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Research Article
Role of Probiotic Mixture with and Without Green Tea Extract in Prevention of Hepatorenal Syndrome in Rat Model

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Abstract

Background and Objective: Hepatorenal syndrome (HRS) is a major public health problem in which both liver and kidney dysfunctions are encountered. The present research aimed to investigate the beneficial use of micro-encapsulated probiotic alone (*Bifidobacterium bifidum*, *Lactobacillus delbrueckii* and *Streptococcus thermophilus* mixture) or with green tea alcohol extract in HRS model in rats. **Materials and Methods:** Flavonoids content and *in vitro* antioxidant activity of the extract were assessed. The animal experiment consisted of 4 groups; control healthy, control with HRS and two test groups with HRS and treated with either the encapsulated probiotic mixture alone or with green tea extract. After 3 weeks; urinary creatinine was determined in 24 h rat urine samples. Colonic microbiota was assessed in faeces. Plasma malondialdehyde, nitrite, C-reactive protein, creatinine, uric acid, urea and the activity of transaminases, catalase (CAT) and angiotensin-1 converting enzyme (ACE-1) were determined with calculation of creatinine clearance. **Results:** Results showed significant increase in all biochemical parameters of HRS control except for ACE-1, CAT and creatinine clearance that experienced significant reduction along with dysbiosis compared to healthy control. Test groups showed improvement in all biochemical parameters with superiority to probiotic-green tea extract combination. Both treatments produced significant increase in fecal *B. bifidum*, *S. thermophilus* and *L. bulgaricus* and reduction of Staphylococci and Coliform. The effect of probiotic-green tea extract combination was more pronounced concerning the last three. Flavonoids and antioxidant activity of the extract were 1.325±0.01 mg quercetin/g and 98±1.66%, respectively. **Conclusion:** Administration of micro-encapsulated probiotic with or without alcohol green tea extract exerted significant prevention of HRS in rat with superiority to probiotic-green tea extract combination.

Key words: *Bifidobacterium bifidum*, *Lactobacillus delbrueckii*, *Streptococcus thermophilus*, probiotic-green tea exract, Hepatorenal syndrome, green tea extract, rats

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hepatorenal syndrome (HRS) is a state of advanced liver disease associated with acute renal failure. The pathophysiolgy of such syndrome is still not well understood. Severe liver failure might induce inhibition of renal blood flow pointing to renal vasoconstriction which could lead to renal dysfunction however the reduction in the filtration rate of the glomeruli is not always proportion as to renal blood flow referring to involvement of other events in the pathogenesis of HRS. Elevated nitric oxide synthase during such syndrome as well as elevated malondialdehyde (MDA) denote the involvement of high oxidative stress and inflammation in HRS[1,2]. Changes in colonic microflora were recently linked to chronic diseases including liver, kidney and inflammatory diseases[3-5], so imbalance of gut microbiota could have a hand in development of HRS. As the exact mechanism of HRS is still ignored, therapy of such syndrome has not yet been fully established. Nutraceuticals and probiotic could have a role to overcome, manage or protect from HRS.

Green tea is one of the major nutritional botanical sources of nutraceuticals that have diverse health benefits. Green tea is rich in phenolic compounds such as epigallocatechin gallate, epicatechin gallate, epicatechin, epigallocatechin, gallic, protocatechuic, catechin, syringic, vanillic, sinapic, cummaric, cinnamic and chrysin[6,7]. It was reported that green tea possesses antioxidant, antinflammatory and optimizing bone health activity[8]. Green tea extracts proved to protect the liver during exposure to high oxidative stress[9]. Alcohol green tea extract was shown previously to possess both reno and hepato-protective effect in hepatorenal syndrome model in rats[7].

The application of probiotics to kidney health is an emerging area of nutraceuticals and functional foods. Probiotics represented by *S. thermophilus, L. acidophilus, B. bifidum. B. longum* and others were reported to possess renoprotective effect[4]. On the other hand, some probiotic represented by *Lactobacillus plantarum* CCFM639 proved efficient in improving liver health which was ascribed to its antioxidant activity[5]. So, it is hypothesized that if a blend of probiotics was combined with green tea extract they could afford an optimum protective effect towards HRS. The objective of the current research was to assess the hepato and reno-protective effect of a mixture of encapsulated probiotics (*Bifidobacterium bifidum*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) with or without alcohol extract of green tea in HRS model in rats. Colonic microbiota and biochemical changes were followed to study the possible mechanism of action. The aim of this study included the determination of total flavonoids and *in vitro* antioxidant activity of the green tea extract.

MATERIALS AND METHODS

This work was carried out in National Research Centre, Egypt and was accomplished in 2018.

Chemicals and plant materials: Green tea was purchased from local markets, Cairo, Egypt. D-(+)-Galactosamine hydrochloride was obtained from Sigma, USA. All other chemicals used in the experiment were of high analytical grade.

Preparation of green tea extract: Green tea was dried in an air-circulated oven at 40°C. The dried powder of green tea was placed in a continuous extraction apparatus (Soxhlet) and subjected to extraction by absolute ethanol. The solvent was completely removed by evaporation under reduced pressure at a temperature not exceeding 40°C. The extract was kept in deep-freeze till used.

Determination of flavonoids content and antioxidant activity of green tea extract: Total flavonoids were determined in green tea extract according to the colorimetric method of Sakanaka *et al.*[10] and the result was expressed as mg quercetin/g extract. The *in vitro* antioxidant activity was assessed for green tea extract using butylated hydroxytoluene (BHT) as reference material[11].

Bacterial strains: *Bifidobacterium bifidum* (*B. bifidum*), *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) and *Streptococcus thermophilus* (*S. thermophilus*) were obtained from Chr. Hansene's Lab., Denmark.

Cultivation and harvesting of bacterial cells: The MRS broth (Oxoid) was used to prepare the cell suspensions for *Lactobacillus bulgaricus*. The medium was inoculated with 2% active *Lactobacilli* cells and incubated at 37°C for 48 h. *Streptococcus thermophilus* was activated by inoculating 2% of its culture on M17 broth and incubated at 30°C for 72 h. Also, *Bifidobactirum bifidum* was activated by inoculating 2% of its culture in MRS broth medium supplemented with 0.05% L-Cysteine-HCL. Cells were harvested by centrifugation at 5000 rpm for 15 min at 4°C and were washed twice with saline and used to prepare micro-capsules.

Preparation of micro-encapsulated cells culture: Micro-encapsulation by alginate was prepared as indicated

previously[12] with some modifications. Suspensions of cells (mixture from the three aforementioned strains) were mixed with an equal volume of sodium alginate (4%) and an equal volume of green tea extract. The mixture was added drop-wise into solution of calcium chloride (0.5 mol L^{-1}) and magnetically stirred at 200 rpm till alginate beads were formed. So, two encapsulated forms were prepared; mixture of 3 probiotics alone (A) and encapsulated 3 probiotic with green tea crude ethanol extract (B). Both forms were freeze dried. Form A: Each 1 g contains 2×10^9 probiotic mixture. Form B: Each gram contains 2×10^9 probiotic mixture+100 mg green tea alcohol extract.

Diet: Balanced diet composed of 12% casein, 10% corn oil, 70.5% starch, 3.5% salt mixture, 1% vitamin mixture and 3% cellulose was prepared as reported previously[13] with minor changes and used for feeding rats of all groups for 3 weeks.

Animal experiment: Male Sprague Dawley rats weighing 100-120 g were used in the present study. Animals were obtained from animal house of National Research Centre, Cairo, Egypt. Animals were kept individually in stainless steel metabolic cages; water and food were given *ad-libtium*. The animal experiment was carried out according to the Ethics Committee of the National Research Centre, Cairo, Egypt and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals. (Publication No. 85-23, revised 1985).

Twenty four rats were divided into 4 groups, each of 6 rats. All rats were fed on balanced diet. Rats of group 1 and 2 were fed on balanced diet and provided orally by 0.5 g from micro-encapsulated form A and B/rat/day, respectively. The 3rd group was considered as hepatorenal syndrome control (HRS control). The 4th group was healthy normal control, where rats received no treatments. During the experiment, body weight and food intake were recorded weekly. At the 21st day all rats except normal control received 1.1 g of D-(+)-Galactosamine hydrochloride/kg rat body weight via intraperitoneal injection as 200 mg mL^{-1} solution in saline to induce HRS[1]. The control normal rats were given only intraperitoneal saline. Twenty four hours urine samples were collected after the galactosamine hydrochloride injection, measured and urinary creatinine was determined[14]. Blood samples were collected from all rats after an overnight fast for the determination of plasma malondialdehyde MDA[15] as indicator of lipid peroxidation. Plasma nitrite (NO) and catalase (CAT) activity were assessed by applying colorimetric methods[16,17]. Plasma C-reactive protein (CRP) was determined as an inflammatory biomarker[18]. Plasma activity of aspartate transaminase (AST) and alanine transaminase (ALT) were estimated as indicator of liver function[19]. Plasma creatinine, uric acid and urea were assessed[14,20,21], referring to kidney function. Plasma angiotensin-1 converting enzyme (ACE-1) was determined by enzyme linked immuno-sorbent assay[22]. Creatinine clearance, total food intake, body weight gain and food efficiency ratio (Body weight gain/total food intake) were calculated.

Microbiological counts of microbiota: The faeces samples (1 g) were placed in a 9 mL diluted Ringer solution homogenized and serially diluted with 10 fold dilution using pre-reduced buffered peptone (20 g L^{-1} bufferedpeptone Oxoid CM509). *Bifidobacterium bifidum* was counted as reported previously[23] using modified MRS agar (Oxoid) supplemented with 0.05% L. Cysteine-HCL (Merck, Germany). *Lactobacillus delbrueckii* subsp. *bulgaricus* count was carried out using MRS medium while M17 was used for *Streptococcus thermophilus* count. The coliform count was carried out using MacConkey agar while a *Staphylococci* count was implemented using Baird-Parker medium base, supplemented with egg yolk and potassium tellurite. The plates for anaerobic bacteria were incubated for 48 h at 37°C in an anaerobic atmosphere using Gen Kits in Oxoid jars. The plates for aerobic bacteria were incubated aerobically for 48 h at 37°C. At the mentioned time, the Colony Forming Units (CFU) on each plate were counted and calculated with reference to the original weight of the samples. The results were expressed as $\log_{10}$ CFU g^{-1} of faeces.

Statistical analysis: All data were expressed as the mean±SE. The results of animal experiment were analyzed statistically using one-way analysis of variance followed by Tukey test. In all cases $p<0.05$ was used as the criterion of statistical significance.

RESULTS

Flavonoid content and *in vitro* antioxidant activity of green tea extract: Flavonoid content of green tea extract was found to be 1.325±0.01 mg quercetin/g. *In vitro* antioxidant activity of green tea extract was shown to be 98±1.66% while that of BHT as reference antioxidant was108±0.3% (Table 1).

Table 1: Total flavonoid content and the percentage of *in vitro* antioxidant activity of green tea alcohol extract

Parameters	Total flavonoids (mg quercetin/g extract)	Antioxidant activity (%)
Alcohol green tea extract	1.325±0.01	98±1.66%
BHT	-	108±0.3%

BHT: Butylated hydroxytoluene

Animal experiment

Biochemical changes: The data in Table 2 showed the biochemical changes of different experimental groups. The ALT and AST plasma activities were shown to be significantly high in HRS control compared to control healthy group, indicating liver dysfunction. Treatment with encapsulated probiotic or encapsulated probiotic with green tea extract reduced the activity of ALT and AST significantly compared with HRS control but still significantly higher than normal control. Plasma levels of creatinine, urea and uric acid were significantly high in HRS control rats compared to normal rats, while ACE-1plasma levels decreased significantly in HRS control compared to normal control indicating kidney dysfunction. Supplementation with encapsulated probiotic or encapsulated probiotic with green tea extract significantly suppressed the elevation observed in the plasma levels of creatinine, urea and uric acid to reach levels similar to normal control, while plasma levels of ACE-1 significantly increased in both treated groups but still significantly lower than normal control. Plasma MDA and CRP demonstrated significant high level in HRS control when compared to normal healthy control referring to elevated lipid peroxidation and inflammation, respectively. Also plasma nitrite (NO) level was increased significantly in HRS control compared with normal control, while plasma catalase activity reduced significantly in HRS control compared with normal control. Both treatments showed significant improvement in plasma MDA, CRP, NO and catalase compared to HRS control group. Creatinine clearance was reduced significantly in HRS control group compared to normal control. Encapsulated probiotic with or without green tea extract showed significant increase in creatinine clearance levels similar to normal control. Rats treated by encapsulated probiotic with green tea extract showed significant improvement in ACE-1, NO, CAT and CRP compared to those treated by encapsulated probiotic alone.

Microbiological counts of microbiota: In Table 3, the count of *B. bifidum* and *S. thermophilus* in fecal samples of HRS control rats were significantly reduced while *Staphylococci* and *Coliform* were significantly elevated with non significant change in *L. bulgaricus* compared to control healthy. Treatment with either probiotic alone or with green tea extract produced significant increase in fecal *B. bifidum*, *S. thermophilus* and *L. bulgaricus* and significant reduction of Staphylococci and Coliform compared to HRS control. The effect of encapsulated probiotic with green tea extract was more pronounced concerning the last three.

Nutritional parameters: The results in Table 4 illustrated the different nutritional parameters of the studied groups. It

Table 2: Biochemical parameters of different experimental groups

Parameters	Normal control	HRS control	Encapsulated probiotic	Encapsulated probiotic with green tea extract
Plasma parameters				
Creatinine (mg dL^{-1})	0.753±0.031^{a}	1.110±0.066^{b}	0.805±0.029^{a}	0.785±0.009^{a}
Urea (mg dL^{-1})	29.800±0.909^{a}	43.800±1.077^{b}	30.500±0.764^{a}	30.000±0.577^{a}
Uric acid (mg dL^{-1})	1.100±0.055^{a}	1.230±0.021^{b}	1.100±0.029^{a}	1.030±0.036^{a}
ALT (U L^{-1})	33.700±1.429^{a}	77.500±2.045^{b}	70.200±2.725^{c}	64.800±2.522^{c}
AST (U L^{-1})	41.300±1.085^{a}	91.300±2.260^{b}	75.300±1.626^{c}	67.800±2.088^{c}
ACE-1 (pg mL^{-1})	544.500±5.376^{a}	379.200±7.349^{b}	454.000±6.805^{d}	498.300±6.008^{c}
NO (µmol L^{-1})	10.000±0.578^{a}	19.200±0.749^{b}	14.300±0.558^{d}	12.300±0.558^{c}
CAT (U L^{-1})	415.000±9.218^{a}	199.200±5.687^{b}	341.000±4.829^{c}	392.200±8.907^{a}
MDA (nmol mL^{-1})	8.700±0.558^{a}	17.700±0.803^{b}	13.000±0.632^{c}	11.800±0.477^{c}
CRP (ng mL^{-1})	0.755±0.035^{a}	1.330±0.061^{b}	0.933±0.036^{d}	0.775±0.017^{a}
Urine parameter				
Creatinine clearance (mL min^{-1})	0.973±0.019^{a}	0.548±0.016^{b}	0.865±0.033^{a}	0.909±0.037^{a}

Values are expressed as Mean±SE, the sample size(n) = 6, HRS: Hepatorenal syndrome, in each row same superscript letters means non-significant difference, different letters means significant difference at 0.05 probability

Table 3: Microflora of different experimental groups (log_{10} CFU g^{-1} feces)

Parameters	Normal control	HRS control	Encapsulated probiotic	Encapsulated probiotic and green tea extract
B. bifidum	5.5±0.299^{b}	4.5±0.223^{a}	7.9±0.616^{c}	8.4±0.566^{c}
S. thermophilus	4.7±0.203^{b}	3.4±0.138^{a}	7.4±0.421^{c}	7.5±0.232^{c}
L. bulgaricus	4.0±0.076^{a}	4.1±0.299^{a}	6.8±0.152^{c}	7.3±0.159^{d}
Staphylococci	3.6±0.402^{a}	6.4±0.749^{c}	5.2±0.219^{b}	3.0±0.299^{a}
Coliform	3.7±0.165^{b}	5.8±0.641^{c}	3.8±0.375^{b}	1.7±0.386^{a}

Values are expressed as Mean±SE, the sample size(n) = 6, HRS: Hepatorenal syndrome, in each row same superscript letters means non-significant difference, different letters means significant difference at 0.05 probability

Table 4: Nutritional parameters of different experimental groups

Parameters	Normal control	HRS control	Encapsulated probiotic	Encapsulated probiotic and green tea extract
Initial body weight (g)	109.3±3.971[a]	109.5±2.872[a]	109.7±1.819[a]	109.3±2.246[a]
Final body weight (g)	143.0±2.529[a]	143.3±4.772[a]	143.5±2.952[a]	142.3±2.800[a]
Body weight gain (g)	33.7±2.347[a]	33.8±3.419[a]	33.8±2.914[a]	33.0±1.183[a]
Total food intake (g)	263.3±2.231[a]	262.2±4.928[a]	263.8±6.117[a]	263.5±3.190[a]
Food efficiency ratio	0.128±0.009[a]	0.128±0.011[a]	0.127±0.008[a]	0.125±0.004[a]

Values are expressed as Mean±SE, the sample size(n) = 6, HRS: Hepatorenal syndrome, in each row same superscript letters means non-significant difference, different letters means significant difference at 0.05 probability

could be noticed that there were no significant changes in final body weight, body weight gain, total food intake and food efficiency ratio when the experimental groups were compared with each others. Food efficiency ratio of the group treated by the encapsulated probiotic with green tea extract showed the least value.

DISCUSSION

The improvement in both liver and kidney function in the present study on administration of either probiotic alone or combined with green tea alcohol extract could be related to the reduction of inflammation and oxidative stress translated in reduction of CRP, MDA and NO and elevation in CAT in the *in vivo* study. Also, the *in vitro* antioxidant activity of green tea extract supported the *in vivo* effect. In addition, restoring microbiota profile by both treatments might be an aid in protection from HRS. The current study showed the presence of flavonoids in green tea extract that reported previously to possess antioxidant and anti-inflammatory activity. Epigallocatechin gallate (EGCG) and quercetin prepared from green tea were shown to possess anti-inflammatory effect. Epigallocatechin gallate inhibit inflammation through reduction of generation of free radicals in macrophages[24]. The anti-inflammatory activity of quercetin is related to regulation of NF-κβ associated mechanisms[25] and inhibition of proinflammatory signals[26] and circulating inflammatory cytokines[27]. Green tea extract was reported to improve liver function and to have antioxidant effect in patulin induced oxidative stress in mice[9]. Green tea polyphenol inhibited necrosis and glomerular collapse and globally reduce renal injury and oxidative stress with subsequent improvement of kidney function after ischemia/reperfusion in rabbits[28].

The EGCG from green tea ameliorate microbiota to favorable effect[29] which agreed with the present study. Dysbiosis in gut microbiota has been shown to have an intimate relation to pathogenesis of chronic diseases. It is worth to mention that dysbiosis is present in chronic kidney disease that develops sustained inflammation and further decline in both cardiac and renal function[30]. So, it could be speculated that probiotic and prebiotic administration could improve renal dysfunction through improving dysbiosis. Both prebiotic and probiotic are considered as functional food or nutraceuticals. In the present study, green tea extract represents a prebiotic. In patient with peritoneal dialysis given probiotics; there was a reduction in proinflammatory cytokines[31]. A recent study[32] showed that continual consumption of yogurt and/or probiotic was associated with reduction of the risk of kidney disease.

Reduction of ACE-1 in HRS model in the present study could point to acute renal failure related to glomerular filtration rate and creatinine clearance. The wide use of *Lactobacillus* in fermented food has been shown to have various health benefits[33] mediated by the release of bioactive peptides during the fermentation process[34] or the maintenance of gut microbiota balance as reported previously[35]. Probiotic administration could improve endothelial dysfunction, vascular inflammation, vascular oxidative stress, cardiac and renal hypertrophy and maintain normal blood pressure[34] through restoring gut microbiota. Also, supplementation of a mixture of 6 probiotic strains was reported to improve kidney function[36].

A previous study showed *Lactobacillus plantarum* CCFM639 to elevate antioxidant biomarkers and to reduce MDA in liver of mice treated by Aluminium[5]. This finding agreed with the result of the present study. Probiotic can reduce oxidative stress through inhibiting NO synthase expression[37] and free radical scavenging activity besides regulating trace element level[38]. The ALT, AST, blood urea nitrogen and creatinine were reported to be improved by probiotic[5].

Lactobacillus plantarum and *Bacillus coagulans* were demonstrated to improve liver and kidney function and to reduce oxidative stress through ameliorating antioxidant enzymes[39]. Improving intestinal endotoxemia and restoring gut microbiota profile could participate in improving liver injury in the present study as reported previously[40].

The aforementioned effect of different probiotics and green tea constituents as antioxidant, anti-inflammatory,

anti-hypertensive and protector of renal and hepatic function as well as restoring gut microbiota can explain the synergistic protective effect of the combined micro-encapsulated probiotic mixture and green tea extract towards HRS in the present study.

CONCLUSION

Administration of microencapsulated probiotic with or without alcohol green tea extract produced significant improvement of both liver and kidney functions through amelioration of HRS changes represented by oxidative stress and inflammation biomarkers, angiotensin converting enzyme-1 and gut microbiota. Micro-encapsulated probiotics combined with green tea extract showed superiority in improving HRS compared to probiotic alone which could be due to synergistic action.

SIGNIFICANCE STATEMENTS

This study discovered that probiotics in combination with alcohol green tea extract can be beneficial for protection from HRS with a mechanism involved restoring gut microbiota. This study introduced a new complementary therapy from a safe natural source that could aid in the management of HRS which is a great public health problem. Based on this discovery researchers could develop an innovated remedy as a final solution for curing HRS.

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