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A Potential Novel Probiotic Strain and its Comparative Antagonism Evaluation with a Multi-Strain Probiotic Combination along with an Innovative Approach for Quantifying the Viable Microbiota

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A Potential Novel Probiotic Strain and Its Comparative Antagonism Evaluation with a Multi-Strain Probiotic Combination Along with an Innovative Approach for Quantifying the Viable Microbiota

Running Title: Antagonism Comparison and Quantifying the Viable Microbiota

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Abstract: The goal of the current study was to discover a novel potential probiotic strain of *Lactobacillus* spp. with anti-*Escherichia coli* activity from locally produced yogurt in Tongi, Gazipur, Bangladesh, compare its antagonistic activity with a commercial probiotic mixture of several strains, and approve a novel method for confirming the viability and relative abundance of the microbial community in a probiotic mixer. We carried out 16S sequencing, 16S metagenomics, Transmission Electron Microscopy (TEM) analysis, and other *in vitro* laboratory experiments to reach this objective. The strain TY-11 was identified as *Lactobacillus delbrueckii* subsp. *indicus* (16S sequence accession number OQ652026). It was gram-positive, anaerobic, lactose fermenting, and round-ended rod that typically measured 0.7 to 1.3 μm by 2.2 to 9 μm. In addition to having seven probiotic characteristics, it also showed an antagonistic impact on six different pathogens, but what's more noteworthy is that *E. coli* was the pathogen it inhibited most strongly (inhibition zone diameter was 18.88±0.18 mm). The most important and ground-breaking finding of this work was determining the probiotic features of a new probiotic strain, TY-11, whose antibacterial activity was virtually as effective as that of the probiotic combination with three different strains. Furthermore, the results of 16S high throughput sequencing and the conventional plate count method demonstrated a strong correlation (0.999) at the genus level, indicating that the use of both of these approaches in combination may be a practical way to assess the relative abundance of the microbial community and their viability in commercially available probiotic blends.

Keywords: *Lactobacillus delbrueckii* subsp. *indicus* TY-11; TEM; anti-*E. coli* activity; 16S metagenomics

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Introduction:

The human digestive tract can harbour probiotics, inhibiting pathogens including *Escherichia coli*, *Salmonella typhimurium*, *Streptococcus aureus*, and others, which can reside in the intestine too [1, 2]. These probiotics are used to prevent diarrheal illnesses [3]. According to the most recent Global

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Burden of Disease Study, around 2.39 billion individuals worldwide had diarrheal disease, and every year, 0.53 million children under the age of five perished. Incidence and case-fatality ratios are significantly greater in lower and middle-income (LMI) countries than in developing nations, particularly in Bangladesh [3]. The average episode cost in Bangladesh was 67.18 US dollars, whilst the average costs of inpatient and outpatient were 110.51 and 23.62 US dollars, respectively. In contrast to 4.21% income of the wealthy individuals, the cost was significantly higher for the more impoverished families, accounting for 21.45% of their living expenses [4]. A probiotic can be an affordable choice for protecting billions of consumers worldwide from sickness caused by diarrhoea and other food-related illnesses linked to harmful *E. coli*.

The elimination of infections in the intestinal tract depends on promising probiotic strains. Conventional fermented milk products are highly concentrated in wild LAB, because they are typically naturally fermented with no use of artificial starters and local bacteria from raw milk. Their distinctive qualities, native flavour, and firmness are greatly influenced by this community of microbes [5, 6]. It can be an excellent plan to create novel cultures for starters and probiotics by isolating and identifying such wild LAB derived from conventional dairy meals [7]. Most advertised probiotics in Bangladesh originate from other countries. In Bangladesh, there hasn't been much research done on probiotics [8]. To strengthen the immune system of the majority of the population in this region, it is important to isolate prospective probiotics from sources of natural origin in Bangladesh. Additionally, it has been demonstrated that probiotic effectiveness varies by species or even strain since various probiotic strains have varying effects on hosts [9]. Before marketing a strain as a probiotic, its potential probiotic attributes must be evaluated [8, 10].

In the present investigation, we had three targets. First of all, we identified a novel probiotic strain as *Lactobacillus delbrueckii* subsp. *indicus* TY-11, which had anti-*E. coli* activity. In spite of the fact that *Lactobacillus delbrueckii* subsp. *indicus* is typically regarded as risk-free for human consumption, a number of *in vitro* studies have been conducted to identify these bacteria and screen its potential probiotic

properties [11, 12]. The species *Lactobacillus delbrueckii* currently comprises of six subspecies: *delbrueckii, lactis, bulgaricus, indicus, sunki*, and *jakobsenii* [13]. For appropriate identification, 16S sequencing and a detailed phylogenetic tree with all six subspecies of *Lactobacillus delbrueckii* are required. A novel strain should also be evaluated for its antimicrobial characteristics, antibiotic susceptibility, acid-tolerant behaviour, bile salt resilience, cold and heat shock endurance capability, exopolysaccharide production, and metabolism of carbohydrates, among other factors [8, 10,11]. Therefore, this work primarily aimed to isolate and identify a potential probiotic strain with anti-*E. coli* activity and to give a more thorough evaluation of the probiotic qualities of the isolate acquired from traditional yogurt in Bangladesh by various *in vitro* experiments. Furthermore, we chose another objective because many businesses now provide probiotic mixtures with many strains with the belief that additional strains will increase the likelihood of effectiveness in treating human illnesses [14]. The selection of a suitable probiotic needs to be focused on effectiveness rather than the number of strains in the product. That is why, we targeted the commercially available probiotic blend of three strains: *Lactobacillus acidophilus, Lactobacillus delbrueckii* subsp. *bulguricus,* and *Bifidobacterium bifidum,* and we compared antimicrobial action of the isolated single probiotic strain *Lactobacillus delbrueckii* subsp. *indicus* TY-11 with above mentioned probiotic combination against six pathogens especially *E. coli*. Such a comparison of antibacterial activity had not previously been supported by scientific data. Finally, this study had an extra goal because it can be challenging to determine the viability of probiotic combinations, count each strain separately, and control for contamination. As of right now, this problem has no published solution. We explored a novel approach for the issue—a plate count method and 16S metagenomics approach together. As far as we are aware, probiotic combos have never before been approached using these combination methods.

Materials and Methods

Isolation of bacterial samples

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Probiotic bacteria were isolated from a variety of yogurt specimens in Tongi, Gazipur, Bangladesh. 20 to 25 mL of MRS (de Man Rogosa Sharpe, Oxoid, UK) agar medium was overlaid on 1 mL of a 10-6 decimal diluted sample, which was then incubated at 35°C for 24 to 72 hours. Numerous colonies of bacteria from MRS agar medium were chosen based on morphology and their closeness to genus *Lactobacillus.* The isolates were streaked on the same media in order to purify colonies, and then the pure colonies were transferred to MRS broth with 15% glycerol for additional investigation.

We also observed acid production capacity of the isolates in MRS broth after 24 hours' incubation at 35°C. The anti-*E. coli* activity of the isolates were examined in Tryptic Soy Agar (TSA) medium. 100µl supernatant of the cell suspension broth of the isolates (24 hours incubation at 35℃) was placed in a 7mm diameter well in TSA media having a lawn of *Escherichia coli* ATCC 8739. We preferred 3 isolates for 16S sequencing based on morphology, acid secretion efficiency, and anti-*E. coli* activity.

16S sequencing, phylogenetic study, and molecular identification of bacterial samples

Following the product's instructions, the MRS-agar grown cultures of three isolates (TY-11, TB-3, TY-3) were put to use for the Phenol chloroform chemical lysis procedure to isolate genomic DNA. The quantity and purification of the DNA were assessed using a Nanodrop® spectrophotometer ND2000 (Thermo Scientific, USA) following the extraction process for DNA. The pureness of the DNA was determined by looking at the 260/280 ratio, or absorbance at 260 and 280 nm. For pure DNA, the ratio is more than 1.8. In accordance with the technique previously reported by Rahman et al. (2017) [15], 16S rDNA amplification and sequencing were carried out in this work. The following were the standard 16S rRNA primers sequence for PCR (polymerase chain reaction) amplification: 1492r (5'- GGTTACCTTGTTACGACTT-3') and 27f (5'-AGAGTTTGATCCTGGCTCAG-3').

According to the identification by 16S sequencing, we decided only the isolate TY-11 for further investigation. The phylogenetic tree for the isolate TY-11 was constructed using the UPGMA (unweighted pair group method with arithmetic mean) [16] implemented in MEGA X [17].

Colony morphology of the isolate TY-11

We examined the colony morphology of the isolate TY-11 under The Leica MZ9.5 (Germany), a powerful stereo microscope with an excellent 9.5:1 zooming ratio including magnification levels up to 480x.

Gram staining test, and cellular morphology analysis of the isolate TY-11 under TEM

The chosen culture of the TY-11 was analyzed employing the Coica (2005) method's gram staining protocol, and afterwards viewed under a light microscope with a 100X magnification [18]. For TEM analysis, samples were positioned on carbon-coated EM grids that had been thoroughly prehydrophilized before being negatively stained with a 1.8% uranyl acetate liquid. A VELETA CCD Camera (Olympus Soft Imaging Solutions) attached to a JEM 1010 electron transmission microscope (JEOL) was employed for taking the images.

Probiotic potentiality evaluation of the isolate TY-11

1. EPS production

EPS production the isolate TY-11 was measured following the method of Changjun et al. (2022) with some modification [11]. The isolate TY-11 was cultured in 50 mL MRS broth for 48 h. 2 ml of 0.1 M EDTA was added to the broth at the conclusion of incubation, and the liquid was then gently agitated on a shaker machine with a 200 rpm rotation for 4 hours. After that, the mixer was centrifuged for 10 minutes at 5000 rpm to collect the cells. By adding three times as much ethanol (99.5%) to the cell-free supernatants, the EPSs were precipitated. Precipitates were obtained by centrifuging at 5000 rpm for 10 minutes after vortex and storing for an overnight period at 4°C. The pellet was gathered, allowed to air dry, and weighed.

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2. Anaerobic growth

The culture of TY-11 was streaked on MRS agar media and incubated for 48h at 35°C in aerobic and anaerobic conditions. Anaerobic condition was maintained in an anaerobic jar containing anaerobic kit with 7-15% CO_2 (AnaeroGen™ 2.5L, Oxoid Ltd., UK). The MRS broth inoculated with the strain TY-11 was incubated for 48h at 35°C in aerobic condition.

3. Carbohydrate metabolism

The isolate TY-11 was grown aerobically for 24 hours at 35°C in MRS broth, which contained dextrose (D-glucose) as the carbon source. The strain was tested for lactose, glucose, mannose and sucrose fermentation by BioMérieux's API biochemical kit (USA).

4. Acid tolerance

The acid tolerance of the isolate TY-11 was tested following the method of Hassanzadazar et al. (2012) with some modifications [19]. The isolate TY-11 was grown in MRS broth for 24 hours at 35℃ and the cells were suspended after 24 hours incubation by slow vortex. Then 1% cell suspension broth was added to the MRS broth having pH 2.49±0.01, and pH 1.83±0.03. Few drops of 1.0 M HCl (Stock solution) were used to make the pH of MRS broth (pH 2.49±0.01, and pH 1.83±0.03). This final solution was used for analysing the capability of the isolate TY-11 in our study to tolerate acidity (pH <3, and pH <2). After 2 hours, and 4 hours' incubation at pH 2.49±0.01, and pH 1.83±0.03; the Colony Forming Unit (CFU) of the isolate TY-11 was counted by culturing in petridishes by spread plate method for 5 days at 35°C. The growth of the isolate TY-11 measured at 600 nm wavelength by UV spectrophotometer (UV-1800, Shimadzu, Japan), and the light absorbance was compared between initial time and 24 hours' incubation at 35°C. The MRS broth with 1% cell suspension without adding any HCl stock solution (final pH 6.55±0.15) was used as a control. The tests were performed with three replicates.

5. Bile salt tolerance

The bile tolerance of the isolate TY-11 was tested following the method of Hassanzadazar et al. (2012) with some modifications [19]. The isolate TY-11 was grown in MRS broth for 24 hours at 35℃ and the cells were suspended after 24 hours incubation by slow vortex. Then 1% cell suspension broth was added to the MRS broth containing bile salt (0.5% and 0.25% oxgall). This final solution was used for analysing the capability of the isolate TY-11 in our study to tolerate bile salt (0.5% and 0.25% oxgall). Following 8 hours of incubation, the isolate TY-11's viability was counted through culturing in petridishes using the spread plate technique, which incubated for 5 days at 35°C. By using a UV spectrophotometer (UV-1800, Shimadzu, Japan), the growth of the isolate TY-11 was monitored at 600 nm, and the absorption of light was contrasted between the beginning time and 24 hours of incubation at 35°C. The cell suspension broth without adding any bile salt (after 24 hours incubation at 35℃) was used as a control. The tests were performed with three replicates.

6. Heat shock and cold shock tolerance

The heat shock and cold shock tolerance of the isolate TY-11 was tested following the method of Prasad et al. (2003) and Mojtaba et al. (2016) with some modifications [20, 21]. The isolate TY-11 was grown in MRS broth for 24 hours at 35℃ and the cells were suspended after 24 hours incubation by slow vortex. This final cell suspension broth was used for analysing the capability of the isolate TY-11 in our study to tolerate heat shock for 1 hour and then cold shock for additional 1 hour. The viability of the isolate TY-11 was counted by spread plate technique after 2 hours' heat shock and cold shock. Through the use of a UV spectrophotometer (UV-1800, Shimadzu, Japan), the isolated strain TY-11's proliferation at 600 nm wavelength was assessed after 2 hours' heat shock and cold shock. Moreover, the wavelength of the light absorbency was then compared between the initial time and 24 hours of incubation at 35°C. The cell suspension broth without any heat shock and cold shock (after 24 hours incubation at 35℃) was used as control. The tests were conducted three times.

Safety evaluation of the isolate TY-11

1. Antibiotic sensitivity test by agar well diffusion method

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An antibiotic sensitivity test was conducted against the probiotic isolate TY-11 following the method of Chetan et al. (2017) with some modifications. 8 mL MRS agar ($1.93\pm0.12\times10^7$ CFU/mL of the isolate TY-11) overplayed on previously solidified MRS agar [22]. 100 µL of Ampicillin (10 µg / 100 µL), Vancomycin (30 µg / 100 µL), Gentamicin (10 µg / 100 µL), Kanamycin (30 µg / 100 µL), Streptomycin (10 µg / 100 µL), Erythromycin (15 µg / 100 µL), Tetracycline (30 µg / 100 µL), Chloramphenicol (30 µg / 100 µL), Tylosin (30 µg / 100 µL), and Clindamycin (10 µg / 100 µL) was filled in a 7mm diameter well in MRS agar media with an upper layer of the probiotic bacterial strain. The test Petri dishes were incubated for 48 hours at 35°C. Antibiotic sensitivity for every antibiotic was tested thrice.

2. Hemolysis test

The plates made from blood agar (BD, USA) were streaked with MRS broth containing cultures of the TY-11 strains and incubated at 37 °C for 72 hours to test for hemolytic function. The plates were then examined for the development of any greenish (α-hemolysis) or clean (β- hemolysis) hemolytic zones, or no such zone (γ-hemolysis), surrounding the *Lactobacillus* colonies.

A novel approach for assessing the relative abundance of the viable microbial community in a probiotic mixer

We collected three sources of the most popular probiotic blend in Bangladesh from a local market in Gazipur, Bangladesh (*Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulguricus*, and *Bifidobacterium bifidum*). The samples were processed for 16s metagenomics studies in IlluminaiSeq 100 system. This work was a part of next generation sequencing for v3 and v4 variable regions of 16s gene, which had a workflow, comprised of three steps, such as, library preparation, sequencing and data analysis. To guarantee the accuracy of the results of the microbial abundance in the three sources, the data produced from the throughout sequencing process was compared with that of the plate count approach. Total aerobic microbial count (TAMC) was conducted on Tryptic Soy Agar (TSA) and total yeast and mold count (TYMC) was observed on Sabouraud Chloramphenicol Agar (SCA). For counting *L. acidophilus* and *L. delbrueckii* subsp. *bulguricus*, diluted samples were spread on MRS (de Man Rogosa Sharpe, HIMEDIA, India) agar medium. For counting *Bifidobacterium* spp. in the samples, Bifidobacterium Agar Modified supplemented with propionic acid (HIMEDIA, India) was used.

Antagonistic test and comparison

Antimicrobial activity test of the isolate TY-11 and the probiotic combination was conducted following the method of Chidre et al. (2017) with some modifications [10]. For comparison, we cultured separately the isolate TY-11 and the probiotic combination for 24 hours at 35°C in MRS broth. We cultured collectively the probiotic mixer of *L. acidophilus*, *L. delbrueckii* subsp. *bulguricus*, and *B. bifidum*. Antagonistic effects against six pathogenic microorganisms were tested in TSA (Tryptic Soy Agar) media. 100µl supernatant of the cell suspension broth of the isolate TY-11 and the probiotic combination (24 hours incubation at 35℃) was placed in a 7mm diameter well in TSA media having a lawn of pathogenic microorganism. *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC6538, *Escherichia coli* ATCC 8739, *Bacillus subtitlis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 142, and *Candida albicans* ATCC 10231 were bought from a local supplier in Dhaka, Bangladesh.

The cell suspension broth of the isolate TY-11 and the probiotic combination was prepared twice for testing antimicrobial activity against six pathogens. Two antagonistic tests (each with 2 replications) were conducted against each pathogen. The CFU for the isolate TY-11, control probiotic combination and the pathogens in TSA media was counted **(Table 3)**. The pH of supernatant broth for the isolate TY-11 and control probiotic combination was measured as well.

Results and Discussion

Isolation of bacterial samples

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We selected 15 colonies on MRS (de Man Rogosa Sharpe, Oxoid, UK) agar medium from several yogurt samples in Tongi, Gazipur, Bangladesh. The colonies were large, glossy on top, irregular-edged, and opaque [11]. From 15 isolates, 60% bacterial strains (9 strains) demonstrated excellent acid production capacity and anti-*E. coli* activity. This suggested that yogurt from Bangladesh would be an appropriate substitute for treating the infection with *E. coli*. The inhibition zone against *Escherichia coli* ATCC 8739 and acid production capacity of 9 isolates have been cited in the table (**Supplement 1: Table S1)**. We conducted this antagonistic test for primary selection of our target probiotic strains. From the view point of morphology, acid secretion, and anti-*E. coli* activity, we selected 3 strains: TY-11, TY-3, and TB-3, which demonstrated the ZOI of 21.33±1.53 mm, 21.0±1.0 mm, and 17.17±1.44 mm respectively.

16S sequencing, phylogenetic study, and molecular identification of bacterial samples

Among three isolates (TY-11, TB-3, TY-3), the isolate TY-11 was identified as *L. delbrueckii* subsp. *indicus*. The GenBank accession number of the isolate TY-11 was OQ652026. 16S ribosomal RNA of TY-11 showed 99.84% identity with the closest 16S ribosomal RNA of *L. delbrueckii* subsp. *indicus* strain NCC725 (subject and query sequence length were 1515bp and 1283bp respectively). In the tree, the strain TY-11 was at the top, and the most closely related strain was *L. delbrueckii* subsp. *indicus* strain NCC725 (**Figure 1**).

Colony morphology of probiotic TY-11 from yogurt

The colonies of TY-11 were large, opaque, white, umbonate (possessing knobby protuberances) and shiny in surface, as well as irregular in edge (**Figure 2**). Only one study (Changjun et al., 2022) described previously the colony morphology of *L. delbrueckii* subsp. *indicus* [11]. Our observation was slightly different from Changjun et al. (2022), because we examined the morphology by a different stereo microscope, Leica MZ9.5 (Germany) besides visual inspection.

Gram staining test and cell morphology of probiotic TY-11 from yogurt

The cells were gram-positive and long rod under light microscopy with 100X resolution [11, 12]. Figure 3 displays TEM images of the *L. delbrueckii* subsp. *indicus* TY-11 strain. The cells were round-ended rods that typically measured 0.7 to 1.3 μm by 2.2 to 9 μm. They might be found single, in pairs, or in small chains. Under TEM image processing, no polar fibers or flagella were seen. Two TY-11 cells were joined together by substances resembling exopolysaccharides (**Figure 3**). These characteristics are the same as those of *L. delbrueckii* subsp. *indicus* described by Dellaglio *et al.* (2005), and Changjun et al. (2022) [11, 12]. Binary fission was also observed in dividing bacterial cells.

Probiotic potentiality evaluation

1. EPS production

We harvested 2.73±0.05 mg EPS per 1 mL MRS broth after 48 h incubation. Comparing with previous studies, the potential to create EPS in TY-11 (0.06±0.0 mg/mL/h) was substantially lower than in *L. delbrueckii* subsp. *indicus* WDS-7 (0.11mg/mL/h), and higher than in *L. delbrueckii* subsp. *bulgaricus* (0.02±0.0 mg/mL/h), *as well as Ligilactobacillus salivaris UCO* (0.03 mg/mL/h) [11, 23, 24].

2. Anaerobic growth

Excellent growth was seen in anaerobic conditions in the MRS agar media of Petri dishes, but minimal growth was observed in aerobic environments. In MRS broth, the bottom of the broth showed significant growth whereas the top of the liquid displayed slight development (**Supplement 2: Figure S1**). It indicated that the microbial culture was facultative anaerobic [11]. This facultative anaerobic growth is suitable for the anaerobic environment of the human intestine [25].

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3. Carbohydrate metabolism

The strain TY-11 was grown in MRS broth, which contained dextrose (D-glucose) as as the carbon source. After 24 hours pH was 3.97±0.03 and colony count was 17.87±10.19 x10^7 CFU/mL. The strain was tested positive by BioMérieux's API biochemical kit for lactose, glucose, mannose and sucrose fermentation. This result is cosistent with the previous experiment of Changjun et al. (2022) [11]. As this strain can grow on and degrade lactose in lactose enriched media, the amount of lactose in lactose intolerance sufferers can be lowered in their intestines.

4. Acid tolerance

Low pH conditions in the human intestines can impede metabolism and lessen *Lactobacilli* growth and survival [19]. In our experiment, we cultivated the strain on petridishes to ascertain whether the cells remained alive, and we observed the growth of the strain on petridishes after two hours, as well as four hours. When the pH was less than three (pH2.49±0.01), the CFU/mL dropped from (1.74±1.17)×10^6 to (2.0±1.73)×10^5 after two hours, and to (1.08±1.66)×10^5 after four hours. It fell from (4.4±0.53)×10^6 CFU/mL to (4.57±0.66)×10^4 CFU/mL at <pH2 (pH 1.83±0.03) after two hours, and then to (1.14±0.99)×10^1 CFU/mL after four hours **(Supplement 1: Table S3)**. Other previous experiments supported this finding, showing that after three hours of incubation, exposure to stomach acid with a pH ≤ 2 significantly reduced the number of bacteria that were viable [19].

We assessed wavelength absorbance at 24 hours to compare the increase in growth. After 24 hours' incubation at <pH3 (pH2.49±0.01), it had an increase in absorbance of wavelength 600 that was 3.42 times greater than it had been at the beginning (0 hour). On the other hand, the absorbance climbed 2.69-fold at <pH2 (pH 1.83±0.03) over the course of 24 hours, from 0.51±0.14 at initial time to 1.61±0.79 after 24 hours. When <pH3 was used instead of <pH2, the absorbance rose by 1.27-fold. The absorbance of the control MRS broth (containing 1% cell suspension) went up to 9.48-fold over a 24 hours' time period **(Supplement 1: Table S2)**. This absorbance-based conclusion contradicted the results of the colony count technique, as after some time, the overall mass of cells grew while the number of viable cells declined. According to this finding, the bacterial isolate TY-11 was able to cope with and even develop at <pH 2 level, however, its growth was slower than usual **(Supplement 1: Table S2)**. Therefore, it is expected that this strain will be able to pass the acidic environment of the human stomach easily when it will be consumed.

5. Bile salt tolerance

Probiotics, which are part of the healthy bacteria in the human colon, have to adapt to bile salt. We evaluated our isolate TY-11's bile salt endurance by oxgall because it is normally used for this type of test [26]. We cultivated the isolate on plates after 8 hours' incubation, and the growth was observed on petridishes. The CFU/mL reduced from (4.4±0.53)×10^6 to (1.91±2.69)×10^5 when 0.025% oxgall was added, and to (2.96±4.38)×10^4 when 0.5% oxgall was added **(Supplement 1: Table S3)**. The results corroborated those reported by Ding et al. (2007), who observed that following eight hours of incubation, the probiotic strains in their study declined [26].

As for the absorbance, after 24 hours of incubation, the absorbance increased 10.10 times with 0.025% oxgall, but the absorbance in control broth increased 9.48 times within the same time period. The rise in absorbance (4.07 fold) was roughly half of the amount achieved with 0.5% oxgall as opposed to the typical growth (9.48 fold). The findings revealed that it could withstand 0.5% oxgall even though the organism's growth was reduced by half and could grow normally at 0.025% oxgall **(Supplement 1: Table S2)**. Hassanzadazar et al. (2012) chose several probiotic cultures for a bile sensitivity test in MRS broth with bile concentrations of 0% as the control and 0.3% as the test. This experiment supported our findings, even though there were far more live bacteria present in our study than in their study [19]. Thus, this strain TY-11 is perfect for growing in the human gut surrounded by bile salt.

6. Heat shock and cold shock tolerance

The readings of three replicating tests of heat and cold shock for initial time, 2 hours and 24 hours were demonstrated in the figure (**Supplement 2: Figure S2**). A 1-hour heat shock followed by a 1-hour cold shock significantly decreased the viable cells. Because some cells were likely injured

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and shrunk as a result of this treatment, we evaluated absorbance after two hours (0.36±0.34) and found that it had decreased by 50% from the initial reading (0.67±0.32). Similar findings were noted for the plate count method, whereby heat and cold shock caused the CFU/mL to drop from (2.33±0.35)×10^6 CFU/mL to (1.19±1.57)×10^6 CFU/mL. This finding is very close to what Ding et al. (2007) discovered [26]. The result of our experiment was much better than the finding of Changjun et al. (2022). The rate of survival for the WDS-7 strain in subspecies *indicus* was around 0% after treatment in a water bath at 70°C and 80°C for 3 minutes. The strain had a survival percentage of 12.382.33% after three minutes at 60°C [11]. The cultures of *L. rhamnosus* HN001 showed differences, according to Prasad et al. (2003). The OD610 values of the control cultures were 1.25 at 2 hours after heat shock, while the OD610 of the 55°C-shocked culture was just 0.54. [20].

Some cells of the TY-11were able to endure the heat and cold shock in the broth and grew on petridishes through streak culture. After 24 hours of broth incubation, the absorbance doubled (1.46±0.62) from initial reading (0.67±0.32) compared to a 9.48-times increase in control broth that received no treatment. The colonies in the control broth rose by approximately 10 times using the plate count method, from an initial count of 2.33±0.35) ×106 CFU/mL to (2.03±1.11)×107 CFU/mL. In summary, the treatment we used was only marginally tolerated by our isolate TY-11. The probiotics should be tolerable heat and cold environment, because the commercial probiotic strain has to pass a long shelf life in diverse environment when it is used as a nutrient supplement or, drug. Our probiotic strain was capable to endure this environment.

Safety evaluation of the isolate TY-11

1. Antibiotic sensitivity test

The isolated strain TY-11 in our study was inhibited by antibiotics with a Zone of Inhibition (ZOI) larger than 20mm (diameter). According to the description of the Clinical and Laboratory Standards Institute (CLSI), it was susceptible to Ampicillin (10 µg / 100 µL), Vancomycin (30 µg / 100 µL), Gentamicin (10 µg / 100 µL), Kanamycin (30 µg / 100 µL), Streptomycin (10 µg / 100 µL), Erythromycin (15 µg / 100 µL), Tetracycline (30 µg / 100 µL), Chloramphenicol (30 µg / 100 µL), Tylosin (30 µg / 100 µL), and Clindamycin (10 µg / 100 µL) **(Table 1)** [10, 27]. It suggested that this strain didn't contain any effective resistant gene for the tested antibiotics. It is an ideal character of a probiotic.

Table 1. Antibiotic sensitivity test.

Antibiotic Name	Antibiotic Concentration	Diameter of ZOI (mm)
Ampicillin	10 µg / 100 µL	49.67±4.04
Vancomycin	30 µg / 100 µL	39.17±1.04
Gentamicin	10 µg / 100 µL	28±2.65
Kanamycin	30 µg / 100 µL	20±1
Streptomycin	10 µg / 100 µL	23±2
Erythromycin	15 µg / 100 µL	34.5±2.18

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Tetracycline	30 µg / 100 µL	44±1
Chloramphenicol	30 µg / 100 µL	41.33±1.53
Tylosin	30 µg / 100 µL	48.33±4.04
Clindamycin	10 µg / 100 µL	49.67±0.58

2. Hemolysis test

Hemolytic activity was not observed for the strain TY-11. The isolate was identified as γ-hemolytic or non-hemolytic because there was no distinct transparency or greenish zone encircling their colonies on the blood agar Petri-dishes. A probiotic with these qualities is perfect [28].

A novel approach for assessing the relative abundance of the viable microbial community in a probiotic mixer

There are a lot of probiotic combinations worldwide. One of the most well-known probiotic trio in the world is made up of L. acidophilus, *L. delbrueckii* subsp. *bulguricus*, and *B. bifidum*. At present there is no published method for determining the count, relative abundance of the mixed probiotic strains with their contaminations, and their viability. In this study, we examined three sources of probiotic combination made up of the aforementioned species using 16S metagenomics and the conventional plate count method with a variety of selective and non-selective medium. Because each strategy has certain downsides, we combined two separate methodologies. Identification and separate microbial count for closely related microbial species is not possible by conventional plate count method. Contamination cannot be identified by this technique. The decimal serial dilution, which is the greatest obstacle to data repeatability, is the fundamental drawback of the plate count method. Pipetting and vortex mistakes are possible. In addition, totally different microbial proliferation may be seen due to varying medium composition. On the other hand, by 16S amplicon sequencing technique, viable and non-viable microorganism cannot be detected and species level report is not accurate [29]. PCR technique is applicable for determining the target species in a probiotic mixer, although it is not appropriate for non-target contamination in the mixer. This disadvantage can be overcome by 16S amplicon sequencing.

The result of high throughout 16S amplicon sequencing was obtained from genus level for comparing with plate count method. Only two genera of lactic acid bacteria were found in the probiotic sources. *Lactobacillus* spp. (65.63%, 88.80%, and 90.71% in Source-1, Source-2, and Source-3 respectively) was the most abundant genus, whereas *Bifidobacterium* spp. (27.08%, 3.66%, and 3.29% in Source-1, Source-2, and Source-3 respectively) was the second most genus in the probiotic sources according to 16S high throughout sequencing data. The percentage of *Lactobacillus* spp. (75.27%, 100%, 100% in Source-1, Source-2, and Source-3 respectively) and *Bifidobacterium* spp. (24.72%, 0.0%, 0.0% in Source-1, Source-2, and Source-3 respectively) in plate count method is strongly correlated (0.999) with the relative abundance of throughout sequencing **(Table 2)**. One possible reason for this is that large number of probiotic bacteria was present in the tested sample. Also, according to regression analysis result, plate count result is strongly predictable (R^2=0.999) from 16S metagenomics **(Table 2, Figure 4)**. We considered the following equation **(Figure 4)**:

$$\mathbf{y = 1.1576x - 3.4046}$$

Here, y= percentage of this genus in plate count method, x= percentage of this genus in16S metagenomics. To take an example, if the 30% *Bifidobacterium* spp. is present in metagenomics method, the percentage of this genus will be 30.77 in plate count technique (y=1.1576×30-3.4046

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=31.32). Regression analysis and correlation both provide compelling evidence for the validity of the methods' respective findings.

Table 2. Comparison of conventional plate count method with amplicon sequencing.

Source of probiotic combination	Classification	Number of Reads	Colony Count x10^{10}CFU/g	% Total Reads	Plate Count %	Correlation	Regression Statistics
Source-1	*Lactobacillus*	197,829	129.37 ±0.89	65.63 %	75.27%	0.999	Multiple R=0.999, R Square =0.998, Adjusted R Square =0.998, Standard Error=1.878.
	Bifidobacterium	81,617	42.50 ±3.53	27.08 %	24.72%		
	Unclassified at Genus level	13,728	-	4.55 %	-		
	Contamination	8258	0.0	2.74 %	0.0%		
Source-2	*Lactobacillus*	89,911	22.0±0.0	88.80 %	100%		
	Bifidobacterium	3,705	0.0	3.66 %	0.0%		
	Unclassified at Genus level	5,762	-	5.69 %	-		
	Contamination	1870	0.0	1.85%	0.0%		
Source-3	*Lactobacillus*	111,424	14.50±0.0	90.71 %	100%		
	Bifidobacterium	4,041	0.0	3.29 %	0.0%		

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	Unclassified at Genus level	5,090	-	4.14 %	-		
	Contamination	2280	0.0	1.86%	0.0%		

Note: - indicates not applicable. The "Contamination" category in this table is other than *Lactobacillus* spp. and *Bifidobacterium* spp. Total Genus-level Taxonomic Categories was identified as follows: Source: 1=210 Genus, Source: 2=167 Genus, and Source: 3=208 Genus. Correlation and regression were conducted between the two colour rows, where 'Unclassified at Genus level' was excluded.

The microbial abundance other than *Lactobacillu*s spp. and *Bifidobacterium* spp. was considered contamination in terms of genus level data. In Source-1, Source-2, and Source-3, respectively, contamination from high throughout sequencing results was reported at 2.74%, 1.85%, and 1.86%. This contamination probably arrived through nucleic acid extraction reagents and ultrapure water systems in high throughout sequencing process, or from probiotic sources directly. The former route is the most possible cause of contaminations, because the contaminations were Streptococcus, Mycobacterium, Enterococcus, Amycolatopsis and so on. Streptococcus, Mycobacterium, Enterococcus and many other genera are frequent contaminants of nucleic acid extraction reagents and ultrapure water systems [30]. Although these bacteria can grow on TSA media [31, 32, 33], no growth other than *Lactobacillus* was observed on this media from morphological and biochemical observations. One explanation for this might be that when the pollutants were grown on TSA medium, they were few, completely missing, or completely dead **(Table 2)**.

These findings lead us to the conclusion that 16S high throughput sequencing and the plate count approach, when used alongside one another, can be trustworthy methods for determining the count, relative abundance of the multi probiotic strains with their contaminations, and their viability in commercially available probiotic blends at the genus level. As far as we know, this combined method was not used previously in case of probiotic combinations.

According to the Certificate of Analysis (COA) of three sources, only the pure cultures of three species were mixed in the probiotic blend: *B. bifidum, L. acidophilus,* and *L. delbruecki* subsp. *bulguricus.* Although these three species were identified accurately in this study by 16S metagenomics, the main disadvantage observed was that many other species (mostly unexpected *Lactobacillus* ssp.) were detected by this process as well [29] (**Supplement 3:** 16S metagenomics of source-1, **Supplement 4:** 16S metagenomics of source-2, and **Supplement 5:** 16S metagenomics of source-3). The ratio of *B. bifidum* (22.85%), *L. acidophilus* (12.82%), and *L. delbrueckii* (10.69%) was found to be around 2:1:1 in source-1 of the species level data from 16S metagenomics. In source-1, the ratio of the three target species was detected in adequate level as compared to other sources. Therefore, we selected this source for comparative antagonistic analysis.

Antagonistic activity test and comparison

After 10-12 hours' incubation, the zone of inhibition was measured. The inhibition zones of more than 20mm, 10-20mm, and less than 10 mm were accepted as strong, intermediate, and low inhibition respectively. In our study the strain TY-11 had an intermediate inhibition against tested pathogens. We might get a better result than this if we would carry out our experiment by the simple spot-on lawn agar, because the Zone of Inhibition (ZOI) of a simple spot-on lawn agar is larger than that of the well diffusion method [34]. In another point, longer than 24-hour incubation of the probiotic culture from which we removed the supernatant for the antimicrobial efficacy test could also result in bigger ZOI. We found that the inhibition zone diameter was 18.88±0.18 mm (19±0.0 mm, and 18.75±0.35 mm) against *E. coli* ATCC 8739, which was the widest ZOI by agar well diffusion method. The inhibition zone diameter against *C. albicans* ATCC 10231 was 14.5±0.36 mm (15.0±0.0 mm, and 14.50±0.71 mm), which was the lowest ZOI **(Table 3)**. The result of this experiment suggested that the

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probiotic TY-11 might be used as a low-cost alternative for recovering *Escherichia coli* mediated diarrhoea and other foodborne illnesses around the world, especially in developing nations like Bangladesh.

Table 3. Antagonistic test.

Number of tests for probiotic strain→	2 tests (each with 2 replications) for the strain TY-11 ↓		2 tests (each with 2 replications) for the probiotic combination ↓			
pH→	pH3.97±0.01	pH3.99±0.0	pH 4.18±0.01		pH 4.09±0.01	
Probiotic count x10^7 CFU/mL→	23.5± 10.61	24.0± 8.49	La, Lb	Bb	La, Lb	Bb
			95.15±3.75	52.4±3.40	90.0±4.24	56.5±17.68
Supernatant quantity → (Probiotic culture)	100 µL	100 µL	100 µL		100 µL	
Diameter of ZOI (mm) →	Diameter of ZOI (mm) for the strain TY-11↓		Diameter of ZOI (mm) for probiotic combination (La, Lb, Bb) ↓			
Salmonella typhimurium ATCC 14028 (100µL of 1.90±4.24 x10^{5}CFU/mL) →	15.50±0.71	15.25±0.35	16.5±1.41		14.75±0.35	
Staphylococcus aureus ATCC6538 (200 µL of 1.18±17.68 x10^5 CFU/mL) →	15.50±0.71	15.0±1.41	13±0.0		13.0±0.0	
Escherichia coli ATCC 8739 (100 µL of 1.70±4.24 x10^5 CFU/mL) →	19±0.0	18.75±0.35	16.75±0.35		18.0±0.0	
Bacillus subtitlis ATCC 6633 (200 µL of 3.16±3.11 x10^{5}CFU/mL) →	16.5±0.0	15.75±1.06	13.25±0.35		15.0±0.0	
Pseudomonas aeruginosa ATCC 1427 (100 µL of 1.0±0.0 x10^5 CFU/mL) →	15.75±1.06	15.50±0.71	19.0± 0.71		15.0±0.0	
Candida albicans ATCC 10231 (200 µL of 1.49±6.36 x10^{5}CFU/mL) →	15.0±0.0	14.50±0.71	13.75±1.06		13.25±1.76	

Note: La indicates L. acidophilus, Lb indicates L. delbrueckii subsp. bulguricus, and Bb indicates B. bifidum.

Numerous earlier researches had demonstrated how the three probiotics (Source-1) can coexist and thrive at the same time [35, 36, 37]. They are the normal flora of the human intestine, and can grow mutually as well as suppress pathogens in the intestine. Although they show mutual growth, they may have minor internal inhibition that causes them to secrete more anti-pathogenic substances. Thus, we cultured this probiotic combination (Source-1) altogether in MRS broth for 24 hours at 35℃ to compare this with the TY-11's antimicrobial activity. After incubation, although the total count of probiotic combination (Source-1) was sufficient for our experiment, we counted *B. bifidum* separately in BD Bifidobacterium Agar, Modified; in contrast, *L. acidophilus*, and *L. delbrueckii* subsp. *bulguricus* were counted collectively on MRS agar. In our current investigation, it was not able to

separately count *L. acidophilus* and *L. delbrueckii* subsp. *bulguricus* either by plate counting or by amplicon sequencing. This limitation didn't affect our objective of comparative antimicrobial activity test. But after incubation, we independently quantified the CFU of TY-11. In the first and second tests, the probiotic combination count exceeded the TY-11 count by 6.27 and 6.10 times, respectively (**Table 3**). After incubation, the media's pH significantly altered (it was around pH 4.0 in both cases, probiotic combination and TY-11), implying that secreted acid was the cause of the pathogens' growth inhibition. Although it was not explored in our work, additional inhibitory substances might potentially be to blame.

In comparison to strain TY-11, which had 15.38±0.53 mm ZOI (15.50±0.71mm, and 15.25±0.35mm) against *Salmonella typhimurium* ATCC 14028; the probiotic combination had a slightly higher ZOI of 16.63±0.88 mm (16.5±1.41mm, and 14.75±0.35 mm). This minor increase in ZOI of probiotic combination also appeared against *Pseudomonas aeruginosa* ATCC 1427. In every other instance, the ZOI of the strain TY-11 was just marginally greater than the probiotic combination's ZOI. Overall, the joint antimicrobial effectiveness of this probiotic combination against six tested pathogens was almost equal (avoiding negligible change) to the antimicrobial activity of the single probiotic TY-11 (**Table 3, Supplement 2: Figure S3**). That indicated that the isolate TY-11 alone had the same ability to combat pathogens as the probiotic combination. This was the special godsend antimicrobial property of the strain TY-11. To battle infections, several probiotic manufacturers produce probiotic combinations today, albeit it might be challenging to maintain their viability for the specified shelf life and carefully manage quality. Our research demonstrated that a single effective probiotic—rather than many—might be sufficient to combat the target infections.

Conclusion

According to the results of our study, yogurt in Bangladesh can be a valuable source of probiotics with anti-*E. coli* action. In the current research project, we uncovered the probiotic characteristics of our isolated novel probiotic strain, TY-11 with anti-*E. coli* function. We relied on biochemical evaluation and resemblance to the antimicrobial characteristics of other probiotics, which concluded a number of prominent and unique qualities of the probiotic *L. delbrueckii* subsp. *indicus* TY-11 that were undoubtedly significant for explaining its intestinal inhabitancy and probiotic functions. The probiotic TY-11 may be utilized as an inexpensive substitute for preventing foodborne infections such diarrhoea caused by *E. coli*, particularly in developing countries like Bangladesh. Moreover, our study revealed that rather than using multiple probiotics, one powerful probiotic may be enough to treat the target infections. Previously, there has been no scientific evidence to justify such a specific comparison of antibacterial activity. Furthermore, we proved that, when combined, plate counting and 16S high-throughput sequencing can provide accurate estimates of the number, relative abundance, and viability of multiple probiotic strains, along with any contaminations they may have, in commercially available probiotic blends. To our knowledge, this combined method has never been used previously when it comes to probiotic combinations. Beside *in vitro* laboratory tests, probiotic features should be unveiled by *in vivo* tests in follow-up research.

Supplementary information: Supplement 1: Tables (Table S1, Table S2, Table S3); Supplement 2: Figures (Figure S1, Figure S2, Figure S3); Supplement 3: 16S metagenomics of Source-1; Supplement 4: 16S metagenomics of Source-2; Supplement 5: 16S metagenomics of Source-3.

Availability of data and materials: The 16S metagenomics data analysed during the current study are available at NCBI under the Project PRJNA1008867 at https://www.ncbi.nlm.nih.gov/sra/PRJNA1008867. The associated Sequence Read Archive (SRA) accession numbers are SRS18718052, SRS18718053, & SRS18718054 and BioSample accession numbers are SAMN37131996, SAMN37131997, & SAMN37131998. The accession number of 16S sequence for the isolate TY-11 is OQ652026.

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