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EVALUATION OF ANTIMICROBIAL ACTIVITY OF PROBIOTIC BACTERIA ISOLATED FROM DIFFERENT NATURAL AND COMMERCIAL PRODUCTS

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ABSTRACT

Probiotics are defined as live microorganisms which when consumed in a sufficient amount could confer a health benefits. Probiotics are made of good live bacteria and yeast that naturally living in your body you constantly have both good and bad bacteria in body, when you get any infection, there is more bad bacteria knocking your system out of balance. Probiotics shows antimicrobial, anti-oxidant, and recently discovered that probiotics also contains anti-fungal property they also inhibit the growth of fungus.(Lactobacillus rhamnoses). Commonly claimed benefits of probiotics include the decrease of potentially pathogenic gastrointestinal microorganisms the reduction of gastro-intestinal discomfort, strengthening of immune system, improvement of skin function improvement of bowel regulator resistance to pollen allergens decrease in body pathogens the protection of DNA maintaining the stomach health when receiving antibiotics etc. Present paper discuss about isolation of probiotic bacteria from different natural sources fruits and vegetables kiwi, cabbage and capsicum and commercial sample like yoghurt etc. A total of four samples (kiwi, yoghurt, capsicum and cabbage) samples were collected from the local market. Fruit sample was inoculated into 100 ml of sterile MRS broth and incubated at 37^0C and the growth of isolates were sub cultured on nutrient agar plates and kept for incubation. After incubation, colonies were randomly selected, picked up and purified for further characterization. Then the isolates were screened for physiological characterization and biochemical characterization. Physiological characterization studies were performed at different temperature, pH, NaCl concentration. Isolates were exhibited growth at various conditions. Biochemical characterization were performed for catalase activity, carbohydrate fermentation test, arginine hydrolysis, starch hydrolysis citrate utilization and urea utilization. The antimicrobial properties of the probiotic bacteria were determined using agar well diffusion and the disc diffusion method using *Bacillus subtills*. Probiotic isolates exhibits antimicrobial activity for *Bacillus subtilis.*

KEYWORDS: Probiotic bacteria, Physiological characterization, Biochemical characterization, MRS broth, Citrate utilization test, Antimicrobial studies.

INTRODUCTION

Probiotics are living microorganisms that mitigate the negative effects of antibiotics by restoring the body's natural homeostasis. Probiotics are identified by their genus, species, and a strain-specific identifying name (e.g. Lactobacillus rhamnosus GG) and are available in a range of formulations, including yoghurt drinks, pills, and nutritional supplements. Microorganisms have been part of the human diet for centuries, but the concept of "probiotics" is relatively new. In 1907, the Russian-born Nobel laureate Elie Metchnikoff proposed using helpful bacteria to replace dangerous microbes and pathogens in the human stomach. The majority of probiotic bacteria belong to the genera Lactobacillus and Bifidobacterium. Other bacteria and yeasts possess probiotic characteristics as well. Etymologically the term probiotic is derived from the Greek language meaning "for life" but the definition of probiotics has evolved over time simultaneously with the increasing interest in the use of viable bacterial supplements and in relation to the progress made in understanding their mechanisms of action. The term was originally used to describe substances produced by one microorganism that stimulated the growth of others and was later used to describe tissue extracts that stimulated microbial growth and animal feed supplements exerting a beneficial effect on animals by contributing to their intestinal flora balance. Until recently the most widely used definition which contributed to the development of the probiotic concept in several ways was that of Fuller: "probiotics are live microbial feed supplements which beneficially affect the host animal by improving microbial balance". The definition used at present was given by the Food and Agriculture Organization of the United Nations World Health Organization, according to which probiotics are redefined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." In relation to food the definition can be

adjusted by emphasizing that the beneficial effect is exerted by the microorganisms "when consumed in adequate amounts as part of food". The continuous growing demand for organic farming and the reduction of antimicrobial usage in food producing animals obviously necessitate the intense search for novel alternatives, including new probiotic strains with more effective properties most especially in curtailing diseases and improving production. With the increasing use of dairy and non-dairy products in the food industry, it has become increasingly important to search for and isolate probiotic bacteria from cheap and alternative or novel sources with no adverse health consequences; therefore, food crop plant leaves were investigated in this study for their potential as sources of beneficial bacteria. Probiotic strains have always originated in the human body and isolated from traditionally fermented foods and drinks; however, food crop plant leaves may be a potential source of bacterial genus such as *Lactobabillus*, *Enteroccocus*, and *Weissela*. Presently, different commercial probiotic products marketed globally are available for poultry. Nevertheless, some of them may not be highly potent due to insufficient examination of the specific beneficial properties of the probiotic strains formulated in the product. Moreover, most manufacturers lack the patience to conduct an in depth study of each strain to ascertain its full probiotic potentials before commercializing as most industries are after profits maximization with minimal expense. Isolation of lactic acid bacteria (LAB) from non intestinal and nondairy sources can offer better health advantages. Probiotics originating from fruits and vegetables may suit the gut environment of vegans more than those from different sources. Vegetables provide nutrients such as vitamins, minerals high carbohydrate content and acidic microenvironment which favours the growth of LAB. Hence, the objectives of this research were to identify and investigate the potential probiotic bacteria isolated from different fruits, vegetables and commercial yoghurt sample and to evaluate their physiological biochemical and antimicrobial properties.

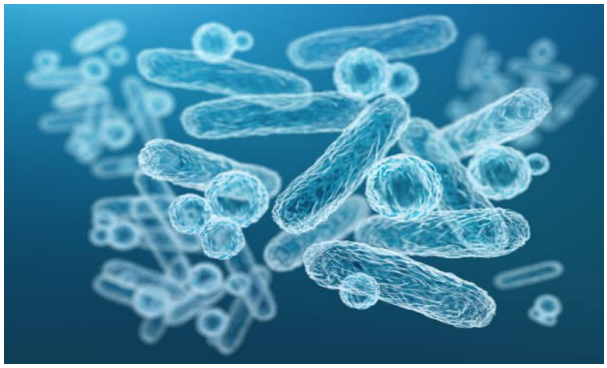


Fig 1: Lactobacillus.

Fig 2: Bifidobacterium.

MATERIALS AND METHODS

Collection of materials: A total of four samples (kiwi, yoghurt, capsicum and cabbage) samples were collected from the local market and washed thoroughly with the tap water following rinsing with 70% ethanol for 30 second and finally rinsed two times with double distilled water. The fruits and vegetables were chopped and crushed manually with sterile cutter under aseptic condition and stored.

Fig 3a: CABBAGE.

Fig 3b: CAPSCIUM.

Fig 3c: KIWI.

Fig 3d: YOGHURT.

Fig 3: vegetables fruits and yoghurt for isolation of probiotic bacteria.

Isolation of bacteria: 10 g of fruit sample was inoculated into 100 ml of sterile MRS broth and incubated at 37^{o}C for 48 hours in rotary shaker at 120 rpm and kept for incubation. The growth of bacteria was observed. 0.1 ml of inoculum was spread on to the nutrient agar plate. The plates were incubated in an inverted position for 24-48 hours at 37^{0} C. After incubation, colonies were randomly selected, picked up and purified for further characterization by streak plate technique on nutrient agar plate and incubated for 24-48 hours at 37^{0}C and transferred to slant of MRS and nutrient agar, maintained in refrigerator at 4^{0}C for further analysis.

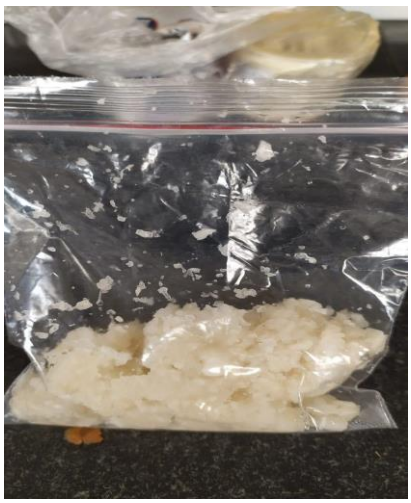


Fig 4a: CABBAGE EXTRACT.

Fig 4b: CAPSICUM EXTRACT.

Fig 4c: KIWI EXTRACT.
Fig 4: Processing of the samples.

Fig 5a: Cabbage sample.

Fig 5b: Capsicum sample.

Fig 5c: Kiwi sample.

Fig 5d: Yoghurt sample.
Fig 5: Samples in MRS broth media.

PHYSIOLOGICAL CHARACTERIZATION OF ISOLATES

Growth of bacterial isolates at different temperature:

The over night grown cultures were inoculated into nutrient agar slants and incubated for 24-48 hours at 10oc 30c, 40c.

Growth of bacterial isolates at different pH: To the nutrient agar medium the acid solution HCL is added into 4 test-tubes and the bacteria is inoculated into it and incubated under 30^0c in the incubator. To the nutrient agar medium the basic solution NaOH is added into each test tube. Then the bacteria is streaked onto the medium and incubated under the 42^0C For 24-48 hours.

Growth of bacterial isolates at different NaCl concentration: To the nutrient agar medium different concentrations of Nacl 10%, 15%, 25% is added into different test-tubes and then the bacteria is streaked and incubated under 42^0C for 48 hours.

BIOCHEMICAL CHARACTERIZATION OF ISOLATES

Catalase activity: Inoculum from the pure cultures was streaked on nutrient agar slant and incubated at 37^0C for 24hours. Two drops of 3% hydrogen peroxide solution is added on to clean glass slide then with the help of nichrome wire loop cells from the center of well isolated colony were transferred onto drop of hydrogen peroxide.

Carbohydrate fermentation test: Active bacterial cultures were inoculated into media containing 0.5% yeast extract supplemented with 2% mannitol, sucrose, glucose, xylose, and lactose along with Andrade's indicator and the tubes were incubated at 37^oC for 24 hours.

Urea utilization test: Overnight grown culture of isolates were inoculated into urea broth and incubated at 37^oC for 24 hours.

Citrate Utilization test: Isolates were streaked on simmon citrate agar slant and incubated at 37^oC for 24 hours and the colour change was observed.

Arginine Hydrolysis test: Overnight grown cultures were inoculated into Arginine broth medium and the tubes were incubated at 37^oC for 24 hours.

Starch hydrolysis test: The isolates were streaked on starch agar plate and incubated at 37^oC for 24 hours, after incubation the plates were flooded with Lugo's iodine solution and observed for transparent zone surrounding the colony.

EVALUATION OF ANTIMICROBIAL ACTIVITY OF PROBIOTICS BACTERIA

DISK DIFFUSION AGAR METHOD

In this well-known procedure, agar plates are inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (about 6 mm in diameter), containing the test compound at a desired concentration, are placed on the agar surface. The Petri dishes are incubated under suitable conditions. Generally, antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones are measured.

WELL DIFFUSION AGAR METHOD

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts. Similarly to the procedure used in disk-diffusion method, the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip, and a volume (20–100 µL) of the antimicrobial agent or extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested.

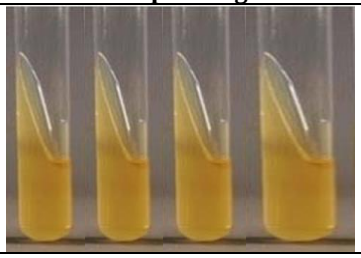
RESULTS AND DISCUSSION

PHYSIOLOGICAL CHARACTERIZATION

Growth of bacterial isolates at different temperature, pH and NaCl concentration:

There is no bacterial growth was observed at 10^0C, 20^0C and bacterial growth was observed at 40^0C in all the four samples. Under acidic conditions the bacterial growth was observed and in alkali conditions the bacterial growth was not observed. At 10%, 15% NaCl concentration bacterial growth was observed and 20% NaCl concentration bacterial growth was not observed.

Table 1: Growth of bacterial isolates at different temperature.

Temperature	Observation	Sample image
AT 10℃	There is no bacterial growth in all the four samples	

At 20℃	There is no bacterial growth was observed in all the 4 samples.	
At 40℃	The bacterial growth was observed in all the 4 samples.	

Table 2: Isolates at different pH conditions.

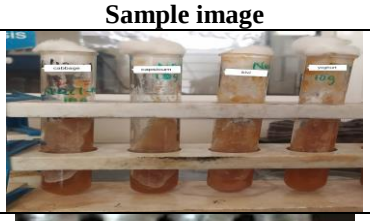
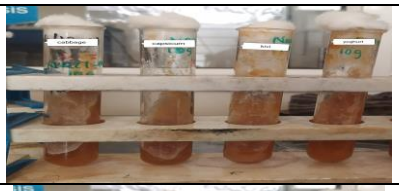
Isolates at different ph	Observation	Sample image
Under acidic condition	The bacterial growth was observed	
Under basic condition	There is no bacterial growth was observed	

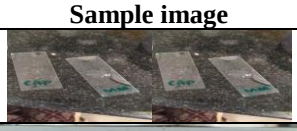
Table 3: Isolation at different NaCl solution concentration.

Isolates at different nacl concentration	Observation	Sample image
At 10%NaCl concentration	Bacterial growth was observed	
At 15% NaCl concentration	Bacterial growth was observed	
At 20%NaCl concentration	No bacterial growth was observed	

BIOCHEMICAL CHARACTERIZATION OF ISOLATES

Catalase activity is one important criterion for the selection of probiotic bacteria. In the present study effervescence was observed and isolates utilized the catalase activity. In carbohydrate fermentation test media was changed to pink colour indicates positive for carbohydrate test. During the citatre utilization test blue colour was formed indicates the utilization of citrate as the energy source. In urea utilization test orange red colour was observed indicates the hydrolysis of urea. In starch hydrolysis test transparent zone around the colony was observed confirms the hydrolysis of starch. For arginine hydrolysis test yellow colour was observed indicates negative to the test.

Table 4: Biochemical charcterization of bacterial isolates.

Chemical test	Observation	Reference	Sample image
Catalase activity	Efferenvense was observed	The isolates utilizes the catalase in it	
Carbohydrates fermentation test	Pink colour media was formed	Indicates the presence of carbohydrates	
Citrate utilization test	Blue colour was observed	The isolates utilizes citrate as energy source	

ANTIMICROBIAL ACTIVITY OF PROBIOTICS BACTERIA

Antibacterial activity of probiotic isolates was performed using two methods (i.e., Agar well diffusion method and Disc diffusion method.

In agar well diffusion method, the antibacterial activity of probiotic isolates was performed against bacterial cultures (i.e., Bacillus subtills species) using Azithromycin as standard. Probiotic bacteria showed antimicrobial activity against both Bacillus subtills exhibits clear zone of inhibition.

In Disc diffusion method, the antibacterial activity of probiotic bacteria was performed against bacterial cultures (i.e., Bacillus subtills species using Azithromycin as standard. Probiotic bacteria showed antibacterial activity against Bacillus subtilils exhibits clear zone of inhibition.

Fig 6a Capsicum.

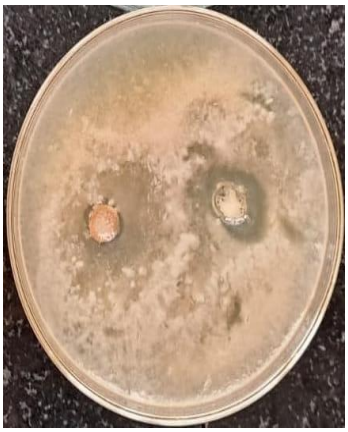


Fig 6b Cabbage.


Fig 6c Kiwi. **Fig 6d Yoghurt.**

Fig 6: Agar well diffused plates containing both test solution and standard.

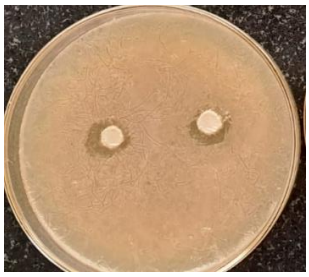



Fig 6a Capsicum. **Fig 6b Cabbage.**

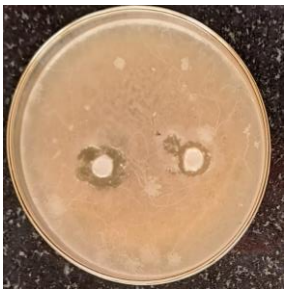



Fig 6c Kiwi. **Fig 6d Yoghurt.**

Fig 7: Disc diffused plates containing both test solution and standard.

Table 5: Zone of inhibition against *Bacillus subtilis* (agar well diffusion method)

S.no	Sample	Standard
1.	1.1 cm	1.3 cm
2.	2 cm	2.3 cm
3.	1.8 cm	2 cm
4.	3.1 cm	3.4cm

Table 6: Zone of inhibition against *Bacillus subtilis* (Disc diffusion method)

s.no	sample	standard
1.	1.2cm	1.5cm
2.	1.8cm	2cm
3.	2cm	2.3cm
4.	1.6cm	1.8cm

CONCLUSION

Probiotics are designed to meet food safety, shelf life, technological effectiveness and economic feasibility criteria. Isolate from cabbage kiwi, capsicum and yoghurt showed probiotic activity. However, all the desirable properties of probiotics may not be present in single isolate. There is a further need to assess their probiotic potential in detail for its exploitation in food products. Further, molecular identification of these samples needs to be carried out to confirm the identity of the bacterial isolates. Thus, it may be concluded that the probiotic isolates from fruits and vegetables have potential application in the development of probiotic food products.

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