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# Molecular identification of *Bifidobacterium* sp. from local yoghurt and evaluation of growth inhibition activity against pathogenic bacteria

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## Abstract

Yoghurt is a potential source of probiotic bacteria including *Bifidobacterium* sp. In this context, sour yoghurt sample was collected from local market in Rajshahi for molecular identification and characterization of *Bifidobacterium* sp. with promising antagonistic activity against pathogenic bacteria. Isolation was done on Luria broth agar media for molecular identification and revealed that isolated bacterium showed 90% similarity with *Bifidobacterium* sp. Antibiotic sensitivity test result revealed that isolated *Bifidobacterium* sp. was sensitive to erythromycin, kanamycin, gentamycin, tetracycline, ciprofloxacin, doxycycline out of eleven commercially used antibiotics. Moreover, antagonistic activity of *Bifidobacterium* sp. was evaluated in our present study against four pathogenic bacteriathrough disc diffusion method. *Bifidobacterium* sp. had relatively strong antagonistic effect (inhibition zone ≥15 mm) against *Salmonella* sp. with 16mm and 19mm zones of inhibition at doses of 150 and 200 µg/disc, respectively. Similarly, the isolate showed strong growth inhibitory activity against *Acinetobacter* sp. and *E. coli* with inhibition zone of 17 mm and 16 mm at dose of 200 µg/disc while moderate growth inhibitory activity was observed against *Aeromonas* sp. at applied four doses. Furthermore, present investigation showed that the isolated *Bifidobacterium* sp. had the utmost effect against *Salmonella* sp. and exhibited growth inhibition of understudy pathogens in such pattern *Salmonella* sp.>*Acinetobacter* sp.> *E. coli*> *Aeromonas* sp.

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## Introduction

Probiotics are live microorganisms intended to provide health benefits when consumed in adequate amounts, generally by improving or restoring the gut flora (Hill *et al*., 2014). Probiotics can be defined as 'viable microorganisms' that are used as food additives with beneficial effects on health by setting microbial balance in gastrointestinal tract (Hassanzadazar *et al*., 2012). Most common probiotic bacterial strains include *Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus plantarum, Bifidobacterium infantis, Bifidobacterium longum, Streptococcus thermophilus, Enterococcus faecium* etc (Fijan, 2016). Probiotic bacteria play an important role in the protection of the organism against harmful microorganisms and strengthen the host's immune system when administered in appropriate quantities (Pundir *et al.* 2013). Nowadays, probiotic microorganisms have been added into food as dietary adjuncts for improving human health as well as prevention of many diseases (Reyhaneh *et al*., 2018).

Bifidobacteria are generally considered to be food-grade organisms that do not impose health risks on the consumer. They are found in the intestinal tract of human and animal (Baivati *et al*., 2000) and have probiotic properties for promoting health (Matsuki *et al.,* 2004). These organisms are frequently applied as probiotics in health-promoting dairy products and dried food supplements (Gomes and Malcata, 1999) because they play an essential role in maintaining the microbial balance of a healthy intestinal tract.

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Moreover, inco-administration with antibiotics in order to restore the intestinal health of the host, the presence of antimicrobial resistance in probiotic Bifidobacterium strains might be regarded as a desirable trait to allow their survival in the gastrointestinal tract. In addition, molecular identification has been used as a worldwide acceptable technique to identify any bacterium up to genus level across all major phyla. Generally, the identification is done using 16S rRNA which is considered as the most reliable tools for identification of microorganism species (Shah, 2012). After isolation and characterization of bacteria it is necessary to identify the bacteria because only morphological and biochemical characterization cannot confirm the isolated species. The 16S rRNA is the most conserved gene in all cells and portions of the 16S rRNA sequence from distantly related organisms are remarkably similar.

Recent concerns on the rampant and indiscriminate use of antibiotics for disease treatments, growth promotion of livestock and the development of antibiotic resistant pathogens have led to increase interest in the application of probiotics and their antimicrobial metabolites as alternative antimicrobial strategies for treatment and prevention of infections. Similarly,strong antibacterial activity of bifidobacteria towards gram negative bacteria, *Salmonella* and *E. coli* strains with the production of lactic and acetic acids as well as the inhibition of growth of gram positive bacteria with the production of a bacteriocin were reported (Makras and Vuyst, 2006).

Dairy product such as yogurt, is one of the best known foods which is mostly used as probiotic sources for transporting probiotic microorganisms to the human gastrointestinal tract (Tabanelli *et al*., 2016). In Bangladesh, yoghurt locally known as dahi is produced and consumed in large amount across the country (Shahnaz *et al.,* 2004). However, despite growing interest in probiotics, there is a paucity of scientific research regarding emerging uses of yoghurts as probiotic sources in Bangladesh (Sultana *et al*., 2017). Development of probiotic products such as yoghurt with defined probiotic culture would be able to confer health benefits for mass and common people of Bangladesh. Therefore, our present research work was carried out to isolate, identify and characterize bacteria from yoghurt as potential probiotics with antimicrobial activity against microorganisms that are pathogenic to human.

## Materials and methods

### *Sample collection*

Sour yoghurt sample was purchased from local market in Saheb Bazar, Rajshahi, prior to the expiry date during October, 2018. Sample was aseptically collected in sterile plastic container and transported to the Microbiology Laboratory, Dept. of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh, in an insulated box with ice to maintain a temperature ranging from 4°C to 6°C. Sample was processed within 6 hours of its collection.

### *Enrichment of yoghurt bacteria*

The collected yoghurt sample was filtered by filter paper in a beaker. 5 ml of each filtered yoghurt sample was added to 100 ml LB liquid medium for enrichment as well as selection and incubated for 48-72 hr at 37°C with shaking. Then the bacterial isolate was screened on nutrient agar (NA) plates. Plates were incubated at 37°C for 24 hr and colonies differing in morphological characteristics were selected and used for further studies.

### *Isolation and maintenance of pure culture*

Plating is essential to get single colony from mixed bacterial culture. Individual bacterial population was isolated from the above mentioned enrichment cultures by streak plate method on Luria Broth (LB) agar and plates were incubated at 37°C for 24 hr. The single colonies from these plates were sub cultured onto replicate plates and colonies from these eventually transferred into LB liquid medium. Pure strains were maintained by weekly passage in LB liquid medium and also by weekly subculture onto the LB agar medium.

### *Molecular identification*

Molecular identification and characterization of the isolated bacterium was performed through the following steps: extraction of chromosomal DNA, amplification of 16S rRNA gene, purification of PCR products, cycle sequencing, purification of cycle sequencing products, detection of nucleotides and sequence analysis. The genomic DNA of the isolated bacterium was extracted using phenol/chloroform method. The 16S rRNA genes were amplified by PCR using 16S rDNA specific forward primer 27F 5′-AGAGTTTGATCMTGGCTCAG-3′and reverse primer 1492R 5′-GGTTACCTTGTTACGACTT-3′. The PCR reactions were carried out in thermal cycler (Applied Biosystem 9700, USA) using following amplification conditions: an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and the final extension at 72°C for 10 min. The PCR products were purified and were sequenced on both strands on genetic analyzer (Prism 310, USA). The sequences were then edited by bioinformatics software Chromas.

*Antibiotic sensitivity test*

Antibiotic sensitivity and resistance pattern of the isolated probiotic bacterium was assayed according to the Kirby- Bauer disc diffusion method (Bauer *et al*., 1966). Commercially available and frequently prescribed antibiotics namely; penicillin (10units/ disk), amoxicillin (10µg/ disk), erythromycin (15µg/ disk), ampicillin (10µg/ disk), kanamycin (30µg/ disk), ceftazidime (30µg/ disk), gentamycin (10µg/ disk), tetracycline (30µg/ disk), ciprofloxacin (5µg/ disk), cefuroxime (30 µg/ disk) and doxycycline (30 µg/ disk) were used as antibiotic disks.

*Preparation of media and culture plate*

The isolated bacterial strains were grown overnight in nutrient broths through orbital shaker at 37°C and 160 rpm for the antibiotic sensitivity test. At first, LB agar medium was prepared, then for making culture plates, the sterile liquid medium was distributed in sterile petridishes. Approximately, (15-20) ml of the medium was poured in each petridish and left in the laminar airflow cabinet for solidification. 1ml of the overnight grown LB culture (O.D. = 0.5) for the isolated bacterium was poured into each nutrient plate and dried up. After overnight incubation at 37°C, the zones were observed on the plates and were measured with the help of mm scale.

*Growth inhibition activity of isolated bacterium against pathogenic bacteria*

Four pathogenic bacteria such as *Aeromonas* sp., *Acinetobacter* sp., *Salmonella* sp. and *Escherichia coli* were used as test microorganisms for this experimentbecause these bacteria cause serious diseases in human body. In this study, Luria broth (LB) liquid and Luria broth (LB) agar medium were used as culture medium for the growth of bacteria. For preparation of culture plates, Luria broth agar medium was prepared and autoclaved at 121°C for 20 min. Then the sterilized medium was poured in equal amount (about 20 ml) in each autoclaved petri-dish and were allowed to cool and solidify. Each test bacterial fresh culture was transferred to separate petri-dish with the help of micropipette in an aseptic condition and were smeared gently to assure homogenous distribution of the test bacteria. The discs (6mm diameters) were made by punching the Whatman No. 1 filter paper. For testing the growth inhibition activity of isolated probiotic bacteria against one pathogenic bacterial sample, paper discs were soaked with different concentrations of isolate (50, 100, 150, 200 µl). By means of a pair of sterile forceps, the dried discs were placed gently on the solidified agar plates seeded with the test bacteria to ensure contact with the medium. The plates were then kept in an incubator at 37°C for 24 h. After incubation, growth inhibition activities of isolated bacterium was determined by measuring the diameter of inhibitory zones with a millimeter scale. The experiment was repeated three times for the accuracy of results.

**Results and discussion**

*Isolation of probiotic bacteria*

From all mixed bacterial culture plates, one colony was isolated and identified which was cream colored colony. That colony was isolated by sub-culturing onto fresh LB plates and purified by restreaking further on nutrient agar medium (Fig.1) and incubated for 18-24 hr at 37°C. Following overnight incubation, the isolate was preserved in 30% glycerol at -20°C.

**Fig. 1. Streaking plate of isolated bacterial pure culture**

*PCR amplification and sequencing of 16S rRNA genes*

The homology of the 16S rRNA gene sequence was checked with the 16S rRNA gene sequences of other organisms using the BLASTN (http:// www. ncbi.nih. gov/ BLAST) algorithm.Isolated bacteria produced 90% identity with the 16S rRNA gene sequencing of *Bifidobacterium* sp. DNA quantification analysis and PCR band of isolated bacterium was shown in Fig. 2 and Fig. 3, respectively.

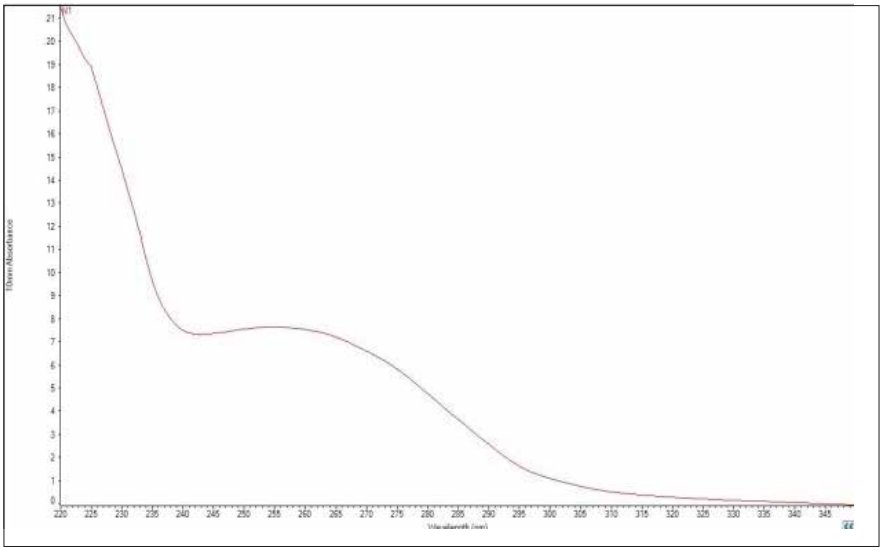

**Fig. 2. DNA quantification analysis of the isolate**

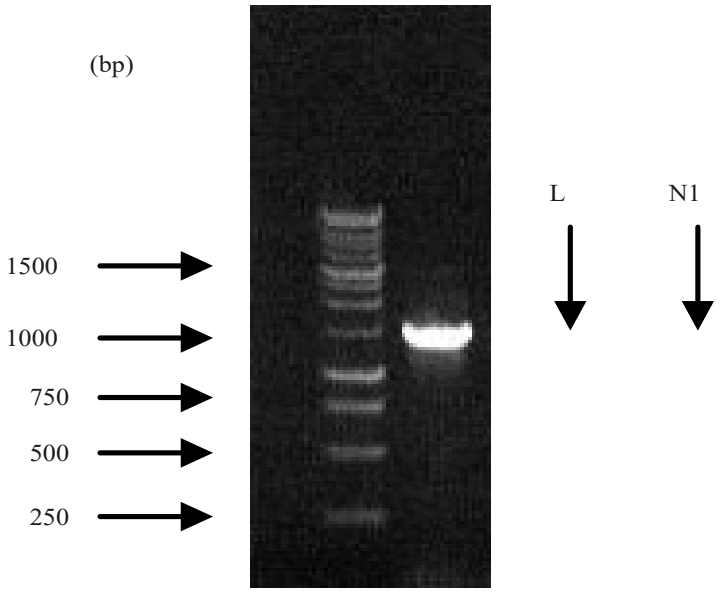


**Fig. 3. 16S rRNA gene profiling of the isolate (N1) using 27F and 1492R primers. L denotes 1kb DNA ladder (marker)**

*Antibiotic sensitivity test*

The sensitivity of probiotics to conventional antibiotics is a fundamental health-promoting characteristic. In our present study, *Bifidobacterium* sp. was sensitive to erythromycin, kanamycin, gentamycin, tetracycline, ciprofloxacin, doxycycline and resistant against penicillin, amoxicillin, ceftazidime, cefuroxime (Table I and Fig. 4). Masco *et al*. (2006) found that *Bifidobacterium* sp. from probiotic products exhibited high sensitivity to tested antibiotics. In their study, *Bifidobacterium* sp. isolated from probiotic products were sensitive to amoxicillin, ciprofloxacin, erythromycin, rifampicin, tetracycline and resistant to gentamycin, polymixin B. It was noteworthy that antibiotic susceptible

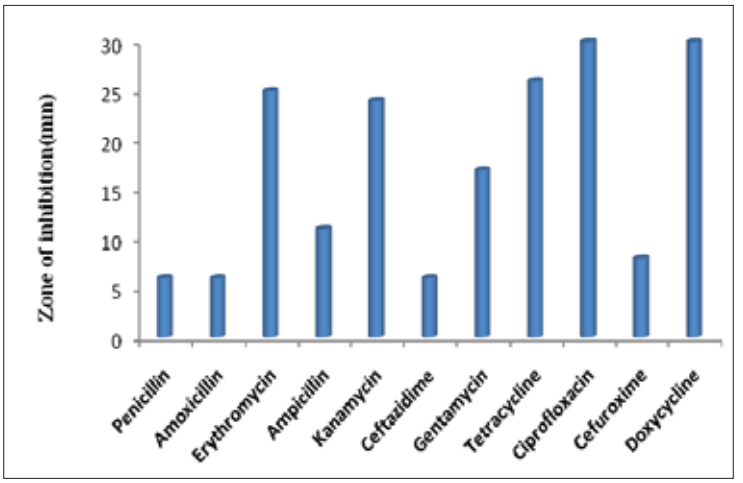


**Fig. 4. Antibiotic sensitivity pattern of the isolate identified as *Bifidobacterium* sp.**

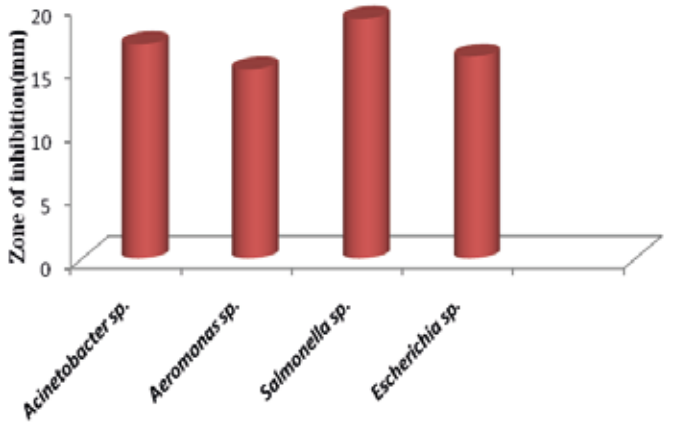


**Fig. 5. Growth inhibition activity of the isolate identified as *Bifidobacterium* sp. against pathogenic bacteria at dose of 200 µl/disc**

*Bifidobacterium* sp. was isolated from homemade yoghurt (Ralitsa *et al*., 2015). According to their investigation, all isolates were sensitive to amoxocillin, gentamycin, erythromycin and tetracycline. Our result showed similarity with previous findings.

*Growth inhibition activity of isolated bacterium against pathogenic bacteria*

Antimicrobial activity is a very important criterion for selection of starter and probiotic culture as natural antagonists of potentially harmful bacteria. The present study explored the growth inhibition activity of isolated *Bifidobacterium* sp. against pathogenic bacteria with four different doses like 50, 100, 150 and 200 µl/disc. In case of isolated bacterium, *Acinetobacter* sp. and *Escherichia* sp. were susceptible at dose of 200 µl/disc with 17 mm and 16 mm zones of inhibition, respectively (Table II and Fig. 5). In addition to that, *Salmonella*sp. was susceptible with inhibition zones of 16 mm and 19 mm at doses of 150 and 200 µl/disc, respectively (Table II and Fig. 5). But isolated *Bifidobacterium* sp. exhibited no susceptible zone of inhibition against *Aeromonas* sp. (Table II and Fig.5). According to Denkova *et al.* (2005) *Bifidobacterium* sp. isolated from different sources were able to inhibit the growth of *Escherichia coli*, *Salmonella*sp and *Klebsiella*

**Table I. Summarized result of antibiotic sensitivity test of isolate identified as *Bifidobacterium* sp.**

| Name of antibiotic | Zone of inhibition (mm) | Resistance pattern |
|---|---|---|
| Penicillin | 6 mm | Resistant |
| Amoxicillin | 6 mm | Resistant |
| Erythromycin | 25 mm | Susceptible |
| Ampicillin | 11 mm | Intermediate resistant |
| Kanamycin | 24 mm | Susceptible |
| Ceftazidime | 6 mm | Resistant |
| Gentamycin | 17 mm | Susceptible |
| Tetracycline | 26 mm | Susceptible |
| Ciprofloxacin | 30 mm | Susceptible |
| Cefuroxime | 8 mm | Resistant |
| Doxycycline | 30 mm | Susceptible |

Note: Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15

**Table II. Growth inhibition activity of the isolate identified as *Bifidobacterium* sp. against four pathogenic bacteria**

| Name of test bacteria | Dose (µl/disc) | Zone of inhibition (mm) *Bifidobacterium* sp. | Resistant pattern *Bifidobacterium* sp. |
|---|---|---|---|
| *Acinetobacter* sp. | 50 | 9 | Resistant |
| | 100 | 11 | Intermedia te resistant |
| | 150 | 14 | Intermediate resistant |
| | 200 | 17 | Susceptible |
| *Aeromonas* sp. | 50 | 6 | Resistant |
| | 100 | 8 | Resistant |
| | 150 | 11 | Intermediate resistant |
| | 200 | 15 | Intermediate resistant |
| *Salmonella* sp. | 50 | 11 | Intermediate resistant |
| | 100 | 13 | Intermediate resistant |
| | 150 | 16 | Susceptible |
| | 200 | 19 | Susceptible |
| *Escherichia coli* | 50 | 7 | Resistant |
| | 100 | 10 | Resistant |
| | 150 | 13 | Intermediate resistant |
| | 200 | 16 | Susceptible |

*pneumonae.* Another study of Bayar *et al.* (2018) confirmed that probiotic lactic acid bacterial strains showed antimicrobial activity against *Escherichia coli*, *Salmonella typhimurium, Staphylococcus aureus* species by producing zones of inhibition ranged from 10 mm to 22 mm diameter at dose of 100 µl/disc. Our result had resemblance with previous findings. From the result it was clear that isolated probiotic strains can produce antimicrobial product which can restrain the growth of pathogenic bacteria.

**Conclusion**

From the present study, it was observed that isolated probiotic bacterial strain had growth inhibition activity against human pathogens. It should be studied further as biotherapeutic agents for treatment of specific disease conditions. In addition, sensitivity or intrinsic resistance of the majority of the strains to a recommended set of antibiotics make them safe for use in different products for human or animal consumption.

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