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EFFECTS OF THE SELECTED PROBIOTIC MIXTURE IN FREE AND IMMOBILIZED FORMS ON THE PATHOPHYSIOLOGY OF SODIUM DEXTRAN SULPHATE-INDUCED ULCERATIVE COLITIS AND ASSOCIATED MOOD DISTURBANCES

Guevorkyan A.G.[1], Alchujyan N.Kh.[2*], Madoyan R.A.[3], Sargsyan H.H.[2], Hovhannisyan M. R.[2], Aghababova A.A.[2], Aznauryan A.V.[1], Aghajanov M. I.[1]

[1] Department of Biochemistry, Yerevan State Medical University after M. Heratsi,Yerevan, Armenia
[2] Department of Pathological Biochemistry, Institute of Biochemistry after H. Buniatyan of the National Academy of Sciences of the Republic of Armenia, Yerevan, Armenia
[3] VITAMAX-E, LTD Co, Yerevan, Armenia

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Abstract

Experimental ulcerative colitis was developed in 3-month old male mice fed with sodium dextran sulphate in drinking water for 7 days. Sodium dextran sulphate-induced ulcerative colitis was characterized by acute moderate-to-severe ulcerative colitis accompanied by symptoms of the anxiety disorders and depression, changes in microbiota and morphology of colon and brain, activation of lipid peroxidation processes in the colon, blood and brain regions responsible for cognitive function, emotion and mood. Two-week daily administration to ulcerative colitis-mice the mixture of the selected strains of probiotics with antipsychotic and antifungal activities in free form and immobilized on the micronized composition of chemically modified natural minerals (80% zeolite, 10% diatomite, and 10% bentonite) decreased ulcerative colitis disease activity index score, improving some of the symptoms associated with disease, effectively restored gut microbiota balance, prevented signs of microbial translocation, alleviated symptoms associated with depression/anxiety and histopathological changes in colon and brain, and protected against oxidative stress via inhibition of lipid peroxidation in the colon, blood and regions of corticolimbic system.

Nevertheless, further study is needed to confirm whether free and/or immobilized probiotics are effective in managing ulcerative colitis patients in combination with conventional drugs to avoid or delay step-up therapies with drugs burdened by potentially serious side effects.

Keywords: *anxiety, chemically modified natural minerals, corticolimbic system, leukocyte, plasma, depression, Linex forte, lipid peroxidation, selected strains of probiotic, sodium dextran sulphate, ulcerative colitis*

Introduction

Ulcerative colitis (UC) is characterized by the colonic mucosa inflammation and disturbances in the immune response to gut microbiota with an incidence of approximately 10-20 per 100.000 per year [*Danese S, Fiocchi C, 2011*]. Alternatives for the treatment of ulcerative colitis are needed, because existing interventions are not effective for all ulcerative colitis patients, and there are adverse effects with current therapies [*Furfaro F et al., 2015*]. Gut microbiota alterations are involved in the pathophysiology of ulcerative colitis and can not only unbalance the gastrointestinal immune responses but also influence distal effector sites leading to central nervous system disorder (including affective disorders) complicating the course and treatment of ulcerative colitis [*Ochoa-Repáraz J, Kasper LH, 2014*; *Sampson TR, Mazmanian SK, 2015*]. Based on this microbiota is considered as a perspective therapeutic target in acute and chronic gastrointestinal diseases accompanied by mental

Address for Correspondence:
Nelly Kh. Alchujyan, Ph.D.,
H. Buniatian Institute of Biochemistry NAS RA
5/1 P. Sevak Street, Yerevan 0014, Armenia
Tel.: (+374 98) 22-71-70
E-mail: alchujyan@mail.ru

disorders but these issues are not sufficiently elucidated [*Cawthorpe D, Davidson M, 2015*].

Probiotics (PB) are live microbial feed supplements that may improve intestinal microbial balance, through enhancing gut barrier function eliminating microbial translocation and improving local immune response [*Mallon P et al., 2007*]. Moreover, probiotics via correction of microbiota may prevent gut-brain alterations relevant to mood and cognitive processes [*Bravo J et al., 2012; Benros M et al., 2015*]. Preliminary data suggest the efficiency of probiotics for the treatment of ulcerative colitis, but strong data are still lacking [*Bibiloni R et al., 2005*]. However, major concern for the use of probiotics *in vivo* is that they must survive and sustain transit through the detrimental factors of the gut (high acidity (low pH), bile salts, molecular oxygen in case of obligatory anaerobic microbes, bacteriophages, and chemical as well as antimicrobial agents) in large quantities to facilitate their colonization in the host to confer the probiotics *in vivo* health benefits. Immobilization of probiotics protects from the harmful environmental factors, enabling their transport and normal functioning in gut [*Ding WK, Shah N, 2007; Singh PK, Kaur IP, 2012*]. Natural minerals, zeolites, diatomite and bentonite, containing several macro- and micro elements exhibit absorbent and ion-exchange properties and effectively used as growth promoters and bacterial carriers [*Mery C et al., 2012*]. They can replenish the need of organism for minerals and also serve as enterosorbents improving metabolism absorbing toxins in the intestine and from the blood preventing toxins diffusion through the intestine [*Weiß S et al., 2013*]. Drugs based on natural minerals do not exert mutagenic effects; they are non-toxic, effective, versatile and economical. Moreover, bentonite and diatomite are food additives, E558 and E551 respectively, approved in EU as anti-caking agents. Composition of the chemically modified natural minerals was created and used by us for and immobilization of probiotics and their effective culturing, as well as for treatment of stressed rats to modulate microbiota and decrease cognitive deficit and depression/anxiety [*Barsegyan KA et al., 2013*]. The aim of this investigation was to assess whether using the mixture of the selected strains of probiotics with psycho-, antifungal activities in free form and immobilized on the micronized composition of previously used zeolite-based (PBZ) composition of natural minerals, it would be possible to attenuate the ulcerative colitis injuries, accelerate recovery processes, and improve ulcerative colitis-associated mood disorder, as well as to assess effects of the selected probiotics and the advantages of the probiotics immobilized with PBZ compared to Linex forte, which beneficial effects are demonstrated in the experimental and clinical studies in gastroenterology [*Ushkalova EA, Gushchina YuSh, 2015*].

Material and Methods

Chemicals and reagents: Dextran sulphate 40 sodium salt, Mr~40000, and bovine serum albumin were from Carl Roth, dextran (Mr~70000) (Serva, Heidelberg Germany), Linex forte (LEK d.d., Slovenia). Sodium citrate anticoagulant, thiobarbituric acid, HEPES, 1,4-Dithiothreitol, Hematoxylin and Eosin were purchased from Sigma-Aldrich Chemical Co.

Animals and treatments: All procedures involving animals were in accordance with the International Laboratory Animal Care and the European Communities Council Directive (86/809/EEC) and approved by the respective local committee on biomedical ethics (H. Bunyatyan institute of biochemistry, Yerevan, NAS RA). The 2-to 3-month-old male mice from our breeding colony were used. All animals were maintained on a 12 *h* light/dark cycle at normal room temperature and housed in groups of 6/cage with free access to food and tap water.

Experimental design: *Ulcerative colitis induction.* The animals were randomly divided into groups (n=12): I. control group - native mice; and experimental groups with sodium dextran sulphate-induced ulcerative colitis [*Okayasu I et al., 1990*]: II - mice received ad libitum 2.5% sodium dextran sulphate dissolved in regular tap water for 7 days and examined immediately at the end of sodium dextran sulphate treatment; III - a group of sodium dextran sulphate-induced ulcerative colitis mice left untreated for the duration of 2-weeks in parallel to treated ones and referred as self-recovery to assess self-healing mechanism; IV-VI - mice, that were fed with 2.5 % sodium dextran sulphate dissolved in regular tap water for 7 days and then given daily per os (through a flexible polypropylene gavage) separately probi-

otics, and/or PBZ, and/or Linex forte (6 x 10^9 *CFU/ml*) for two weeks respectively. Control mice were given water only. On day 8, mice were sacrificed and monitored for colitis.

Probiotic strains and growth conditions: A commercially available probiotic, a concentrated source of naturally occurring microorganisms were used. *L. rhamnosus* strain B-6778 and *Bifidobacterium bifidum* strain AC-1666 (RF), as well as *L. plantarum* strain *B*-2353 (RF), *L. acidophilus* Er317/402 "Narine" (RA), *L. salivarius* B-7701 possessing fungicidal activity, and *E. coli* Nissle 1917 strain were rehydrated in sterile 0.85% NaCl and routinely propagated at 37° C in MRS medium (Hi Media, India) pH of 6.5 ± 0.2. Limiting dilution assay (based on the method of McCrady) was used for the separation, characterization, and quantitation of bacteria [*Aristovskaya TV et al., 1962*]. Viability was confirmed by culturing 1 *ml* of the rehydrated mixture on plates supplemented with MRS + agar at 37°C for 24 *h*. For immobilization of probiotics the culture medium was enriched with 5% of the chemically modified micronized natural mineral composition (vide infra). The probiotic mixture contained equal quantities the mentioned microorganisms (6 x 10^9 *CFU/ml* in total). Linex forte which active ingredients are Lactobacillus acidophilus (LA-5) and Bifidobacterium animals subsp. lactis (BB-12) (2 x 10^9 *CFU/ml*) was also studied for ulcerative colitis treatment.

***Natural mineral composition*:** Natural minerals mined in Armenia were used: zeolites – from Noyemberyan, bentonite – from Ijevan, diatomite – from Jadzor. Their multi-elemental composition at the trace and ultra-trace levels was determined and chemical modification was previously performed and composition (zeolite (80%), diatomite (10%), and bentonite (10%)) used as efficient stimulators and carriers of the probiotics [*Barsegyan KA et al., 2013*]. High pressure micro-powder mill was used to produce about 50μm powder from the mentioned minerals.

Evaluation of experimental colitis: The animals were weighed and monitored for the appearance of diarrhea and blood in the stool throughout the experimental period. The overall disease severity was assessed by a clinical scoring system [*Melgar S et al., 2005*], and the disease activity index score was calculated for each animal. Assessment includes 3 parameters: difference in weight before and after receiving DSS (0 = weight gain ≥ 1*g*: 1 = weight gain <1*g*; 2 = weight loss <1*g*; 3 = weight loss ≥ 1*g*); stool consistency (0 = normal stool; 1 = soft but formed stool, 2 = liquid stool); the presence of blood in the perianal region (0 = no sign of blood; 1 = signs of blood in perianal region; 2 = presence of blood in perianal region). The minimum score is 0, maximum is 7. After decapitation colon is excised opened longitudinally, rinsed with saline, and the length was measured and considered as an additional index for colitis, as well as macroscopic damage of the colonic mucosa scored based on the degree of inflammation and the presence of edema and/or ulcerations (0 = normal; 1 = slight inflammation; 2 = moderate inflammation and/or edema; and 3 = heavy inflammation and/or ulcerations) [*Mitrovic M et al., 2010*].

Toward the end of the treatment, before decapitation, all of the animals underwent a behavioral testing in open field and elevated plus-maze.

Behavioral examination: *Open field test.* The mice were placed singly into an open field (diameter 1*m*, divided by 2 concentric circles into 16 equal sections on the floor of the arena) and observed in 3 *min* to measure locomotor activity (the number of sectors crossed with all paws (crossing), the number of rears (posture sustained with hind-paws on the floor), grooming (including washing or mouthing of forelimbs, hind-paws, body and genitals) (exploratory behavior) and boluses (anxiety) counted manually/visually [*Buresh Ya et al., 1991*].

Elevated plus-maze test. Immediately after the open field test the mice were placed singly into a common central platform (10 *cm* × 10 *cm*) of elevated plus-maze comprised two open and two closed arms (45 *cm* x 10 *cm* x 10 *cm*) and elevated to a height of 80 *cm* above the floor. During a 3-*min* observation period, the following parameters were measured: number of open arms entries and number of closed arm entries. Exploration (grooming and rearing) and risk assessment (number of hanging over the open arms) were also examined [*Augustsson H, Meyerson B, 2004*]. At the end of each trial, the open field and elevated plus-maze were wiped clean with ethanol.

Microbiota: Each animal was opened aseptically; samples of feces from the lower part of the

gut, and brain washout were immediately placed into an anaerobic chamber for bacteriological analysis. After decapitation trunk blood was collected and analyzed. For identification light microscopy and/or the culturing method were used for all samples. Cultures were incubated in sucrose broth at 37 °C for 24*h* (blood was diluted by 1:5 *v/v*), then examined by microscopy, inoculated to the solid culture media, agar plates (Endo, sucrose, and blood agar), and incubated for 24*h*; blood samples incubated for 5 days to facilitate a growth of microbes daily monitored. The characteristics (such as morphology and color) of the colonies, as well as hemolysis, plasma-coagulation, aerobic fermentation of mannitol were examined for identification of microorganisms [*Holdeman, LV, Moore WEC, 1972; Pokrovsky VI et al., 1984*].

Histological examination: Samples for histology were excised from the brain and distal 6–8 *cm* of the colon, fixed in 10% buffered formalin, and embedded in paraffin blocks. Slices with 6 μm sections were stained with hematoxylin and eosin (H&E), and scored for histological damage [*Dieleman LA et al., 1998*]. Pathological diagnosis of each specimen was assessed and analyzed by specialized histopathologist in a blinded manner.

Isolation of leukocyte and plasma from blood: Blood was drawn into 3.8% sodium citrate anticoagulant, mixed with dextran (6 % solution in 0.9 % NaCl) and incubated at 37° C for 60 *min* for gravity sedimentation of erythrocyte from the fresh blood, the decanted layer was centrifuged at 1000*g* for 5 *min*, the resultant cell pellet fraction containing leucocytes after washing with buffer was homogenized with a Potter homogenizer (1500 *rpm*, 3 *min*) in buffer (20 *mM* HEPES, 0.2 *mM* DTT pH 7.4,), while supernatant obtained was centrifuged at 4 °C (6000 *rpm*, 10 *min*) and blood plasma was separated from platelets in supernatant [*Frik G et al., 1979*].

Isolation of the colon and the brain regions: Colon was scraped with a glass slide, rinsed, cut into pieces and (1:10, *w/v*) homogenized in ice-cold buffer (20 *mM* HEPES containing 0.2 *mM* DTT pH 7.4) (1:10, *w/v*) (1500 rpm, 3-5 *min*). Brains were rapidly removed from the skulls, placed on cold plate, and prefrontal cortex, striatum, hippocampus and hypothalamus were dissected and homogenized in the same conditions.

Indices of oxidative stress referring to lipid peroxidation processes were established by measuring malondialdehyde (MDA) using thiobarbituric acid [*Vladimirov YuA, Archakov AI 1972*]. Samples were deproteinized with 10 % TCA, centrifuged at 15000 *rpm* for 3 *min*, and supernatants were mixed with 0.6 *N* HCl and 0.72% TBA, heated for 15 *min* in boiling water bath with the resultant formation of pink-colored secondary product of MDA which absorbance was measured at 535 *nm* wavelength against reagent blank containing all the reagents minus the sample.

Protein was determined using crystalline bovine serum albumin as standard [*Lowry OH et al., 1951*].

Statistical analysis: All data were analyzed using a one-way analysis of variance (ANOVA) followed by post hoc Holm-Sidak test (SigmaStat 3.5 for Windows). Data are expressed as the mean ± S.E.M. Statistical significance is accepted at the level $p < 0.05$.

Results

Before presenting the major experimental results, we would like to briefly discuss reasons of selection the probiotics used to treat ulcerative colitis. These are the psychoactive *L. rhamnosus* strain B-6778 and *Bifidobacterium bifidum* strain AC-1666 (RF), as well as *L. plantarum* strain *B*-2353 (RF), *L. acidophilus* Er317/402 "Narine" (RA), *L. salivarius* B-7701 possessing fungicidal activity, and *E. coli* Nissle 1917 strain, named Mutaflor that used in medicine mainly for treatment of gastrointestinal diseases (also in infant children) and plays pivotal role in the modulation of microbiota and maintaining homeostasis [*Hudault S et al., 2001; Lodinová-Zádníková R et al., 2003*]. Recently Mutaflor dose-dependent protective effect has been discovered in ulcerative colitis mice model [*Sha S et al., 2014*]. Bifidobacteria have been shown to improve psychological symptoms in patients with functional gastrointestinal disorders and its specific strain may alter serotonin and dopamine turnover in areas of the brain associated with depression and anxiety [*Desbonnet L et al., 2008; Urita Y et al., 2015*]. The probiotic strains with antifungal activities were included in the probiotics mixture, as the widespread use of probiotics has been associated with cases of fungemia in immunocompromised patients, with increasing in-

cidence in the past years [*Roy U et al., 2017*]. It should be also noted that a mixture of probiotic strains was preferentially used, as it may be more beneficial than single-strain probiotic due to their synergistic effects and complementary actions within the gut [*Yoo SR et al., 2013*].

Effect of the selected probiotics in free and immobilized forms and Linex forte on the gut microbiota and disease activity in mice following DSS-induced ulcerative colitis

Here we represent the effect of two-week treatment with the mixture of probiotics in free (probiotics), immobilized (probiotics) forms, and Linex forte (see *Material and methods*) administered immediately at the end of sodium dextran sulphate drinking with induction of moderate-to-severe ulcerative colitis and compare to self-healing processes. The analysis of gut microbiota showed that ulcerative colitis was accompanied by an overgrowth of *Candida albicans*, manifestation and overgrowth of *Staphylococcus aureus* and hemolytic *E. coli*, the changes that, possibly, contributed to the reduction in the number of beneficial bacteria observed [*Bondarenko VM, Rybalchenko OV, 2009*]. Moreover, single colonies of lactose negative *E. coli* were found in blood, and in some mice single colonies of *C. albicans* were seen in the brain indicating bacterial translocation. Both probiotics and PBZ completely prevented bacterial translocation and normalized microbiota on 14 day, while in the blood of Linex forte-treated mice single colonies of C. albicans were found, which number per g feces was of a 1.44-fold higher compared to control, but *S. aureus* and hemolytic *E. coli* were not detected. Notably, *L.* salivarius included in the probiotics mixture may display the capacity of self-aggregation and coaggregation with *Candida albican* and exert considerable antifungal activity, some of them (e.g. *L. salivarius* BGHO1) may produce multiple bacteriocins and exhibit a broad inhibitory spectrum both against Gram-positive and Gram-negative pathogens [*Hronek M et al., 2005*]. sodium dextran sulphate-induced dysbiosis was ameliorated in all the treated groups. This is in line with the others findings that the reductions in *Lactobacillus*, *E. rectale*, and *Bacillus* were reversed by probiotic administration in sodium dextran sulphate-treated mice [*Emge J et al., 2016*].

Acute colitis manifested as diarrhea and bloody feces, in some cases rectal prolapse was occurred as a complication in colitis models using sodium dextran sulphate [*Byrnes J et al., 2009*]. PBZ shortened the duration of diarrhea in one day, whereas it was lasted longer approximately for 1-2 and 2-3 days during probiotics and Linex forte treatment respectively. This is presumably, because of a species-specific efficacy of probiotics in the treatment of acute diarrhea, and *L. rhamnosus* included in the probiotics mixture is the effective one [*Mantegazza C et al., 2018*]. Body weight of animals (29.67 ± 1.97 *g*) was decreased during sodium dextran sulphate-induction of ulcerative colitis up to 13.5 - 16.9 % at the end of sodium dextran sulphate administration, and began to reverse by 10 days post-SDS and tendency to normal weight gain was observed until mice were sacrificed on day 14 post-DSS. At the same time weight gain in the treated groups were 1.83 ± 0.71*g* (Linex forte), 2.42 ± 1.31 (PB), and 4.83 ± 0.72 (PBZ). Thus, all the tested probiotics prevented diarrhea, and bleeding in one-two days, as well as efficiently restored the weight gain suppressed during ulcerative colitis that is coincided with findings of the other authors [*Shi LH et al., 2016*].

Colon length and disease activity index were also significantly improved suggesting ongoing recovery from disease. As shown on Fig. 1A, on day 14 post-SDS the colon length of ulcerative colitis mice was (6.2 ± 0.3 *cm*) shorter than that of control mice (8.9 ± 0.8 *cm*). Treatment with Linex forte and probiotics attenuated colon shortening about equally (7.13 ± 0.32 *cm* and 7.41 ± 0.43 *cm* respectively), while it was normalized by PBZ. The DAI calculated by diarrhea, visible fecal blood weight loss, as well as colon length and macroscopic damage additional indices for colitis (see *Material and methods*) reached up to 8-11 in the DSS-treated group, it was significantly lowered of about thrice in the self-healing processes on day 14 post-DSS, when all three treatment groups showed complete recovery. The above mentioned parameters correlated with histological grading of colitis based on the amount and depth of inflammation, as well as the amount of crypt damage or regeneration, and the percentage involvement by the disease process (Fig. 1B).

Histopathological changes observed in the colon and brain regions of corticolimbic system

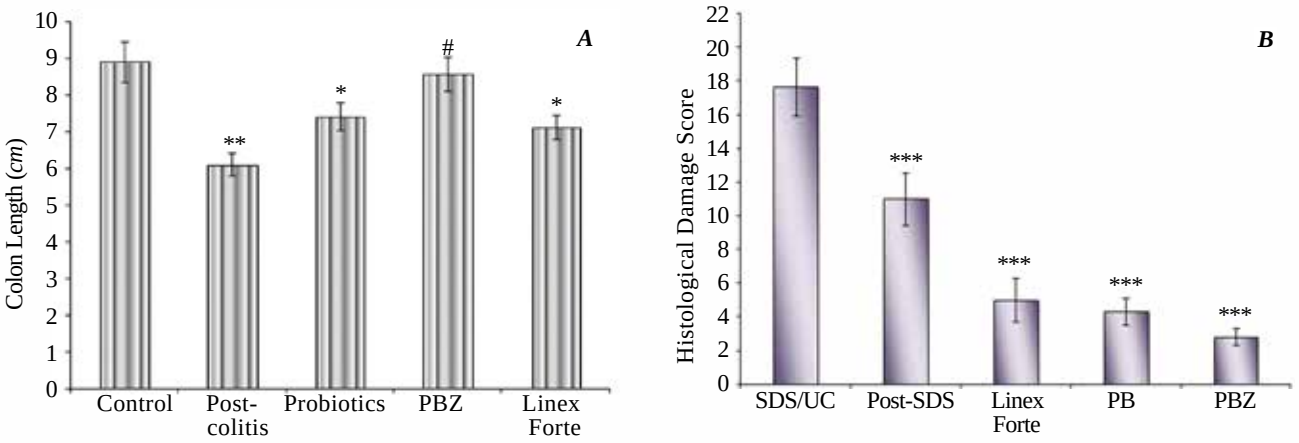

Figure 1. *Probiotics ameliorate sodium dextran sulphate-induced ulcerative colitis. as indicated by increased colon length (**A**), and decreased histological damage score (**B**). Data are expressed as M ± SD, n=12. The confidence probability (p) of parameters evaluated compared to the colitis group and designated as # p >0.05, * p <0.05, ** p <0.01, *** p<0.001.*

were examined using H&E staining. As in many other findings microscopic features of sodium dextran sulphate-induced ulcerative colitis was characterized by epithelial damage, disruption of crypt architecture, and inflammation [*Dieleman LA et al., 1998*]. Inflammatory cell infiltration in the areas of focal lesions, and edema in the colonic submucosal layer were detected (Fig. 2 A, B, C). It should be noted that acute inflammatory or infectious process are accompanied by vasodilatation with increased capillary permeability and extravasation of protein-aqueous fluid in a loosely packed submucosal layer resulted in the edema relative to a thickened echogenic submucosal layer, thereto mucosal hyperplasia seen in ulcerative colitis mice is an initial step in the formation of polyps, which were also found, indicating an acute disease process [*Frisoli JK et al., 2000*].

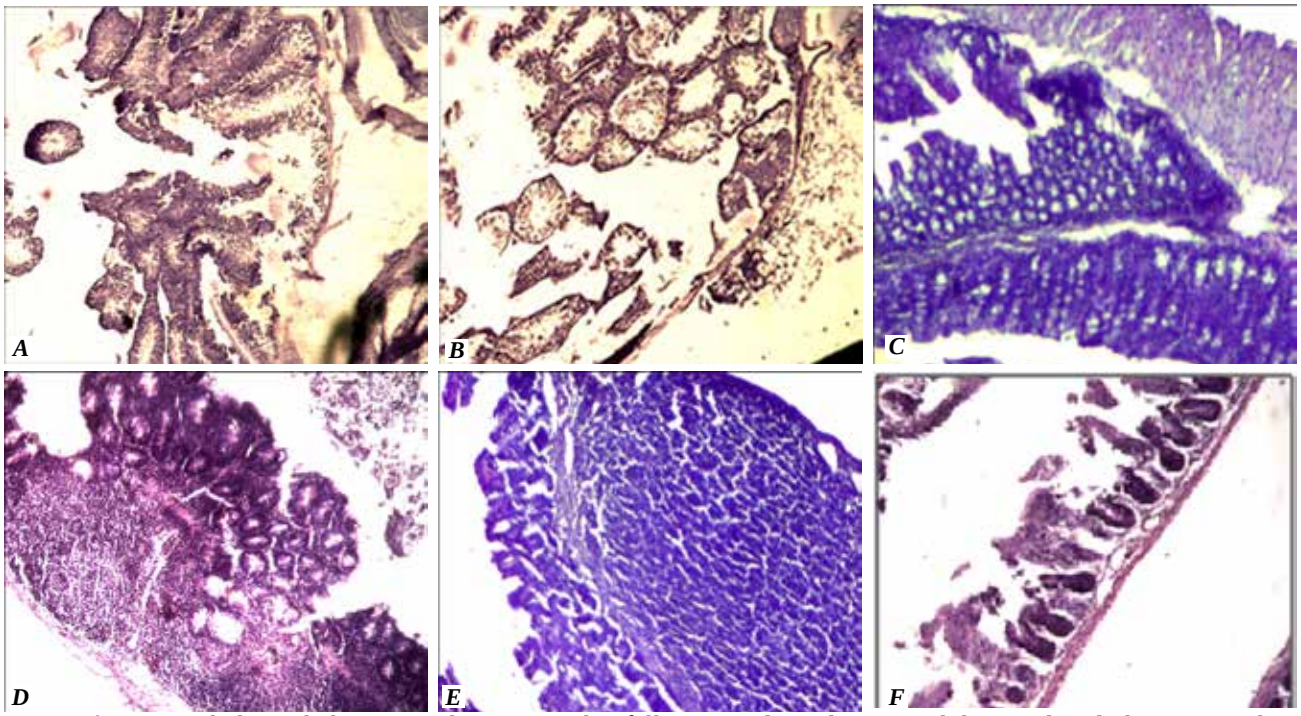

Figure 2. *Histopathological changes in the mouse colon following sodium dextran sulphate-induced ulcerative colitis (upper row) and 14 days post-colitis (lower row). Erosions in the colonic mucosa, edema and inflammation in the subepithelial layer (**A, B**) x100, H&E stain; the intestinal wall with the mucous glandular hyperplasia and formation of the hyperplastic polyp (**C**) x100, H&E stain; focal glandular hyperplasia of the mucosa (**D**) x100, H&E stain; erosions in the colonic mucosa, edema and inflammation in the subepithelial layer (**E**) x100 H&E stain; intact mouse colonic mucosa with not wide subepithelial layer (**F**), x100 H&E stain.*

Two weeks after stopping sodium dextran sulphate, the recovery phase of sodium dextran sulphate-induced ulcerative colitis was associated with a reduced bacterial translocation to extra-intestinal sites, limited epithelial apoptosis and disruption of the mucosa, the submucosa contained some macrophages, and lymphoid microfollicles were seen in lamina propria, as well as the colonic epithelial cell proliferation that involved in the regeneration processes [*Edelblum KL et al., 2008*]. However, someplace edema and erosions and focal glandular hyperplasia in the colonic mucosa were observed, epithelium was incompletely regenerated (Fig 2, D, E).

Of interest, the sodium dextran sulphate-induced ulcerative colitis caused edema and proliferation of glial cells in the mouse brain tissues lasted for the post-SDS period with manifestation of gangliomatous atypical nervous cells (Fig. 3, A-F).

Probiotics-treatment attenuated hyperplastic changes and prevented the polyp formation in the colon, someplace mucosal glandular hyperplasia and moderate inflammation were detected (Fig. 4, A). Notably, hyperplasia may be due to any number of causes, including chronic inflammatory response, compensation for damage or disease elsewhere [*Porth CL, 2011*]. The probiotics-treated mice showed increased crypt height compared to self-healing post-SDS mice (Fig. 4, B). Surprisingly, the brain regions of probiotics-treated mice were preserved and did not undergo deleterious changes observed in both sodium dextran sulphate-induced ulcerative colitis and post-SDS period (Fig. 4, C).

On the preparations of the PBZ-treated mice slight inflammation and proliferation of epithelial cells were observed, and someplace deformed crypt structures occurred (Fig. 5, A, B). However, the layers of the mucosa are preserved, and in the colonic lamina propria the clusters of lymphoid structures are presented both in the form of folicles and in the form of diffusely located lymphoid elements, among which plasma cells and macrophages were found (Fig. 5, C). Gut-associated lymphoid tissue is known play a pivotal role in the repair mechanisms. In inflammatory conditions, increased number, diameter and density of isolated lymphoid follicles suggest their involvement in immune surveillance, their presence is also indispensable in normal mucosal regeneration of the colon, as they serve as a regenerative pool of stem cells in case of mucosal damage, and/or contribute

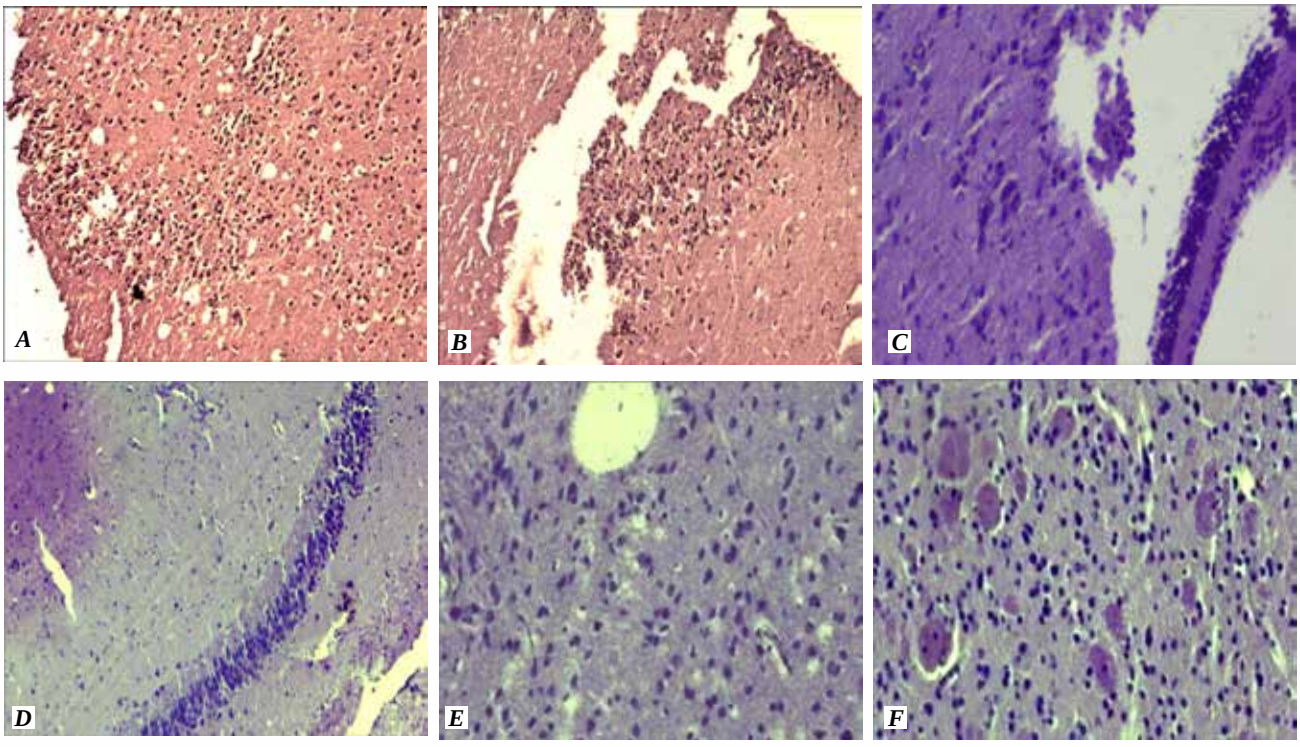

FIGURE 3. *Histopathological changes in the mouse brain following sodium dextran sulphate-induced colitis (upper row) and 14 days post-colitis (lower row). Edema and proliferation in the brain tissues (**A, B**); intensive focal proliferation of glial cells, and edema (**D, E**); manifestation of gangliomatous cells (**F**). (H&E stain,100X)*

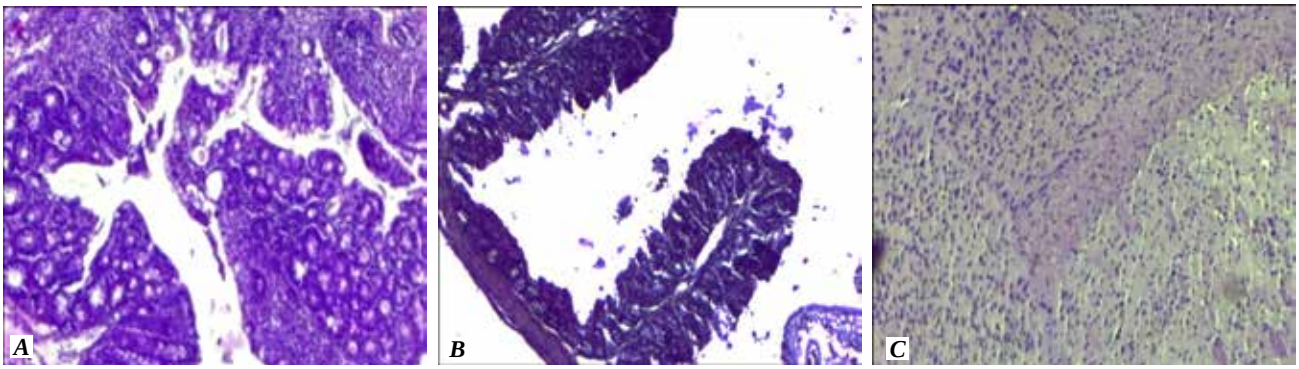

Figure 4. *Effect of probiotic-treatment on the histopathological changes in colon and brain following sodium dextran sulphate-induced ulcerative colitis. Someplace mucosal glandular hyperplasia and mild inflammation in the colon (**A**) x 40, H&E stain; an increased crypt height in the colonic mucosa (**B**), x 100, H&E stain; in the brain no changes detected (**C**), x 40, H&E stain*

to the optimal cytokine milieu for the differentiation of immigrating stem cells reviewed elsewhere [*Sipos F et al., 2010*].

Beneficial effect of PBZ treatment was found in the brain, where in some areas detected the ganglion cells surrounding the nerve structures that play a protective role in nervous tissues. At the same time, in the brain of some PBZ-treated mice we observed signs of a negligible dystrophy, and someplace still widened full-blooded vessels pointed to the possibility of development the perivascular edema that may be involved in the the formation of the perivascular exudative cuff, which plays dual role on one side it can be implicated in disruption of the blood-brain barrier, on the other can be considered as a protective mechanism of strengthening vascular wall from the outside [*Semenov IV, 1984*]. Notably, the similarity between perivascular spaces in human and rodent brains and their significance as anatomical routes for edema fluid drainage from damaged brain tissues has been demonstrated [*Kida S, Kato T, 2015*].

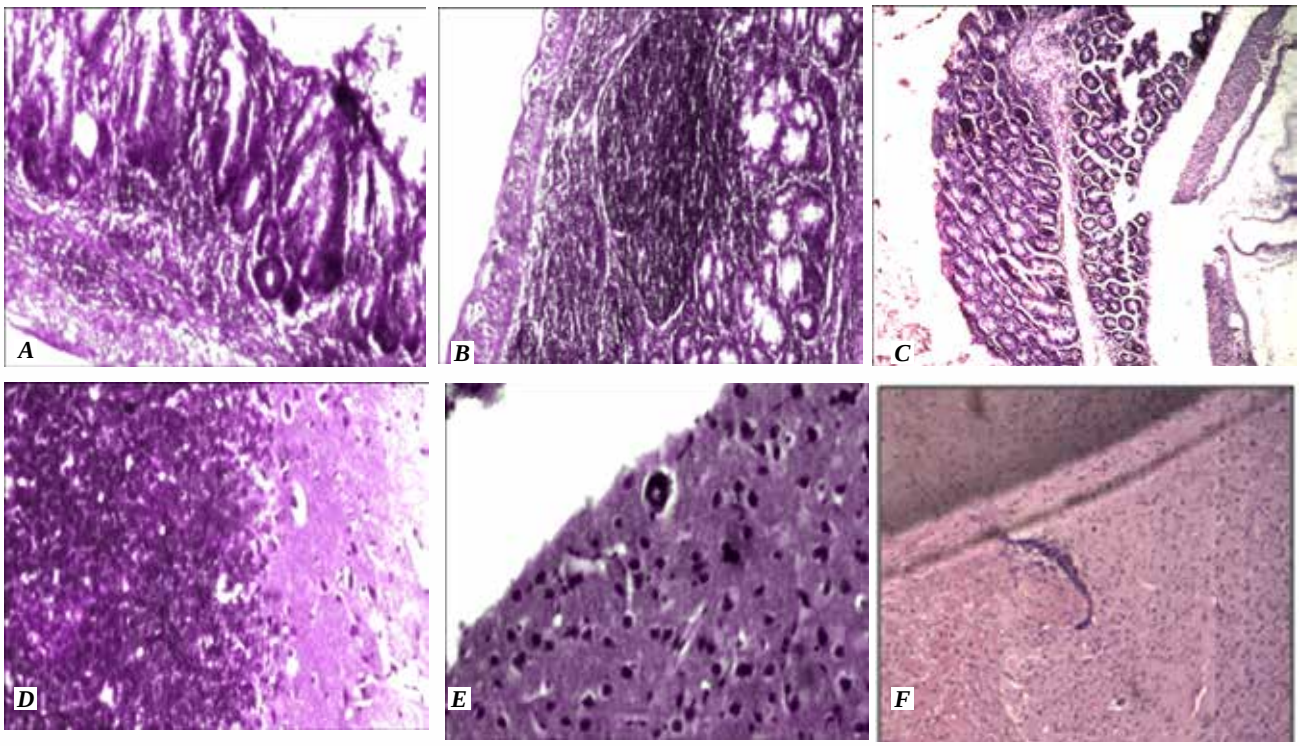

Figure 5. *At lower magnification PBZ-treatment presented the colonic wall with slight signs of inflammation, epithelial cell proliferation (deformed crypt seen) (**A**), x 40, H&E stain; the lymphoid macrofollicles in the colonic mucosa (**B**), x 100 H&E stain in the subepithelial layer (**C**) x 100 H&E stain; focal proliferation in the brain (**D**) x 40, H&E stain); someplace full-blooded vessels (**E**) x100, H&E stain; intact mouse brain (**F**) x 40, H&E stain*

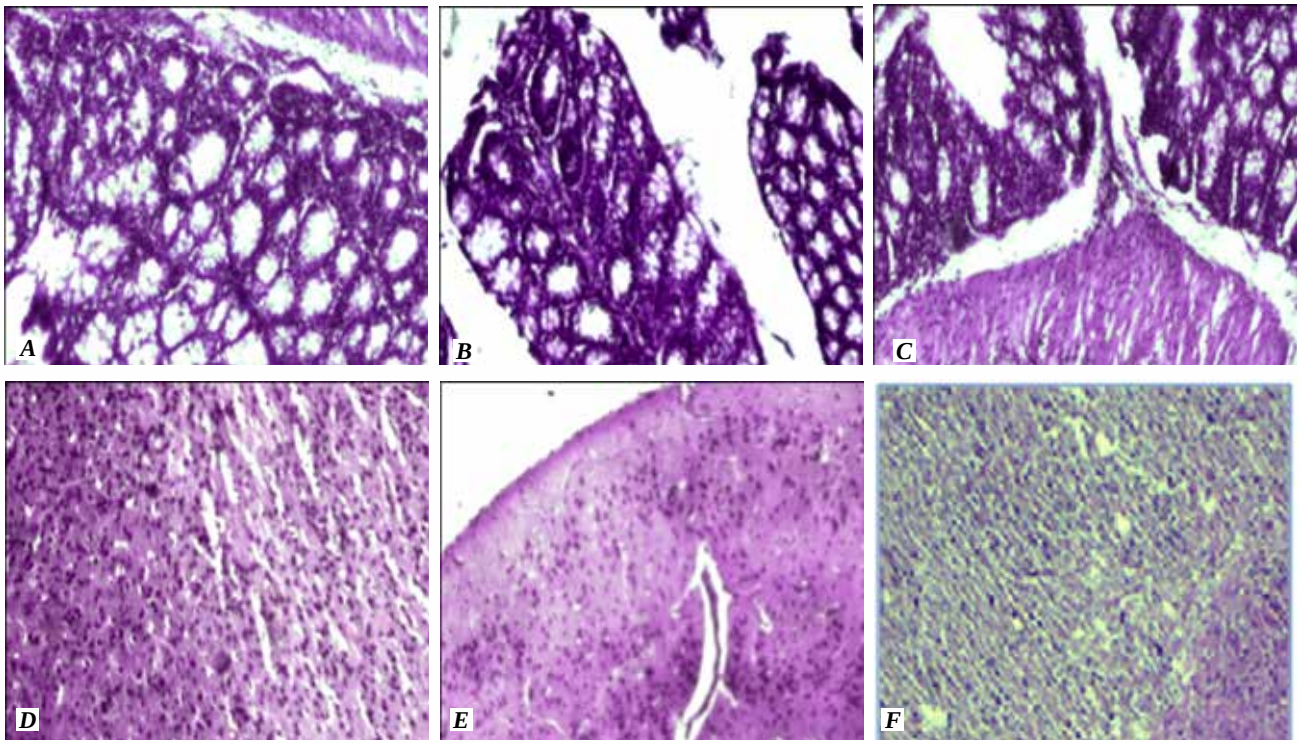

FIGURE 6. *Linex forte-treatment presented with focal mucosal glandular hyperplasia without cellular atypia (**A**); someplace the formation of small papillary structures (**B**); edema in the colonic submucosal layer (**C**). x40 H&E stain; the brain tissues with slight signs of cell proliferation (**D**), x 40, H&E stain; someplace perivascular edema (**E**), x100 H&E stain, brain edema (**F**) x 40, H&E stain.*

Linex forte-treatment presented in the colon with focal mucosal glandular hyperplasia without cellular atypia, but someplace small papillary structures were detected, as well as edema in the colonic submucosal layer (Figure 6 A-F). Histostructures of the colonic mucosa were reduced/contracted, and the lymphoid structures in the intrinsic layer were mainly diffused.

In the different parts of the Linex forte-treated mouse brain (cortex, cerebellum, hypothalamus, as well as neurostructures of locus coeruleus which involved in the physiological response to stress and anxiety) were observed moderate dystrophy, and in some areas signs of perinuclear and perivascular edema and chromatolysis, after which neuronal recovery can occur, but most often it contributes to apoptosis [*Stoica B, Faden A, 2010*]. The mentioned features indicate insufficiently successful recovery processes in the colon and brain of mice received Linex forte, compared with those shown for both probiotics and PBZ treated mice.

Effect of the selected probiotics in free and immobilized forms and Linex forte on the behavior and mood of mice following DSS-induced ulcerative colitis

Depression-like behavior of mice was observed immediately after sodium dextran sulphate-induced ulcerative colitis. Animals displayed a decreased motor activity in the open field mimicking human psychomotor retardation [*Wilner P, 1997*]. Another major symptom of depression that was detected in the open field and the elevated plus-maze a decreased number of rearing relevant to exploratory behavior showing a "refractory loss of interest" [*Katz RJ et al., 1981*]. Reduced grooming was also observed and reflects decreased self-care and motivation, another trait of depression-like behavior [*Brenes Saenz JC et al., 2006*]. ulcerative colitis mice exhibited also a decreased number of hanging (a risk-assessment) in the elevated plus-maze . It has been shown that sodium dextran sulphate colitis modifies the behavioral responses in mice via impact on cerebral expression of stress-related neuropeptide systems and level of pro-inflammatory cytokines in the limbic system [*Cruz AP et al., 1994*]. We have previously reported that behavior was no longer impacted at 14 days post-SDS in mild-to-moderate ulcerative colitis, but in severe colitis post-SDS non-treated animals still exhibited attenuated levels of depression and anxi-

Table 1.

Effect of treatment on the mice behavior in the open field following sodium salt-induced ulcerative colitis.

Groups	Motor activity	Rearing	Gruming	Defecation boli
Control (healthy)	47.29 ± 5.68	7.18 ± 1.15	4.33 ± 1.56	1.19 ± 0.65
Sodium salt-induced	15.3 ± 1.4***	1.1 ± 0.3 **	1.03 ± 0.2*	0.02 ± 0.02***
Post self-recovery	17 ± 2.85**	1.6 ± 0.8**	1.45 ± 0.71*	0.69 ± 0.58#
Probiotic-treated	50.33 ± 6.34#	2.42 ± 1.08*	2.75 ± 1.55#	0.42 ± 0.52#
Zeolite-immobilized probiotics treated	64.83 ± 16.72#	7.9 ± 2.5#	6.08 ± 1.13#	0.69 ± 0.93#
Linex forte-treated	55 ± 12.23#	4.92 ± 1.31#	3.17 ± 0.72#	0.58 ± 0.52#

Notes: *M ± SD (n=12). # p >0.05, * p<0.05, ** p <0.01, *** p <0.001 (compared to control)*

ety that disappeared completely in all the three groups of treated mice (Table 1, 2). Number of entries into open arms, percentage of open/total arm entries might also be earned to anxiety resembling human anxiety/depression, while number of enclosed arm entries, total number of arm entries and rearing probably related to motor activity [*Reichmann F et al., 2015*]. According to above mentioned, anxiety/depression was for Linex forte > PB > PBZ (44.8, 47.5, and 59.3 % respectively), and motor activity assessed as a sum of variables the OF and the elevated plus-maze was for PBZ > Linex forte > PB (96.06, 80.92, 66.92, respectively). The number of defecation boli of treated mice from post-SDS group was similar to those of from non-treated group in the open field test. Contrary, both treated and non-treated mice from post-SDS groups showed normalized number of boli in the elevated plus-maze test. Thus, hardly to explain psycho-emotional activity of tested animals with respect to this variable, because of its opposite values determined in two tests used.

However, the other variables in both tests suggested the modulatory effects of all the probiotics, and the most beneficial effect of PBZ on motor and psycho-emotional activity of ulcerative colitis-mice. This is in line with findings on regulation emotional behavior by Lactobacillus strain in a mouse via the vagus nerve was demonstrated [*Bravo JA et al., 2012*]. Correlation between the changes in microbiota and depression was also shown [*Naseribafrouei A et al., 2014*]. As we mentioned above probiotics via correction of microbiota may enhance gut barrier, modulate immune response, as well as prevent gut-brain alterations relevant to mood and emotions [*Bibiloni*

Table 2.

Effect of treatment on the mice behavior in the elevated plus-maze following sodium salt-induced ulcerative colitis

Groups	Rearing	Gruming	Number of Entries into the open arms	Number of Entries into the closed arms	Hanging over the arms	Defecation boli
Control (healthy)	6.6 ± 1.04	3.62 ± 0.5	4.29 ± 0.98	3.71 ± 0.78	4.93 ± 0.88	1.14 ± 0.34
sodium salt-induced	0.18 ± 0.4**	0	0	0.63 ± 0.5*	0	0
Post self-recovery	2.5 ± 0.76*	0.18 ± 0.4**	0.09 ± 0.3**	1.45 ± 0.83*	0	0
Probiotic-treated	4.0 ± 0.85*	2.67 ± 1.5#	3.17 ± 0.72#	3.5 ± 0.8#	2.75 ± 1.29#	0.67 ± 0.49#
Zeolite-immobilized probiotics treated	8.91 ± 3.32#	2.83 ± 0.85#	6.08 ± 3.08#	4.17 ± 2.37#	5.66 ± 1.4#	0.59 ± 0.64
Linex forte-treated	7.42 ± 3.55#	2.92 ± 1.38#	3.92 ± 0.9#	4.83 ± 1.4#	1.5 ± 0.67*	0.42 ± 0.67#

Notes: *M ± SD (n=12). # p >0.05, * p<0.05, ** p <0.01, *** p <0.001 (compared to control)*

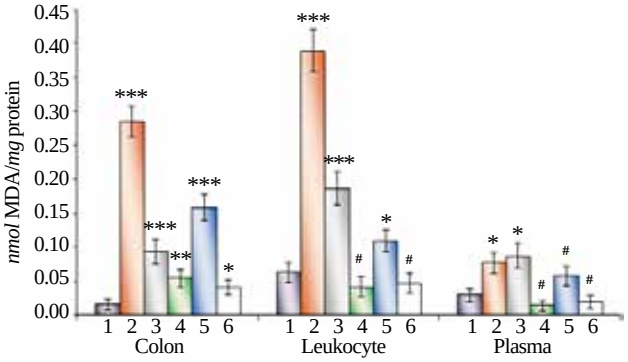

FIGURE 7. *Effect of probiotic mixture in free and immobilized forms and Linex forte on the lipid peroxidation processes in the colon, and blood following sodium dextran sulphate-induced ulcerative colitis.*

NOTES: *(1) - control, (2) - SDS/UC, (3) - Post SDS, (4) - PB, (5) - PBZ, (6) - Linex Forte, Data are expressed as M ± SEM, n=12. The confidence probability (p) of parameters evaluated compared to the control and designated as (#) - p >0.05, (*) - p <0.05, (**) - p <0.01, (***) - p<0.001.*

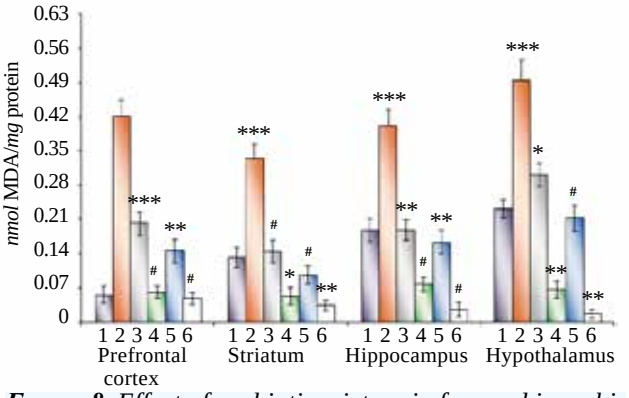

FIGURE 8. *Effect of probiotic mixture in free and immobilized forms and Linex forte on the lipid peroxidation processes in the brain corticolimbic regions following sodium dextran sulphate-induced ulcerative colitis.*

NOTES: *(1) - control, (2) - SDS/UC, (3) - Post SDS, (4) - PB,(5) - PBZ, (6) - Linex Forte, Data are expressed as M ± SEM, n=12. The confidence probability (p) of parameters evaluated compared to the control and designated as (#) - p >0.05, (*) - p <0.05, (**) - p <0.01, (***) - p<0.001.*

R et al., 2005; Bondarenko VM, Rybalchenko OV., 2009; Bravo JA, et al., 2012]. Probiotic bacteria lactobacillus and bifidobacterium attenuate inflammation in sodium dextran sulphate-induced colitis in mice and may be used for induction of remission in ulcerative colitis [*Mallon P et al., 2007; Toumi R et al., 2014*].

Effect of the selected probiotics in free and immobilized forms and Linex forte on the lipid peroxidation processes following DSS-induced ulcerative colitis

Maintaining a redox balance is crucial to preserve gut homeostasis. The gut cells respond to commensal microbiota or pathogens with immune tolerance and proinflammatory signals respectively. Reactive oxygen species produced by mucosa-resident cells or by newly recruited innate immune cells are essential for antimicrobial responses and regulation of signalling pathways including processes involved in wound healing. Overproduction of reactive oxygen species due to up-regulation of oxidases or altered mitochondrial function is linked to ulcerative colitis [*Aviello G., Knaus UG, 2017*]. sodium dextran sulphate-induced ulcerative colitis is also accompanied by stimulation of lipid peroxidation processes i.e., system-wide oxidative stress response [*Guevorkian AG et al., 2017*]. As shown in Fig. 7, the colonic MDA level was significantly increased in the sodium dextran sulphate/UC group (0.29 ± 0.023 *nmol MDA/mg protein*) compared to control (0.017 ± 0.008 *nmol MDA/mg protein*) ($p < 0.001$).

Colonic MDA content decreased in post-SDS group, remaining 5.6 times higher than control values. Treatment with probiotics and Linex forte reduced it approximately equally by half compared to self-healing processes, while PBZ interfered with suppression of lipid peroxidation in self-recovery group which requires further study. Notably, probiotics, PBZ and Linex forte practically normalized MDA levels increased by 6 and 2.6 times in the blood leucocyte and plasma following ulcerative colitis (Fig. 8).

At the same time, MDA level was increased by 7.5, 2.5, 2.1, and 2.1 times in the prefrontal cortex, striatum, hippocampus and hypothalamus respectively. Two weeks after stopping sodium dextran sulphate, it was normalized in the striatum and hippocampus, but was by 3.6 and 1.3 times high in the prefrontal cortex and hypothalamus compared to control. Of interest probiotics and Linex forte normalized the MDA content in prefrontal cortex, and dropped it drastically below the control: PB from 2.5 to 3.5 times, and Linex forte 3.8, 7.1, 13.7 times in the striatum, hippocampus and hypothalamus respectively). This may cause a decrease in the physiological level of oxidant challenge that is essential for governing life processes through redox signaling [*Sies H, 2017*]. In this respect the antioxidant effect of PBZ is more acceptable.

However, probiotics significantly inhibit lipid peroxidation preventing oxidative damage in the regions of corticolimbic system that may also contribute to their attenuation of histopathological changes in the brain and prevention mood disturbances following sodium dextran sulphate-induced ulcerative colitis.

In conclusion, the selected strains of probiotics with psycho-, antifungal activities in free form and immobilized on the micronized zeolite based composition of chemically modified natural minerals may decrease ulcerative colitis disease activity index score, improve some of the symptoms associated with UC, effectively restore gut microbiota balance, prevent signs of microbial translocation, alleviate symptoms associated with depression/anxiety and histopathological changes in colon and brain, and protected against oxidative stress via inhibition of lipid peroxidation in the colon, blood and regions of corticolimbic system following sodium dextran sulphate-induced UC. Nevertheless, further study is needed to confirm whether probiotics and/or PBZ are effective in managing UC patients in combination with conventional drugs to avoid or delay step-up therapies with drugs burdened by potentially serious side effects.

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