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Detection and characterization of *Bifidobacteria* from indigenous poultry and their *in vitro* evaluation as probiotic

Md. Abu Taher[1, 3]*, Md. Mahabub Raihan[1], Md. Firoz Ahmed[1], Mir Himayet Kabir[1], Ashish Kumar Das[2] and M.M.R. Khalil[2]

[1]*Department of Microbiology, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh.*
[2]*International Centre for Diarrhoeal Disease Research, Bangladesh, Mohakhali, Dhaka-1212, Bangladesh.*
[3]*Incepta Pharmaceuticals Ltd., Dewan Idris Road, Bara Rangamatia, Zirabo, Savar, Dhaka-1341, Bangladesh.*

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Corresponding Author:

Md. Abu Taher
E-mail: mataher37@gmail.com
Phone: +880-1812- 668571

ABSTRACT

Probiotics are live microorganisms which when ingested or locally applied in sufficient numbers confer one or more health benefits for the host. Recently, growing application of *Bifidobacteria* as probiotic in many food industries has prompted the researchers to screen for even better isolates. In this study, *Bifidobacteria* were isolated from indigenous chicken and evaluate these isolated for their potential use as probiotic. A total of 230 organisms were isolated from fecal materials of chickens and ducks. Preliminary identification of the *Bifidobacteria* was carried out on selective MRS agar medium and 30 isolates were confirmed as *Bifidobacteria* by biochemical tests. Antimicrobial susceptibility, antibacterial activity, haemolytic activity and acid tolerance of the isolated organisms were tested to determine the suitability of these isolates as probiotic. Among the isolates 36.67% were found to be resistant to at least four tested drugs of them, C2(2), C6(6), C10(6), C11(3), D6(1) and D7(6) were resistant to five antibiotics; C7(7) was resistant to six antibiotics and D3(6) was resistant to seven antibiotics. Higher resistance was recorded against commonly used penicillin (60%). The isolates of *Bifidobacteria* showed good antibacterial activity against *Escherichia coli* (43.33%) and *Enterococcus faecalis* (36.67%) compared to other potent intestinal pathogens (<15%) namely *Vibrio cholerae, Salmonella typhi* and *Shigella dysenteriae*. The isolates were non haemolytic and were able to grow at pH 2.0. Thus three isolates C7(7), C9(4) and D3(6) can be used as probiotics in the food, feed and dairy industries. However, further studies are needed to characterize these probiotic *Bifidobacteria* in order to use in *in vivo* application.

Introduction

Probiotics have been described as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” (Fuller, 1989). Typically, probiotics are associated with two main genera of lactic acid bacteria: *Lactobacillus* & *Bifidobacterium* and the less common probiotic bacteria include *Leuconostoc, Propionibacterium, and Bacillus* (Fuller, 1992). They are helpful in maintaining a proper balance in the intestinal flora, playing a protective role against potential pathogens and putrefactive bacteria; hence they have been included in the probiotics group (Biavati et al., 2000).

The genus *Bifidobacterium* can generally be characterized as gram-positive, pleomorphic with V and Y shape, non-spore forming and non-motile anaerobes that are catalase-negative and saccharolytic (Scardovi, 1986). These bacteria play an important role in maintaining human health. They suppress harmful bacteria by controlling the pH of the large intestine (Gibson and Wang, 1994). *Bifidobacteria* have anticarcinogenic (Reddy & Rivenson, 1993), anticholesterolemic (Pereira & Gibson, 2002) and immune system activation effects (Mitsuoka, 1992). Other effects that have been ascribed to this genus are alleviation of lactose intolerance and vitamin production (Fooks et al., 1999). In recent years, the growing interest for application of *Bifidobacterium* in many fermented dairy foods has prompted starter industry to screen for new isolates from culture collections or human colonic flora (Mayer et al., 2007).

Methods to accurately and rapidly identify these microorganisms remain largely insufficient and it is difficult to discriminate among different *Bifidobacterium* species (Youn et al., 2008). Genus *Bifidobacterium* can be distinguished from other bacterial groups by exhibiting the activity of fructose-6-phosphate phosphoketolase (EC 4.1.2.22; F6PPK), a key enzyme in bifidobacterial metabolic pathway (Scardovi, 1986). Biochemical tests for the identification of *Bifidobacteria* are now largely superseded by the use of the genus-specific (Kok et al., 1996) and species-specific PCR primers (Matsuki et al., 1999; Ventura, 2001). Several methods have been used to identify *Bifidobacteria* namely carbohydrate fermentation and enzymatic tests (Gavini et al., 1991; Crociani et al., 1994), rDNA restriction patterns (Mangin et al., 1994), amplified ribosomal DNA restriction analysis (Langendijk et al., 1995), denaturing gradient gel analysis of amplified 16S rDNA, oligonucleotide probes (Kaufmann et al., 1997), recA (Kullen et al., 1997) and 16S rDNA targeted PCR (Matsuki et al., 1999; Ventura, 2001).

However, studies relating to animal/poultry probiotics are relatively scarce despite their widespread use especially in poultry industries (Patterson et al., 2003). Even in Bangladesh, the use of probiotics in poultry is gradually being increased. But surprisingly, there is no local probiotic for the huge poultry industry in Bangladesh. Here, we assume the Bangladeshi indigenous poultry as the potential source of probiotic bacteria considering their natural resistance to gastrointestinal tract (GIT) infection. Appreciably, it could be assumed that the indigenous poultry is more resistant to the diseases of GIT. They are scavenging here and there, taking feeds from natural

sources, even from rotten materials and animal feces but rarely getting GIT infection. These give the idea that they may naturally possess more beneficial bacteria (i.e. probiotic bacteria) in their GIT than the other commercial poultry. In the light of these apparent findings, this is rational to assume that the indigenous poultry could be a potential source of probiotic bacteria which upon isolation and identification might be used for commercial implementation. If probiotic could be developed from our native poultry, this is obviously would save the huge amount of money that is spent every year for the importation of such materials. Therefore, objective of this study was to isolate *Bifidobacteria* from indigenous chicken and evaluate these isolated for their potential use as probiotics.

Materials and methods

Specimen collection and preservation

Indigenous chickens including Naked Neck and Hilly and ducks namely Ginding were used in the study. The fecal material from ceca was collected aseptically in sterile eppendorf tubes by operating the upper end of cecum and stored at -20 °C until further study.

Isolation of potential *Bifidobacterium* isolates

Sample processing and inoculum preparation
Faecal samples stored at -20 °C were allowed 20 minutes for thawing. Next, 50 mg of feces was weighed in a sterile eppendorf tube and 450 µl phosphate buffer saline (PBS) was added to make a tenfold dilution and subsequently diluted to 10^{-2}, 10^{-3} and so on.

Selective inoculation on MRS agar medium
A loopful of the diluted sample was streaked on petri plates containing MRS (De Man, Rogosa and Sharpe) agar (Difco, USA) medium and incubated at 37 °C for 24 h in CO_2 incubator to maintain anaerobic condition. Circular, white, slimy colonies were streaked further on MRS agar medium to obtain pure culture of presumed *Bifidobacterium* strains.

Identification of *Bifidobacteria* isolates

Gram staining and phenotypic characterization
A thin and uniform smear of circular white and slimy colony from MRS plate was prepared on microscopic slide. The slide was heat fixed and allowed for cooling. Then the slide was flooded with crystal violet, kept for 1 minute and rinsed with water. Next, the slide was flooded with gram's iodine, kept for 1 minute then rinsed with water. Gently decolorized with acetone alcohol for 20 seconds and rinsed with water. Finally the slide was flooded with counter stain safranin and kept for 30-60 seconds and rinsed with well water, gently blotted and allowed to dry. Finally the slide was viewed under light microscope (Olympus, Japan) using immersion oil.

Catalase test
A clean glass slide was divided into two sections. One section was labeled as "test" and the other as "control". A small drop of normal saline was placed on each area and a small amount of the culture was picked up from MRS agar plate and emulsified to make a smooth suspension. With a micro-pipette, one drop of hydrogen peroxide (3%) was placed over the test smear. The fluid over the smears was observed for the appearance of gas bubbles.

Carbohydrate fermentation test
Solutions of phenol red (0.1%), peptone water (1.5%), and sugars (1%) including glucose, sucrose, lactose, galactose, maltose and mannitol were prepared and sterilized at 115 °C for 15 minutes. Each properly labeled screw capped tube was dispensed with 1.5 ml of each of the sugar solutions, 3.5 ml of the peptone water and two drops of phenol red. Each tube was inoculated aseptically with gram positive and catalase negative isolates. Durham tubes were inserted at inverted position into all tubes to observe gas production. The tubes were incubated at 24 hours at 37 °C under anaerobic condition.

Determination of probiotic properties of the isolated *Bifidobacteria* strains

Antimicrobial susceptibility
Susceptibility test was performed using disc diffusion according to the guidelines of the EUCAST (EUCAST, February 2012) with necessary modifications. Commercially available antibiotics discs (Oxoid, England) were used for the test. The antibiotic used in this study (with their potency) were Ampicillin (10 µg), Azithromycin (15 µg), Ceftriaxone (30 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Penicillin (10 µg).

Antibacterial activity
Antibacterial activity of *Bifidobacteria* strains were tested against gram-positive *Staphylococcus aureus* and Gram-negative including *Escherichia coli*, *Enterococcus faecalis, Vibrio cholerae, Salmonella typhi* and *Shigella dysenteriae* (Identified in Bacteriology Laboratory of BLRI) for their potential inhibitory activity with the well diffusion assay.

Haemolytic activity
500 ml of distilled water was weighed using a measuring cylinder. The distilled water transferred into a 1 litre conical flask. 20g of Blood agar base (Difco, USA) was weighed using a weighing balance. The measured blood agar base was suspend into the 500 ml of distilled water and mixed thoroughly and finally autoclaved at 121°C for 15 minutes. The autoclaved blood agar was allowed to cool to 45-50 °C and then aseptically 25 ml (5%) of sterile sheep blood was added and mixed thoroughly. The Blood agar was poured on petri plates and allowed for solidification. Each bacterial suspension was streaked on the Blood agar plates and incubated at 37 °C for 24 h under anaerobic condition and the plates were examined for sign haemolysis.

Acid tolerance
The tolerance of the strains to simulated gastric juices (10 ml of phosphate buffer saline adjusted to pH 2, 3, 4, and 5 with 1 N HCl) was tested. Stationary phase cells grown in MRS broth under anaerobic atmosphere at 37 °C were harvested by centrifugation (at 6000 rpm for 5 min) then suspended in phosphate buffer saline and washed. Then the washed cell suspension was harvested by high speed centrifugation (at 6000 rpm for 5 min) and cultures were inoculated in 10 ml of PBS adjusted to pH 2.0, 3.0, 4.0, and 5.0 with 1 N HCl.

Samples were incubated at 37 °C for 3 h under anaerobic atmosphere. Cells were then serially diluted to 10-fold dilution by phosphate buffer with pH 7.0. The dilution was plated on MRS agar and incubated at 37 °C under anaerobic conditions for determination of viable cells after 48 h of incubation. Isolates which grown on the agar were considered to be acid tolerant strains. The survival rate was calculated as the percentage of colonies grown on MRS agar compared to the initial cell concentration.

Table 1. Results of phenotypic characterization, catalase test and carbohydrate fermentation test for the identification of *Bifidobacteria*.

Sample ID	Gram Reaction	Cell Morphology	Catalase Test	Gas production	Acid Production
C1(4)	+	Rod with V and Y shape	-	-	+
C1(7)	+	Rod with V and Y shape	-	-	+
C2(2)	+	Rod with V and Y shape	-	-	+
C2(3)	+	Rod with V and Y shape	-	-	+
C5(2)	+	Rod with V and Y shape	-	-	+
C5(7)	+	Rod with V and Y shape	-	-	+
C6(3)	+	Rod with V and Y shape	-	-	+
C6(6)	+	Rod with V and Y shape	-	-	+
C7(2)	+	Rod with V and Y shape	-	-	+
C7(7)	+	Rod with V and Y shape	-	-	+
C8(4)	+	Rod with V and Y shape	-	-	+
C8(5)	+	Rod with V and Y shape	-	-	+
C9(1)	+	Rod with V and Y shape	-	-	+
C9(4)	+	Rod with V and Y shape	-	-	+
C10(6)	+	Rod with V and Y shape	-	-	+
C10(7)	+	Rod with V and Y shape	-	-	+
C11(1)	+	Rod with V and Y shape	-	-	+
C11(3)	+	Rod with V and Y shape	-	-	+
D1(1)	+	Rod with V and Y shape	-	-	+
D1(2)	+	Rod with V and Y shape	-	-	+
D3(3)	+	Rod with V and Y shape	-	-	+
D3(6)	+	Rod with V and Y shape	-	-	+
D4(7)	+	Rod with V and Y shape	-	-	+
D4(8)	+	Rod with V and Y shape	-	-	+
D5(5)	+	Rod with V and Y shape	-	-	+
D5(6)	+	Rod with V and Y shape	-	-	+
D6(1)	+	Rod with V and Y shape	-	-	+
D6(2)	+	Rod with V and Y shape	-	-	+
D7(2)	+	Rod with V and Y shape	-	-	+
D7(6)	+	Rod with V and Y shape	-	-	+

Table 2. Result of carbohydrate fermentation test by isolates of *Bifidobacteria.*

Sample ID	Sugar fermentation test					
	Dextrose	Maltose	Lactose	Galactose	Sucrose	Manitol
C1(4)	+	+	+	+	+	-
C1(7)	+	+	+	+	+	+
C2(2)	+	+	+	+	+	+
C2(3)	+	+	+	+	+	-
C5(2)	+	+	+	+	-	+
C5(7)	+	-	+	+	+	+
C6(3)	+	+	+	+	+	-
C6(6)	+	+	-	+	+	+
C7(2)	+	-	+	+	+	+
C7(7)	+	+	+	+	+	-
C8(4)	+	+	+	+	+	+
C8(5)	+	+	+	+	+	+
C9(1)	+	-	+	+	-	+
C9(4)	+	+	+	+	+	+
C10(6)	+	+	+	+	+	-
C10(7)	+	+	-	+	+	+
C11(1)	+	+	+	+	+	-
C11(3)	+	+	+	+	+	-
D1(1)	+	+	+	+	+	+
D1(2)	+	+	+	+	+	+
D3(3)	+	+	+	+	-	+
D3(6)	+	+	+	+	+	-
D4(7)	+	+	+	+	+	+
D4(8)	+	+	+	+	-	-
D5(5)	+	+	+	+	+	-
D5(6)	+	+	-	+	+	+
D6(1)	+	+	+	+	+	+
D6(2)	+	+	-	+	+	+
D7(2)	+	+	+	+	+	-
D7(6)	+	+	+	+	+	+

Here, (+) indicates positive reaction and (-) indicates negative reaction.

Results and discussion

In the present study, a total of 230 isolates were isolated from indigenous chickens and ducks using selective MRS agar medium and based on the phenotypic characterizations such as colony morphology, gram staining and cell morphology. Finally, 30 isolates were identified as *Bifidobacteri*a based on catalase test and analysis of carbohydrate fermentation profile (Table 2). In the current study 30 isolates were found to be gram positive, short rod with Y and V shape that morphologically differentiate *Bifidobacteri*a from *Lactobacillus*, catalase negative, acidification of most of the sugars without gas production and anaerobic growth indicated the typical basic characteristics of *Bifidobacteria* strains. The identified 30 isolates of *Bifidobacteria* were subjected to the evaluation of probiotic properties included determination of antimicrobial susceptibility, antibacterial activity, haemolytic activity and acid tolerance (Table 1).

Eight commonly used antibiotics were tested for the evaluation of antimicrobial susceptibility of the *Bifidobacteria* isolates based on the principle that the bacteria should be resistant to the common antibiotics so that they can remain viable to be acted as probiotic even in the presence of those antibiotics.

Table 3. Antibacterial activity and the inhibition zone produced by the *Bifidobacteria* isolates against pathogenic bacteria.

Sample ID	Inhibition zone (mm) of indicator microorganisms					
	Staphylococcus aureus	*Escherichia coli*	*Enterococcus Faecalis*	*Salmonella typhi*	Shigella dysenteriae	*Vibrio cholerae*
C1(4)	-	-	-	-	-	-
C1(7)	-	+	-	+	-	-
C2(2)	-	-	-	-	-	-
C2(3)	+	++	+	-	-	+
C5(2)	-	-	-	-	-	-
C5(7)	-	-	+	-	-	-
C6(3)	-	-	-	-	-	-
C6(6)	-	+	+	-	-	+
C7(2)	-	-	++	-	-	-
C7(7)	-	-	-	-	-	-
C8(4)	+	+	-	+	-	-
C8(5)	-	-	-	-	-	-
C9(1)	-	-	-	-	-	-
C9(4)	+	+	+	+	-	-
C10(6)	-	+	+	-	-	-
C10(7)	-	-	-	-	-	-
C11(1)	-	-	-	-	+	-
C11(3)	-	+	+	-	-	-
D1(1)	-	-	-	-	-	-
D1(2)	-	+	+	-	-	-
D3(3)	+	+	-	-	-	-
D3(6)	-	++	+	-	+	-
D4(7)	+	+	+	-	+	-
D4(8)	-	-	-	-	-	-
D5(5)	-	+	-	-	-	-
D5(6)	-	-	-	-	-	+
D6(1)	+	-	-	+	-	-
D6(2)	-	+	-		-	-
D7(2)	-	-	-	-	-	-
D7(6)	-	-	-	-	-	-

Here, (-) indicates no inhibition, (+) indicates inhibition zone between 2 and 6 mm; (++) indicates inhibition zone larger than 6 mm.

Antimicrobial susceptibility

Antimicrobial Susceptibility profile revealed a varying degree of resistance among the tested isolates. Result of antimicrobial susceptibility of the isolates has been recorded in a graphical representation of antimicrobial susceptibility (Figure 1).

Among the 30 isolates only one isolate [C1(7)] was sensitive to all antibiotics and eight isolates revealed a higher degree of resistance, of them 6 isolates [C2(2), C6(6), C10(6), C11(3), D6(1), D7(6)] were resistant to five antibiotics, one isolate [C7(7)] was resistant to six antibiotics and the isolate D3(3) was resistant to seven of the eight tested antibiotics. More than 40% isolates were sensitive to azithromycin, ciprofloxacin, ceftriaxone and gentamicin, intermediate resistance of the isolates to most antibiotics was around 30-35% and a high number of the isolates were resistant to penicillin (60%), ampicillin (46.67%), tetracycline (43.33%) and ceftriaxone (40%). The findings that *Bifidobacteria* were resistant to penicillin, ampicillin, tetracycline and ceftriaxone and susceptible to azithromycin, ciprofloxacin, ceftriaxone but sensitivity to gentamicin. In Bangladesh, a wide ranges of non-specific antibiotic treatment is a common practice, as a result many pathogenic bacteria in its geographical location has gained resistance to several groups of antibiotics.

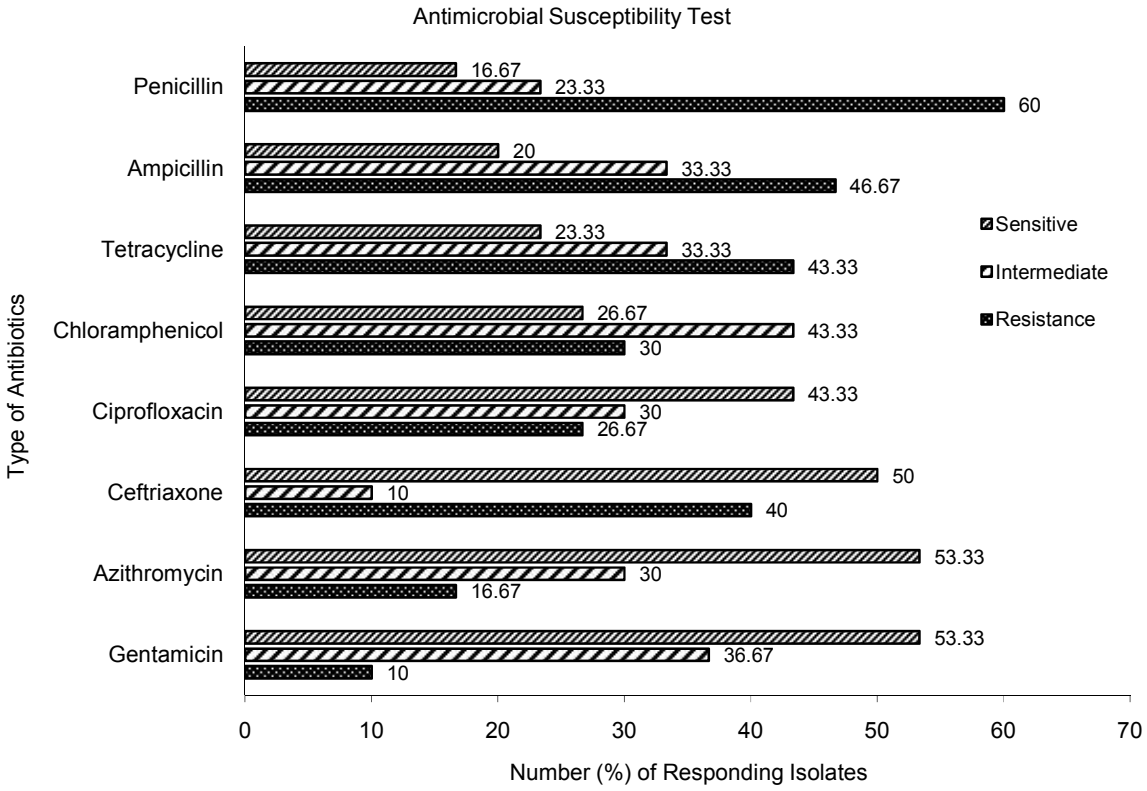

Figure 1. The extent of antimicrobial susceptibility with sensitive (Closed lining bar), intermediate (Spaced lining bar) and resistance response (Dot bar) of isolates to the commonly used antibiotics.

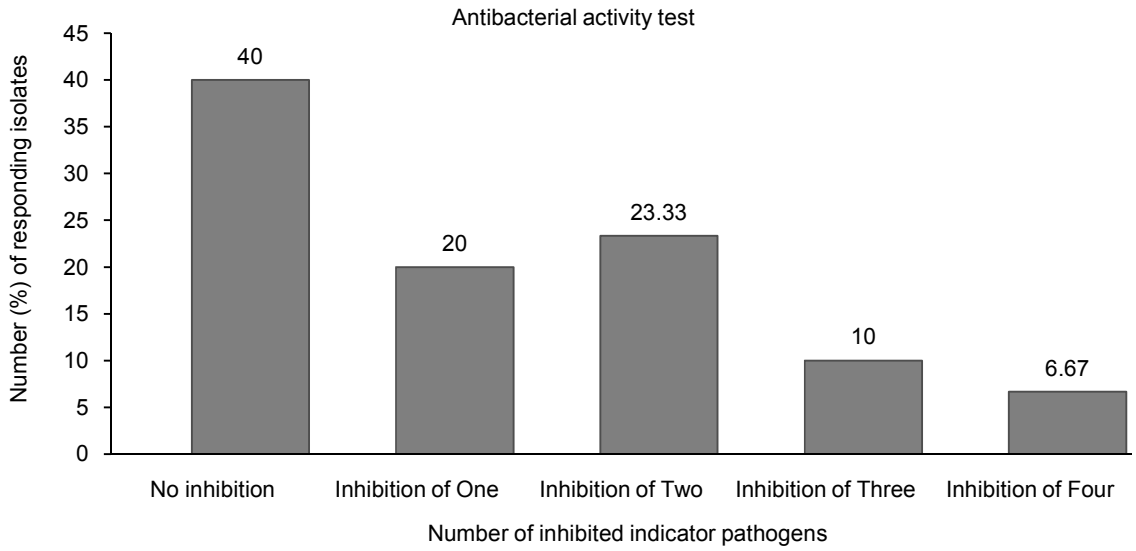

Figure 2. The extent of antibacterial activity of *Bifidobacteria* to the number of inhibiting indicator pathogens.

Antibacterial activity

The *Bifidobacteria* isolates revealed variable antibacterial activity against gastrointestinal pathogenic organisms (Table 3). The extent of antibacterial activity of 30 isolates of *Bifidobacteria* to the number of inhibiting indicator pathogens varied greatly (Figure 2) while the extent of antibacterial activity of 30 isolates of *Bifidobacteria* against the incubator pathogen responded significantly (Figure 3).

In this study, the antibacterial activity of the *Bifidobacteria* isolates was tested for their potential inhibitory activity against six indicator pathogens and variable responses were found. Although, comparatively higher inhibiting activity of the *Bifidobacteria* isolates was obtained to *Escherichia coli* (43.33%) and *Enterococcus faecalis* (36.67%) but the ability of inhibiting the growth of other potent intestinal pathogens namely *Vibrio cholerae, Salmonella typhi* and *Shigella dysenteriae* was very low (<15%). Among 30 isolates of *Bifidobacteria*, 40% isolates showed no inhibition of growth of the indicator pathogens, some isolates inhibited the growth of one or two indicator pathogens (20-23%) and few isolates (6-10%) inhibited the growth of three [Isolates: C6(6), C8(4), D3(6)] or four [Isolates: C2(3), C9(4)] indicator pathogens.

Haemolytic activity

Haemolytic activity of 30 isolates was observed on blood agar medium and all of the isolates were found to be non-haemolytic because they did not break down red blood cells on the media. In this research, all of the isolates of *Bifidobacteria* were found to be non haemolytic indicating that they are unable to cause haemorrhage during colonization in GIT of animals.

Acid tolerance

30 isolates of *Bifidobacteria* were tested at pH 2.0, 3.0, 4.0, 5.0 and all isolates were able to grow at the above pH with variable survival rate. The result of acid tolerance was recorded and survival rate of *Bifidobacteria* was calculated at each pH after 3 hours of acid treatment. Survival rate of *Bifidobacteria* isolates at pH 2.0, 3.0, 4.0 and 5.0 showed that most of the isolates (56.67%) had survival rate less than 50% at pH 2.0, 66.67 % isolates had 50-69% survival rate at pH 3.0 but highest survival rate (70-90%) was found for 43.33% isolates at pH 4.0 and 5.0, and 20% isolates showed more than 90% survival rate only at pH 5.0 (Figure 4). In this research, the strains tested were able to tolerate three hours of acid exposure at pH 2.0, 3.0, 4.0 and 5.0.

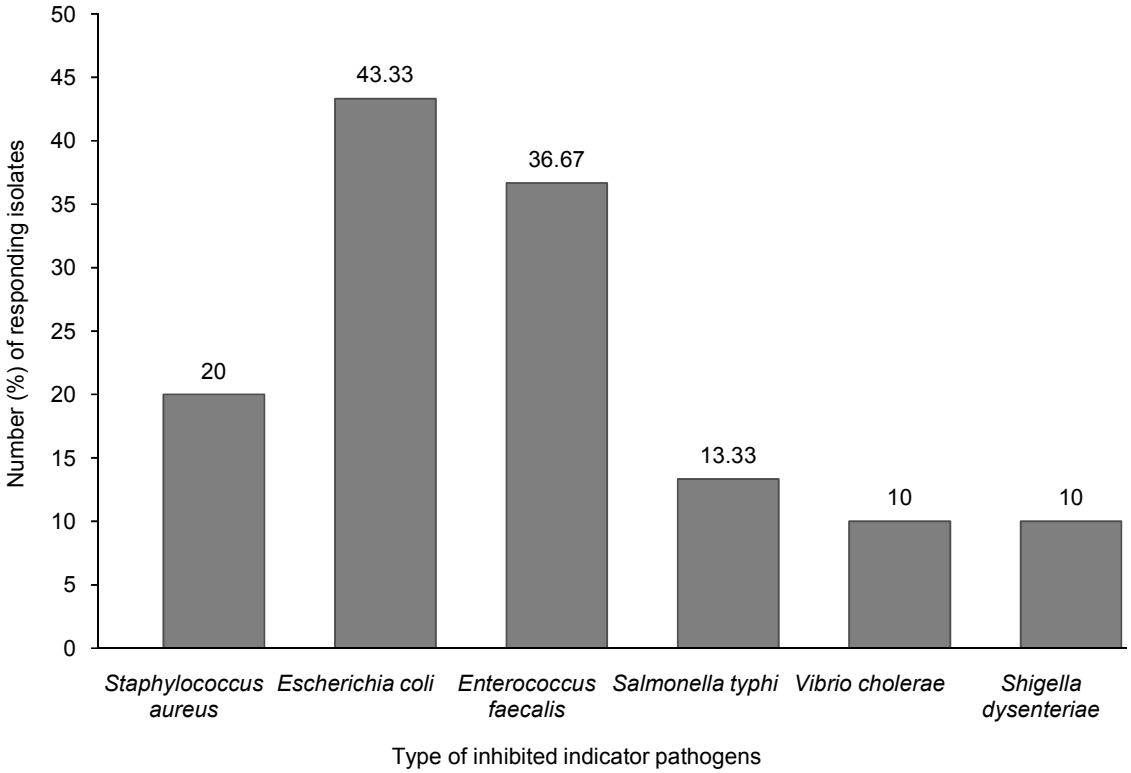

Figure 3. The extent of antibacterial activity of the *Bifidobacteria* isolates against the indicator pathogens.

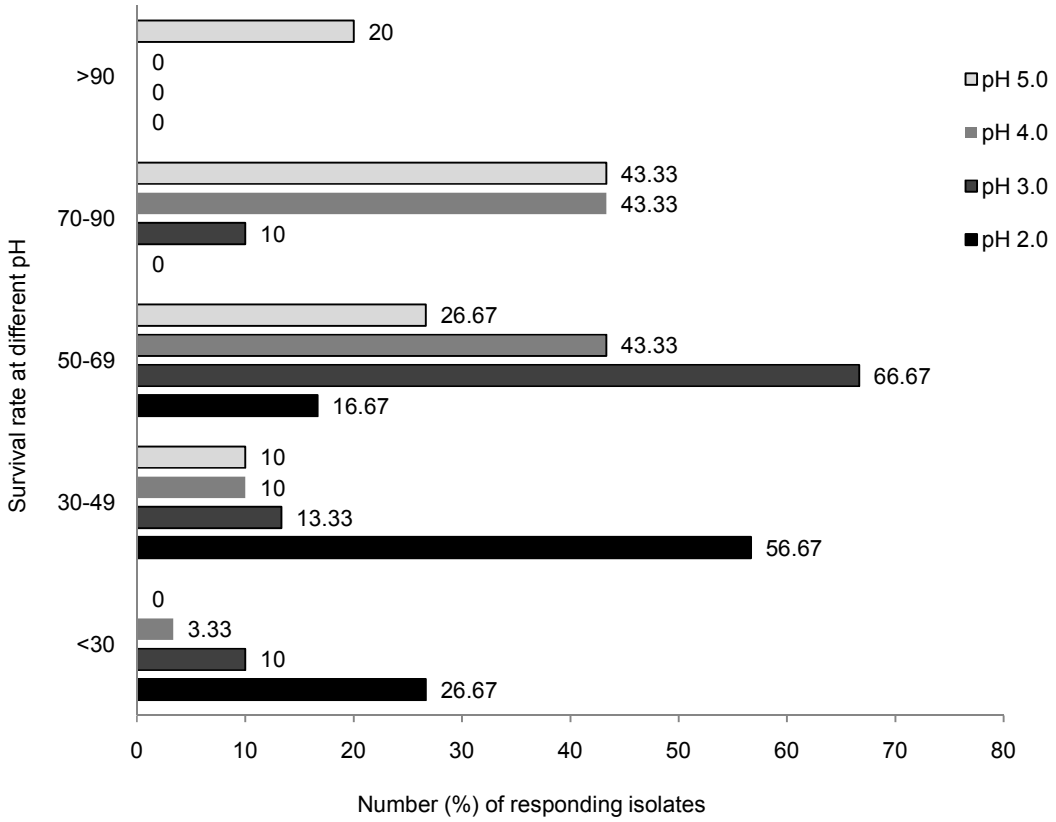

Figure 4. Survival rate of *Bifidobacteria* isolates at pH 2.0 (Blue color), 3.0 (Dark red color), 4.0 (Light Green color) and 5.0 (Purple color) according to Acid Tolerance Test.

Conclusion

To our knowledge, this is the first report that describes the isolation, identification and characterization of *Bifidobacteria* from indigenous poultry in Bangladesh. We tried to isolate *Bifidobacteria* which are able to resist the commonly used antibiotics with enhanced antibacterial activity but non-haemolytic and also able to tolerate low pH to be acted as effective probiotic. A handsome number of isolates were resistant to maximum antibiotics and most were intermediate resistant. Of the antibiotic resistant isolates, C6(6), C9(4) and D3(6) showed good antibacterial activity to the most indicator pathogens. All of the isolates of *Bifidobacteria* were non-haemolytic and able to grow at low pH including pH 2.0 at varying degrees indicating that they will be able to survive at low pH of stomach and small intestine. The isolates C6(6), C9(4) and D3(6) meet all the evaluated probiotic proprieties and these results offer them potentially useful as probiotics in human diet and/or animal feeds which suggests their possible use in the food and feed industry. However, further research is needed to carry out the *in vivo* study of these potential probiotic bacteria.

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