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COMPARATIVE STUDY ON PROBIOTIC BACTERIAL ISOLATES AND FORMULATION INTO PRODUCT

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ABSTRACT

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer health benefits to the host. The term ''probiotic'' includes a large range of microorganisms, mainly bacteria but also yeasts. Because they can stay alive until the intestine and provide beneficial effects on the host health, lactic acid bacteria (LAB), non-lactic acid bacteria and yeasts can be considered as probiotics. LAB is the most important probiotic known to have beneficial effects on the human gastro-intestinal (GI) tract. Probiotic bacteria are used in production of functional foods and pharmaceutical products. They play an important role in promoting and maintaining human health. In order, to make health benefits probiotic strains should be present in a viable form at a suitable level during the product's is shelf life until consumption and maintain high viability throughout the gastrointestinal tract. To improve the survival rates of probiotic microorganisms during gastric transit, microencapsulation is considered to be a promising process. Therefore, the main objective of this study is possible to isolate and identify different types of probiotic microbes from different food samples, formulation of probiotic products as s functional and beneficial effect using fruits, vegetables and food samples.

Keywords: Probiotics; Encapsulation, Lactic Acid Bacteria, Lactobacillus, Probiotic bacteria

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1. INTRODUCTION

The increase in population around the globe increases the demand for food and its availability to satisfy the needs of human is increasing day by day. The United Nations Food and Agriculture Organization estimated that nearly 870 million people of the 7.1 billion people around the world are suffering from chronic under nourishment [1]. The term "probiotics" is derived from two Greek words, "pro" means "for" and "bios" means "life". Probiotics refer to live microorganisms which when administered in adequate amounts, confer a health benefit on its host. The term Probiotics was coined in 1965 by Lilly and Stillwell, who defined them as "microbial derived factors that stimulate growth of other organisms" [2]. Probiotic bacteria may produce various compounds, which are inhibitory to the pathogen's growth, which include organic acids (lactic and acetic acids), bacteriocins, and reuterin. The organic acids not only lower the pH, thereby affecting the growth of the pathogen, but they can also be toxic to the microbes. There is increasing evidence that probiotics are beneficial in gastrointestinal disturbances, such as diarrhoea, dysentery, typhoid etc. [3]. It is important to underline when considering the effectiveness and biological activity of probiotics, probiotics or their combination (symbiotic) that they are food products and not drugs. Furthermore, in many cases, their effects are mainly prophylactic in nature, rather than therapeutic, i.e. preventive rather than curative. Lactic acid bacteria were referred to as probiotics in scientific literature by Lilley and Stillwell [4].

Lactic acid bacteria (LAB) are a group of Gram positive, non-spore forming, cocci or rods which produce lactic acid as major end product from fermentation of carbohydrates. Majority of microorganisms used as probiotics belong to the LAB and Bifidobacteria. Within the group of LAB, *Lactobacillus* species are most commonly utilized group of microorganisms for their potential beneficiary properties as probiotics [5].

The effect of probiotics ranges from regulation of bowel activity and well-being to more specific actions, such as, antagonistic effect on the gastro enteric pathogens like *Clostridium difficile, Campylobacter jejuni, Helicobacter pylori, rotavirus* etc., [6].The most common application of probiotics is for dairy production such as yogurt, cheese, and ice cream. Recently, several studies were done for the use of probiotic bacteria in preventing antibiotic-associated diarrhea and C. *difficile* infections [7]. In addition, many species have been suggested to be effective in alleviating gastrointestinal pathogenic bacterial infections both in vitro and in vivo [8]. Due to the numerous positive effects of probiotics on human and animal health, a number of Probiotic products are now available in market. Probiotic products can either be available as Dairy based products or non-dairy based [9]. The aim of the present study is possible to isolate and identify different types of probiotic microbes from different food samples, formulation of probiotic products as s functional and beneficial effect using fruits, vegetables and food samples.

2. MATERIALS AND METHODS

In this study, samples like curd, Bael fruit, Brinjal were used for isolation of lactic acid bacteria. The curd sample was collected in sterilized reagent bottle from retail outlets in Davanagere, India.

2.1. Isolation of LAB

A 10 g sample of each food sample was homogenized in 100 ml of sterile water; pH 7. 10-fold serial dilutions of the samples were prepared in sterile water. MRS agar was used for the isolation of probiotic bacteria. The number of LAB strains was determined by spread plating the dilutions on MRS (de Man Rogosa Sharpe Agar) agar medium. The plates were incubated at 25ºC for 24-48 hrs. After incubation, single morphologically similar

colonies were isolated, observed microscopically, sub cultured and preserved as MRS agar slant cultures [9, 10].

2.2. Identification of LAB

2.2.1. Gram Staining

The slides are taken and the smears are prepared by heat fixing and allowed them to dry. After that the slides are stained with crystal violet for 30seconds. Rinse the slide with water and then add gram's iodine for 30seconds again rinse the slide with water and washed with 95% ethyl alcohol for 10 to 15seconds counter stain with saffronin. Rinse with water, air dry the smear. Observe under microscope.

2.2.2. Catalase Test

The slides are taken and small amount of inoculum is placed with the help of sterile loop, a drop of 3% H_2O_2 is added and observed for evolution of oxygen bubbles.

2.3. Evaluation of Probiotic Potentials of Isolated Bacterial Cultures

For the screening of the probiotic, it was subjected to following tests like pH tolerance test, Bile tolerance test, temperature tolerance test, antibiotic sensitivity test and antimicrobial test, lactose utilization test, for three samples so that, whether they can tolerate the various stress factors in the gut, and their results were obtained.

2.3.1. pH Tolerance Test

The isolated bacterial cultures were inoculated into sterile MRS broth tubes of varying pH, i.e. pH 2, 3, 4, 5, 6, and 7 and incubated at 37°C for 2-3 days. Then 0.1ml inoculums from each tube was poured to MRS agar medium by spread plate method and incubated at 37°C for 48hrs. The growth of LAB on MRS agar was used to designate isolates as pH tolerant [3].

2.3.2 Bile Salt Tolerance Test

The medium with varying concentrations of bile salt (0.5, 1.0, 1.5 and 2.0%) was inoculated with each selected bacterial culture and incubated at 37°C for 48hrs. Then 0.1ml inoculum was transferred to MRS agar by pour plate method and incubated at 37°C for 48hrs. The growth of LAB cultures on agar plates was used to designate isolates as bile salt tolerant [3].

2.3.3. Temperature Sensitivity Test

The selected bacterial cultures were grown at varying temperatures, i.e. 25°C, 37°C for 48-72 hrs. Then 0.1ml inoculum was transferred to MRS plates by pour plate method and incubated at 37°C for 48hrs. [3].

2.3.4. Lactose Utilization Test

The acid production by selected bacterial cultures was detected by observing the change in colour of the medium. Sterilized fermentation medium (10g peptone, NaCl 15g, phenol red 0.018g, lactose 5g, for 1L distilled water and final pH 7.0) was inoculated with different cultures and incubated at 35°C for 24-48 hrs. Change in colour from red to yellow indicates the production of acid [3].

2.4. Antibiotic Susceptibility Test

The antibiotic susceptibility of isolated LAB was assessed using antibiotic discs diffusion method on MRS agar plates. Broth cultures of LAB were prepared using MRS. A 100µl suspension of freshly grown bacterial cultures was spread on MRS agar plates. The antibiotic discs were placed on the surface of agar and the plates were incubated at 37°C for 48 hrs. The pattern was assessed using Amoxicillin, Oxacillin, Erythromycin discs [3].

2.5. Antimicrobial Activity Test

Each Lactobacillus isolate spread on media by using sterile L shaped glass rod. The wells are drilled by using cork borer and samples of *E.coli and Staphylococcus aureus* are poured into the well and the plates were incubated for (24 – 48) hrs at 37°C. After the incubation the activity was analysed [3].

2.6. Formulation into Product (Chocolate)

2.6.1. Encapsulation of Probiotic Bacteria

2.6.2. Growth, Isolation and Preparation of Probiotic Sample

The probiotic sample was inoculated in 100 ml of MRS broth and incubated for 48 hours at 37°C. At the end of 48 hours, the bacterial cells were isolated by centrifugation at 7000 rpm for 15 minutes. The supernatant was discarded and pellet was taken. Sample was prepared by dilution of the bacterial cell pellet in 1 ml autoclaved water.

2.6.3. Preparation of Capsules/ Beads

Three percent sodium alginate and 0.05 M calcium chloride solution was prepared using deionized water and both the solutions were autoclaved at 121°C (15 psi) for fifteen minutes. To the autoclaved sodium alginate solution, bacterial sample was added and the solution was homogenized using vortex. This solution was added drop wise using a syringe with needle diameter of 1 mm to the calcium chloride solution. The capsules/beads formed were allowed to harden for 10 minutes, washed in deionised water twice and spread on a petriplate to dry.

2.6.4. Preparation of Mold and Making of Chocolate

Milk chocolate (Cadbury) was melted at 50°C in water bath and 1 g of melted chocolate was used to line the walls of the mold of suitable shape. It was cooled for 15 minutes to harden the chocolate. Upon hardening, 2 g of sodium alginate beads containing LAB isolates was added to each mold and it was covered with melted chocolate on the top. The mold was allowed to freeze for 2 hours to set the mixture. As the mixture sets, it was carefully removed.

3. RESULTS

The LAB isolates were identified by cultural, morphological and biochemical characteristics. The characteristics of LAB belonging to genus Lactobacillus should be gram positive, rod shaped, catalase negative, must be acid producing and gas formation may or may not be there from sugars. So, this was confirmed that isolates resembled the characteristics of genus *Lactobacillus* as described by Hutt *et al*. [5].

3.1. Gram Staining

Table 1 Showing results of gram staining and catalase test

Isolates	Gram staining	Catalase test
C1	Gram +ve	-
C2	+	+
C3	+	+
C4	+	+
F1	+	-
F2	+	-
F3	+	+
F4	+	-
V1	+	-
V2	+	+
V3	+	+
V4	+	-

Note: C: Curd sample; F: Fruit sample and V: Vegetable sample

In present research, the selected LAB isolates were gram positive and rod shaped, the results were as shown in table 1.

3.2. Evaluation of Probiotic Potentials of Isolated Bacterial Cultures

For the screening of the probiotic, it was subjected to following tests like pH tolerance test, Bile tolerance test, temperature tolerance test, antibiotic sensitivity test and antimicrobial test, lactose utilization test, for isolates from each sample so that, whether they can tolerate the various stress factors in the gut, and their results were obtained.

3.2.1. pH Tolerance Test

In present study, the selected LAB isolates were able to grow in pH 7.0, 6.0, 5.0, 4.0, 3.0 and 2.0 but some species were unable to grow at pH 7.0 and 4.0 as shown in Table 2 and figure 1.

pH tolerance of Fruit isolate pH tolerance of vegetable isolate pH tolerance of curd isolate

Figure 1 Results of pH tolerance test

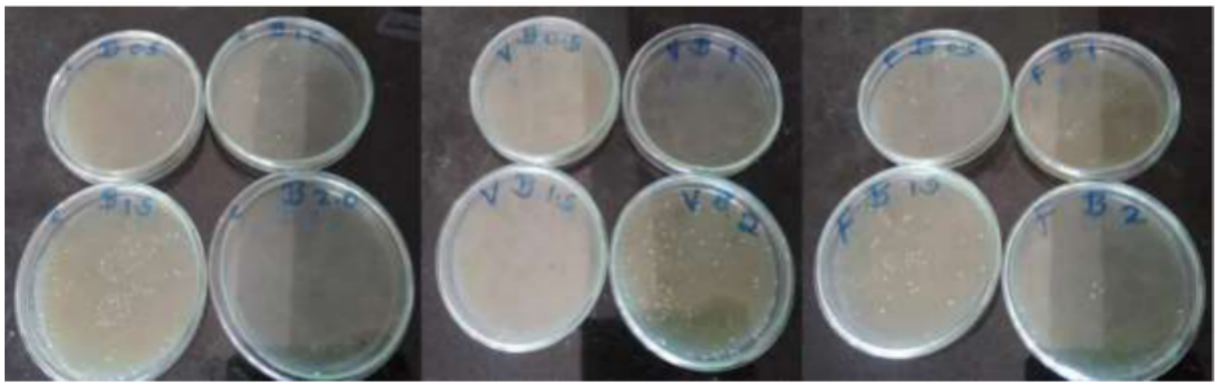
Table 2 Showing results of pH Tolerance test

pH	2	3	4	5	6	7
Isolate C	+++	+++	+++	++	+	-
Isolate V	++	-	++	+	++	+
Isolate F	+	++	-	+	+	+

+ indicates less than 10 colonies; ++ indicates colonies ranging between 10-20; +++ indicates colonies ranging between 20-30; • indicates no colonies

3.2.2. Bile salt Tolerance Test

Bile salt test: In present study, the selected LAB isolates were able to survive in 0.5, 1.0, 1.5 and 2.0 % bile salt concentrations as shown in Table 3 and Figure 2.



Bile tolerance of curd isolate Bile tolerance of vegetable isolate Bile tolerance fruit isolate

Figure 2 Results of Bile salt tolerance test

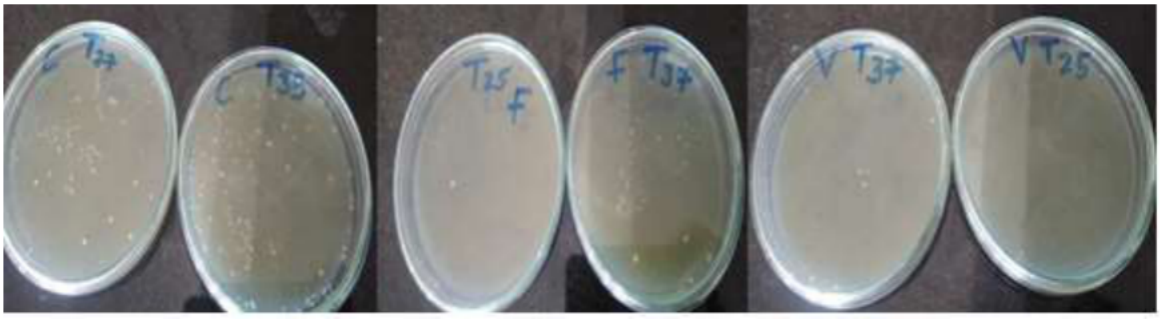
Table 3 Showing results of Bile salt test

Bile%	0.5	1	1.5	2
Isolate C	+++	+++	+++	++
Isolate V	++	-	++	+
Isolate F	+	++	--	+

+ indicates less than 10 colonies; ++ indicates colonies ranging between 10-20**;** +++ indicates colonies ranging between 20-30; • indicates no colonies

3.2.3. Temperature Sensitivity Test

All the selected LAB isolates were able to survive at temperature 25°C and 37°C. The temperature is an important factor which can dramatically affect the bacterial growth. The reason for choosing this temperature range was to detect whether the isolated cultures were able to grow within range of normal body temperature or not. As if the isolates were not able to survive within the selected temperature range then they would not have been able to survive in the human gut, which is an essential factor of probiotics to show their effectiveness. Here we have chosen 25°c so that the potency of isolates is checked during preparation of product. The results obtained were positive for growth at chosen temperature range (Table 4 and Figure 3).



Temperature tolerance of curd isolate.....Temperature tolerance of fruit isolate...Temperature tolerance of vegetable isolate

Figure 3 Results of Temperature sensitivity test

Table 4 Showing results of Temperature sensitivity test

Temperature (°c)	25°C	37° C
Isolate C	++	++
Isolate V	+	+
Isolate F	+	++

+ indicates less than 10 colonies; ++ indicates colonies ranging between 10-20; +++ indicates colonies ranging between 20-30; • indicates no colonies

3.2.4. Lactose Utilization Test

The selected LAB isolates were grown in fermentation medium supplemented with lactose and was observed for change in colour from red to yellow which indicates the production of lactic acid. It was observed that every selected LAB isolate was able to produce lactic acid from lactose as shown in figure 4.

Figure 4 Result of Lactose Utilisation test

3.3. Antibiotic Susceptibility Test

Antibiotic susceptibility pattern of selected LAB isolates was observed by the results as shown in Table 5 and Figure 5. All the isolates were sensitive to drug Erythromycin, Oxacillin and Amoxicillin but isolate F showed mild resistance for Erythromycin. The isolates were found to be sensitive against almost all above antibiotics used.

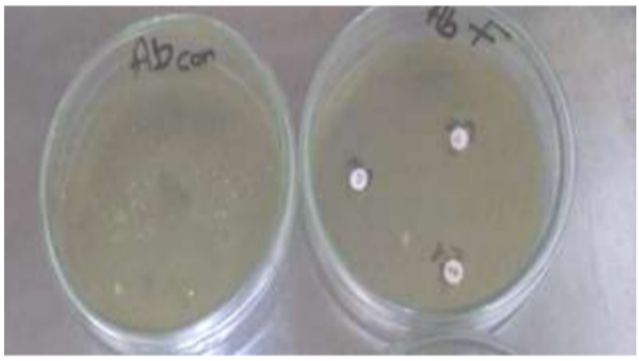


Figure 5 Result of Antibiotic Susceptibility Test

Table 5 Showing results of Antibiotic sensitivity test

Discs / Isolates	Amoxicillin	Oxacillin	Erythromycin
Isolate C	S	S	S
Isolate V	S	S	S
Isolate F	S	S	R

R-Resistant; S-Sensitive

3.4. Antimicrobial Test

The isolates were found to exhibit antimicrobial activity against indicator strains as shown in Table 6 and Figure 6. *Staphylococcus* growth was not observed around the wells, it shows that the isolates inhibited the growth of *Staphylococcus aureas,* whereas the isolates did not show antimicrobial activity towards *E.coli* species.

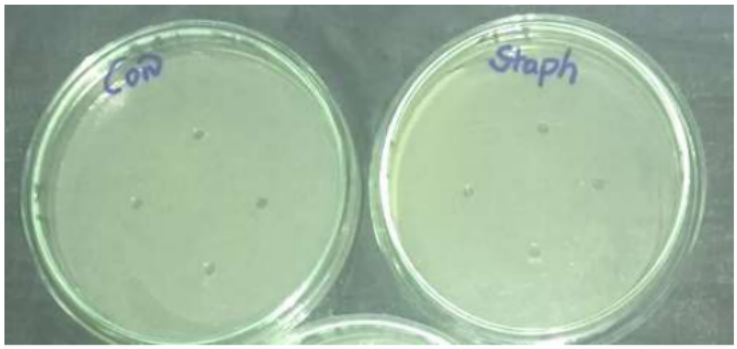


Figure 6 Results of Antimicrobial activity test

Table 6 Showing results of Antimicrobial activity

Antimicrobial activity	*Staphylococcus* spp.	*E.coli*
Isolate C	✓	×
Isolate V	✓	×
Isolate F	✓	×

3.5. Formulation into Product (Chocolate)

We have formulated probiotic isolates into chocolate, by the method of sodium alginate encapsulation. The chocolate prepared were of two types i.e. milk chocolate and dark chocolate (Figure 6).

Figure 6 Preparation of chocolate from LAB encapsulated sodium alginate beads

4. DISCUSSION

Probiotics need to survive the inevitable biological barriers of gut. The primary barrier of microorganisms in the stomach is the gastric acidity (pH of 1.5-3.5). Besides the strong acid condition in the stomach, the probiotic microorganisms taken orally have to defend against the bile salt in the gastrointestinal tract. Hence, bile tolerance is also considered to be one of the important properties required for high survival of the probiotic organism. There is no consensus about the precise concentration to which the selected strains should be tolerant. The physiological concentration of bile salts in the small intestine is between 0.2 and 2.0%.

A probiotic organism must also be able to tolerate and grow at human body temperature of 37ºC and so were these isolates selected and subjected for temperature tolerance test. Most of the probiotic microorganisms are bacteria and many of them are not able to resist or tolerate these antibiotics, hence they must be checked once before administering to patients, as it has been discussed by Syal.p and Vohra [11].

Chocolate can be attractive vehicle for probiotic intake combining both health promoting and mood boosting effects. Monitoring and characterising the survival of the probiotic bacteria in chocolate is of high importance. Patil Liladhar Shivram [12] have prepared probiotic ice-cream. With reference to that, we have prepared probiotic chocolate. From the above study it can be concluded that it is possible to isolate and identify different types of probiotic microbes from different food samples. The organisms isolated as probiotic organisms are a better way to supplement the human body. The probiotic product was formulated using fruits, vegetables and food samples and the obtained product will be a functional food and it has a beneficial effect.

CONFLICT OF INTEREST

We declare that no conflict of interest.

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