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EVALUATION OF THE TOXICOLOGICAL SAFETY OF A FREEZE-DRIED PROBIOTIC PREPARATION

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Abstract: *A combination consisting of selected probiotic strains lactobacilli and bifidobacteria from the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC), Bulgaria was developed. During their joint cultivation the strains demonstrated their ability to multiplicate, accumulating high concentrations of viable cells.*
The toxicogenity of the symbiotic combination was examined by testing the freeze-dryed substance, containing lactobacilli and bifidobacteria, on biological objects (white mice). Each experimental animal was injected intraperitoneally with 5.10[8] CFU viable cells daily in the duration of 10 days.
After the experimental period a post-mortem examination was conducted to analyze the safety of the substance of probiotic lactobacilli and bifidobacteria, which is the basis for the preparation of probiotic combinations. The absence of toxicogenity of the freeze-dried substance of probiotic lactobacilli and bifidobacteria is proven by the lack of any pathological changes in the health status, damages to the parenchymal organs, the peritoneum or the omentum that would otherwise indicate toxicity.

Keywords: *probiotic, toxicology, safety, Lactobacillus, Bifidobacterium*

1. Introduction

Probiotics are biologically active preparations containing high concentrations of viable cells of bifidobacteria and lactobacilli as well as products of their metabolism that confer a health benefit to the host when administered in adequate amounts [1, 2]. There is growing scientific evidence for the number of beneficial effects of live cells of bifidobacteria and lactobacilli on a variety of gastrointestinal, immunological and metabolic conditions [3].
Many strains have obtained the generally recognized as safe (GRAS) status or have been determined to be safe for their traditional food uses [4]. Studies have also demonstrated that most *Lactobacillus* species show no pathogenecity or acute oral toxicity to animals [5, 6] or humans [7, 8]. Although there is a reasonable number of well characterized probiotic strains available for commercial use around the world [9, 10, 11], the isolation and characterization of new strains is still desirable. Although traditional *Lactobacillus* strains have an excellent history of safe use in the formation of dairy products and other foods and some have "generally recognized as safe" (GRAS) status [12], newly isolated organisms often have no previous history of food product use and do not necessarily share the GRAS status of traditional *Lactobacillus* strains.

Therefore, it is necessary to conduct the safety assessment on any new strain or combination of strains prior to being added into foods or used as a dietary supplement [13]. In general, in vitro data alone is not sufficient to describe strains as probiotics, but they still remain valuable and can provide scientific insight into characteristics of probiotic organisms. Therefore, the selection of suitable strains may be further refined by undertaking a series of in vitro experiments [14]. Additionally, in vitro tests are still recommended by the FAO/WHO [15] for an initial screening of potential probiotic bacteria. In addition to the traditional selection procedures, in recent years, knowledge on intestinal microbiota, nutrition, immunity and mechanisms of action has increased dramatically and can now be combined with genomic data to allow the isolation and characterization of new target- or site-specific probiotics [16]. In order for the newly isolated and characterized probiotic strains to be included in the composition of probiotic preparations, both in vitro and in vivo testings need to be conducted.

The aim of the present work was to investigate *in vivo* the toxicogenity of a developed freeze-dried probiotic preparation containing lactobacilli and bifidobacteria in experimental animals - mice.

2. Materials and methods

Preparation of the material for toxicogenity testing. The probiotic strains *Lactobacillus delbrueckii* ssp. *bulgaricus* NBIMCC 3706, *Lactobacillus acidophilus* NBIMCC 2416, *Lactobacillus plantarum* NBIMCC 2415, *Bifidobacterium bifidum* NBIMCC 3601 were propagated in a fermenter, concentrated and freeze-dried in compliance with standard operating procedures (SOP) and quality control (QC) procedures at Probiotical S.p.A (Novara, Italy). Microbiological analyses were done and bacterial culture purity was confirmed immediately after production, at study baseline and throughout the study. The strains were grown in the optimal medium for the optimal time needed for each strain to accumulate 10^{10}CFU/cm^3 and the single strain cultural suspensions were freeze-dried. The freeze-dried single strain products of the four probiotic strains *Lactobacillus delbrueckii* ssp. *bulgaricus* NBIMCC 3706, *Lactobacillus acidophilus* NBIMCC 2416, *Lactobacillus plantarum* NBIMCC 2415, *Bifidobacterium bifidum* NBIMCC 3601 were mixed in a ratio of 1 : 1 : 1 : 1 in order to obtain the material for toxicogenity testing. Thus the toxicogenity of the four probiotic strains in the composition of the tested material can be determined simultaneously.

Toxicogenity testing – study design.

The freeze-dried probiotic substance of lactobacilli and bifidobacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus* NBIMCC 3706, *Lactobacillus acidophilus* NBIMCC 2416, *Lactobacillus plantarum* NBIMCC 2415, *Bifidobacterium bifidum* NBIMCC 3601), was examined in order to determine the concentration of viable cells of lactobacilli according to the ISO 7889 and BS 10945-91 standards in sterile milk to determine the most probable number according to McCrady. The material for toxicogenity testing was diluted ten times in sterile, inert diluent. The toxicogenity analysis was conducted by parenteral (intraperitoneal) daily injection in white mice for a period of 14 days. The white mice were injected intraperitoneally with 0,5 cm^3 of the suspension of the diluted material in one and the same place - the inguinal canal – in order to obtain comparable results.

Experimental groups.
The following groups of experimental animals were used in the experiment:
1. Control group - untreated - 10 mice (5 male and 5 female);
2. Experimental group - treated with diluted material - 20 mice (10 male and 10 female).
Both groups of experimental animals were grown in standard mode with experimental housing food and water ad libitum and were observed (general physical appearance, body weight, food consumption and abnormalities) daily for 10 days prior to the experiment.

Post-mortem examination.
Animal use was approved by IACUC. All mice were humanely sacrificed at the end of the test and a complete necropsy was conducted. After the experimental period post-mortem examination in „LABOREKS" AD - Laboratory „Pathomorphology" was conducted. A macroscopic examination of the internal organs - liver, spleen, kidneys and adrenals – was done. Stomach, small and large intestine, spleen, liver, kidney, adrenals, peritoneum and omentum samples were histologically tested 10 days after the injection of lactic acid bacteria in the peritoneal cavity of the treated animals. After fixing the tissues in 4% neutral formalin the samples were incorporated into paraffin cubes. The obtained microscopic preparations were stained with hemalaun eosin. A transmission electronic microscopic examination on liver, mediastinum and omentum from control and experimental animals was conducted. The abdominal wall from the application site from the experimental animals was also observed.

3. Results and discussion
Suitable strains of lactobacilli and bifidobacteria for incorporation in starter cultures for fermented milk products, probiotics and probiotic foods and drinks were chosen from the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC), Bulgaria. They have the ability to reproduce in the model conditions of digestion, to synthesize lactic and other organic acids, bacteriocins, which inhibit the growth of pathogens, causing toxemia, toxic fungal infections [18]. They also allow the accumulation of high concentrations of viable cells in the processes of fermentation, immobilisation and freeze-drying and also retain viability under storage conditions.
The experiment was performed using the prepared freeze-dried substance of lactobacilli and bifidobacteria (consisting of the strains *Lactobacillus delbrueckii* ssp. *bulgaricus* NBIMCC 3706, *Lactobacillus acidophilus* NBIMCC 2416, *Lactobacillus plantarum* NBIMCC 2415, *Bifidobacterium bifidum* NBIMCC 3601) for the production of probiotic preparations and the used biological objects were white mice. After ten-day monitoring, post-mortem examination was conducted including macroscopic examination, histological findings and a transmission electronic microscopic (TEM) study.
There were no statistically significant differences in body weights and in weekly food consumption between the treatment groups and the control group. No treatment-related adverse effects and no deaths were observed.

Macroscopic Examination
The internal organs - liver, spleen, kidneys and adrenals - showed no visible changes under examination.

Their shape, size and color were unchanged. The stomach, the small and the large intestines were whitish pink, the serous was smooth and shiny. There were no obviously enlarged Peyer's patches. The

peritoneum and the omentum had smooth surfaces without adhesion and nodule formation.

Histological Findings

The stomach, small and large intestine, spleen, liver, kidney, adrenals, peritoneum and omentum samples were histologically tested. The study of these eight organs was conducted 10 days after the infiltration of lactic acid bacteria in the peritoneal cavity of the treated animals.

No changes in the parenchymal organs were established in all the animals included in the study (from both groups – experimental and control) except for the vascular changes indicating acute venous stasis (reaction to euthanasia).

In the experimental group some changes in the serous and the subserous layer of the examined organs were observed - proliferation of histiocytic elements and appearance of macrophages (Fig. 1).

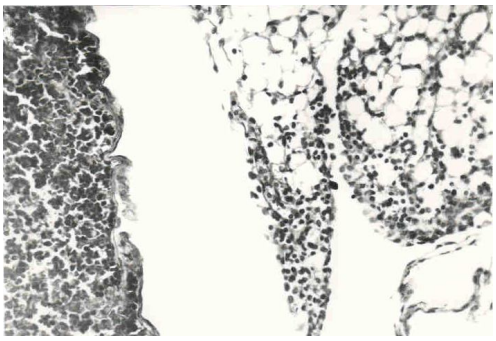

Fig. 1. TEM: A fragment of a spleen and adipose tissue. Magnification 25x. Subserous proliferation of macrophages, some of which have the characteristics of giant macrophages. Adipose tissue with more extensive proliferation of histiocytes and macrophages.

The process was localized and it was less pronounced in the subserous, while larger clusters were found on the serous surface. In the adipose tissue of the peritoneum and the omentum, similar clusters of the described cellular elements were observed as well (Fig. 2).

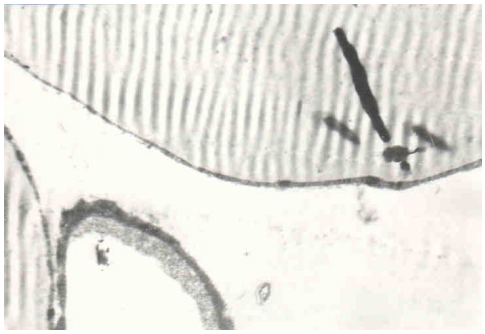

Fig. 2. TEM: Adipose tissue. Treated group. Magnification 4000. Adipocytes, bacteria at different depths in the cell.

The relatively poor bacterial cell proliferation is due to the rapid elimination of lactic acid bacteria.

It is believed that this reaction is a response to the injected lactic acid bacteria (+ inert solvent, which would produce the same response). The observed changes are protective and physiological - a „foreign body" reaction.

In some animals of both sexes two types of bacteria were histologically established - one morphological type of lactic acid bacteria - probably cocci (Fig. 3) and lactobacilli (Fig. 4, Fig. 5).

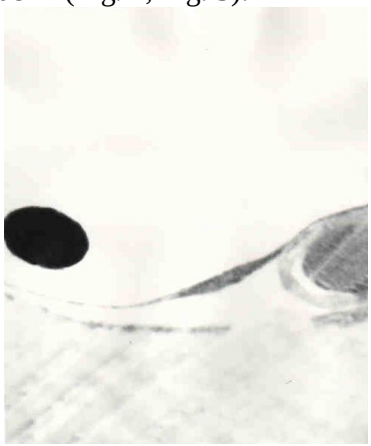

Fig. 3. TEM: Adipose tissue. Tested group. Magnification 4000. Presence of cocci-shaped bacteria.

Most likely, these bacteria are the lactobacilli introduced but it is possible that some of them endogenously penetrate from the intestinal tract.

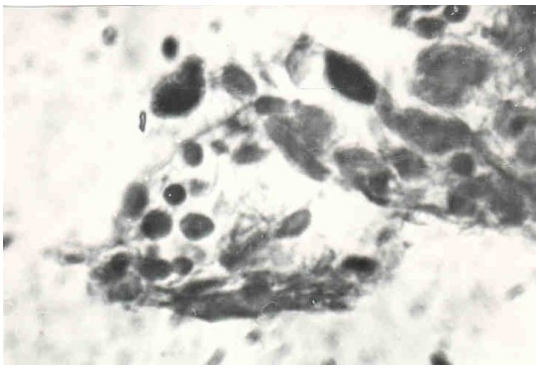

Fig. 4. TEM: Adipose tissue from the omentum.. Magnification 63x. Macrophages with ingested cocci in the cytoplasm.

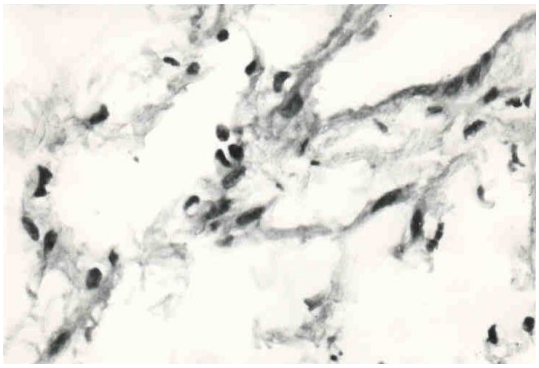

Fig. 5. TEM: Adipose tissue. Magnification 40x. Presence of lactobacilli.

Electron microscopic examination

The ultrastructural study was conducted on liver, mediastinum and omentum from control and experimental animals. The abdominal wall from the injection site from the experimental animals was also observed.

The tissues were fixed in glutaraldehyde and samples were prepared for TEM examination.

Liver - There were no differences between control and experimental groups. The ultrastructure of hepatocytes - nuclear apparatus, mitochondria, granular endoplasmic reticulum, smooth endoplasmic reticulum, Golgi apparatus - was unchanged. In some control and experimental animals tiny fatty droplets were found. No proliferation of Kupfer's cells in the sinuses was found and the μW were normal. Bile ducts showed no pathological changes. A characteristic distinguishing feature of murine liver is the presence of multiple argirophilic granules in the hepatocytes (Fig. 6a, Fig. 6b, Fig. 6c, Fig. 6d).

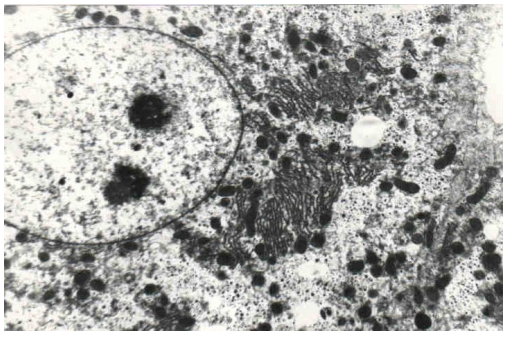

Fig. 6a. TEM: Liver. Control. Magnification 4000. Hepatocyte with a nucleus with two nucleolus. Single fatty droplets, normal cellular ultrastructure.

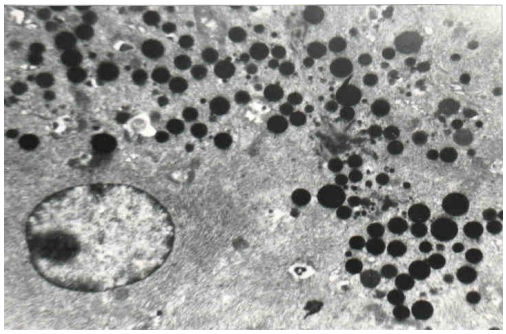

Fig. 6b. TEM: Liver. Control. Magnification 4000. Two border hepatocytes. Many argirophilic granules.

Mediastinum, omentum - In two of the experimental animals groups of two types fully preserved bacteria were found in the striae fat cells.

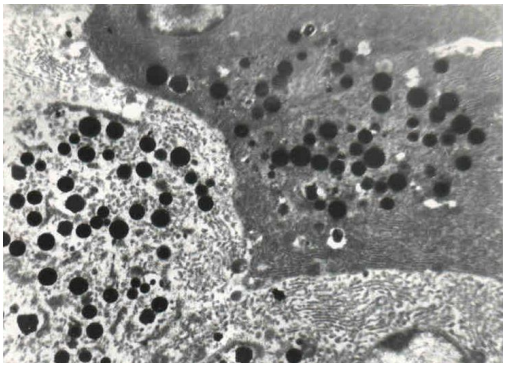

Fig. 6c. TEM: Liver. Tested group. Magnification 4000. Four border hepatocytes, argirophilic granules, preserved ergastoplasm.

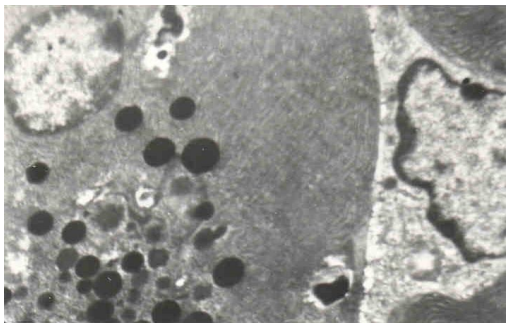

Fig. 6d. TEM: Liver. Tested group. Magnification 5000. A hepatocyte, a smooth sinus, unactivated Kupfer's cell.

Section of the abdominal wall - The examination of the abdominal wall of the experimental animals showed no evidence of lactic acid bacteria (Fig. 7a, Fig. 7b).

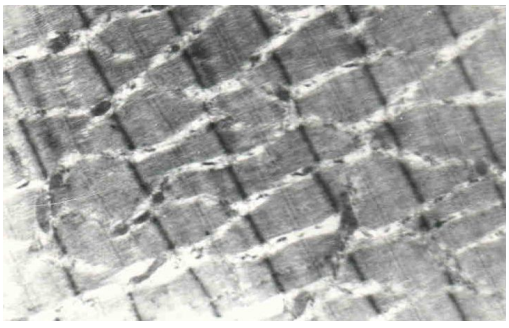

Fig. 7a. TEM: Abdominal wall. Magnification 9000. Longitudinal cut. Striated muscles without ultrastructural changes.

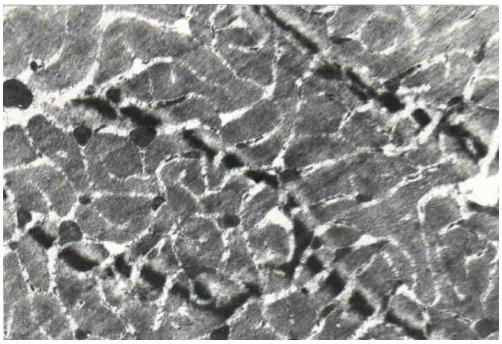

Fig. 7b. TEM: Abdominal wall. Magnification 9000. Transverse section. Striated muscles without ultrastructural changes.

It has been strongly recommended to confirm the safety of any newly isolated stain for developing combinations of strains before their incorporation into food products. As many *Lactobacillus* and *Bifidobacterium* strains have the GRAS classification due to their long history of safe use in food production (Donohue and Salminen, 1996), the studies demonstrated that most of the *Lactobacillus* and *Bifidobacterium* species show no pathogenecity or acute oral toxicity to animals [5, 6, 18] or humans [7, 8] which is consistent with our results.

4. Conclusion

During the whole ten-day experimental period of the tested freeze-dried substance of lactobacilli and bifidobacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus* NBIMCC 3706, *Lactobacillus acidophilus* NBIMCC 2416, *Lactobacillus plantarum* NBIMCC 2415, *Bifidobacterium bifidum* NBIMCC 3601), the control and the experimental animals showed no changes in their behavioral responses. There were no pathological changes in the health status or the mortality rate. The histomorphologic and electron microscopic studies suggest the following conclusion: the only histomorphologic change was the physiological response of the tissue to

injection injury – a "foreign body" response. There was some evidence of local penetration of lactobacilli and cocci-shaped bacteria from the intestinal tract of the experimental animals, which were confirmed by electron microscopic observation. There was no evidence for parenchymal organ, peritoneum and omentum damages indicating toxicity. The overall conclusion for the freeze-dried substance of probiotic lactobacilli and bifidobacteria is the absence of toxicogenity.

5. Acknowledgements

The examined freeze-dried substance of probiotic lactobacilli and bifidobacteria was the basis for the development of a series of the probiotic preparations „Enterosan", Bulgarian patent №103295, [19].

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