Jpn. J. Cancer Res. **93**, 36–41, January 2002

Chemopreventive Effect of *Lactobacillus rhamnosus* on Growth of a Subcutaneously Implanted Bladder Cancer Cell Line in the Mouse

Boon-Kian Lim,[1] Ratha Mahendran,[2] Yuan-Kun Lee[1] and Boon-Huat Bay[3, 4]

[1]Department of Microbiology, [2]Department of Surgery and [3]Department of Anatomy, National University of Singapore, 4 Medical Drive, S117 597, Singapore

Lactic acid bacteria are known to have beneficial effects on the host, such as preventing carcinogenesis. The present study was designed to evaluate the chemopreventive effects of *Lactobacillus rhamnosus* strain GG (LGG) in suppressing bladder cancer formation in a murine subcutaneous model of bladder cancer involving the inoculation of MB49 cells in C57B/L6 mice. After tumor implantation, one group of mice (n=8) was fed LGG immediately. The remaining mice that had tumors between 0.03–0.1 cm^3 were divided into two groups: those fed LGG after 7 days (n=7) and those fed saline (n=7). A second group of mice without any inoculation of MB49 cells was fed either LGG (n=10) or saline (n=10) and served as non-tumor-bearing controls. LGG was administered orally at 1.6×10^8 colony-forming units daily. Mice fed LGG immediately after tumor cell implantation formed smaller tumors and some did not develop tumors (2 out of 8 mice), when the tumor burden was small. The level of spleen CD3, CD4 and CD8a T lymphocytes, as well as natural killer cells in mice fed immediately with LGG was also higher than that in control tumor-bearing mice. There was an increase in lymphocytes and granulocytes in tumor sections, especially from the immediately fed group as compared to the controls. Our results suggest that oral consumption of LGG may prevent tumor growth via modulation of the immune system. The potential of LGG as an adjunct therapy in the treatment of bladder cancer could be further explored.

Key words: *Lactobacillus rhamnosus* strain GG — Bladder carcinogenesis — MB49 cell line — Immunomodulation

Bladder cancer, one of the major cancers worldwide, affects males three times more frequently than females. The usual treatment is transurethral resection of the bladder tumor (TURBT). However, bladder cancers frequently recur with the tumors becoming more malignant with each regrowth.[1)] Thus, prevention of tumor recurrence is of great importance and adjuvant therapy for bladder cancer has been instituted. The best results are obtained from intravesical Bacillus Calmette Guerin (BCG) instillations into the bladder following TURBT.[1, 2)] However, as a significant proportion of patients with bladder cancer do not respond to BCG therapy and side effects are known to occur,[3)] alternative modalities of treatment are being evaluated.

Lactic acid bacteria (LAB) are a group of microorganisms that are considered as probiotics[4)] which have beneficial effects on the host such as enhancing the immune response and possibly preventing carcinogenesis and tumor growth.[5)] Dr. Michio Asano, a Japanese urologist, was the first to propose the use of lactic acid bacteria as anticancer therapy for bladder cancer.[6)] Using an experimental murine bladder tumor model, Asano and colleagues found that consumption of *Lactobacillus casei* could inhibit tumor growth and prevent the recurrence of bladder cancer.[7)] However, the exact mechanism by which lactobacillus inhibits bladder cancer growth is still unclear.

In light of the above findings, the aim of this study was to determine the immunomodulatory effects of orally administered *Lactobacillus rhamnosus* strain GG (LGG), a different species of LAB, on cancer formation arising from the subcutaneous implantation of murine MB49 bladder cancer cells. The favorable effects of LGG are well established[8, 9)] and the consumption of LGG has also previously been shown to inhibit the initiation and promotion of dimethylhydrazine-induced intestinal tumors in rats.[10)]

MATERIALS AND METHODS

***Lactobacillus* strain** LGG (ATCC 53103) was obtained from The National Collections of Industrial and Marine Bacteria, Ltd. (Scotland, UK). The bacterium was propagated in MRS (de Man, Rogosa, Sharpe) broth at 37°C with 5% CO_2 until the culture reached an absorbance ($A_{550\ nm}$) of 7.00 before it was used. All media were obtained from Oxoid, Ltd., Hampshire, England. The bacterial cells were enumerated using an experimentally derived conversion factor.[11)] The conversion factor of LGG used in the experiment was 2.27×10^8 colony-forming units/ml/A.

Cell culture A murine bladder cancer cell line, MB49, provided by Dr. T. L. Ratiff, University of Iowa, USA, was used. All cells were routinely grown as described by

[4] To whom correspondence is addressed.
E-mail: antbaybh@nus.edu.sg

Gan *et al*.[12] Monolayers of MB49 cells were grown in 6-well plates with 1×10^5 cells per well in 4 ml of culture medium containing 50 U/ml penicillin G and 0.05 mg/ml streptomycin or with 50 U/ml Pen G.

Experimental animals and treatment C57BL/6J female mice were fed on a standard mouse diet (Glen Forrest Stockfeeders, WA, Australia) and given water *ad libitum.* The method described by Gan *et al.*[12] was used to establish the subcutaneous murine bladder tumor model. Immediately after tumor inoculation, 8 mice were given orally 100 μl of 1.6×10^9 cells/ml LGG suspension in sterile saline (0.85% NaCl). One week later mice with a tumor size of approximately 0.03–0.1 cm^3 were randomized into 2 groups; the control tumor-bearing group which was fed sterile saline (0.85% NaCl) (n=7) and the treatment group, fed LGG daily (n=7). Non-tumor-bearing mice also received the same treatments (LGG or saline) and served as controls. Mice were monitored for 1.5 months.

Evaluation of anti-tumor activity Tumor size was measured using calipers and the formula: length×width× height was used for the calculation of the tumor volume. The health of the mice was monitored by checking their weight regularly.

Histopathological evaluation Tumors were removed aseptically from the mice and fixed in 10% neutral formalin. After paraffinization, 10 μm sections were cut, stained with hematoxylin and eosin and analyzed under the light microscope. Tumor sections from three mice per group were selected and viewed under a 100× objective. The number of lymphocytes and granulocytes were counted in 10 random fields and expressed as per 10 high power fields (h.p.f.).

Macrophagic activity in peritoneal exudate cells The assay was carried out using *Escherichia coli* strain (*E. coli*) K12, as described by Lee *et al.*,[13] except that incubation was carried out at 37°C, and quenching of the red fluorescence of propidium iodide-stained membrane-bound and non-engulfed *E. coli* was performed using 100 μl of trypan blue (obtained from PhagoTest, OPREGEN, Gesellschaft für Biotechnologische Forschung, Entwicklung und Produktion m.b.H., Heidelberg, Germany).

Evaluation of spleen cell suspension by flow cytometry Mice were sacrificed 24 h after the last treatment. The spleens were processed as described by Gan *et al.*[12] to produce spleen cell suspensions. The suspensions were separately labeled with anti-CD4, anti-CD8 and anti-Pan natural killer (NK) cells antibodies that had been conjugated to R-phycoerythrin (R-PE); and anti-CD3 and anti-Mac-3 that had been conjugated to fluorescein isothiocyanate (FITC) (PharMingen, San Diego, CA) and analyzed by flow cytometry.

Statistical analysis The SPSS statistical software was used. Results were expressed as mean±SD. All differences between treatments were examined for the level of significance using one-way ANOVA followed by the Scheffe statistical test; except for tumor volumes, which was analyzed using repeated measures followed by the Dunnett t test. $P<0.05$ was considered as statistically significant.

RESULTS

Oral feeding with LGG affects tumor formation and growth MB49 cells were implanted subcutaneously and mice were either fed with LGG immediately (immediately

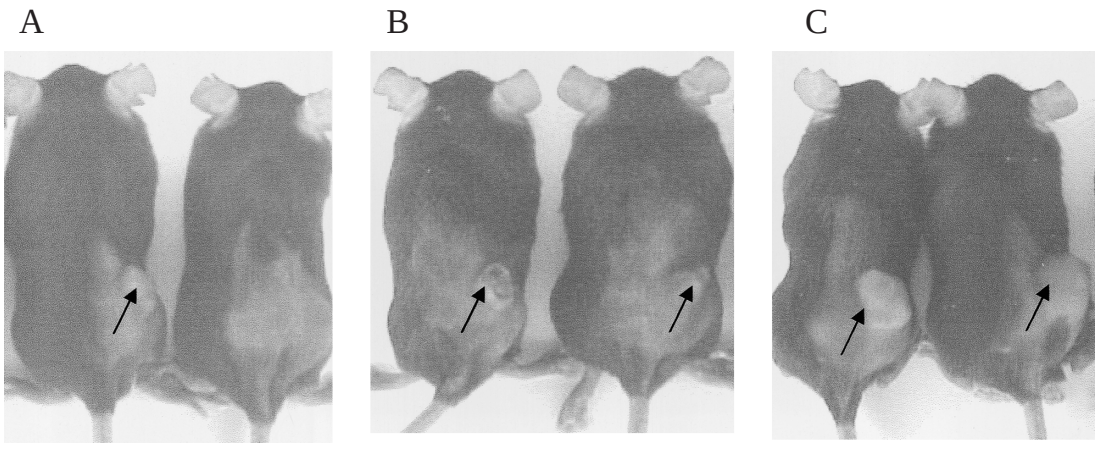

Fig. 1. Subcutaneous tumors (indicated by arrows) in mice from the different treatment groups 35 days after the inoculation of tumor cells. The mice in the immediately fed group had smaller tumors and 2/8 mice did not develop tumors (see mouse on the right) (A). Mice fed after 7 days also developed smaller tumors (B) than control mice (C).

fed) or given LGG 1 week later (fed after 7 days). Control mice bearing tumors were not fed LGG and non-tumor-bearing mice which were either fed with or without LGG served as further controls. For all groups, the mice increased in weight throughout the experiment. All the mice in both the LGG and saline fed after 7 days groups developed subcutaneous tumors from the inoculated MB 49 cells (Fig. 1). However, 2 of the 8 mice (25%) in the immediately fed group did not develop any tumor. Histopathological examination revealed the presence of sheets of plump spindle cells with enlarged vesicular nuclei and occasional distinct nuclei (Fig. 2). The mice that developed tumors in the immediately fed group showed a significantly lower tumor size at 35 days after tumor inoculation when compared to the controls ($P<0.05$; Fig. 3). The fed after 7 days mice had overall smaller tumors than control mice, but larger tumors than the immediately fed mice, although the difference in tumor volumes was not significant.

Spleen cell profiles and phagocytic activity of peritoneal exudate cells T cells, NK cells and macrophage populations in the spleen were analyzed to determine if there were any differences between mice that were fed with LGG and the control mice (Table I). Although non-tumor-bearing mice were observed to have a significantly larger amount of CD3 T cells as compared to the tumor-bearing mice ($P<0.01$), tumor-bearing mice of the immediately fed group had 1.4 times the level of CD3 cells compared to control tumor-bearing mice. The CD4 cell level was observed to be highest in the immediately fed group as compared to all the other groups. In the non-tumor-bearing group, CD4 cells in mice fed with LGG were also 1.2 fold higher than in control mice. In the tumor-bearing groups, the highest number of CD8a cells was observed in the immediately fed group, followed by the fed after 7 days group and control tumor-bearing mice. In the non-tumor-bearing group, mice fed with LGG had 1.6 times the number of CD8a cells as compared to controls.

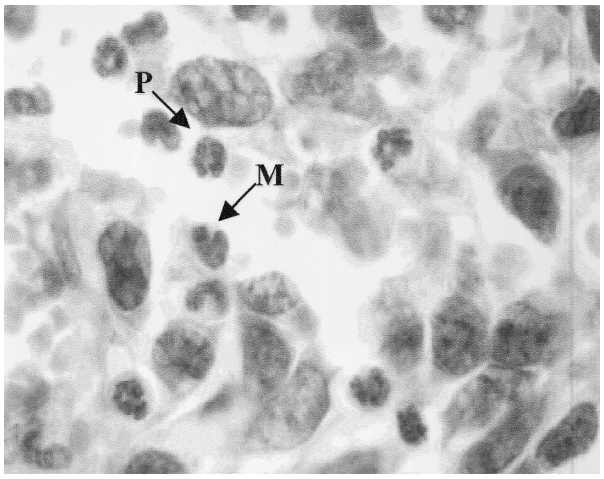

Fig. 2. Histopathological analysis of subcutaneous tumors under light microscopy. Cancer cells in a section taken from the tumor of a LGG-treated mouse together with a monocyte (M) and a polymorph (P) can be seen in this field.

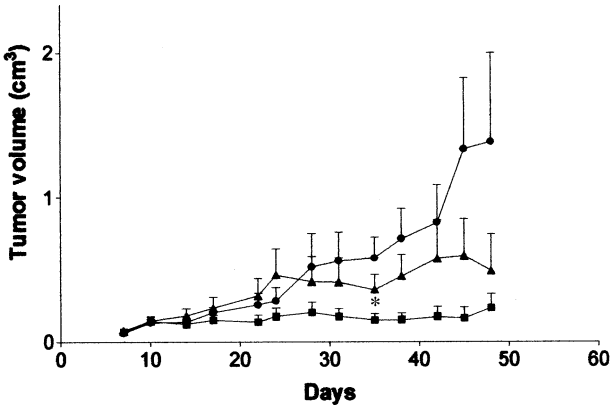

Fig. 3. Quantitation of tumor size in the three tumor-bearing groups. Tumors were measured every 3–4 days starting 1 week after tumor inoculation. By day 35, there were significantly smaller tumors (* indicates $P<0.05$) in the immediately fed group compared to the control group (which was fed saline). ■ immediately fed, ▲ fed after 7 days, ● control.

Table I. Spleen T Cells, NK Cells and Macrophage Populations in Groups of Mice with Different Treatments

	T cells			NK cells	Macrophages
	CD3	CD4	CD8a		
Immediately fed	21.834±7.930	22.581±5.476	14.511±6.462	6.815±1.900	34.711±16.171
Fed (after 7 days)	15.602±2.742	19.660±2.742	13.592±3.859	8.044±3.076	41.070±20.615
Control	15.257±3.315	16.919±3.315	11.883±3.667	7.544±4.365	41.773±21.102
No tumor—fed LGG	30.717±6.891[a)]	21.397±4.873	15.174±8.553	7.496±5.647	21.074±7.616
No tumor—control	30.373±6.050[b)]	17.838±4.217	9.719±4.974	3.060±1.099	18.946±8.207

a) $P<0.01$, *b*) $P<0.01$ compared with CD3 cells in control animals.

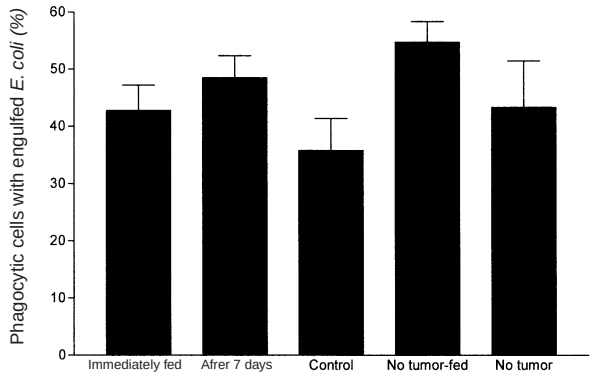

Fig. 4. Flow cytometric analysis of activated phagocytic cells in the peritoneal fluid collected from the different treatment groups. Activated phagocytic cells are identified by their ability to engulf propidium iodide-labeled *E. coli*.

Table II. Immune Cells at Site of Tumor in Different Treatment Groups

	Lymphocytes (per 10 h.p.f.)	Granulocytes (per 10 h.p.f.)
Immediately fed	15.253±1.340[a)]	12.613±3.057
Fed (after 7 days)	8.500±1.819	6.733±2.312
Control	8.766±0.321	7.500±0.265

a) $P<0.01$ compared with control group.

Although the NK and macrophage populations did not vary significantly between the different treatment groups, the NK cell number of the non-tumor-bearing mice fed LGG was higher than that of control mice. In addition, macrophage numbers in the spleens of tumor-bearing mice were found to be far greater than in the non-tumor-bearing mice. There was no enhancement of the phagocytic activity of peritoneal exudate cells in the LGG-treated groups (Fig. 4).

Local immune cell infiltration of the tumors The number of lymphocytes was highest in the immediately fed group, and there was a significant difference between the immediately fed and the control groups ($P<0.01$) (Table II). The numbers of lymphocytes in the fed after 7 days and control groups were found to be comparable. A similar trend was also observed in the granulocytes (polymorphonuclear neutrophils or polymorphs, eosinophils and basophils) infiltrating the tumors (see also Fig. 2).

DISCUSSION

The positive effects of lactic acid bacteria consumption on bladder cancer had been previously studied in a murine model using MBT-2 bladder cancer cells. Asano *et al.*[7] showed the inhibition of bladder tumor growth in mice fed both viable and non-viable *Lactobacillus casei* strain Shirota (LcS). In our study, mice that were fed with a different species of lactobacillus, LGG, had a smaller overall bladder tumor volume compared to mice that were administered saline only. Mice fed immediately with LGG after tumor inoculation had the smallest tumor volume on average. This shows that LGG was more efficacious when the tumor burden was small. Moreover, 2 of the 8 mice in the immediately fed group did not develop tumors, showing that LGG may also prevent implanted cancer cells from establishing a foothold and developing into a tumor mass. It is likely that LGG may not be effective in reducing the tumor size of large tumors or inhibiting expansion of rapidly growing tumors.

Previous studies of the effect of LAB supplements on the enhancement of immune response had been carried out in both animal tumor models and clinical trials. As early as 1984, Yasutake *et al.*[14] suggested that T cells with lactobacillus-potentiated anti-tumor immunity suppressed tumor growth. Perdigon *et al.*[15] found an increase in IgA-producing cells and T lymphocytes in the large intestines of mice with intestinal carcinoma that received oral administration of yoghurt. In this study, we observed that, although CD3 T cells in the tumor-bearing mice were significantly fewer than in non-tumor bearing mice, there was a general increase in all three T cell populations in the immediately fed group when compared to control tumor-bearing mice. Hence it would appear that oral administration of LGG to mice immediately after tumor inoculation could reduce the CD3 T cell depletion observed in tumor-bearing mice.

A study by Matsuzaki and Chin[16] demonstrated increased NK activity in spleen cells of mice fed LcS compared with the control group that were not fed bacteria. In a clinical study, Sawamura *et al.*[17] found that oral administration of LcS to patients with Dukes A colorectal cancer brought about an increase of T helper cells 1 and NK cells in the peripheral blood. It was also recently reported that the cytotoxicity of NK cells and the number of NK cells were increased after LcS treatment in mice treated with 3-methylcholanthrene.[18] However, we did not find any significant differences in the number of NK cells in the tumor-bearing groups although in the non-tumor bearing groups, mice fed with LGG had apparently higher levels of NK cells as compared to the controls.

Further, tumor-bearing mice had higher levels of macrophages compared to the non-tumor-bearing mice. This indicated that the presence of the tumor itself could stimulate an increase in macrophage numbers in the spleen. Matsuzaki[19] reported an increased cytolytic activity of peritoneal macrophages in mice that were treated with LcS. In addition, Perdigon *et al.*[20] also demonstrated that some *Lactobacillus* subspecies administered orally to mice

could activate peritoneal macrophages. However, we did not observe a significant difference between the different treatment groups. There are several possible explanations for the difference in the results, one of which could be the difference in the experimental assay that was used in the evaluation of macrophagic activity.

Although the chemopreventive effect and possible immunomodulation of LGG were clearly demonstrated, it is still unclear as to how the administered LGG exerts its effects on the immune system. It is known that despite recognition and elimination of foreign antigens (LGG in this case) in the body, mediated by the gut-associated lymphoid tissue (GALT), some microorganisms have the ability to cross the gut mucosal barrier.[21] Bacterial translocation can occur in situations where the number of bacteria exceeds a particular threshold.[22, 23] Some mechanisms that enable them to do so are endocytosis by enterocytes, intracellular diffusion, non-specific uptake when there is intestinal damage, or transportation through M cells. Claassen *et al.*[24] proved that LAB could adhere to and be taken up by M cells of the Peyer's patches and thus could be transported to underlying lymphoid follicles where they are subjected to immunological scrutiny by immune cells. In addition, LAB or its products may also be transported to systemic lymphoid tissues and activate other immune cells. Lefrancois[25] showed that lymphocytes at the intestinal epithelium could also be stimulated to secrete cytokines.

This general immune activation may explain the abundance of immune cells at the tumor site. This is evidenced by the finding that the number of lymphocytes and granulocytes in the tumor-bearing mice were highest in the immediately fed group showing increased infiltration of the immune cells at the tumor site. It is also possible that LAB products may be able to stimulate the cytotoxic activity of immune cells against bladder tumor cells. In BCG immunotherapy, it is known that some BCG components can cause the induction of non-major histocompatibility complex restricted bladder tumor cell killing.[26] Yet another possibility is that LGG or its products could be transported to the tumor site, where it either has a direct killing effect, or induces cytokine production that may attract more immune cells.

Like LcS,[7] orally administered LGG can inhibit the development of bladder tumors in mice. Suppression of cancer formation achieved via regulation of the innate immunity by probiotics could become an important mode of cancer control.[18, 27] Thus LGG taken orally could be a potential and convenient adjunct therapy for bladder cancer.

ACKNOWLEDGMENTS

This research was supported by Grant RP3992345 from the Academic Research Fund, National University of Singapore. We would like to thank Dr. Tan Puay Hoon, Senior Consultant Histopathologist (Singapore General Hospital) for the histopathological examination of the tumor sections.

(Received August 16, 2001/Revised September 26, 2001/ Accepted October 15, 2001)

REFERENCES

1) Naeyo, U. O. and Lamm, D. L. Therapy of superficial bladder cancer. *Semin. Oncol.*, **23**, 598–604 (1996).
2) Brosman, S. A. Bacillus Calmette-Guerin immunotherapy: techniques and results. *Urol. Clin. North Am.*, **19**, 557–564 (1992).
3) Zhang, Y., Khoo, H. E. and Esuvaranathan, K. Effects of Bacillus Calmette-Guérin and interferon α-2B on cytokine production in human bladder cancer cell lines. *J. Urol.*, **161**, 977–983 (1999).
4) Lee, Y. K. and Salminen, S. The coming of age of probiotics. *Trends Food Sci. Technol.*, **6**, 241–245 (1995).
5) De Roos, N. M. and Katan, M. B. Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. *Am. J. Clin. Nutr.*, **71**, 405–411 (2000).
6) Aso, Y., Akaza, H., Kotake, T., Tsukamoto, T., Imai, K. and Naito, S. Preventive effect of a *Lactobacillus casei* preparation on the recurrence of superficial bladder cancer in a double blind clinical trial. *Eur. Urol.*, **27**, 104–109 (1995).
7) Asano, M., Karasawa, E. and Takayama, T. Antitumor activity of *Lactobacillus casei* (LC 9018) against experimental mouse bladder tumor (MBT-2). *J. Urol.*, **136**, 719–721 (1986).
8) Isolauri, E., Juntunen, M., Rautanen, T., Sillanauki, P. and Koivula, T. A human *Lactobacillus* strain (*Lactobacillus casei* sp. strain GG) promotes recovery from acute diarrhea in children. *Pediatrics*, **88**, 90–97 (1991).
9) Biller, J. A., Katz, P. J., Flores, A. F., Buie, T. M. and Gorbach, S. L. Treatment of recurrent *Clostridium difficile* colitis with *Lactobacillus* GG. *J. Pediatr. Gastroenterol. Nutr.*, **21**, 224–226 (1995).
10) Goldin, B. R., Gualtieri, L. J. and Moore, R. P. The effect of *Lactobacillus* GG on the initiation and promotion of DMH-induced intestinal tumors in the rat. *Nutr. Cancer*, **25**, 197–204 (1996).
11) Greene, J. D. and Klaenhammer, T. R. Factors involved in adherence of *Lactobacillus* to human Caco-2 cells. *Appl. Environ. Microbiol.*, **60**, 4487–4494 (1994).
12) Gan, Y. H., Zhang, Y., Khoo, H. E. and Esuvaranathan, K. Antitumour immunity of Bacillus Calmette-Guerin and interferon alpha in murine bladder cancer. *Eur. J. Cancer*, **35**, 1123–1129 (1999).
13) Lee, Y. K., Soh, B. S. and Wu, J. H. Quantitative assess-

ment of phagocytic activity of hemocytes in the prawn, *Penaeus merguiensis*, by flow cytometric analysis. *Cytometry*, **43**, 82–85 (2001).
14) Kato, I., Ohwaki, M., Yokokura, T. and Mutai, M. Host-mediated antitumor activity of *Lactobacillus casei* in mice. *Gann*, **75**, 72–80 (1984).
15) Perdigon, G., Alvarez, S., Rachid, M., Aguero, G. and Gobbato, N. Symposium: probiotic bacteria for humans. clinical systems for evaluation of effectiveness. *J. Dairy Sci.*, **78**, 1597–1606 (1995).
16) Matsuzaki, T. and Chin, J. Modulating immune responses with probiotic bacteria. *Immunol. Cell Biol.*, **78**, 67–73 (2000).
17) Sawamura, A., Yamaguchi, Y., Toge, T., Nagata, N., Ikeda, H., Nakanishi, K. and Asakura, A. Enhancement of immuno-activities by oral administration of *Lactobacillus casei* in colorectal cancer patients. *Biotherapy*, **8**, 1567–1572 (1994).
18) Takagi, A., Matsuzaki, T., Sato, M., Nomoto, K., Morotomi, M. and Yokokura, T. Enhancement of natural killer cytotoxicity delayed murine carcinogenesis by a probiotic microorganism. *Carcinogenesis*, **22**, 599–605 (2001).
19) Matsuzaki, T. Immunomodulation by treatment with *Lactobacillus casei* strain Shirota. *Int. J. Food Microbiol.*, **41**, 133–140 (1998).
20) Perdigon, G., De Macias, M. E., Alvarez, S., Oliver, G. and De Ruiz Holgado, A. P. Systemic augmentation of the immune response in mice by feeding fermented milks with *Lactobacillus casei* and *Lactobacillus acidophilus*. *Immunology*, **63**, 17–23 (1988).
21) Brandtzaeg, P., Baklein, K., Bjerke, K., Rognum, T. O., Scot, H. and Valnes, K. Nature and properties of the human gastrointestinal immune system. *In* "Immunology of the Gastrointestinal Tract," ed. K. Miller and S. Nicklin, pp. 1–87 (1987). CRC Press, Inc., Boca Raton.
22) Steffen, E. K. and Berg, R. D. Relationship between caecal population levels of indigenous bacteria and translocation to the mesenteric lymph nodes. *Infect. Immunol.*, **39**, 1252–1257 (1983).
23) Fuller, R. Probiotics in human medicine. *Gut*, **32**, 439–442 (1991).
24) Claassen, E., Van Winsen, R., Posno, M. and Bocrsma, W. J. A. New and safe oral live vaccines based on *Lactobacillus*. *In* "Advances in Mucosal Immunology," ed. M. Mestecky, W. Russell, S. Jackson, S. M. Michalek, H. Tlaskova and J. Stezl, pp. 1553–1558 (1995). Plenum Press, New York.
25) Lefrancois, L. Basic aspects of intraepithelial lymphocyte immunobiology. *In* "Handbook of Mucosal Immunology," ed. P. L. Ogra, J. Mestecky, M. Lamm, W. Strober, J. Bienenstock and J. McGhee, pp. 287–290 (1994). Academic Press, San Diego.
26) Zlotta, A. R., Van Vooren, J. P., Denis, O., Drowart, A., Daffe, M., Lefevre, P., Schandene, L., De Cock, M., De Bruyn, J., Vandenbussche, P., Jurion, F., Palfliet, K., Simon, J., Schulman, C. C., Content, J. and Huygen, K. What are the immunologically active components of Bacillus Calmette-Guerin in therapy of superficial bladder cancer? *Int. J. Cancer*, **87**, 844–852 (2000).
27) Kitagawa, T., Hara, M., Sano, T. and Sugimura, T. The concept of Tenju-gann or 'natural cancer.' *Cancer*, **83**, 1061–1065 (1998).