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Effects of Some Probiotic Lactic Acid Bacteria on Diarrhea, Hematological Parameters and Blood Serum on Mice

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The potential use of Probiotics in restoring the urogenital and gastrointestinal health has received tremendous interest in the last decade, while few safety concerns are still being debated. The effect of 4 probiotic lactic acid bacteria on the ability of pathogenic Escherichia coli to infect mice was determined. Administration of E. coli produced adverse clinical signs, such as diarrhea, weakness and death compared to the control and other treated groups. The administration of probiotic strains along with the pathogen enhanced the immune system functions and increased the body weight gain rates in treated groups of E. coli and Lb. acidophilus P106 or E. coli and all lactic acid bacteria.

Keywords: *Probiotics, feed consumption, E. coli, Enterococcus durans, Lb. plantarum, Enterococcus faecium, Lb. acidophilus.*

INTRODUCTION

For centuries lactic acid bacteria (LAB) have been used in the preservation of food and in other areas of food industry. Most LAB are recognized as being safe for human consumption due to their ubiquitous distribution on the surface of the human body, and in the gut, and because of their long history of safe use in food products [1,2]. There is now a renewed interest in using LAB as probiotic food additives to enhance immune function and prevent gastrointestinal infections. Probiotics are food supplements containing live microorganisms which beneficially affect the host by improving its intestinal microbial balance. [3-5].

Probiotic bacteria may assist in maintaining a health promoting balance of intestinal tract [6]. The ingestion of lactic acid bacteria (LAB) as a probiotics has drawn interest throughout and led to an increase in the consumption of fermented milk products. *Lactobacillus* species are frequently associated with health-promoting effects in the human and animal intestinal tract. Their probiotic effects may include an ability to inhibit pathogenic bacteria, reduce colon cancer, increase the immune response and decrease serum cholesterol [7, 8]. Probiotics, including lactobacilli species, have the potential for use in clinical practice as an alternative to use antibiotics especially for infants, pregnant women and old ageing [9-11].

Bacterial diarrheal diseases remain one of the major causes of morbidity and mortality in developing countries. These diseases are established when pathogenic bacteria produce virulence factors in the enteric environment, causing the loss of the normal activity of intestinal tissues [12]. The virulence factors facilitate colonization and invasion of host cells, avoidance or disruption of host defense mechanisms, injury to host tissue, and stimulate a host inflammatory response [13]. One of the most important enteric bacterial pathogens is diarrheagenic *E. coli* and most strains of *E. coli* are commensal colonizers of mammals [14]. However, some strains are capable of causing intestinal or extra intestinal infection. Strains responsible for intestinal diseases cause much of their pathology in the small intestine [15].

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This pathology is due, in part, to the ability of the strains to adhere to gut epithelial cells. LAB can inhibit the *in vitro* growth of many enteric bacteria, including *Salmonella typhimurium*, *Staphylococcus aureus*, *E. coli*, *Clostridium perfringens*, and *Clostridium difficile*, and have been used in both humans and animals to treat a broad range of gastrointestinal disorders. Sinha [16] reported that the major metabolites of LAB, short chain fatty acids and lactic acid are responsible for their antimicrobial activity against *E. coli* in the intestine.

In order to demonstrate further the safety of the ingestion of probiotics and in considering the concerns rose, this paper reports the effects of feeding different strains of probiotics on general health indicators, hematological parameters and blood serum in mice.

2. MATERIALS AND METHODS

2.1. Tested Microbial Strains

Probiotic strains: *Lb. plantarum* P164, *Lb. acidophilus* P106, *E. durans* P174, *E. faecium* P166 were obtained from healthy, breast- feeding infants (15-90 days old) and used after the selection had been done according to Bergey's Manual of Determinative Bacteriology, 9th edition [17] and characterized by colonial, morphology, and biochemical parameters. To confirm the identification method, SDS-PAGE technique and API System were used for all the strains. While *Lactobacillus* strains were cultivated in MRS (de Man Rogosa Sharpe) broth (Lab M, IDG, UK) and incubated at 37 ^{o}C, *Enterococcus* cultivated in M17broth (Difco, Detroit, USA) at the same temperature. Tested probiotic strains were anaerobically propagated for 48 h at 37 ^{o}C, and then concentrated by centrifugation. The cell pellets were resuspended in 10% skim milk at a concentration of 10^{8}-10^{10} CFU mL^{-1}, the number of viable cells (colony forming units per mL, **CFU** mL^{-1}) was determined by the agar plate method [18]. *E. coli* strain was obtained from the culture collection of NIZO Food Research, Ede, Netherlands.

2.2. Animals and Conditions

Seventy male ICR(CD-I) mice, approximately 10 weeks old, were obtained from Faculty of Science, Department of Zoology, Alexandria University, Alexandria, Egypt. All mice were examined for health status and acclimated to laboratory environment for 2 weeks prior to use. Temperature was maintained at 23 ± 2 ^{o}C, and relative humidity at approximately 50%, with a 12 h: 12 h light: dark photoperiod. Animals were housed in stainless-steel cages and given standard diet and water *ad libitum* throughout the study.

2.3. Treatment of Animals

Male mice were randomly assigned to treatment groups according to an approximately equal mean body weight. Seven groups of ten male mice per each were given an individual probiotic strains in combination with *E. coli*. Group 0 received skim milk only, Group 1, received *E. coli* , Group 2, received *E. coli* + *E. durmas* P174, Group 3, received *E.coli* + *Lb. plantarum* P164, Group 4, received *E. coli* + *E. faecium* P166, Group 5, received *E. coli* + *Lb. acidophilus* P106, Group 6, received *E. coli* + all LAB by oral gavages for 4 weeks (5 days week^{-1}, 20 days) at dose level 10^{8}-10^{10} CFU mL^{-1}for each strain in skim milk as the vehicle. The administered volume of each dose was 1.0 mLkg^{-1} day^{-1}, adjusted daily for recorded body weight changes during the treatment period.

2.4. Animal Observations

Health status of treated male mice was monitored daily throughout the experimental period. The number of animals with diarrhea was recorded daily. Male body weight and feed consumption were recorded weekly.

2.5. Male Mice Blood Collection

After dosing (20 days), males were anesthetized with diethyl ether. Male blood was obtained by cardiac puncture via aspiration through polyethylene tubing attached to a heparinized microhematocrit capillary tube which had been flamed and pulled to a fine point.

2.5.1. Hematological Parameters

For measuring hematocrit value, whole blood was centrifuged in 3 or 5 µL heparinized microhematocrit capillary tubes for 90 sec in a hematocrit centrifuge. The lengths of the total sample and red cell fraction were measured on a standard ruler and the hematocrit value was calculated by dividing the length of the red cell

fraction (in millimeters) by the total sample length. For the assay of hemoglobin concentration, A standard diagnostic colorimetric assay based on formation of cyanomethemoglobin via reaction of hemoglobin with potassium cyanide under alkaline conditions [19, 20] was used to determine hemoglobin concentrations in male mice blood. Five µL of whole blood was incubated with 1.25 mL of Drabkin's solution. After at least 15 min at room temperature, absorbance at 540 nm was measured spectrophotometrically. All samples were analyzed within 4 h of blood collection. Sample hemoglobin levels were determined by interpolation from a concurrently run standard curve of human hemoglobin and fixed with 895 µL of 2.5% paraformaldehyde. An aliquot of 50- 200 µL aliquot of the fixed blood sample was added. The counts of red blood cell (RBC) and white blood cell (WBC) were done using the hemocytometer slide [21]. The absolute values, mean cell value (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentrations (MCHC) were calculated according to Dacie and Lewis [22].

2.6. Cholesterol

The cholesterol was determined by the method of Watson [23].Briefly; 2.5 mL of reagent (acetic anhydride 3.5 mol L^{-1} & acetic acid 5 mol L^{-1}) was added to 100 µL of plasma, mixed well and incubated for 5 minutes in water bath at 20 – 25 °C. Onto this mixture, 500 µL of H_2SO_4 was added and mixed immediately thoroughly under constant cooling and let stand for 20 minutes at the same temperature. The absorbance was measured at 500 nm against blank. The concentration of cholesterol was then calculated as mg/dl using the standard concentration.

2.7. Distribution of Mice Plasma Proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the discontinuous buffer system as described by Laemmli [24]. An appropriate volume of mice plasma was diluted (1:20) with sample treatment buffer (0.0625M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.002% bromophenol blue, with 5% β-mercaptoethanol) and submitted to heat treatment for 5 min in a boiling water bath prior to be applied to the gel. Samples allowed to cool to room temperature, centrifuged at 10000x*g* for 5 min to remove any insoluble materials causing streaking during electrophoresis.

2.8. Statistical Analysis

Data are presented as the mean ± standard deviation, and n represents the number of mice from the probiotics fed groups; *E. coli* fed group and the control. Comparisons were made by use of the student's t-test. Differences were regarded significant with *P* value less than $0.05_{(N=10)}$.

3. RESULTS AND DISCUSSION

3.1. Adverse Clinical Signs

There were three deaths in the males treated with *E. coli* during the course of the study compared to the other treated groups. Administration of *E. coli* produced adverse clinical signs, in particularly, diarrhea and weakness compared to the control and other treated groups. These signs appeared on day 5 of treatment and progressed in the same animals throughout the second half of treatment period. These signs were shown in 80% of treated males (5/7) with *E. coli* and disappeared in other treated groups with probiotic strains. These results are in agreement with those reported previously [25] that *Lb. plantarum* and *Lb. salivarius* inhibited *E. coli*, *S. typhimurium* and *C. perferingens* on chicken feed agar. Also, it has been reported previously [7] that probiotics have beneficial effects on the health of the host. Kikuchi and Yajima [26] showed that probiotic microorganisms' strains are useful in the treatment of disturbed intestinal microflora and diarrheal diseases. Moreover, probiotics might prevent infection because they compete with pathogenic viruses or bacteria for binding sites on epithelial cells or by producing bacteriocins.

3.2. Feed Consumption

Male feed consumption is presented in Table 1. It was significantly reduced in the treated group of *E. coli* at the beginning of the treatment compared to the control and other treated groups. On the other hand, the amount of food was increased at the end of the treatment in mice treated with *E. coli* and *Lb. acidophilus* P106 and *E. coli* and all LAB. No significant effects were observed in feed consumption in the remaining treated groups.

3.3. Body and Organ Weights

Mice body and organ weights are presented in Tables 2 and 3. Body weight gain was significantly reduced in the pathogen-treated group at the end of the treatment compared to the control and other treated groups. On the other hand, male body weights increased in treated groups of *E. coli* and *Lb. acidophilus* P106 and *E. coli* and all LAB. No significant effects were observed in male body weights in all other treated groups (Table 2).

Reduction of male body weights and weight gain can be attributed to infection with *E. coli* which was association with a reduction in feed consumption. Addition of the probiotics to the diet was associated with increased feed consumption and weight gain. This indicates a protective effect of these strains [7] against the adverse effect?

3.4. Hematological Analysis

Blood parameters are presented in Table 3. Haematocrite value in male mice treated by *E. coli* was reduced significantly by 51.1% of control group. No significant difference in the value of Hct in the remaining treated groups compared to the control group.

Haemoglobin content was markedly reduced in the same treated group (*E. coli*) compared to the control and the other treated groups. The reduction in Hb was about 42% of the control value. No significant effects in the values of Hb in the remaining treated groups.

Red blood cells count was pronounced exhibited after treatment with *E. coli* compared to the control. No significant effects were observed in the other treated groups compared to the control. On the other hand, WBC count was significantly increased in the male mice treated with the same microorganism strain (*E. coli*). The value of the WBC count in the other treated groups was similar to the value of the control (Table 3). The mean total WBC's of male mice fed with *E. coli* was higher than the control and other treated groups, indicating that there was induction of peripheral inflammatory response which is associated with infection [27]. On the other hand, when male mice treated with *E. coli* with all the tested probiotic strains, the mean WBC's counts did not change significantly, when compared with control, indicating that these strains prevented peripheral lymphocytosis? in the presence of pathogen. Our results also are in agreement with the reported data investigated that oral injection of the probiotic strains in humans did not lead to cytokine changes beyond normal values [28]. This finding provides evidence for the safety of the probiotic cultures.

The calculated parameters of an individual RBC's characterization: The calculated parameters are presented in Table 4.

Mean Cell Volume (MCV): Significant decrease in the value of MCV was observed in male mice treated with *E. coli* compared to the control group. No significant differences were observed regarding the remaining treated groups.

Mean Cell Haemoglobin (MCH): No significant differences were observed in the calculation value of MCH in any of the treated groups compared to the control.

Mean Cell Haemoglobin Concentration (MCHC): MCHC was increased in the animals treated with *E. coli* compared to the control and the other treated groups. In this study we have shown that treatment of mice with enteropathogenic *E. coli* only produced adverse effects on the hematological values (MCV, MCH and MCHC). Ingestion of probiotic strains *E. durans* P174, *Lb. plantarum* P164, *E. faecium* P166 *Lb. acidophilus* P106 and all LAB had no adverse effects on the hematological parameters. These results are consistent with the published results which indicated that LAB strains had no adverse effects on the hematological parameters [27].

On a final note, this study has demonstrated that the protective ability of these strains to exert a non-alteration of the peripheral blood parameters in healthy subjects. This suggests that ingestion of proven probiotic strains have a safe hematological profile.

3.5. Cholesterol

Serum cholesterol increased in the animals treated with only *E.coli* compared to the control and the other treated groups (Table 4). High level of serum cholesterol has been associated with risks of coronary heart disease. The use of probiotic bacteria in reducing serum cholesterol levels has attracted much attention. It is now thought that cholesterol

Table 1
Feed Consumption (g kg^{-1} d^{-1} $week^{-1}$) of Treated Mice with Enteropathogenic E. Coli and Probiotic Microorganisms

Weeks	*Mice groups*[a]						
	0	*1*	*2*	*3*	*4*	*5*	*6*
1	378.5 ± 0.1	376.4 ± 0.5	409.3± 0.3	373.2 ±0.6	446.3 ± 0.2	428.1± 0.5	388.3± 0.1
2	473.4 ± 0.2	230.9 ± 0.4**	512.8 ± 0.1	499.1± 0.7	497.0 ± 0.6	488.7 ± 0.7	489.9 ± 0.9
3	528.5 ± 0.3	299.6 ± 0.5**	544.3± 0.1	558.6± 0.5	544.9± 0.4	551.1 ± 0.8	572.1 ± 0.7
4	695.7 ± 0.3	307.6 ± 0.6**	670.8± 0.1	644.9± 0.8	665.9 ± 0.4	799.5 ± 0.6*	700.0± 0.6*

Data are presented as mean ± SD
All probiotic strains were added at (10^8-10^{10} CFU mL^{-1}).
[a]0: Skim milk; 1: E. coli; 2: E.coli + E. durans P174; 3: E.coli + Lb. plantarum P164; 4: E. coli + E. faecium P166; 5: E. coli + Lb. acidophilus P106; 6: E. coli + all LAB
*Significantly different from control at $P < 0.05$
**Significantly different from control at $P < 0.01$

Table 2
Body Weight and Weight Gain of Treated Mice with E. Coli and Probiotic Microorganisms

		Mice groups[a]						
	Weeks	*0*	*1*	*2*	*3*	*4*	*5*	*6*
Body weight (g)	1	27.6±0.1	26.7±0.7	26.7± 0.1	27.5± 0.1	28.8± 0.2	26.8± 0.5	27.0± 0.1
	2	32.7 ± 0.2	31.5 ± 0.4	30.6± 0.1	31.6 ± 0.7	30.3 ± 0.6	32.9 ± 0.2	31.7 ± 0.2
	3	37.7 ± 0.3	27.6 ± 0.6**	36.3 ± 0.2	36.9 ± 0.5	36.2 ± 0.4	39.9 ± 0.3*	39.7 ± 0.3*
	4	41.5 ± 0.6	25.7 ± 0.8**	40.4 ± 0.3	40.7 ± 0.8	40.5 ± 0.4	46.5 ± 0.6*	45.3 ± 0.6*
Body weight gain (g)	1-2	5.1± 0.3	4.8 ± 0.5	3.9 ± 0.5	4.1 ± 0.5	1.5 ± 0.2	6.1 ± 0.6	4.7 ± 0.5
	2-3	5.0 ± 0.9	-3.9 ± 0.6**	5.7 ± 0.6	5.3 ± 0.5	5.9 ± 0.4	7.0 ± 0.7	8.0 ± 0.8*
	3-4	3.8± 0.3	-1.9 ± 0.4**	4.1 ± 0.8	3.8 ± 0.5	4.3 ± 0.6	6.6 ± 0.5*	5.6 ± 0.4*
	1-4	13.0± 0.2	-1.0 ± 0.6**	13.7 ± 0.5	13.2 ± 0.6	11.7 ± 0.8	19.7± 0.7**	18.3 ± 0.7**

Data are presented as mean ± SD
All probiotic strains were added at (10^8-10^{10} CFU mL^{-1}).
[a] 0: Skim milk; 1: E. coli; 2: E.coli + E. durans P174; 3: E.coli + Lb. plantarum P164; 4: E. coli + E. faecium P166; 5: E. coli + Lb. acidophilus P106; 6: E. coli + all LAB
*Significantly different from control at $P < 0.05$
**Significantly different from control at $P < 0.01$

Table 3
Blood Analysis of Mice After Treatment with E. coli and Probiotic Microorganism

		Hct value		*Hb content*		*RBC's*		*WBC's*	
Mice groups[a]	*Body weight (g)*	*%*	*% of control*	*g/100 mL*	*% of control*	*X 10^6 / uL*	*% of control*	*X 10^3 / uL*	*% of control*
0	41.5 ± 0.6	41± 3	100	12. 5± 1	100	5.6 ± 1	100	6.5 ± 2	100
1	25.7 ± 0.8**	20 ± 6	48.9	7.2 ± 2	58	3.2 ± 3	58	10.8 ± 1	166
2	40.4 ± 0.3	38 ± 3	92.7	11.3± 1	90	5.3 ± 5	95	6.8 ± 3	105
3	40.7 ± 0.8	37 ± 2	90.2	11.0 ± 2	88	5.0 ± 3	90	6.9 ± 5	106
4	40.5 ± 0.4	35 ± 5	85.4	10.4 ± 3	83	4.9 ± 4	88	6.6 ± 4	102
5	46.5 ± 0.6*	40 ± 2	97.6	12.7 ± 2	102	5.5 ± 6	99	7.0 ± 6	108
6	45.3 ± 0.6*	39 ± 3	95.1	12..9 ± 1	103	5.4 ± 2	98	6.9 ± 2	106

[a] 0: Skim milk; 1: E. coli; 2: E.coli + E. durans P174; 3: E.coli + Lb. plantarum P164; 4: E. coli + E.. faecium P166; 5: E. coli + Lb. acidophilus P106; 6: E. coli + all LAB
Hct value: Haematocrite value; Hb: Haemoglobin; RBC's: red blood cells; WBC's: white blood cells.
*Significantly different from control at $P< 0.05$
**Significantly different from control at $P < 0.01$

Table 4
Size of Red Cells & Cholesterol Content in Blood Mice after Treatment with E. Coli and Certain Probiotic Microorganisms

		MCV		*MCH*		*MCHC*		
Mice groups[a]	*Body weight (g)*	*Fento L (FL)*	*% of control*	*Picogram (Pg)*	*% of control*	*g/L*	*% of control*	*Cholesterol mg/dL*
0	41.5 ± 0.6	73 ± 0.1	100	22.3 ± 1	100	305 ± 1	100	121.85± 7.10
1	25.7 ± 0.8**	63 ± 0.2*	86	22.5 ± 2	101	360 ± 4	118	130.50 ± 8.24
2	40.4 ± 0.3	72 ± 0.4	99	21.3 ± 3	96	297 ± 3	97	115.60± 3.50
3	40.7 ± 0.8	74 ± 0.8	101	22.0 ± 4	99	297 ± 3	97	114.42± 3.30
4	40.5 ± 0.4	71 ± 0.4	97	21.2 ± 3	95	297 ± 4	97	116.21 ± 4.20
5	46.5 ± 0.6*	73 ± 0.6	100	23.1 ± 2	104	318 ± 5	104	114.50 ± 5.20
6	45.3 ± 0.6*	72 ± 0.6	99	23.9 ± 5	107	331 ± 2	109	115.30 ± 2.56

[a] 0: Skim milk; 1: E. coli; 2: E.coli + E.. durans P174; 3: E.coli + Lb. plantarum P164; 4: E. coli + E. faecium P166; 5: E. coli + Lb. acidophilus P106; 6: E. coli + all LAB
MCV: mean cell value, MCH: mean cell hemoglobin and MCHC: mean cell hemoglobin concentration.
*Significantly different from control at P< 0.05
**Significantly different from control at P < 0.01

removal from culture media was a result of precipitation of cholesterol with free bile acids formed in the media because of the activity of the bacterial enzyme bile salt hydrolase. Various studies have shown that some lactobacilli could lower total cholesterol and low-density lipoprotein (LDL) cholesterol [29-32].

3.6. Distribution of Mice Plasma Protein

The changes in blood plasma proteins of mice fed on skim milk (control), skim milk inoculated with *E. coli*+ *E. durans* p174; *E. coli* + *Lb. plantarum* P164; *E. coli* + *E. faecium* P166; *E. coli* + *Lb. acidophilus* P106 and *E. coli* + all LAB were monitored using SDS-PAGE (Figure 1). Results showed that there are marked differences in plasma IgGs concentration among the different groups. The highest concentration of Igs was found in plasma of mice fed on skim milk inoculated with probiotic strains, while, the lowest concentration of IgGs was found with mice fed on skim milk inoculated with *E. coli* as compared with control. This may indicate how the ingestion of probiotic strains enhances immune response. These results are in agrement with those reported by [33-35].

These data also indicate that allbumin concentration was higher in plasma of mice fed on *E. coli and Lb. acidophilus* P106 and *E. coli* and all LAB. This may be related to the high rate of body weight gain of these mice (Table 1). The lowest concentration of plasma albumin was found with mice fed on only *E. coli*, which is related to the loss

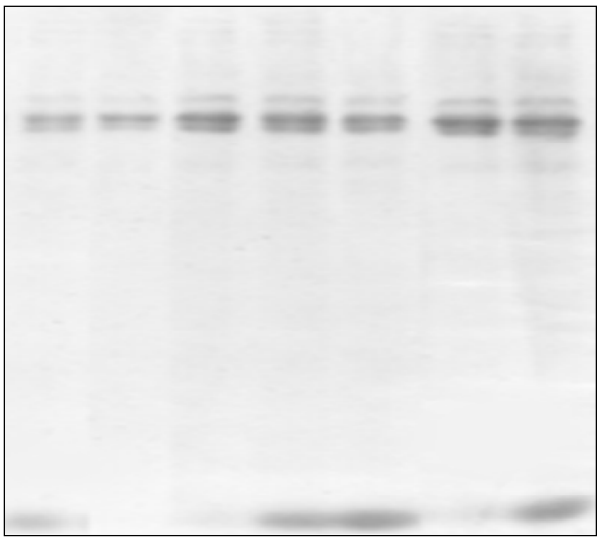

Figure 1: SDS-PAGE (10%T) of mice protreins; Lanes (1-7): Plasma samples of mice fed on skim milk (control); *E. coli*; *E.coli* + *E. durans* P174; 3: *E.coli* + *Lb. plantarum* P164; 4: *E. coli* + *E. faecium* P166; 5: *E. coli* + *Lb. acidophilus* P106: 6: *E. coli* + all LAB, respectively. Ig G HC: Immunoglobulin G heavy chain; IgG LC: Immunoglobulin G light chain; Anode is toward bottom of photo

of body weight of these animals (Table 1). With respect to β-globulin, its highest concentration was found in plasma of mice fed on *E. coli* + *Lb. plantarum* P164, *E. coli* + *E. faecium* P166 and *E. coli* + all LAB, whereas, it was disappered in plasma of mice fed on *E. coli*.

4. CONCLUSIONS

In conclusion, the results of this study have demonstrated that ingestion of probiotic strains had

no adverse effect on health & useful to protect mice from infection by enteropathogenic *E. coli*. In addition, results indicate that ingestion of probiotic strains such as *E. durans* P174, *Lb. plantarum* P164, *E. faecium* P166 and *Lb. acidophilus* P106 produce a safe hematological profile. These results suggest that the probiotics strains studied here are likely to be safe for human consumption.

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