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The Immune Response in Mice Immunized with *Lactobacillus acidophilus* LF221 – A Potential Probiotic Strain

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Summary

Lactobacillus acidophilus LF221, a child's faeces isolate, has some promising probiotic properties. Prior to its commercial use the immunological effects have to be assessed as well. Therefore, the effect of the bacteriocins producing LF221 (bac^+), its mutant (bac^-), and Lactobacillus casei ATCC393 strains on the specific immune response in NIH mice was studied. Mice were immunized intraperitoneally (i.p.) or orally with a dose of $2.4 \cdot 10^{10}$ viable or dead cells. The blood samples were collected on 9^{th}, 11^{th}, 14^{th}, 18^{th}, 21^{st} and 24^{th} day after 1^{st} immunization. The sera of mice immunized i.p. with viable cells of LF221 (bac^+) were screened for specific anti-LF221 (bac^+) IgA, IgG and IgM antibody reaction by a modified DIBA assay. With ELISA assay sera samples from all groups were tested for total and specific anti-lactobacilli IgA, IgG and IgM antibodies. Irrespective of the way of immunization, strain or cells preparation, the concentration of total serum IgA, IgG and IgM antibodies remained on the same level. The highest levels of specific anti-lactobacilli IgG and IgM antibodies after i.p. immunization were detected in mice immunized with viable cells of the strain LF221 (bac^+), while activation of specific humoral immune response with dead cells was low. LF221 does not evoke the specific humoral immune response after oral application and is as such suitable for probiotic application.

Key words: probiotics, bacteriocins, immune response, anti-lactobacilli antibodies

Introduction

Probiotics are defined as viable microorganisms that exhibit a beneficial effect on the health of the host after they are ingested. They are frequently used in fermented dairy products but also in pharmaceutical preparations. Probiotic species such as *Lactobacillus acidophilus* have been safetly used for more than 70 years. However, it cannot be assumed that new strains of probiotic bacteria, often isolated from the human intestinal microflora, share the historical safety of traditional strains. New strains should be carefully tested for safety and efficacy *(1)*.

Previous investigations have shown that the *Lactobacillus acidophilus* LF221, a child's faeces isolate, is bacteriocinogenic, resistant to low *pH* value and bile salts. It inhibits the growth of some lactobacilli, lactococci and pediococci and some strains of *Streptococcus thermophilus, Staphylococcus aureus, Listeria innocua, Clostridium* sp. *(2,3)*. It was found in the faeces of mice, fed with a daily dose of 10^9 viable cells of LF221 *(4)*. Because of these properties the strain is potential probiotic. Immunogenic potential is also one of the probiotic properties. Probiotic lactobacilli can stimulate the immune response but they must not evoke reaction against themselves *(5)*. Many authors *(6–8)* have reported that immunization with lactobacilli stimulates the production of specific serum antibodies. Bearing this in mind, the present work was carried out to determine whether the strain *Lactobacillus acidophilus* LF221 could effect the specific immune sys-

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tem in mice. Besides the bacteriocinogenic strain LF221 (bac$^+$), its nonbacteriocinogenic mutant LF221 (bac$^-$) and *Lactobacillus casei* ATCC393 were tested as well. The *Lactobacillus casei* ATCC393, which was reported to enhance the host's resistance to *Listeria* infection *(9)*, was taken as the control.

Material and Methods

Animals

Female NIH mice weighing 25–30 g (KRKA, Novo Mesto) were housed in plastic cages at room temperature, with 12 h light per day, 55 % relative humidity, and were allowed to adapt to their new environment for a period of 7 days. In each of 14 groups the number of mice was identical, *i.e.* 3 mice per group.

Strains, cultivation and inoculum preparation

Lactobacillus acidophilus LF221 (bac$^+$) – bacteriocins producing strain isolated from child's faeces, *Lactobacillus acidophilus* LF221 (bac$^-$) – non-bacteriocins producing mutant, and *Lactobacillus casei* ATCC393 were cultivated aerobically in MRS broth at 37 °C for 18 hours. Cells were removed by centrifugation at 3500 × g for 10 min and washed three times with sterile saline solution. After washing, cells were re-suspended in a final dose of $2.4 \cdot 10^{10}$ cells/200 μL in sterile saline solution. Dead cells were prepared using the same procedure and heated at 100 °C for 50 min.

Immunization

The mice were immunized i.p. or orally with a dose of $2.4 \cdot 10^{10}$ viable or dead bacterial cells/200 μL, prepared as described above. The i.p. immunization was performed twice. Two weeks after the first immunization, a second immunization with the same dose was done. The oral immunization was performed eight times over a period of eight successive days. Each day the mice were given a dose of $2.4 \cdot 10^{10}$ of viable or dead bacterial cells 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. During the experiment the mice were fed *ad libitum*.

Collection of blood samples

On the 9th, 11th, 14th, 18th, 21st and 24th days after first immunization the blood samples were collected by bleeding of the tail vein with heparinized capillaries. Collected blood was transferred immediately from capillaries into the tubes, allowed to clot at room temperature for 1 hour and left overnight at 4 °C. Tubes were centrifuged at 3000 × g for 10 min. The sera samples were kept at –20 °C until use.

Antibody determination

In our preliminary experiments, only sera samples from mice which were immunized i.p. with viable cells of *Lactobacillus acidophilus* LF221 (bac$^+$) were screened for specific serum IgA, IgG and IgM antibody reaction by modified DIBA (dot-immunobinding assay) method *(10)*. Sera samples from all groups were later screened with ELISA (enzyme-linked immunosorbent assay) method *(11,12)* for total and anti-lactobacilli IgA, IgG and IgM antibodies.

Preparation of lactobacilli for DIBA and ELISA methods

All strains of lactobacilli were inoculated in MRS broth and incubated aerobically at 37 °C for 18 hours. The lactobacilli were then harvested by centrifugation (3500 × g, 10 min) and washed 3 times in phosphate-buffered saline (0.01 M, 0.15 M NaCl, *pH*=7.2) (PBS). Sediment was re-suspended in 0.75 % formaldehyde-PBS solution. After incubation at 4 °C for 24 hours the bacteria were washed 3 times and re-suspended in 10 mL of PBS and diluted (1:100).

Modified indirect DIBA method (10)

A volume of 2 μL of bacterial cells was dotted on the nitrocellulose extra blotting membranes (0.2 nm, Sartorius) and incubated overnight at room temperature. To saturate the remaining binding sites, nitrocellulose strips were incubated in 0.5 % Tween 80-PBS solution (T-PBS) at room temperature for 30 min. The strips were then dipped in sera dilutions at room temperature for 35 min. The sera dilutions (1:10, 1:100, 1:1 000, and 1:10 000) were made with PBS. The strips were then washed 3 times in 0.05 % T-PBS for 10 min and, after washing, incubated in peroxidase conjugated goat anti-mouse IgA (α-chain specific, Sigma A-4789), IgG (Fc-specific, Sigma A-9309) or IgM (μ-chain specific, Sigma A-8786) antibodies diluted 1:250 in 0.05 % T-PBS (100 μL/well) for 2 h at 37 °C. The nitro-cellulose strips were washed twice in 0.05 % T-PBS and once in PBS for 10 min. Final reactions were estimated after the reaction with the horseradish peroxidase substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) (1 mL DAB substrate in 0.1 M tris-buffered saline, *pH*=7.5, 3 mL 0.1 M tris-buffered saline, 4 μL 33 % H_2O_2) in which the nitrocellulose strips were dipped. In the case of positive reaction the reddish-brown colour appeared in two minutes.

The control of the peroxidase conjugated antibodies (PBS instead of serum) and the control of the substrate (PBS instead of serum and peroxidase conjugated antibodies) were also performed.

Total antibody determination (ELISA method)

The total antibody sera titres were determined in polystyrene microtiter plates (NUNC). Plates were first incubated with anti-mouse IgA (α-chain specific, Sigma M-1272; 400 ng/well), or anti-mouse IgG (γ-chain specific, Sigma M-1397; 400 ng/well), or anti-mouse IgM (μ-chain specific, Sigma M-8644; 400 ng/well) overnight at 4 °C. To saturate the remaining binding sites, plates were incubated with 3 % bovine serum albumin in PBS (200 μL/well) for 2 h at 37 °C. After washing 3 times for 10 min with 0.05 % Tween in PBS (200 μL/well), the plates were incubated overnight at 4 °C with dilutions of the sera (50 μL/well). The dilutions (1:100, 1:1 000) were performed in PBS. The plates were then washed 3 times in 0.05 % T-PBS for 10 min and, after washing, incubated in peroxidase conjugated goat anti-mouse IgA

(α-chain specific, Sigma A-4789) or anti-mouse IgG (Fc--specific, Sigma A-9309) or anti-mouse IgM (μ-chain specific, Sigma A-8786) antibodies diluted 1:15 000 in PBS (100 μL/well) for 2 h at 37 °C. The plates were then washed 3 times with 0.05 % T-PBS for 10 min (200 μL/well). The total antibody sera titres were estimated after the reaction with the horse-radish peroxidase substrate o-phenylenediamine (ODP) dihydrochloride (Sigma P-8287) diluted in phosphate-citrate buffer (0.05 M, *pH*=5) with 0.03 % sodium perborate (100 μL/well). After 40 min the absorption was determined at 450 nm with Titertek Multiskan. The control of the peroxidase conjugated antibodies (PBS instead of serum) and the control of the substrate (PBS instead of serum and peroxidase conjugated antibodies) were also performed.

Specific antibody determination (ELISA method)

The specific anti-lactobacilli serum titre was determined on preactivated polystyrene microtiter plates (NUNC). In each well 100 μL of glutaraldehyde (Sigma, 25 % aqueous solution; in final concentration of 0.025 % in PBS 0.01 M, NaCl 0.15 M, *pH*=7) was added. Plates were incubated 2 h at 37 °C and dried for 20 min at 37 °C before incubation with pre-treated and thoroughly washed lactobacilli cells prepared as described above ($1 \cdot 10^7 - 1 \cdot 10^8$ cells/mL; 100 μL/well). After overnight incubation at 4 °C, the ELISA method was performed exactly as indicated for total antibody determination without the first incubation with anti-mouse IgA, IgG or IgM antibodies. All sera samples were diluted with PBS at a ratio of 1 : 100.

Statistical methods

Average values from independent experiments (within each group) were calculated and differences in the level of antibodies between the groups were tested with one-dimensional analysis of variance (F-test, α=0.05).

Results and Discussion

No specific anti-*Lactobacillus acidophilus* LF221 (bac$^+$) sera antibodies of the IgA isotype could be detected after i.p. immunization by DIBA method. The highest level of specific IgM antibodies was detected on the 9th day after the 1st immunization (Fig. 1). The titre was 1:1 000. Later on the concentration of specific IgM antibodies in serum decreased and on the 14th day after the 1st immunization the titre was only 1:10. The level of specific IgM antibodies increased again after the booster. On the 4th day after the booster specific IgM antibodies were observed in dilution of serum 1:100, but later only in dilutions 1:10. In the case of the serum of the control group and in the control of the peroxidase conjugated antibodies and the substrate, the reaction did not flow off.

The concentration of specific serum IgG antibodies increased very slowly after the 1st immunization (Fig. 1). These antibodies were observed in dilution of serum 1:100 on the 9th and 14th day after the 1st immunization. The level of specific serum IgG antibodies after the booster increased more rapidly and the highest level of specific serum IgG antibodies was observed on the 4th day after the booster. The titre was 1:10 000. After that day the concentration slowly decreased.

Irrespective of the way of immunization, strain or cells preparation, the concentration of total serum IgA, IgG and IgM antibodies remained on the same level (results not presented).

After both ways of immunization, the levels of specific serum anti-lactobacilli IgA antibodies from all groups were very close to the control group and the differences were below the sensitivity of our test. That is why these results are not presented in Figures. In contrast, after i.p. immunization, the specific serum IgG and IgM antibody responses were detected with ELISA method. Both IgG and IgM antibody appeared in sera in a way which is typical of the secondary immune response. After i.p. immunization with viable cells (Figs. 2 and 3) the differences in specific IgM and IgG antibodies between groups were significant on all blood sample collecting days ($P > 0.001$). The specific IgG and IgM responses were weaker after immunization with dead cells (Figs. 4 and 5) but the differences between groups were still significant, except for IgG on the day 14th ($P < 0.05$) (Fig. 4). In the comparison of different bacteria as antigens, the highest levels of specific IgG and IgM anti-

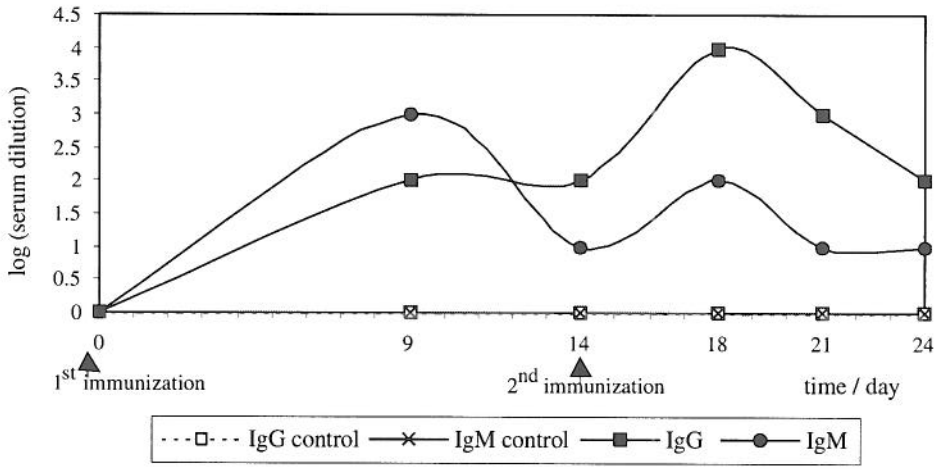

Fig. 1. Titres of sera anti-*Lactobacillus acidophilus* LF221 (bac$^+$) IgG and IgM antibodies after i.p. immunization with viable cells of *Lactobacillus acidophilus* LF221 (bac$^+$), ($2.4 \cdot 10^{10}$ cells/200 μL)

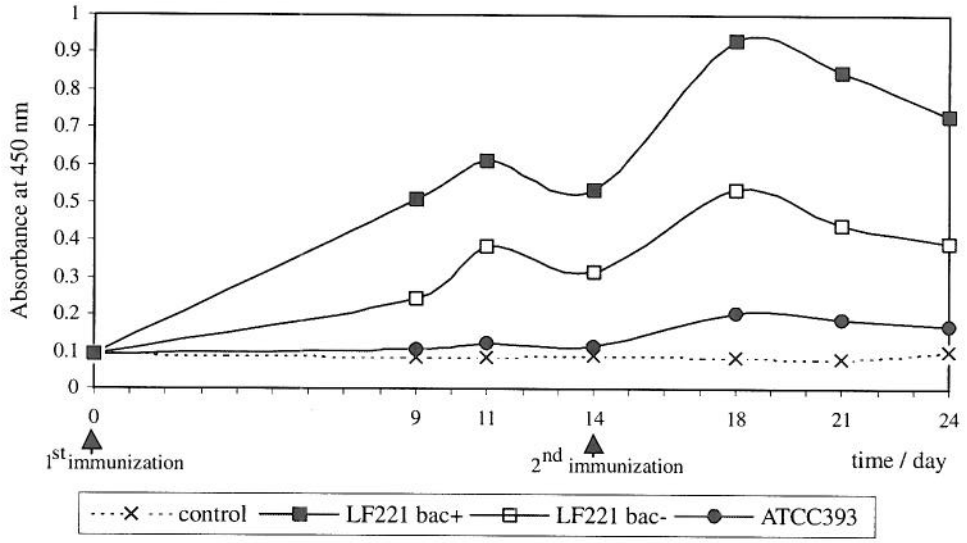

Fig. 2. Detected anti-lactobacilli IgG antibodies in sera diluted 1:100 after i.p. immunization with viable cells ($2.4 \cdot 10^{10}$ cells/200 μL)

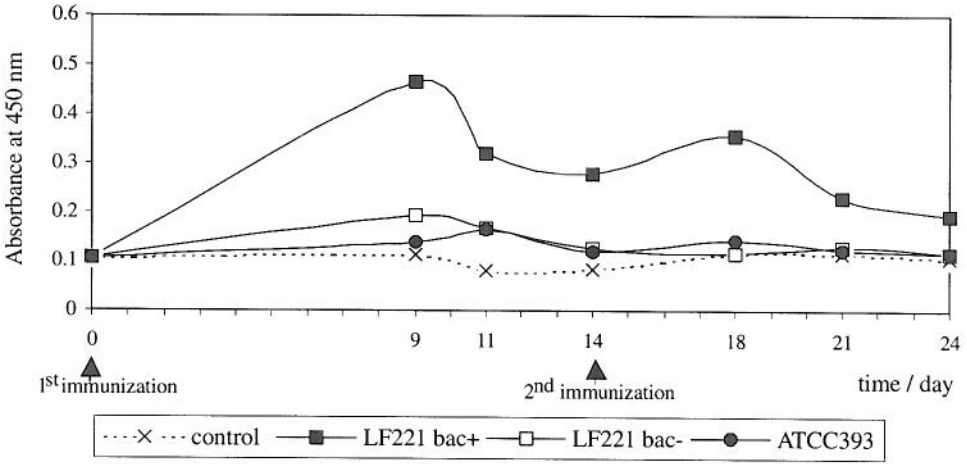

Fig. 3. Detected anti-lactobacilli IgM antibodies in sera diluted 1:100 after i.p. immunization with viable cells ($2.4 \cdot 10^{10}$ cells/200 μL)

bodies were observed after the i.p. immunization with viable cells of *Lactobacillus acidophilus* LF221 (bac$^+$). It is interesting that after immunization with dead LF221 cells the specific anti LF221 (bac$^+$) and LF221 (bac$^-$) IgG and IgM antibody responses were similar (Figs. 4 and 5).

The synthesis of specific IgG and IgM antibodies after the i.p. immunization with viable cells of *Lactobacillus casei* ATCC393 was weak (Figs. 2 and 3). After immunization with dead *Lactobacillus casei* ATCC393 cells, the quantity of IgG antibodies was close to the control group, while the amount of IgM antibodies was higher compared to the other groups (Figs. 4 and 5).

The levels of specific serum IgA, IgG and IgM antibodies after the oral immunization were very low and the differences between the tested groups were not significant. Testing of serum antibodies is not enough to detect specific antibodies after oral immunization. The gut has a complex system of immunological and non-immunological host defence factors. When the pathogen overcomes the non-specific host defence mechanisms, the host activates specific defence mechanisms, producing antibodies, mainly secretory IgA immunoglobulins *(13,14)*. The majority of IgA precursor cells are derived from Peyer's patches. After stimulation they migrate into the circulation and develop into cells with cytoplasmic IgA. From there, these cells are distributed throughout the mucosal tissues with a preference for the organ in which they were induced *(15,16)*. These antibodies can inhibit bacterial colonisation by agglutinating microorganisms in the gut lumen, or by interfering with motility and blocking the bacterial surface structures responsible for adherence to the epithelium *(13)*. It is also important to know that secretory IgA antibodies pass into the periphery blood very slowly and in different forms *(17)*.

The present study demonstrates that the i.p. administered viable cells of *Lactobacillus acidophilus* LF221 (bac$^+$) evoke the specific IgG and IgM antibody response in serum, as could be concluded from the IgG and IgM sera titres after primary and booster injection. We can observe the secondary IgG and IgM immune response,

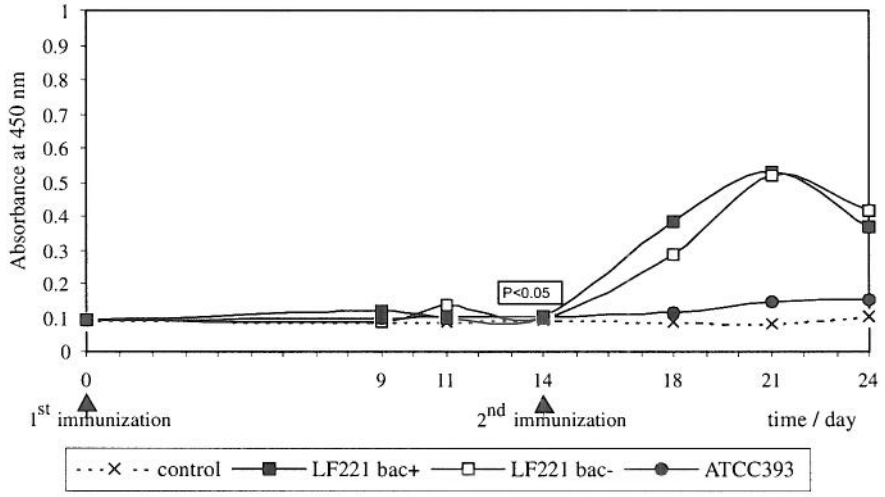

Fig. 4. Detected anti-lactobacilli IgG antibodies in sera diluted 1:100 after i.p. immunization with dead cells ($2.4 \cdot 10^{10}$ cells/200 μL)

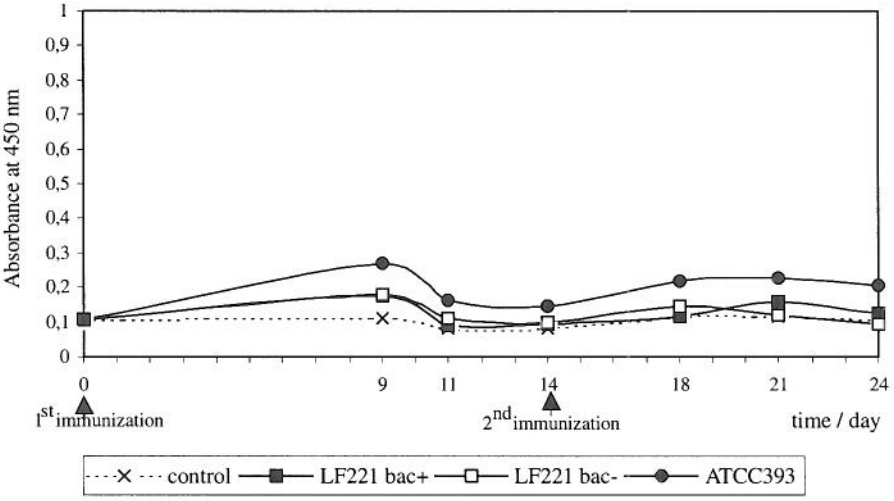

Fig. 5. Detected anti-lactobacilli IgM antibodies in sera diluted 1:100 after i.p. immunization with dead cells ($2.4 \cdot 10^{10}$ cells/200 μL)

which is regarded in IgG as stronger than the primary immune response. It is typical of the adaptive immunity system that it »remembers« each encounter with foreign antigen, so that subsequent encounters stimulate increasingly effective defence mechanisms *(18)*. The detected concentrations of specific anti-*Lactobacillus acidophilus* LF221 (bac^-) IgG and IgM antibodies were lower compared to the specific serum anti-*Lactobacillus acidophilus* LF221 (bac^+) IgG and IgM antibodies. According to these results it can be assumed that the bacteriocin complex (acidocin LF221 A and acidocin LF221 B) could enhance immunogenicity of *Lactobacillus acidophilus* LF221 (bac^+), but this has yet to be proven. The molecular weight of the bacteriocin complex is too small to evoke immunogenicity *(19)*. The complex is reflexive bounded on the bacterial cell wall and in that instance the bacterial cell could be a carrier of antigen – bacteriocin complex and the probability of immunogenicity of the bacteriocin complex is greater.

It was reported that probiotic strain can stimulate the immune response but it must not evoke a reaction against itself *(5)*. From our results it can be concluded that the strain *Lactobacillus acidophilus* LF221 (bac^+) is an immunogenic agent which, after i.p. immunization, stimulates the synthesis of specific anti-*Lactobacillus acidophilus* LF221 (bac^+) IgG and IgM antibodies. In the comparison of total serum antibodies, the specific serum antibodies are very low and specific serum antibodies appear only after the i.p. immunization which is not the case in industrial products using this bacteria. The main application of probiotic preparations is oral. After the oral immunization in our experiment the specific anti-lactobacilli antibody response did not appear. We concluded that the strain LF221 (bac^+) is still of great interest for further experiments as probiotic food additive.

The antigenic stimulation with certain bacteria is important in the maturation of the immune system. The present study shows that the strain *Lactobacillus acidophilus* LF221 (bac^+) administered i.p. activates the adaptive

humoral immune response. The i.p. immunization with this strain evokes a specific IgG and IgM antibody response in serum, while no specific IgA antibodies were observed. Further studies are needed on the immunogenic properties of the bacteriocin complex of the strain *Lactobacillus acidophilus* LF221 (bac$^+$) as well as to determine other immunodominant proteins which are probably common to bac$^+$ and bac$^-$ strain. It should be established whether feeding with strain LF221 (bac$^+$) may induce an enhancement of non-specific IgA, IgG or IgM anti-pathogen response. Another important question is whether the strain LF221 (bac$^+$) induces specific antibodies with cross–reactive properties for other pathogens in the GI tract. An immunoassay with specific antibodies towards the bacteriocin molecule or bacteriocin producing strain, obtained by i.p. immunization, could be a useful tool for monitoring the LF221 (bac$^+$) strain during the food fermentation or colonisation of the GI tract.

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Imunološki odaziv miševa imuniziranih s *Lactobacillus acidophilus* LF221 – potencijalni probiotički soj

Sažetak

Lactobacillus acidophilus LF221, izoliran iz dječjeg fecesa, izgleda da ima određena probiotička svojstva. Prije njegove komercijalne uporabe potrebno je utvrditi imunološko djelovanje. Stoga je istražen utjecaj bakteriocinogenog soja LF221 (bac$^+$), njegova mutanta (bac$^-$) i soja Lactobacillus casei ATCC393 na specifični imuni odaziv u NIH miševima. Miševi su bili imunizirani intraperitonealno (i.p.) ili oralno s dozom od $2{,}4 \cdot 10^{10}$ živih ili mrtvih stanica. Nakon prve imunizacije uzimani su uzorci krvi 9., 11., 14., 18., 21. i 24. dana. Uzorci seruma miševa, imunizirani i.p., sa živim stanicama LF221 (bac$^+$), ispitani su na specifične reakcije IgA, IgG i IgM protutijela na LF221 (bac$^+$) pomoću modificiranog postupka DIBA. Postupkom ELISA u uzorcima seruma svih skupina ispitana su ukupna i specifična IgA, IgG i IgM protutijela na laktobacil LF221. Bez obzira na način imunizacije, soj ili stanični pripravak, koncentracija ukupnih serumskih protutijela IgA, IgG i IgM bila je na istoj razini. Najviša razina specifičnih IgG i IgM protutijela na laktobacil, nakon i.p. imunizacije, utvrđena je u miševa imuniziranih sa živim stanicama soja LF221 (bac$^+$), dok je aktivacija specifičnog humoralnog imunosnog odgovora s mrtvim stanicama bila slaba. LF221 ne uzrokuje specifični humoralni imunosni odgovor nakon oralne uporabe te je stoga prikladan za probiotičku primjenu.