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"COMPARISON OF BIOLOGICAL ACTIVITIES OF NATURAL AND COMMERCIAL PROBIOTICS"

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ABSTRACT

The aim of this paper is to study and compare the biological characteristics of natural and commercial probiotic. The probiotics that were studied were Lactic Acid Bacteria. They were isolated from cow milk and Bifilac. The information obtained from this study could contribute to the potential use of these lactobacilli isolates in the food and pharmaceutical industries.

Keywords: Probiotics, Lactobacilli, GI Tract

INTRODUCTION

Probiotics are the live bacteria and yeasts that are good for the health and especially the digestive system. Beneficial bacteria include *Lactobacillus* and *Bifidobacterium* spp. and other lactic acid bacteria. There are enough evidences for the role of probiotics in gastrointestinal infections, irritable bowel syndrome and inflammatory bowel diseases. In human medicine, much research has been performed, and the list of documented health benefits is growing, ranging from amelioration of irritable bowel symptoms in children to improvement of oral health or prevention of recurrent urinary tract infections in adult.

One of the main aspects of the definition of a probiotic is that it should provide beneficial effects to the host when administered in adequate amounts.

Despite some of its limitations, the potential therapeutic effects of probiotics should not be dismissed, particularly given the positive effects documented for prevention and treatment of a wide range of human diseases.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Materials for Isolation of Lactobacillus

Cow milk, commercially available probiotic (Bifilac), glass wares, distilled water, pipette, MRSA media, and spreader.

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2.1.2 Materials for identification of *Lactobacillus* species

Fresh culture, crystal violet, Gram's iodine, 95% ethyl alcohol, safranin stain, H_2O_2 and glass wares.

2.1.3 Materials for biochemical characterization of *Lactobacillus*

Cultured cells, Nacl, tryptone broth, Kovac's reagent, MRVP broth, methyl red indicator, Barritt's A reagent, Barritt's B reagent, Simmon's citrate agar, sugar fermentation broth, Durham tubes, nutrient gelatin media and glass wares.

2.1.4 Materials for determination of probiotic properties of *Lactobacillus*

MRSA media, buffer, antibiotic discs (Gentamycin), standard indicator strains (*Staphylococcus*, *Pseudomonas*), glass wares.

2.2 Methods

2.2.1 Isolation of Lactobacillus

The samples, cow milk and Bifilac were collected from a local farm in Dehradun and Bifilac was obtained from a pharmacy, respectively. Appropriate dilutions of the collected milk sample and the commercial probiotic were made in distilled water up to 10^{-5}. 1 ml aliquots of three dilutions of each sample was pour plated on MRSA media and incubated at 37°C for 48 to72 hours. At the end of 72 hours, when the colonies became predominant, morphologically distinct and well isolated, the colonies were randomly picked and inoculated in new MRSA plates by streaking. Colonies of lactobacilli were further transferred into MRS broth for further enrichment. The pure isolates were preserved for further works.

2.2.2 Identification of *Lactobacillus* species

The identification of bacterial colonies was done by Gram staining followed by catalase test to confer whether the bacteria possess the enzyme catalase or not.

2.2.2.1 Gram Staining

Aseptically bacterial colonies were transferred into clean slide and a thin smear was made. The smear was heat fixed to which crystal violet was added and the dye was then washed under running water after 60 seconds. The smear was again flooded with Gram's iodine and the slide was washed after another 60 seconds. Further the decolorizing agent, 95% ethyl alcohol was added to the smear. After 15 seconds the counterstain safranin was added to the smear and washed after keeping it for 60 seconds. The slide was then cleaned off the excess stain using a blotting paper. The prepared slide was microscopically observed under oil immersion for morphological identification.

2.2.2.2 Catalase Test

To perform the catalase test bacterial colonies were transferred onto a clean slide under aseptic condition. To the isolated cells 1-2 drops of H_2O_2 was added. The isolates were then observed for formation of O_2 gas bubbles.

2.2.3 Biochemical characterization of *Lactobacillus*

The following biochemical tests were run:-

2.2.3.1 Growth at different temperature

The growth of the natural and commercial bacteria was observed by plating them on MRSA media and incubating them at varied temperatures for 48 hours.

2.2.3.2 Growth at different NaCl concentrations

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The bacterial isolates of the probiotics were grown on varied salt concentrations to observe their maximum growth. The micro-organisms were inoculated on MRSA media containing……. Salt concentrations and incubated for up to 7 days.

2.2.3.3 Indole production test

The test was run to determine whether the bacteria could oxidise the tryptophan into indole, pyruvic acid and ammonia. Tryptone broth was prepared which was taken in sterile test tubes. The bacterial isolates were inoculated in two test tubes whereas the other two test tubes were left uninoculated to serve as controls. The tubes were incubated at 37℃ for 48 hours. Following incubation 5 drops of Kovac's reagent was added to the incubated broth. The tubes were then observed for the formation of any red layer over the broths.

2.2.3.4 Methyl Red test

Methyl red test detects the ability of the bacteria to oxidise glucose with the production of concentrated acid and acidic products. MRVP broths were taken in test tubes which were then inoculated with the bacterial colonies and controls were left uninoculated. The tubes were incubated for 48 hours. To the incubated test tubes 4-5 drops of methyl red indicator was added. The tubes were observed for any change in colour.

2.2.3.5 Voges Proskauer test

The Voges Proskauer test differentiates the bacteria on the basis whether they could ferment the pyruvic acid to further a neutral product acetoin. The MRVP broth was suspended with test culture and incubated for 48 hours. 5-10 drops of Barritt's A reagent followed by Barritt's B reagent was added to the incubated tubes. The tubes were observed for any colour change.

2.2.3.6 Citrate utilisation test

Simmon's citrate test was performed on Simmon's citrate agar to check whether the bacteria could convert the citrate present in the media to pyruvate. Slants were prepared of the above mentioned media. Aseptically bacterial isolates of the probiotics were streaked onto the agar slants and incubated for 48 hours. After two days the incubated tubes were observed.

2.2.3.7 Carbohydrate utilisation test

This test determines the ability of the microbes to ferment a carbohydrate. To perform the test phenol red glucose broth was prepared. The prepared broth was taken in test tubes to which Durham's tubes were inserted in inverted positions. The test micro-organisms were then aseptically inoculated in the broth and incubated for 48 hours. The broths were then observed for any change in their colour. Change in their colour from red to yellow would indicate a positive reaction.

2.2.3.8 Gelatin hydrolysis test

This test determines whether a microbe could hydrolyse gelatin. The nutrient gelatin media was used for this test. The media was transferred in test tubes and left for solidification. The test bacteria were stab inoculated in the media and incubated at optimal temperature for one week. The incubated tubes were checked every day for gelatin liquefaction by keeping them in refrigerator for some time.

2.2.4 Determination of probiotic properties of *Lactobacillus*

The probiotic properties of the test micro-organisms were determined by examining their resistance to pH, antimicrobial activity and antibiotic resistance.

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2.2.4.1 Resistance to pH

The differential media MRS agar was prepared with pH values 2, 4, and 5. Pouring the media on different petri dishes the isolated cells of both the probiotics were inoculated. The plates were incubated at 37°C for 48 hours.

2.2.4.2 Antimicrobial activity

The appointed indicator strains were inoculated on a nutrient agar media. The test micro-organisms were centrifuged at 10,000 rpm for 20 minutes to prepare the cell free extract for the determination of antimicrobial activity. The extract was obtained in the supernatant. The test was done agar well diffusion method. Wells were dug on the prepared NAM plates which were the filled with the cell free extract. The plates were incubated at 37°C for 2 days for the growth of the indicator strains. After 2 days the zone of inhibition of the bacterial growth was measured.

2.2.4.3 Antibiotic resistance

For determining the antibiotic resistance of the bacterial isolates of the natural and commercial probiotics, the antibiotic Gentamycin was used. Disc diffusion method was employed to examine the antibiotic resistance. The test cultures were inoculated in their differential media MRSA. Small wells were dug on the plates to impregnate the antibiotic discs. After placing the discs using a disc dispenser the plates were incubated for 24 hours at 37°C. The clear zones around the bacterial colonies were observed for depicting their antibiotic resistance.

RESULTS & DISCUSSION

3.1 Isolation of Lactobacillus

The bacteria from the natural probiotic and the commercial probiotic were isolated by serial dilution. Both the samples were serially diluted using distilled water upto a range of 10^{-5} from $10^{-1.}$

Samples of various concentrations were poured on MRSA plates and incubated at 37°C for 72 hours.

The Cultures Were Re-Streaked for Preservation:

3.2 Identification of Lactobacillus

Table 3.2 The identification was done by Gram staining and catalase test.

Bacterial Isolates	Cell Shape	Cell Arrangement	Gram Staining	Catalase Test
Natural	Rod	Single, Chain	Positive	Negative
Commercial	Rod	Clustered	Positive	Negative

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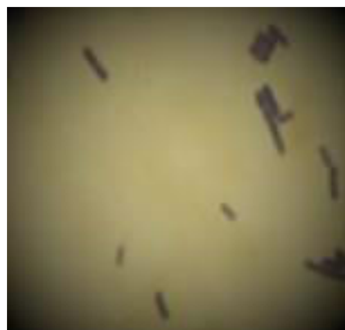
3.2.1 Gram staining



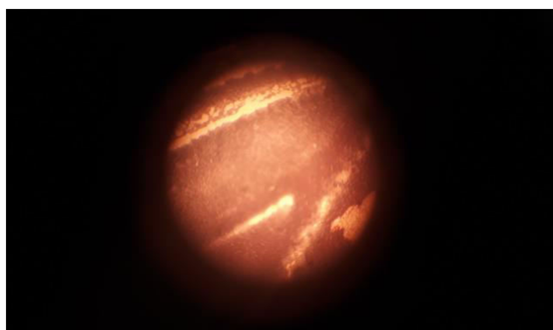


Fig. Natural probiotic **Fig.** Commercial probiotic

3.2.2 Catalase test

3.3 Biochemical Characterization

The following results were obtained from the bio chemical tests performed:

3.3.1 Growth at different NaCl concentrations

Table 3.3.1 Both the probiotics were grown on the following salt concentrations and the result is mentioned below:

Bacterial Isolates	NaCl Concentrations			
	0.1g	**0.3g**	**0.5g**	**0.7g**
Natural	+ve	+ve	+ve	-ve
Probiotic	-ve	-ve	-ve	-ve

3.3.2 IMViC test

Table 3.3.2 IMViC test results as demonstrated by the natural and commercial probiotic:

Isolates	IMViC Test			
	Indole	**MR**	**VP**	**Citrate**
Natural	+ve	-ve	-ve	-ve
Commercial	+ve	+ve	-ve	-ve

3.3.3 Sugar utilisation test

Table 3.3.3 Sugar utilisation test of natural and commercial probiotic:

Bacterial isolates	Sucrose
Natural	+ve
Commercial	+ve

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3.3.4 Gelatin hydrolysis test

Table 3.3.4 Gelatin hydrolysis test in natural and commercial probiotic:

Bacterial Isolates	**Gelatin Hydrolysis Test**
Natural Probiotic	+ve
Commercial Probiotic	+ve

3.4 Determination of Probiotic Properties

3.4.1 Resistance to pH

Table 3.4.1 Resistance to varied pH concentrations of natural and commercial probiotics:

Bacterial Isolates	**pH Concentrations**		
	pH 2	**pH 3**	**pH 4**
Natural	**+ve**	**++ve**	**+ve**
Commerial	**-ve**	**-ve**	**-ve**

3.4.2 Antimicrobial activity

Table 3.4.2 Zone of inhibition produced by probiotics:

Bacterial isolates	**Wells**	**Diameter of zone of inhibition (mm)**	
		Staphylococccus	Pseudomonas
Natural	A	4	No zone
	B	12	No zone
	C	No zone	4
	D	7	13
Commercial	A	No zone	No zone
	B	3	No zone
	C	9	No zone
	D	11.3	13.5

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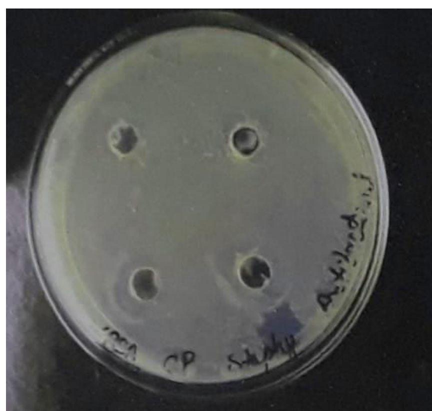

Fig. (A)

Fig. (B)

Fig. (C)

Fig. (D)

Fig. (A) Antimicrobial activity of Commercial probiotic against *Staphylococcus*

Fig. (B) Antimicrobial activity of Natural probiotic against *Staphylococcus*

Fig. (C) Antimicrobial activity of Natural probiotic against *Pseudomanas*

Fig. (D) Antimicrobial activity of Commercial probiotic against *Pseudomonas*

3.4.3 Antibiotic resistance

Table 3.4.3 Antibiotic resistance of natural and commercial probiotic against Gentamycin:

Isolates	**Wells**	**Zone of inhibition due to Gentamycin (mm)**
Natural	A	9
	B	10
Commercial	A	6
	B	4

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Fig. Zone of inhibition due to Gentamycin in natural and commercial probiotics

The isolated bacterial cultures were subjected to Gram staining for identification. While viewing the prepared slides with Gram stain under compound light microscope, it was observed in both the probiotics that the bacteria were purple, rod shaped and formed single to long chained arrangements. Hence it was inferred, the bacteria present in the samples were Gram positive. *Lactobacillus* sp. are common probiotics known for their efficiency in the enhancement of digestive system.

The bacteria were streaked again onto new MRSA plates for preservation and stored in the refrigerator. Since Gram staining is not the only criteria for identification, it was tested whether the bacteria possessed the enzyme catalase. The catalase test was performed, where oxygen evolves in the form of bubbles when the bacterial isolates come in contact with the hydrogen peroxide if the micro-organism possesses the catalase enzyme. In this case when the test was performed for both the probiotic bacteria no such bubbles were formed which clearly indicated the bacteria were catalase negative i.e., the bacteria did not possess catalase enzyme. Since the concerned were both Gram positive and catalase negative and also one of them was isolated from dairy product it was concluded that the bacteria were LAB.

Further biochemical tests were performed on the bacteria. The bacterial isolates were for IMViC, carbohydrate fermentation and gelatin hydrolysis. While some of the results of the IMViC test were positive, the carbohydrate fermentation and gelatin hydrolysis test was positive for both the probiotics. Hence it was deduced that the test probiotic bacteria could ferment sugars and they produce the extracellular enzyme gelatinase that hydrolyses gelatin.

The bacteria were grown on varied salt concentrations. The natural probiotic bacteria could grow in a low to moderate salt concentration but not in a high salt concentration. While the natural probiotic could tolerate salt concentration up to a point the commercial probiotic bacteria could not grow in any given salt concentration.

The LAB are acid resistant. They were grown on culture media with a pH range of 2-4. In the petri plates containing natural probiotic cultures were observed proving that they can tolerate and grow in an acidic environment. While the growth in acidic media was low in pH 2 and high in pH 3 and 4, there was minimal to no growth of the commercial probiotics. Thus it was considered that the commercial probiotic bacteria were not efficient enough to grow in high acidic environment.

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CONCLUSION

In the present world probiotics are consumed by a large population for its varied known health benefits. The probiotics are confirmed to provide a generous amount of comfort in case of gastrointestinal problems. In the study of commercial and natural probiotics it was seen that both showed similar results almost in each test performed except the growth of the bacteria in different pH.

The growth of the natural probiotic was quite high compared to the commercial probiotic. The natural bacteria were more acid resistant hence could grow in high acidic environment. Therefore, it can be concluded that the natural probiotic is better for consumption compared to the commercial probiotic. Considering the easy availability of natural probiotics and its greater benefit the human population must start to have them regularly.

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