Antibiotic Resistance in Commercially Available Probiotics in Bangladesh

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of B.Sc. in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

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Abstract

"Probiotic" can be defined as live microorganism with the ability to promote health befits in the host body when consumed in adequate amount. It is well known that probiotics improve intestinal health, boost immune system, prevent diarrhea and other allergic diseases, cancer, maintain cholesterol level, hypertension, inflammatory bowel disease etc. The health benefits of probiotics gain most attention while the potential risk factors are ignored. In our research, we aim to highlight the presence of antibiotic resistance in commercially produced probiotics, mostly yogurt and supplements along with making a comparison between the leading brands in Bangladesh. Five samples of yogurt and two dietary supplements from leading brands were collected from local supermarkets (Shawpno, Daily shopping etc.) in Dhaka for the isolation of probiotics. Identification and characterization of *Bifidobacterium bifidum, Bacillus coagulans, Lactobacillus acidophilus, Lactobacillus rhamnosus and Enterococcus faecium* probiotics were done using gram staining and series of other biochemical tests. In order to identify multidrug resistance, antibiotic profiling was done using eleven different antibiotics. *Bifidobacterium bifidum, Bacillus coagulans, Lactobacillus acidophilus, Lactobacillus rhamnosus and Enterococcus faecium* showed 45%, 59%, 36%, 23% and 45% resistance respectively. While most of the probiotics demonstrated sensitive results, a lot of them showed resistance to the antibiotics which result in serious health issues if incorporation of antibiotic resistant genes occurs in pathogenic bacteria. According to the antibiotic susceptibility result of samples from different companies, the most resistance of 55% was found in yogurt 3 sample and yogurt 2 showed the most sensitivity of 73%. Sample of Yogurt 1, supplement 1 and supplement 2 demonstrated 53%, 23% and 18% resistance in that order. Meanwhile, no active probiotic was found in the samples of yogurt 4 and yogurt 5. The presence of these antibiotic resistant probiotics could be a potential source of antibiotic resistance in humans.

Keywords: Probiotic, Yogurt, Dietary supplement, Multidrug resistant, *Bifidobacterium bifidum, Bacillus coagulans, Lactobacillus acidophilus, Lactobacillus rhamnosus and Enterococcus faecium.*

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Chapter 1

1.1 Introduction

The term “Probiotic” means “for life” and was derived from the Greek language. However, with time the definition of probiotic has changed as more and more knowledge about such bacteria were gained (Kechagia et al., 2013). Most widely used definition of probiotics was given by Fuller “probiotics are live microbial feed supplements which beneficially affect the host animal by improving microbial balance” which is the most widely used definition until today (Afrc, 1989). And the most recent definition of probiotic was provided by the Food and Agriculture Organization of the United Nations World Health Organization “live microorganisms which when administered in adequate amounts confer a health benefit on the host.”(Food and Agriculture Organization of the United Nations & World Health Organization, 2006).

It is well known that, probiotics provide a wide range of health benefits mostly to those directly related to intestinal health, including regulation of gut microbiota, boost the immune system, prevent diarrhea and other allergic diseases, cancer, maintain cholesterol level, hypertension, inflammatory bowel disease, etc (Toh et al., 2012).

It was claimed through many research projects that probiotic organisms can stimulate the immune system. These probiotics can increase non-specific cellular immune response such as activation of the macrophages, natural killer cells or NK cells, and antigen-specific cytotoxic T-lymphocytes. Moreover, the probiotics can also induce the release of various cytokines depending on what strains of probiotics are being used in what manner(Ashraf & Shah, 2014). Probiotics that are being consumed through fermented milk or yogurt can increase the number of IgA^+ cells, along with cytokine-producing cells in the effector site of the intestine, thus increasing the gut mucosal immune system (Ashraf & Shah, 2014).

To be considered as a probiotic, it must have a few characteristics, like- have to be isolated from humans, resistance to pH, bile and digestive enzymes, ability to prevent pathogen or food antigens from binding to the epithelial cell, have anti-microbial activity to harmful bacteria, viruses, fungi and parasites, and have importance for clinical use (Toh et al., 2012). Most common strains of probiotics are from the genera *Lactobacillus* and *Bifidobacterium*. (Toh et al., 2012). A table containing the names of microorganisms considered as probiotics is given below-

***Lactobacillus* species**	***Bifidobacterium* species**
L. acidophilus	*B. adolescentis*
L. casei	*B. animalis*
L. crispatus	*B. bifidum*
L. gallinarum	*B. breve*
L. gasseri	*B. infantis*
L. johnsonii	*B. lactis*
L. paracasei	*B. longum*
L. plantarum	
L. reuteri	
L. rhamnosus	

Table 1: List of microbes considered as probiotics from *Lactobacillus* species and *Bifidobacterium* species (Kechagia et al., 2013).

Other lactic acid bacteria	**Non-lactic acid bacteria**
Enterococcus f ae ca li s	*Bacillus cereus var.* toyo*i*
E. faecium	*Escherichia coli* strain nissle
Lactococcus lactis	*Propionibacterium freudenreichii*
Leuconostoc mesenteroides	*Saccharomyces cerevisiae*
Pediococcus acidilactici	*S. boulardii*
Sporolactobacillus inulinus	
Streptococcus thermophilus	

Table 2: List of probiotics from lactic acid and non-lactic acid-producing bacteria (Kechagia et al., 2013).

Lactic acid bacteria (LAB) or probiotic strains are a group of gram-positive bacteria, non-spore-forming, rod or cocci shaped bacteria which produce lactic acid as the main end product. In Bangladesh, fermented milk for instance, yogurt, cheese etc. along with fermented rice, pickles and dietary supplements are the most common sources of probiotics (Shahriar et al., 2019).

The health benefits of probiotic gain most attention while the potential risk factors are ignored. A

few studies have pointed out that probiotics can have some adverse effects on host health such as bacteremia, brain fogginess and antibiotic resistance gene transfer (Li et al., 2020). Though, some probiotic bacteria such as *Lactobacilli, Lactococci, Bifidobacterium*, and yeast are popular for their health benefits along with being safe for human consumption. However, other probiotics such as *Enterococcus, Bacillus, Streptococcus* and spore-forming bacteria are not considered safe for human consumption. The presence of these probiotics could be a potential source of antibiotic resistance in human. The resistance of these probiotics towards antibiotics can be harmful for human consumption. In addition to that, the chance of these probiotics consumed by humans containing antibiotic resistant genes can be a huge threat. Use of probiotic bacteria in association with antibiotic can result in incorporation of antibiotic resistant gene in probiotic bacteria(Zheng et al., 2017).

In the year of 2019, a group of researchers from Bangladesh conducted an experiment to find out about the multi-drug resistant traits in probiotics from fermented milk products. In this research, used samples were freshly made yogurt and cheese from the local market of Dhaka. They identified three different strains of probiotics - *Lactobacillus acidophilus, Lactobacillus bulgaricus* and *Streptococcus thermophilus,* and all three strains were sensitive to both bacitracin and penicillin g. However, *L. acidophilus* and *S. thermophilus* showed high resistance against amikacin, amoxicillin, azithromycin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, kanamycin, tetracycline, and vancomycin. And the researchers argued that in previous studies, all three of the isolated species of probiotic strains showed resistance against bacitracin(Shahriar et al., 2019).

In a study conducted in China, 41 strains of lactic acid bacteria were isolated from a range of samples like, commercial dairy, pharmaceutical products and probiotic products from Shanghai, China. Among these 41 strains of probiotic stains 35 of them showed resistance to different antibiotics and few showed multi-drug resistance also in disk-diffusion test. Later, resistant strains were selected and specific primer pairs were designed to amplify 57 different resistant Determinants and antibiotic-resistant genes were detected in five strains (Liu et al., 2009).

In another study published in September 2015, a group of researchers used five commercially available dietary supplements to identify antibiotic resistance against different classes of antibiotics. The result of this experiment was, probiotics from all the batches showed resistance against vancomycin. In case of batch-dependent result, it demonstrated resistance towards

streptomycin, aztreonam, gentamycin and/or ciprofloxacin antibiotics and the probiotic strains were from the brands called Bi and Bn, Bg. L. isolated from the brand Cn showed resistant towards gentamycin, streptomycin and ciprofloxacin. Furthermore, the number of bacteria found through the experiment was different from what the companies claimed to be available in the supplements (Wong et al., 2015).

In our research, we aim to highlight the presence of antibiotic resistance in commercially produced probiotics mostly yogurt and supplements along with making a comparison between the leading brands in Bangladesh. There have been a lot of researches on this topic in other countries including detection, implication, propagation and possible preventions of antibiotic resistance in probiotics. Nevertheless, there aren't many studies on probiotics that were done in Bangladesh and most of them were on cow's milk while very few of them included fermented dairy products such as yogurt and cheese, and even fewer research was done in Bangladesh that included local dietary supplements.

1.2 Objective

Our goal is to investigate the bacterial strains found in commercially available probiotics in Bangladesh contain any antibiotic resistance or not.

Chapter 2

Materials and methods

2.1 Sample collection:

For this particular thesis project, 7 probiotic samples were taken focusing mostly on yogurt and dietary supplements. The yogurt samples were Yogurt 1, Yogurt 4, Yogurt 2, Yogurt 5, Yogurt 3, whereas, probiotic capsules from Supplement 2 and Supplement 1 were used. The samples were collected from local shops and supermarkets while probiotic capsules were collected from Pharmacy.

All the microbial tests were performed in the Microbial Research Laboratory of Mathematics and Natural Science Department of BRAC University while maintaining proper precautions and

safety guidelines.

2.2 Serial Dilution:

Serial dilution of all the samples were done by taking 1 gram of yogurt sample in 9ml saline solution and diluted up to 10^6 dilutions. As, most of the samples didn't give any growth in selective media initially, thus, the samples were enriched in MRS Broth. Later, 1g sample was diluted in 9ml MRS broth and incubated in shaker incubator for 48hours. Again, serial dilution was done up to 10^6 dilutions.

2.3 Enrichment media:

MRS broth was used to enrich all the samples. 1gram of raw sample were mixed in the MRS broth and then incubated for 48 hours at 37^{o}C. The purpose of this particular step was to enrich the samples.

2.4 Growth on Selective Media:

Above mentioned diluted samples were then inoculated in different selective media, such as, Nutrient Agar, Xylose Lysine Deoxycholate Agar or XLD agar, MacConkey agar, Mannitol salt agar or MSA agar, HiCrome UTI Agar, Thiosulfate-citrate-bile salts-sucrose agar, or TCBS agar and Luria-Bertani agar plates. In case of XLD, TCBS and MSA media raw sample was inoculated. And for Nutrient Agar, MacConkey agar, HiCrome UTI and Luria-Bertani agar10^3 to 10^6 dilution of the sample was used.

2.5 Identification of bacterial strains

For any successful project, it is very important to identify the bacterial strain samples that wa isolated. To do so, different biochemical tests were performed for the identification of the bacterial strains, such as-

2.5.1 The Triple Sugar Iron (TSI) test

TSI agar media was inoculated by stabbing the agar media with a straight inoculation needle and followed by streaking the surface for each sample and incubated for 48hours at 37^{o}C. Three different interpretation is possible for this test. Positive result is indicated either by red slant/yellow butt, or yellow slant/yellow butt, while cracks may form in the agar media and sometimes blackening of the media may also occur. A negative result is indicated by red slant/red butt media.

2.5.1 Gram Staining

It is one of the most important tests for the identification of the bacteria based on the structure of their cell wall. Through this test, bacteria is classified to two different groups- Gram positive (stains violet) and Gram negative (strains pink) bacteria.

2.5.2 Oxidase test

Freshly cultured samples were used for this test. The cotton swab was dipped into reagent (1% dimethyl-*p*-phenylenediamine dihydrochloride) and then touched with the desired sample. Positive result is indicated by a color change within 10 seconds, otherwise it was considered as a negative result.

2.5.3 Catalase test

Small amount of freshly cultured isolates were taken on fresh microscopic slides. A drop of 3% H_2O_2 was added on top. Positive result was indicated by the formation of bubbles and no bubbles were formed in case of negative result.

2.5.4 Methyl red test

For this test, broth containing glucose and phosphate buffer was used and isolated freshly cultured strains were inoculated and inoculated at 37℃ for 48 hours. Four drops of methyl red were added afterwards. In a positive reaction, the color of the medium turned red, whereas negative result was indicated by yellow color of the media.

2.5.5 The Voges-Proskauer (VP) test

Glucose phosphate broths were used here and freshly cultured bacterial strains were inoculated and inoculated at 37℃ for 48 hours. 10 drops of alpha-naphthol (Barritt's A) were added first and then ten drops of potassium hydroxide (Barritt's B) were added. In a positive reaction, the color of the medium turned red and the yellow color indicated negative result.

2.5.6 Motility Indole Urea or MIU test

MIU agar was used for this test and with the help of a needle a single freshly cultured isolate was taken and stabbed inside the media, leaving 1/3 part from the bottom of a tube and incubated at 37℃ for 48 hours. After incubation, color change from yellow-orange to pink-red indicates the positive test result for urease test and no color change of the media indicates negative result. For positive motility test, the medium gets cloudy surrounding the inoculating line and growth within the inoculating line indicates negative result. Lastly, for positive indole test, a pink-red color ring will appear after addition of Kovac's reagent and no formation of ring indicates negative result.

2.5.7 Citrate Utilization

Desired organisms were inoculated into a slope of in this test, simmon's citrate agar was used and microbe was inoculated on the slope of the agar media and incubated at 37℃ for 48 hours. The change in color from green to blue indicates positive result and no color change indicates negative test result.

2.5.8 Indole Production test

In the tryptophan broth, bacteria isolate was inoculated and incubated at 37℃ for 24 hours. After

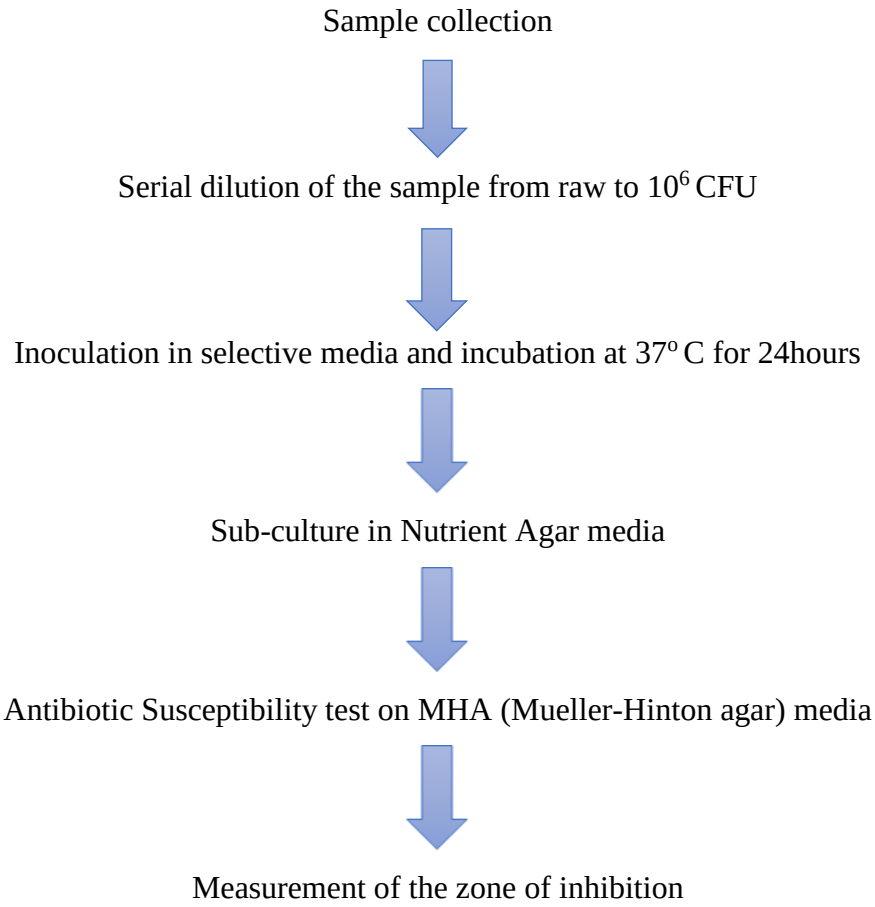
incubation, 0.5 ml of Kovac's reagent was added to each test tube. Positive result demonstrated red ring like structure at the top of the broth and negative result did not show any ring like structure.

2.5.9 Nitrate Reduction test

In the nitrate broth, bacteria isolate was inoculated and incubated at 37℃ for 48 hours. After incubation, 6-8 drops of nitrite reagent A and add the 6-8 drops of nitrite reagent B. If the color of the broth changes to red color, it indicates positive result. Zinc powder is added if no color change takes place. After adding the zinc powder, if the color does not change to red, it indicates positive result, however, if the color changes to red it indicates negative result.

Different biochemical tests were performed for the identification of the bacterial strains. Named as-

2.6 Experimental Work Flow



For the AST or Antibiotic Susceptibility test, 11 different antibiotic disks were used-

1. Penicillin (P)
2. Chloramphenicol (C)
3. Azithromycin (AZM)
4. Ciprofloxacin (CIP)
5. Tetracycline (TE)
6. Vancomycin (VA)
7. Rifampicin (RIF)
8. Meropenem (MEM)
9. Cefuroxime (CXM)
10. Colistin (CT)
11. Cefixime (CFM)

Chapter 3

Result:

To identify the strains 9 different biochemical tests were performed and 5 different strains were identified. Identified Strains are-

To identify the strains 9 different biochemical tests were performed and 5 different strains were identified. Identified Strains are-

Sample name	Name of the organism
Yogurt sample1 (LB)	*Lactobacillus acidophilus*
Yogurt sample1 (NA)	*Bifidobacterium bifidum*
Yogurt sample1 (HP)	*Bacillus coagulans*
Yogurt sample1(HB)	*Enterococcus faecium*
Yogurt sample1(HW)	*Bacillus coagulans*
Supplement sample1 (NA)	*Bifidobacterium bifidum*
Supplement sample1 (LB)	*Bifidobacterium bifidum*
Yogurt sample3 (NA)	*Lactobacillus acidophilus*
Supplement sample2 (NA)	*Lactobacillus rhamnosus*
Yogurt sample2 (LB)	*Lactobacillus rhamnosus*

Table 3: Name of identified organisms

Name of the organism	Sample Initial	Gram staining	Citrate	Indole	Catalase	Oxidase	MR	VP	Nitrate reduction	Motility	Shape
Lactobacillus acidophilus	YOGURT 1 (LB)	+	-	-	-	-	-	-	-	-	rod-shaped
Lactobacillus rhamnosus	YOGURT 2 (LB)	+	-	-	-	-	-	-	-	-	rod-shaped
Bifidobacterium bifidum	SUPPLEMENT 1 (NA)	+	-	-	-	-	-	-	-	-	Pleomorphic rods
Bacillus coagulans	YOGURT 1 (HP)	+	-	-	-	-	+	+	+	+	rod-shaped
Bifidobacterium bifidum	YOGURT 1 (NA)	+	-	-	-	-	-	-	-	-	Pleomorphic rods
Bifidobacterium bifidum	SUPPLEMENT 1 (LB)	+	-	-	-	-	-	-	-	-	Pleomorphic rods
Enterococcus faecium	YOGURT 1 (HB)	+	-	-	-	-	-	+	+	-	coccal shaped
Lactobacillus acidophilus	Yogurt 3 (NA)	+	-	-	-	-	-	-	-	-	rod-shaped
Lactobacillus rhamnosus	SUPPLEMENT 2 (OR)	+	-	-	-	-	-	-	-	-	rod-shaped
Bacillus coagulans	YOGURT 1 (HW)	+	-	-	-	-	+	+	+	+	rod-shaped

Table 4: Results of biochemical tests for bacterial identification

Few results of sample growth after dilution in Nutrient Agar (NA) and Luria-Bertani media (LB) are given below-

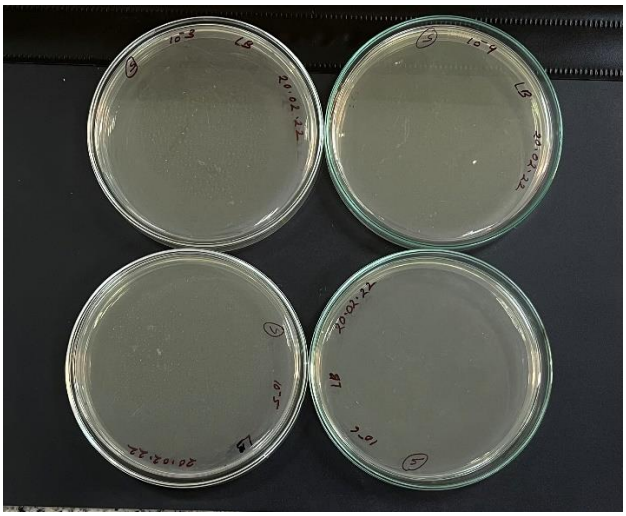


Figure 1: Yogurt 5 yogurt sample showed no growth in LB media.

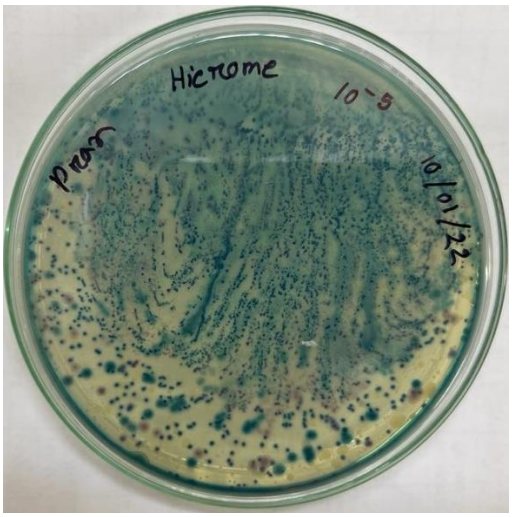


Figure 2: Growth of Yogurt 1 yogurt on Hicrome UTI media at 10^{-5} dilution factor.

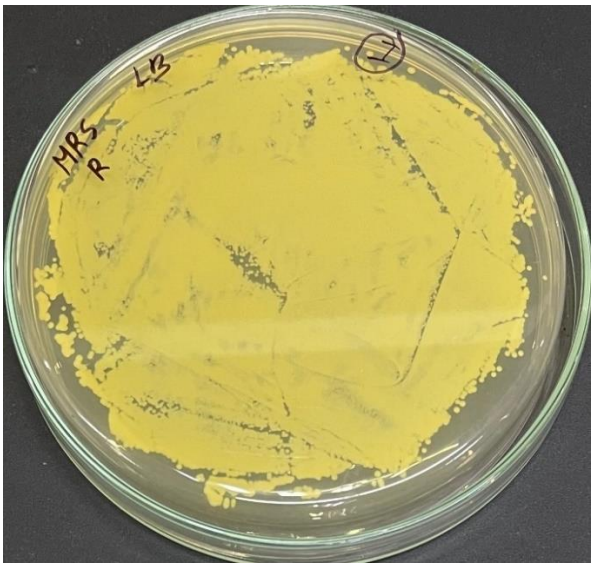


Figure 3: Growth of raw Supplement 1 sample on LB media.

Figure 4: Growth of raw Supplement 2 sample on LB media.

Table 5: Colony forming unit (CFU) of each bacterial strains

Sample initial	Name of organism	Number of visible colonies in different dilution						CFU/ml
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
YOGURT 1 (LB)	*Lactobacillus acidophilus*	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	_
Yogurt 2 (LB)	*Lactobacillus rhamnosus*	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	_
Supplement 1 (NA)	*Bifidobacterium bifidum*	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	_
YOGURT 1 (HP)	*Bacillus coagulans*	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	_
YOGURT 1 (NA)	*Bifidobacterium bifidum*	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	_
Supplement 1 (LB)	*Bifidobacterium bifidum*	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	_
YOGURT 1 (HB)	*Enterococcus faecium*	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	_
Yogurt 3 (NA)	*Lactobacillus acidophilus*	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	_
Supplement 2 (OR)	*Lactobacillus rhamnosus*	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	_
YOGURT 1 (HW)	*Bacillus coagulans*	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	_

*TNTC: Too numerous to count

Antibiotic susceptibility test result of each samples is given below-

Name of organism	Sample initial	Antibiotic	Interpretation
Bifidobacterium bifidum	Supplement 1 (NA)	Penicillin	Resistant
		Chloramphenicol (C)	Sensitive
		Azithromycin (AZM)	Sensitive
		Ciprofloxacin (CIP)	Sensitive
		Tetracycline (TE)	Sensitive
		Vancomycin (VA)	Sensitive
		Rifampicin (RIF)	Sensitive
		Meropenem (MEM)	Sensitive
		Cefuroxime (CXM)	Sensitive
		Colistin (CT)	Resistant
		Cefixime (CFM)	Sensitive

Table 6: Antibiotic susceptibility test for *Bifidobacterium bifidum* (Supplement 1 (NA))

Name of organism	Sample initial	Antibiotic	Interpretation
Bifidobacterium bifidum	Yogurt 1 (NA)	Penicillin	Resistant
		Chloramphenicol (C)	Resistant
		Azithromycin (AZM)	Resistant
		Ciprofloxacin (CIP)	Resistant
		Tetracycline (TE)	Resistant
		Vancomycin (VA)	Resistant
		Rifampicin (RIF)	Resistant
		Meropenem (MEM)	Sensitive
		Cefuroxime (CXM)	Resistant
		Colistin (CT)	Resistant
		Cefixime (CFM)	Sensitive

Table 7: Antibiotic susceptibility test for *Bifidobacterium bifidum* (Yogurt 1 (NA))

Name of organism	Sample initial	Antibiotic	Interpretation
Bifidobacterium bifidum	Supplement 1 (LB)	Penicillin	Resistant
		Chloramphenicol (C)	Sensitive
		Azithromycin (AZM)	Resistant
		Ciprofloxacin (CIP)	Sensitive
		Tetracycline (TE)	Sensitive
		Vancomycin (VA)	Sensitive
		Rifampicin (RIF)	Sensitive
		Meropenem (MEM)	Sensitive
		Cefuroxime (CXM)	Sensitive
		Colistin (CT)	Resistant

Table 8: Antibiotic susceptibility test for *Bifidobacterium bifidum* (Supplement 1 (LB))

Name of organism	Sample initial	Antibiotic	Interpretation
Bacillus coagulans	Yogurt 1 (HW)	Penicillin	Resistant
		Chloramphenicol (C)	Sensitive
		Azithromycin (AZM)	Intermediate
		Ciprofloxacin (CIP)	Sensitive
		Tetracycline (TE)	Resistant
		Vancomycin (VA)	Resistant
		Rifampicin (RIF)	Resistant
		Meropenem (MEM)	Sensitive
		Cefuroxime (CXM)	Resistant
		Colistin (CT)	Resistant
		Cefixime (CFM)	Resistant

Table 9: Antibiotic susceptibility test for *Bacillus coagulans* (Yogurt 1 (HW))

Name of organism	Sample initial	Antibiotic	Interpretation
Bacillus coagulans	Yogurt 1 (HP)	Penicillin	Resistant
		Chloramphenicol (C)	Sensitive
		Azithromycin (AZM)	Resistant
		Ciprofloxacin (CIP)	Sensitive
		Tetracycline (TE)	Sensitive
		Vancomycin (VA)	Resistant
		Rifampicin (RIF)	Resistant
		Meropenem (MEM)	Sensitive
		Cefuroxime (CXM)	Resistant
		Colistin (CT)	Resistant
		Cefixime (CFM)	Sensitive

Table 10: Antibiotic susceptibility test for *Bacillus coagulans* (Yogurt 1 (HP))

Name of organism	Sample initial	Antibiotic	Interpretation
Lactobacillus acidophilus	Yogurt 1 (LB)	Penicillin	Sensitive
		Chloramphenicol (C)	Sensitive
		Azithromycin (AZM)	Sensitive
		Ciprofloxacin (CIP)	Sensitive
		Tetracycline (TE)	Sensitive
		Vancomycin (VA)	Sensitive
		Rifampicin (RIF)	Resistant
		Meropenem (MEM)	Sensitive
		Cefuroxime (CXM)	Sensitive
		Colistin (CT)	Sensitive
		Cefixime (CFM)	Resistant

Table 11: Antibiotic susceptibility test for *Lactobacillus acidophilus* (Yogurt 1 (LB))

Name of organism	Sample initial	Antibiotic	Interpretation
Lactobacillus acidophilus	Yogurt 3 (NA)	Penicillin	Resistant
		Chloramphenicol (C)	Resistant
		Azithromycin (AZM)	Resistant
		Ciprofloxacin (CIP)	Sensitive
		Tetracycline (TE)	Sensitive
		Vancomycin (VA)	Resistant
		Rifampicin (RIF)	Resistant
		Meropenem (MEM)	Sensitive
		Cefuroxime (CXM)	Sensitive
		Colistin (CT)	Resistant
		Cefixime (CFM)	Sensitive

Table 12: Antibiotic susceptibility test for *Lactobacillus acidophilus* (Yogurt 3 (NA))

Name of organism	Sample initial	Antibiotic	Interpretation
Lactobacillus rhamnosus	Supplement 2 (NA - OR)	Oxacillin	Resistant
		Chloramphenicol (C)	Sensitive
		Azithromycin (AZM)	Sensitive
		Ciprofloxacin (CIP)	Intermediate
		Tetracycline (TE)	Sensitive
		Vancomycin (VA)	Sensitive
		Rifampicin (RIF)	Sensitive
		Meropenem (MEM)	Sensitive
		Cefuroxime (CXM)	Sensitive
		Colistin (CT)	Resistant
		Cefixime (CFM)	Sensitive

Table 13: Antibiotic susceptibility test for *Bifidobacterium bifidum* (Supplement 2 (NA - OR))

Name of organism	Sample initial	Antibiotic	Interpretation
Lactobacillus rhamnosus	Yogurt 2 (LB)	Penicillin	Sensitive
		Chloramphenicol (C)	Sensitive
		Azithromycin (AZM)	Resistant
		Ciprofloxacin (CIP)	Sensitive
		Tetracycline (TE)	Sensitive
		Vancomycin (VA)	Sensitive
		Rifampicin (RIF)	Resistant
		Meropenem (MEM)	Sensitive
		Cefuroxime (CXM)	Sensitive
		Colistin (CT)	Sensitive
		Cefixime (CFM)	Resistant

Table 14: Antibiotic susceptibility test for *Lactobacillus rhamnosus* (Yogurt 2 (LB))

Name of organism	Sample initial	Antibiotic	Interpretation
Enterococcus faecium	Yogurt 1 (HB)	Penicillin	Resistant
		Chloramphenicol (C)	Sensitive
		Azithromycin (AZM)	Intermediate
		Ciprofloxacin (CIP)	Sensitive
		Tetracycline (TE)	Sensitive
		Vancomycin (VA)	Resistant
		Rifampicin (RIF)	Resistant
		Meropenem (MEM)	Sensitive
		Cefuroxime (CXM)	Resistant
		Colistin (CT)	Resistant
		Cefixime (CFM)	Sensitive

Table 15: Antibiotic susceptibility test for *Enterococcus faecium* (Yogurt 1 (HB))

Few results of antibiotic susceptibility test is given below-

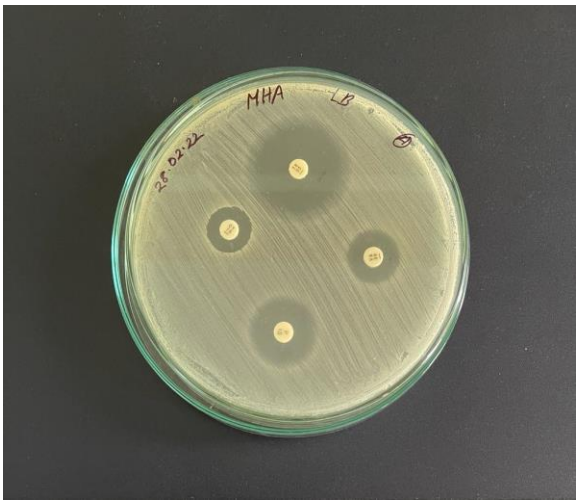


Figure 5: AST of Yogurt 2 (LB) or *Lactobacillus rhamnosus* in MHA media.

Figure 6: AST of Yogurt 1 (NA) or *Bifidobacterium bifidum* in MHA media.

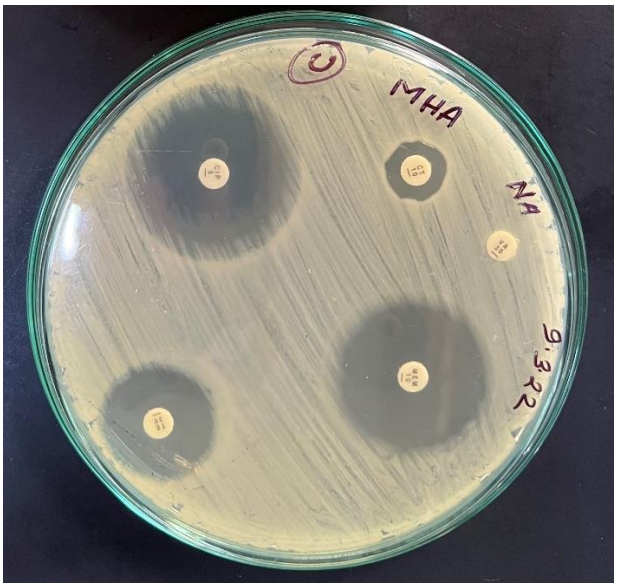


Figure 7: AST of Yogurt 3 (NA) or *Lactobacillus acidophilus* in MHA media.

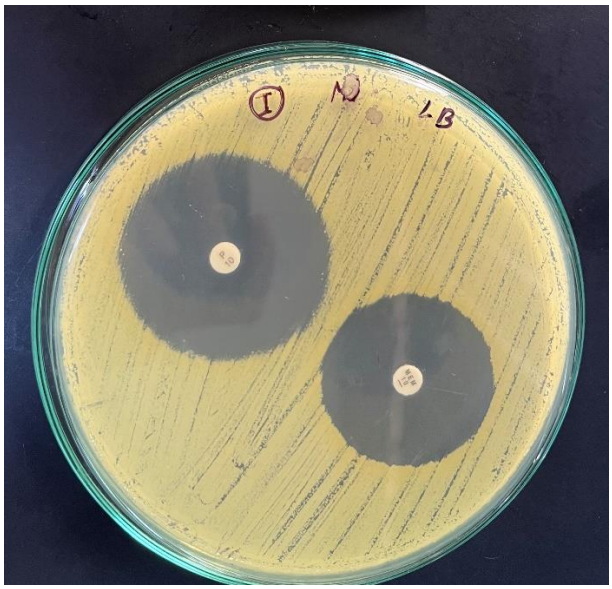


Figure 8: AST of Supplement 1 (LB) or *Bifidobacterium bifidum* in MHA media.

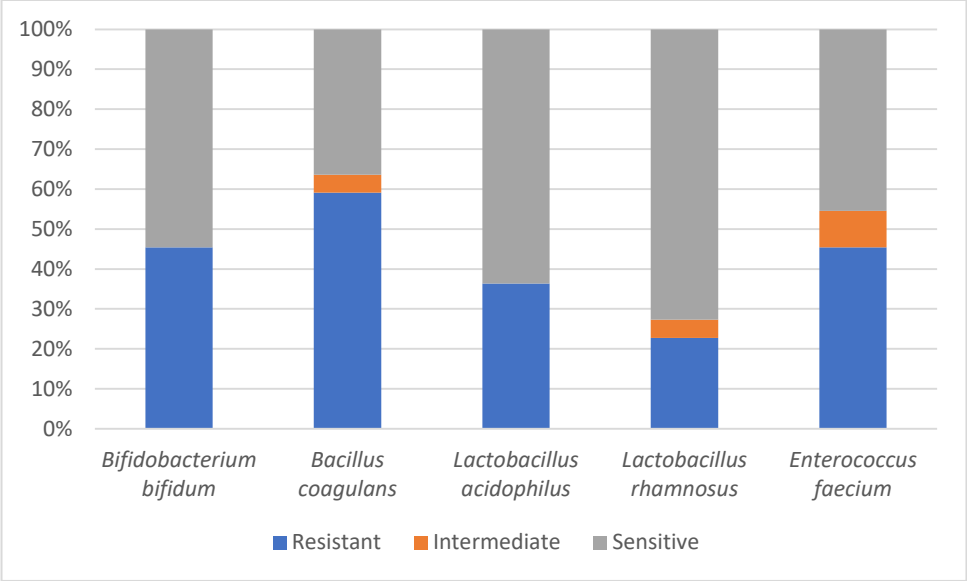


Figure 9: Comparison of AST result between different bacteria showed that *Bacillus coagulans* was found to have the highest (59%) resistance among all the bacteria, resistance of *Bifidobacterium bifidum* and *Enterococcus faecium* was found 45%, *Lactobacillus acidophilus* showed 36% resistance and *Lactobacillus rhamnosus* showed the least resistance (23%). *Enterococcus faecium*, *Lactobacillus rhamnosus*, *Bacillus coagulans* gave 9%, 5% and 5% intermediate result respectively.

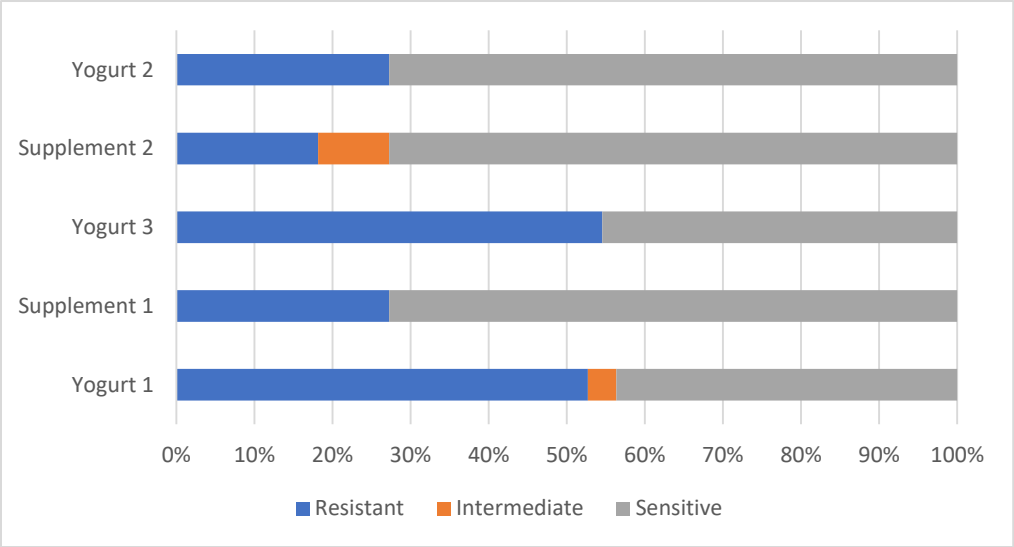


Figure 10: A comparison of AST results of samples from different companies was showed on this graph. Here, yogurt 3 demonstrated most (55%) resistance, yogurt 2, yogurt 1, supplement 1

and supplement 2 showed 27%, 53%, 23% and 18% resistance respectively. Supplement 2 and Yogurt 1 was 9% and 4% intermediate in that order.

Chapter 4

Discussion:

Through this research, we aimed to make a compression of antibiotic resistance between leading probiotic brands in Bangladesh. Among the samples, Yogurt 3 showed the most resistance of 55%. While, Supplement 1, Supplement 2 and Yogurt 2 showed the most sensitivity of 73%. In our research *Bifidobacterium bifidum*, *Bacillus coagulans*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus and Enterococcus faecium* were identified.

In our study *Bacillus coagulans* was found to have the highest (59%) resistance among all the bacteria, *Bifidobacterium bifidum* and *Enterococcus faecium* also had moderately high resistance (45%), *Lactobacillus acidophilus* comparatively lower resistance (36%) and *Lactobacillus rhamnosus* showed the least resistance (23%).

In our study, we had to use enrichment media for most of the samples including Yogurt 2 and Yogurt 3 as they did not show any growth in growth media possibly because of their low concentration of viable cell. Previous studies showed, in order to serve beneficial effects probiotics must be present in high concentration, have strong survival properties along with having high viability, typically $10^6 – 10^7$ cfu/g (Shah et al., n.d.). A study done on supplements in Bangladesh showed that, the claimed number of viable cells were three to four log cycle

higher than what was found in their research (Begum et al., 2015). Sensitivity to antimicrobial substances, acid production during storage, acidity and oxygen level of product were found to be some of the reasons for loss of viability of probiotics (Dave & Shah, 1997). However, inhibitory activity was determined by using inhibitory bacterial strains such as *Salmonella typhi, Vibrio cholerae and Shigella sp.* etc. Despite having lower concentration of viable probiotic cells than claimed, the probiotics showed good inhibitory activity (Begum et al., 2015). In this study, all the probiotic strains claimed by the manufacturer of the supplements were not found. In our study, only *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* probiotics were identified contradicting the claim of the supplement manufacturers which also included *Fructo-Oligasaccharides* and *Lactobacillus acidophilus*.

One of our most interesting finds was, that no probiotics were found in Yogurt 4 and Yogurt 5 samples even after enriching the sample in MRS broth for 48 hr. From this, we could come to the conclusion that no active probiotics were present in them. The possible reasons for not getting any bacterial cultures in our study, specifically in Yogurt 5 and Yogurt 4 yogurt samples, can be-

As most probiotics have poor thermostability, improper transportation and storage can cause bacterial cell death. Transportation at a very low temperature can be a possible risk for thawing of the microorganism which can lead to cellular injury and result in inactivation. Dehydration methods including freeze-drying, spray drying, and fluidization drying can be used to maintain the viability of micro-organisms. In addition to that, when the certain critical water content in microbial biomass exceeds, it can cause dehydration inactivation which can lead to biochemical reactions as water works as a substrate for such reactions in microorganisms. Also, the removal of microorganisms' water under a certain level restricts the maintenance of metabolic functions and eventually leads to cell death (Goderska, 2012).

It has been proved that the survival rate of probiotics is highly dependent on the storage condition. Huge viability loss takes place when the probiotic source is stored in room temperature (Ferdousi et al., 2013). Heat-treatment or heating the yogurt following pasteurization method is used to kill pathogenic bacteria or inactivate enzymes so that the spoilage of the product can be avoided. However, heat-treatment after the product has been

manufactured, can also kill the beneficial probiotic cultures. As a result, our desired bacterial cultures will also be dead along with the pathogenic bacteria. In research, it was found that storing the yogurt sample at 2^{o}C or below 5^{o}C, results in the loss of viability of the bacterial culture (Mortazavian et al., 2007). Different probiotic strains offer different benefits and in probiotics, different type of bacterial cultures is used. Depending on the strain, the storage temperature varies. For example in a study, it was found that *L. acidophilus* had more viability than *L. casei* or *L. reuteri* when stored at 5°C for 35days. Thus, storing at wrong temperature can also be a possible reason(Mani-López et al., 2014).

Another important find was that among all the samples only Yogurt 1, Supplement 1 and Supplement 2 showed probiotic growth without needing to be enriched in enrichment media. As no growth in selective media was shown by other samples, enrichment media was used in order to determine if there was any active probiotic present or not. Since our initial aim was to isolate lactic acid bacteria (LAB), MRS Broth was used as enrichment media. However, MRS broth does not support the growth of all probiotic cultures or all LAB cultures(Hayek et al., 2019). While this can be considered as a limitation of this study, we reckon there shouldn't be a requirement for the probiotic sources to be enriched in the first place. Moreover, we would like to emphasize on the fact that the need to be enriched in order to grow can be a possible drawback for these probiotic sources since they are consumed directly and the human stomach does not provide the same environment as enrichment media for the survival of the probiotics. Furthermore, the acidic environment of the human gastrointestinal tract and alkaline condition of the intestine along with different digestive enzymes will make it very difficult for the fragile probiotics to survive.

Chapter 5

Conclusion

To summarize, probiotics are proved to have a lot of health benefits which help to increase the gut health of human over the period. However, it is quite possible that commercially available probiotics may not contain any probiotic strains whatsoever. In our research, no bacterial growth was found in two of the yogurt samples despite being enriched. Thus, it is impossible to get any probiotic strain in the stomach as the gut environment is way too acidic or alkaline (intestine). Moreover, most of the yogurt samples were required to be enriched in enrichment media as no bacterial growth was found otherwise. This may have happened due to the poor storage condition of the local markets. Additionally, manufacturing and marketing probiotic foods in order to meet the increasing demand is not enough if the quality of the probiotics are not maintained. In addition to that, regulation and legislation should be maintained for misleading consumers with inaccurate health claims by the manufactures. Furthermore, the possibility of incorporation of antibiotic resistant genes in pathogenic bacteria should not be taken lightly, as in this study, indication of increasing antibiotic resistance of probiotic strains was found.

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