Research Paper

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In Vitro Assesment of Tolerance of Lactobacillus Spp. To Gastrointestinal Tract Enviornment

Microbiology

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ABSTRACT

In order to act as a potential Probiotic, exogenously administered organism (belonging to Lactobacillus and Bifidobacterium species) have to pass through the gastrointestinal tract, and hence during their passage through gastrointestinal tract these organism must tolerate the high pH of the stomach and high bile salt concentration in the small intestine, also they must compete with the other microorganisms in the colon. In the present study five isolates of the Lactobacillus spp. isolated from the curd fermented idly batter and Cheese samples were studied for their tolerance to gastric pH, 0.3% bile salt, and also other characters like ability to utilize prebiotic and their antimicrobial action against the other intestinal inhabitant. All isolates were survived up to the 90 mins. In gastric pH (2.5) except isolate IV In addition these isolates survived and grew in the medium supply with 0.3% bile salt except isolate IV. Furthermore all isolates were found to reduce the cholesterol and exerted antimicrobial activity against common intestinal inhabitants. Isolates V was able to utilize Inulin as a prebiotic in addition to all above characteristics including Inulin utilization ability so it may be good candidate for Probiotic preparations.

INTRODUCTION

Probiotics are the live microbial food supplements of bacteria, such bacteria are live in the human intestine and control the balance of intestinal microflora and finally elicit physiological and beneficial effects on health of the host have recently been named 'probiotics' Within every human being is a flourishing, living colony of approximately four pounds of bacteria. Most of these bacteria reside in the human digestive tract although some live elsewhere (i.e., oral cavity, throat, etc.). Absence of a sufficient number of friendly lactic acid bacteria, known as "probiotics," human life could not exist. When a human fails to maintain a sufficient number of good bacteria in the body, disease usually occurs and death often follows. Life on planet Earth requires adequate colonies of friendly lactic acid bacteria to survive (Dr Ohhira *et. al.* 2000). Alteration of the microbial flora of the intestine such as may occur with antibiotic use, disease and aging, this is the negatively affect of antibiotics.

The gastrointestinal tract represents a complex ecosystem in which a delicate balance exists between the intestinal microflora and the host. It is thought that more than one hundred species and more than 10 billion bacteria coexist in the human intestine. The microflora is principally comprised of facultative anaerobes and obligate anaerobes. Approximately 95% of the intestinal bacterial population in humans is comprised of obligate anaerobes, including *Bifidobacterium, Clostridium, Eubacterium, Fusobacterium, Peptococcus*, *Peptostreptococcus* and *Bacteroides*. Approximately 1% to 10% of the intestinal population is comprised of facultative anaerobes, including *Lactobacillus, Escherichia coli, Klebsiella, Streptococcus, Staphylococcus* and *Bacillus.* Within them *Lactobacillus* species represent indigenous organisms of the mammalian gastrointestinal (GI) tract and have been used as Probiotic agents for the treatment of GI infections and inflammatory bowel disease (IBD).

Probiotics is live microbial food supplements that beneficially affect the host by improving its microbial balance. As new findings emerged, several definitions of Probiotics have been proposed. Fuller gave a precise definition of Probiotics which is still widely referred as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance (Verschuere *et.al.*2000). More recently the meaning of this word has been refined by a United Nations and World Health Organization Expert Panel as 'live microorganisms, which when consumed in adequate amounts, confer a health effect on the host' (Morelli *et. al.* 2000).

Probiotic bacteria, which beneficially affect the host by improving the intestinal microbial balance, may affect the immune response. *Lactobacillus rhamnosus* GG, ATCC 53103, a probiotic strain of human origin with widely documented health effects, influences immune response, both specifically by stimulating antibody production and nonspecifically by enhancing the phagocytic activity of the blood leucocytes. It promotes recovery from rotavirus diarrhoea and reduces the incidence of diarrhoea associated with use of antibiotics in children (Suvarna *et. al.* 2005).

Fuller (1989) listed the following as features of a good Probiotic like, It should be a strain, which is capable of exerting a beneficial effect on the host animal, e.g. increased growth or resistance to disease, non-pathogenic, non-toxic, Exert a beneficial effect on the host, Adhere to the intestinal epithelial cell lining, Survive transit through the intestinal tract such as exposure to stomach and bile acids, Produce antimicrobial substances towards pathogens, Stabilize the intestinal micro flora, Accepted by the host's immune response, present as viable cells, preferably in large numbers, capable of surviving and metabolizing in the gut environment e.g. resistance to low pH and organic acids. The present work was undertaken with following objectives, Isolation and characterization of lactobacillus species, study ability of organisms to tolerate gastric pH (2.5), check tolerance of bile salt by organisms, detect antimicrobial activity of organisms, study ability of organisms to utilize prebiotic, study reduction of cholesterol by organisms

MATERIALS AND METHODS:

1. Isolation of the *Lactobacillus species:*

Curd Milk Cheese and fermented idly batter were used for the isolation of the *Lactobacillus species.*1g of curd and milk were added to 10ml of sterile distilled water. 0.1ml of this sample were spread on the lactobacillus selective de Man Rogosa Sharpe's (MRS) media containing: peptone, 10g/lit; beef extract, 10g/lit; yeast extract, 5g/lit; glucose, 20g/lit; Tween 80, 1ml/lit; Na_2HPO_4, 2g/lit; sodium acetate, 5g/lit; triammonium citrate, 2g/lit; $MgSO_4.7H_2O$, 0.2g/lit; $MnSO_4.4H_2O$, 0.2g/lit; agar, 15g/lit and pH adjusted to 6.2 to 6.6 (de Man *et.al.* 1960). Culture of *Lactoba-*

cillus casei (NCIM-2125/ATCC-12116) and *Lactobacillus plantarum* (NCIM-2083/ATCC-8014) were obtained from National collection of industrial microorganisms, NCL, Pune and were used as standard Lactobacillus. All isolates are preserved and maintain on MRS media.

2. Identification and characterization of *Lactobacillus species:* Organism showing visible colonies on selective MRS media within 24 hrs. of incubation at 37^{0}C were selected. Organisms were identified and characterized on the basis of their Gram character, motility, ability to ferment sugar like Glucose, Lactose and Mannitol, Catalase test, Oxidase test, Nitrate reduction test, gas production from Glucose and Gelatin liquefaction test (Lopez *et. Al. 1997*)

3. Tolerance to Gastric p^H

Overnight grown cultures containing 10^{6}cells/ml were inoculated in MRS broth previously adjusted to pH 2.5 with 1M HCl. This inoculated broth was placed into the anaerobic Jar at the room temperature and 10µl of the sample was plated on the MRS agar plates after every 30mins of incubation until 180mins (0, 30, 60, 90 and 180mins) and finally after 24hrs to observe survival of the cultures at 2.5 pH (Jacobsen *et.al.*1999)

4. Tolerance to the Bile salt:

1% inoculums of cultures of every test organism containing 10^{6}cells/ml was inoculated in MRS medium supplemented with 0.3% bile (Sodium taurocholate) (Kimoto *et.al.* 2002). Changes in absorbance at 620nm were measured after every 1hr time interval till 6hrs and then after 24hrs of incubation at 37°C, control was maintain without inoculation. Survival was also tested by plating out 100µl of broth on MRS agar plates after every hour and finally after 24hrs of incubation at 37°C (Jacobsen *et.al.*1999, Pereira *et.al.*2002).

5. Antimicrobial activity.

The antimicrobial activity of the *Lactobacillus* against normal inhabitants of gastrointestinal tract and other pathogenic bacteria were analyzed with giant colony technique on MRS agar plates without triammonium citrate and sodium acetate (pH 7.2). A single line of lactobacilli culture grown in MRS broth for 48hrs was seeded in the middle of the agar plate. *Lactobacillus* culture were then cultivated for 48hrs at 37˚C. Test bacteria (*Ecscherichia coli, Salmonella typhimurium* and *Klebsiella pneuminiae*) were cultured in nutrient broth for 24hrs at 37˚C and seeded in duplicate perpendicular to the streak line of *lactobacilli*. To check the susceptibility to the antimicrobial compound produced by *Lactobacillus species*. Those test organisms arte inhibited by the *Lactobacillus* were used for the further antimicrobial bioassay carried out with the agar diffusion method. The antimicrobial assay was performed as followes-0.1ml of 24 hrs old culture of test organisms were spread on Muller-Hinton agar plates and 6 mm wells were bored on the plates. Then the 48 hrs old culture of each of the Lactobacillus was centrifuge at 5000 rpm for 10 min and 100µl of the supernatant was poured in the wells. The plates were incubated for 24 hrs at 37^{0}C and zone of inhibition (mm) around the well was measured. (Annuk *et. Al 2002)*

9 Utilization of prebiotic:

The lactobacillus cultures were propagated in MRS broth twice and cultures obtained after 12hrs of growth at 37°C were centrifuged (at 5000xg for 3 min). The pellets were suspended in phosphate buffer saline (0.8% NaCl, 0.02%KH_2PO_4, 0.115% Na_2HPO_4 [pH 7.4]). This suspension was spot inoculated on mMRS agar plates containing the appropriate energy source (1%w/v) and 50mg/liter of Neutral red is used as a colour indicator. Inulin was used as prebiotic and glucose was used as control. The plates were incubated at 37˚C for 48hrs. Plates were checked for yellow zone around the developing colonies against red background (Makras *et.al.* 2005).

10. Cholesterol Reduction:

The ability of the cultures to reduce cholesterol was determined by performing the cholesterol assay. The test medium used for screening cultures for cholesterol uptake was sterile modified MRS broth (mMRS) supplemented with bile (Sodium taurocholate) and cholesterol. The composition of the mMRS broth was: peptone, 10g/lit; beef extract, 8g/lit; yeast extract, 4g/lit; Tween 80, 1ml/lit; K_2HPO_4, 2g/lit; triammonium citrate, 2g/lit; sodium citrate, 3g/lit; $MgSO_4.7H_2O$, 0.2g/lit; $MnSO_4.H_2O$, 0.04g/lit; sodium thioglycolate, 2g/lit; glucose, 10g/lit. Two different bile concentrations were used, 0.2% and 0.3% (wt/vol) sodium taurocholate. The final concentration of cholesterol in the medium was 200mg/liter. The cultures were added to 5ml of this mMRS broth. Bacterial growth was monitored hourly (0min, 1hr, 2hrs, 3hrs, 4hrs, 5hrs, 6hrs and 24hrs) by measuring the absorbance of the culture broth at 560nm. Uninoculated broth was used as the control. Each culture was tested in triplicates. After incubation the bacterial cells were removed by centrifugation (1000Xg, for 10min) the suspended broth and the control broth was then assayed for their cholesterol content. The dry weight of the cultures was determined after drying the centrifuged cells to a constant weight in an 80°C oven (Pereira *et.al.*2002, Pereira *et.al.* 2003).

Cultures were compared for cholesterol reduction in terms of their specific cholesterol uptake after 24hrs incubation period, according to the following equation:

$$(\%\ \text{cholesterol}) * g\ (\text{dry weight})/1 = [B - T / B * 100]/W$$

Were *B* is cholesterol content in the uninoculated control (milligrams/liter), *T* is cholesterol in the culture medium (milligrams/liter), and *W* is cells (dry weight [grams]) after 24hrs of incubation.

RESULT:

Isolation and characterization of lactobacilli :

From curd sample five morphologically different translucent colonies were selected after 48 hrs of incubation on MRS agar plates at 37˚C. Of these isolates three were found to be Gram positive rods which were non motile. From idly batter two morphologically different colonies were selected after incubation of 48 hrs on MRS agar plates at 37˚C. One of these isolates was found to be Gram positive rods which were non-motile. All the isolates were identified as the *Lactobacillus species* using Bergey's manual of determinative Bacteriology (Holt *et.al.* 1994).

2 Tolerance to gastric pH:

Tolerance to gastric pH was checked for 24 hrs at 37˚C. Absorbance of the MRS broth (pH 2.5) inoculated with culture was taken at 650 nm after 30 min interval for first 180 min and then after 24 hrs of incubation at 37˚C. It was found that absorbance remained constant for all the cultures which indicate that there was no growth. Survival in gastric environment was studied by plating out 100 µl of broth after 30 min interval on MRS agar plates. After incubation of plates at 37˚C for 24 hrs most of the plates showed no growth. Two of the test organisms (Isolate II and V) and control *L.casei* survived for 90 min in MRS broth (p^H: 2.5) but none of the isolates was able to grow under this conditions.

4. Antimicrobial activity:

All the test organisms were tested for antimicrobial activity against normal inhabitants of gastrointestinal tract and pathogenic organisms. After incubation at 37˚C for 24hrs, plates were observed for inhibitory effect of lactobacillus against target organism. It was found that most of the test organisms showed zone of inhibition of target organisms used. It was found that there was variation in inhibitory activity of different test organisms. *E.coli S.pneumonie and S.typhimurium* were inhibited by the test organisms except isolate IV, Isolate V and control show-

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ing inhibitory effect with highest degree.

Table: 2 Tolerance of test organisms to Gastric p^H and Bile salt.

Test organism	Survival in MRS broth (p^H:2.5)	growth in MRS broth (p^H:2.5)	Survival in MRS broth (Bile 0.3%)	growth in MRS broth (Bile 0.3%)
L.casei	+	-	+	+
L.plantarum	+	-	+	+
Isolate I	+	-	+	+
Isolate II	+	-	+	+
Isolate III	+	-	+	+
Isolate IV	-	-	-	-
Isolate V	+	-	+	+

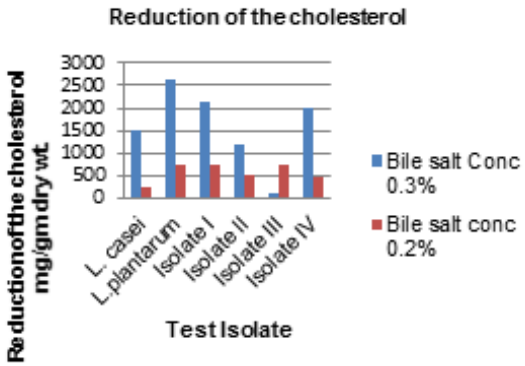
Negative test + Positive test

5. Utilization of prebiotic:
All of the test organisms employed in the work showed and observed the yellow zone around the colony against red background on mMRS agar plate

supplemented with 1% Inulin after 48 hrs incubation at 37^{0}C. This result shows that only Isolate V have the ability to ferment the Inulin other test organism showed growth on mMRS agar but they does not utilizes Inulin.

6 .Cholesterol reduction by *Lactobacillus spp.:*
All of the test organisms reduced cholesterol to some extent in MRS broth after incubation at 37˚C for 24 hrs. Variations were seen in amount of cholesterol reduced by different test organisms. Amount of cholesterol reduced ranges from approximately 27 to 2423 mg gm^{-1} dry weight in presence of 0.2% bile and from 12 to 2652 mg gm^{-1} dry weight in presence of 0.3% bile. In presence of 0.2% bile Isolate V showed maximum reduction of cholesterol and in presence of 0.3% bile *L.plantarum,* Isolate V showed maximum cholesterol reduction. It was found that amount of cholesterol reduced in presence of 0.3% bile was significantly higher than that in presence of 0.2% bile. (Graph:1)

DISCUSSION:
Probiotics, live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance (Verschuere *et.al.* 2000), are gaining interest as functional foods. Several criteria are used to evaluate various characters of potentially probiotic organisms. These include tolerance to low pH of stomach and bile acids. Probiotic bacteria should produce antimicrobial substances towards pathogens and it should be present as viable cells, preferably in large numbers (Kalpan *et.al.*2000). In present study several *in vitro* methods were used to select potentially Probiotic organisms.



Graph: 1 Reduction of the cholesterol

In tolerance to gastric acidity MRS broth adjusted to pH 2.5 showed survival of test organisms for different time intervals. However none of the test organism was able to grow under these conditions. In another study, similar results were obtained where only 29 of 44 strains survived and none of them were able to grow (Jacobsen *et.al.* 1999).Pereira *et.al.* used MRS broth adjusted at pH 2 and found that most of the strains were viable for less than 15 min (Pereira *et.al.*2002).

In this study, variations were seen in bile salt tolerance in the test organisms. Most of the test organisms showed delay of 1 to 2 hr in growth in presence of bile salt. Jacobsen and coworkers observed similar variation in bile salt tolerance but there was delay of 1 to 4 hrs in growth of the strains under study (Jacobsen *et.al.*1999). Pereira and coworkers also observed variations in bile salt tolerance (Pereira and Gibson 2002). In present study Isolate VI was found to be most sensitive to bile salt and could not survive in presence of bile salt. Other isolates showed significantly higher resistance to bile salt and were able to grow in its presence.

Several selection criteria have been used for novel probiotic strains which include safety, functional aspects and technological aspects (Annuk *et.al.* 2002). Production of antimicrobial compounds is one of the major functional criteria used. Lactobacilli show antimicrobial activity due to production of various compounds which include certain organic acids, hydrogen peroxide (H_2O_2), carbon dioxide, diacetyl, acetaldehyde, reuterin and bacteriocin (Ouwehand 1998). In the present study, it was found that all the test organisms showed antimicrobial activity against normal gastrointestinal tract inhabitants and pathogenic organisms to some extent. This might be due to production of any of the antimicrobial compounds mentioned above. Among the pathogenic organisms tested *S. typhi* was inhibited to highest degree by all of the test organisms. Variations were seen with the test organism in antimicrobial activity against different cultures tested. Annuk *et. al.* reported varying degree of inhibition of test organisms due to production of organic acids and hydrogen peroxide (H_2O_2) (Annuk *et.al.* 2002).

Prebiotics are known to selectively increase growth of probiotic bacteria. In the present study, ability of test organisms to utilize inulin was studied. It was found that none of the test organism employed in this study was able to ferment inulin whereas glucose which was used as control was readily utilized by all of them. It has been reported that *L. paracasei sub. paracasei* 8700:2 degrades inulin type fructans whereas none of other lactobacilli strains employed was found to degrade inulin (Makras *et.al.* 2005).

To study cholesterol reduction ability MRS broth supplemented with 100 mg/lit cholesterol and varying concentrations of bile salt (0.2 %and 0.3%) was used. Bile salt was added in the medium to mimic the approximate levels in the gastrointestinal tract. It was found that cholesterol uptake in presence of 0.3% bile salt was significantly higher than that in presence of 0.2% bile salt for most of the test organisms. Similar results were reported using 0.4% bile salt which showed higher amount of cholesterol uptake than 0.2% bile salt by most of the strains studied (Pereira *et.al.* 2002).

CONCLUSION:
As per the guidelines given in Bergey's manual all the isolates were identified and characterized up to genus level (Holt *et.al.* 1994). All the isolates might be lactobacilli. Another studied character is the stability of the test organisms at the gastric P^H, these condition states that minimum time which require to passing from the gastric p^H, these condition states that minimum time which require to passing from the gastric P^H environment up to this time all the test organisms are stable in the

P^H. A next important thing is the growth and the stability of the test organisms at the gastrointestinal tract environment (bile salt concentration 0.2 to 0.3%). This study shows that all the test organisms able to grow in the presence of 0.3% bile salt. All the test organisms show the antimicrobial activity against *E.coli S.pneumonie and S.typhimurium* to varying degrees. This proves that they are have the ability to modified the commensal flora of the intestine Isolate V have ability to ferment Inulin (prebiotic). Another study found that the all test organisms reduces the cholesterol within them Isolate V and *L.plantarum* reduces the higher quantity of the cholesterol.

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