Immunomodulatory effect of *Lactobacillus rhamnosus* GG from low fat fresh cheese "BioAktiv LGG"

Stjepan Mahnet, Slavko Kirin, Blaženka Kos, Jadranka Frece, Jagoda Šušković

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Summary

Various immune responses have been influenced by probiotics and these immunomodulatory effects have been proposed for several potential applications such as the prevention of infectious diarrhoea, alleviation of hypersensitivity reactions and tumour suppression. The new probiotic product from LURA d. d., low fat fresh cheese "BioAktiv LGG", contains well-known probiotic strain ***Lactobacillus rhamnosus*** *GG (LGG). The viable count of* ***L. rhamnosus*** *GG remained stable and has been $1.3x10^8$ CFU/g of low fat fresh cheese "BioAktiv LGG" during storage of 16 days at 4 ^{0}C. The effect of* ***L. rhamnosus*** *GG from this product on the immune response, as an important point in relation to immunomodulation, and its survival/residence time in the gastrointestinal tract in Swiss albino mice was also studied. During the feeding of mice with* ***L. rhamnosus*** *GG, with a daily dose of $2.2x10^{10}$ cells, the number of lactobacilli in faeces was increased and reduction of enterobacteria and sulphite-reducing clostridia was observed. The similar results were obtained in homogenates of large intestine of mice in the 1st and in the 14th day after feeding with* ***L. rhamnosus*** *GG, which pointed out the adherence ability of examined strain. Furthermore, the oral immunization of Swiss albino mice was performed with a dose of $2.2x10^{10}$* ***L. rhamnosus*** *GG cells per day, for eight consecutive days. The blood samples were collected on the 4th, 8th, 10th, 14th and 21st day after the 1st immunization. The sera of mice were tested for total and specific anti-lactobacilli IgA, IgG and IgM antibodies by ELISA assay.* ***L. rhamnosus*** *GG slightly stimulated the total humoral immune response but didn't evoke reaction against itself, which confirms its probiotic properties and make new probiotic product, low fat fresh cheese "BioAktiv LGG", as good carrier for this probiotic strain.*

Key words: ***Lactobacillus rhamnosus****, probiotic properties, adhesion, immunomodulation, antibodies, ELISA*

Introduction

Probiotic-containing products come in a variety of formats: conventional foods (probiotic-containing yoghurts, fluid milk and cheese, consumed primarily for nutritional purpose, but also for probiotic benefits), food supplements or fermented milks (food formulations whose primary purpose is a delivery vehicle for probiotic bacteria and their fermentation end products, consumed for health effects, in monoculture or mixed culture, and dietary supplements (capsules and other formats designed to be taken by healthy individuals looking to enhance health) (Sanders and Huis in't Veld, 1999). Only a few studies have been published about probiotics in cheese. However, high levels of fat or salt have not been considered in the development of probiotic cheeses (Ryhänen *et al.* 2001). The LURA d.d. developed a new type of low fat fresh cheese "BioAktiv LGG" with *Lactobacillus rhamnosus* GG (LGG) which is claimed to have probiotic properties. Probiotics have been reported to have several healthy benefits such as balancing of intestinal microflora, stimulation of the immune system, prevention of diarrhoea and anticarcinogenic activity (Šušković *et al.* 2001).

When pathogenic bacteria and viruses invade the body from the exterior, the body has the capability to recognize these as foreign substances and act to remove them. This capability is generally termed the "immune system", which includes the humoral and the cellular immune systems. Lactic acid bacteria and its components can act on the immune system within the body, and in many cases enhance the immune response (Yasui *et al.* 1999). The aim of this work was to determine the viable count of *L. rhamnosus* GG (LGG), in low fat fresh cheese "BioAktiv LGG" from LURA d.d. during storage, survival and adhesion of this strain in the gastrointestinal tract of mice and to assess its immunomodulatory effect.

Materials and Methods

Determination of the viable count of *L. rhamnosus* GG during storage

To assess the viability of *L. rhamnosus* GG in "BioAktiv LGG" low fat fresh cheese, during refrigerated storage, with appropriate dilutions from cheese were prepared and spread on MRS agar plates containing vancomycin. The plates were incubated for 48 h at 37 ^{o}C.

Determination of the viable count of *L. rhamnosus* GG during the transit trough gastrointestinal (GI) tract of mice

Survival of *L. rhamnosus* GG during transit trough GI tract of mice was determined in faeces samples. Fresh faecal samples were taken from single Swiss albino mice, which had been isolated in plastic cages. The mice had been fed by probiotic strain *L. rhamnosus* GG with a dose of $2.2 \cdot 10^{10}$ of viable bacterial cells. Each faecal sample was used to determine faecal microflora. Samples of approximately 1 g (wet weight) were suspended in 1 mL sterile water. After homogenisation an appropriate dilutions (100 μL) were prepared, and inoculated onto non-selective media (Peptone yeast extract glucose agar for aerobic and anaerobic counts), and four selective media: MRS-agar for *Lactobacillus* counts, Violet red bile glucose agar and Eosin methylene blue agar for *Enterobacteriaceae* counts, and sulphite agar for sulphite-reducing clostridia counts.

In vitro test for adhesion of *L. rhamnosus* GG

Adhesion of *L. rhamnosus* GG to the intestinal epithelium of the mouse was performed using the method of Mäyrä-Mäkinen *et al.* (1983). The standard microscope slides were stained by method for gram-positive and gram-negative bacteria identification according to Brown and Brenn (Švob, 1974). Slides were examined and photographed by light microscope Nikon Mikrophot-FXA.

In vivo adherence ability of *L. rhamnosus* GG

Adherence ability of examined probiotic strain was determined in homogenates of large intestine of Swiss albino mice on the 1^{st} and 14^{th} day after feeding with *L. rhamnosus* GG with a daily dose of $2.2 \cdot 10^{10}$ viable cells for eight consecutive days. Appropriate dilutions of homogenates (100 μl) of large intestine were prepared, and inoculated onto non-selective media (Peptone yeast extract glucose agar for aerobic and anaerobic counts), and four selective media: MRS-agar for *Lactobacillus* counts, Violet red bile glucose agar and Eosin methylene blue agar for *Enterobacteriaceae* counts, and sulphite agar for sulphite-reducing clostridia counts.

Immunization of mice with *L. rhamnosus* GG and determination of antibodies by ELISA assay

The oral immunization was performed eight times over period of eight successive days. Each day a mice was given a dose of $2.2 \cdot 10^{10}$ viable bacterial cells of *L. rhamnosus* GG in sterile saline solution, by automatic 200 μL pipette, directly into the mouth. The control group was challenged with 200 μL of sterile saline solution. On the 4^{th}, 8^{th}, 10^{th}, 14^{th} and 21^{st} days, after first immunization, the blood samples were collected by bleeding of the tail vein directly into the tubes, allowed to clot at room temperature for 3 hours and then centrifuged at 3000g for 10 min. The sera samples were kept at -20 ^{o}C until use. The total and specific anti-lactobacilli (IgA, IgG and IgM) antibodies were determined by ELISA (Enzyme-Linked Immunosorbent Assay).

Results and Discussion

The "BioAktiv LGG" low fat fresh cheese, from LURA d.d., contains well-known probiotic strain *Lactobacillus rhamnosus* GG (LGG). It seams reasonable to assume that adequate numbers of the probiotic bacteria need to be consumed with probiotic cheese to exert a health-promoting effect for the consumer. It has been suggested that, in order to have any therapeutic effect, the minimal number of probiotic bacteria in a product should be at 10^6-10^8 per gram (Kullen and Klaenhammer, 1999). Moreover, it is important that the lactobacilli remain viable during refrigerated storage of the product for a certain period as fresh cheese may be consumed after storage in the refrigerator for several weeks. The results of Schillinger (1999) indicated that not all products, available on the market in Germany and other European countries, contain probiotic bacteria in high numbers and that not all of them show good survival characteristics. *L. rhamnosus* GG viable counts obtained from low fat fresh cheese during 16 days of storage at 4 ^{o}C showed high numbers (above 10^8 per gram) even at the end of the storage period and therefore survived well in the examined cheese (Fig. 1). The effect of *L. rhamnosus* GG from this product on the immune response, and its survival/residence time in the gastrointestinal tract in Swiss albino mice was also examined, as this is an important point in relation to immunomodulation.

I*n vitro* adhesion ability of *L. rhamnosus* GG was studied as a prerequisite for *in vivo* adhesion of this strain in Swiss albino mice. Results observed by *in vitro* adhesion test have shown very good adhesive ability of an examined strain to intestinal epithelial cells of mice (Fig. 2). These results are in

agreement with adhesion results observed on the porcine gastric epithelial cells, performed by the same method (Mahnet *et al.* 2002). Survival of *L. rhamnosus* GG during transit trough GI tract of mice was determined in faeces

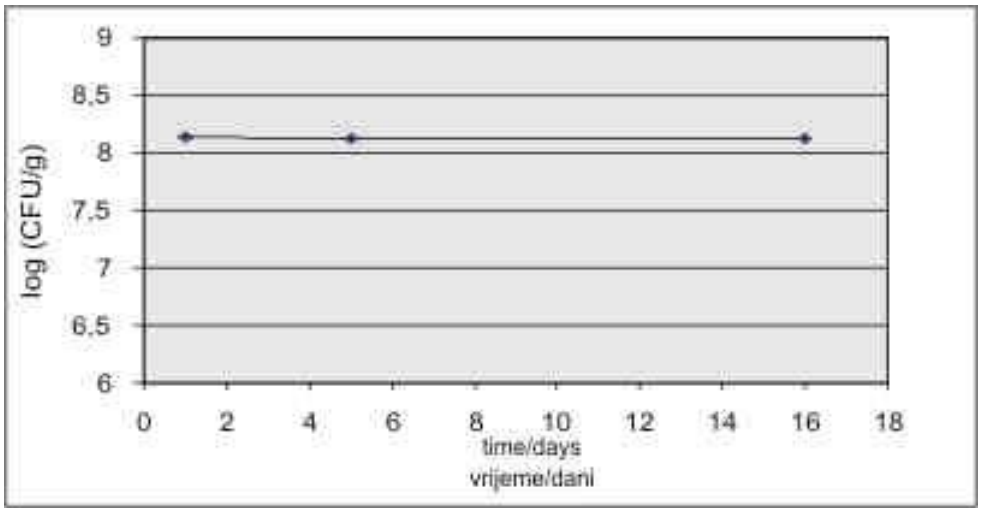

Fig. 1: Survival of ***Lactobacillus rhamnosus*** *GG in low fat fresh cheese "BioAktiv LGG" during storage at 4 °C*

Slika 1: Preživljavanje ***Lactobacillus rhamnosus*** *GG u svježem posnom siru "BioAktiv LGG" tijekom skladištenja pri 4 °C*

A B

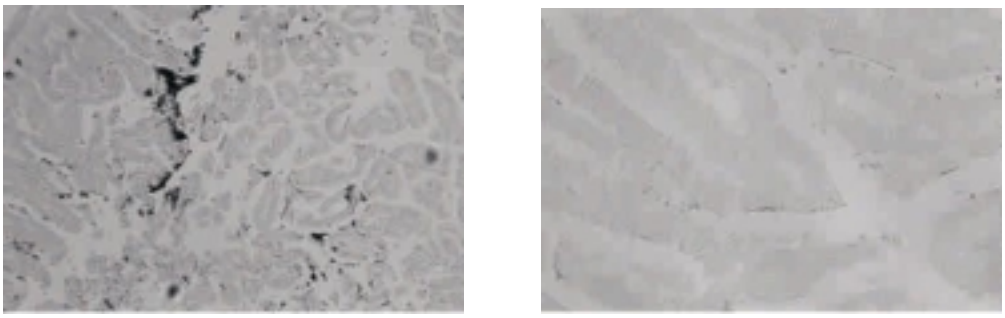

Fig 2: Adhesion of ***Lactobacillus rhamnosus*** *GG to the intestinal epithelial cells of the mouse. A - Magnification, x 250; B - Magnification, x 1000.*

Slika 2: Adhezija stanica ***Lactobacillus rhamnosus*** *GG na epitelne stanice ileuma miša. A – Povećanje, x 250; B – Povećanje, x 1000.*

samples. The number of lactobacilli was increased, and reduction of enterobacteria and sulphite-reducing clostridia was observed (Fig 3). These results suggested capability of *L. rhamnosus* GG to survive transit through the mouse gastrointestinal tract, and to interact and compete with other

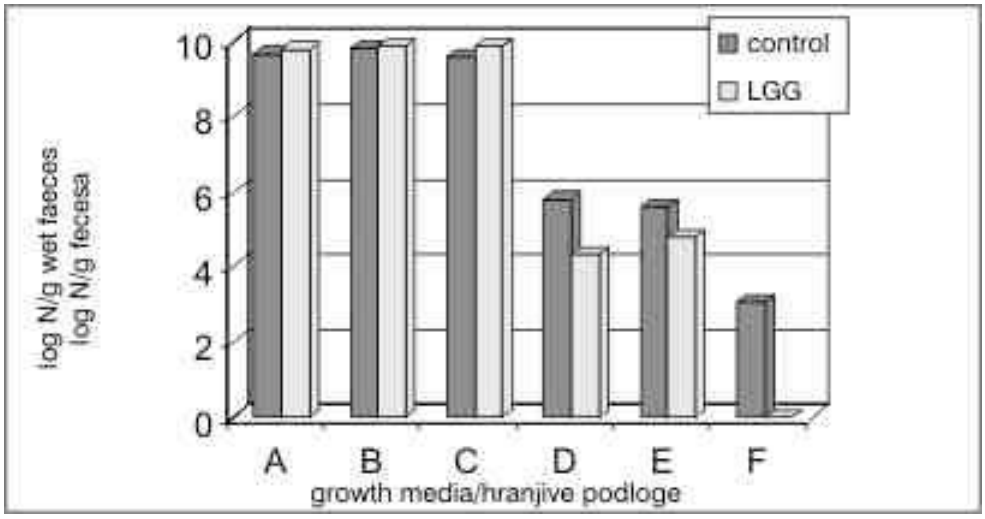

Fig. 3: Comparison of the microbial population in mice faeces before and after feeding with ***Lactobacillus rhamnosus*** *GG. A – aerobic bacteria; B – anaerobic bacteria; C – lactic acid bacteria; D –* ***Enterobacteriaceae*** *from Violet Red Bile Glucose agar; E –* ***Enterobacteriaceae*** *from Eosin Methylene Blue agar; F – sulphite-reducing clostridia*

Slika 3: Usporedba mikrobne populacije u fecesu miševa prije i nakon hranjenja miševa s ***Lactobacillus rhamnosus*** *GG. A – aerobne bakterije; B – anaerobne bakterije; C – bakterije mliječne kiseline;* ***Enterobacteriaceae*** *na ljubičasto-crvenom žučnom agaru; E –* ***Enterobacteriaceae*** *na eozin metilen plavom agaru; F - sulfito-reducirajući klostridiji*

microorganisms within the gut environment. In our previous study, *L. rhamnosus* GG from different types of BioAktiv LGG yoghurts, a high degree of antimicrobial activity against some enteropathogens, which could influence its ability to survive and to adhere in the intestinal tract and operate as an effective probiotic by suppressing some harmful bacteria (Mahnet *et al*. 2002), was determined.

In vivo adherence ability of *L. rhamnosus* GG was determined in homogenates of large intestine of Swiss albino mice. The increased number of lactobacilli in large intestine, especially one day after feeding with *L. rhamnosus* GG, pointed out the adherence ability of examined probiotic strain. The composition of other bacterial strains in the large intestine was also strongly influenced by administrated *L. rhamnosus* GG - the number of enterobacteria and sulphite-reducing clostridia was decreased. (Fig. 4). These results confirm the good survival and binding of administrated probiotic strain to the intestinal epithelial cells. This observation is supported by the results of a human study completed by Goldin *et al.* (1992) where it was demonstrated that 60-80 % of individuals consuming *L. rhamnosus* GG excreted this strain

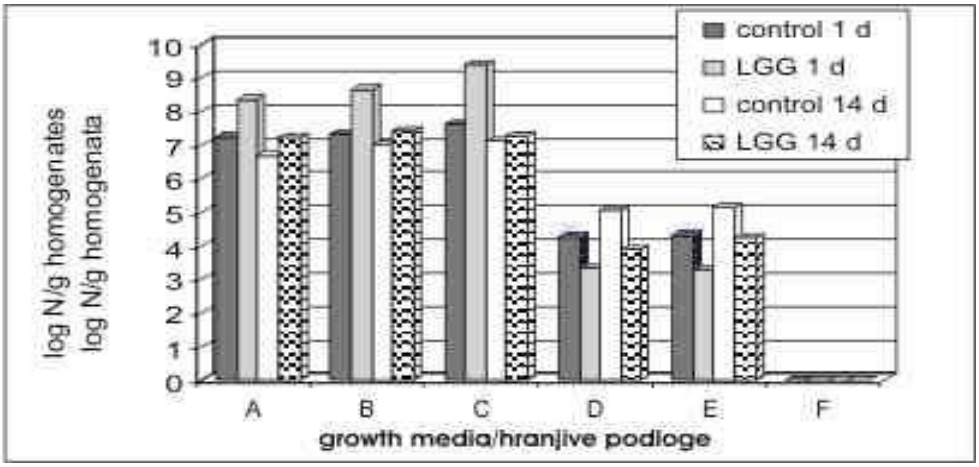

Fig. 4: Microbial counts in large bowel of mice at 1 and 14 days after feeding with ***Lactobacillus rhamnosus*** *GG. A – aerobic bacteria; B – anaerobic bacteria; C – lactic acid bacteria; D –* ***Enterobacteriaceae*** *from Violet Red Bile Glucose agar; E –* ***Enterobacteriaceae*** *from Eosin Methylene Blue agar; F – sulphite-reducing clostridia*

Slika 4: Određivanje broja mikroorganizama u debelom crijevu miševa, 1. i 14. dan nakon prestanka hranjenja s ***Lactobacillus rhamnosus*** *GG. A – aerobne bakterije; B – anaerobne bakterije; C – bakterije mliječne kiseline; D –* ***Enterobacteriaceae*** *na ljubičasto-crvenom žučnom agaru; E –* ***Enterobacteriaceae*** *na eozin metilen plavom agaru; F - sulfito-reducirajući klostridiji*

for 3-4 days, but only 33 % of the population after 7 days. Therefore, it appears likely that daily administration of the preferred strain is necessary for maintenance of high probiotic levels.

Many authors have reported that immunization with lactobacilli can stimulate immune response, by production of total and specific serum antibodies (Fuller, 1992;1997; Fuller and Perdigon, 2000; Shu, 2003). However, the immunomodulatory effect of probiotics is a strain-dependent characteristic and species speciality in the host (Perdigón and Pesce de Ruiz Holgado, 2000; He *et al.* 2000). Therefore, a further objective of this study was to investigate immunomodulating capacity of *L. rhamnosus* GG, which means induction of antibody production in Swiss albino mice by this strain. *L. rhamnosus* GG slightly stimulates the total immune response, demonstrating its positive effect on the host immune system (Figs. 5-7). This strain didn't evoke reaction against itself, which could be seen on the Figs. 8-10.

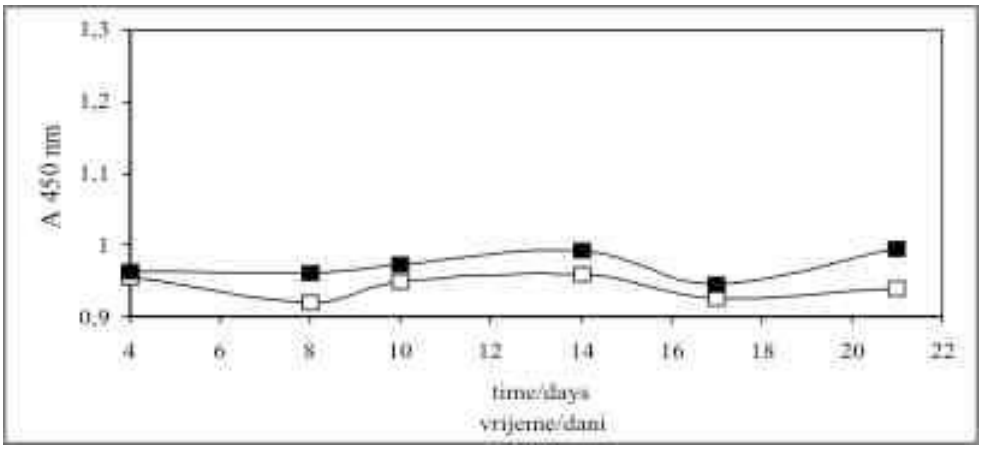

Fig. 5: Determination of total IgA antibodies in 1:100 diluted sera after oral imunization of mice with viable cells of ***Lactobacillus rhamnosus*** *GG by ELISA method.* □ *control;* ■ *LGG.*

Slika 5: Ukupna IgA antitijela određena u serumima (razrjeđenje 1:100), nakon oralne imunizacije miševa sa živim bakterijskim stanicama ***Lactobacillus rhamnosus*** *GG, ELISA metodom.* □ *kontrola;* ■ *LGG.*

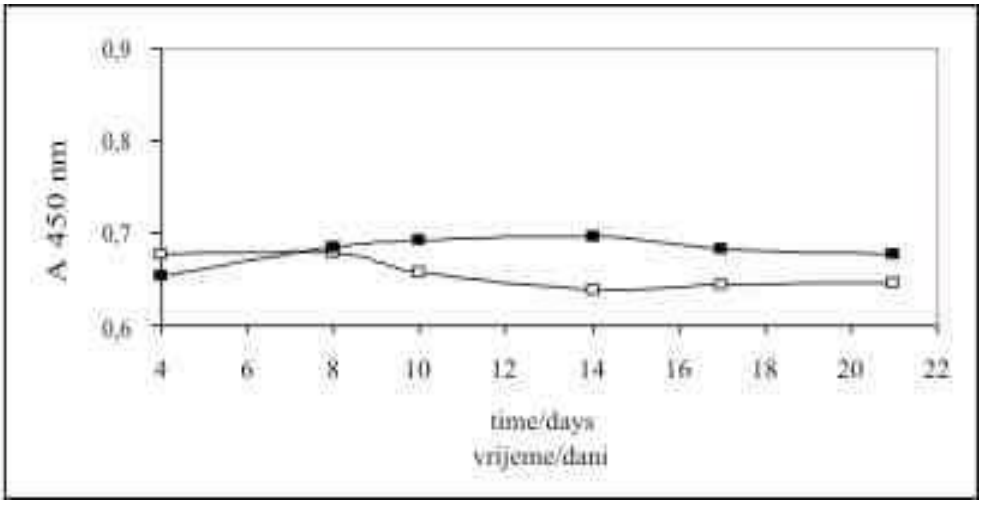

Fig. 6: Determination of total IgG antibodies in 1:100 diluted sera after oral imunization of mice with viable cells ***of Lactobacillus rhamnosus*** *GG by ELISA method. □ control; ■ LGG.*

Slika 6: Ukupna IgG antitijela određena u serumima (razrjeđenje 1:100), nakon oralne imunizacije miševa sa živim bakterijskim stanicama ***Lactobacillus rhamnosus*** *GG, ELISA metodom. □ kontrola; ■ LGG.*

This means that specific humoral immune response with examined strains was low. The observed total humoral immune enhancement by *L. rhamnosus* GG after oral application in mice could be related to its viability and maintenance in the mouse intestine. This statement is supported by the results of Kaila *et al.* (1995). They found out that both, active and inactive forms of *L. rhamnosus* GG have significantly shortened the duration of rotavirus-induced diarrhoea. However, only active *L. rhamnosus* GG cells significantly increases the level of specific IgA-secreting cells to rotavirus. Similarly a trend towards increased *Salmonella* specific IgA-levels was found in subjects who received combination of *L. rhamnosus* GG and oral *Salmonella* vaccination (He *et al.* 2000). All these results suggest that, in addition to the beneficial effect related to competition in colonization, immuno-enhancing properties of probiotics may also be important for protection from infections.

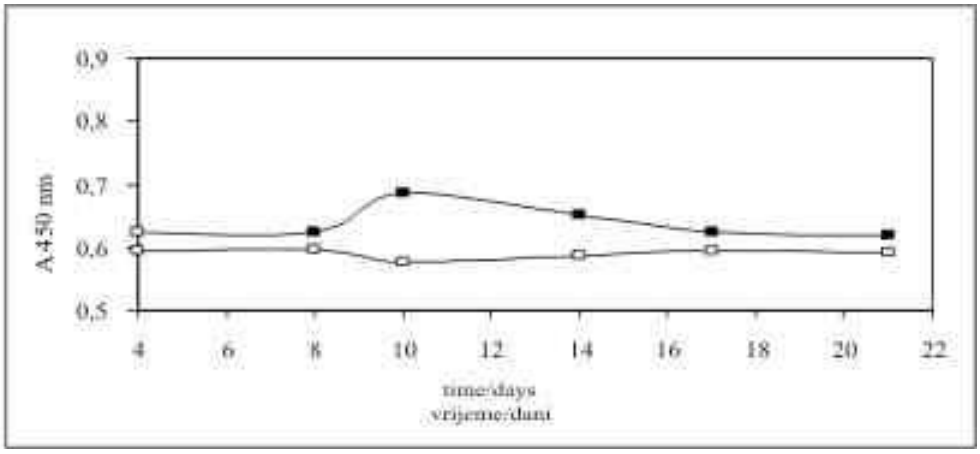

Fig. 7: Determination of total IgM antibodies in 1:100 diluted sera after oral imunization of mice with viable cells of ***Lactobacillus rhamnosus*** *GG by ELISA method. □ control; ■ LGG.*

Slika 7: Ukupna IgM antitijela određena u serumima (razrjeđenje 1:100), nakon oralne imunizacije miševa sa živim bakterijskim stanicama ***Lactobacillus rhamnosus*** *GG, ELISA metodom. □ kontrola; ■ LGG.*

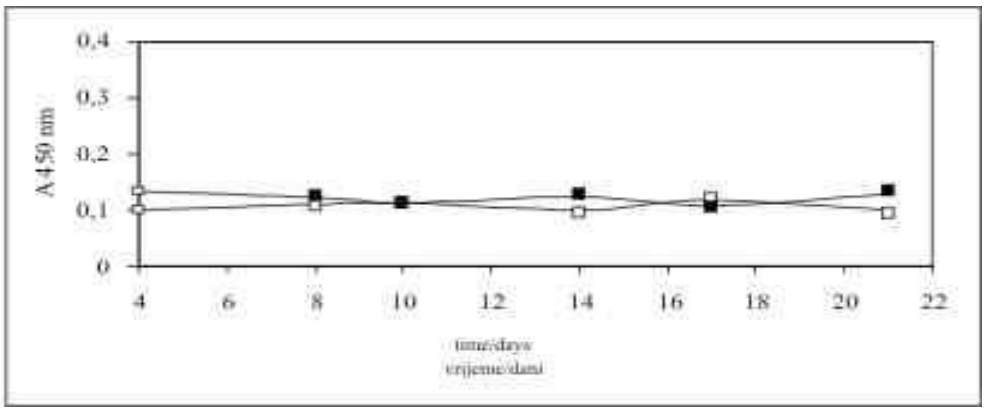

Fig. 8: Determination of anti-lactobacilli IgA antibodies in 1:100 diluted sera after oral imunization of mice with viable cells of ***Lactobacillus rhamnosus*** *GG by ELISA method. □ control; ■ LGG.*

Slika 8: Specifična anti-laktobacili IgA antitijela određena u serumima (razrjeđenje 1:100), nakon oralne imunizacije miševa sa živim bakterijskim stanicama ***Lactobacillus rhamnosus*** *GG, ELISA metodom. □ kontrola; ■ LGG.*

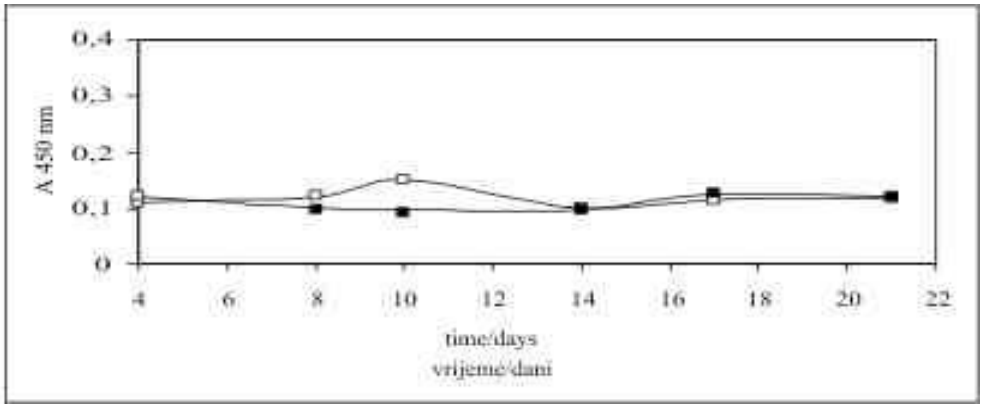

Fig. 9: Determination of anti-lactobacilli IgG antibodies in 1:100 diluted sera after oral imunization of mice with viable cells of ***Lactobacillus rhamnosus*** *GG by ELISA method. □ control; ■ LGG.*

Slika 9: Specifična anti-laktobacili IgG antitijela određena u serumima (razrjeđenje 1:100), nakon oralne imunizacije miševa sa živim bakterijskim stanicama ***Lactobacillus rhamnosus*** *GG, ELISA metodom. □ kontrola; ■ LGG.*

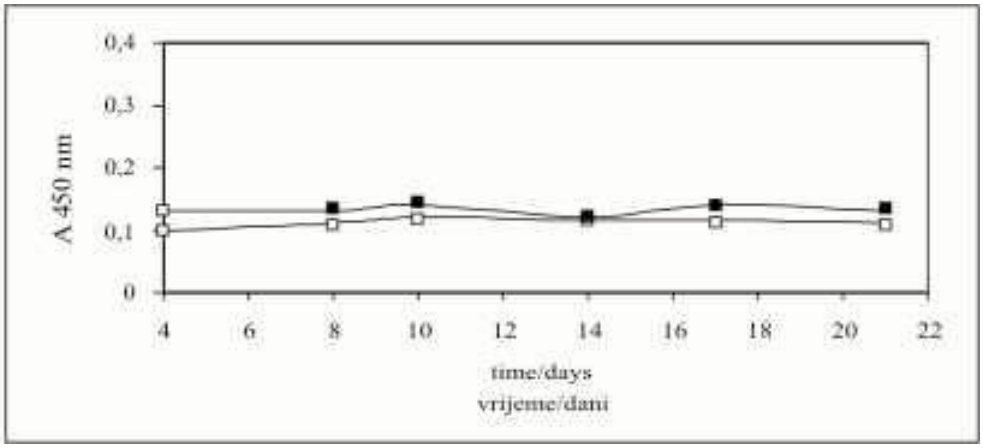

Fig. 10: Determination of anti-lactobacilli IgM antibodies in 1:100 diluted sera after oral imunization of mice with viable cells of ***Lactobacillus rhamnosus*** *GG by ELISA method. □ control; ■ LGG.*

Slika 10: Specifična anti-laktobacili IgA antitijela određena u serumima (razrjeđenje 1:100), nakon oralne imunizacije miševa sa živim bakterijskim stanicama ***Lactobacillus rhamnosus*** *GG, ELISA metodom. □ kontrola; ■ LGG*

Conclusion

Low fat fresh cheese "BioAktiv LGG" appeared as an attractive carrier for probiotic strain *L. rhamnosus* GG and its viability and probiotic activity. Strain *L. rhamnosus* GG, from low fat fresh cheese "BioAktiv LGG", has the capacity to influence the microbial ecology of the host and, by doing so, beneficially affect total humoral immune response.

*IMUNOMODULACIJSKI UČINAK **LACTOBACILLUS RHAMNOSUS** GG IZ SVJEŽEG POSNOG SIRA " BioAktiv LGG"*

Sažetak

*Poticanje imunološkog odgovora probiotičkim mikroorganizmima i njihov imunomodulacijski učinak mogu se primijeniti u prevenciji infektivne dijareje, sprječavanju hipersenzitivnih reakcija i suzbijanju tumora. Novi probiotički proizvod tvrtke LURA d.d., svježi posni sir "BioAktiv LGG", sadrži probiotički soj **Lactobacillus rhamnosus** GG (LGG), koji ima najviše znanstvenih dokaza o korisnim učincima na ljudsko zdravlje. Broj živih stanica **L. rhamnosus** GG u svježem posnom siru "BioAktiv LGG" iznosio je 1,3 x 10^8 CFU/g, i ostao je stabilan tijekom 16 dana skladištenja na 4 ^oC. U ovom radu istražen je utjecaj **L. rhamnosus** GG, izoliranog iz svježeg posnog sira "BioAktiv LGG", na imuno odgovor, te njegovo preživljavanje/zadržavanje u probavnom sustavu Swiss albino miševa, kao važan preduvjet za imunomodulacijsko djelovanje. Tijekom hranjenja miševa s 2,2 x 10^{10} stanica **L. rhamnosus** GG dnevno, povećao se ukupan broj laktobacila, a smanjio broj enterobakterija i sulfito-reducirajućih klostridija u fecesima miševa. Sličan rezultat dobiven je određivanjem sastava crijevne mikroflore u homogenatima tankog i debelog crijeva miševa, prvi i četrnaesti dan nakon prestanka hranjenja miševa s **L. rhamnosus** GG, što ukazuje na adhezijska svojstva ispitivanog probiotičkog soja. Također je provedena i oralna imunizacija Swiss albino miševa, s dnevnom dozom od 2,2 x 10^{10} stanica L. rhamnosus GG kroz period od 8 dana. Uzorci krvi Swiss albino miševa sakupljani su 4., 8., 10., 14., 17. i 21. dana nakon prve imunizacije. U priređenim serumima određivana su ukupna i specifična anti-laktobacili IgA, IgG i IgM antitijela, ELISA metodom. **L. rhamnosus** GG stimulirao je ukupni humoralni imuno odgovor, a nije izazvao specifični imuno odgovor, što potvrđuje njegov imunomodulacijski učinak, i*

čini novi probiotički proizvod, svježi posni sir "BioAktiv LGG", dobrim matriksom za ispitivani probiotički soj.

Ključne riječi: ***Lactobacillus rhamnosus****, probiotička svojstva, adhezija, imunomodulacija, antitijela, ELISA*

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Author's addresses – Adrese autora:

Stjepan Mahnet, dipl. ing.
Slavko Kirin, dipl. ing.
LURA d. d. Food Industry, Vukovar street 271, 10 000 Zagreb, Croatia
Dr. sc. Blaženka Kos
Mr. sc. Jadranka Frece, dipl. ing.
Dr. sc. Jagoda Šušković, izv. prof.
Faculty of Food Technology and Biotechnology, University of Zagreb,
Pierottijeva 6, 10 000 Zagreb, Croatia

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