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Health and Nutritional Benefits from Wild Probiotic Strains Isolated from Human Breast Milk, Zabady and Laben Rayb

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ABSTRACT: The objective of this study was to investigate in vivo scientific validation of seven wild selected potentially probiotic lactic acid bacteria (LAB) strains isolated from human breast milk, Zabady and Laben Rayb. These strains were used in cultured fermented milk as a vehicle for delivery of beneficial bacteria for five weeks to seven Albino rats groups. Feeding cultured milk products increased rats' body weight compared to control without significant change in body organs' weights. Hematology parameters of treated rats were comparable to control. All tested probiotic strains showed a hypolipidemic effect either by reducing triglycerides (TG) or by reducing LDL-Ch and atherogenic indices. There was no remarkable effect on oxidative stress in treated rats according to superoxide dismutase (SOD) and thiobarbituric acid reactive substances (TBARS) determining results and histological examination. The rats' groups fed cultured milk fermented using mothers' breast milk originated *Enterococcus* spp. cultures resulted in higher intestinal and fecal LAB comparing to control group. There was considerable suppression in intestinal and fecal contents of *Staphylococcus* spp. and coliforms among all treated rats' groups. Safety considerations of these probiotic strains were confirmed when carcinoembryonic antigen (CEA) levels and histological examination of liver tissues showed no changes comparing to control.

INTRODUCTION

Probiotic strains are components of the commensal microbial flora of the human gastrointestinal tract (GIT). These bacterial species play an important role in the enhancement of immunity, in maintenance of intestinal microbial balance and in the prevention of GIT infection.[1] The ability of several probiotics to positively modulate host immune responses has been demonstrated in many in vitro experiments and animal models.[2,3] Immunologic enhancement includes increased nonspecific immunity[4] and

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natural killer cell activation,[5] humeral immunity (antibody production[4]), and cellular immunity (lymphocyte proliferation[6]). It is well established that the most successful host immune responses involve the activation of both natural and acquired (antibody and cell-mediated) immune responses; therefore, probiotic strains that are able to affect a wider array of immune functions are likely to be more beneficial for human health.[7] The ability of probiotics to influence metabolic activities (e.g. cholesterol assimilation, lactase activity and vitamin production) and production of antimicrolbial substances have been confirmed.[8,9] The beneficial effects of probiotics including lactobacilli and bifidobacteria have stimulated interest in finding new strains able to enhance immunity for human health. The beneficial effects of probiotics *Enterococcus* spp. in different hosts have previously been reported.[10] Bacteriocin-producing *Enterococcus* spp. could be explored in food biopreservation to provide safety, mainly due to the antimicrobial activity of the enterocins.[11]

The purpose of the present study was to investigate scientific validation for the in vivo efficacy of the tested potentially probiotic seven selected LAB strains, RM732 (*St. thermophilus)*, ZP7411 (*E. faecium)*, ZP653 (*Lb. plantarum)*, HM72M1 (*E. faecalis1),* HT74II4 (*E. faecalis2)*, HT714 (*E. faecalis3)* and HT741 (*L. lactis* subsp*. lactis)* on health promoting represented in their influences on physiological status in treated animals. In recognition of the importance of assuring safety, the evaluation of safety considerations of potential probiotic strains was studied in vivo through Carcinoembryonic Antigen (CEA) blood test and histological studies to evaluate the convenience of recommending these newly selected LAB probiotics for human consumption as starter cultures in fermented dairy industries.

MATERIALS AND METHODS

Seven selected potential probiotic LAB

isolated strains were identified on molecular level by using 16s rRNA approach.[12]; Four strains were originated from healthy mother-breast milk; HM72M1, HT74II4, HT714 (*E. faecalis*) and HT741 (*L. lactis* subsp*. lactis*). Two strains were originated from Zabady; ZP653 (*Lb. plantarum*) and ZP7411 (*E. faecium*). One strain was originated from Laban Rayeb; RM732 (*St. thermophilus*). These strains were used in cultured fermented milk as a vehicle for delivery of beneficial bacteria for forty male Albino rats (4-5 weeks old, approximately 65-70 g body weight) that were used in this study. The rats were bred and maintained in a colony at Physiology Department, Faculty of Medicine, Alexandria University, Egypt. They were acclimatized on commercial chow (Atmida) for one week before starting experiment at room temperature (22^{o}C ± 2). The chemical composition of the chow was as follows; protein 18.5 %, fat 2.8 %, fiber 11.2 %. Animals were arranged in 8 groups, 5 rats each. Group (G1): was the control group fed the commercial chow diet and drank pasteurized milk (3% fat, 8.5% SNF). Groups from (G2) to (G8); were fed the commercial chow diet and drank seven cultured milk prepared with single strains of RM732 (*St. thermophilus)*, ZP7411 (*E. faecium)*, ZP653 (*Lb. plantarum)*, HM72M1 (*E. faecalis),* HT74II4 (*E. faecalis)*, HT714 (*E. faecalis)* and HT741 (*L. lactis* subsp. *lactis),* respectively. Diet and milk/ cultured milk were provided for five weeks. At the end of the experiment, final weights were recorded, and rats were sacrificed after overnight fasting under light diethyl ether anesthesia. Blood samples were collected from abdominal aorta and rats' organs; liver, kidneys, brain and spleen were dissected out carefully and weighed. The rats' livers fixed in 10% formol saline at 4°C for 48 h. Liver tissue was further embedded in paraffin blocks and sectioned into 5-6µm sections for serial specimens, mounted on glass slides and stained with hematoxylin and eosin (H & E) stains for histological light microscopic evaluation.[13] Hematology

parameters were determined using (Cell-Dyn® 6000 Hematology analyzer), lipid profile and Carcinoembryonic Antigen (CEA) were determined with (Hitachi 7600 Biochemistry Autoanalyzer) and antioxidative enzymes (SOD and TBARS) were determined using Enzyme-linked immunosorbent assay (ELISA).[14] All blood tests were analyzed at Mabarret El-Asafra Laboratories, Alexandria, Egypt. The rats' small and large intestines were taken off and washed with 20 ml sterilized saline. Fecal samples were collected from rectum and analyzed immediately according to Klaver and colleagues.[15] The bacterial count of intestinal samples washing saline was determined as described by Patel and colleagues.[16] For counting the lactobacilli group; the MRS pH 5 agar (Biolife) was used for 48 h at 37°C. For counting the cocci LAB; the M17 agar (Biolife) was used for 48 h at 37°C. *Staphylococci* spp. was enumerated on Staph 110 media (Biolife) for 48 h at 37°C. Coliforms were counted on Violet Red Bile agar (Biolife) at 37°C for 20 h. SPSS® 13.0 Analytical Software.[17] was used to investigate significance basing on control group, differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

All animals remained healthy for the duration of the study. Table (1) illustrates that all rats fed the cultured milk showed increase in body weight gain at the end of the experiment compared to control group. No significant differences were observed among all groups in relative liver, spleen, kidney and brain weights (g organ per 100 g body weight). Increases in body weight may be related to fermentation of milk by LAB increases its protein availability and its nutritional value, which agreed with Anon,[18]

Table (2) shows hematology parameters of treated rats. White blood cells or leukocytes are classified into two main groups of reticulocytes: granulocytes (polymorphonuclears or neutrophils, eosinophils and basophils) and nongranulocytes (monocytes and

lymphocytes). No significant differences were found between all rat groups concerning hematology parameters compared with control, except the increase in WBCs count in G3, G5, G6 and G7. However, the WBCs count of these four groups was within the normal range according to Lang.[19] The increases of WBCs count in response to bacterial infection or inflammation is usually accompanied with increase in neutrophils, monocytes.[20] which is not exhibited in results where neutrophils, monocytes in all groups were comparable to control or even less. Only lymphocytes that showed significant increase, which indicate for immune response. The immune system was reported to respond in a regulated fashion to microbes and eliminates them, but it does not respond to self-antigens.[19]

In comparison to control group, no remarkable differences were found in plasma lipid among all tested groups concerning High-density lipoprotein (HDL) and total cholesterol concentrations (Table 3). Although some cultured milk strains showed higher concentrations in TG and total lipids but this was accompanied with a significant suppression in LDL-C concentrations by (31-87.5%) which is a good indicator for their hypolipidemic effect. While the dietary groups fed *St. thermophilus* and *L. lactis* subsp. *lactis* tended to have hypolipidemic effect by lowering TG and total lipids concentrations but they did not significantly affect neither total cholesterol nor LDL-C levels. Most of probiotic strains in cultured milk had a hypolipidemic effect by reducing atherogenic indices up to 88% comparing with control. Hypolipidemic effects of lactic cultures have been reported earlier.[21] Lipid peroxidation of unsaturated fatty acids is a frequently used indicator of increased oxidative stress and subsequent oxidative damage[22] which very commonly detected by the measurement of TBARS

as an end-product. Table 4 shows the activities of antioxidative enzymes and Carcinoembryonic Antigen concentrations. No significant differences between control group diet and all treated groups concerning the specific activities of SOD, except, G4 and G6 which received ZP653 (*Lb. plantarum)* and HT74II4 (*E. faecalis)* showed slight suppression in SOD levels. These two groups showed significantly lower LDL concentrations and atherogenic indices as well as the suppression of SOD levels didn't significantly affect the production of thiobarbituric acid reactive substances (TBARS) in rats as a reflection of insufficiency of antioxidant defenses. The results depict remarkable double impact role; LDL-lowering and antioxidative potential of *E. faecium* where the insignificant enhanced the specific activity of SOD accompanied with significant decrease in LDL-C concentrations and atherogenic indices. Similar observations were reported.[23] The rats' G7 fed HT714 (*E. faecalis)* was the only group that showed slight significant increase in TBARS production. In contrast, receiving this probiotic showed other preferable interactions such as; significant suppression in LDL-C concentrations and atherogenic indices as well as it had no significant effect on SOD specific activities. These conflicting findings do not allow conclusions to be drawn to indicate the effect of HT714 (*E. faecalis)* as a probiotic strain of decreasing antioxidant status but the histological examination declared that all strains had no effect on oxidative stress showed in liver cells (see below). The effect of other tested probiotic cultures on SOD specific activities and TBARs content was not markedly different from that observed in the control group. The results also revealed that none of the used culture strains caused the production of the CEA protein.

The histological examination of rats' livers revealed normal architecture of hepatocytes radiating from central vein and separated by blood sinusoids. Hepatocytes appeared polyhedral in shape with central vesicular (active) nuclei and prominent nucleoli, surrounding by vacuolated acidophilic cytoplasm. Blood sinusoids showed normal calibre and lined by flat endothelial cells and Kupffer cells (macrophages). Normal intact cell membrane of hepatocytes indicates no effect on oxidative stress caused by ingested tested probiotic strains where oxidative stress reported to occur when free oxygen species bind with high affinity to cell membranes and cytoplasmic membranous structures causing alterations in the target cell function.[24] These findings confirm earlier similar observations by biochemical parameters results.

Influences of tested probiotic culture strains on intestinal microbiota and fecal population of LAB (rod and cocci) are shown in Table (5). Comparing with control G1, there were considerable variations among rat groups in their small intestinal count of LAB, *Staphylococcus* spp. and coliforms. Enterococci are normal inhabitants of the gastrointestinal tracts of both humans and animals; in the human intestine, *Enterococcus faecium* and *Enterococcus faecalis* are the two predominant species**.**[25] The results showed that in small intestine, population in rats of received *Enterococci* spp. resulted in higher LAB counts compared to control group and other groups especially *Enterococci* spp. isolated from mothers' breast milk. All dietary groups of rats that received cultured milks exhibited reduction in *Staphylococcus* spp. counts compared to control. The coliforms count decreased in treated groups compared to the control, especially G2 received RM732 (*St. thermophilus*) where the count decreased by (30%). The inhibition role of tested LAB probiotics against *Staphylococcus* spp. and

coliforms may be relayed to several metabolic compounds produced by LAB including; organic acids, fatty acids, hydrogen peroxide, and diacetyl, that have antimicrobial effects.[26]

The large intestinal microflora in treated rats groups exhibited increase in LAB counts comparing to control especially groups; G5 and G6 that showed remarkable increase by 41.5% and 43.7% respectively. *Staphylococcus* spp. and coliforms counts in treated rats groups showed decrease than control. The effect of the cultured milk on rats' intestinal microflora is reflected on their feces. Feeding rats on different cultured milk resulted in significant increase in LAB counts in their feces which was more pronounced in G6 received HT74II4 (*E. faecalis*) where the count increased by (46.7%) in LAB population. Staphylococci counts decreased in feces of all groups comparing to control especially G3 which received *E. faecium* and showed suppression in *Staphylococcus* spp. count by (50.3 %). Other tested probiotic *Enterococci* spp. also showed significant suppression in *Staphylococcus* spp. and coliforms counts in feces. *Enterococci* are used as probiotics may improve the microbial balance of the intestine or can be used in the treatment of gastroenteritis in humans and animals.[27] The bacteriocins produced by *enterococci* (referred to as enterocins) are particularly active against pathogenic bacteria such as *Clostridium* spp., and *Staphylococcus* spp.[28] The coliforms count decreased in treated groups compared to the control especially groups G4 and G2 that received *Lb. plantarum* and *St. thermophilus,* respectively, which scored remarkable suppression in coliform counts by 46 and 41.7 %. These results are in agreement with Sarkar and Misra.[29]

In conclusion, in vivo tests results confirm positive validation of the seven tested probiotic LAB strains by proving

their safety, efficacy and high performance in gastrointestinal tract which encourages their applicability in fermented milk products. These strains were applied as probiotic cultures in different pro-yoghurt products and showed good results of chemical, microbiological and organoleptic properties and can be recommended for human consumption (under publication).

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Table (1): Growth performances and relative organs' weights of rats

Dietary groups	Initial body wt.	Final body wt.	Body wt. gain	% of relative organ weight			
	(g)	(g)	(g)	Liver	Spleen	Kidney	Brain
G1 Control	68.33	153.5	85.17	3.40	0.34	0.65	0.80
G2 RM732 *(St. thermophilus)*	64.67	166.00	101.33	3.91	0.58	0.69	0.78
G3 ZP7411 *(E. faecium)*	68.33	164.00	96.00	4.34	0.56	0.68	0.91
G4 ZP653 *(Lb. plantarum)*	72.33	175.00	102.67	3.94	0.51	0.67	0.91
G5 HM72M1 *(E. faecalis)*	90.00	186.67	96.67	3.83	0.63	0.64	0.78
G6 HT74II4 *(E. faecalis)*	71.67	173.67	102.00	4.17	0.61	0.73	0.84
G7 HT714 *(E. faecalis)*	72.67	164.00	91.33	3.88	0.46	0.67	0.85
G8 HT741 *(L. lactis* subsp. *lactis)*	62.00	167.33	105.33	3.81	0.52	0.85	0.83

Data are the mean for 5 rats per group.

Table (2): Hematological parameters of rats

Parameters[a]	G1 (control)	G2 RM732 (*St. thermophilus*)	G3 ZP7411 (*E. faecium*)	G4 ZP653 (*Lb. plantarum*)	G5 HM72M1 (*E. faecalis*)	G6 HT74II4 (*E. faecalis*)	G7 HT714 (*E. faecalis*)	G8 HT741 (*L. lactis* subsp. *lactis*)
WBCs	8.900^{a}	11.300^{b}	21.400^{c}	9.800^{d}	27.600^{e}	22.600^{f}	22.700^{f}	13.600^{g}
RBCs	$6.630.000^{a}$	$6.650.000^{a}$	$6.560.000^{a}$	$7.180.000^{b}$	$6.080.000^{a}$	$7.040.000^{b}$	$8.150.000^{c}$	$7.110.000^{b}$
Hb content	12.1	11.2	12.6	12.3	12.1	13.6	14.6	12.6
Hb content%	84^{a}	78^{b}	88^{a}	86^{a}	84^{a}	95^{c}	102^{d}	88^{a}
Ht	41^{a}	42^{a}	42^{a}	42^{a}	42^{a}	43^{a}	49^{b}	41^{a}
Platelets	773.000^{a}	885.000^{b}	540.000^{c}	750.000^{a}	557.000^{c}	448.000^{d}	668.000^{e}	682.000^{e}
Reticulocytes:								
Poly	15^{a}	15^{a}	12^{b}	13^{ab}	12^{b}	9^{c}	5^{d}	9^{c}
Mono	7^{a}	4^{b}	3^{b}	8^{a}	3^{b}	4^{b}	2^{b}	3^{b}
Band	1	1	1	1	1	1	1	1
Eosino	3	1	1	1	1	1	1	1
Lymph	74^{a}	79^{a}	83^{b}	77^{a}	83^{b}	85^{b}	91^{c}	86^{b}
Baso	0	0	0	0	0	0	0	0

[a]: WBCs, White blood cells; RBCs, Red blood cells; Hb, Hemoglobin; Ht, Hematocrit; Poly, Polymorphonuclears or neutrophils; Mono, Monocytes; Bands, Less mature neutrophils; Eosino, Eosinophils; Lymph, Lymphocytes; Baso, Basophils.

Data are the mean for 5 rats per group.

^{A,b,c,} ..Means values in the same row marked with unlike letters are significantly different ($p<0.05$).

Table (3): Plasma lipid profile and atherogenic indices of treated rats

Parameters[a]	G1 (Control)	G2 RM732 (*St. thermophilus*)	G3 ZP7411 (*E. faecium*)	G4 ZP653 (*Lb. plantarum*)	G5 HM72M1 (*E. faecalis*)	G6 HT74II4 (*E. faecalis*)	G7 HT714 (*E. faecalis*)	G8 HT741 (*L. lactis* subsp. *lactis*)
Total Ch (mg/dl)	86^{a}	88^{a}	84^{a}	88^{a}	73^{ac}	101^{b}	96^{b}	88^{a}
HDL-Ch (mg/dl)	51	56	48	54	44	56	51	56
LDL-Ch (mg/dl)	16^{a}	17^{a}	2^{b}	4^{b}	11^{b}	8^{b}	2^{b}	18^{a}
VLDL-Ch (mg/dl)	19^{a}	15^{a}	34^{b}	29^{b}	19^{a}	37^{bc}	43^{c}	14^{a}
Triglycerides (mg/dl)	96^{a}	73^{b}	172^{c}	149^{d}	94^{a}	186^{c}	216^{e}	72^{b}
Total lipid (mg/dl)	282^{a}	261^{a}	356^{b}	337^{b}	267^{a}	387^{c}	412^{d}	261^{a}
Atherogenic indices								
Friedwald formula	0.31	0.30	0.04	0.07	0.25	0.14	0.04	0.32
LDL-Ch/Total Ch	0.19	0.19	0.02	0.05	0.15	0.08	0.02	0.20

[a]. Ch, Cholesterol; HDL, High Density Lipoprotein; LDL, Low Density Lipoprotein; VLDL, Very Low Density Lipoprotein; Friedwald formula, LDL-Ch/HDL-Ch.
Data are the mean for 5 rats per group.
^{A,b,c}...Means values in the same row marked with unlike letters are significantly different ($p<0.05$).

Table (4): Activities of antioxidative enzymes and Carcinoembryonic Antigen

Parametersa	G1 (control)	G2 RM732 (St. thermophilus)	G3 ZP7411 (E. faecium)	G4 ZP653 (Lb. plantarum)	G5 HM72M1 (E. faecalis)	G6 HT74II4 (E. faecalis)	G7 HT714 (E. faecalis)	G8 HT741 (L. lactis subsp. lactis)
Antioxidative enzymes								
SOD (μM/mg proteins)	53^{a}	51^{a}	57^{a}	36^{b}	48^{a}	43^{ab}	45^{a}	55^{a}
TBARS (μM)	23^{a}	27^{a}	25^{a}	27^{a}	32^{a}	21^{a}	37^{b}	31^{a}
Carcinoembryonic Antigen (CEA)								
CEA (mg/dl)	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2

[a]: SOD, Superoxide Dismutase; TBARS, Thiobarbituric acid reactant substances; CEA, Carcinoembryonic Antigen.
Data are the mean for 5 rats per group.
[A],[b]Means values in the same row marked with unlike letters are significantly different ($p<0.05$).

Table (5): Influence of probiotic cultures on intestinal microbiota and fecal population

count (Log CFU ml^{-1})	G1 (Control)		G2 RM732 *(St. thermophilus)* cocci	G3 ZP7411 *(E. faecium)* cocci	G4 ZP653 *(Lb. plantarum)* Rod	G5 HM72M1 *(E. faecalis)* cocci	G6 HT74II4 *(E. faecalis)* cocci	G7 HT714 *(E. faecalis)* cocci	G8 HT741 *(L. lactis* subsp.*lactis)* cocci
	Rod	cocci							
Small intestines									
LAB	8.00^{a}	8.78^{c}	9.95^{d}	10.07^{d}	9.60^{b}	10.11^{d}	10.88^{e}	11.05^{e}	10.39^{e}
Staphylococcus	4.04^{a}		3.67^{a}	3.62^{a}	3.66^{a}	3.54^{a}	3.60^{a}	3.69^{a}	3.47^{a}
Coliforms	3.95^{a}		2.78^{b}	3.60^{a}	3.30^{a}	3.84^{a}	3.53^{a}	3.47^{a}	3.39^{a}
Large intestines									
LAB	7.48^{a}	7.90^{c}	9.00^{d}	10.15^{e}	9.48^{b}	11.18^{f}	11.36^{f}	9.60^{d}	10.08^{e}
Staphylococcus	4.98^{a}		4.57^{a}	4.41^{a}	4.00^{b}	4.57^{a}	4.23^{a}	4.43^{a}	4.11^{b}
Coliforms	4.43^{a}		3.70^{a}	4.32^{a}	4.28^{a}	4.34^{a}	4.32^{a}	4.15^{a}	4.08^{a}
Feces									
LAB	7.00^{a}	7.70^{c}	9.00^{d}	8.58^{d}	8.00^{b}	8.85^{d}	11.29^{e}	8.70^{d}	9.00^{d}
Staphylococcus	4.99^{a}		4.08^{b}	2.48^{c}	2.85^{c}	4.79^{a}	4.81^{a}	4.28^{b}	3.78^{d}
Coliforms	4.26^{a}		2.48^{b}	2.60^{b}	2.30^{b}	3.08^{bc}	3.00^{bc}	2.70^{b}	3.30^{c}

Data are the mean for 5 rats per group.

^{A,b,c},...Means values in the same row marked with unlike letters are significantly different ($p<0.05$).

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