



SAFETY ASSESSMENT OF POTENTIAL PROBIOTIC STRAINS *LACTOBACILLUS RHAMNOSUS* 231 AND *LACTOBACILLUS RHAMNOSUS* V92 IN MOUSE MODEL

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Abstract: Two human lactobacilli strains *L. rhamnosus* 231 (Lr 231) and *L. rhamnosus* V92 (Lr V92) exhibits potential probiotic properties. In the present study we evaluated safety of these strains by using (i) *in vitro* test; non haemolytic and antibiotic sensitivity (ii) *in vivo* trials using mouse model by an orally feeding 1×10^9 cfu of Lr 231 and Lr V92 to Swiss albino mice for 2 weeks. During the experimental period, probiotic strains did not produce any adverse effect on the general health status, feed intake and body weight. No viable Lr 231 or Lr V92 cells were recovered from blood or tissue (liver and spleen) of mice. No treatment-associated illness or death was observed. Microscopic observation of liver and spleen sections did not show any sign of inflammation, degeneration or necrosis. The potential probiotic strains Lr 231 and Lr V92 possess no hemolytic activity, antibiotic sensitivity pattern was evaluated and are non-toxic to mice and appear to be safe for human use.

Keywords: Bacterial Translocation, Bacteremia, Lr 231, Lr V92, Safety, Swiss albino mice

INTRODUCTION

Probiotics are live microbial food supplements that can beneficially affects the host by improving its intestinal microbial balance¹. Lactic acid bacteria (LAB) and bifidobacteria are widely used as probiotics and are consumed in fermented food products with long safe history. They exhibit several health-promoting effects; antimicrobial activity (AMA) against human pathogens and food spoilage organisms, immunomodulating properties, decrease the serum cholesterol levels, stress induced biofilm formation and reduce the incidence of the colon cancer on a regular consumption of fermented food products¹⁻⁶. With growing consumer awareness about diet and health, probiotic research has gained lot interest in both food and medical science⁷⁻⁸. As a result, new and more specific lactobacilli strains with probiotic attributes are being introduced into the food products, which do not necessarily share the generally regarded as safe (GRAS) status of traditional LAB strains.

Hemolysis is process in which RBC membrane is degraded by hemolysin, a bacterial protein. As consequences haemoglobin will leak from the RBC and thereby affects the immune system. Many types of bacteria exhibit hemolytic activity⁹.

The over usage of antibiotics in last one century led to the spread of antibiotic resistant microorganisms. Moreover it has been shown that genes coding for antibiotics resistance can be transferred among bacteria of different genera,

consequently pathogenic bacteria is becoming resistant to more number of antibiotics¹⁰. The potential health risks that could result from the transfer of antibiotic resistance genes among different bacteria in the resident human gut microflora could be possibility. For approval of microorganisms as feed additives or plant protection agents in European Food Safety Authority (EFSA), it is mandatory to provide information on antibiotic resistant profile of the bacteria¹¹.

According to Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO)¹², the phenotype and genotype of probiotic strains should be established followed by determining safety and functional properties of probiotics using *in vitro* assays and animal studies. Later, probiotics have to be tested using standard methods in two clinical evaluations: phase 1 (safety assessment) and phase 2 (efficacy assessment) studies. After confirming efficacy and safety of a probiotic strain in clinical studies, it can be marketed as a probiotic food. When a claim is made that a probiotic can alter a disease state, then a phase 3 study must be performed. This claim should be based on sound scientific evidence.

With growing concerns for safety of new probiotic strains due to few cases of infections associated with some indigenous lactobacilli strains, it is important to evaluate safety of potential probiotics strains¹³⁻¹⁶. In



our laboratory, we isolated two potential probiotic lactobacilli strains viz *L. rhamnosus* 231 (Lr 231) and *L. rhamnosus* V92 (Lr V92) having (i) *in vitro* antimicrobial activity against human pathogens and food spoilage organisms; antimicrobial activity was attributed to cell free culture filtrate and extracellular protein concentrate¹⁷ (ii) *in vitro* mutagens binding and antimutagenic activity¹⁸ and (iii) administration of viable Lr 231 protected rats from MNNG-induced colon inflammation¹⁹.

The present study is undertaken (a) to analyze the *in vitro* hemolytic and antibiotic sensitivity pattern of the two lactobacilli strains and (b) by an orally feeding 1×10^9 cfu of Lr 231 and Lr V92 to Swiss albino mice.

MATERIAL AND METHODS

Chemicals:

De Man Rogosa Sharpe (MRS) agar and octadiscs containing different antibiotics were purchased from Himedia, Mumbai, India. All other solvents used were of analytical grade and purchased from Merck, India.

Bacteria:

Lactobacilli strains, Lr 231 and Lr V92 were isolated from human feces and female vagina and pure cultures were preserved in 10% skimmed milk broth at 4°C. These frozen cultures were grown on MRS agar and incubated at 37°C for 48 h. Single colonies were inoculated into 5 mL MRS broth and sub-cultured three times to ensure actively growing cells. A 1-mL aliquot of each culture was inoculated in 10 mL MRS broth and incubated at 37 °C for 24 h. Cells were then harvested by centrifugation at 5000 rpm at 4°C for 15 min; washed twice with 10 mM phosphate buffer saline (PBS), pH 7, and resuspended in the same ($OD_{600}=1$).

In vitro hemolytic activity:

Hemolytic activity was studied by streaking fresh cultures on blood agar plate containing 10% (w/v) human blood and incubating it at 37°C for 48 h. Blood agar plates were examined for signs of α -haemolysis (green-hued zones around colonies), β hemolysis (clear zones around colonies), or γ -hemolysis (no zone around colonies)²⁰.

Antibiotic resistance:

For testing antibiotic resistance, Lactobacilli strains were inoculated (1%) in molten MRS agar before pouring into petri plates and octadisc was placed on the surface of MRS agar and incubated at 37°C for 24 h. Resistance and susceptibility against antibiotics was determined by measuring the zone of inhibition.

Animals:

Experimental protocol approved by the Saurashtra University animal ethical committee was followed (CP6EA/CH/RF/ACK-2003). Mice were acclimatized for 2

weeks to the experimental conditions, 20 Swiss albino mice were randomly assigned into 3 groups containing 5 animals each. Group 1 served as control group and received only 0.1 ml of PBS buffer. Group 2 and Group 3 were probiotic groups and were fed with 0.1 ml (equivalent to 1×10^9 cfu/ day) of Lr 231 and Lr V92 respectively. All three groups were fed with PBS or probiotic strains using sterile disposable syringe for 15 days. During the entire experimental period, activity and behavior of animals was observed and recorded daily. Feed intake and body weight were measured daily. On the 15th day, all mice from each group were euthanasia humanely by chloroform treatment and their blood and tissue samples were collected aseptically and weighed.

Bacterial translocation:

One drop of blood from each organ was directly inoculated and spread on MRS agar and incubated at 37°C for 48 h to detect bacterial translocation and bacteremia²¹. The spleen and liver was excised, homogenized using glass homogenizer (Borosil, India) and plated on MRS agar and incubated at 37°C for 48 h to determine bacterial translocation.

Histology:

Small pieces of liver and spleen fixed in a Bouin's fluid for 16 h and washed overnight¹⁹. Tissues were then subjected to serial dilutions of alcohol (10-100%) and alcohol-xylene mix; and thereafter, embedded in paraffin blocks and sectioned for histopathological study. Tissue sections were prepared and stained with haematoxylin and eosin. Sections were observed for inflammation, oedema, leucocyte infiltration, etc. and samples were analyzed by a single blinded pathologist.

RESULTS

Hemolytic activity:

None of the strains used in the study exhibited β -hemolysis when grown on blood agar plates. Both lactobacilli strains were γ -hemolytic (i.e. no haemolysis).

Antibiotic resistance profile:

Antibiotic resistance profile of Lr 231 and Lr V92 were evaluated (Table.1). Tested antibiotics, amoxicillin, bacitracin, ciprofloxacin, colistin, fosfomycin, nalidixic acid, polymyxin B and vancomycin were not effective against Lr V92 while co-trimoxazole, colistin, fosfomycin, nalidixic acid, polymyxin B and vancomycin were observed to be non-effective against Lr 231.

General health status of mice fed with Lr 231 and Lr V92:

Throughout the experimental period, no noticeable behavioral or activity changes were

observed in the any of the animal groups, and no treatment related illness or death occurred. Feed intake was similar among control group and groups fed with lactobacilli (Fig.1a). No significant difference was observed in body weight of treated groups in comparison to control group (Fig.1b).

Table.1: Antibiotic susceptibility and resistance pattern of *Lactobacillus rhamnosus* 231 and *Lactobacillus rhamnosus* V92

Antibiotics	Zone of inhibition (mm)	
	n=4	
	LAB V92	LAB 231
Amoxycillin (10 µg)	-	12±1
Ampicillin (10 µg)	17.7±1.5	16.2±3.3
Bacitracin (10 U)	-	16±1
Carbenicillin (100 µg)	14±4.2	18±8.9
Cephalothin (5 µg)	17±1	13±1.4
Chloramphenicol (30 µg)	22.9±4.9	25.5±1.9
Ciprofloxacin (5 µg)	-	13±1.4
Clindamycin (2 µg)	26±1	28±2.6
Cloxacillin (5 µg)	12±1	21±1.2
Co-Trimoxazole (25 µg)	10±1	17.6±4.2
Colistin (10 µg)	-	-
Erythromycin (15 µg)	15.5±6.4	23±3.6
Fosfomycin (200 µg)	-	-
Gentamicin (10 µg)	15±7.1	17±1.9
Kanamycin (30 µg)	11±1	12.3±4.16
Lincomycin (2 µg)	19±1	24±1
Neomycin (30 µg)	12±	12.5±0.7
Nalidixic acid (30 µg)	-	-
Nitrofurantoin (300 µg)	22.5±0.7	20±5.4
Novobiocin (30 µg)	18±1	27±1
Ofloxacin (5 µg)	nd	21±1
Penicillin (1 U)	20.5±1.5	28±1.8
Polymyxin B (300 U)	-	-
Streptomycin (25 µg)	16±1	17.1±2.31
Tetracycline (30 µg)	22.3±5	25.1±5.6
Vancomycin (30 µg)	-	-

no inhibition, nd not determined

Bacterial translocation:

Lactobacilli were not observed in the visceral swab cultures plated on MRS agar, indicating a non-contaminated visceral surface. However, chalky white colonies were observed in control and treated groups from sections of liver and spleen, plated on MRS agar. Nevertheless, Gram staining confirmed that they were cocci and not gram positive rods. No bacteraemia was detected in any of the groups.

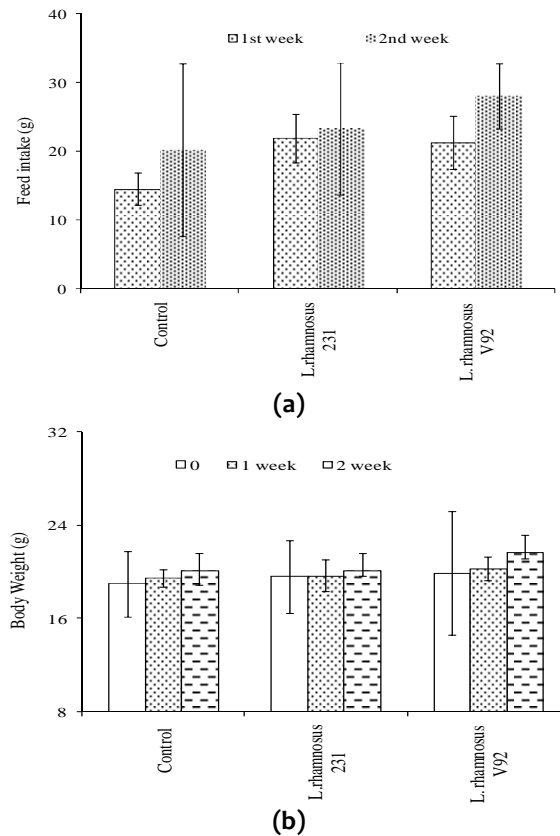


Fig.1: Overall changes in weekly (a) feed intake and (b) weight gain among mice orally administrated with Lr 231, Lr V92 or control group for 2 weeks

Histology:

Macroscopic examination did not reveal any obvious variation in the size and appearance of visceral organs between the groups. No Hepatomegaly or splenomegaly occurred. There was no statistically significant difference in the spleen weight index (SWI) of the mice in comparison to control (Fig.2). Microscopic observation did not show any sign of inflammation, degeneration or necrosis of liver in the treated or control groups as determined by a blinded histopathology (Fig.3).

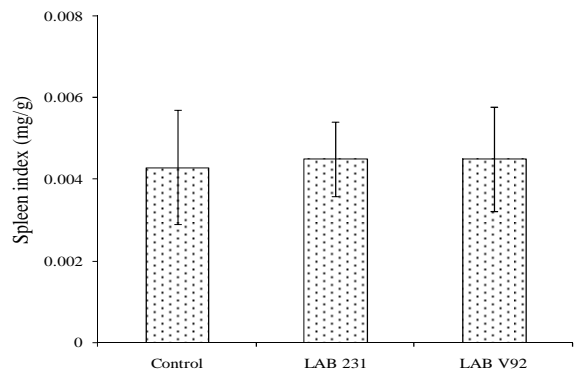


Figure.2: Spleen weight index (SWI) of mice orally inoculated with probiotic strains Lr 231 and Lr V92 for 2 weeks. The values included in this figure are the spleen weight (mg) / body weight (g).

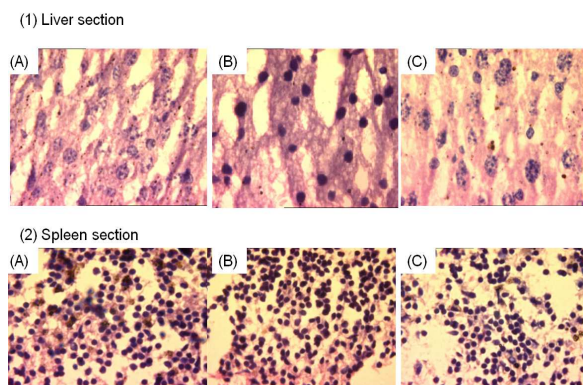


Figure.3: Histological sections of (1) liver and (2) spleen (a) Group 1: Control group (b) Group 2: Lr 231 and (c) Group 3: Lr V92. Sections were viewed at 100x magnification.

DISCUSSION

In the present study we evaluated safety of potential probiotic human strains Lr 231 and Lr V92 using *in vitro* and mouse model. Hemolytic activity is one of the important undesirable metabolic activities of strain contributing for increased pathogenicity. Lr 231 and Lr V92 do not exhibit hemolytic activity and confirms non-pathogenicity of the lactic isolates. In a report, except few strains, all the strains of *Lb. paracasei* subsp. *paracasei*, *Lactobacillus* spp. and *Lb. casei* showed γ -haemolysis²². Recently, four strains of *Lb. pentosus* were reported to exhibit α -haemolysis²³.

Lr 231 and Lr V92 exhibited antibiotic resistance against only few antibiotics including vancomycin. These properties of these strains could be exploited to restore gut microbiota during antibiotic therapy²⁴. However, it is remained to be determined whether antibiotic resistance gene is plasmid or chromosomal encoded. Lactobacilli display a wide range of antibiotic resistance naturally, but in most cases antibiotic resistance is not of the transmissible type²⁵. Many intrinsically vancomycin resistant strains of lactobacilli have a long history of safe use as probiotics and there is no indication that vancomycin resistant lactobacilli would transfer the resistance to other bacteria. According to earlier reports *Lb. plantarum*, three *L. rhamnosus* strains (HN001, HN067 and GG) were resistant to vancomycin and *Lb. casei* species showed native resistance to vancomycin²⁶⁻²⁹.

Appetite, activity and live weight gain are the valuable indicators to evaluate general health status for animals. Feed intake and weight gain was either higher or equal to control group demonstrating that lactic isolates are non-toxic²¹.

Bacterial translocation is a prerequisite for pathogenicity for most opportunistic indigenous lumen strains.³⁰ Bacterial translocation is a phenomenon caused by a diminished intestinal barrier, resulting in the passage of bacteria (or bacterial components or

products) across the mucous membrane and epithelium³¹. Potential probiotic strains Lr 231 and Lr V92 did not translocate to other organs including blood, thus do not possess invasive properties and hence are safe for consumption.

Splenomegaly and hepatomegaly are indirect indicators of infection, but we did not find any macroscopic and microscopic changes in the spleen or liver morphology in probiotics groups compared to control group. Furthermore, animals in the test and control groups had similar SWIs. This result suggests that feeding mice with Lr 231 and Lr V92 for 2 weeks did not cause any infection or adverse effect. Earlier we have shown that Lr 231 supplementation protected the rats from MNNG-induced inflammation¹⁹.

To summarize, Lr 231 and Lr V92 possess no hemolytic activity and antibiotic sensitivity pattern was evaluated. Feeding mice with Lr 231 and Lr V92 for 2 weeks had no adverse effects on feed intake activity, weight gain and general health status. Fed lactic isolates neither caused histological damage to gut mucosa nor translocated to other organs. Thus, it can be concluded that Lr V92 and Lr 231 are safe for animal and human consumption and ready for human clinical trials.

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