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Regular Article

Qualitative and Quantitative Analysis of Native Food Products for Probiotic Properties

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ABSTRACT: Certain properties relevant to probiotic action, e.g. resistance to acid, bile tolerance, antibacterial activity, and antibiotic susceptibility were investigated of lactobacilli isolated from three kinds of Indian traditional fermented foods. Media of pH = 2.0–7.0 and bile salt concentrations of 0.3–1.0% were used as stress conditions. Antibacterial activity of the probiotic lactobacilli was determined by means of the Well diffusion test. The results revealed that the antibacterial activity of the three selected lactobacilli could inhibit all test pathogenic bacteria however at different inhibition levels. Among 7 isolates, only 3 strains (two from paneer and one from Ragi porridge) showed extremely high survival rates under stress caused by acid or bile salts. The three strains inhibited test pathogenic bacteria to different extents. They were sensitive to chloramphenicol, quinupristin, Chloramphenicol, Tetracyclin, Rifampicin and Streptomycin but resistant to Gentamicin, Vancomycin, Ciproflaxin and Penicillin G

Introduction

Traditional fermented food is the product of a biotechnological process. It is produced by taking advantage of the natural microbiota associated with fresh food materials. It is one of the most practical, economic, and widely applied empirical methods for preserving and often enhancing organoleptic and nutritional quality of fresh food. It has been unique to historical countries in different parts of the world, e.g. milk fermentation "yoghurt" in Bulgaria, vegetable fermentation "suan cai" in China, "kimchi" in Korea, "natto" and "mizo" in Japan, "Nham" and "Miang" in Thailand. In India, we have been consuming various fermented foods from ages. Mostly they are milk products or cereals porridges. We have narrowed down our research to only 3 such products, viz., Ragi Porridge, Shrikhand and Paneer.

Although lactobacilli show a high impact on effective protection to human health, there is obvious evidence that lactobacilli from different origins possess probiotic properties at different levels. In order to survive and colonize in the gastrointestinal tract, the bacteria should express high tolerance to acidic media and bile and should be able to adhere to the intestinal surfaces. The antibiotic resistance of pathogenic bacteria is an increasing medical problem, and raises the question of antibiotic resistance among desired probiotic strains. Therefore, the antibiotic susceptibility test therefore should be incorporated for the safety assessment of the desired property of the promising probiotic lactobacilli. The present work was undertaken to provide practical data on lactobacilli of fermented food origin.

Lactobacillus have been used as a flavouring and texturizing agent as well as a preservative in food for centuries and are now added as starters in food. LAB, such as lactobacilli inhibits food spoilage and pathogenic bacteria and preserves the nutritive qualities of raw food material for an extended shelf life. Recently, the use of metabolites of *Lactobacillus* as biological preservatives in food packaging materials has been discussed. The antimicrobial effect of *Lactobacillus* is mainly due to their lactic and organic acid production, causing pH of the growth environment to decrease. Low pH induces organic acids to become lipid soluble and diffuse through the cell membrane into the cytoplasm

Materials and Methods

1) Glasswares: conical flasks (100 ml, 250 ml and 500 ml), beakers (100 ml, 250 ml and 500 ml), slides, L-rod, petriplates, pipettes (10 ml and 2 ml) and test tubes.
2) Sterilization:
   a) .Sterilization of glasswares
   b) Sterilization of culture media and reagents
3) Sample collection
4) Bacterial culture method:
   a) Isolation from Paneer samples
   b) Isolation from Shrikhand samples
   c) Isolation from Ragi Porridge Samples
5) Preliminary test to confirm LAB'S
   a) Gram staining test
   b) Catalase test
   c) Oxidase test
   d) Motility test-Hanging drop method
6) Preservation of LAB
   a) Slant culture technique
   b) Glycerol stock method
7) Biochemical test to confirm LAB
   a) Voges Proskauer test
   b) Methyl Red Test
   c) . Citrate *Utilization Test*
   d) Growth at 15°C, 37°C and 45°C temperatures
8) Antibiotic resistance test- Kirby Bauer test
9) Antimicrobial activity test-Well diffusion test
10) Acid tolerance test
11) Bile tolerance test
12) Use of prebiotics as growth enhancers
   a) Spirulina
   b) Psyllium Husk\

Results and Discussions

Preliminary analysis to confirm *Lactobacilli*

Colony Morphology

Samples (Paneer, Shrikhand and Ragi Porridge) were spread plated and streaked on MRS medium to get the pure culture and the morphology of the colony is observed. Colonies were found to be circular in shape, white to creamish in colour and measured 1-3 mm in size mostly.

Gram's staining

The colonies obtained from spread plate method and streaking were either Gram-positive or Gram-negative. Morphologically these colonies were bacilli, coccobacilli or cocci. Gram-negative colonies were rejected as they would either be enteric or pathogens and have no beneficial role. From these mixed cultures obtained, the Gram-positive rods were subjected to further studies as this would yield *Lactobacillus* species, the results are presented in Table 1.

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Table 1: Strains isolated from the samples

S.No.	Sample name	Strains number	Morphology
1.	Paneer	pb,pc	Large gram +ve coccobacilli in chains and pairs
2.	Shrikhand	sa, sb	Small gram +ve bacilli in chains and pairs.
3.	Ragi Porridge	ra, rb, rd	Large gram+ve bacilli in chains

Catalase test

All the strains tested showed catalase negative reaction

Table 2: Catalase test

S.No.	Strains	Catalase test results
1.	pb, pc	-ve
2.	sa, sb	-ve
3.	ra. rb, rd	-ve

Oxidase test

All the strains tested showed oxidase negative reaction.

Table 3: Oxidase test

S.No.	Strains	Oxidase test results
1.	pb, pc	-ve
2.	sa, sb	-ve
3.	ra. rb, rd	-ve

Motility test

Hanging drop method was performed and the strains were found to be non-motile.

Biochemical characterization test:

1. Voges- Proskauer Test: After 15 minutes of incubation, the test tubes, each inoculated with single strain of *Lactobacillus* isolated, showed a deep red colour ring at the top thus indicating the presence of acetylmethylcarbinol and hence a positive result obtained.

Fig. 4. Voges-Proskauer Test - Red colour ring at the surface

2. Methyl Red Test:

After 15 minutes of incubation upon the addition of methyl red indicator, the test tubes, each inoculated with a single strain of *Lactobacillus* isolated, showed a red colouration, thus indicating a positive result.

3. Citrate Utilization Test:

Following incubation, no microbial growth at the surface of the slants was seen in all the test tubes and the medium remained green thus indicating a negative result.

Growth at 45°C, 37°C, 15°C

As seen in Table 4, all the strains were subjected to these different temperatures namely 45ºC, 37ºC and 15°C.Growth was observed for all the strains at 45°C and 37°C. All the strains except sa, ra and rb showed growth at 15ºC.

In this experiment, Gram's staining showed that most of the strains are gram positive, catalase and oxidase negative, as Lactobacillus are generally gram positive rods and negative for catalase and oxidase so further test were performed with these colonies. In biochemical characterization test, most of the strains showed positive for Voges Proskaeur and Methyl Red test, negative for citrate utilization and were able to grow at 45ºC, 37ºC and 15ºC. This is in accordance to previous study which showed the same results (Fontech.A.Florence et al., 2008). From the results obtained above, all the isolated strains were confirmed as lactobacillus and further antibiotic resistance tests, antimicrobial tests, acid tolerance and bile tolerance were performed to determine the potential probiotics among the 7 strains.

Table 4

S.No.	Strains	Growth @45°C	Growth @37°C	Growth @15°C
1.	pb	+	+	+
2	pc	+	+	+
3.	sa	+	+	-
4.	sb	+	+	+
5.	ra	+	+	-
6.	rb	+	+	-
7.	rd	+	+	+

Test for Probiotics
Antibiotic resistance test:

Table 5: Resistance to Antibiotics

Antibiotic	pb	pc	sa	sb	ra	rb	rd
Gentamycin – 30 mcg/disc	R	IN	R	R	R	R	R
Streptomycin – 10 mcg/disc	S	S	IN	R	R	S	S
Rifampicin – 5 mcg/disc	S	S	IN	S	R	S	S
Vancomycin – 30 mcg/disc	R	R	R	R	R	R	R
Tetracyclin – 30 mcg/disk	R	S	IN	IN	S	S	S
Ciprofloxacin – 10 mcg/disc	R	R	R	R	R	R	R
Chloramphenicol – 30 mcg/disc	S	S	R	R	S	S	IN
Penicillin G – 10 mcg/disc	R	R	R	R	R	R	R

Sensitive, S(≥21mm); Intermediate, IN(16-20mm); Resistant, R(≤15mm)

From results we conclude that these strains have a better chance of survival during normal antibiotic medications during infection, with exception to few antibiotics to which they are sensitive, which is an acceptable characteristics of probiotic strain

Fig.5: Antibiotic susceptibility tests of isolated strains from different samples

Antimicrobial resistance test

Table 6: Inhibitory spectrum of isolated strains

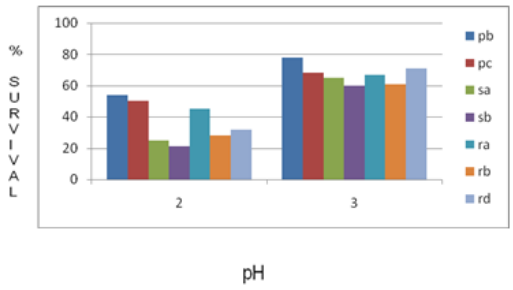
S.No	Strains	Test Bacteria		
		E. Coli	*S.aureus*	*S. Typhi*
1.	pb	++	++	+++
2.	pc	+	++	+
3.	sa	+	++	++
4.	sb	+	++	++
5.	ra	++	+	++
6.	rb	+++	++	++
7.	rd	++	++	++

Degree of inhibition:
+++ = very strong inhibition of zone (15-18mm);
++ = strong inhibition of zone (10-14mm);
+ = moderate inhibition of zone (6-9mm);
- = no inhibition of zone;

The good probiotics should present their antimicrobial actions particularly to the pathogens in the GI system. In this study, *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli* were used as the test bacteria because they are occasionally found as food borne microorganisms that might cause gastroenteritis. A differential pattern in inhibition potential was obtained, the difference in inhibition potential among the three selected strains was considered to be due to the different intrinsic factors induced by different food origins.

Acid Tolerance Test

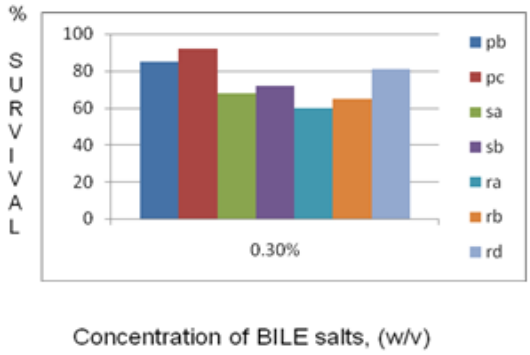
Graph 1. Survival % of Lactobacilli at pH 2 and 3



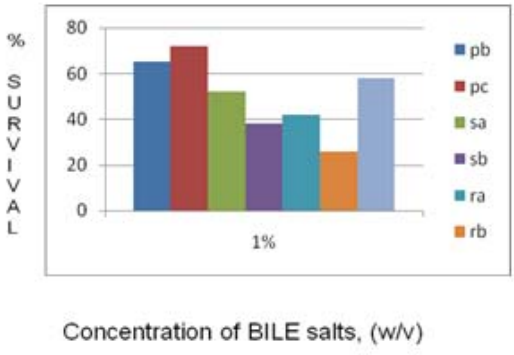
The effect of pH ranging from 2.0 to 7.0 on the survival rate of the 7 selected strains was studied. It was found that most strains could survive approximately less than 50% in pH 2.0 as shown in Graph 1. Only 2 strains, both from Paneer could survive to an extent of more than 50%. The most tolerant strain was the pb which was isolated from Paneer. This strain could survive in pH 2.0 at the survival rate of 53.6%. When the pH was raised to 3.0, more than 50% of isolates from all kinds of food origins exhibited a survival rate higher than 60%. When the pH was up to 7.0, all isolates could survive 100%. When pH was raised to pH 3.0, the test *Lactobacillus casei* showed higher survival than in pH 2.0. (Mishra and Prasad, 2004). The effect of food source on bacterial acid tolerance was clearly observed when the percentage of viable strains from each origin was plotted against pH. It was shown that the isolates from different origins demonstrated different acid resistance patterns. The isolate from paneer showed the highest acid resistance at extremely low pH (pH 2.0), followed by those isolated from ragi porridge. This suggests that the food origin ecosystem play an important role for the bacterial cells to be able to adapt to the stress conditions.

Bile Tolerance Test

Graph 2. Survival % of Lactobacilli at 0.30 % conc. of Bile salts, (w/v)



Graph 3. Survival % of Lactobacilli at 1 % conc. of Bile salts, (w/v)



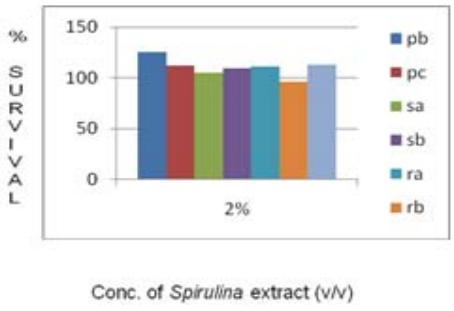
Beside the strong acid media in the stomach, the probiotic microorganisms taken orally have to defend against the bile salt in the gastrointestinal tract. Hence, bile tolerance is considered to be one of the important properties required for high survival and as a consequence for a probiotic activity. There is no consensus about the precise concentration to which the selected strain should be tolerant. The physiological concentration of bile salts in the small intestine is between 0.2 and 2.0% (Gunn JS., 2000). In this study, concentrations of 0.3 and 1.0% bile salts were used. All strains could survive more than 60% in 0.3 bile salt solutions (Graph 2.).

The isolates from our experiment showed stronger bile tolerance than those reported by other investigators (Papamanoli E, 2003). The gradually decrease of viable cells was observed when the concentration of bile salt was increased up to 1.0% (Graph 3.). It was considered that bile salt causes the increase in permeability of bacterial cell membranes, as the membranes are composed of lipids and fatty acids. Some strains isolated from Paneer showed high tolerance even in the extremely high salt media. The most important probiotic property of desirable bacteria is dependent on their ability to remain viable in acid and bile in gastrointestinal tract ecosystem. Among 7 strains, only 3 strains showed significant resistance to the acid and bile in the extremely high concentration. These 3 strains are, 1 isolated from Ragi Porridge, rd and 2 isolated from Paneer, pb and pc.

Prebiotic Effect

Spirulina:

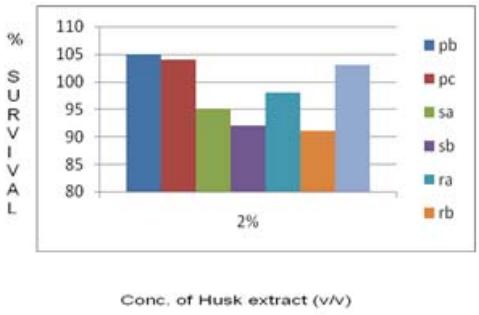
Graph. 4. Survival % of Lactobacilli at 2 % conc. of *Spirulina platensis* Extract, (v/v)



The effect of the addition of the *Spirulina* extract could be seen on the enhanced growth of the Lactobacilli. Only one strain i.e. rb showed growth less than 100 %. The strains pb, pc, sb and rd showed growth more than 110 %. Maximum growth was observed in pb. It depicts that the extracellular extract of *Spirulina* has growth enhancing effect on lactobacillus.

Psyllium Husk:

Graph. 5. Survival % of Lactobacilli at 2 % conc. of Psyllium husk Extract, (v/v)



The effect of the addition of the Psyllium Husk extract could be seen on the enhanced growth of the Lactobacilli. The strains sa, sb, ra and rb showed growth less than 100 %. Maximum growth was observed in pb. It depicts that the extract of Psyllium Husk has growth enhancing effect on lactobacillus.

Conclusion

The discovery of probiotics has been an important one in the history of medical sciences. It is unusual for microbes to aid higher organisms directly; however, this is not in case with probiotic microbes which do aid the higher organisms directly. They do this simply by living within them in mutual symbiotic relationship. It also plays a vital role in giving the benefits to human by its eminent activities like role of cholesterol reduction, in the treatment of irritable bowel syndrome, anticancer activities, antibiotic associated diahorrea and many more *Lactobacillus* spp produce antimicrobial factors and bacteriocins. Due to the production of diverse antimicrobial agents it may be consider for the treatment and prevention of various infectious diseases.

From these experiments the *Lactobacillus* strains viz., pb, pc, sa, sb, ra, rb and rd were isolated, characterized, and were analyzed for probiotic properties and the results reveals that these strains having potent probiotics activity and are more beneficial to human and animals which assist in re-establishing normal gut microflora. It was found that the strains from Paneer, viz pb and pc had highest acid and bile tolerance in addition to elevated antibacterial properties. The antimicrobial substances produced by these probiotics combat the growth of pathogenic microorganisms. The strains isolated could be used in new formulations in Food Biotechnology and Nutraceutical sector.

In addition to it, Prebiotics such as Spirulina and Psyllium Husk showed a growth enhancing effect on Lactobacillus and hence, help to restore normal gut microflora. Further research needs to focus on characterization and identification of the novel prebiotic compounds which can be used along with these strains in various food processes.

In a climate of increasing consumer awareness that diet and health are linked, research in probiotics remains more than ever a fascinating challenge. Despite the scientific problems that still exist, many researchers in the field are gradually accepting the idea that probiotics will help many patients in the future. A number of clinicians have started to use probiotics, not as an alternative treatment, but often as an additional factor, minimizing negative aspects of traditional treatment procedures. The use of probiotics in a medical context, however, is still hampered by the relatively low number of properly registered preparations.

The formats of applications can vary between simple dietary adjustments to the therapeutic use of probiotics attempting to modify the intestinal flora in a desired way.Research efforts will need to be maintained in order to support these and possibly new applications of probiotics. Genomics and proteomics approaches will most probably help to gain insight in the cross-talk between the intestinal microflora and the host intestinal epithelium and immune system.

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