isolates were catalase and oxidase negative; these features have already been mentioned by several authors. Ewa *et al.* (2003) carried out studies proved that Bifidobacteria species differ significantly in morphology and biochemical activity. Among 750 our study samples, though we found 186 samples of *Lactobacillus* species positive through carbohydrate fermentation, biochemical properties, colony characteristics, cell morphology in distinguished media, Gram's staining and Polymerase Chain Reaction analysis but we confirmed about 23 samples that were *L. acidophilus* species with carbohydrate fermentation, biochemical properties and mostly trustworthy target gene based PCR. Identification of the *Lactobacillus* isolates with genus specific primers designed by Magne *et al.* (2006). As we have demonstrated here, amplification of the spacer regions by PCR can be carried out with suspensions of whole *Lactobacillus* cells, so colonies picked from agar plates can be used directly in identification of an isolate. For the majority of our *Lactobacillus* isolates, clear species identification could be made on the basis of percent similarity to GenBank sequences (97.5 to 100.0% similarity). The use of 16S-23S spacer sequences in the identification of *Lactobacilli* promises to be a valuable aid in advancing our knowledge of the species composition of *Lactobacillus* populations (Tannock *et al.*, 1999). Kamrun *et al.* (2016) used a genus specific primer prepared by analyzing similarities between the nucleotide sequences of the spacer region between the 16 and 23S ribosomal RNA genes of *Lactobacillus*. The specificity of this genus specific primer combined with a universal primer was tested against 23 strains of *Lactobacillus* of varied origin. An expected sharp band of 200 bp amplicon was found for each sample which was corresponding to the 16-23S rRNA intergenic spacer region of *Lactobacillus* species. Thus all isolates were confirmed at genus level as *Lactobacillus* species. Amit *et al.* (2004) reported that Langendijk *et al.* (1995) also had supported the PCR-based techniques targeting the bacterial 16S rDNA to identify different bacterial species in fecal samples and others. However, the simultaneous use of universal primers directed at overall bacteria DNA had allowed them to extend this technique to estimate the relative proportions of different species present in the intestinal lumen of chickens. Recent advances in ribosomal DNA-based molecular techniques make it possible to identify different bacterial populations in environmental samples without cultivation. In this study, the intestinal microflora was examined using 16S ribosomal DNA (rDNA) targeted probes from bacterial DNA isolated from intestinal and cecal contents of chickens at 4, 14, and 25 d of age. Massi *et al.* (2004) used the primers 16-1A and 23-1B to amplify the ribosomal fragments comprising the internal transcribed spacers (ITS) and the flanking 16S and 23S rDNA regions of all *Lactobacillus* strains and in case of *L. acidophilus* the amplicon size was 199 bp. Chen *et al.* (2007) had commented that PCR amplification of the 16S–23S rRNA gene intergenic spacer region (IGSR) was found to be a useful tool for bacterial species-specific typing because of the considerable variability in size and sequence among organisms. Although combined biochemical and genetic analysis allowed the identification of the isolates to the genus level as *Bacillus* species, the API results and 16S rRNA sequences were not always in complete agreement (Teresa, 2005). Among 750 our study samples though we found 102 samples of *Bacillus* species positive through carbohydrate fermentation, biochemical properties, colony characteristics, cell morphology in distinguished media, Gram's staining and Polymerase Chain Reaction analysis but we confirmed about 14 samples that were *Bacillus subtilis* species with carbohydrate fermentation, biochemical properties and mostly credible target gene based PCR. We have followed detection of *Bacillus* isolates with genus specific primers described by Kim *et al.* (2010). This study finding is similar to Barbosa *et al.* (2005). However, some of the *Bacillus* strains almost share equal 16S rRNA gene sequences (99.2–99.6 %) which usually illustrate limited diversity for members of closely related strains (Nakamura *et al.*, 1999). To overcome the above-mentioned obstacles, sequence analysis of housekeeping protein-coding genes and the multilocus sequence analysis (MLSA) have also been reported to be a helpful alternative in discriminating between closely related species (Kwon *et al.*, 2009; Glazunova *et al.*, 2009). Bacterial identification and distinction of these species using 16S rDNA sequencing also has major drawbacks. Analysis of 16S rRNA gene sequences could not be used to distinguish *B. subtilis* and *B. amyloliquefaciens* because sequence similarities greater than 99% were found (Ash *et al.*, 1991; Nakamura *et al.*, 1999). Alternative phylogenetic markers have recently been described for differentiating closely related *Bacillus* species using protein-encoding genes housekeeping protein-coding genes like *apr-E* genes, *rpoB*, *gyrA*, *gyrB*, *vrroA* genes (Ki *et al.*, 2009). Isolates of *Bacillus subtilis* detection at species level was carried out based on housekeeping protein-coding *apr-E* genes according to Alireza *et al.*, 2012. Final

PCR product was loaded in 1.5% agarose gels with BenchTop™ 100 bp DNA Ladder (consists of 11 double-stranded DNA fragments with sizes from 100-1500 bp). These limitations for identification of species of *Bacillus* were discussed in detail previously (Van 2011) and the 16S rRNA genes sequences of species within the *B. subtilis* group were found to be too highly conserved to enable discrimination among species and subspecies (Shaver *et al.* 2002). This restriction prompted the search for and development of alternative conserved genetic markers having a greater degree of variability among strains compared with the 16S rRNA gene. The use of essential housekeeping genes that evolve more quickly than the 16S rDNA gene have proven to be useful for taxonomic classification (Palys *et al.*, 2000). Sequence variations in the 16S rRNA genes may exist between some *Bacillus* strains within species and several *Bacillus* species were reclassified based on alignment of these sequences (Wu *et al.*, 2006). However, the presence of highly conserved sequences in the 16S rRNA gene does not permit the discrimination among some species and subspecies of this group (Shaver *et al.* 2002). This observation was also true for the 10 reference strains of *B. subtilis* group in the present study that clustered together on 16S rRNA gene sequence analysis (Catia *et al.*, 2008). We pointed out 220 samples of *Enterococcus* species positive among total 750 study samples, through carbohydrate fermentation, biochemical properties, colony characteristics, cell morphology in distinguished media, Gram's staining and Polymerase Chain Reaction analysis but we confirmed about 31 samples that were *Enterococcus faecium* species with carbohydrate fermentation, biochemical properties and mostly authorized target gene based PCR. The amplicon size of genus of *Enterococcus* was targeting the 16S-23S *ISR* rDNA according to Ke *et al.* (1999). Molecular detection of *E. faecium* from suspected isolates were performed according to Dukta-Malen *et al.* (1995) targeting *ddl* gene species specific PCR. We used genus and species specific authenticated target gene from mostly précised previous works of many researchers for molecular detection. We also demarcated 112 samples of *Streptococcus* genus positive through carbohydrate fermentation, biochemical properties, colony characteristics, cell morphology in distinguished media, Gram's staining and Polymerase Chain Reaction analysis but we confirmed about 19 samples that were *S. thermophilus* species with carbohydrate fermentation, biochemical properties and mostly trustworthy target gene based PCR. Molecular detection of *Streptococcus* genus using PCR, primers pair specific to 16S rDNA specific to *Streptococcus* was performed according to Sato *et al.* (2003). The target amplicon size which is similar to Rabeah *et al.* (2008). In where, the detection of the genus *Streptococci* was performed by using primer pair specific to 16S rDNA of the genus *Streptococci*. The positive amplified PCR product (1505 bp size), representing *Streptococcus* genus, was detected. All isolates of suspected *Streptococcus thermophilus*, species-specific primers Stp-TH-FW and Stp-TH-REV were selected for confirming the correction assignation of the species to the genus *Streptococcus*. *Streptococcus thermophilus* species were isolated from the all samples according to Jeanne *et al.* (2005). We delineated 33 samples of *Bifidobacterium* species positive through carbohydrate fermentation, biochemical properties, colony characteristics, cell morphology in distinguished media, Gram's staining and Polymerase Chain Reaction analysis but we resolved about 09 samples that were *B. bifidum* species with carbohydrate fermentation, biochemical properties and mostly authenticated target gene based PCR. *Bifidobacterium* isolates with genus specific primers have described by Langendijk *et al.* (1995). In where, target gene from all suspected *Bifidobacterium* isolates were amplified by PCR using Bif164 forward and Bif662 reverse primers with sequence for *Bifidobacterium* genus identification. Delcenserie *et al.* (2005) reported that most of these molecular methods have been applied to detect *Bifidobacterium* species in human feces, rather than in the detection of Bifidobacteria of animal origin. Moreover, the 16S rRNA sequences are well conserved among the Bifidobacteria and there are multiple copies of the 16S rRNA gene per chromosome. The *hsp60* gene, has been sequenced in most *Bifidobacterium* species (Jian and Dong, 2002). This gene presents species-specific sequences. Several molecular methods that alleviate this inconvenience have recently been described: PCR targeting the 16S rRNA (Bonjoch *et al.*, 2004). Delcenserie *et al.* (2005) commented that four possibilities of results they observed (i) Only the target was positive (217 bp fragment), (ii) The target (217 bp) and the internal control (315 bp fragment) were positive. In these two cases, PCR was considered as positive for Bifidobacteria detection. Isolates of *Bifidobacterium bifidum* detection at species level was carried out according to Matsuki *et al.* (1998). Target segment of *BIFI* gene with FW and REV primers with sequence was detected and PCR product was loaded in 2% agarose gels with BenchTop 100 bp DNA Ladder (consists of 11 double-stranded DNA fragments with sizes from 100-1500 bp, Promega, Madison, WI, USA) and ran at 80 Volt for 25 minutes.

## 5. Conclusions

We used reference strains to cross check out our studied five bacterial species on context of carbohydrate fermentation, biochemical properties, colony characteristics, cell morphology in distinguished media, Gram's staining and Polymerase Chain Reaction analysis. The significance of this study is at first time in Bangladesh,

we isolated and identified five selected potential probiotic bacteria species which belongs to five genera at a repercussion, from the gastro-intestinal tract of broiler chickens and their environmental sources like litter, feeder and water drinker by linkage not only traditional cultural, biochemical techniques but also modern genus and species specific trustworthy target gene based molecular detection (PCR).

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### Conflict of interest

None to declare.

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