

ASSESSMENT OF ANTIOXIDANT
AND HEPATOPROTECTIVE ACTIVITY OF PROBIOTIC
FOOD LDB-ST AGAINST PARACETAMOL-INDUCED
LIVER DAMAGE IN RATS

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Abstract

Recently, the probiotics' therapeutic influence on different hepatic disorders has been established. Given the fact that free radicals are involved in the toxic liver injury pathogenesis, it was important to investigate whether the probiotic cocktail LDB-ST could be used as an antioxidant and a hepatoprotective agent.

The antioxidant and hepatoprotective activity of the probiotic food in question against Paracetamol (PCM) induced liver injury in 24 male Wistar rats (divided into four groups, $n = 6$) were evaluated.

The PCM-treatment (1200 mg/kg b. w., p. o.) resulted in serious hepatotoxic injury and increase in alanine aminotransferase, aspartate aminotransferase, total bilirubin and gamma-glutamyl transferase levels. On the other hand, the animals pre-treated with LDB-ST (1600 mg/kg b. w., p. o.) for 60 days prior to PCM-gavaging, showed significant improvement of the biochemical markers. The methodical LDB-ST-treatment also led to maintaining the reduced glutathione level to 71% and also reduced malondialdehyde production by 14%. The conducted histopathological examination reaffirmed the hepatoprotective effect of the probiotic food against PCM-induced liver damage, as there was no fatty degeneration observed and fewer focal necrosis, acidophilic necrosis, and nonspecific granulomas established (vs. PCM-overdosed group).

The oral long-term treatment with LDB-ST resulted in a significant protection against lipid peroxidation and oxidative stress in the applied animal model.

Key words: probiotics, LDB-ST, toxic liver injury, ROS, antioxidant, paracetamol

Introduction. The liver is the most metabolically active organ, which is involved in the biotransformation of some xenobiotics to toxic metabolites. This can lead to acute toxic liver injury and deleterious effect on liver functions [1, 2].

Acute toxic liver injury is characterized by membrane damage, massive hepatocytes necrosis, infiltration of parenchyma by neutrophils, and hepatic stellate cells activation. It is followed by liberation of proinflammatory cytokines, formation and accumulation of reactive oxygen species (ROS), as the latter are the main factor that cause liver cells damage [3]. In response to ROS, the human organism has developed different antioxidant defences. However, once the free radicals and ROS overcome the regulatory ability of the organism, a state of oxidative stress (OS) is established [4].

In the last decades, it was assumed that the intestinal microbiota may influence therapeutically different hepatic disorders [5]. In this regard, it has been proven that beside the popular probiotics' indications, they show a protective effect on liver diseases, such as hepatocellular carcinoma [6], liver cirrhosis [7], etc. In addition, a potential antioxidant activity of probiotics was reported, which should be researched in detail, given the harmful effect of ROS [4]. Thus, the aim of the present study was to assess the hepatoprotective and antioxidant effect of the time-tested probiotic cocktail LDB-ST (*Lactobacillus delbrueckii* subsp. *bulgaricus* strain DWT1); CCM 7992 (LDB) and *Streptococcus thermophilus* strain DWT4; CCM 7992 (ST), in a model of paracetamol-induced hepatotoxicity in rats.

Materials and methods. Substances. The bacterial cell culture LDB-ST was kindly provided by Daflorn Ltd. 1.0 mg of the substance contained 25×10^6 live and latent CFU *Lactobacillus delbrueckii* subsp. *bulgaricus* DWT1, *Lactobacillus helveticus* DWT2, *Lactobacillus lactis* DWT3, and *Streptococcus thermophilus* DWT 4, 5, 6, 7, 8. Paracetamol (PCM, $\geq 99.0\%$), diethyl ether, 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol, TBA), 10% neutral buffered formalin, and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were provided by Merck. Saline (NaCl 0.9%), Potassium dihydrogen phosphate and Trichloroacetic acid (TCA) were supplied by B. Braun, Scharlau Chemie SA and Valerus, respectively.

Animals. Twenty-four male Wistar rats (body weight (b.w.) 350 ± 20 g) were housed under standard hygienic conditions, in plastic cages with wood shavings as bedding. They were healthy and not genetically modified. A controlled environment (ambient temperature $23 \pm 5^\circ\text{C}$, relative humidity $50 \pm 10\%$, and alternating 12 h light/dark cycles) with ad libitum access to a standard pelletized

rat chow and water was provided. There were not any changes in animals' location during the experiments. Rats were monitored twice daily for signs of health problems as worsening of the physical condition, dyspnea or paralysis, that were determined as humane endpoints. In order to minimize potential confounders, rodents were treated at the same time and in the same order every day.

All experiment procedures were ethically approved by the *Commission of Foods Safety in the Bulgarian Ministry of Agriculture and Foods* (permission No. 175) and were conducted in accordance with the National Policies and the Council Directive (2010/63/EU) [8].

Design of PCM-induced liver toxicity model. After acclimatizing, all twenty-four animals were randomly divided into four groups ($n = 6$; to ensure statistical significance of the obtained results). All of the specimens were orally gavaged (via FTP-13-90 polypropylene plastic feeding tubes, Prime Bioscience PTE LTD) with provided for the purpose pre-dissolved in water substances, as follows:

Group 1 (Control group): animals, p. o. treated with normal saline for 60 days;

Group 2: animals, p. o. treated with normal saline for 60 days; 90 min after the last treatment (on the 60th day) the group was p. o. gavaged with PCM (1200 mg/kg b. w.) [9];

Group 3: animals, orally pre-treated with LDB-ST (1600 mg/kg b. w.) for 60 days; 90 min after the last treatment (on the 60th day) the group was p. o. gavaged with PCM (1200 mg/kg b. w.);

Group 4: animals, pre-treated with LDB-ST (1600 mg/kg b. w., p. o.) for 60 days [10].

All PCM-treated animals were strictly observed for lethality for a 24 h period.

Collection of blood and organs. At the end of the in vivo experiment (on the 61st day), all animals were sacrificed under diethyl ether anesthesia. Blood samples were collected from each specimen with the aim of the planned hematology and biochemical assays conduction.

Liver samples were also isolated. Half of them (from each group) were separated for histopathological examination and the other half for antioxidant power analysis. Then they were kept on dry ice during the transfer to the laboratory and were analysed as rapidly as possible.

Blood biochemical assay. The serum separation was conducted in accordance with the protocol of MAHMOOD et al. [11]. Then the samples were frozen at -20°C for the estimation of biochemical parameters. A series of serum biochemical tests of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and gamma-glutamyl transferase (GGT), were performed by a BA-88 Mindray serum - chemistry analyzer.

Assessment of lipid peroxidation of organs. The production of malondialdehyde (MDA) in the rats' tissue homogenates was evaluated and used as

a marker of lipid peroxidation [12]. The analysis was performed as follows: the extracted liver was homogenized with 0.1 M potassium buffer (pH 7.4) and 1.0 mL liver homogenate was mixed with 1.0 mL 25% TCA and 1.0 mL 0.67% TBA. The amount of MDA was spectrophotometrically measured at $\lambda = 535$ nm [13]. MDA quantity was measured by molar extinction coefficient $155 \text{ mM}^{-1}\text{cm}^{-1}$ and was expressed as nmol/g liver.

Assessment of non-enzyme antioxidant defense system. The glutathione (GSH) depletion was set as a marker of non-enzyme antioxidant defense system. The livers were subjected to procedures, involving extraction, washing out with a cold saline solution, weighing and homogenizing with appropriate buffers. The homogenates were centrifuged at 4000 rpm for 20 min with 5% TCA. The supernatant (1.0 mL) was mixed with potassium buffer (1.0 mL 0.1 M, pH 8) and 1.0 mL DTNB solution. Then, the reduced GSH level was spectrophotometrically determined at $\lambda = 412$ nm and expressed as nmol/g liver [14].

Histopathology. The organs intended for histopathological analysis were immediately dissected, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5.0 μm thick slices. Straining methods have been used according to the standard Hematoxylin-Eosin (HE) stain protocol, according to the methods of Bio-Optica staining kits [15]. All preparations were analyzed under light microscope (Leica DM 1000 LED) on 200 $\times$ magnification.

Statistical analysis. A statistical software package MedCalc was used for the data analysis. The obtained results were expressed as mean SD for six experiments. The significance of the data was assessed using the Mann–Whitney test ($p < 0.05$ was considered statistically significant). Statistical evaluation was performed using the GraphPad Prism 5.0 software.

Results and discussion. The probiotic cocktail LDB-ST was the focus of the present study, because of its recently proven modulation properties on the processes of inflammation, immune response and tumour progression [16–18].

Influence of LDB-ST on PCM-induced serum biochemical parameters. The PCM-treatment (1200 mg/kg b. w., p. o.) resulted in a serious hepatotoxic injury, evidenced by notable increase in AST, ALT, total bilirubin and GGT. On the contrary, the animals pretreated with LDB-ST (1600 mg/kg b. w., p. o.) for 60 days prior to PCM-gavaging showed significant improvement of the AST ($p < 0.01$), ALT ($p < 0.001$), GGT ($p < 0.001$) and the total bilirubin ($p < 0.01$) (vs. PCM-treated group) (Table 1).

Influence of LDB-ST on OS parameters in liver. A reduction in GSH levels by 48% ($p < 0.001$, vs. control group) and increasing of MDA by 14% ($p < 0.01$, vs. control group) were observed in the PCM-treated animals. Meanwhile, the long-term treatment with LDB-ST (prior PCM-intoxication) maintained the GSH levels to 71% ($p < 0.01$) and reduced MDA production by 14% ($p < 0.05$), both vs. PCM-treated animals. It may be assumed that the probiotic cocktail could exert hepatoprotective and radical scavenging activities by preventing the

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Effects of LDB-ST (1600 mg/kg b. w., p. o.) on AST, ALT, total bilirubin and GGT values in a model of PCM-induced toxicity in male Wistar rats

Parameter	Group 1	Group 2	Group 3
AST (U/L)	44.01 ± 7.64	80.16*** ± 2.27	55.83 ⁺⁺ ± 1.70
ALT (U/L)	50.25 ± 3.51	119.88*** ± 11.08	68.50 ⁺⁺⁺ ± 7.26
Total bilirubin (μmol/L)	1.35 ± 0.08	1.87** ± 0.13	1.23 ⁺⁺ ± 0.14
GGT (U/L)	1.857 ± 0.09	4.02*** ± 0.35	1.83 ⁺⁺⁺ ± 0.09
** <i>p</i> < 0.001, *** <i>p</i> < 0.001 vs. Control			
⁺⁺ <i>p</i> < 0.01, ⁺⁺⁺ <i>p</i> < 0.001 vs. PCM treatment			

formation of free radicals and peroxidation products, as well as an increase of the antioxidant defenses. The possibility of CYP2E1-inhibition should also not be overlooked, bearing in mind the ability of some probiotic strains to modulate the enzyme activity [19].

Histopathological examination. The data, obtained from the histopathological study correlated with the biochemical and hematological results reported above. The control group of animals demonstrated normal lobular architecture and hepatic cells (with intact cytoplasm), and well-defined sinusoids (Fig. 1A). The LDB-ST-gavaged animals showed well-preserved histological structure without any pathological changes too (Fig. 1B). As expected, the PCM-induced toxicity (Group 2) was observed in the form of severe hepatic cell damage (Fig. 2). Focal and acidophilic necroses in all liver lobes were established. Nonspecific granulomas and small-droplet steatosis were also detected. On the other hand, the LDB-ST pre-treatment (1600 mg/kg, b. w., p. o.) resulted in significantly lower histopathological changes after the PCM-gavaging (1200 mg/kg b. w., p. o.). The

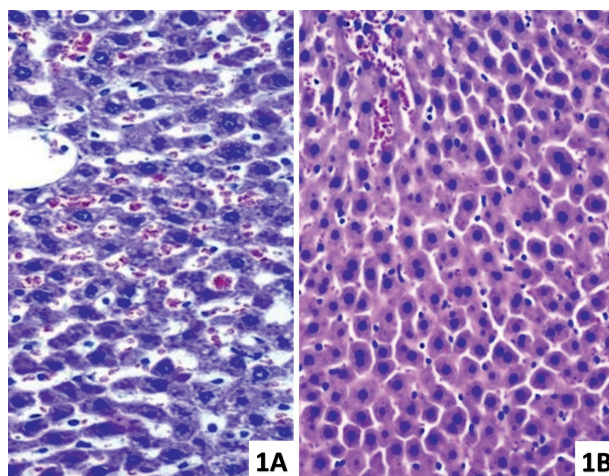


Fig. 1. Histopathological results of Group 1 and Group 4. 1A) Control group – normal liver architecture; 1B) Group 4. Normal liver architecture. HE staining – 1A, 1B × 200

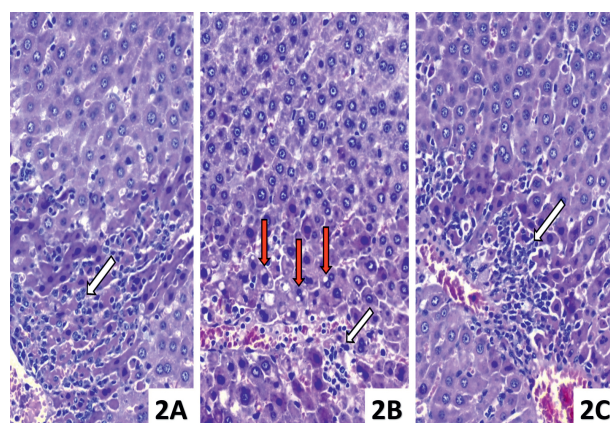


Fig. 2. Group 2 – PCM-overdosed animals (1200 mg/kg, b. w.). Impaired architecture of the liver; 2A) Degenerative altered hepatocytes and dense infiltrate of inflammatory cells (white arrow); 2B) Fatty degeneration (red arrows) and inflammatory infiltrate (white arrow); 2C) Damaged hepatocytes (white arrow) and dense inflammatory infiltrate mainly around *V. centralis*; HE staining – 2A, 2B, 2C $\times 200$

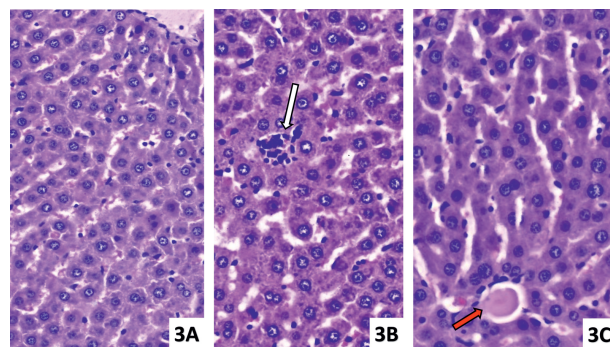


Fig. 3. Group 3 – Liver treated with PCM and LDB-ST; 3A) Normal liver architecture; 3B) Scarce inflammatory infiltrate in the liver parenchyma (white arrow); 3C) Apoptotic body surrounded by inflammatory cells (red arrow). HE staining – 3A, 3B, 3C $\times 200$

damage degree was notably reduced, as there were fewer focal necrosis, acidophilic necrosis, and nonspecific granulomas. In addition, no fatty degeneration was detected (Fig. 3).

All reported results support the initial hypothesis about the potential antioxidant and hepatoprotective properties of LDB-ST probiotic food.

Conclusion. The oral long-term treatment with the probiotic cocktail resulted in a significant protection against lipid peroxidation and OS in the applied model of PCM-induced liver injury. The use of bacterial strains minimized hepatic morphological changes and necrosis, proving once again that LDB-ST can contribute to prevention of OS and liver toxicity. The obtained results were especially valuable due the fact that PCM is a widely used analgesic and antipyretic, whose overdosing causes hundreds of deaths per annum in all industrialized countries.

REFERENCES

- [1] YANG Y. S., T. H. AHN, J. C. LEE et al. (2008) Protective effects of Pycnogenol on carbon tetrachloride-induced hepatotoxicity in Sprague-Dawley rats, *Food Chem. Toxicol.*, **46**(1), 380–387.
- [2] SINGH R., S. KUMAR, A. C. RANA, N. SHARMA (2012) Different models of hepatotoxicity and related liver diseases: A review, *Int. Res. J. Pharm.*, **3**, 86–95.
- [3] RAŠKOVIĆ A., N. PAVLOVIĆ, M. KVRGIĆ et al. (2015) Effects of pharmaceutical formulations containing thyme on carbon tetrachloride-induced liver injury in rats, *BMC Complement. Altern. Med.*, **18**(15), 442.
- [4] MISHRA V., C. SHAH, N. MOKASHE et al. (2015) Probiotics as potential antioxidants: a systematic review, *J. Agric. Food Chem.*, **63**(14), 3615–3626.
- [5] CHASSAING B., L. ETIENNE-MESMIN, A. T. GEWIRTZ (2014) Microbiota-liver axis in hepatic disease, *Hepatology*, **59**, 328–339.
- [6] LI J., C. Y. J. SUNG, N. LEE et al. (2016) Probiotics modulated gut microbiota suppresses hepatocellular carcinoma growth in mice, *Proc. Natl. Acad. Sci. USA*, **113**(9), 1306–1315.
- [7] BRENNER D. A., Y. H. PAIK, B. SCHNABL (2015) Role of gut microbiota in liver disease, *J. Clin. Gastroenterol.*, **49**(1), 25–27.
- [8] European Parliament (2010) Directive 2010/63/EU on the protection of animals used for scientific purposes, *Official Journal of the European Union*, **276**, 33–79.
- [9] AKINTONWA A., A. R. ESSIEN (1990) Protective effects of *Garcinia kola* seed extract against paracetamol-induced hepatotoxicity in rats, *J. Ethnopharmacol.*, **29**(2), 207–211.
- [10] GEORGIEVA M., E. SOFTOVA, M. GABROVSKA et al. (2008) Influence of the “Bios-tim LBS” probiotic on morphological liver changes in experimentally induced hepatotoxicity, *Acta Morphol. Anthropol.*, **13**, 149–153.
- [11] MAHMOOD N. D., S. S. MAMAT, F. H. KAMISAN et al. (2014) Amelioration of paracetamol-induced hepatotoxicity in rat by the administration of methanol extract of *Muntingia calabura* L. leaves, *Biomed Res. Int.*, 695678.
- [12] CHAVKO M., A. L. HARABIN (1996) Regional lipid peroxidation and protein oxidation in rat brain after hyperbaric oxygen exposure, *Free Radic. Biol. Med.*, **20**, 973–978.
- [13] DEBY C., R. GOUTIER (1990) New perspectives on the biochemistry of superoxide anion and the efficiency of superoxide dismutases, *Biochem. Pharmacol.*, **39**, 399–405.
- [14] FAU D., A. BERSON, D. EUGENE et al. (1992) Mechanism for the hepatotoxicity of the antiandrogen, nilutamide. Evidence suggesting that redox cycling of this nitroaromatic drug leads to oxidative stress in isolated hepatocytes, *J. Pharmacol. Exp. Ther.*, **263**, 69–77.
- [15] HEMATOXYLIN EOSIN STAINING KIT for AUS 240 stainer with isoparaffinic solvent 21 – A140EI. Bio-Optica Milano s.p.a. CND Code: W01030708 [updated 2017 May 02; cited 2021 Jan 08]. Available from: https://bio-optica.it/ftp/Sito/technical_datasheet/A140EI_en.pdf (Accessed February 08, 2021).
- [16] GEORGIEVA M., K. GEORGIEV, P. DOBROMIROV (2015) Probiotics and Immunity, Immunopathology and Immunomodulation, InTech, <https://doi.org/10.5772/61337>.

- [17] GEORGIEV K., M. GEORGIEVA, I. ILIEV et al. (2015) Antiproliferative effect of Bulgarian spring water probiotics (Laktera nature probiotic®) against human colon carcinoma cell line, *World J. Pharm. Pharm. Sci.*, **4**(6), 130–136.
- [18] GUHA D., A. BANERJEE, R. MUKHERJEE et al. (2019) A probiotic formulation containing *Lactobacillus bulgaricus* DWT1 inhibits tumor growth by activating pro-inflammatory responses in macrophages, *J. Funct. Foods*, **56**, 232–245.
- [19] MATUŠKOVÁ Z., M. ŠILLER, A. TUNKOVA et al. (2011) Effects of *Lactobacillus casei* on the expression and the activity of cytochromes P450 and on the CYP mRNA level in the intestine and the liver of male rats, *Neuroendocrinol. Lett.*, **32**(1), 8–14.

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