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Oral toxicity evaluation of probiotic strains isolated from Finger millet [*Eleusine coracana* (L.) Gaertn.] in Wistar rat models (*in vivo*)

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Abstract

This study evaluates the oral toxicity of five probiotic strains recently isolated from fermented flour of finger-millet (*Eleusine coracana*) varieties of Sri Lanka. Probiotic strains; *Lactobacillus plantarum* MF405176, *Lactobacillus fermentum* MF033346, *Lactococcus lactis* subspecies *lactis* MF480428, *Enterococcus faecium* MF480431 and *Pediococcus acidilactici* MF480434 were evaluated for acute and sub-chronic oral toxicity in Wistars. Three individual doses (10^8 CFU/g, 10^{10} CFU/g and 10^{12} CFU/g) of each probiotic strain at single oral dose of 5000 mg/kg bw were orally administered to rats and observations were done till 14th day. Since no animals demonstrated signs of toxicity as a result of the administered probiotics strains, repeated dose sub-chronic oral toxicity study was conducted by oral administration of three doses (10^8 CFU/g, 10^{10} CFU/g, 10^{12} CFU/g) of each probiotic strain at 1000 mg/kg bw/day for consecutive 90 days. Administration of probiotic strains to rats did not cause mortality in any of the tested doses. No changes in animal behavior, feed or water intake and negative effects on body weight observed. Probiotic feeding did not cause changes in analyzed biochemical and hematological parameters attributed to toxicity. Bacteremia, bacterial translocation and histopathological changes in rat organs were not observed. No significant difference in liver enzymes observed in treatment groups compared to control. In conclusion, all tested probiotic strains are nonpathogenic therefore could be considered as safe for human consumption.

1. Introduction

Probiotics are live microorganisms, when administered in adequate amounts confer health benefits to the host (FAO/WHO, 2002). Probiotic bacteria consist of several genera of lactic acid bacteria (LAB) that are Gram-positive, non-spore-forming, anaerobic or facultative aerobic cocci or rods producing lactic acid during carbohydrate metabolism (Fenster *et al.*, 2019; Quinto *et al.*, 2014). Among LAB, *Lactobacillus* is the largest genus and Generally Recognized as Safe (GRAS). Applications of the selected strains of Genus *Enterococcus* as probiotics are also well documented. Probiotics are broadly classified under functional food, therefore extends its role beyond providing adequate nutrients to improving health and preventing the risk of diseases including certain non-communicable diseases (NCD) such as cancer, hypertension, hypercholesterolemia, etc. Ability of probiotics to modulate

physiological functions resulting in prevention of diseases is widely reported (Kumar *et al.*, 2015). Beneficial effects of probiotics as antimicrobial agents against pathogenic, carcinogenic and conditionally pathogenic microorganisms are strain-specific. Antimicrobial activity involves competitive exclusion via competition for adhesion sites, competition for substrates and limiting resources, synthesis of anti-microbial substances and inhibition of toxin expression in pathogens (Denkova *et al.*, 2017). Role of probiotics in cancer therapy may also be strain dependent and associated with their immunomodulatory effects and expression of different genes involved in cell transformation, migration and invasion (Motevaseli *et al.*, 2017). Anti-oxidant properties of probiotics are caused by metal ion chelating ability, presence of anti-oxidant enzyme system, production of anti-oxidant metabolites, regulation of anti-oxidant signaling pathways, and regulation of enzymes producing Reactive Oxygen Species and modulating the

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gut microbiota (Wang et al., 2017). Understanding reduction of lipid and cholesterol levels in human subjects by probiotics that occur through bile salt hydrolase activity and cholesterol assimilation ability has received wide attention in recent years (Duchesneau et al., 2014). Due to absence of side effects compared to drugs, probiotics are becoming an effective alternative in managing preferment for human health (Gionchetti et al., 2007; Tripathi et al., 2014). In addition, they find application in technological advancement in food processing such as ripening, shelf-life improvement and aroma development.

Consequently, a number of new bacterial strains are being identified as probiotics and incorporated into the food and pharmaceutical formulations globally. However, assessing safety of a new probiotic strain intended to be incorporated in to food or supplement, is crucial (Conway, 1996). In this study, five new probiotic strains; *Lactobacillus plantarum* MF405176, *Lactobacillus fermentum* MF033346, *Lactococcus lactis* subspecies *lactis* MF480428, *Enterococcus faecium* MF480431 and *Pediococcus acidilactici* MF480434, previously isolated from fermented flour of finger-millet (*Eleusine coracana*) varieties cultivated in Sri Lanka (Divisekera et al., 2019), were investigated for oral toxicity. Probiotic strains under study exhibited preliminary requirements of survival in simulated conditions of the human gut, could aggregate and adhere to intestinal cells, free from virulence causing enzymes responsible for hemolysis, DNAs and gelatin hydrolysis and demonstrated antibiotic susceptibility (Divisekera et al., 2019). Further, these strains have already demonstrated efficacy (anti-bacterial, anti-cancer, anti-oxidant and cholesterol assimilation) in-vitro. The study envisioned to authenticate the safety (acute and sub-chronic oral toxicity) of five potential probiotic strains to establish their suitability as future probiotics.

2. Material and methods

Probiotic strains

Five probiotic strains; *Lactobacillus plantarum* MF405176, *Lactobacillus fermentum* MF033346, *Lactococcus lactis* subspecies *lactis* MF480428, *Enterococcus faecium* MF480431 and *Pediococcus acidilactici* MF480434 isolated from fermented flour of finger-millet (*Eleusine coracana*) varieties of Sri Lanka were selected for the study.

2.1 Oral toxicity evaluation of probiotic strains in Wistar rats

Experimental animals and housing conditions

Pathogen free Wistar rats of both sexes (aged 4-6 weeks, male and female) bred at the animal breeding unit of the ICCBS, University of Karachi, Pakistan. The animals were acclimated for one week before starting experiment. Animals were housed in stainless-steel cages (5 per cage, segregated by gender) with 12 h light/ dark cycle (8.30 am to 8.30 pm) in a controlled atmosphere (temperature 24 ± 2 °C, humidity $55 \pm 2\%$). Animals were given access to standard rat diet (LabDiet®) and potable tap water *ad libitum*. Animal cage beddings were changed weekly. The study has been approved by the institutional animal care and user committee of the International Centre for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan (Ethical clearance certificate number is 2016-0001). The study was conducted according to the ARRIVE guidelines (Sert et al., 2020) and is in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines.

Single dose acute oral toxicity study

Wistar rats were randomly divided into sixteen groups (fifteen treatment groups and one control group for each test probiotic, three treatment groups were assigned (each group receiving different dose of test probiotic, similarly three groups received three doses of each test probiotic). Likewise, for the five test probiotics under study, fifteen treatment groups were assigned. Each group consisted of 5 male and 5 female rats housed based on their gender. Body weights at randomization were 200–220 g for males, and 200–215 g for females. These doses; 10^8 CFU/g, 10^{10} CFU/g and 10^{12} CFU/g of each probiotic candidates were prepared by inoculating in to 1% skim milk, stored in ice prior to administration (Zhou et al., 2000). A single oral dose of 5000 mg/kg bw of each test article (three different doses of five probiotic strains) was orally administered to treatment groups, while control group was administered with 1% (w/v) skim milk only. During the experiment, animals' health, behaviors, mortality (if any) were observed daily for consecutive 14 days using a three-scale method; lazy, weak and sleepy-1, intermediate movements and interactions with each other-2 and active movements and interactions with each other-3. Observations including changes in feed and water intake, sleeping pattern, skin and fur, eyes and mucous membranes, respiratory, somatomotor activity, behavior pattern, breathing, tremors, convulsions, salivation, diarrhea, sleep and changes in gait and posture were recorded daily from 1st to 15th day, using three scales; + (normal), ++ (intermediate), +++ (severe). On the 15th day, live weights of all the animals were recorded. Katamine 30 mg/kg combined with medetomidine 1 mg/kg was used as the anesthetic drug and doses were calculated based on the body weights of animals, and administered intra-muscularly. Surgery was performed in accordance to guidelines given in the animal care and use course derived by The American Association for Laboratory Animal Science of the ICCBS, University of Karachi, Pakistan. Animals were observed for perception of pain prior to perform non survival surgical procedure. Surgical areas were cleaned with 70% ethanol (v/v), incision sites were clipped. Animal hearts were punctured using sterile needles and blood was drawn. From each animal, 2 ml of blood was collected to individual vacutainers containing EDTA and 4 ml of blood was collected to vacutainers containing clot activator with gel. The vacutainers were stored at 4 ± 1 °C until analyzed. Animal organs portions (kidney, liver and intestine) were excised aseptically washed with sterile 10% PBS and preserved in 10% v/v formaldehyde solution. Rats were euthanized in a CO₂ chamber. Animal blood was tested for hematological parameters including hemoglobin, erythrocyte count (RBC), hematocrit (HCT/PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leukocyte count (WBC) and platelet count using an automated hematological analyzer (HA 6700, Hawksley, UK). Series of serum biochemistry tests were performed by using an automatic clinical chemistry analyzer (BIOBASE-Emerald, china) including random blood glucose test, liver function tests (total bilirubin, direct bilirubin, Alkaline phosphatase, gamma-glutamyl transferase and alanine transaminase (ALT) and lipid profile (cholesterol, triglycerides, high density lipoproteins, low density lipoproteins and very low-density lipoproteins). Histopathological examination of rat organs was performed. Bacterial translocation in blood was investigated by streaking a loop full of each blood sample on individual sterile de Man Rogosa and Sharpe (MRS) agar plates in triplicate. Bacterial translocations in organs were investigated by culturing 1 g of tissues of animal organs; liver, intestine, mesenteric lymph node and kidney on individual MRS agar plates in triplicate. MRS agar plates containing blood and organs were incubated at 37 ± 1 °C for 48 h.

Repeated dose sub chronic oral toxicity study

Wistar rats were randomly divided into sixteen groups (fifteen treatment groups and one control group). For each test probiotic, three treatment groups were assigned (each group receiving different dose of test probiotic, similarly three groups received three doses of each test probiotic). Likewise, for the five test probiotics under study, fifteen treatment groups were assigned. Each group consisted of 10 male and 10 female rats. Body weights at randomization were 210-225 g for males, and 200-215 g for females.

Doses of 10^8 CFU/g, 10^{10} CFU/g, 10^{12} CFU/g at 1000 mg/kgbw/day was administered orally for consecutive 90 days. Body weights of animals were measured weekly. During the experiment, animals' health, behaviors, mortality (if any) was observed daily. Observations including changes in feed and water intake, sleeping pattern, skin and fur, eyes and mucous membranes, respiratory, somatomotor activity, behavior pattern, breathing, tremors, convulsions, salivation, diarrhea, sleep and changes in gait and posture was also recorded weekly. Anesthesia and surgery was performed on 91st day as described in acute oral toxicity study. Prior to surgery, animals were fasted for 16 h. Surgery was performed in accordance to guidelines given in the animal care and use course derived by The American Association for Laboratory Animal Science of the ICCBS, University of Karachi, Pakistan. Hematology and biochemistry of rat blood was evaluated as per the parameters mentioned in acute oral toxicity study. Histopathological examination of rat organs including tests and control was performed. Bacterial translocation in blood and organs of rats was studied using MRS agar as mentioned under the methodology of acute oral toxicity study.

2.2 Statistical analysis

The mean and standard error of the data obtained from parallel experiments were calculated using Minitab 14. One-way ANOVA (unstacked) followed by the multiple comparisons using Tukey's family error rate was performed to analyze the data. Values $P < 0.05$ were considered as significant.

3. Results

3.1 Evaluation of single dose acute oral toxicity of probiotic candidates

During the acute toxicity study, oral administration of the three doses; 10^8 CFU/g, 10^{10} CFU/g, 10^{12} CFU/g of probiotic strains *Lactobacillus plantarum* MF405176, *Lactobacillus fermentum* MF033346, *Lactococcus lactis* subspecies *lactis* MF480428, *Enterococcus faecium* MF480431 and *Pediococcus acidilactici* MF480434 did not cause abnormal changes in sleeping pattern, skin and fur, eyes and mucous membranes, respiratory, somatomotor activity, behavior, breathing, tremors, convulsions, salivation, diarrhea, gait and posture. Furthermore, no treatment-related illness or animal death was shown. Intake of probiotics, at administered doses, did not interrupt the usual pattern of feed and water intake in both male and female rats, neither did it cause significant difference in body weight evolution between experimental and control groups.

Results of hematological analysis of whole blood revealed significant differences ($P < 0.05$) in hemoglobin content in both male and female animals orally received *L. plantarum* MF405176 and *L. fermentum* MF033346. While others did not demonstrate significant difference. While significant differences ($P < 0.05$) in platelet count was observed in all female animals fed with tested probiotic strains, *L. plantarum* MF405176, *L. fermentum* MF033346, *L. lactis* subspecies *lactis* MF480428, *E. faecium*

MF480431 and *P. acidilactici* MF480434 (Table 1). Lipid profile and liver function tests of both male and female rats received test probiotic strains revealed no significant difference (Tables 2, 3). No abnormal histopathological observations in animal organs (kidney, liver and intestine) were detected. In all experimental groups, neither bacteremia in blood nor bacterial translocation in organs observed.

3.2 Repeated dose sub chronic oral toxicity evaluation of probiotic strains

In the repeated dose sub-chronic oral toxicity study, oral administration of tested doses; 10^8 CFU/g, 10^{10} CFU/g, 10^{12} CFU/g of probiotic strains did not cause abnormal changes in sleeping pattern, skin and fur, eyes and mucous membranes, respiratory, somatomotor activity, behavior, breathing, tremors, convulsions, salivation, diarrhea, gait and posture in both male and female rats. Further, no treatment-related illness or animal deaths were befallen. Oral intake of probiotics did not interrupt the usual pattern of feed and water intake in both male and female rats. Significant increment ($P < 0.05$) in mean body weights was observed at the end of feeding (90th day) compared to day 01 (Table 4).

With regard to the hemoglobin content, male rats, except animals administered with *L. lactis* subspecies *lactis* MF480428 and *E. faecium* MF480431 and female rats, except animals administered with *L. lactis* subspecies *lactis* MF480428, others demonstrated significant difference ($P < 0.05$) (Table 5). With regard to the RBC content, all animals both male and female except females orally administered with *L. plantarum* MF405176, *L. fermentum* MF033346, *L. lactis* subspecies *lactis* MF480428 demonstrated significant difference ($P < 0.05$) (Table 5). With regard to the HCT/PCV, MCV and MCH content, all male and female animals except males administered with *L. lactis* subspecies *lactis* MF480428 demonstrated significant difference ($P < 0.05$) (Table 5). Significant difference ($P < 0.05$) in WBC content was observed in males administered with *E. faecium* MF480431 and females administered with *E. faecium* MF480431 and *L. lactis* subspecies *lactis* MF480428 (Table 5). Significant difference ($P < 0.05$) in platelet content was observed in all animals except females administered with *P. acidilactici* MF480434 (Table 5).

With regard to the lipid profile, all animals except males administered with *L. lactis* subspecies *lactis* MF480428, *E. faecium* MF480431 and *P. acidilactici* MF480434 and females administered with *L. fermentum* MF033346 demonstrated significant difference ($P < 0.05$) in cholesterol content (Table 6). Except males administered with *P. acidilactici* MF480434, others demonstrated significant difference ($P < 0.05$) in triglyceride content. All animals except females administered with *L. fermentum* MF033346, others demonstrated significant difference ($P < 0.05$) in HDL and LDL content (Table 6). Significant difference in VLDL content was observed in all animals except males administered with *E. faecium* MF480431 (Table 6).

With regard to the liver function tests, all animals demonstrated significant difference ($P < 0.05$) in SGPT and alkaline phosphatase (Table 7). While no significant difference in Gamma GT was observed in any of the treated animals compared to control. Except males administered with *E. faecium* MF480431 and *P. acidilactici* MF480434, and females administered with *L. plantarum* MF405176, *L. fermentum* MF033346, *E. faecium* MF480431 and *P. acidilactici* MF480434 others demonstrated significant difference ($P < 0.05$) in total bilirubin content. All animals except males administered with *L. plantarum* MF405176 and females administered with *L. plantarum* MF405176, *L. fermentum* MF033346, *E. faecium* MF480431 demonstrated significant difference ($P < 0.05$) in direct bilirubin (Table 7).

In the sub-chronic toxicity study, none of the experimental groups administrated with different doses of test probiotic strains demonstrated necrosis, fibrosis, loss of normal architecture, atrophy or inflammation in any of the examined organs i.e., kidney, liver, intestine in both male and female rats

indicating no histopathological abnormalities were caused by oral administrated probiotics under study. None of the animals exhibited bacteremia in blood and/or demonstrate bacterial translocation in organs.

Table 1 Hematology of rat blood in acute oral toxicity study

Probiotic Candidate	Dose		Hematological Parameters						
			Hb (g/dl)	RBC (Million/ μ l)	HCT/PCV (%)	MCV (fl)	MCH (pg)	WBC ($\times 10^9$ /l)	Platelet ($\times 10^9$ /l)
		Contr ol(M)	9.60 \pm 0.00 ^a	5.09 \pm 0.50 ^a	29.80 \pm 0.00 ^a	58.50 \pm 0.00 ^a	19.03 \pm 0.08 ^a	3.87 \pm 0.03 ^a	590.33 \pm 0.33 ^a
<i>L.plantarum</i> MF405176	D1	M	10.93 \pm 1.10 ^a	5.85 \pm 0.72 ^a	31.00 \pm 3.80 ^a	57.70 \pm 0.90 ^a	19.80 \pm 0.30 ^a	3.73 \pm 0.07 ^a	662.0 \pm 17.0 ^a
	D2		12.37 \pm 0.52 ^b	6.84 \pm 0.13 ^a	30.10 \pm 0.90 ^a	57.17 \pm 2.09 ^a	18.57 \pm 0.72 ^a	3.40 \pm 0.10 ^a	901.0 \pm 91.0 ^a
	D3		12.60 \pm 0.23 ^b	6.67 \pm 0.08 ^a	30.53 \pm 0.87 ^a	57.80 \pm 0.95 ^a	18.90 \pm 0.25 ^a	3.33 \pm 2.64 ^a	957.7 \pm 63.2 ^a
<i>L.fermentum</i> MF033346	D1	M	11.70 \pm 0.00 ^a	6.11 \pm 0.00 ^a	27.30 \pm 0.00 ^a	57.80 \pm 0.00 ^a	19.07 \pm 0.03 ^a	3.70 \pm 0.05 ^a	1211 \pm 1.00 ^a
	D2		12.60 \pm 1.00 ^a	6.71 \pm 0.21 ^a	27.30 \pm 0.0 ^a	58.10 \pm 0.10 ^a	18.93 \pm 0.17 ^a	3.27 \pm 0.27 ^a	956.0 \pm 35 ^a
	D3		13.20 \pm 0.29 ^b	6.00 \pm 0.09 ^a	27.00 \pm 0.20 ^a	58.70 \pm 0.76 ^a	18.90 \pm 0.63 ^a	3.10 \pm 0.87 ^a	1050 \pm 176 ^a
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	M	11.40 \pm 0.00 ^a	6.13 \pm 0.00 ^a	34.60 \pm 0.00 ^a	56.40 \pm 0.00 ^a	18.73 \pm 0.13 ^a	3.47 \pm 0.03 ^a	1134.7 \pm 3.33 ^a
	D2		12.57 \pm 0.81 ^a	6.64 \pm 0.38 ^a	30.63 \pm 2.71 ^a	58.07 \pm 0.93 ^a	18.87 \pm 0.31 ^a	4.90 \pm 1.67 ^a	799.3 \pm 86.3 ^a
	D3		12.23 \pm 0.13 ^a	6.62 \pm 0.39 ^a	30.77 \pm 0.87 ^a	58.77 \pm 2.03 ^a	18.57 \pm 0.83 ^a	3.77 \pm 0.03 ^a	1010.7 \pm 14.3 ^a
<i>E.faecium</i> MF480431	D1	M	12.00 \pm 0.34 ^a	5.83 \pm 0.15 ^a	30.27 \pm 0.5 ^a	60.60 \pm 0.70 ^a	20.60 \pm 0.40 ^a	3.60 \pm 0.91 ^a	868.3 \pm 58.3 ^a
	D2		11.50 \pm 0.17 ^a	6.46 \pm 0.09 ^a	30.63 \pm 0.92 ^a	59.77 \pm 0.99 ^a	19.37 \pm 0.29 ^a	2.10 \pm 0.31 ^a	956 \pm 129 ^a
	D3		11.47 \pm 0.17 ^a	6.68 \pm 0.34 ^a	30.40 \pm 1.20 ^a	56.00 \pm 1.00 ^a	17.00 \pm 0.50 ^a	3.73 \pm 0.63 ^a	1070.7 \pm 90.3 ^a
<i>P.acidilactici</i> MF480434	D1	M	11.37 \pm 0.26 ^a	6.52 \pm 0.14 ^a	30.27 \pm 0.83 ^a	57.20 \pm 0.15 ^a	19.00 \pm 0.21 ^a	6.17 \pm 2.09 ^a	1080 \pm 146 ^a
	D2		11.87 \pm 0.13 ^a	6.96 \pm 0.22 ^a	30.27 \pm 1.63 ^a	56.93 \pm 0.79 ^a	18.50 \pm 0.40 ^a	5.20 \pm 2.45 ^a	892.3 \pm 76.7 ^a
	D3		12.07 \pm 0.03 ^a	6.09 \pm 0.22 ^a	31.83 \pm 0.27 ^a	58.20 \pm 2.04 ^a	18.33 \pm 0.53 ^a	5.43 \pm 1.73 ^a	898.3 \pm 58.4 ^a
		Contr ol(F)	9.40 \pm 0.00 ^a	5.39 \pm 0.30 ^a	30.20 \pm 0.60 ^a	58.10 \pm 0.56 ^a	18.60 \pm 0.30 ^a	3.30 \pm 0.05 ^a	589.67 \pm 1.33 ^a
<i>L.plantarum</i> MF405176	D1	F	10.53 \pm 0.29 ^a	5.80 \pm 0.10 ^a	27.47 \pm 0.27 ^a	57.53 \pm 1.27 ^a	19.33 \pm 0.17 ^a	3.57 \pm 0.03 ^a	893.33 \pm 9.82 ^b
	D2		13.97 \pm 0.09 ^b	6.38 \pm 0.29 ^a	27.93 \pm 2.97 ^a	60.20 \pm 1.10 ^a	19.07 \pm 0.53 ^a	3.73 \pm 1.67 ^a	849.3 \pm 24.7 ^b
	D3		13.47 \pm 0.27 ^b	6.31 \pm 0.15 ^a	26.67 \pm 0.33 ^a	56.53 \pm 0.77 ^a	18.60 \pm 0.30 ^a	3.00 \pm 1.00 ^a	938 \pm 110 ^b
<i>L.fermentum</i> MF033346	D1	F	10.40 \pm 0.31 ^a	6.91 \pm 0.17 ^a	29.57 \pm 0.81 ^a	56.57 \pm 0.82 ^a	18.57 \pm 0.23 ^a	5.20 \pm 0.94 ^a	526.0 \pm 24.0 ^a
	D2		10.00 \pm 0.20 ^a	6.01 \pm 0.16 ^a	29.77 \pm 1.39 ^a	56.40 \pm 0.95 ^a	18.53 \pm 0.19 ^a	2.23 \pm 0.47 ^a	1079 \pm 124 ^b
	D3		13.10 \pm 0.59 ^b	6.93 \pm 0.47 ^a	29.90 \pm 2.47 ^a	57.50 \pm 0.36 ^a	18.30 \pm 0.25 ^a	3.20 \pm 1.44 ^a	1169 \pm 105 ^b
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	F	10.97 \pm 0.39 ^a	6.12 \pm 0.03 ^a	30.57 \pm 0.95 ^a	57.00 \pm 1.17 ^a	18.20 \pm 0.47 ^a	3.50 \pm 0.66 ^a	1070.3 \pm 55.2 ^b
	D2		10.20 \pm 0.40 ^a	6.74 \pm 0.52 ^a	32.00 \pm 1.60 ^a	57.53 \pm 1.23 ^a	18.13 \pm 0.53 ^a	3.13 \pm 0.03 ^a	975 \pm 139 ^a
	D3		11.87 \pm 0.13 ^a	6.35 \pm 0.17 ^a	32.43 \pm 0.30 ^a	55.70 \pm 0.85 ^a	17.27 \pm 0.27 ^a	3.37 \pm 0.41 ^a	1048.0 \pm 113 ^{ab}
<i>E. faecium</i> MF480431	D1	F	10.33 \pm 0.20 ^a	5.49 \pm 0.39 ^a	30.50 \pm 0.55 ^a	58.00 \pm 1.18 ^a	19.70 \pm 0.38 ^a	3.77 \pm 0.63 ^a	853.7 \pm 29.1 ^a
	D2		10.60 \pm 0.30 ^a	5.86 \pm 0.35 ^a	30.87 \pm 1.47 ^a	59.57 \pm 1.03 ^a	19.37 \pm 0.63 ^a	3.40 \pm 0.20 ^a	996.0 \pm 60.2 ^b
	D3		10.53 \pm 0.27 ^a	6.42 \pm 0.29 ^a	30.47 \pm 1.23 ^a	56.37 \pm 0.68 ^a	18.60 \pm 0.20 ^a	5.00 \pm 0.90 ^a	921.3 \pm 89.7 ^{ab}
<i>P.acidilactici</i> MF480434	D1	F	9.01 \pm 0.07 ^a	6.51 \pm 0.26 ^a	31.10 \pm 0.10 ^a	55.07 \pm 0.03 ^a	18.33 \pm 0.33 ^a	3.90 \pm 0.32 ^a	1107.7 \pm 4.33 ^b
	D2		10.53 \pm 0.27 ^a	6.62 \pm 0.02 ^a	32.50 \pm 0.90 ^a	59.70 \pm 1.50 ^a	19.27 \pm 0.07 ^a	2.90 \pm 0.40 ^a	969.0 \pm 35.8 ^b
	D3		10.87 \pm 0.33 ^a	6.52 \pm 0.0 ^a	35.43 \pm 0.97 ^a	60.03 \pm 1.37 ^a	19.73 \pm 0.47 ^a	5.43 \pm 1.47 ^a	864 \pm 128 ^b

Data is expressed as mean \pm SEM, n=5. Within a column containing three doses of each probiotic candidate compared to control, mean values superscripted with different letters are significantly different ($P < 0.05$). (D1) Dose 1: 10^8 CFU/ml, (D2) Dose 2: 10^{10} CFU/ml, (D3) Dose 3: 10^{12} CFU/ml. Male (M), Female (F). Hemoglobin (Hb), Erythrocyte count (RBC), Hematocrit/Packed Cell Volume (HCT/PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Total leucocyte count (WBC).

Table 2 Lipid profile of rat blood in acute oral toxicity study

Probiotic candidates	Dose	Control /Sex	Lipid profile parameters				
			Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
		Control (M)	57.67 ± 2.67 ^a	48.33 ± 1.33 ^a	47.00 ± 1.15 ^a	10.00 ± 1.00 ^a	9.57 ± 0.12 ^a
<i>L.plantarum</i> MF405176	D1	M	63.70 ± 14.8 ^a	59.30 ± 12.7 ^a	41.0 ± 12.70 ^a	15.00 ± 3.00 ^a	15.67 ± 2.33 ^a
	D2		51.00 ± 3.06 ^a	47.00 ± 7.64 ^a	42.67 ± 4.06 ^a	10.00 ± 2.52 ^a	15.00 ± 1.53 ^a
	D3		48.33 ± 6.98 ^a	48.70 ± 11.0 ^a	42.00 ± 3.21 ^a	11.33 ± 0.88 ^a	14.00 ± 4.93 ^a
<i>L.fermentum</i> MF033346	D1	M	67.67 ± 1.45 ^a	50.00 ± 8.62 ^a	46.67 ± 1.76 ^a	14.00 ± 1.00 ^a	9.67 ± 1.67 ^a
	D2		64.67 ± 6.69 ^a	47.33 ± 9.84 ^a	47.33 ± 1.76 ^a	14.33 ± 1.76 ^a	9.67 ± 0.67 ^a
	D3		48.00 ± 8.50 ^a	46.70 ± 12.7 ^a	44.00 ± 5.51 ^a	12.33 ± 1.45 ^a	9.00 ± 2.65 ^a
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	M	58.67 ± 1.67 ^a	49.67 ± 8.33 ^a	38.67 ± 2.67 ^a	12.33 ± 1.86 ^a	09.67 ± 1.86 ^a
	D2		51.00 ± 3.00 ^a	58.00 ± 2.08 ^a	35.00 ± 3.51 ^a	13.00 ± 1.15 ^a	11.47 ± 0.2 ^a
	D3		50.70 ± 10.3 ^a	41.33 ± 8.51 ^a	40.33 ± 7.31 ^a	09.67 ± 0.88 ^a	08.20 ± 1.72 ^a
<i>E.faecium</i> MF480431	D1	M	63.33 ± 6.84 ^a	59.70 ± 16.8 ^a	44.33 ± 3.18 ^a	11.33 ± 2.85 ^a	11.67 ± 3.18 ^a
	D2		60.33 ± 3.67 ^a	49.67 ± 8.69 ^a	42.00 ± 3.00 ^a	14.00 ± 1.0 ^a	08.33 ± 0.33 ^a
	D3		57.33 ± 1.20 ^a	45.67 ± 2.03 ^a	47.33 ± 2.33 ^a	8.00 ± 1.15 ^a	13.33 ± 4.33 ^a
<i>P.acidilactici</i> MF480434	D1	M	58.33 ± 2.67 ^a	48.30 ± 15.2 ^a	37.00 ± 2.08 ^a	10.67 ± 1.76 ^a	15.33 ± 3.18 ^a
	D2		50.00 ± 2.89 ^a	46.33 ± 1.20 ^a	36.67 ± 2.67 ^{ab}	5.67 ± 0.67 ^a	12.00 ± 0.5 ^a
	D3		48.00 ± 3.61 ^a	71.00 ± 20.4 ^a	38.67 ± 3.93 ^a	08.33 ± 1.45 ^a	14.07 ± 4.12 ^a
		Control (F)	57.00 ± 2.00 ^a	49.67 ± 2.67 ^a	48.67 ± 3.67 ^a	11.00 ± 2.00 ^a	09.93 ± 0.53 ^a
<i>L.plantarum</i> MF405176	D1	F	63.00 ± 5.51 ^a	46.67 ± 1.67 ^a	47.33 ± 3.18 ^a	17.00 ± 1.00 ^a	15.33 ± 3.76 ^a
	D2		58.00 ± 11.0 ^a	65.00 ± 6.00 ^a	48.33 ± 8.67 ^a	12.00 ± 3.51 ^a	13.00 ± 1.00 ^a
	D3		47.00 ± 4.93 ^a	90.70 ± 24.5 ^a	46.67 ± 2.73 ^a	08.33 ± 2.19 ^a	18.00 ± 5.03 ^a
<i>L.fermentum</i> MF033346	D1	F	64.67 ± 9.13 ^a	47.33 ± 2.85 ^a	44.33 ± 7.31 ^a	13.00 ± 1.53 ^a	13.53 ± 0.53 ^a
	D2		68.00 ± 6.56 ^a	49.00 ± 5.51 ^a	41.00 ± 3.46 ^a	18.33 ± 1.67 ^a	07.33 ± 1.20 ^a
	D3		49.33 ± 0.33 ^a	61.30 ± 17.7 ^a	46.33 ± 2.33 ^a	11.33 ± 0.67 ^a	11.67 ± 3.33 ^a
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	F	68.33 ± 9.61 ^a	39.00 ± 2.00 ^a	45.00 ± 1.53 ^a	13.00 ± 0.58 ^a	10.33 ± 2.60 ^a
	D2		34.70 ± 13.7 ^a	41.00 ± 5.00 ^a	25.70 ± 11.7 ^a	07.30 ± 3.33 ^a	10.00 ± 32.5 ^a
	D3		46.33 ± 5.33 ^a	38.67 ± 4.67 ^a	47.00 ± 5.00 ^a	08.67 ± 0.33 ^a	07.67 ± 0.67 ^a
<i>E.faecium</i> MF480431	D1	F	70.67 ± 2.33 ^a	75.67 ± 8.11 ^a	44.67 ± 2.73 ^a	13.00 ± 1.00 ^a	14.47 ± 1.79 ^a
	D2		51.33 ± 9.84 ^a	50.30 ± 14.9 ^a	33.67 ± 4.41 ^a	14.33 ± 2.19 ^a	10.00 ± 3.00 ^a
	D3		51.67 ± 0.33 ^a	48.67 ± 5.33 ^a	40.33 ± 0.33 ^a	12.00 ± 1.00 ^a	09.00 ± 1.00 ^a
<i>P.acidilactici</i> MF480434	D1	F	53.33 ± 2.67 ^a	74.00 ± 14.4 ^a	40.00 ± 8.02 ^a	05.67 ± 0.67 ^a	17.00 ± 3.51 ^a
	D2		59.33 ± 3.53 ^a	56.33 ± 6.33 ^a	39.67 ± 3.38 ^a	10.33 ± 0.88 ^a	15.67 ± 2.60 ^a
	D3		56.33 ± 6.98 ^a	50.67 ± 2.60 ^a	40.67 ± 3.76 ^a	14.00 ± 2.08 ^a	10.00 ± 0.58 ^a

Data is expressed as mean ± SEM, n=5. Within a column containing three doses of each probiotic candidate compared to control, mean values superscripted with different letters are significantly different (P < 0.05). D1) Dose 1: 10⁸ CFU/ml, ((D2) Dose 2: 10¹⁰ CFU/ml, (D3) Dose 3: 10¹² CFU/ml. Male (M), Female (F). High Density Lipids (HDL), Cholesterol, Triglycerides, Low-Density Lipid (LDL) and Very Low-Density Lipid (VLDL).

Table 3 Liver function of rat blood in acute oral toxicity study

Probiotic Candidate	Dose		Liver function parameters				
			Total bilirubin(mg/dl)	Direct bilirubin(mg/dl)	ALT (U/l)	Alkaline phosphatase (U/L)	Gamma GT(U/l)
		Control (M)	0.13 ± 0.00 ^a	0.01 ± 0.00 ^a	74.00 ± 0.00 ^a	79.00 ± 27.00 ^a	<03 ± 0.00 ^a
<i>L.plantarum</i> MF405176	D1	M	0.08 ± 0.03 ^a	0.01 ± 0.01 ^a	67.67 ± 3.18 ^a	76.01 ± 2.13 ^a	<03 ± 0.00 ^a
	D2		0.13 ± 0.01 ^a	0.03 ± 0.01 ^a	74.00 ± 7.57 ^a	60.67 ± 5.84 ^a	<03 ± 0.00 ^a
	D3		0.10 ± 0.01 ^a	0.03 ± 0.01 ^a	61.67 ± 7.13 ^a	58.00 ± 10.8 ^a	<03 ± 0.00 ^a
<i>L.fermentum</i> MF033346	D1	M	0.09 ± 0.01 ^a	0.04 ± 0.02 ^a	55.33 ± 6.17 ^a	78.33 ± 0.02 ^a	<03 ± 0.00 ^a
	D2		0.10 ± 0.02 ^a	0.01 ± 0.00 ^a	50.7 ± 12.0 ^a	49.00 ± 7.37 ^a	<03 ± 0.00 ^a
	D3		0.09 ± 0.00 ^a	0.02 ± 0.01 ^a	57.67 ± 6.84 ^a	62.33 ± 9.60 ^a	<03 ± 0.00 ^a
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	M	0.14 ± 0.03 ^a	0.03 ± 0.01 ^a	59.00 ± 5.86 ^a	70.0 ± 5.01 ^a	<03 ± 0.00 ^a
	D2		0.10 ± 0.01 ^a	0.02 ± 0.01 ^a	89.67 ± 5.36 ^a	63.33 ± 1.86 ^a	<03 ± 0.00 ^a
	D3		0.13 ± 0.05 ^a	0.01 ± 0.00 ^a	80.00 ± 9.07 ^a	83.00 ± 11.0 ^a	<03 ± 0.00 ^a
<i>E.faecium</i> MF480431	D1	M	0.09 ± 0.01 ^a	0.03 ± 0.0 ^a	61.33 ± 8.25 ^a	73.33 ± 3.01 ^a	<03 ± 0.00 ^a
	D2		0.10 ± 0.01 ^a	0.02 ± 0.01 ^a	71.00 ± 8.50 ^a	64.00 ± 22.0 ^a	<03 ± 0.00 ^a
	D3		0.11 ± 0.02 ^a	0.03 ± 0.01 ^a	93.67 ± 2.85 ^a	75.30 ± 11.1 ^a	<03 ± 0.00 ^a
<i>P.acidilactici</i> MF480434	D1	M	0.09 ± 0.01 ^a	0.02 ± 0.00 ^a	62.67 ± 8.99 ^a	78.67 ± 8.99 ^a	<03 ± 0.00 ^a
	D2		0.12 ± 0.00 ^a	0.02 ± 0.00 ^a	64.33 ± 4.91 ^a	67.67 ± 4.18 ^a	<03 ± 0.00 ^a
	D3		0.09 ± 0.01 ^a	0.02 ± 0.01 ^a	75.67 ± 9.82 ^a	72.00 ± 4.73 ^a	<03 ± 0.00 ^a
		Control (F)	0.12 ± 0.01 ^a	0.01 ± 0.00 ^a	71.33 ± 2.67 ^a	55.00 ± 3.00 ^a	<03 ± 0.00 ^a
<i>L.plantarum</i> MF405176	D1	F	0.11 ± 0.01 ^a	0.04 ± 0.00 ^a	59.33 ± 7.67 ^a	56.67 ± 1.67 ^a	<03 ± 0.00 ^a
	D2		0.15 ± 0.02 ^a	0.02 ± 0.00 ^a	79.00 ± 1.00 ^a	61.67 ± 8.65 ^a	<03 ± 0.00 ^a
	D3		0.19 ± 0.30 ^a	0.02 ± 0.01 ^a	60.33 ± 7.06 ^a	53.33 ± 4.48 ^a	<03 ± 0.00 ^a
<i>L.fermentum</i> MF033346	D1	F	0.08 ± 0.00 ^a	0.03 ± 0.00 ^a	61.33 ± 9.96 ^a	78.30 ± 16.0 ^a	<03 ± 0.00 ^a
	D2		0.08 ± 0.01 ^a	0.01 ± 0.00 ^a	62.33 ± 4.98 ^a	50.33 ± 7.26 ^a	<03 ± 0.00 ^a
	D3		0.10 ± 0.01 ^a	0.04 ± 0.01 ^a	58.67 ± 3.33 ^b	79.00 ± 0.00 ^a	<03 ± 0.00 ^a
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	F	0.08 ± 0.01 ^a	0.03 ± 0.01 ^a	42.33 ± 2.91 ^a	78.30 ± 19.9 ^b	<03 ± 0.00 ^a
	D2		0.08 ± 0.01 ^a	0.02 ± 0.00 ^a	69.00 ± 8.00 ^a	90.30 ± 36.7 ^a	<03 ± 0.00 ^a
	D3		0.09 ± 0.01 ^a	0.02 ± 0.01 ^a	54.67 ± 9.67 ^a	59.00 ± 0.00 ^a	<03 ± 0.00 ^a
<i>E.faecium</i> MF480431	D1	F	0.10 ± 0.00 ^a	0.03 ± 0.00 ^a	59.67 ± 2.67 ^a	65.33 ± 4.67 ^a	<03 ± 0.00 ^a
	D2		0.10 ± 0.01 ^a	0.03 ± 0.00 ^a	86.33 ± 3.76 ^a	79.67 ± 6.39 ^a	<03 ± 0.00 ^a
	D3		0.09 ± 0.01 ^a	0.03 ± 0.01 ^a	53.67 ± 4.67 ^a	53.67 ± 1.33 ^a	<03 ± 0.00 ^a
<i>P.acidilactici</i> MF480434	D1	F	0.10 ± 0.02 ^a	0.02 ± 0.00 ^a	55.67 ± 4.67 ^a	61.67 ± 2.60 ^a	<03 ± 0.00 ^a
	D2		0.12 ± 0.01 ^a	0.04 ± 0.01 ^a	94.50 ± 14.5 ^a	65.00 ± 2.08 ^a	<03 ± 0.00 ^a
	D3		0.09 ± 0.01 ^a	0.02 ± 0.01 ^a	69.33 ± 6.84 ^a	49.67 ± 7.33 ^a	<03 ± 0.00 ^a

Data is expressed as mean ± SEM, n=5 Within a column containing three doses of each probiotic candidate compared to control, mean values superscripted with different letters are significantly different (P < 0.05). D1) Dose 1: 10⁸ CFU/ml, ((D2) Dose 2: 10¹⁰ CFU/ml, (D3) Dose 3: 10¹² CFU/ml. Male (M), Female (F). ALT (alanine transaminase).

Table 4 Body weight gain of rats during the sub-chronic oral toxicity study

Probiotic candidate	Control/Dose	Weight gains of rats	
		Male	Female
	Control	61.00 ± 10.4	52.00 ± 4.08
<i>L.plantarum</i> MF405176	D1	61.00 ± 9.3	50.10 ± 9.0
	D2	60.20 ± 11.0	54.92 ± 9.1
	D3	62.10 ± 10.0	53.30 ± 11.6
<i>L.fermentum</i> MF033346	D1	61.20 ± 10.5	50.10 ± 8.5
	D2	59.90 ± 12.0	53.45 ± 9.1
	D3	64.50 ± 11.5	54.80 ± 10.3
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	61.10 ± 12.0	51.15 ± 11.55
	D2	61.30 ± 9.5	59.08 ± 10.50
	D3	60.00 ± 8.4	56.25 ± 8.56
<i>E.faecium</i> MF480431	D1		
	D2	62.20 ± 9.0	51.06 ± 9.0
	D3	66.52 ± 11.0 64.15 ± 9.8	51.18 ± 8.1 54.31 ± 11.0
<i>P.acidilactici</i> MF480434	D1		
	D2	64.40 ± 11.2	55.55 ± 8.0
	D3	60.15 ± 12.2 68.10 ± 11.0	48.10 ± 9.7 51.75 ± 5.5

Data is expressed as mean ± SEM, n=10. D1) Dose 1: 10⁸ CFU/ml, ((D2) Dose 2: 10¹⁰ CFU/ml, (D3) Dose 3: 10¹² CFU/ml.

Table 5 Hematology of rat blood in sub-chronic oral toxicity study

Probiotic Candidate	Dose	Hematological Parameters						
		Hb (g/dl)	RBC (Million/ μ l)	HCT/PCV (%)	MCV (fl)	MCH (pg)	WBC ($\times 10^9$ /l)	Platelet ($\times 10^9$ /l)
	Control (M)	13.50 \pm 0.53 ^b	7.54 \pm 0.48 ^b	44.73 \pm 2.23 ^b	59.50 \pm 1.05 ^b	18.00 \pm 0.55 ^b	6.80 \pm 1.07 ^b	1103.5 \pm 95.7 ^b
<i>L.plantarum</i> MF405176	D1	12.98 \pm 0.32 ^b	8.10 \pm 0.24 ^a	43.25 \pm 1.04 ^b	55.80 \pm 0.31 ^{bc}	17.50 \pm 0.17 ^b	6.18 \pm 0.44 ^b	1052.5 \pm 67.0 ^b
	D2	13.75 \pm 0.35 ^b	7.96 \pm 0.37 ^{ab}	46.88 \pm 1.47 ^{ab}	69.25 \pm 6.08 ^a	19.56 \pm 0.40 ^a	6.85 \pm 0.68 ^b	1048.0 \pm 5.97 ^b
	D3	14.23 \pm 0.23 ^a	7.73 \pm 0.33 ^b	43.13 \pm 1.36 ^b	53.43 \pm 0.51 ^c	16.03 \pm 0.09 ^c	7.54 \pm 1.18 ^b	984.10 \pm 50.4 ^b
<i>L.fermentum</i> MF033346	D1	13.18 \pm 0.55 ^b	7.88 \pm 0.17 ^b	42.75 \pm 1.63 ^b	54.20 \pm 1.10 ^c	16.73 \pm 0.41 ^c	7.45 \pm 2.37 ^b	1104.8 \pm 51.1 ^b
	D2	13.53 \pm 0.37 ^b	6.69 \pm 0.54 ^b	44.48 \pm 1.98 ^b	56.13 \pm 0.77 ^b	17.03 \pm 0.28 ^b	6.73 \pm 1.60 ^b	980.30 \pm 40.30 ^b
	D3	14.20 \pm 2.17 ^a	6.65 \pm 0.75 ^b	40.28 \pm 4.61 ^b	64.40 \pm 0.58 ^a	20.80 \pm 2.16 ^a	6.70 \pm 1.81 ^b	892.90 \pm 54.8 ^{bc}
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	13.78 \pm 0.37 ^b	7.94 \pm 0.27 ^{ab}	44.30 \pm 1.00 ^b	56.68 \pm 0.58 ^b	17.75 \pm 0.12 ^b	6.50 \pm 1.22 ^b	918.5 \pm 30.6 ^{bc}
	D2	13.65 \pm 0.19 ^b	7.87 \pm 0.08 ^b	44.45 \pm 0.63 ^b	56.50 \pm 0.58 ^b	17.35 \pm 0.27 ^b	7.55 \pm 0.63 ^b	1086 \pm 126 ^b
	D3	13.40 \pm 0.51 ^b	7.45 \pm 0.24 ^b	44.57 \pm 1.38 ^b	59.48 \pm 0.95 ^b	17.44 \pm 0.36 ^b	7.47 \pm 1.10 ^b	976.9 \pm 27.0 ^{bc}
<i>E.faecium</i> MF480431	D1	16.38 \pm 0.28 ^a	9.91 \pm 0.12 ^a	53.60 \pm 0.51 ^a	54.10 \pm 0.82 ^c	16.96 \pm 0.10 ^c	7.45 \pm 1.14 ^b	1137.5 \pm 59.5 ^{ab}
	D2	15.38 \pm 0.38 ^a	7.32 \pm 1.55 ^b	44.95 \pm 8.22 ^b	64.03 \pm 4.07 ^a	19.73 \pm 8.97 ^a	7.90 \pm 0.87 ^b	1200.5 \pm 27.6 ^{ab}
	D3	14.35 \pm 0.12 ^a	8.40 \pm 0.10 ^a	50.02 \pm 0.80 ^a	58.82 \pm 0.71 ^b	16.53 \pm 0.38 ^b	9.80 \pm 2.18 ^a	1028.8 \pm 50.2 ^b
<i>P.acidilactici</i> MF480434	D1	13.60 \pm 0.25 ^b	8.03 \pm 0.31 ^a	44.10 \pm 1.15 ^b	55.03 \pm 1.01 ^{bc}	17.00 \pm 0.35 ^b	7.25 \pm 2.85 ^b	1016.0 \pm 64.9 ^b
	D2	14.55 \pm 0.35 ^a	8.31 \pm 0.27 ^a	49.85 \pm 1.07 ^{ab}	64.03 \pm 0.74 ^a	19.53 \pm 0.20 ^a	6.33 \pm 1.19 ^b	1079.8 \pm 34.40 ^b
	D3	13.65 \pm 0.14 ^b	6.81 \pm 0.57 ^b	44.37 \pm 2.56 ^b	59.64 \pm 1.32 ^b	17.24 \pm 0.75 ^b	6.46 \pm 1.11 ^b	1276.2 \pm 42.6 ^a
	Control (F)	13.10 \pm 0.53 ^b	7.10 \pm 0.13 ^b	42.13 \pm 1.25 ^b	54.56 \pm 1.43 ^b	18.00 \pm 0.19 ^b	6.06 \pm 1.73 ^b	993.6 \pm 49.9 ^b
<i>L.plantarum</i> MF405176	D1	12.00 \pm 0.62 ^b	6.74 \pm 0.41 ^b	38.93 \pm 2.31 ^b	57.77 \pm 0.45 ^{ab}	19.38 \pm 0.19 ^a	6.85 \pm 0.43 ^b	1030.0 \pm 60.6 ^b
	D2	13.23 \pm 0.36 ^a	6.82 \pm 0.13 ^b	40.38 \pm 1.45 ^b	59.13 \pm 1.18 ^a	20.53 \pm 0.33 ^a	6.03 \pm 0.23 ^b	1118.0 \pm 27.1 ^{ab}
	D3	12.78 \pm 0.31 ^b	6.90 \pm 0.10 ^b	39.95 \pm 1.10 ^c	44.50 \pm 12.5 ^c	16.82 \pm 0.38 ^c	7.80 \pm 1.42 ^b	1003.5 \pm 87.5 ^b
<i>L.fermentum</i> MF033346	D1	12.15 \pm 0.22 ^b	6.36 \pm 0.17 ^b	38.50 \pm 0.70 ^c	58.85 \pm 0.93 ^a	19.57 \pm 0.46 ^a	6.15 \pm 0.42 ^b	1013.0 \pm 24.5 ^b
	D2	12.78 \pm 0.18 ^b	7.00 \pm 0.15 ^b	40.15 \pm 1.08 ^b	57.38 \pm 0.52 ^{ab}	18.23 \pm 0.13 ^b	6.83 \pm 0.68 ^b	1070.3 \pm 25.20 ^b
	D3	13.20 \pm 0.17 ^a	7.09 \pm 0.14 ^b	43.25 \pm 0.62 ^b	61.00 \pm 0.89 ^a	19.65 \pm 0.43 ^a	6.68 \pm 0.51 ^b	895.3 \pm 61.0 ^b
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	12.37 \pm 0.36 ^b	7.00 \pm 0.08 ^b	40.58 \pm 1.53 ^b	57.88 \pm 1.51 ^{ab}	17.65 \pm 0.32 ^b	7.28 \pm 1.10 ^a	931.3 \pm 78.5 ^b
	D2	13.18 \pm 0.37 ^b	6.97 \pm 0.21 ^b	42.53 \pm 1.15 ^b	61.10 \pm 0.94 ^a	19.93 \pm 0.31 ^a	5.30 \pm 0.53 ^b	990.3 \pm 45.50 ^b
	D3	12.45 \pm 0.31 ^b	6.89 \pm 0.17 ^b	42.73 \pm 1.06 ^b	61.30 \pm 0.62 ^a	19.30 \pm 0.11 ^a	5.98 \pm 1.20 ^b	963.3 \pm 49.8 ^b
<i>E. faecium</i> MF480431	D1	13.15 \pm 0.29 ^b	7.41 \pm 0.11 ^{ab}	42.53 \pm 1.00 ^b	57.35 \pm 0.56 ^{ab}	17.75 \pm 0.22 ^b	6.45 \pm 0.66 ^b	1059.5 \pm 28.7 ^b
	D2	12.57 \pm 0.19 ^b	7.01 \pm 0.18 ^b	41.75 \pm 0.52 ^b	59.65 \pm 0.80 ^a	19.98 \pm 0.47 ^a	7.38 \pm 1.34 ^a	1131 \pm 143 ^{ab}
	D3	14.35 \pm 0.20 ^a	8.33 \pm 0.19 ^a	50.68 \pm 0.42 ^a	57.68 \pm 0.79 ^{ab}	18.73 \pm 0.24 ^b	6.03 \pm 0.03 ^b	721 \pm 211 ^c
<i>P.acidilactici</i> MF480434	D1	12.47 \pm 0.28 ^b	7.12 \pm 0.16 ^b	40.02 \pm 0.66 ^b	56.20 \pm 0.48 ^b	17.52 \pm 0.22 ^b	7.01 \pm 0.97 ^b	1012.5 \pm 91.5 ^b
	D2	12.83 \pm 0.49 ^b	6.99 \pm 0.21 ^b	44.00 \pm 1.57 ^{ab}	61.03 \pm 1.58 ^a	19.38 \pm 0.54 ^a	6.55 \pm 0.82 ^b	978.8 \pm 45.20 ^b
	D3	11.20 \pm 0.12 ^{bc}	5.36 \pm 0.21 ^c	46.20 \pm 0.12 ^a	62.20 \pm 0.90 ^a	19.50 \pm 0.96 ^a	6.03 \pm 0.03 ^b	1012.3 \pm 82.4 ^b

Data is expressed as mean \pm SEM, n=10. Within a column containing three doses of each probiotic candidate compared to control, mean values superscripted with different letters are significantly different (P < 0.05). D1) Dose 1: 10^8 CFU/ml, (D2) Dose 2: 10^{10} CFU/ml, (D3) Dose 3: 10^{12} CFU/ml. Male (M), Female (F). Hemoglobin (Hb), Erythrocyte count (RBC), Hematocrit/Packed Cell Volume (HCT/PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Total leucocyte count (WBC).

Table 6 Lipid profile of rat blood in sub-chronic oral toxicity study

Probiotic candidates	Dose	Control /Sex	Lipid profile parameters				
			Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
		Control (M)	70.50 ± 7.19 ^a	85.25 ± 9.07 ^a	53.00 ± 5.40 ^a	16.50 ± 3.84 ^a	17.00 ± 1.87 ^a
<i>L.plantarum</i> MF405176	D1	M	57.50 ± 5.39 ^b	78.5 ± 12.2 ^a	39.50 ± 2.53 ^a	19.25 ± 3.09 ^a	15.25 ± 2.43 ^a
	D2		68.00 ± 4.67 ^a	74.0 ± 13.5 ^a	32.75 ± 2.39 ^{ab}	24.00 ± 1.35 ^b	14.50 ± 2.60 ^a
	D3		67.88 ± 4.83 ^a	62.1 ± 21.7 ^{ab}	26.71 ± 3.15 ^b	20.86 ± 3.62 ^{ab}	24.14 ± 4.36 ^{ab}
<i>L.fermentum</i> MF033346	D1	M	66.25 ± 8.00 ^{ab}	89.5 ± 10.7 ^a	54.00 ± 2.68 ^a	26.50 ± 2.53 ^b	17.75 ± 2.17 ^a
	D2		67.75 ± 0.75 ^a	93.0 ± 14.1 ^a	34.75 ± 1.31 ^{ab}	22.50 ± 1.50 ^b	15.50 ± 2.87 ^b
	D3		63.88 ± 3.36 ^a	84.3 ± 17.5 ^{ab}	25.00 ± 2.28 ^b	21.25 ± 4.22 ^{ab}	17.33 ± 3.46 ^a
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	M	62.00 ± 4.81 ^a	90.5 ± 18.6 ^a	46.75 ± 2.29 ^a	17.25 ± 1.70 ^a	18.00 ± 3.76 ^a
	D2		73.75 ± 5.96 ^a	88.3 ± 18.9 ^a	41.75 ± 5.50 ^{ab}	21.25 ± 3.71 ^{ab}	17.75 ± 3.71 ^a
	D3		79.50 ± 7.97 ^a	67.0 ± 15.3 ^{ab}	22.25 ± 2.01 ^b	23.13 ± 4.97 ^b	21.00 ± 3.02 ^{ab}
<i>E.faecium</i> MF480431	D1	M	62.00 ± 6.77 ^a	62.50 ± 5.17 ^{ab}	46.50 ± 1.66 ^a	35.75 ± 3.71 ^c	16.00 ± 0.91 ^a
	D2		95.50 ± 3.38 ^a	69.25 ± 1.4 ^{ab}	26.75 ± 1.11 ^b	22.75 ± 2.36 ^b	15.50 ± 0.29 ^a
	D3		82.00 ± 2.61 ^a	99.00 ± 5.50 ^a	29.33 ± 2.06 ^b	29.17 ± 0.75 ^b	18.00 ± 1.83 ^a
<i>P.acidilactici</i> MF480434	D1	M	63.50 ± 1.94 ^a	83.50 ± 8.65 ^a	37.25 ± 3.52 ^{ab}	16.25 ± 1.80 ^a	16.55 ± 1.66 ^a
	D2		67.50 ± 4.01 ^a	84.75 ± 6.12 ^a	24.00 ± 0.71 ^b	15.00 ± 3.32 ^a	16.50 ± 1.19 ^a
	D3		70.13 ± 1.55 ^a	83.00 ± 9.81 ^a	25.50 ± 2.60 ^b	22.00 ± 1.15 ^b	26.00 ± 1.73 ^{ab}
		Control (F)	70.50 ± 7.19 ^a	55.25 ± 9.07 ^a	41.75 ± 4.15 ^a	16.80 ± 3.84 ^a	17.00 ± 1.87 ^a
<i>L.plantarum</i> MF405176	D1	F	70.00 ± 4.92 ^a	92.00 ± 22.2 ^c	41.50 ± 2.18 ^a	17.00 ± 1.47 ^a	38.00 ± 4.34 ^c
	D2		67.50 ± 7.40 ^a	70.00 ± 21.7 ^b	40.50 ± 3.38 ^a	12.00 ± 1.41 ^b	13.75 ± 4.42 ^a
	D3		56.25 ± 3.35 ^b	97.30 ± 24.6 ^c	53.25 ± 6.16 ^b	11.50 ± 1.94 ^c	29.75 ± 4.84 ^b
<i>L.fermentum</i> MF033346	D1	F	64.00 ± 2.58 ^a	75.80 ± 15.5 ^b	42.00 ± 0.71 ^a	10.50 ± 0.50 ^b	15.00 ± 3.03 ^a
	D2		71.3 ± 14.9 ^a	88.80 ± 25.2 ^c	48.00 ± 6.75 ^a	10.75 ± 2.63 ^b	17.50 ± 4.87 ^a
	D3		63.75 ± 4.40 ^a	89.50 ± 8.18 ^c	40.00 ± 3.46 ^a	16.00 ± 1.78 ^a	27.00 ± 1.73 ^b
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	F	87.25 ± 4.96 ^c	97.50 ± 62.8 ^c	40.30 ± 10.6 ^a	19.25 ± 4.05 ^a	59.30 ± 12.8 ^c
	D2		183.5 ± 31.3 ^c	79.80 ± 42.5 ^b	52.50 ± 17.6 ^{ab}	14.50 ± 3.97 ^a	39.80 ± 17.2 ^{ab}
	D3		70.50 ± 5.33 ^a	75.30 ± 23.1 ^b	19.75 ± 1.65 ^c	89.00 ± 7.04 ^d	26.50 ± 4.63 ^b
<i>E.faecium</i> MF480431	D1	F	67.00 ± 6.36 ^a	64.30 ± 22.7 ^{ab}	35.25 ± 2.53 ^{ac}	12.75 ± 1.18 ^b	22.50 ± 4.59 ^{ab}
	D2		45.50 ± 10.9 ^b	72.25 ± 2.50 ^b	20.25 ± 3.75 ^c	44.75 ± 1.31 ^c	16.00 ± 0.58 ^a
	D3		82.50 ± 3.18 ^c	73.25 ± 8.06 ^b	31.00 ± 1.73 ^{ac}	11.75 ± 2.63 ^b	23.00 ± 3.08 ^{ab}
<i>P.acidilactici</i> MF480434	D1	F	65.80 ± 15.9 ^a	53.00 ± 48.8 ^a	24.25 ± 3.47 ^c	11.50 ± 3.23 ^b	36.25 ± 9.66 ^b
	D2		58.80 ± 13.9 ^{ab}	66.00 ± 188 ^{ab}	37.25 ± 7.09 ^a	17.25 ± 5.11 ^a	15.50 ± 1.55 ^a
	D3		68.25 ± 1.31 ^a	68.25 ± 8.14 ^b	32.00 ± 1.35 ^{ac}	15.00 ± 1.47 ^a	23.25 ± 3.09 ^{ab}

Data is expressed as mean ± SEM, n=10. Within a column containing three doses of each probiotic candidate compared to control, mean values superscripted with different letters are significantly different (P < 0.05). D1) Dose 1: 10⁸ CFU/ml, (D2) Dose 2: 10¹⁰ CFU/ml, (D3) Dose 3: 10¹² CFU/ml. Male (M), Female (F). High Density Lipids (HDL), Cholesterol, Triglycerides, Low-Density Lipid (LDL) and Very Low-Density Lipid (VLDL).

Table 7 Liver function of rat blood in sub-chronic oral toxicity study

Probiotic Candidate	Dose		Liver function parameters				
			Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)	ALT (U/l)	Alkaline phosphatase (U/L)	Gamma GT(U/l)
		Control (M)	0.10 ± 0.01 ^a	0.05 ± 0.01 ^a	77.00 ± 1.78 ^a	83.00 ± 27.9 ^a	<03 ± 0.00 ^a
<i>L.plantarum</i> MF405176	D1	M	0.12 ± 0.01 ^a	0.05 ± 0.01 ^a	72.75 ± 4.66 ^a	83.50 ± 31.3 ^a	<03 ± 0.00 ^a
	D2		0.12 ± 0.00 ^a	0.04 ± 0.01 ^a	68.00 ± 4.95 ^{ab}	93.80 ± 18.1 ^a	<03 ± 0.00 ^a
	D3		0.13 ± 0.02 ^a	0.05 ± 0.01 ^a	70.43 ± 3.68 ^a	117 ± 19.0 ^{ab}	<03 ± 0.00 ^a
<i>L.fermentum</i> MF033346	D1	M	0.12 ± 0.20 ^a	0.04 ± 0.02 ^a	66.00 ± 8.50 ^b	89.30 ± 15.9 ^a	<03 ± 0.00 ^a
	D2		0.11 ± 0.02 ^a	0.02 ± 0.00 ^b	85.50 ± 4.50 ^{ac}	87.00 ± 4.12 ^a	<03 ± 0.00 ^a
	D3		0.13 ± 0.02 ^a	0.02 ± 0.00 ^b	74.67 ± 2.79 ^a	92.00 ± 25.7 ^a	<03 ± 0.00 ^a
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	M	0.12 ± 0.01 ^a	0.05 ± 0.01 ^a	54.25 ± 1.75 ^b	84.25 ± 7.12 ^a	<03 ± 0.00 ^a
	D2		0.11 ± 0.01 ^a	0.03 ± 0.00 ^b	70.00 ± 11.0 ^a	121.3 ± 10.8 ^b	<03 ± 0.00 ^a
	D3		0.11 ± 0.01 ^a	0.03 ± 0.01 ^b	81.38 ± 5.35 ^{ac}	128.1 ± 21.0 ^b	<03 ± 0.00 ^a
<i>E.faecium</i> MF480431	D1	M	0.11 ± 0.02 ^a	0.05 ± 0.01 ^a	31.25 ± 1.75 ^{bc}	200.0 ± 24.3 ^c	<03 ± 0.00 ^a
	D2		0.11 ± 0.01 ^a	0.05 ± 0.00 ^a	44.00 ± 2.83 ^{bc}	139.7 ± 8.52 ^b	<03 ± 0.00 ^a
	D3		0.08 ± 0.02 ^b	0.05 ± 0.00 ^a	74.33 ± 8.27 ^{bc}	159.2 ± 27.1 ^{bc}	<03 ± 0.00 ^a
<i>P.acidilactici</i> MF480434	D1	M	0.12 ± 0.01 ^a	0.03 ± 0.02 ^b	59.25 ± 9.41 ^b	160.8 ± 23.0 ^{bc}	<03 ± 0.00 ^a
	D2		0.10 ± 0.01 ^b	0.05 ± 0.00 ^a	82.00 ± 5.83 ^a	172.8 ± 27.0 ^{bc}	<03 ± 0.00 ^a
	D3		0.07 ± 0.02 ^b	0.05 ± 0.00 ^a	84.63 ± 1.62 ^{ac}	130.0 ± 15.6 ^b	<03 ± 0.00 ^a
		Control (F)	0.10 ± 0.01 ^a	0.05 ± 0.01 ^a	57.00 ± 1.78 ^a	78.0 ± 21.4 ^a	<03 ± 0.00 ^a
<i>L.plantarum</i> MF405176	D1	F	0.12 ± 0.03 ^a	0.05 ± 0.00 ^a	65.25 ± 7.38 ^a	74.80 ± 25.6 ^a	<03 ± 0.00 ^a
	D2		0.09 ± 0.00 ^b	0.04 ± 0.02 ^a	45.25 ± 4.31 ^b	62.00 ± 10.4 ^b	<03 ± 0.00 ^a
	D3		0.12 ± 0.04 ^a	0.04 ± 0.01 ^a	66.50 ± 4.56 ^a	74.80 ± 11.7 ^a	<03 ± 0.00 ^a
<i>L.fermentum</i> MF033346	D1	F	0.11 ± 0.12 ^a	0.05 ± 0.01 ^a	77.50 ± 3.20 ^c	71.50 ± 41.3 ^a	<03 ± 0.00 ^a
	D2		0.09 ± 0.01 ^b	0.03 ± 0.00 ^a	40.75 ± 9.81 ^b	54.75 ± 2.29 ^b	<03 ± 0.00 ^a
	D3		0.06 ± 0.03 ^b	0.04 ± 0.00 ^a	67.50 ± 1.19 ^a	73.50 ± 2.06 ^a	<03 ± 0.00 ^a
<i>L.lactis</i> subspecies <i>lactis</i> MF480428	D1	F	0.12 ± 0.01 ^a	0.04 ± 0.00 ^a	78.00 ± 17.9 ^c	71.50 ± 60.3 ^a	<03 ± 0.00 ^a
	D2		0.13 ± 0.02 ^a	0.10 ± 0.01 ^b	85.00 ± 28.8 ^c	75.50 ± 27.1 ^a	<03 ± 0.00 ^a
	D3		0.11 ± 0.02 ^a	0.04 ± 0.01 ^a	87.50 ± 10.2 ^c	75.50 ± 4.52 ^a	<03 ± 0.00 ^a
<i>E.faecium</i> MF480431	D1	F	0.13 ± 0.01 ^a	0.05 ± 0.00 ^a	58.50 ± 2.72 ^a	78.00 ± 15.1 ^a	<03 ± 0.00 ^a
	D2		0.13 ± 0.01 ^a	0.01 ± 0.00 ^c	64.00 ± 10.7 ^{ac}	78.50 ± 65.2 ^a	<03 ± 0.00 ^a
	D3		0.07 ± 0.03 ^b	0.01 ± 0.00 ^c	54.00 ± 2.04 ^a	74.25 ± 5.75 ^a	<03 ± 0.00 ^a
<i>P.acidilactici</i> MF480434	D1	F	0.11 ± 0.00 ^a	0.04 ± 0.01 ^a	41.75 ± 5.45 ^b	64.80 ± 10.8 ^{ab}	<03 ± 0.00 ^a
	D2		0.10 ± 0.01 ^a	0.02 ± 0.01 ^{ac}	72.50 ± 5.04 ^{bc}	71.50 ± 15.4 ^a	<03 ± 0.00 ^a
	D3		0.06 ± 0.03 ^b	0.01 ± 0.00 ^c	84.50 ± 2.60 ^c	56.50 ± 0.87 ^b	<03 ± 0.00 ^a

Data is expressed as mean ± SEM, n=10. Within a column containing three doses of each probiotic candidate compared to control, mean values superscripted with different letters are significantly different (P < 0.05). D1) Dose 1: 10⁸ CFU/ml, (D2) Dose 2: 10¹⁰ CFU/ml, (D3) Dose 3: 10¹² CFU/ml. Male (M), Female (F). ALT (alanine transaminase).

4. Discussion

Consumer demand for new probiotics with potential applications in alternative therapy, especially in treating several non-communicable diseases is increasing (Shokryazdan et al., 2017). With the increasing health awareness among consumers, attraction towards healthy probiotic functional food is growing. Factors such as milk protein allergy, lactose intolerance, high fat content and drift towards vegetarianism are the major limitations associated with dairy based probiotics. Hence research is being continued in developing alternate solutions to

dairy based probiotic products and preference for non-dairy based probiotic products especially using cereals as major substrate is a choice attraction (Divisekera et al., 2019b). Absence of difference in terms of health benefits irrespective of the source of probiotic isolation (dairy, non-dairy) revealed in literature further supports this emerging trend (Kumar et al., 2015). Assessment of safety attributes is necessary before considering efficacy proven new probiotic strains in food and pharmaceutical applications.

Five probiotic candidates under study, *Lactobacillus plantarum* MF405176, *Lactobacillus fermentum* MF033346, *Lactococcus*

lactis subspecies *lactis* MF480428, *Enterococcus faecium* MF480431 and *Pediococcus acidilactici* MF480434 were investigated for acute and sub-chronic oral toxicity (*in-vitro*) at different doses of 1×10^8 (lower dose), 1×10^{10} , 1×10^{12} (high dose) CFU/ g), using Wistar rat models consisting of both male and female rats. The cell concentrations of these probiotic strains under study were in the range of 10^8 - 10^{12} CFU/g, while it is suggested that positive effects on the host could be achieved by maintaining a concentration of cells at least between 10^6 - 10^8 CFU/g (Zielinska et al., 2018).

Due to physiological similarities, rat is considered as standardized physiological and toxicological model (Iannaccone and Jacob., 2009). Single dose acute toxicity study provided initial toxicological information that further enabled to determine appropriate doses for future repeated-dose toxicity studies. Further, sub-chronic 90-day repeated-dose toxicity study provided information on the potential health hazards likely to arise due to repeated exposure to the selected probiotic candidates, over substantial period of time. In the acute oral toxicity study, no treatment associated mortality was observed in any of the tested doses of probiotic candidates. Further, no weight losses, no changes including animal behavior, hematological and biochemical parameters of blood, histopathological changes in organs were observed. Absence of bacteremia and bacterial translocation further confirms the preliminary data indicating oral safety of probiotic candidates, at tested concentrations.

Based on the results of the repeated-dose sub-chronic oral toxicity study, it is concluded that none of the tested probiotic candidates caused any sign of toxicity even at the highest tested dose of 10^{12} at a concentration of 1000 mg/kgbw/day, for 90 days indicating the safety of probiotics. Significant increment ($P < 0.05$) in mean body weights and uninterrupted feed and water intake indicate that, animal digestion and absorption was not altered due to introduced probiotics. The hematopoietic system is one of the most sensitive targets for toxic substances and is a good measure of the pathological and physiological state in animals and humans. In the current study, results on the hematological parameters did not demonstrate contrary pattern indicating free from adverse effects on the circulating blood cells and their production. Liver is the main organ in the detoxification and metabolism of chemicals. Therefore, changes in quantity of liver enzymes and bilirubin can be used as good indicators for liver function (Steffen et al., 2011).

No adverse effects on liver function of rats observed during the study indicating that none of the tested probiotic strains inducing damage to hepatic cells. Absence of histopathological changes in organs indicated that oral feeding of probiotic candidates had not caused any adverse effects on animal organs. Bacteremia and bacterial translocation were not observed as an outcome of the oral administration of probiotics under study. Bacterial translocation being the first step in the pathogenesis process for numerous opportunistic strains (Steffen and Berg., 1983) hence suggested as an indicator (Lara-Villoslada et al., 2007) while declaring the safety of probiotics.

5. Conclusion

In conclusion, all five probiotic strains under study possessed potential safety for food and pharmaceutical applications. Therefore, this study provides preliminary data related to safety of tested strains. However, further pre-clinical and clinical trials using appropriate food formulations are required to further validate the safety of these strains before they can be used as probiotics for human consumption.

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Declaration of interest

The authors have no conflicts of interest.

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