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THE EFFECTS OF PROBIOTIC ISOLATES AGAINST ENTERIC PATHOGENIC MICROORGANISMS IN MICE MODEL

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Abstract: Probiotics are one such kind of bacteria that are ingested as a supplementary food to maintain or restore beneficial bacteria of the digestive tract which has a significant impact on living being and improves the gastrointestinal microflora inside the body. This research work assessed the properties of probiotic bacteria through isolation, biochemical analysis and the antimicrobial efficacy of isolated probiotic bacteria. To conduct the research, yogurt was considered as test samples and probiotic bacteria was isolated from these samples from different Divisions (Dhaka, Chattagram, Cumilla, Khulna, Sylhet, Mymensingh and Rajshahi) of Bangladesh. Morphological, biochemical and physiological analysis showed rod shaped morphology by colony morphology observation, Gram positive properties by Gram staining technique, non-motile properties by motility test and coagulase positive and catalase negative properties by coagulase test and catalase test respectively. These properties facilitated the identification of probiotic bacteria. To identify the presumptive probiotics, sugar fermentation pattern, 2-6% NaCl test, 0.4% phenol tolerance level test, 0.3% bile tolerance level test and pH resistance test were considered in which the probiotics showed excellent growth. Antimicrobial activity was observed using isolated bacteria against eight enteric pathogens. For determining the antimicrobial efficacy, pathogen-induced mice model trail was conducted by feeding with probiotic yogurt at different concentrations. At the end of the fifth week of probiotic treatment, the 3rd Treatment group (150 ml yogurt per kg body weight) showed significant ($p<0.001$) antimicrobial activity. In comparison to 2nd Treatment group (100 ml yogurt per kg body weight) and 3rd Treatment group (150 ml yogurt per kg body weight) with Control group**,** the mean difference was found significant ($p<0.001$) for the growth of inhibition zone of pathogenic bacteria. No significant difference was observed while the Standard group was compared to 3rd Treatment group especially for the measurement of IgG level. However, 3rd Treatment group exhibited significant ($p<0.001$) difference than the 2nd Treatment group and Control group. In the case of the body weight measurement, when the 3rd Treatment group was compared with Control group, the 3rd Treatment group gained a significant ($p<0.001$) body weight**.** In case of white blood cell count and determination of total protein level, albumin level and hemoglobin level, the treatment groups showed significant value than Control group. Molecular identification of isolated probiotic bacteria would be worthy to investigate for further development of probiotic product.

Keywords: Probiotic, Antimicrobial Activity, Mice Model, Pathogen, Lactic Acid Bacteria

1. INTRODUCTION

Probiotics are a large beneficial group of live microorganisms (FAO/WHO, 2019) and is also expressed as a term "for life" (Ozen *et al.*, 2015) that promotes an excellent health protection on the host when administered in an appropriate amount (Nadia *et al.*, 2019). To conduct any probiotic related applications accurately, probiotic species (*Lactobacillus, Enterococcus, Bifidobacterium* and *Lactococcus* etc.) must be genetically regarded as safe and commercially affordable (Gharaei-Fathabad

et al., 2011 and Hill *et al.*, 2014), bile and acid tolerant (Stellah *et al.*, 2020), capable of showing antimicrobial activity against pathogens and susceptible to antibiotics (Fijan, 2016). *Bifidobacterium and Lactobacillus* are well-known probiotics (Doron & Snydman, 2015). These probiotics express antibacterial activity against enteric pathogens by protecting and enhancing the whole immune system. Probiotic strain also produces antimicrobial compounds (bacteriocins, antioxidants etc.) that basically perform synergistically to increase the antimicrobial titer of the specific microorganisms (Diego *et al.*, 2021). In recent times, antibiotics are randomly used in the treatment of diseases and for this reason drug resistance is increasing day by day which is creating an alarming issue (Yang *et al.*, 2019). So, we can conclude that microbial therapy like yogurt and yoghurt like product-based therapy are promising approaches to defense against infectious diseases now a days. (Silva *et al.*, 2019; Aabbas and Jafri, 1992). There are many approaches such as *in vivo* models to determine the properties of antimicrobial activities containing probiotic bacteria through different clinical trials. (Mazaya *et al.*, 2015). Our research programme was planned to find out the efficiency of antimicrobial attributes of the required probiotic isolates by analyzing the biochemical characteristics in pathogen-induced animal (mice) model.

2. METHODOLOGY

2.1 Sample Collection

Collected yogurt samples were preserved in esky box containing ice from seven sweet shops namely Muslim Sweets and Confectionary (MSC), Shitol Vander (SV), Satkhira Ghosh Dairy (SGD), Flavors (FVS), Asia Sweet Meat & Cold Drinks (ASMCD), Modhubon (MB), Krisna Cabin (KC) from seven Divisions (Dhaka, Cumilla, Khulna, Chattagram, Rajshahi, Sylhet, and Mymensingh) in Bangladesh. 4°C is appropriate for storing the samples but -20°C was considered to protect them from contamination.

2.2 Media Preparation

De Man, Rogosa and Sharpe agar (MRS) media was prepared to increase the growth of probiotic bacteria e.g., Lactic Acid Bacteria. To make 100 ml solution, 100 ml distilled water was mixed with all the ingredients and autoclaved at 121°C for 15 minutes and then it was transferred to petri dishes**.** After thawing the sample, seven times serial dilution was done by using peptone water. Pour plate technique was used to prepare primary culture. After spreading the solution on the medium into each petri dishes, the petri plates were incubated at 37°C for 24 hours - 48 hours.

2.3 Preparation of Primary Subculture and Pure Isolates

The single colonies were observed after incubation period and sub-culture was done into other plates which were further incubated at 37°C for 24 hours - 48 hours. This sub-culturing process was performed for five to six times to acquire pure isolates and the pure isolated bacterial strains were identified by morphological, biochemical and physiological properties.

2.4 Morphological Characterization

After developing the bacterial colony, the colony morphology was observed in the naked eye, but microscopic examination would be better to get appropriate separated colonies.

2.5 Gram Staining

Light microscopic examination was used to observe the reaction. For Gram staining test, after picking up the single colony, smearing and drying process was done aseptically for 5 minutes and heat fixed. 1 ml crystal violet solution was placed on heat fixed smeared slide, then was washed away with water, afterwards, iodine solution was added and washed with clean water. 95% ethanol was added for 30 seconds and after washing it, safranin was placed. It was then washed with clean water and dried with cotton towel gently. After Gram staining and examined under 40X light microscope, Gram negative bacteria appeared red in color and Gram-positive bacteria as blue in color.

2.6 Method of Motility Testing

Semi solid medium such as MIL medium was considered to get the better result for motility testing. After overnight culturing, the isolates were incubated under the conditions which favored motility. After 24 hours of incubation, a single colony was inoculated into 10 ml sterilized MIL semi solid broth medium and stabbed in every test tube. The needle was entered and removed at the same line. Again, incubation

was done at 37°C for 24 hours. After observation, it was concluded that Lactic Acid Bacteria were non-motile and produced only the stabbed line.

2.7 Catalase Test

Slide method was preferred to conduct the catalase test. A little quantity of culture was transferred from petri dish to glass slide. Then, 3% H_2O_2 solution was placed in the sterile glass slide**.** After observation, no bubbles were found there. So, it was concluded that Lactic Acid Bacteria were catalase negative.

2.8 Maintenance of Lactic Acid Bacteria

Subculture was done for 2-3 weeks for the further use of bacteria. For the maintenance of bacteria, sterilized glycerol (20%) in MRS medium was used and the isolates were preserved in -20°C.

2.9 Probiotic Property Analysis

Probiotic property analysis was done by the following biochemical test:

2.9.1 pH Resistance Test

After overnight culture in MRS medium, the growth of isolates was observed by adjusting at pH 3.0 with 5N HCl and then incubated at 37°C for 24 hours. Absorbance was determined at 620 nm at every 4-hour interval which helped to calculate the low tolerance level of pH. Control group (Positive) was prepared by inoculating bacterial cells and Control group (Negative) was prepared without inoculating bacterial cells into normal MRS media.

2.9.2 Bile Level Tolerance Test

To determine the tolerance level of bile, this experiment utilized the sterilized medium (15 ml MRS broth) containing 0.3% bile. After inoculation of this bile with overnight grown isolates culture (20 µl), the incubation was done for the culture at 37°C for 24 hours and absorbance was determined at 620 nm at every 4 hours interval for 4 times (total 16 hours).

2.9.3 NaCl Tolerance Test

Different NaCl concentrations (2%, 4%, 6% and 8%) were used in MRS broth medium. At first, sterilization was done and then 1% overnight grown culture (Lactic Acid Bacteria) was added in each test tubes. After inoculation, incubation was done at 37ºC for 24 hours. After that, the medium with different salt concentrations were inoculated into 20 µl fresh culture media in 15 ml broth. After incubation, absorbance was determined at 620 nm to identify the NaCl tolerance of the isolates at every 4 hours interval.

2.9.4 Phenol Tolerance Test

Different amount of phenol concentrations (0.1%, 0.2%, 0.3% and 0.4%) were used in MRS medium. Then, 1% overnight grown culture was added and the incubation temperature was done at 37°C for 24 hours. Subsequently, absorbance was measured at 620 nm to monitor the growth of the isolates at every 4 hours interval.

2.9.5 Coagulase Test

Isolates (20 µl) were inoculated into 10 ml cow's milk (sterilized at 121°C). After incubation period was over, it was found that coagulation of milk led to the presence of Lactic Acid Bacteria.

2.9.6 Sugar Fermentation Test

Fructose, glucose, sorbitol, sucrose, maltose, lactose, galactose, xylose, mannitol and raffinose were chosen to conduct this assay. 5% solution of these sugars were added in MRS broth (pH 6.5) and then autoclaved at 121°C for 15 minutes. After that, incubation was done at 37°C for 24 hours. At that time, the color of the medium changed and gas formation occurred due to the production of acid which ensured the presence of desired bacteria (Lactic Acid Bacteria).

2.9.7 Antimicrobial Property Test

Antagonistic activity was tested for the Lactic Acid Bacteria isolates against eight bacterial pathogens *viz. Bacillus megaterium***,** *Vibrio cholerae***,** *Micrococcus*, *Staphylococcus aureus***,** *Salmonella paratyphi*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi*. After

inoculating the Lactic Acid Bacterial strains in MRS medium, centrifugation was done at 5000 rpm for 15 minutes in order to remove bacterial cells. Then, NaOH (0.1 N) was added in the supernatant and pH was set at 6.5. Cellulose acetate was used for the filtration of the supernatant and the supernatant was preserved at 4°C. Supernatant was preferred than the pellet for antagonistic test.

Disc diffusion assay was conducted to determine the antimicrobial activity of the selected probiotic. The selected pathogenic microorganisms were spread over the surface of MRS solid media. After 1 hour, sterile blank paper discs were placed on the agar plate. 20 µl of supernatant of indicator pathogenic microorganism and pellets of probiotic isolates were inoculated on agar plate determining the antimicrobial activity. The plates were allowed to diffuse at 4°C for 30 minutes and the incubation was done at 37°C for 24 hours. Control group (Positive) was prepared using the antibiotic streptomycin and the discs dipped into sterile water were used as Control (Negative). The zone of inhibition was measured in millimeters (mm).

2.10. Animal Trial

2.10.1 Yogurt Preparation

1liter fresh cow milk was taken in a beaker and was heated at 100ºC for 15 minutes. Then, the cow milk was cooled at room temperature for 15 minutes and afterwards 5% yogurt bacteria (Lactic Acid Bacteria) was added into it. It was then mixed with the help of a glass rod and fermented at 37ºC for 12 hours. After fermentation is completed, it was preserved in a storage tank at 4ºC.

2.10.2 Feeding Trial Programme in Animal Model

36 Swiss albino mice with average weights of 18-22 gm were taken and divided into 6 groups, each group having 6 mice. All the mice were provided by International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b). The experimental groups were randomly selected for feeding trial. All the mice groups were provided with their respective oral treatment. The feeding programme has been described in Table 1.

Table 1: Feeding programme in mice

Group Name	1st Week of Feeding Trial	2nd Week of Feeding Trial	3rd Week of Feeding Trial	4th Week of Feeding Trial	5th Week of Feeding Trial
Control group (positive and negative group) (No. of Mice = 6)	Balanced diet and water	Balanced diet and water	Balanced diet and water	Balanced diet and water	Balanced diet and water (Negative control) Balanced diet and water + *Escherichia coli* (Positive control)
Standard group (No. of Mice = 6)	Balanced diet and water	Balanced diet and water	Balanced diet and water	Balanced diet and water	Balanced diet and water + preferred antibiotic group (Ciprofloxacin 500 mg containing Fluoroquinolone) + *Escherichia coli*
1st Treatment group (No. of Mice = 6)	Balanced diet and water	Balanced diet and water + 50 ml yogurt (Lab prepared probiotic)	Balanced diet and water + 50 ml yogurt (Lab prepared probiotic)	Balanced diet and water + 50 ml yogurt (Lab prepared probiotic)	Balanced diet and water + 50 ml yogurt (Lab prepared probiotic) + *Escherichia coli*
2nd Treatment Group (No. of Mice = 6)	Balanced diet and water	Balanced diet and water + 100 ml yogurt (Lab prepared probiotic)	Balanced diet and water + 100 ml yogurt (Lab prepared probiotic)	Balanced diet and water + 100 ml yogurt (Lab prepared probiotic)	Balanced diet and water + 100 ml yogurt (Lab prepared probiotic) + *Escherichia coli*
3rd Treatment group (No. of Mice = 6)	Balanced diet and water	Balanced diet and water + 150 ml yogurt (Lab prepared probiotic)	Balanced diet and water + 150 ml yogurt (Lab prepared probiotic)	Balanced diet and water + 150 ml yogurt (Lab prepared probiotic)	Balanced diet and water + 150 ml yogurt (Lab prepared probiotic) + *Escherichia coli*

2.10.3 Blood Collection

Blood was collected from all the mice which were sacrificed after 12 hours fasting at the end of the 5th week. Then, Blood serum was centrifuged at 1500 rpm for 15 minutes.

2.10.4 Parameter of Antimicrobial Activity Analysis

2.10.5 Body Weight

Body weight of all mice were measured at every 1-week interval. The data were recorded for statistical analysis.

2.10.6 Complete Blood Count (CBC) Test

For CBC test, total red blood cell (RBC) test and total white blood cell (WBC) test were performed.

2.10.6.1 Total Red Blood Cell (RBC) Count

RBC fluid (3 gm sodium citrate, 1 ml formalin and 100 ml distilled water) was taken in a RBC pipette (range 0.5 and 101). Then, RBC fluid was mixed properly with blood serum sample and we waited for 5-10 minutes. The pipette was shaked well and 2-3 drops of mixed sample were discarded from the mixture and 1 drop of mixed sample was placed in an improved neubauer chamber. After 1-2 minutes, it was observed under a microscope. RBC was counted using standard protocol from the following formula:

Calculation of RBC/Cu mm = Number of cells counted × Blood dilution × Chamber depth / Area of chamber counted.

2.10.6.2 Total White Blood Cell (WBC) Count

Blood serum sample and WBC fluid were taken in a WBC pipette (range 0.5 and 11). Then, WBC fluid was mixed properly with blood serum sample and we waited for 4-5 minutes. A special cover slip was placed in an improved neubauer chamber. The WBC pipette was shaked well and first ½ drop was discarded and 1 drop was placed in the joint section between neubauer chamber and special cover slip. After ½ minute, it was observed under a microscope. WBC was counted using standard protocol from the following formula:

Calculation of WBC/Cu mm = Number of cells counted × Blood dilution × Chamber depth / Area of chamber counted.

2.10.7 Immunoglobulin G (IgG) Test: ELISA Method

20 µl serum was collected from blood sample and 100 µl conjugate enzyme was added into it. Then, we waited for 1 hour and the mixture was washed for 5 times with distilled water. As a result, 100 µl substrate was formed from the reaction. Then, we waited for 20 minutes and 50 µl stock solution was prepared for reading in Multiscan FC hormone analyzer machine.

2.10.8 Determination of Total Protein, Albumin and Globulin Level

End point method was used to determine the both the total protein level and albumin level. Protein liquicolor and albumin liquicolor were used as reagent for protein and albumin level determination respectively. For colorimeter, the wavelength was set at 530 nm and for analyzer, the wavelength was set at 564 nm.

2.10.9 Leishman Stain

Leishman stain is used to test the viability of red blood cell, white blood cell and platelets. 5-10 drops of blood sample were placed on a glass slide and we waited for 2 minutes. Then, 10-20 drops of distilled water were added into the glass slide. Afterwards, the glass slide was incubated for 10 minutes at 37°C. The glass slide was rinsed with the distilled water until it produces purple pinkish color and it was observed under microscope.

2.10.10 Pathogen Used for Antimicrobial Activity Test

DifoTM MacConkey broth without salt of pH 7.1 was used for the growth of *Escherichia coli*. The culture was grown at 37°C for 18 hours in order to reach the final amount of 10^2 CFU/ml. Mice models were infected with 0.1 ml of these bacterial cells. At the second day of post-infection, the feces of the infected mice of Control group and infected mice of the probiotic supplemented groups were collected. 1 gm fecal sample was collected from every group and 10-fold serial dilution was performed. 0.1 ml diluted

solution from each group were plated on difoTM MacConkey agar without salt of pH 7.1. After culturing, produced colonies were observed and counted using the following equation:

No. of bacteria/ml in sample = No. of colonies (CFU)/Dilution no. × Amount of sample plated

3. Results

3.1 Physiological and Biochemical Analysis of Lactic Acid Bacteria

Yogurt samples were derived from Dhaka, Cumilla, Khulna, Chattagram, Rangpur, Rajshahi and Sylhet Divisions. The seven isolates were characterized by physiological and biochemical analysis. The results are given below (Table 2).

Table 2: The properties of probiotic isolates characterized by biochemical and physiological analysis from selected samples of seven different Divisions in Bangladesh.

Name of the Division	Biochemical Test: Result of Gram Staining	Biochemical Test: Result of Catalase Test	Biochemical Test: Result of Coagulase Test	Physiological Property Test: Result of Motility Test
Dhaka Division Muslim Sweets and Confectionary (MSC)	+	-	+	-
Cumilla Shitol Vander (SV)	+	-	+	-
Khulna Satkhira Ghosh Dairy (SGD)	+	-	+	-
Chattagram Flavors (FVS)	+	-	+	-
Rajshahi Asia Sweet Meat & Cold drinks (ASMD)	+	-	+	-
Sylhet Modhubon (MB)	+	-	+	-
Mymensingh Krisna Cabin (KC)	+	-	+	-

* (+) means reaction positive; (-) means reaction negative

3.2 Identification of Probiotic Isolates

3.2.1 pH Resistance Test

The MSC, SV, SGD, FVS, ASMD, MB and KC strains were observed for cell viability at pH 3.0. Result have been shown in Figure 1 and in Table 3.

3.2.2 Bile Salt Tolerance Level Test

The MSC, SV, SGD, FVS, ASMD, MB and KC isolates exhibited tolerance level of 0.3% bile salt. Optical density was measured at 620 nm. Result have been shown in Figure 2 and in Table 3.

3.2.3 NaCl Tolerance Test

The selected isolates of bacteria had the ability to tolerate up to 6% NaCl concentration. Result have been shown graphically in Figure 3, Figure 4, Figure 5, Figure 6 and enlisted in Table 3.

3.2.4 Phenol Tolerance Test

All the isolates showed positive effects on phenol tolerance test. Results are shown graphically in Figure 7, Figure 8, Figure 9, Figure 10 and enlisted in Table 3.

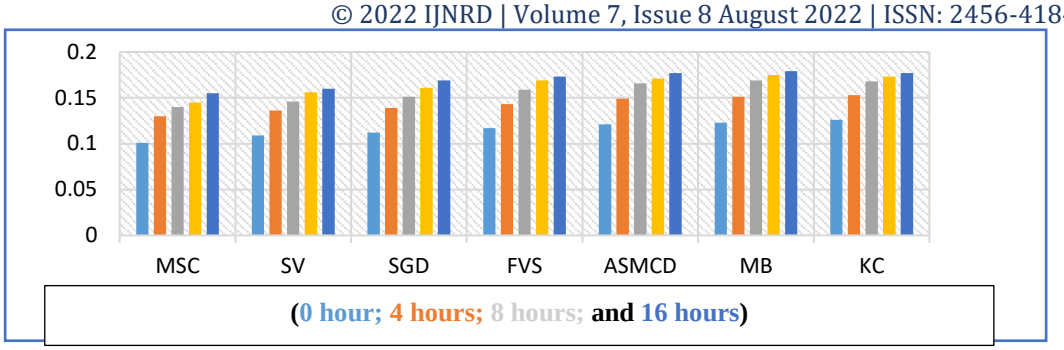


Figure 1: pH tolerance test of the isolates obtained in every 4 hours intervals from selective samples of seven Divisions in Bangladesh.

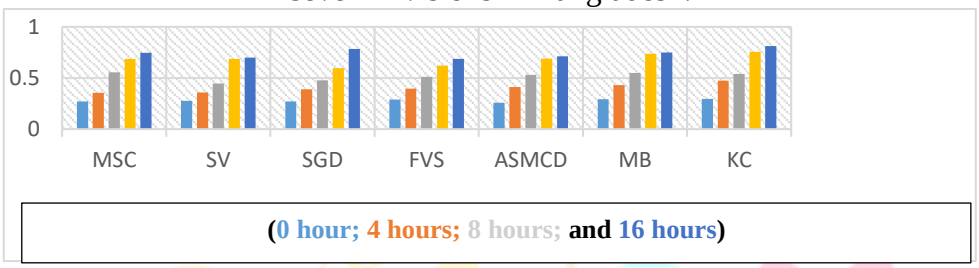


Figure 2: The tolerance level test of bile salt of the required isolates obtained in every 4 hours intervals from selective samples of seven Divisions in Bangladesh.

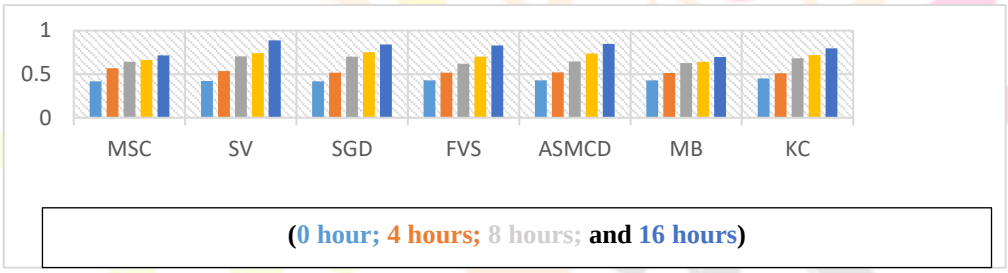


Figure 3: 2% NaCl tolerance level test of the isolates obtained from seven Divisions of Bangladesh.

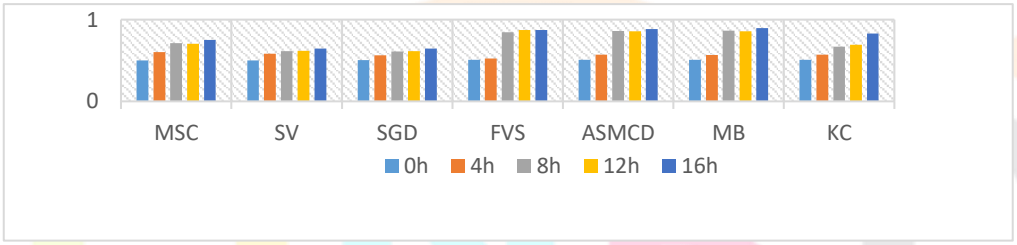


Figure 4: 4% NaCl tolerance test of the probiotic isolates obtained from seven Divisions of Bangladesh.

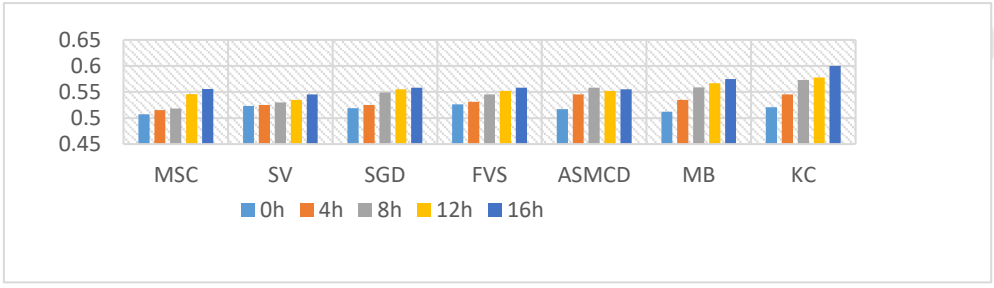


Figure 5: 6% NaCl tolerance test of the probiotic isolates obtained from seven Divisions of Bangladesh.

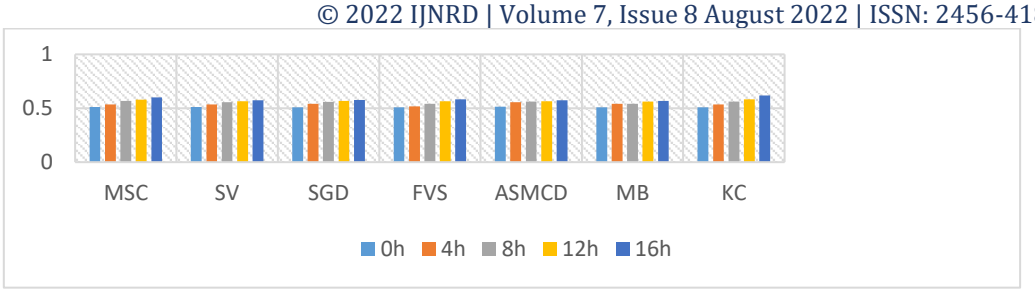


Figure 6: 8% NaCl tolerance test of the probiotic isolates obtained from seven Divisions of Bangladesh.

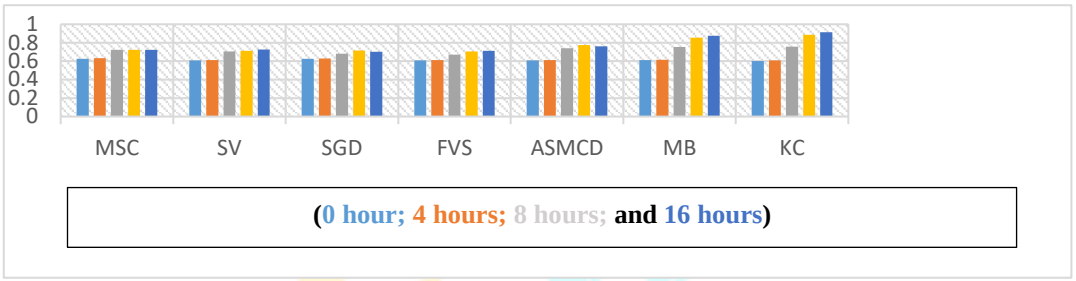


Figure 7: 0.1% Phenol tolerance test of the probiotic isolates obtained from seven Divisions of Bangladesh.

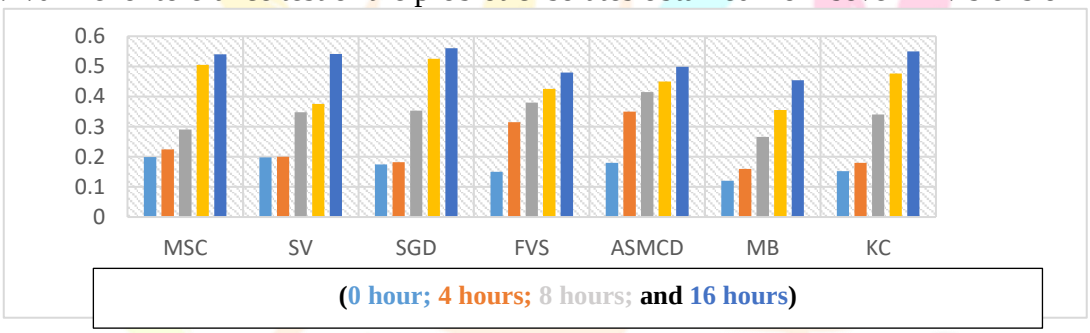


Figure 8: 0.2% Phenol tolerance test of the probiotic isolates obtained from seven Divisions of Bangladesh.

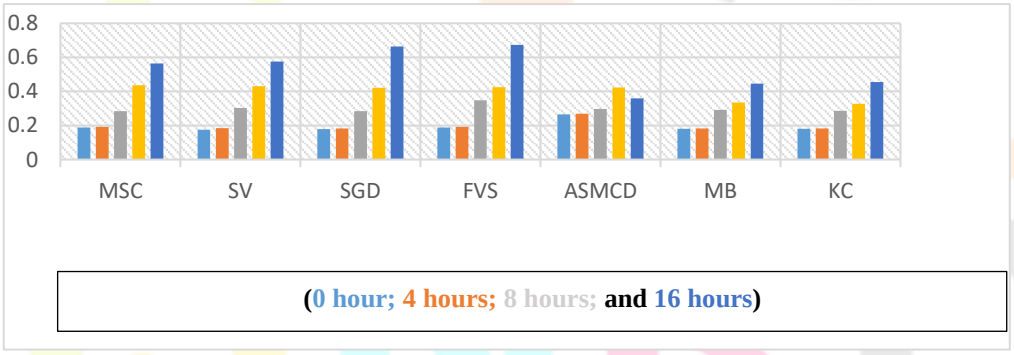


Figure 9: 0.3% Phenol tolerance test of the probiotic isolates obtained from seven Divisions of Bangladesh.

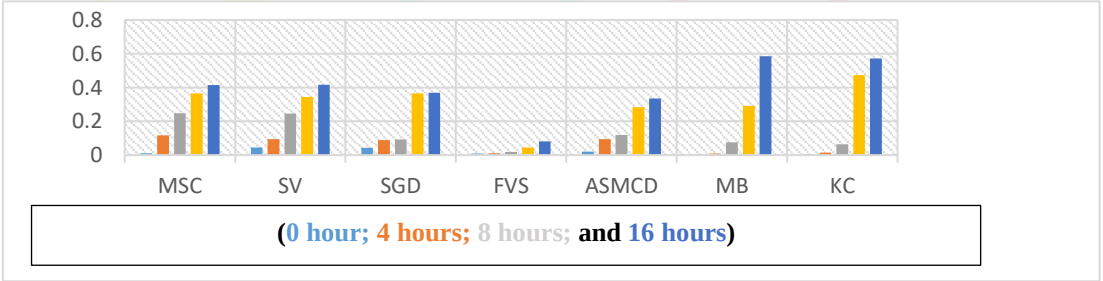


Figure 10: 0.4% Phenol tolerance test of the probiotic isolates obtained from seven Divisions of Bangladesh.

Table 3: Probiotic properties of isolates from selected yogurt samples obtained from Seven Divisions of Bangladesh

Name of Isolates	Identification of Probiotic Property									
	Result of Low pH Tolerance Test	Result of Bile Level Tolerance Test	Result of NaCl Tolerance Test				Result of Phenol Tolerance Test			
			2% NaCl Conc.	4% NaCl Conc.	6% NaCl Conc.	8% NaCl Conc.	0.1% Phenol Conc.	0.2% Phenol Conc.	0.3% Phenol Conc.	0.4% Phenol Conc.
Dhaka Muslim Sweets and Confectionary (MSC)	+	+	+	+	-	-	+	+	+	+
Cumilla Shitol Vander (SV)	+	+	+	+	-	-	+	+	+	+
Khulna Satkhira Ghosh Dairy (SGD)	+	+	+	+	-	-	+	+	+	+
Chattagram Flavors (FVS)	+	+	+	+	-	-	+	+	+	+
Rajshahi Asia Sweet Meat & Cold Drinks (ASMCD)	+	+	+	+	-	-	+	+	+	+
Sylhet Modhubon (MB)	+	+	+	+	-	-	+	+	+	+
Mymensingh Krisna Cabin (KC)	+	+	+	+	-	-	+	+	+	+

* (+) means reaction positive; (-) means reaction negative

3.2.5 Sugar Fermentation Test

MSC, SV, SGD, FVS, ASMD, MB, KC isolates showed positive results in the sugar fermentation test against 8 different sugars except maltose and sorbitol. After inoculation, the broths' color changed from purple to yellow due to the presence of acidic environment.

3.2.6 Antimicrobial Property Test

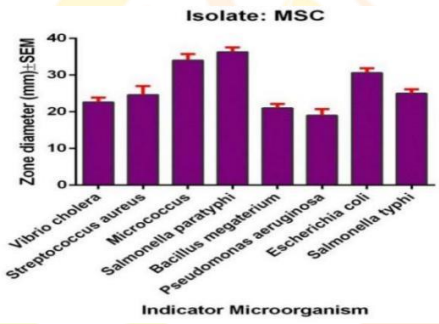
Almost all the isolates showed sensitivity against pathogens. Isolates from Cumilla (SV) showed negative result against *Micrococcus, Salmonella typhi* and *Escherichia coli.* Isolates from Rajshahi (ASMCD) were resistant to *Micrococcus*. Again, Isolates from Mymensingh (KC) showed resistance against *Pseudomonas aeruginosa* (Figure 11)

Figure 11: Antimicrobial activity of Dhaka (MSC) isolates against *Pseudomonas aeruginosa*

Table 4: Antimicrobial property test of selected probiotic isolates against pathogens

Name of the probiotic isolates	Name of the pathogenic microorganism							
	Escherichia coli	*Pseudomonas aeruginosa*	*Salmonella paratyphi*	*Streptococcus aureus*	*Salmonella typhi*	*Bacillus megaterium*	*Micrococcus*	*Vibrio cholera*
MSC	+	+	+	+	+	+	+	+
SV	-	+	+	+	-	+	-	+
SGD	+	+	+	+	+	+	+	+
FVS	+	+	+	+	+	+	+	-
ASMCD	+	+	+	+	+	+	-	+
MB	+	+	+	+	+	+	+	+
KC	+	-	+	+	+	+	+	+

(+) means the isolates showed antimicrobial activity and (-) means isolates didn't show antimicrobial activity.



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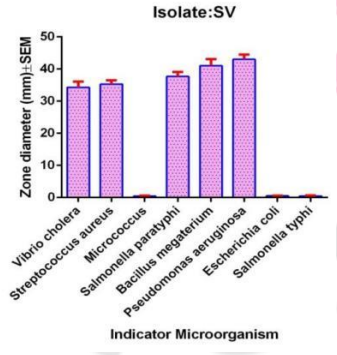

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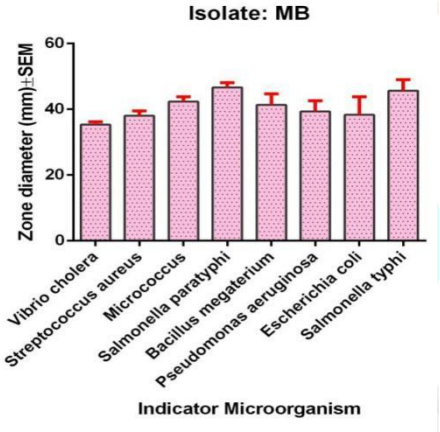
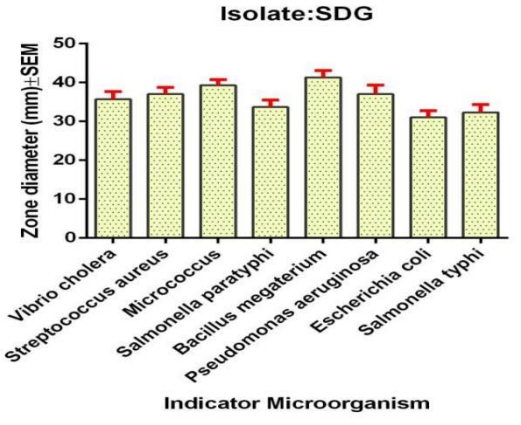
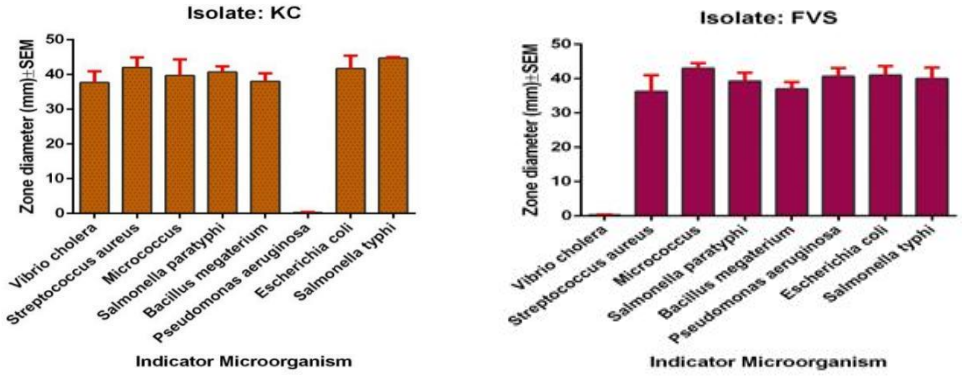


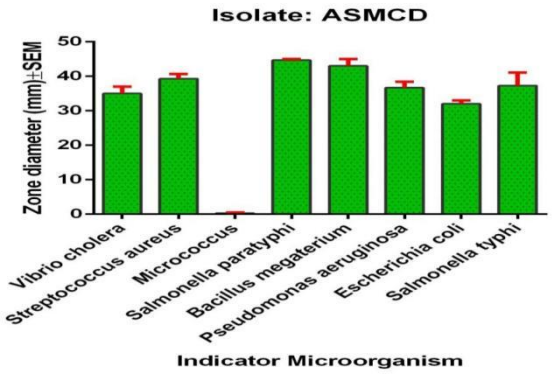
(3)



(4)



(5) (6)

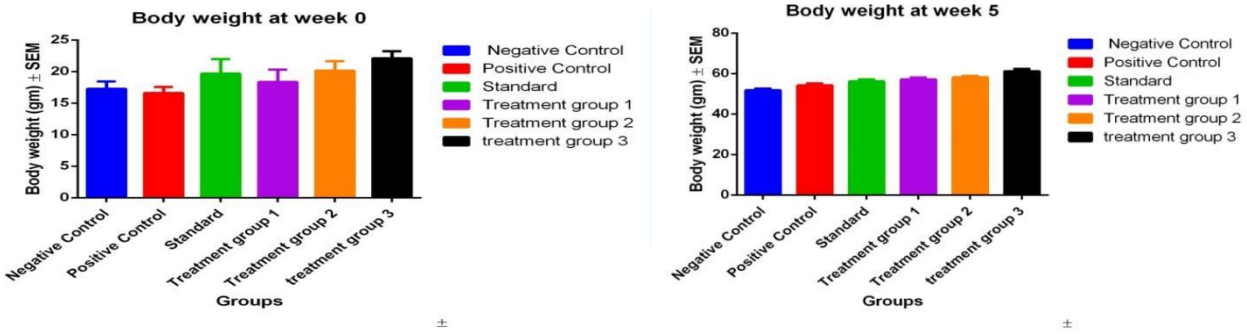


(7)

Figure 12: Antimicrobial activity test of the probiotic isolates obtained from (1) Dhaka (MSC), (2) Cumilla (SV), (3) Sylhet (MB), (4) Khulna (SDG), (5) Mymensingh (KC), (6) Chattagram Division (FVS) and (7) Rajshahi (ASMCD).

3.2.7 Impacts of Probiotics on the Body Weight Gain in Mice

18-22 gm weighted mice at the 0 week were considered for probiotic treatment. Feeding programme was designed for five weeks. Body weight of all mice were measured at every 1-week interval after starting the programme. Our study reveals that the body weight of mice increased significantly after inducing with probiotics of different concentration. At the end of the 5th week, it was found that the body weight (mean weight) of the 3rd treatment group was highest than Control group and other treatment groups. After the completion of the treatment programme, all the treatment groups exhibited significant differences ($p<0.001$) than the Control group.



i) (ii)

Figure 13: (i) Body weight measurement at the 0 week and (ii) Body weight measurement at the 5th week

3.2.8 Impacts of probiotics in Pathogen-infected Mice Model

Different parameters such as total protein level, total albumin level, total globulin level, total white blood cell (WBC) count, total hemoglobin level and total I_gG level were considered to find out the effects of probiotic in mice model through a feeding trial. Control group (Positive) was compared with Control group (Negative), Standard group and other treatment groups. Again, Control group (Negative) was compared with Control group (Positive), Standard group and other treatment groups. The analysis was done by One-way ANOVA method. The experimental data have been given below:

Table 5: Comparison of total protein (g/l) level of Control group (Negative) with Control group (Positive), Standard group, 1st Treatment group, 2nd Treatment group and 3rd Treatment group by One-way ANOVA method

Groups	Mean Difference ± SD
Control group (Negative) vs Control group (Positive)	-25.34±1.1
Control group (Negative) vs Standard group	1.5±1.26
Control group (Negative) vs 1st Treatment group	2±0.31
Control group (Negative) vs 2nd Treatment group	1.83±0.23
Control group (Negative) vs 3rd Treatment group	-2.34±0.66

Table 6: Comparison of total protein (g/l) level of Control group (Positive) with Control group (Negative), Standard group, 1st Treatment group, 2nd Treatment group and 3rd Treatment group by One-way ANOVA method

Groups	Mean Difference ± SD
Control group (Positive) vs Control group (Negative)	25.34±1.1
Control group (Positive) vs Standard group	26.84±0.16
Control group (Positive) vs 1st Treatment group	27.3±0.79
Control group (Positive) vs 2nd Treatment group	27.17±0.87
Control group (Positive) vs 3rd Treatment group	23±0.44

Table 7: Comparison of albumin (g/l) level of Control group (Negative) with Control group (Positive), Standard group, 1st Treatment group, 2nd Treatment group and 3rd Treatment group by One-way ANOVA method

Groups	Mean Difference ± SD
Control group (Negative) vs Control group (Positive)	-37.1±1.2
Control group (Negative) vs Standard group	4.9±1.3
Control group (Negative) vs 1st Treatment group	4.4±0.41
Control group (Negative) vs 2nd Treatment group	2.73±0.38
Control group (Negative) vs 3rd Treatment group	-0.93±0.53

Table 8: Comparison of albumin (g/l) level of Control group (Positive) with Control group (Negative), Standard group, 1st Treatment group, 2nd Treatment group and 3rd Treatment group by One-way ANOVA method

Groups	Mean Difference ± SD
Control group (Positive) vs Control group (Negative)	37.1±1.2
Control group (Positive) vs Standard group	42.0±0.1
Control group (Positive) vs 1st Treatment group	41.5±1.61
Control group (Positive) vs 2nd Treatment group	39.83±1.58
Control group (Positive) vs 3rd Treatment group	36.17±1.73

Table 9: Comparison of globulin (g/l) level of Control group (Negative) with Control group (Positive, Standard group, 1st Treatment group, 2nd Treatment group and 3rd Treatment group by One-way ANOVA method

Groups	Mean Difference ± SD
Control group (Negative) vs Control group (Positive)	-5.67±2.72
Control group (Negative) vs Standard group	2.0±0.62
Control group (Negative) vs 1st Treatment group	4.67±0.7
Control group (Negative) vs 2nd Treatment group	3.17±2.28
Control group (Negative) vs 3rd Treatment group	2.17±0.43

Table 10: Comparison of globulin (g/l) level of Control group (Positive) with Control group (Negative), Standard group, 1st Treatment group, 2nd Treatment group and 3rd Treatment group by One-way ANOVA method

Groups	Mean Difference ± SD
Control group (Positive) vs Control group (Negative)	5.67±2.72
Control group (Positive) vs Standard group	7.67±2.1
Control group (Positive) vs 1st Treatment group	10.34±2.02
Control group (Positive) vs 2nd Treatment group	8.84±0.44
Control group (Positive) vs 3rd Treatment group	7.84±3.15

Table 11: Comparison of white blood cell (WBC)/Cu mm of Control group (Negative) with Control group (Positive), Standard group, 1st Treatment group, 2nd Treatment group and 3rd Treatment group by One-way ANOVA method

Groups	Mean Difference ± SD
Control group (Negative) vs Control group (Positive)	-1.9±0.56
Control group (Negative) vs Standard group	0.3±1.0
Control group (Negative) vs 1st Treatment group	0.8±0.73
Control group (Negative) vs 2nd Treatment group	0.4±0.83
Control group (Negative) vs 3rd Treatment group	0.1±0.96

Table 12: Comparison of white blood cell (WBC)/Cu mm level of Control group (Positive) with Control group (Negative), Standard group, 1st Treatment group, 2nd Treatment group and 3rd Treatment group by One-way ANOVA method.

Groups	Mean Difference ± SD
Control group (Positive) vs Control group (Negative)	1.9 ±0.56
Control group (Positive) vs Standard group	2.2± 0.44
Control group (Positive) vs 1st Treatment group	2.7± 0.17
Control group (Positive) vs 2nd Treatment group	2.3± 0.27
Control group (Positive) vs 3rd Treatment group	2.0±0.4

Table 13: Comparison of hemoglobin (gm/dl) level of Control group (Negative) with Control group (Positive), Standard group, 1st Treatment group, 2nd Treatment group and 3rd Treatment group by One-way ANOVA method.

Groups	Mean Difference ± SD
Control group (Negative) vs Control group (Positive)	-1.27±.03
Control group (Negative) vs Standard group	1.22±.06
Control group (Negative) vs 1st Treatment group	2.83±0.18
Control group (Negative) vs 2nd Treatment group	2.68±0.16
Control group (Negative) vs 3rd Treatment group	1.92±.12

Table 14: Comparison of hemoglobin (gm/dl) level of Control group (Positive) with Control group (Negative), Standard group, 1st Treatment group, 2nd Treatment group and 3rd Treatment group by One-way ANOVA method

Groups	Mean Difference ± SD
Control group (Positive) vs Control group (Negative)	1.27±0.03
Control group (Positive) vs Standard group	2.49±.03
Control group (Positive) vs 1st Treatment group	4.0±0.15
Control group (Positive) vs 2nd Treatment group	3.95±0.16
Control group (Positive) vs 3rd Treatment group	3.19±0.15

Table 15: Comparison of IgG (g/l) level of Control group (Negative) with Control group (Positive), Standard group, 1st Treatment group, 2nd Treatment group and 3rd Treatment group by One-way ANOVA method

Groups	Mean Difference ± SD
Control group (Negative) vs Control group (Positive)	-4.67±0.52
Control group (Negative) vs Standard group	-4.57±0.08
Control group (Negative) vs 1st Treatment group	-4.41±0.37
Control group (Negative) vs 2nd Treatment group	-5.51±0.11
Control group (Negative) vs 3rd Treatment group	-7.25±0.86

Table 16: Comparison of IgG (g/l) level of Control group (Positive) with Control group (Negative), Standard group, 1st Treatment group, 2nd Treatment group and 3rd Treatment group by One-way ANOVA method

Groups	Mean Difference ± SD
Control group (Positive) vs Control group (Negative)	4.67±0.52
Control group (Positive) vs Standard group	0.1±0.44
Control group (Positive) vs 1st Treatment group	0.26±0.89
Control group (Positive) vs 2nd Treatment group	-0.83±0.41
Control group (Positive) vs 3rd Treatment group	-2.58±0.34

3.2.9 Identification of Zone of Inhibition of Enteric Pathogenic Microbes

Among all the trial groups, the treatment groups exhibited significant zone of inhibition of *Escherichia coli* compared to the Control group (Positive) (Table 17). The Control group (Positive) contained 6 million (cfu/ml) pathogenic microbes. The 3rd Treatment group containing 2.65 million (cfu/ml) pathogenic microbes showed the largest zone of inhibition. Therefore, the overall result was significant ($p<0.001$). The mean difference of 2nd Treatment group and 3rd Treatment were significant ($p<0.001$) than the Control group (Positive). The mean difference of 1st Treatment group was not significant with respect to the Control group

Table 17: Zone of inhibition of pathogenic microorganism (*Escherichia coli*) in Control group (Positive), Standard group and different treatment groups.

Groups	Mean ± SD
Control group (Positive)	6.2±0.87
Standard group	2.6±0.50
1st Treatment group	3.85 ±0.51
2nd Treatment group	2.75 ±0.60
3rd Treatment group	2.65±0.35

4. DISCUSSION

All the isolates were irregular, small, rod shaped with brownish or whitish colour which corresponds to the species of *Lactobacillus*. Tallapragada *et al.*, 2018 reported that *Lactobacillus* spp. contain morphological properties like small, rod-shaped bacteria which were morphologically similar to the results of this study. According to Soni *et al*., 2021 and Hensyl, 1994, strains of *Lactobacillus* are non-motile, catalase negative and Gram positive which are supported by the findings of biochemical of this study. Coagulase test was found to be positive and the isolates also showed positive results for sugar fermentation test for all the selected sugars except maltose and sorbitol. All the biochemical tests were carried out according to Bergey's manual and the overall results were similar to the reported results of Prabhurajeshwar and Kelmani (2019).

The experimental findings showed that the probiotic isolates were resistant to pH 3.0. Isolates from Sylhet (MB) was most resistant to low pH among all the isolates. But isolates from Cumilla (SV), Dhaka (MSC), Khulna (SGD), Rajshahi (ASMCD), Chattagram (FVS) and Mymensingh (KC) showed a slight decrease in optical density after 16 hours of observation. According to Jin *et al.*, 1998 isolates from chicken intestine were moderately resistant at pH 3.0. Gilliand *et al.*, 1984 & Graciela and Maruia, 2001 reported that maximum 0.3% bile salt level was tolerable for consumption. Our research study reveals that all the selected isolates exhibited tolerance to 0.3% bile salt level after 16 hours of observation. Mymensingh (KC) and Khulna (SGD) were found to be highly tolerant to 0.3% bile salt level which indicates that the isolates can be considered as potential probiotic. In our study, in case of phenol tolerance test (0.1%-0.4%), all the isolates showed no significant result at the end of 16 hours of observation, But, after 4 hours of observation, the isolates from Chattagram (FVS) exhibited lessen growth at 0.4% phenol level which is similar to the reported findings of Yadav *et al.*, 2016. Different NaCl concentrations (2%, 4%, 6% and 8%) were considered as inhibitory substance in order to test the tolerance level of probiotic isolates. The probiotic isolates showed significant level of tolerance against 2% and 4% concentrations of NaCl and moderate level of tolerance against 6% and 8% concentrations of NaCl after16 hours of observation. Soni *et al.*, 2021 reported that *Lactobacillus* spp. of yogurt or other dietary products can tolerate 1-10% concentrations of NaCl. At the end of the 5th week of feeding programme with probiotic isolates, it was found that the body weight (mean weight) of the 3rd treatment group was highest than Control group and other treatment groups.

Nahanshon *et al.*, 1992 reported that the gut microbes of animals improve the digestion pattern as well as the feed absorption. So, it can be concluded that dietary changes have a great impact on gaining body weight. Most of the probiotic isolates (Lactic Acid Bacteria) showed antagonistic performance in *in vitro* condition against enteric pathogens. The zone of inhibition was measured in diameter. It was found that isolates from Cumilla (SV) showed negative result against *Micrococcus*, *Salmonella typhi* and *Escherichia coli*. Isolates from Rajshahi (ASMCD) and Mymensingh (KC) showed resistance against *Micrococcus* and *Pseudomonas aeruginosa* respectively.

Olivares *et al.*, 2005 and Angelis *et al.*, 2006 reported that probiotic isolates showed antagonistic activity and create significant zone of inhibition against *Escherichia coli, Staphylococcus aureus* and *Salmonella spp*. Quwehand and Vesterlund (2004) also reported that *Lactobacillus* showed antimicrobial activity in *in vitro* condition. Our study is supported by these findings.

In order to evaluate the antimicrobial activity, parameters like IgG level, white blood cell (WBC) count, hemoglobin level, total protein level, albumin level and globulin level were considered for the pathogen-infected mice model.

In case of IgG level, Treatment group 3 showed significant results ($p<0.001$) than the other groups. The significant elevation of IgG in the treatment groups occurred due to the presence of probiotics in the gut microbiome which act as a immune booster to stimulate the production of IgG. Mangell *et al.*, 2002 reported that mice induced with *Escherichia coli* O157:H7 and fed with *Lactobacillus rhamnosus* HN001 showed low mortality and bacterial transformation rate and provided increased immunity against *Escherichia coli*. Ogawa *et al.*, 2001 also reported that *Lactobacillus casei* provided immune response against *Escherichia coli*. Our study is supported by these findings also.

In case of WBC count and hemoglobin level determination, Standard group and other treatment groups showed significant variance ($P>0.0001$) when compared with Control group (Positive). But Standard group and other treatment groups showed no significant variance when compared with Control group (Negative). Nurliyani *et al.*, 2011 reported that probiotic induced rat contains little number of neutrophils and basophils than the Control group**.** He also reported that high amount of WBC causes infection which makes the immune system vulnerable. The result is similar with the reported results of Nurliyani *et al.*, 2011.

In case of total protein level, globulin level and albumin level determination, Standard group and other treatment groups showed significant variance ($P>0.0001$) when compared with Control group (Positive). But, Standard group and other treatment groups showed no significant variance when compared with Control group (Negative). Busanello *et al*., 2015 reported that dehydration, malnutrition, infectious diseases are caused by extreme level of albumin and globulin. He also reported that the Control group was more affected by infectious diseases than the probiotic treatment groups in piglets. The result is also similar with the reported findings of Busanello *et al*., 2015.

As probiotic yogurt was not included in the Control group as a dietary supplement, the presence of total number of pathogenic bacteria (*Escherichia coli*) was high. In the treatment groups, the number of pathogenic bacteria reduced significantly as the mice were fed with probiotic yoghurt. In the treatment groups, the number of pathogenic bacteria reduced significantly as the mice were fed with probiotic yoghurt. Rosenfeldt *et al.*, 2003 reported that probiotic provides a favorable environment for the gut microbiota by increasing formic acid, bacteriocins which provides protection against pathogenic microorganisms. Our results are supported by the reported findings of this study.

5. CONCLUSION

Lactic Acid Bacterial isolates from Dhaka, Cumilla, Sylhet, Mymensingh, Rajshahi, Chattagram and Khulna Divisions of Bangladesh showed that yogurt was an excellent source of probiotic bacteria. Biochemical, physiological and morphological analysis along with antimicrobial property test, bile salt tolerance test, low pH tolerance test and sugar fermentation test proves them as a perfect probiotic candidate for different important sectors. This research work concludes that all the probiotic isolates belong to the *Lactobacillus* genus. It was found that all the probiotic isolates were Gram positive, non-motile, catalase negative and tolerant to 0.3% bile salt, 2-6% NaCl and 0.1-0.4% phenol and pH resistance level was 3.0. They were able to ferment sugar which is another important criterion of probiotic bacteria. Isolated bacteria also demonstrated antimicrobial activity which provide defense against eight enteric pathogens. Probiotic bacteria should have low pH resistance and should be bile tolerant according to World Health Organization (WHO) and Food and Agriculture Organization (FAO) which is supported by the findings of the present study. Although the standard group didn't demonstrate significant difference in the IgG test, Treatment group 3 showed significant ($p<.001$) result than the other groups. In case of hemoglobin level, total protein level, albumin level and globulin level determination and white blood cell (WBC) count, Treatment group 3 showed significant ($p<.001$) result than the other groups.

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