Oral Microbiology Immunology 2007: 22: 140–144
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Short communication

Lactobacillus plantarum for oral peptide delivery

Y. Oh[1], P. Varmanen[2], X. Y. Han[3], G. Bennett[4], Z. Xu[1], T. Lu[1], A. Palva[2]
[1]Department of Thoracic/Head & Neck Medical Oncology, UT M.D. Anderson Cancer Center, Houston, TX, USA, [2]Section for Veterinary Microbiology, Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland, [3]Department of Laboratory Medicine, UT M.D. Anderson Cancer Center, Houston, TX, USA, [4]Department of Biochemistry, Rice University, Houston, TX, USA

Oh Y, Varmanen P, Han XY, Bennett G, Xu Z, Lu T, Palva A. Lactobacillus plantarum *for oral peptide delivery.*
Oral Microbiol Imunol 2007: 22: 140–144. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard.

Aims: To evaluate strains of lactobacilli for their ability to persist and secrete heterologous protein in the oral cavity.
Methods and results: Four different strains of common oral lactobacilli, *Lactobacillus brevis*, *Lactobacillus johnsonii*, *Lactobacillus murinus* and *Lactobacillus plantarum*, were transformed with the plasmid pKTH2121, which contains a secretion cassette for beta-lactamase. Lactobacilli isolated from the mouth of host mice were also transformed with pKTH2121 for later feeding. *Lactococcus lactis*, transformed with pKTH2121, was also fed to mice as a negative control. All transformed isolates were fed to C57Black mice in varying schedules. The number of transformed bacteria persisting in the mouth was reported as a percentage of total oral bacteria recovered by swabbing.
Conclusions: The transformed *L. lactis*, *L. brevis*, *L. johnsonii*, *L. murinus*, and the endogenous murine lactobacillus strain failed to persist in the mouth. Transformed *L. plantarum*, however, persisted in the mouth and comprised up to 25% of the total lactobacilli at 18 h and 10% at 24 h after feeding. *L. plantarum* recovered after feeding retained its ability to secrete beta-lactamase into culture medium efficiently. Beta-lactamase activity could be detected in oral secretions at 8 h after feedings. After repeated feedings, however, the *L. plantarum* containing pKTH2121 gradually lost its ability to persist after feedings. This experiment demonstrates that *L. plantarum* can transiently colonize the oral mucosa in large numbers, while continuously secreting foreign proteins, raising the possibility of using lactobacilli as a vector for delivery of oral mucosal peptides.

Key words: beta-lactamase; *Lactobacillus plantarum*; oral colonization

Yun Oh, Department of Thoracic/Head & Neck Medical Oncology, Unit 432, UT M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA
Tel.: +1 713 792 6363;
fax: +1 713 796 8655;
e-mail: ywoh@mdanderson.org
Accepted for publication August 9, 2006

Many ailments of the oropharynx, from cancer to gingivitis, might be amenable to prevention or treatment using topical therapies. Topical therapies for the oropharynx have traditionally consisted of medicated mouthwashes, which are limited by their transient time of contact with the oral mucosa, requiring frequent administration. One potential solution for the delivery of therapeutic peptides to the oropharynx might be to engineer resident oral bacteria to secrete them. If these resident bacteria could persist on the oropharyngeal mucosa in sufficient numbers, they could deliver therapeutic peptides continuously.

Lactic acid bacteria have already been used as live vectors for the delivery of biologically useful peptides (17, 18). The consumption of lactic acid bacteria as probiotics in the forms of yogurt and other fermented foods promotes health by displacing pathogenic organisms in the gastrointestinal tract (16). At least part of this effect is the result of the secretion of antimicrobial peptides, such as nisins, which are toxic to other species (2). Peptides and peptidoglycans on the surface of at least some lactobacilli act as non-specific immunoadjuvants that have led to the development of mucosal vaccines (6, 9, 13–15, 18, 23). *Lactococcus lactis* engineered to secrete interleukin-10 has been shown to reduce bowel abnormalities in experimental models of colitis (21).

All these examples of lactic acid bacteria as protein delivery vectors depend on their ability to colonize the gastrointestinal tract transiently at concentrations that would have an appreciable effect (8). Similar results, unfortunately, have not yet been achieved with lactic acid bacteria in the upper aerodigestive tract because colonization efficiencies appear to be low (10). *Streptococcus gordonii* was engineered to secrete peptides to impair dental adhesion by the cariogenic bacteria *Porphyromonas gingivalis* but the engineered

bacteria could not achieve a therapeutic effect because they could not colonize the oropharynx in sufficient numbers (7). We sought to investigate whether lactobacilli with natural oral tropism could be engineered for foreign protein secretion and could transiently colonize the murine oropharynx in sufficient numbers for protein secretion to be detected.

Materials and methods

Lactobacillus secretion system

The plasmid pKTH2121 contains an expression cassette for secreting *Escherichia coli* beta-lactamase (cDNA from the pUC 19 plasmid) utilizing promoter and signal sequences from the *Lactobacillus brevis S-layer protein A* gene (*slp-A*) (17). It contains both erythromycin- and chloramphenicol-resistance genes and has previously demonstrated the ability to transform several lactic acid bacteria for beta-lactamase secretion (17). Plasmid pKTH2121 was propagated in *L. lactis* strain (MG1614), the bacterial pellets were then resuspended in plasmid midiprep (Qiagen, Valencia, CA) phosphate buffer according to the manufacturer, but proteinase K or lysozyme (Sigma, St. Louis, MD) was added at 1 mg/ml for 30 min at 50°C to digest the cell wall. The digested pellets were then processed according to the remainder of the manufacturer's protocol. The plasmid was then electroporated into *L. lactis* and into lactobacillus strains as previously described (5, 26). Bacteria transformed with pKTH2121 were selected on agar plates of the appropriate medium containing erythromycin 10 μg/ml (Sigma). Presence of pKTH2121 in the transformants was confirmed by polymerase chain reaction using primers P1 and P4 flanking the secretion cassette (17).

Beta-lactamase expression was analysed by western blot and by enzymatic assays. For western analysis, bacterial pellets and culture supernatants of *Lactobacillus plantarum*, either with or without the pKTH2121 plasmid, were run on polyacrylamide gels and blotted onto nitrocellulose membranes, then beta-lactamase was detected using a polyclonal antibody to beta-lactamase (AB3738, Chemicon International, Temecula, CA) and the enhanced chemiluminescence non-radioactive detection system (Amersham, Pittsburgh, PA) as previously described (24). For enzymatic analysis, nitrocefin-impregnated disks (Cefinase, Difco, San Jose, CA) following an assay described elsewhere (19) were exposed to supernatant from different bacteria washed with phosphate-buffered saline (to eliminate discoloration from the medium) or were swabbed against the oropharynx of mice fed the same bacteria.

Lactic acid bacterial strains

The strains tested were *L. lactis* strain (MG1614), *L. plantarum* strain (NCDO 1193), *L. brevis* (ATCC 8287), *L. johnsonii* (ATCC 11506), and *Lactobacillus murinus* (ATCC 35020). To isolate endogenous murine oral lactobacillus strains, Calgiswab cultures of oral mucosa were inoculated onto minimum required strength agar plates (as described below) and grown anaerobically for 48 h to eliminate contaminating yeast. Isolates were categorized by gram-positive rod and colony morphology, and the two most common isolates were subcultured on minimum required strength plates aerobically at 37°C. *L. lactis* was grown in GM17 medium (5% glucose in M17 medium, Difco) at 37°C, and all lactobacillus strains were grown in minimum required strength medium (Difco) at 37°C. Transformed bacteria were grown in their optimal medium with erythromycin 10 μg/ml.

Mice for bacterial feed and harvest

C57Black mice (Charles River Laboratories, Wilmington, MA) at age 21 days were used for all experiments according to a protocol approved by our institutional Animal Care and Use Committee. No toxicity was noted from any bacterial feedings or collections.

To harvest bacteria, mice were anesthetized with pentobarbital (Nembutal, Abbott Pharmaceuticals, Austin, TX) 50–90 mg/kg by intraperitoneal injection. Type 4 Calgiswabs (Puritan Medical Supplies, Houston, TX) were dipped in citrate collection buffer and swabbed over the buccal, palatal and tongue mucosa gently for 20 s, dissolved in citrate buffer, then cultured as previously described (12). The Calgiswab collections were cultured in serial dilutions on minimum required strength agar plates with or without erythromycin 10 μg/ml to distinguish transformed bacteria from total lactobacilli. Mucosal swabs were taken no more frequently than every 72 h to allow recovery of a stable mucosal flora.

To feed bacteria to the mice, the mice were initially anesthetized with pentobarbital as above, and the tongue was scrubbed with chlorhexidine 0.1% (Peridex, Zila Pharmaceuticals, La Jolla, CA) using a Microbrush dental applicator (Microbrush, WI, Earth City, MD) then scrubbed again with phosphate-buffered saline. Ten microliters of bacteria from overnight plate cultures resuspended in culture medium were administered intraorally at a concentration of 10^9 colony-forming units/ml. All subsequent feedings were given by simple oral administration to unanesthetized animals. All feedings were performed on groups of three or four animals with or without a subsequent application of honey over the tongue. Any animals not involved in the same feedings were segregated to separate cages.

Collecting mucosal antibodies for recognition of *L. plantarum* pKTH2121

For tracheopulmonary lavage, mice were sacrificed by lethal intraperitoneal pentobarbital injection, then underwent immediate tracheal intubation with a 22- or 24-gauge intravenous catheter and lavage with 100 μl phosphate-buffered saline at room temperature (27).

Saliva samples were harvested from mice (11) after anesthetization with pentobarbital injected intraperitoneally at 50 μg/kg. Saliva was collected over a period of 5 min from each animal, followed by animal sacrifice. Immediately after collection, samples were centrifuged at 2000 ***g*** to remove particulate matter, then stored at −80°C until use.

Enzyme-linked immunosorbent assay to detect anti-beta-lactamase mucosal antibodies

Dilutions of bronchopulmonary lavage and saliva samples were tested separately. Exponentially growing *L. lactis* pKTH2121 in 100 ml GM17 broth cultures were pelleted and washed once with cold phosphate-buffered saline. The phosphate-buffered saline supernatant protein was seeded onto enzyme-linked immunosorbent assay wells at 4°C and allowed to adsorb overnight. Supernatant from wild-type *L. lactis* was used as a negative control antigen target. The polyclonal antibody against beta-lactamase used for western blotting was used as a positive control antibody for enzyme-linked immunosorbent assay. Lavage and saliva samples were tested at dilutions between 1 : 4 and 1 : 32.

Immunfluorescence microscopy

L. plantarum pKTH2121, wild-type *L. plantarum*, and control bacteria, including

a mixture of endogenous oral lactobacilli cultured from mice before *L. plantarum* pKTH2121 feeding, were all grown on minimum required strength plates anaerobically then heat fixed onto glass slides *in situ*. Polyclonal antibody used for western blotting was used as a positive control primary antibody at a 1 : 50 dilution in conjunction with biotinylated murine anti-rabbit immunoglobulin G antibody and visualized with fluorescein isothiocyanate-avidin, according to the manufacturer's protocol (Chemicon International). Bronchopulmonary lavage and saliva samples were tested as primary antibodies at dilutions of 1 : 4 to 1 : 32 and visualized using goat anti-mouse immunoglobulin G in the Mouse Extraavidin Peroxidase Staining kit, according to the manufacturer's protocol (Sigma Chemicals).

Results

All bacterial strains could be transformed with pKTH2121, but only *L. lactis*, *L. brevis* and *L. plantarum* demonstrated beta-lactamase secretion; *L. murinus* and *L. johnsonii* did not. Three strains of endogenous lactobacilli, isolated from the host mice and separated based on their colony and microscopic morphology, showed poor transformation efficiency with pKTH2121 and demonstrated poor expression of beta-lactamase. Beta-lactamase secretion of *L. plantarum* pKTH2121 is shown by western blot analysis in Fig. 1. Of all the transformed strains, only *L. plantarum* could be recovered by oral swab within 4 h after feeding. Persistence of *L. plantarum* pKTH2121 in the oropharynx was just as successful with simple intraoral feeding as it was after scrubbing the tongue with chlorhexidine or after applying a coat of honey to the tongue. If any of the other strains had managed to persist in the mouth below our threshold of detection, their frequency would have been less than 0.01% of total oral bacteria.

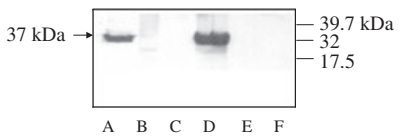
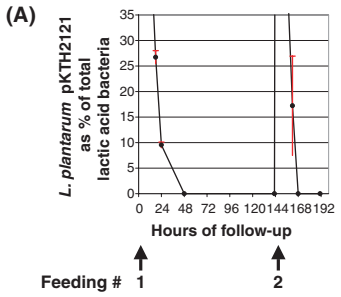
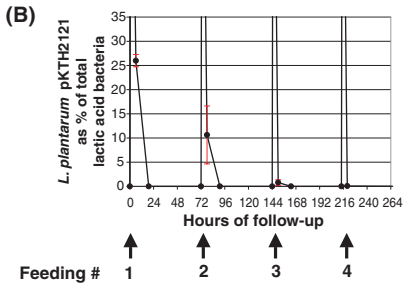


Fig. 1. Western blot analysis of lactobacilli for beta-lactamase. Lane A, cell pellet *Lactobacillus plantarum* pKTH2121; lane B, cell pellet *L. plantarum*; lane C, cell pellet *L. murinus* pKTH2121; lane D, supernatant of *L. plantarum* pKTH2121 culture; lane E, supernatant of *L. plantarum*; lane F, supernatant of *L. murinus* pKTH2121.



Time-point after feeding	Feeding # 1	Feeding # 2
18 h	26.7 (1.28)	17.2 (9.72)
24 h	9.55 (0.55)	0 0



Time-point after feeding	Feeding # 1	2	3	4
5 h	26.0 (0.128)	10.6 (5.98)	0.79 (0.59)	0 0
18 h	0 0	0 0	0 0	0 0

Fig. 2. Persistence of *Lactobacillus plantarum* pKTH2121 after repeated oral feedings. Bacterial persistence as a percentage of total lactobacilli recovered from mouse oropharynx at different time-points after sequential feedings is shown both graphically and in table format. Immediately after each feeding, *L. plantarum* pKTH2121 approached 100% of all total lactobacilli recovered. Arrows on the graph indicate times of feeding. (A) In mice fed for the first time, the percentage of *L. plantarum* pKTH2121 cultured was as high as 27% at 18 h and 10% at 24 h. However, after repeat feeding 6 days later, *L. plantarum* pKTH2121 persistence dropped to 17% at 18 h and 0 at 24 h. (B) In mice that were rechallenged with *L. plantarum* pKTH2121 feedings from 1 to 3 weeks later, these bacteria partially regained their ability to persist in the oropharynx, but the level of persistence waned rapidly with each subsequent feeding given every 3 days. After the fourth feeding, no *L. plantarum* pKTH2121 could be detected in the oropharynx by culture.

Eighteen hours after feeding, *L. plantarum* pKTH2121 represented 27% (SEM 1.3%) of total lactobacilli recoverable from oral mucosa (Fig. 2A). At 24 h after feeding, *L. plantarum* pKTH2121 still represented 10% (SEM 0.55%) of the total oral lactobacilli. The recovery of pKTH2121-positive bacteria from the mouth at various times after feeding was confirmed by polymerase chain reaction for the plasmid secretion cassette. *L. plantarum* pKTH2121 could be recovered equally from all mucosal surfaces separately, including buccal, palatal, lingual and gingival surfaces. If the *L. plantarum* pKTH2121 feedings began again after a hiatus of 1–3 weeks, the bacteria partially regained their ability to persist on the oral mucosa; however, the percentage of *L. plantarum* pKTH2121 surviving after feeding dropped progressively until none could be detected at 5 h following the fourth feeding, 12 days after the refeeding began.

The presence of secreted beta-lactamase in the mouths of mice after *L. plantarum* pKTH2121 feeding was detected by swabbing the oral mucosa with nitrocefin-impregnated indicator disks (Cefinase) (Fig. 3). Beta-lactamase activity could be detected up to 8 h following the first feeding with *L. plantarum* pKTH2121, and at 4 h following all subsequent feedings. Minimal activity could be discerned at 24 h, and no activity could be detected at 36 h or more following feedings or in animals that were not fed.

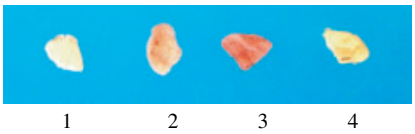


Fig. 3. Cefinase disk fragments after exposure to: 1, supernatant from washing *Lactobacillus plantarum* culture pellet scraped from an agar plate; 2, oral swab of mouse 8 h after feeding *L. plantarum* pKTH2121; 3, supernatant from washing *L. plantarum* pKTH2121 scraped from an agar plate; 4, oral swab of mouse 8 h after feeding *L. plantarum*.

As a preliminary attempt to detect a possible immunological response against *L. plantarum* pKTH2121 that might mediate clearance of these bacteria, mucosal antibodies against *L. plantarum* pKTH2121 or beta-lactamase were sought in saliva and bronchopulmonary lavage samples. Enzyme-linked immunosorbent assay for beta-lactamase antibodies was unsuccessful because two distinct monoclonal antibodies for beta-lactamase are

not available, and a purified beta-lactamase protein was not available as a positive control. Soluble beta-lactamase in the supernatant of *L. lactis* pKTH2121 was used as target antigen with supernatant from wild-type *L. lactis* as a negative control. These protein sources were impure and gave significant background signal when polyclonal anti-beta-lactamase antibody was applied to the negative control antigen. Not surprisingly, bronchoalveolar lavage and saliva samples applied in this setting also gave uninterpretable background signal (data not shown).

Immunofluoresence microscopy was also inconclusive for mucosal antibodies specific for *L. plantarum* pKTH2121. Samples of lavage or saliva were tested as primary antibodies *in situ* on *L. plantarum* pKTH2121 or control lactobacilli, including *L. plantarum* and endogenous oral lactobacilli, followed by indirect immunofluorescence microscopy. Nonspecific signal was seen for both wild-type and pKTH2121-transformed *L. plantarum*, as well as endogenous oral lactobacilli with saliva samples, but no signal was seen for bronchoalveolar lavage fluid samples, suggesting the presence of endogenous salivary mucosal antibodies binding oral lactobacilli (data not shown).

Discussion

Although the enteral persistence of orally administered probiotics has been studied, similar studies for oral persistence of such probiotics have not been reported (8, 16). Our data demonstrate that a strain of *L. plantarum* transformed with pKTH2121 fed to mice is able to persist in the oropharynx and continue to secrete beta-lactamase. The ability to recover relatively large numbers of pKTH2121-transformed bacteria from the oral mucosa 24 h after feeding indicates that these bacteria are able to co-mingle with the local microbial ecosystem and adhere to mucosa for short periods of time. The recovery of pKTH2121-positive bacteria from the mouth after feeding was not a result of plasmid transfer from *L. plantarum* to other organisms, because the orally recovered bacteria retained the original colony and microscopic morphology of the original *L. plantarum.* Also, if the plasmid were being propagated to other bacteria, the number of pKTH2121-positive organisms would be expected to remain stable or increase rather than actually decrease with repeated *L. plantarum* pKTH2121 feedings.

The inability to recover other lactobacillus strains transformed with pKTH2121 after feeding suggests that either these strains lack the ability to adhere to murine oral mucosa or that the erythromycin-resistance marker in pKTH2121 is lost. The inability of *L. murinus* and endogenous lactobacilli isolated from the host mice to persist in the oropharynx after feeding was particularly surprising, because *L. murinus* has been demonstrated to comprise up to 75% of total oral bacteria in other inbred mice (12) and endogenous lactobacilli would be naturally adapted for oral persistence. The low transformation efficiency and beta-lactamase expression of endogenous lactobacilli suggest that the reason they cannot be recovered after oropharyngeal feeding is that they may be rapidly shedding or degrading the pKTH2121 after transformation or unable to survive selection by erythromycin resistance. The rapidity with which the other lactobacillus strains are unrecoverable after feeding (within 4 h), despite the stability of pKTH2121-transformed lactobacilli *in vitro*, suggests that these bacteria fail to adhere to the mucosa. This is the case for *L. lactis*, which is able to adhere to intestinal mucosa but not oral mucosa (8). Mechanical methods to enhance the chances of bacterial adherence, such as opening binding sites on the epithelial mucus by killing resident microbes with chlorhexidine (25) or such as applying honey to improve growth conditions and prolong contact with epithelium (16), were not helpful in promoting bacterial adherence for any of the strains tested.

The loss by *L. plantarum* pKTH2121 of its ability to persist in the mouth with repeated feedings over 3 weeks suggests the possibility of bacterial clearance via the development of an immunological response. A sandwich enzyme-linked immunosorbent assay immobilizing secreted beta-lactamase and assaying salivary and bronchoalveolar fluid from multiply-fed mice resistant to *L. plantarum* pKTH2121 colonization was not specific enough to demonstrate the development of specific IgA or immunoglobulin G immunity to beta-lactamase (data not shown). Likewise, *in situ* immunostaining of *L. plantarum* pKTH2121 with salivary and bronchoalveolar lavage antibodies from both multiply-fed mice resistant to *L. plantarum* pKTH2121 and multiply-fed mice resistant to *L. plantarum*-naive mice was inconclusive to demonstrate any mucosal antibodies specific for the recombinant bacteria, but did suggest the presence of salivary antibodies against oral lactobacilli. These results do not rule out an immune-mediated clearance of multiply-fed mice resistant to *L. plantarum*, because the antibody response may be too small to detect against existing antibodies to lactobacilli (4), and because cellular immunity was not assayed. Considerable precedent already exists for killed *L. plantarum* as an immune adjuvant (13, 14) and for live *L. plantarum* as both a vector and immune adjuvant in oral vaccines (15).

Other explanations for the loss of persistence by *L. plantarum* pKTH2121 may be that this bacteria loses its ability to adhere because of changes on the mucosal surface or that the bacteria are displaced by a change in endogenous flora over time. Feeding our bacteria may induce a change in salivary mucus that is able to better clear away *L. plantarum* or inhibit its adhesion to mucosa (20).

Strong mucosal adherence and oral persistence of other exogenously delivered oral bacteria, *S. gordonii* and *Streptococcus sanguis*, have been attributed previously to such factors as multiple different adhesion molecules, pili, and even immunoglobulin A1 protease (1, 3, 10, 20, 22). However, these streptococci can only colonize in numbers too small for their secreted proteins to reach biologically significant levels in the oropharynx. Here we present an *L. plantarum* bacterial secretion system that can persist in the oropharynx transiently. Measures to improve mucosal adherence and possibly to induce immunological tolerance of these transformed bacteria may lead to an efficient oral peptide delivery system.

Acknowledgment

We thank Dr Ilkka Palva for his advice and guidance in engineering and characterizing our *L. plantarum* clones.

References

1. Bolken TC, Franke CA, Jones KF et al. Inactivation of the srtA gene in *Streptococcus gordonii* inhibits cell wall anchoring of surface proteins and decreases *in vitro* and *in vivo* adhesion. Infect Immun 2001: **69**: 75–80.
2. Cheigh CI, Pyun YR. Nisin biosynthesis and its properties. Biotechnol Lett 20054: **27**: 1641–1648.
3. Cole MF, Evans M, Fitzsimmons S et al. Pioneer oral streptococci produce immunoglobulin A1 protease. Infect Immun 1994: **62**: 2165–2168.
4. Deslauriers N, Seguin J, Trudel L. Differential recognition of oral indigenous bacteria by salivary immunoglobulins A and G. Microbiol Immunol 1987: **31**: 199–209.

5. Holo H, Nes IF. Transformation of *Lactococcus* by electroporation. Methods Mol Biol 1995: **47**: 195–199.
6. Grangette C, Muller-Alouf H, Geoffroy M, Goudercourt D, Turneer M, Mercenier A. Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: impact of strain viability and in vivo persistence. Vaccine 2002: **20**: 3304–3309.
7. Kataoka K, Amano A, Kawabata S, Nagata H, Hamada S, Shizukuishi S. Secretion of functional salivary peptide by *Streptococcus gordonii* which inhibits fimbria-mediated adhesion of *Porphyromonas ginigivalis*. Infect Immun 1999: **67**: 3780–3785.
8. Klijn N, Weerkamp AH, deVos WM. Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. Appl Environ Microbiol 1995: **61**: 2771–2774.
9. Lee Y, Lee TS. Enhancement in ex vivo phagocytic capacity of peritoneal leukocytes in mice by oral delivery of various lactic-acid-producing bacteria. Curr Microbiol 2005: **50**: 24–27.
10. Li L, Tanzer JM, Scannapieco FA. Identification and analysis of the amylase-binding protein B (AbpB) and gene (abpB) from *Streptococcus gordonii*. FEMS Microbiol Lett 2002: **212**: 151–157.
11. Lin AL, Johnson DA, Wu Y, Wong G, Ebersole JL, Yeh CK. Measuring short-term γ-irradiation effects on mouse salivary gland function using a new saliva collection device. Arch Oral Biol 2001: **46**: 1085–1089.
12. Marcotte H, Lavoie MC. Comparison of the indigenous oral microbiota and immunoglobulin responses of athymic (nu/nu) and euthymic (nu/+) mice. Oral Microbiol Immunol 1997: **12**: 141–147.
13. Murosaki S, Muroyama K, Yamamoto Y, Yoshikai Y. Antitumor effect of heat-killed *Lactobacillus plantarum* L-137 through restoration of impaired interleukin-12 production in tumor-bearing mice. Cancer Immunol Immunother 2000: **49**: 157–164.
14. Murosaki S, Yamamoto Y, Ito K et al. Heat-killed *Lactobacillus plantarum* L-137 suppresses naturally fed antigen-specific IgE production by stimulation of IL-12 production in mice. J Allergy Clin Immunol 1998: **102**: 57–64.
15. Reveneau N, Geoffroy MC, Locht C, Chagnaud P, Mercenier A. Comparison of the immune responses induced by local immunizations with recombinant *Lactobacillus plantarum* producing tetanus toxin fragment C in different cellular locations. Vaccine 2002: **20**: 1769–1777.
16. Salminen S, ed. *Lactic acid bacteria: microbiology and functional aspects of food science and technology*. New York: Marcel Dekker, Inc., 1998: 85 pp.
17. Savijoki K, Kahala M, Palva A. High level heterologous protein production in *Lactococcus* and *Lactobacillus* using a new secretion system based on the *Lactobacillus brevis* S-layer signals. Gene 1997: **186**: 255–262.
18. Scheppler L, Vogel M, Zuercher AW et al. Recombinant *Lactobacillus johnsonii* as a mucosal vaccine delivery vehicle. Vaccine 2002: **20**: 2913–2920.
19. Sibakov M, Koivula T, von Wright A, Palva I. Secretion of TEM b-lactamase with signal sequences isolated from the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl Environ Microbiol 1991: **57**: 341–348.
20. Slomiany A, Nishikawa H, Slomiany BL. Screening and modulation of extracellular signals by mucous barrier. Serum glycosylphosphatidylinositol phospholipase D (GPI-PLD) releases protective mucous barrier from oral mucosa. J Physiol Pharmacol 2002: **53**: 21–38.
21. Steidler L, Hans W, Schotte L et al. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science 2000: **289**: 1352–1355.
22. Takahashi Y, Konishi K, Cisar JO, Yoshikawa M. Identification and characterization of *hsa*, the gene encoding the sialic acid-binding adhesin of *Streptococcus gordonii* DL1. Infect Immun 2002: **70**: 1209–1218.
23. Thole JE, van Dalen PJ, Havenith CE et al. Live bacterial delivery systems for development of mucosal vaccines. Curr Opin Mol Ther 2000: **2**: 94–99.
24. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979: **76**: 4350–4354.
25. Vaahtomieni LH, Raisanen S, Stenfors LE. Effect of chlorhexidine and toothbrushing on the presence of bacteria on gingival and buccal epithelial cells. Oral Microbiol Immunol 1994: **9**: 315–317.
26. Walker DC, Klaenhammer TR. Isolation of a novel IS3 group insertion element and construction of an integration vector for *Lactobacillus* spp. J Bacteriol 1994: **176**: 5330–5340.
27. Zhou Z, Kozlowski J, Schuster DP. Physiologic, biochemical, and imaging characterization of acute lung injury in mice. Am J Resp Crit Care Med 2005: **172**: 344–351.