Screening the Antibacterial Potential of Endosymbiotic Bacteria Identified from Different Animal Milk Along with the Antagonistic Efficacy of Commercially Available Probiotic Against the Clinical Pathogens

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ABSTRACT
Probiotics are offered as food to guarantee that the gut flora's balance is maintained. Probiotics have been used for a long time in human and animal feed components without causing any negative side effects. *Lactobacilli*, one kind of probiotic, inhibits the growth of several infections that affect both humans and animals. The objective of this investigation was to assess the antimicrobial potentials of isolated bacteria derived from different milk (Cow, Horse, Goat) samples and commercially available probiotics against *E. coli, Klebsiella* spp. *Serratia marcescens*, *Pseudomonas* spp. *and Staphylococcus aureus.* The isolation and identification of the probiotics bacterium from milk were performed through cultural, microscopical and biochemical analysis. The antimicrobial properties of the probiotic's bacteria and commercial probiotic was determined through agar well diffusion, drop test and overlay methods. The probiotic bacteria isolated from the milk samples were found to be effective against the bacteria such as *E. coli, Klebsiella* spp. *Serratia marcescens*, *Pseudomonas* spp. *and Staphylococcus aureus*. In case of *Klebsiella* spp., *Serratia marcescens, Pseudomonas* spp., and *Staphylococcus aureus* the zone diameter gave the highest zone of inhibition ranging from 21-27mm while lowest ranging from 12-14mm were recorded only against *E. coli* strain. Commercial probiotics (Harmogut, Pro-B, Welgut and Rebio) were also tested where the zone diameter was noticed within the range 13-31mm against the pathogens through drop test method while overlay method test range 13-25mm against the pathogens. The lowest zone of inhibitions 13-18mm against *Pseudomonas* spp. and *Staphylococcus* spp. produced by welgut and rebio. The current findings suggest the usefulness of probiotic bacteria from natural sources and provided the potential activity of commercially available probiotics. Probiotic bacteria are considered as safeguard against the propagation of multi drug resistant bacterial infection and can be one of the best candidates as alternative of conventional antibiotics.
Key words: Endosymbiotic bacteria, Probiotics, Pathogen

Introduction
As antibiotics may inhibit the growth of living microorganisms or kill them, they have a long history of usage in the treatment of bacterial infections [1] Concerns have been expressed over the short-term efficacy of today's antibiotic repertory due to the current spread of antibiotic resistance genes into pathogenic bacteria. The World Health Organization has classified antimicrobial and antibiotic resistance concerns as an unanticipated global health danger with widespread, multisector implications to food, environment, animal, and human safety. These problems have expanded throughout the world [2]. By 2050, it is predicted that the number of fatalities caused by antibiotic-resistant pathogens will reach 10 million annually globally [3]. As a result, several strategies for combating bacterial infections have been promoted, such as using therapeutic drugs to treat illnesses directly or using live, helpful microorganisms known as probiotics to indirectly alter the gut microbial community [4]. probiotic is a live microbial supplement that should have certain qualities, such as being extremely dominant against human infections, having the ability to tolerate acid, not being pathogenic, and adhering to cells [5]. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) state that Lactic Acid Bacteria, Bifido bacterium, Propioni bacterium, and Saccharomyces boulardii are the most prevalent probiotic bacteria [6,7–9].

The antibacterial action of probiotics against numerous pathogenic bacteria, such as the MDR strain of S. aureus, E. faecalis, K. pneumonia, P. aeruginosa, Escherichia coli, S. typhii, and various Salmonella spp., has been highlighted in a number of previous studies [10, 11]. Recent research has demonstrated that probiotics and antibiotics work cooperatively to potentially prevent many bacterial infections while also providing the host with numerous of other health benefits [12, 13]. Additionally, the gut flora becomes more defensive when probiotic microbes are consumed orally. Probiotics have been shown through a variety of investigations and studies to have numerous positive impacts on the host body during illnesses caused on by gut microflora, while its medical applications are yet unknown [7,14,15]. Probiotics have the potential to protect hosts against a range of intestinal illnesses and significantly increase the amount of beneficial bacteria in the gut while doing so [16, 17]. In spite of the many advantages of probiotic usage, it is important to take attention while taking the recommended dosages of probiotics depending on the age of the patient and any physical concerns to avoid the complications related to probiotic use [18]. Probiotics are now more in demand in industrial areas in addition to being used for medicinal and clinical purposes [19]

By utilizing the advantageous bacteria, many commercially available enzymes, medicine, and some fermented foods are produced, which are currently widely marketed as probiotic products [7,20,21]. Additionally, a few human and animal clinical trials and experimental investigations have been published in the literature, suggesting that probiotics may be used as an antibiotic replacement to prevent or eradicate the bacteria that cause a variety of illnesses [22, 23].

According to experimental research, raw goat milk contains new Lactobacilli strains that have the probiotic ability to suppress harmful gut microbiota. A significant amount of the natural microbiota found in the human gut is composed of lactobacilli. When present in sufficient quantities, these probiotic organisms can establish an equilibrium between the gut's beneficial and possibly harmful microflora by preventing the formation of common intestinal pathogens [24,25].

The aim of this study was to isolates bacteria with probiotic potential from animal milk to observe their antibacterial efficacy against clinical pathogenic bacteria as well as to determine the efficacy of commonly used commercial probiotics against the same clinical bacteria.

Materials and methods

Sampling and sample processing

Sample of horse milk was collected from animal farm located in Ghatail, Tangail, Dhaka Bangladesh. Cow milk, goat milk and Horse milk sample were used in this study and four (4) types of commercially available probiotics such as Harmogut, Pro-B, Welgut and Rebio samples were randomly collected from different drug house in Dhaka city. Sterile bottle was used to collect the sample. The sample was then transported with the temperature of 4-8^{0}C within the next 5hrs. Further examinations were conducted on the sample.

Physical examination of milk samples and isolation of milk native bacteria

Physical parameters such as color odor and appearance of the collected milk samples was analyzed. Milk samples were serially diluted up to 10^{-5} and introduced the samples on MSA and MRS agar. For enrichment, 1ml milk samples were added on MRS broth and incubated 4-6 hours and then the 0.1ml sample was again spread on to the MRS agar After incubation of 48hrs at 37^{0}C. White color colonies was observed on De Man-Rogosa-Sharpe Agar.

Cultural, Microscopical and Biochemical Examination of milk and milk native bacteria

Observed the colonies color, size and morphology from the incubated plate. Individual colonies observed than one colony selected on the plate was transformed into slide and then subjected to gram staining to know that the test yield either gram positive or gram-negative bacteria. Finally, the standard biochemical tests were performed for the identification of the isolate found in the sample. For this specific purpose, Triple Sugar Iron media (TSI), Simmons Citrate agar, Oxidase test, Motility, MR-VP, and Indole tests were performed [26].

List of the identified probiotic bacterium from milk

Lactobacillus spp. and *Lactococcus* spp. were found in cow milk, *Carnobacterium* spp. was isolated from horse milk and *Bifidobacterium* were isolated from goat milk samples.

Collection of experimental bacteria against probiotic bacterium from milk and commercial probiotics

The most common clinical pathogens (*Escherichia coli Klebsiella* spp. *Serratia marcescens, Pseudomonas* spp., *Staphylococcus aureus*.) were collected from Medinova hospital, Dhaka Bangladesh. The bacterial culture plates were transported into the research laboratory (Microbiology research lab SUB) in thermal stabilizing box.

Evaluation of antibacterial activity of probiotic bacterium from milk by disk-diffusion method

Antibacterial activity of the isolated bacteria from milk samples were assessed by disk diffusion method in Mueller Hinton agar medium. Bacterial inoculums of the pathogenic isolates were lawned on Mueller-Hinton agar plates through swab-cotton. Next, empty Whatman discs were impregnated with different concentration of *Lactobacillus* spp. and *Lactococcus* spp. *Carnobacterium* spp. and *Bifidobacterium* and were placed on the plates. The plates were anaerobically incubated at 37 °C for 24 to 48 h. The diameters of inhibition zones were investigated using calipers.

Evaluation of antibacterial activity of commercial probiotic by Drop test and Overlay –method on TSA agar

Drop test

Commercial probiotics were also used to observe their antimicrobial activity against the sample pathogenic bacteria. The drop test assay on Trypto-Casein-Soy agar (TSA, BIO RAD) plates containing 20 gl^{-1} NaCl was carried out with slight modifications. Briefly, drops of 4 different probiotic suspensions were spotted (5mm) onto the three agar plates containing a confluent lawn of our three-target strain (dried for 30 min at 30°C) and then incubated at 37°C [27]. Formula: R = (d Inhib – d Spot) /2 ("d Inhib": the diameter of clear zone around the "d Spot"; and "d Spot":) [28].

Overlay –method: Overlay assay was performed with slight modification in this study where the suspensions of 4 different probiotics were spot inoculated, separately, onto the MRS agar plates and the inoculated plates were kept to air dry at 30◦C for 1 h. The MRS agar plates containing the probiotics in spot form (5 mm diameter) were thereafter overlaid with soft Muller-Hinton agar (0.8% agar) pre-mixed with 10^5 CFU of the pathogenic strains and incubated, after solidification of the overlaid agar medium, at 37◦C for 24hr [29].

Result and Discussion

It is a long time that scientists are trying to substitute synthetic drugs with natural products. Nowadays, various natural materials and methods are used to prevent or treat diseases [30]. The use of probiotics is one of these methods. Lactobacilli and bifidobacteria are normal intestinal flora which by preventing intestinal infection, lowering cholesterol, stimulating the immune system, and reducing the risk of colon cancer play an important role in human health. Probiotic bacteria produce lactic acid and organic acids, reduce the pH environment, and try to prevent the growth of many bacteria. These bacteria produce antimicrobial compounds such as bacteriocin which can be used as natural preservatives.

Physical examination of Milk samples

Milk samples from different animal showed different appearance, density and odor (**Figure 1)**. Here, cow milk was in yellowish white color and thick in density while horse milk was in white color and thin in density, but the goat milk was in white-cream color and thicker than both of the sample's milk. The cow milk was odorless while the horse milk had a hay-like smell, and the goat milk gave the goaty aroma (**Table 1**).

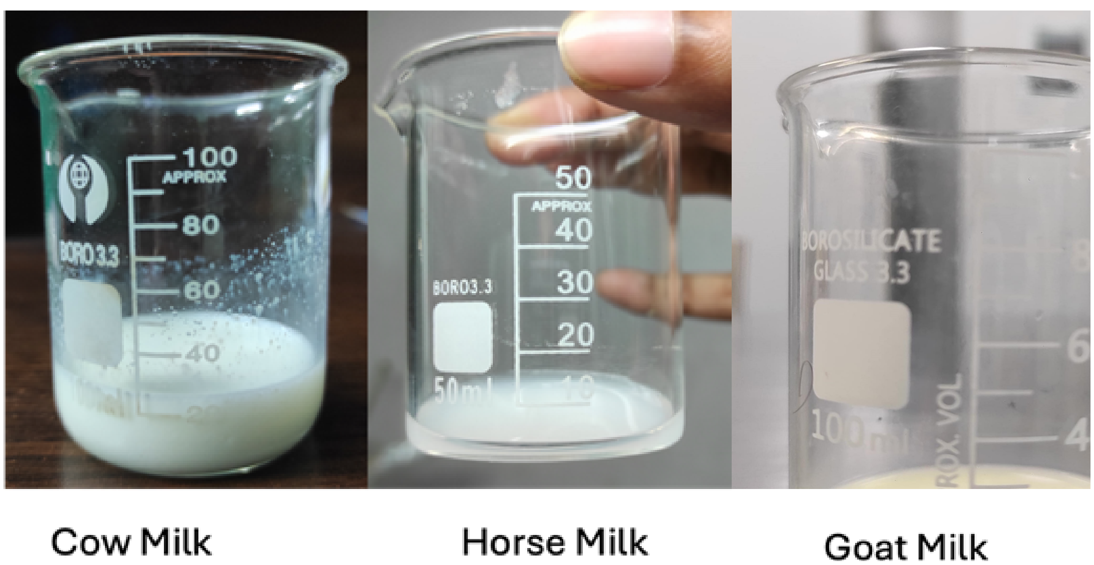

Fig 1: Different types of milk samples

Table 1: Physical examination of Milk samples

Milk Samples	Appearance	Density	Odor
Cow Milk	Yellowish White color	Thick	No odor
Horse Milk	White color	Thin	Hay-like smell
Goat Milk	White-cream color	Thick	Goaty or muttony aroma

Cultural and morphological identification of the isolates:

Identified four isolates showed different cultural and morphological characteristics of the where *Lactobacillus* spp. showed smooth, raised and white color colonies while *Lactobacillus lactis* showed smooth, shiny and off-white color colonies on MRS agar media. *Bifidobacterium* spp. showed purple-brown color and convex shape colonies on bifidobacterium selective media. On the contrary, *Carnobacterium* spp. showed small gray color colonies with irregular edges on blood agar media. All the isolated gave gram positive rod-shaped morphological characteristics except for *Bifidobacterium* spp. which showed short, curved rods (**Table 2**).

Table 2: Morphological or Cultural and Microscopic characteristics of the isolates:

Sample	Cultural Characteristics	Morphological Characteristics
***Lactobacillus* spp.**	Smooth, raised, and often white to cream-colored colonies	Gram-positive, rod-shaped
Lactococcus lactis	Smooth, shiny colonies, round shape, off-white color	Gram-positive, rod-shaped
***Bifidobacterium* spp.**	Purple-brown, convex shape	Gram-positive, short, curved rods,
***Carnobacterium* spp.**	Small, gray, and granular, with irregular edges.	Gram-positive, rod-shaped morphology

Biochemical identification of the isolates from milk samples:

Biochemical tests such as TSI test, Indole test, MR-VP test, Citrate test, Oxidase test and motility test were done to confirm the isolates where presumptive organisms are *Lactobacillus* spp., *Lactobacillus lactis*, *Bifidobacterium* spp. and *Carnobacterium* spp. **(Table 3)**

Table 3: Biochemical Characteristics of the isolate:

Assumed Organism	TSI			H_2S creation	Indole test	MR test	VP test	Citrate test	Motility	Oxidase test
	slant	butt	gas							
***Lactobacillus* spp.**	K	K	-	-	-	+	-	-	-	-
Lactobacillus lactis	A	A	-	-	-	-	+	+	-	-
***Bifidobacterium* spp.**	A	A	+	+	-	+	-	+	-	-
***Carnobacterium* spp.**	K	A	+	+	+	+	-	-	-	-

TSI= Triple Sugar Iron Test, A= Acidic, K= Alkaline, (+)= Positive, (-)= Negative.

Antimicrobial activity of the probiotic bacterium from milk samples against the clinical isolates

After the confirmation of the probiotic isolates from the milk samples, those isolates were further taken to test their antimicrobial activities against clinically important pathogens such as *E. coli*, *Klebsiella* spp., *Serretia mercescens*, *Pseudomonas* spp. and *Staphylococcus* spp. It was shown that probiotic isolate *Lactobacillus* spp. gave lowest zone of 12mm against *E. coli* while highest zone was against *Pseudomonas* spp. of 27 mm. while 25mm zone was noticed against *Serretia mercescens* and *Staphylococcus* spp. and 21mm zone was recorded against *Klebsiella* spp. On the contrary, *Lactobacillus lactis* as probiotic strain gave 14 mm zone against *E. coli* and the zone 27mm and 25mm was produced by *Lactobacillus lactis* against *Pseudomonas* spp. and *Staphylococcus* spp. isolates respectively. The growth of *Serretia mercescens* and *Klebsiella* spp. were inhibited by same as *Lactobacillus lactis* with the zone dieter 22 and 26mm respectively. However, *Carnobacterium* spp. also gave the similar range of zone diameter ranging from 14mm-27mm against the clinical pathogens. *Bifidobacterium* spp. gave the lowest zone of 12mm against *E. coli* while highest zone was 27mm against *Pseudomonas* spp. Others were in range of 21 mm-25 mm zone. Distill water was kept as negative control for the test while gentamycin broad spectrum antibiotic was used as a positive control. It was noticeable that the zone of inhibition of probiotic isolates were in similar range of zone of inhibition of the positive control gentamycin antibiotic which shows that the isolated probiotic strains from milk samples also contain antimicrobial properties to kill the pathogenic isolates (**Table 4).** The study of Boris et al. showed that lactobacilli strains isolated from dairy products were able to inhibit the growth of *P. aeruginosa, E. coli, Salmonella typhimurium, and S. aureus*, the latter was in the highest inhibitory effect [31].

Table 4: Antimicrobial activity of probiotic bacteria from milk samples against pathogens

Probiotic	Zone diameter in mm				
	Escherichia coli.	*Klebsiella* spp.	*Serratia marcescens*	*Pseudomonas* spp.	*Staphylococcus aureus.*
Lactobacillus spp.	12± 0.66	21± 1.63	25± 2.66	27± 2.77	25± 2.33
Lactococcus lactis	14± 0.76	22± 1.65	26± 2.63	27± 1.63	25± 2.33
Carnobacterium spp.	14± 0.76	21± 1.63	26± 2.63	27± 1.60	26± 2.63
Bifidobacterium spp.	12± 0.66	21± 0.63	25± 1.61	27± 2.63	25± 2.33
Negative control (DW)	0± 0	0± 0	0± 0	0± 0	0± 0
Positive Control (Gen)	19± 2.11	24± 1.77	28±2.88	27± 2.77	25± 2.33

The experiments were conducted three times independently. Mean ± SD values have been shown here. The zone diameter of inhibition (ZDI) values was measured and interpreted following Shokryazdan et al. where the ZDI >20 mm, 10–20 mm and < 10 mm were considered as strong, intermediate and weak inhibitions, respectively.

Effects of commercial probiotic sample against the same clinical pathogenic isolates

Commercially available probiotics were then taken to observe the antimicrobial properties against the clinical pathogens. In this study all the probiotics samples were found to be effective against the bacteria such as *E. coli, Klebsiella spp., Serratia marcescens, Pseudomonas* spp. *and Staphylococcus* spp. by drop test and overlay method. Here the satisfactory zone diameter was recorded varies from 13-31mm against the pathogens through drop test method **(Table 5)** while overlay method test range 13-25mm against the pathogens **(Table 6).** It was noteworthy that, the lowest zone of inhibitions (13mm) was recorded by Rebio against the *Pseudomonas* spp. and the highest zone (31mm) was noticed by Pro-B against *Klebsiella* spp. Zone diameter (26mm) was recorded by Harmogut, Pro-B, Wegut and Rebio against *E. coli* and *Serratia marcescens* **(Table 5).** In case of *Pseudomonas* spp., the inhibition range was recorded 13mm-15 by all the probiotic bacteria. We also introduced overlay methods, here we found all the commercial probiotics produced zone diameter 25mm against *E. coli*, *Klebsiella* spp., and *Serratia marcescens*. The lowest zone diameter was recorded 13mm against *Pseudomonas* spp. by Rebio while 18mm-22mm range of zone was recorded against *Staphylococcus* spp. **(Table 6)**. According to the findings all the probiotics could kill the pathogens as the zone diameter of inhibition (ZDI) values obtained were measured and interpreted following Shokryazdan et al. where the ZDI >20 mm, 10–20 mm and < 10 mm were considered as strong, intermediate, and weak inhibitions, respectively. The "R" (width of clear zone) values were also determined as per the formula stated earlier**.** It was noteworthy that, *Pseudomonas* spp. and *Staphylococcus* aureus gave the lowest zone against the commercial probiotics while gave higher zone for the probiotics isolated from the milk samples. Overall, our study reveals that the different animal milks contain probiotics which are beneficial to the consumers as probiotics are good for our health.

For many years, dairy products have been recognized as valuable products to human health. 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. Microorganisms such as lactobacilli and many other bacteria can eliminate pathogens through multiple mechanisms including competitive elimination that results in food safety [20,31].

Table-5: Antagonistic activity of commercial probiotics against pathogens through Drop-test method

Probiotics	Zone diameter in mm				
	E. coli	*Klebsiella* spp.	*Serratia marcescens*	*Pseudomonas* spp.	*Staphylococcus aureus*
Harmogut	26± 2.63	26± 2.22	26± 2.63	15± 0.63	22± 1.63
Pro-B	26± 2.63	31± 3.63	26± 2.62	15± 0.63	20± 2.63
Welgut	26± 2.63	21± 1.62	26± 2.76	15± 0.63	17± 0.67
Rebio	26± 2.63	21± 1.63	26± 2.63	13± 0.60	18± 2.63

The experiments were conducted three times independently. Mean ± SD values have been shown here.

Table-6: Antagonistic activity of commercial probiotics against pathogens through overlay method

Probiotics	Zone diameter in mm				
	E. coli	*Klebsiella* spp.	*Serratia marcescens*	*Pseudomonas* spp.	*Staphylococcus aureus*
Harmogut	25± 2.33	25± 2.33	25± 2.33	25± 2.33	22± 1.33
Pro-B	25± 2.33	25± 2.33	25± 2.33	15± 0.63	20± 1.22
Welgut	25± 2.33	25± 2.33	25± 2.33	15± 0.63	17± 0.73
Rebio	25± 2.33	25± 2.33	25± 2.33	13± 0.73	18± 0.73

The experiments were conducted three times independently. Mean ± SD values have been shown here.

Conclusion

The current study revealed that most of the isolated bacteria including *Lactobacillus* spp*, Lactococcus* spp, *Carnobacterium* spp*, and Bifidobacteria* spp. from cow, goat and house milk were found to be antagonistic against the clinical pathogens as probiotic. In our study, we demonstrated experiments on the commercially available probiotic against the same clinical pathogens and found effective zone diameter. Probiotics are well known for their ability to strengthen the immune system, support digestive health, and reduce the risk of several diseases. Therefore, probiotic formulations may serve as a significant biotherapeutic resource for fighting microbial infections in order to address the global problem of bacterial antibiotic resistance. To ascertain the precise effectiveness of various probiotic strains more thorough investigation is strongly advised. Current study could not determine the other bioactive compound from milk samples due to resource limitation. The health and economic benefits of the identified probiotic bacteria should be given more attention in order to improve community health. The possible application of these bacteria as probiotics should be further tested on different species of experimental animals.

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