



Prevalence of Multi-Drug Resistance Traits in Probiotic Bacterial Species from Fermented Milk Products in Bangladesh

**Asif Shahriar¹, Mahenaj Alo¹, Md. Fahad Hossain¹, Talha Bin Emran^{2,3*},
Md. Zia Uddin² and Arkajyoti Paul^{3,4}**

¹*Department of Microbiology, Stamford University Bangladesh, 51 Siddeswari Road, Dhaka-1217, Bangladesh.*

²*Department of Pharmacy, BGC Trust University Bangladesh, Chandanaish, Chattogram-4381, Bangladesh.*

³*Drug Discovery, GUSTO A Research Group, Chattogram-4000, Bangladesh.*

⁴*Department of Microbiology, Jagannath University, Dhaka-1100, Bangladesh.*

Authors' contributions

This work was carried out in collaboration among all authors. Authors TBE, AS, MA and MFH together planned and designed the research. Authors TBE, AS, MA, MZU and AP arranged the whole facilities for the research. Authors AS, MA and MFH conducted the entire laboratory works. Author AS imparted in study design and interpreted the results putting efforts on statistical analysis with authors MA, MZU, AP and TBE. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Matured nourishment like yogurt and cheese are great wellsprings of probiotic living beings. The present study was carried out for isolation, identification and antibiotic profiling of some important bacteria in, local fermented milk-products sold in the market of Dhaka, Bangladesh.

Methodology: Twenty types of yogurt and fifteen types of cheese samples were collected from two different local market. De Man, Rogosa and Sharpe agar (MRS agar) and *Streptococcus*

*Corresponding author: E-mail: talhabmb@gmail.com; talha-bgctub@bgctrustbd.org

thermophilus agar (ST agar) were used for the isolation of probiotic micro-organisms from these different fermented dairy products. Characterization of lactic acid bacteria belonging to the species of *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* was done by using morphological feature, Gram staining, biochemical tests (catalase test, some carbohydrate fermentation tests, alpha haemolysis test, starch hydrolysis test, bile salt tolerance test). The antibiotic profiling was performed by utilizing twelve distinct antibiotics: amikacin (10 µg), amoxicillin (15 µg), azithromycin (30 µg), bacitracin (30 µg), chloramphenicol (30 µg), ciprofloxacin (10 µg), erythromycin (30 µg), gentamicin (30 µg), kanamycin (30 µg), penicillin g (30 µg), tetracycline (15 µg), vancomycin (15 µg) to identified the multi-drug resistance of probiotic micro-organisms.

Results: The outcomes demonstrated that specific bacterial species (*L. acidophilus*, *L. bulgaricus* and *S. thermophilus*) were utilized in the production of yogurt and cheese samples, that may have unnecessary antibiotic resistances which can be a risk if they transfer antibiotic resistance genes to pathogenic microbes. Notably, *L. bulgaricus* was effectively susceptible against bacitracin and penicillin g.

Conclusion: Yogurt and cheese are one kind of probiotic source, made up by using probiotic starter cultures (*Lactobacillus* spp. and *Streptococcus thermophilus*) which were identified in this study. Most of the probiotic micro-organisms were resistant against these widely accepted commercial antibiotics. These resistant lactic acid bacteria may transfer their resistance genes to pathogenic microorganisms. It can be a significant cause of concern for human and animal health.

Keywords: *Lactobacillus* spp.; *Streptococcus thermophilus*; probiotic food; fermented milk products; yogurt; cheese; drug resistance.

1. INTRODUCTION

Probiotics are nonpathogenic microbes that live inside the host's guts and improve the gut flora, that have beneficial effects on their host's health [1-3]. They are used in the production and consumption of fermented food and beverages [4,5]. Probiotic bacteria are widely distributed in nature and can be used in the food industry [6]. Fermented milk products such as yogurt, cheese, etc. are the main food sources of probiotics in Bangladesh [7].

Lactic acid bacteria including *Lactobacillus* spp. are a group of non-spore-forming Gram-positive bacteria that produce lactic acid as the main end product among the fermentation of carbohydrates and are utilized as starter culture [8]. Lactic acid bacteria (LAB) or probiotic strains have been indexed as a part of human microbiota and it is widely employed as starter cultures [9,2]. The most evident benefits of *Lactobacillus* spp. fermentation include accumulated food acceptability and improved shelf life [9]. *Lactobacillus* spp. are mostly accepted and generally recognized as safe (GRAS) [10], because they are able to produce bacteriocins and their consumption confers several health benefits, such as controlling intestinal infections, improving lactose utilization, lowering blood ammonia levels, providing efficient resistance against gastric acid and bile [11,12-16], influencing the immune system, and

lowering serum cholesterol levels [17,18]. *Lactobacillus* spp. are also adhering to the gastrointestinal tract and confer pathogen inhibition [11,19,20]. Interestingly, the presence of *Lactobacillus* spp. (LAB) resulted in no change or small changes in the abundance of other intestinal microbial groups [21].

Bacteria used in food productions could contain antibiotic resistance genes [22,5]. In recent years, studies were done to establish the antibiotic resistance probiotic in clinically related species [23]. Recently it was speculated that the fermented milk products may act as reservoirs of antibiotic-resistant bacteria and may transfer these antibiotic-resistant bacteria to the human body [24,25]. In Bangladesh, most of the probiotic studies were done on cow's milk [26,7], but very little is known about the probiotic status of the fermented products of milk available in the local market particularly yogurt and cheese. *Lactobacillus* spp. were evaluated for their functional traits, probiotic properties, and ability to inhibit the growth of pathogenic and food poisoning microorganisms (*Campylobacter*, *Clostridium botulinum*, *E. coli* O157:H7, *Listeria monocytogenes*, *Shigella*, *Salmonella* and Norovirus, etc.).

In this study, we isolated and identified the probiotic bacteria from fermented dairy products (yogurt and cheese) obtained from the different areas of the capital city Dhaka, Bangladesh.

Furthermore, we studied their antimicrobial susceptibility to identify the multidrug-resistant strains of these probiotic microorganisms.

2. MATERIALS AND METHODS

2.1 Sample Collection

Twenty types of yogurt and fifteen types of cheese samples were collected for this study. Yogurt samples were collected from the local market of Rampura Bazar, Dhaka, and cheese samples were collected from the New-market, Dhaka, Bangladesh. Both fresh probiotics foodstuffs were made with cow milk. These samples were collected using sterile spoons and preserved in 4°C to avoid contamination and deterioration. Approximately 1 g of each sample was diluted with 100 ml of nutrient broth medium and was incubated at 37°C for overnight.

2.2 Isolation of Probiotic Micro-Organisms

Nutrient agar is one of the most common growth media which is used for isolation of all kind of living micro-organisms. On the other hand, De Man, Rogosa and Sharpe agar (MRS agar) (Oxoid™, Thermo Fisher Scientific, USA) were used to isolate and determine the growth of *Lactobacillus* species and to inhibit the growth of unwanted pathogenic microbes [27]. *Streptococcus thermophilus* agar (ST agar) was used to isolate *Streptococcus thermophilus* species [28]. After incubation at 37°C for 24 h, characteristic colonies of probiotic micro-organisms were enumerated on specific agar media [27,28].

2.3 Morphological Characterization of Isolates by Gram staining

Gram staining method was used to distinguish the morphological characterization of the isolates. Under the microscope, stained slides were observed to differentiate between Gram-positive and Gram-negative bacteria and to distinguish the *bacilli* and the *cocci* [29,30].

2.4 Biochemical Tests

Lactobacillus spp. was isolated and identified morphologically and was characterized by a set of biochemical techniques (catalase test, carbohydrate fermentation tests including glucose, lactose, maltose, mannitol and sucrose test) according to Bergey's manuals [31].

Besides these biochemical tests, the alpha hemolysis (α -hemolysis) test and the starch hydrolysis test are also performed to identified hydrogen peroxide producing bacteria (*Streptococcus thermophilus* and a group of oral *streptococci*) and to demonstrate the hydrolytic activities of the exo-enzymes that are led to process in glycolysis [32,33].

2.5 Identification of the Isolates

Blood agar was used for alpha hemolysis test to identify *Streptococcus thermophilus* and a group of oral streptococci. Starch hydrolysis test was done to observed the enzyme activity of the bacteria which can hydrolyze starch on starch agar media. Nutrient broth with (2%) NaCl has been used to determine the tolerance level of the bacteria and its growth. MRS broth media with NaCl (2%) is a highly selective media for *Lactobacillus* spp. 5% Bile salt tolerance test was followed. Bile salt kills or inhibits the Gram-positive bacteria. But some probiotic group like *Lactobacillus acidophilus* can tolerate the high level of bile salt. This tests help to determine the *Lactobacillus acidophilus* from the other species [27-29,30,32,33].

2.6 Antibiotic Profile Determination

2.6.1 The Kirby-Bauer disk diffusion method

The media used in Kirby-Bauer testing was Mueller-Hinton agar at only 4 mm deep and was poured into 100 ml to 150 ml Petri dishes [34]. The pH level of the plates was between 7.2 and 7.4. Inoculation was made with a broth culture diluted to match a 0.5 McFarland turbidity standard, which was roughly equivalent to 150 million cell/ml. Plates were incubated overnight at an incubation temperature of 37°C. Commercially available 12 different antibiotics: amikacin (10 μ g), amoxicillin (15 μ g), azithromycin (30 μ g), bacitracin (30 μ g), chloramphenicol (30 μ g), ciprofloxacin (10 μ g), erythromycin (30 μ g), gentamicin (30 μ g), kanamycin (30 μ g), penicillin g (30 μ g), tetracycline (15 μ g), vancomycin (15 μ g) were applied on the surface of inoculated plates with appropriate technique. The result was interpreted by following the CLSI guidelines of antibiogram [35].

2.6.2 Diameters of inhibition zones measurements

After 16 to 18 hours of incubation, each agar plate was examined. If the plate was

satisfactorily streaked, the resulting zones of inhibition were uniformly circular and there was a confluent lawn of growth and the inoculum was correct in size and shape. If individual colonies were apparent, the inoculum was too light, and the test had been repeated. The diameters of the zones of complete inhibition were measured, including the diameter of the disc [34,35].

3. RESULTS

3.1 Enumeration of Probiotic Micro-Organisms

The bacterial load was very high in the yogurt samples beside the cheese samples. The total viable bacteria and probiotic micro-organisms (*Lactobacillus* spp. and *Streptococcus thermophilus* sp.) growth were found in yogurt and cheese samples, nearly 8 log CFU/ml. Total viable bacteria were also present in yogurt and cheese samples up to 8 log and 6.5 log CFU/ml, respectively. In case of specific probiotic bacteria like *Lactobacillus* spp. and *Streptococcus thermophilus* sp. were present within the range of 4.5-7.0 log CFU/ml in yogurt samples whereas the cultural growth of *Lactobacillus* spp. and *Streptococcus thermophilus* sp. were present within the range of 3.0-5.5 log CFU/ml in cheese samples. The yogurt samples showed more probiotic microbial contamination beside the cheese samples (Fig. 1). However, two different species of *Lactobacillus* such as *Lactobacillus acidophilus*

and *Lactobacillus bulgaricus* were observed in both categories of samples.

3.2 Identification of Isolates

Identification of the isolates was done based on their biochemical activity. The identification was followed by the Bergey's manual for systematic bacteriology. Catalase test, carbohydrate fermentation tests including glucose, lactose, maltose, mannitol and sucrose tests were positive, which is indicative for the *Lactobacillus* spp. Alpha hemolysis (α -hemolysis) test and the starch hydrolysis test were performed, and the hydrolytic activities of the exo-enzymes are positively led to process in glycolysis (Fig. 2).

Furthermore, the Gram staining was performed to identify the morphological characteristics of isolated micro-organisms under the light microscope (Fig. 2).

The identifications showed that the three species of probiotic bacteria *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were found from the strains 1a, 2a, 3b, (Figs. 1 and 2).

3.3 Differentiation between the Two Strains of *Lactobacillus*

Out of the three micro-organisms, two were strains of *Lactobacillus*. They were morphologically similar. The two *Lactobacillus* strains were differentiated by the following biochemical tests (Table 1).

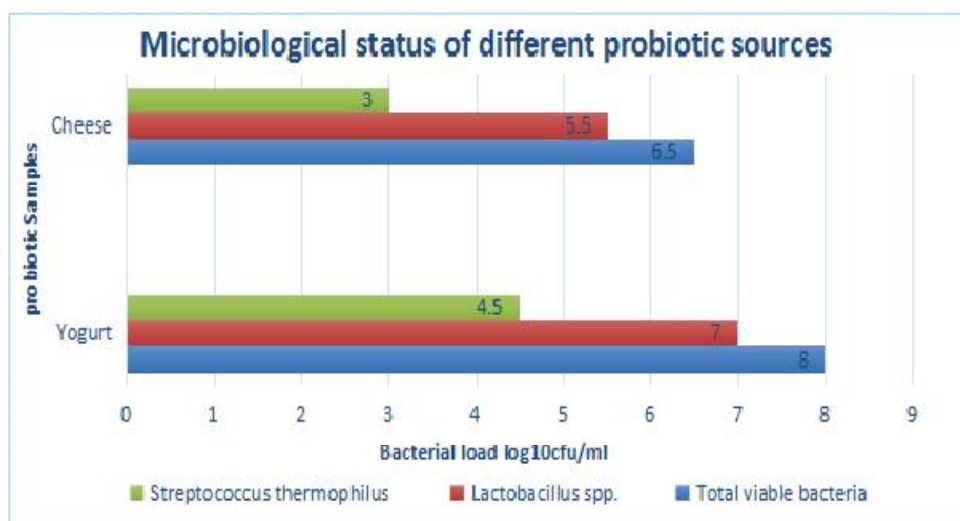


Fig. 1. Microbiological status of different probiotic samples. Probiotic micro-organisms (*Lactobacillus* spp. and *Streptococcus thermophilus*) were isolated

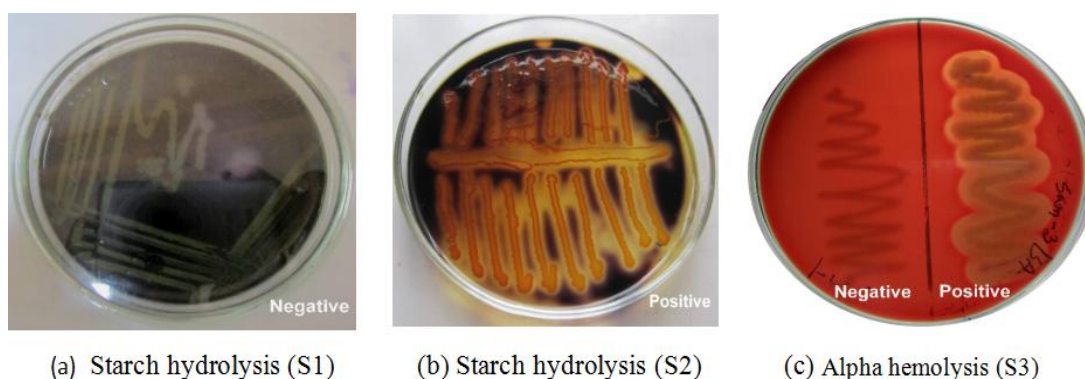


Fig. 2. Biochemical confirmation test for *Streptococcus* spp. (a) Starch hydrolysis test was negative, that means there were no *Streptococcus* spp. found in sample 1 but in sample 2, *Streptococcus* spp. were observed (b). In addition, (c) Alpha hemolysis was also positive that indicate the presence of *Streptococcus* spp. in sample 3

3.4 Antibiotic Profile

3.4.1 On agar plate

The inhibition zones of antibiotic activities were measured to the nearest whole millimeter, using sliding calipers or a ruler which was held on the back of the inverted Petri dish. The Petri dish was held a few inches above a black, nonreflecting background and illuminated with reflected light. If blood was added to the agar base, the zones were measured from the upper surface of the agar illuminated with reflected light, with the cover removed (Figs. 3 and 4).

3.4.2 Antibiotic profile of probiotic bacteria

Among these antibiotics, the *Lactobacillus acidophilus* species were found to be resistant to amikacin (n=8, 25 nm); amoxicillin (n=2, 29 nm); azithromycin (n=4, 29 nm), bacitracin (n=2, 16 nm); chloramphenicol (n=4, 34 nm); ciprofloxacin

(n=8, 29 nm), erythromycin (n=4, 27.5 nm), gentamicin (n=6, 24 nm), kanamycin (n=8, 26 nm), penicillin g (n=2, 21 nm), tetracycline (n=2, 30 nm) and vancomycin (n=8, 17 nm) (Fig. 5). Also antibiotics, amikacin (n=8, 22 nm); amoxicillin (n=2, 14 nm); azithromycin (n=4, 30 nm), chloramphenicol (n=4, 31 nm); ciprofloxacin (n=8, 26 nm), erythromycin (n=4, 27 nm), gentamicin (n=6, 23 nm), kanamycin (n=8, 19 nm), tetracycline (n=2, 24 nm), vancomycin (n=8, 15 nm) were effectively shown as inactive against *Lactobacillus bulgaricus* (Fig. 5). On the other hand, amikacin (n=8, 4.5 nm); amoxicillin (n=2, 21 nm); azithromycin (n=4, 26.5 nm), bacitracin (n=2, 6 nm); chloramphenicol (n=4, 32 nm); ciprofloxacin (n=8, 30 nm), erythromycin (n=4, 26 nm), gentamicin (n=6, 25 nm), kanamycin (n=8, 21 nm), penicillin g (n=2, 13 nm), tetracycline (n=2, 25 nm), vancomycin (n=8, 16 nm) were found as effectively inactive against *Streptococcus thermophilus* (Fig. 5).

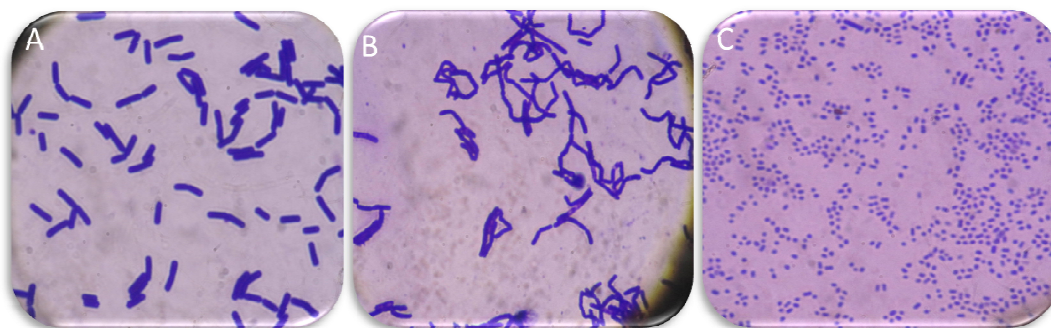


Fig. 3. (A–C). Gram staining (A) Gram-positive rod (Sample 1) (B) Gram-positive rod (Sample 2) (C) Gram-positive coccus (Sample 3). The Gram staining technique was used to identify the *Lactobacillus* spp.

Table 1. Biochemical tests between the two strains of *Lactobacillus* spp.

Source of probiotic	Isolated Micro-organisms	Biochemical tests					
		Starch hydrolysis	Glucose fermentation	Lactose fermentation	Maltose fermentation	Sucrose fermentation	5% bile tolerance
Yogurt	<i>Lactobacillus acidophilus</i>	+ve	+ve	+ve	+ve	+ve	+ve
	<i>Lactobacillus bulgaricus</i>	-ve	+ve	+ve	-ve	-ve	-ve
Cheese	<i>Lactobacillus acidophilus</i>	+ve	+ve	+ve	+ve	+ve	+ve
	<i>Lactobacillus bulgaricus</i>	-ve	+ve	+ve	-ve	-ve	-ve

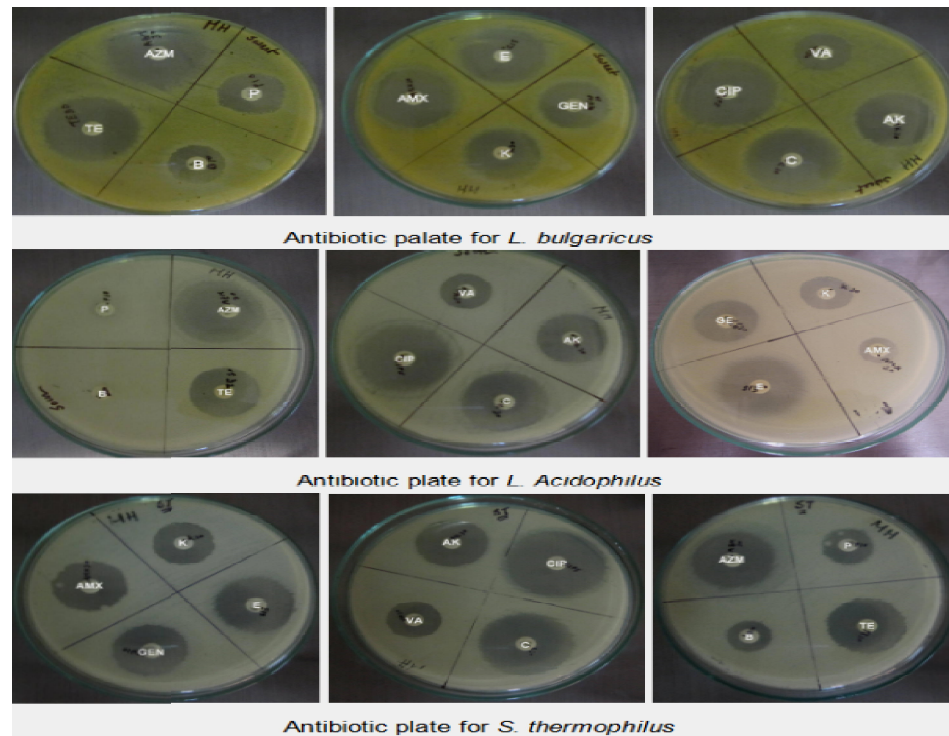


Fig. 4. Inhibition zones obtained from antibiotic disks activities on Petri dishes

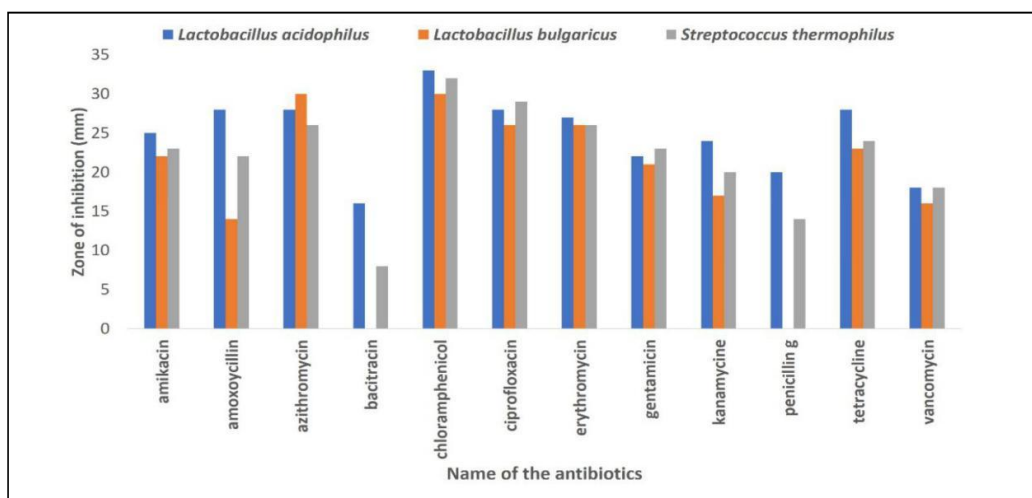


Fig. 5. Zone of inhibition indicating antibiotic resistance pattern of *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* against amikacin (10 µg), amoxicillin (15 µg), azithromycin (30 µg), bacitracin (30 µg), chloramphenicol (30 µg), ciprofloxacin (10 µg), erythromycin (30 µg), gentamicin (30 µg), kanamycin (30 µg), penicillin g (30 µg), tetracycline (15 µg), and vancomycin (15 µg) (in mm)

All the experiments have been done in triplicate. One representative data have been shown. All data were found to be significant ($p < 0.1$)

Notably, *Lactobacillus bulgaricus* species were found to be sensitive against bacitracin (30 µg) and penicillin g (30 µg); and all isolated pathogens of probiotic food were extremely sensitive against bacitracin (30 µg) (Fig. 5).

4. DISCUSSION

The study was designed for characterization and determination of probiotic micro-organisms and their antibiotics profiles from fermented food. The fermented food (Yogurt, Cheese) contained three probiotic micro-organisms. *L. acidophilus*, *L. bulgaricus* and *S. thermophilus* which were identified by biochemical methods. Antimicrobial profiles were determined by observing the antibiotic activities against these three micro-organisms and measurements of the zones of inhibition. *Lactobacilli* antimicrobial effect against pathogens is mainly because of the production of organic acids and pH reduction in co-culture with pathogenic bacteria even though they produce some other substances [36]. *L. bulgaricus*, *L. acidophilus* and *S. thermophilus* species were notably found to be sensitive against bacitracin (30 µg) and penicillin g (30 µg) respectively whereas *L. acidophilus* and *S. thermophilus* species were highly resistant against amikacin (10 µg), amoxicillin (15 µg), azithromycin (30 µg), bacitracin (30 µg), chloramphenicol (30 µg), ciprofloxacin (10 µg), erythromycin (30 µg),

gentamicin (30 µg), kanamycin (30 µg), penicillin g (30 µg), tetracycline (15 µg), and vancomycin (15 µg) (in mm). But in previously published results these three species which were isolated from the probiotics showed resistance to bacitracin [37] while *L. bulgaricus* and *S. thermophilus* was resistant to penicillin g, in addition, *L. bulgaricus* was resistant to amoxicillin also which is evident from previous results [38]. In recent years, many scientists have isolated and identified LAB and lactobacilli from traditional products worldwide and have evaluated their antagonistic effects against various pathogens. Micro-organisms such as lactobacilli and many other bacteria can eliminate pathogens through multiple mechanisms, including competitive elimination that results in food safety [39].

L. acidophilus, *L. bulgaricus* and *S. thermophilus* are the three well-known fermented food probiotic bacteria. Probiotic bacteria are increasing the gut micro-organisms and help in the digestive system, along with enhancing the immune system [40]. Isolation and identification of these probiotic bacteria will need molecular techniques for further study. For phenotypic identification, different phenotypic tests such as morphological examination, resistance to different salt concentrations, gas production from glucose and determination of sugar fermentation patterns were applied for phenotypic

identification. Determination of such characteristics would be helpful for industrial applications in the future. The food products like yogurt and cheese showed beneficial organisms and beneficial activity also, which enables good health and improves immune status. Whatever the reason, results show that certain species which would otherwise be used in the manufacture of yogurt may have numerous unnecessary antibiotic resistance [41].

The production of probiotic foods and fermented milk products were produced by the probiotic bacteria which have an important role in the increasing of gut microflora. It also helps in the digestive system in a proper way with increasing immune response against pathogens in human health. These bacteria can be raised to produce various kinds of food and pharmaceutical products. They can also be used to produce new functional foods. Therefore, increasing use of dairy products containing probiotics, identification and production of foods containing the highest and most effective lactobacilli are recommended in daily diet. Another study concluded that Lactobacilli have intrinsic resistance to bacitracin, kanamycin, gentamicin, metronidazole, streptomycin, sulfamethoxazole and vancomycin [39]. The balance and composition of the intestinal microbiota are imperative for the prosperity and the ability of our organism to resist the invasion of pathogens. To increase the natural resistance of the host to infections, probiotic bacteria such as *Lactobacilli* and bifidobacteria can be devoured.

The major risk associated with these microbes is that they may transfer resistance genes to pathogenic bacteria, which will be a major cause of concern to human and animal health [42]. Although no studies have reported attempts to demonstrate genetic exchange mechanisms in yogurt and cheese cultures, the evidence is accumulating that such mechanisms exist for other bacteria commonly found in dairy products.

5. CONCLUSION

Finally, we recommend to the fermented milk products' manufacturers to use probiotic strains that are able to reduce an unnecessary distribution of antibiotic-resistance. People consume probiotic for benefits and the antibiotic profile of these probiotic helps to understand the beneficial metabolism they procure to those people who take antibiotics and probiotics simultaneously. Future studies may involve

molecular work to identify antimicrobial resistance genes in these probiotic species.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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