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ORIGINAL ARTICLE

Isolation and identification of *Bifidobacterium* from dairy products and screening its antibacterial activity

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ABSTRACT

Introduction:
Bifidobacterium is one of the most used probiotic microorganisms in the food industry due to its health-enhancing benefits.

Methods:
In this study, twenty samples from five different dairy products were collected. *Bifidobacterium* was isolated on Man Rogosa Sharpe (MRS) agar and identified through several tests, including Gram staining, catalase test, oxidase test, triple sugar iron test and testing its susceptibility to mupirocin. Antimicrobial activity against 3 Gram-negative and 1 Gram-positive bacteria which includes *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* respectively was evaluated by disc diffusion method and agar well diffusion method on Mueller-Hinton agar (MHA) medium.

Results:
Out of the 20 isolates from dairy products, 6 were identified as *Bifidobacterium*. All of the 6 isolates showed some antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*, and none against *Klebsiella pneumoniae*.

Conclusion:
The use of *Bifidobacterium spp.* as an adjunctive agent to therapeutic pharmaceutical agents is a promising approach against multi-drug resistant bacteria due to its potential for antibacterial activity as observed in this study.

Keywords
Antibacterial activity, *Bifidobacterium*, dairy products, health-enhancing benefits, probiotics

Introduction

The concept of probiotics was first introduced by Elie Metchnikoff, known as the "Father of Probiotics", in the early 20th century. His theory suggested that the ingestion of host-friendly microorganisms could have significant health-enhancing benefits to the host. [1] The function of probiotics was first understood to be the opposite of antibiotics. That was due to the findings that probiotics' secretion had unique characteristics that enhanced the growth of other microorganisms. The most accepted definition of probiotics and the one used by the World Health Organization (WHO) is given by the Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO) expert committee in 2001. According to which, probiotics are: "Live microorganisms, which when administered in adequate amount confer a health benefit on the host". [1]

It is widely known that probiotics are linked to dairy products, and this concept was introduced by Metchnikoff as well. He believed that a host's autointoxication could be reversed or inhibited by microorganisms that produce lactic-acid (LAB). He endorsed a particular regimen which included the daily intake of probiotics through the ingestion of "soured milk" which is now known as yoghurt. [2] This has contributed to the concept that fermented foods and beverages contain host-friendly bacteria that enhance the host's health. Most of the microorganisms classified as probiotics are bacteria such as *Lactobacillus*, *Bifidobacterium*, *Enterococcus faecium*, *Streptococcus thermophillus*, *Pediococcus*, *Leuconostoc*, *Bacillus coagulans* and *Escherichia coli*. [3] From the microorganisms mentioned, *Lactobacillus* and *Bifidobacterium* are the most significant and used probiotic microorganisms in the food industry and supplementary pharmaceutical preparations. [1] It was shown that the abundant presence of *Bifidobacterium* in the host's gastrointestinal tract is a marker of a healthy micro-flora for the reason that its level fluctuates in humans throughout their lifespan in which the highest numbers are seen in breast-fed infants as breast milk contains bifidogenic substances, which promote the growth of *Bifidobacteria*. Additionally, reduced levels of *Bifidobacterium* are seen in elderly and immune-compromised individuals. [4]

This study's primary focus is *Bifidobacteria*, they are Gram-positive, non-sporulating rods that are anaerobic. The genus consists of more than 50 species, in which only ten are found in the human oro-gastrointestinal tract and vagina, where they contribute beneficial effects. The other species are naturally occurring in fermented products including dairy products, kimchi, and pickles. This study focuses on *Bifidobacterium* found in dairy products widely available and consumed regularly by the public. It aims to determine the antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*. The above bacteria can cause various infections in humans such as infections in the urinary tract, respiratory tract, and gastrointestinal tract. By determining the action of *Bifidobacterium* against the mentioned bacteria, it can be considered a supplementary agent for the management of numerous infections.

Methods

This laboratory study includes the isolation and identification of *Bifidobacterium* from four different dairy products and testing its antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*.

Sample collection

The dairy products used in this study were carefully selected to satisfy the following criteria: a) products need to be popular amongst the population and consumed regularly and b) for the commercial products used, the availability of *Bifidobacterium* in the products needs to be confirmed by the manufacturer. The commercial products used in this study included yoghurt powder, pasteurized yoghurt drink, and cow's milk kefir, apart from homemade yoghurt. All of the samples were procured from local suppliers except for homemade yoghurt. From each product, five samples were taken for further processing which amounted to 20 samples

Isolation of *Bifidobacterium* from dairy products

A small inoculum from each sample was transferred and inoculated into an MRS agar plate as the selective medium using an inoculation loop. It was then incubated anaerobically at 37 ℃ for 48 hours, and subsequent sub-culturing was performed to maintain the culture's live state. [5]

Identification of *Bifidobacterium*

The isolates were identified by Gram staining, catalase test and oxidase test performed by standard procedure. They were subsequently confirmed by their ability to ferment sugar in Triple Sugar Iron (TSI) agar, as well as their susceptibility to Mupirocin.

Preparation of discs with supernatant from cultures of *Bifidobacterium*.

Bifidobacterium isolated from the previous steps was grown on tryptic soy broth supplemented with Tween 80 and incubated anaerobically at 37 ℃ for 48 hours. Fresh cultures of the isolated *Bifidobacterium* were centrifuged for 15 minutes at 8000 rpm, followed by removing the supernatant. Sterile Whatman No. 1 filter paper discs (6mm in diameter) were charged with supernatant of *Bifidobacterium* culture. These discs were placed in a 96 well microplate and charged with 40 µl of supernatant, then allowed to soak overnight. Subsequently, the discs were used to perform disc diffusion method to evaluate the antibacterial activity of *Bifidobacterium*. [5, 6]

Evaluation of the antibacterial activity of *Bifidobacterium* using disc diffusion method

The test strains were inoculated on Mueller-Hinton Agar (MHA), followed by the placement of supernatant charged discs along with positive and negative controls on the surface of the plate. The plates were then incubated at 37 ℃ for 24 hours. The antibacterial activity was indicated by the density of bacterial growth around the discs. [7]

Evaluation of the antibacterial activity of *Bifidobacterium* using agar well diffusion method.

A sterile glass dropper was used to punch six wells each on four MHA plates inoculated with test strains, in which 40 µl of supernatant from each sample was loaded in the wells using a sterile dropper. The plates were then incubated at 37 ℃ for 24 hours. The antibacterial activity was indicated by the density of bacterial growth around the discs. [7]

Results

Isolation of *Bifidobacterium* from samples (Table 1)

Table 1: Isolation of Bifidobacterium from samples

Source	Isolate	Isolation of *Bifidobacterium*
Milk Kefir	1A	-
	1B	-
	1C	-
	1D	-
	1E	-
Pasteurized Yogurt Drink	2A	-
	2B	-
	2C	-
	2D	+
	2E	-
Homemade Yogurt	3A	+
	3B	+
	3C	+
	3D	+
	3E	+
Yogurt Powder	4A	-
	4B	-
	4C	-
	4D	-
	4E	-

Identification tests

Out of the twenty samples tested, six isolates identified as *Bifidobacterium* were taken from 4 different sources. Five isolates were obtained from homemade yoghurt, and one was obtained from the pasteurized yoghurt drink. The morphology from the Gram stain observation of *Bifidobacterium* isolates showed the key structural characteristics of *Bifidobacterium*, which included branched bifurcated Y-shaped rods structure (Fig. 1). *Bifidobacterium* having a club-shaped rod structure was notably seen in the Gram stain observation for samples taken from the pasteurized yoghurt drink (Fig. 1).

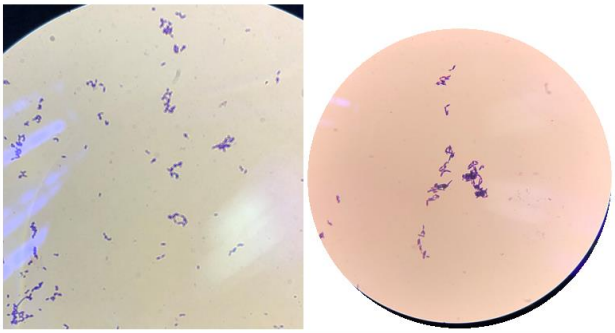

Figure 1: Bifurcated Gram-positive rods isolated from homemade yogurt (100x magnification)

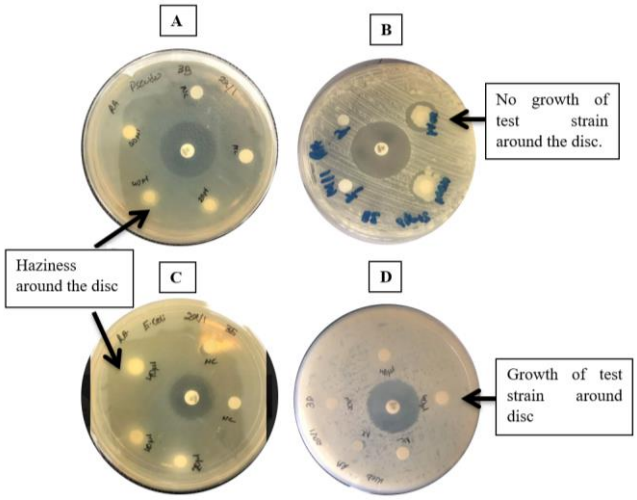

Figure 2: Screening for the antibacterial effect of *Bifidobacterium spp.* by disc diffusion method (A = Isolate 3B against *Pseudomonas aeruginosa*, B = Isolate 3B against *S. aureus*, C = Isolate 3E against *E. coli*, D = Isolate 3D against *Klebsiella pneumoniae*)

Antibacterial activity screening test by disc diffusion method

Antibacterial activity was recorded when there was no growth or haziness around the disc, which indicated decreased density in the growth of test strains, while growth around the disc was indicated to have no antibacterial activity. Isolates 3B, 3C, 3D, and 3E have shown some antibacterial growth against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Whereas 2D and 3A did not show any antibacterial activity against any of the test strains. There was no activity against *Klebsiella pneumoniae* (Fig. 2).

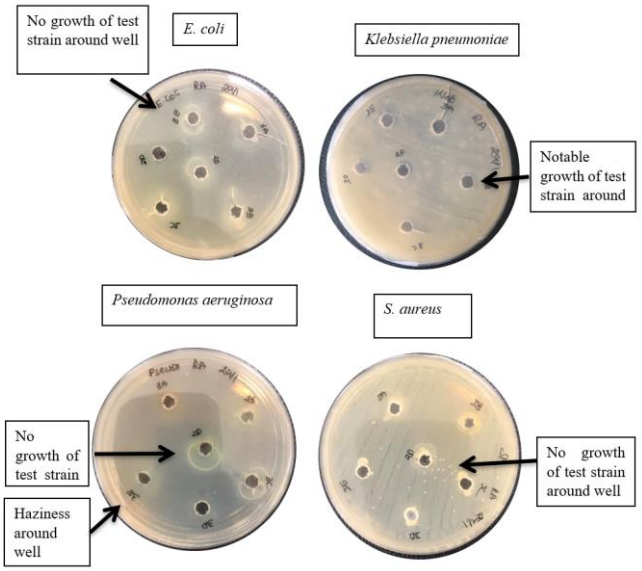

Figure 3: Screening for the antibacterial effect of *Bifidobacterium spp.* by agar well diffusion method

Antibacterial activity screening test by agar well diffusion method

Out of the six isolates identified as *Bifidobacterium*, isolates 2D, 3A, 3B, 3C, and 3E have shown antibacterial activity against *Escherichia coli*. Whereas for *Pseudomonas aeruginosa*, isolates 2D, 3C, and 3E have shown antibacterial activity, however, isolates 3A, and 3B have exerted some antibacterial activity. In addition, isolates 2D, 3A, and 3B have shown antibacterial activity against *Staphylococcus aureus*. However, isolates 3C, 3D, and 3E have exerted some antibacterial activity by agar well diffusion method against *Staphylococcus aureus*. Isolate 3D did not have any inhibitory activity against *Pseudomonas aeruginosa* by agar well diffusion method and none of the isolates had antibacterial activity against *Klebsiella pneumoniae* (Fig 3).

Discussion

In this study, six out of twenty isolates identified as *Bifidobacterium* and were screened for antibacterial activity using the culture supernatant. As seen in Figure 2 and Figure 3, the screening was achieved by disc diffusion and agar well diffusion methods which resulted in the formation of different patterns of zones of inhibition. The methods did not show the same results, which might be due to the disc's preparation, and there is no method to measure the absorbance capacity of the discs used. Therefore, it is not fully known precisely how much of the suspended supernatant was absorbed by the blank disc and the filter paper discs.

The results from other similar studies suggest that the compounds responsible for the antibacterial activity of *Bifidobacterium spp.* are released in their supernatant [7]; nevertheless, these compounds cannot be quantified. One of the factors that contribute to the release of a sufficient amount of these compounds is having the appropriate environment for the growth of *Bifidobacterium* and the release of the antibacterial compounds. Thus, inducing factors that have the possibility of increasing the release of these antibacterial compounds should be looked into.

The findings from the current study indicate that *Bifidobacterium* has a more significant inhibitory effect against *Staphylococcus aureus* than the rest of the test strains. This result comes in accordance with the results obtained from a study. [7] The mentioned study has investigated the antibacterial activity of *Bifidobacterium spp.* isolated from dairy products against bacteria that is commonly found in patients with cardiac catheterization. The antibacterial effect was assessed by the agar well diffusion method, in which each well was filled with 0.5 ml of cultivated *Bifidobacterium* rather than using its supernatant. The results indicated a notable inhibitory action of the isolated *Bifidobacterium spp.* against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Serratia marcescens*. Nonetheless, the results obtained from the current study did not show any inhibitory effect against *Klebsiella pneumoniae*. [7]

A study conducted by Khaleel and Hannon assessed the inhibitory effect of *Bifidobacterium spp.* isolated from various sources, including animal faeces, cow's milk, soil, and vaginal swabs taken from cows. [9] This study targeted the antibacterial effect against *Staphylococcus aureus*. The zone of inhibition produced by each isolate differed in diameter; however, all isolates identified as *Bifidobacterium* showed moderate to strong antibacterial activity against *Staphylococcus aureus*. The assessment was achieved by using 50 µl of *Bifidobacterium* culture that was inoculated in MRS broth, conversely, 40 µl of *Bifidobacterium* supernatant was used in the current study to screen for antibacterial activity. However, the findings from both the studies show similar observations in the case of antibacterial activity against *Staphylococcus aureus*. [9] The fact that *Bifidobacterium* was isolated from sources other than dairy products, it is highly likely that the isolates were of different species as the one from the current study. In addition, the species that are present in dairy products may have different actions from the ones in the normal microbiota, as the benefits of *Bifidobacterium* are species-specific. Therefore, the species isolated by Khaleel and Hannon may possess different antibacterial activity when compared to the species isolated from dairy products. [9]

A recent study by Shamsuddin and Khan investigated the antibacterial and antibiotic sensitivity of lactic acid-producing bacteria isolated from various fermented food. [10] In this study, species from *Bifidobacterium* and *Lactobacillus* were tested for antibacterial activity against pathogenic microorganisms that include *Bacillus subtilis, Salmonella typhimurium, Bacillus cereus, Escherichia coli*, and *Staphylococcus aureus*. The method used to assess

antibacterial activity was the agar well diffusion method in which *Bifidobacterium* strains showed the highest degree of inhibitory effect against all pathogens studied. [10] The inhibition zones produced by the supernatant produced showed similar hazy zones of inhibition as the ones seen in the current study.

Out of the six isolates identified as *Bifidobacterium* and were screened for antibacterial activity, five were isolated from homemade yoghurt and presented different antibacterial activity against the test strains. That may be because homemade yoghurt is a natural source of *Bifidobacterium* and is assumed a diverse species with different antibacterial effects would be present. That was indicated by the different levels of inhibitory effects that the five isolates showed. In addition, only 1 sample out of 5 identified as *Bifidobacterium* from the samples taken from the pasteurized yoghurt drink. Therefore, that would indicate that different samples from the same source could grow different species.

Considering that this is a screening test, further research is necessary to determine to what extent *Bifidobacterium* can have as an inhibitory effect. For that purpose, it may require the stimulation and induction of *Bifidobacterium* to produce antimicrobial compounds more abundantly. The fact that the induction of *Bifidobacterium* was not done in the current study may have resulted in the insufficient release of antibacterial compounds which would be a limitation of this study.

Conclusion

The resistance of various Gram-positive and Gram-negative bacteria against antibiotics has become a significant obstacle in the health industry that calls for the discovery of new strategies and approaches that can overcome this obstacle. Probiotic microorganisms are being extensively investigated as a new supplementary approach.

In conclusion, the use of *Bifidobacterium spp.* as an adjunctive agent to therapeutic pharmaceutical agents is a promising approach against multi-drug-resistant bacteria due to its potential for antibacterial activity as observed in this study.

Limitation and future scope

As revealed from previous research, the beneficial effects of *Bifidobacterium* and other probiotic microorganisms are species-specific; therefore, further research should be dedicated to identifying which species of *Bifidobacterium* is responsible for the antibacterial effect against pathogenic bacteria. That will lead to better utilization of *Bifidobacterium spp.* by the food industry and the health industry in terms of determining which strains to be included in supplementary products.

Abbreviations

Man Rogosa Sharpe (MRS), Mueller-Hinton Agar (MHA), Triple Sugar Iron (TSI)

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Authors' contribution

a. Study planning: RE, BK, NV
b. Data collection: RE
c. Data analysis/ interpretation: RE, BK, NV
d. Manuscript writing: RE
e. Manuscript revision: RE, BK, NV
f. Final approval: RE, BK, NV
g. Agreement to be accountable for all aspects of the work: RE, BK, NV

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Availability of data and materials

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Competing interests

There is no conflict of interest for any author of this manuscript.

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