Swine Intestinal Immunity via Toll-like Receptors and Its Advanced Application to Food Immunology

Swine Intestinal Immunity via Toll-like Receptors and Its Advanced Application to Food Immunology

Haruki KITAZAWA, Takeshi SHIMOSATO, Masanori TOHNO and Tadao SAITO

Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Aobaku, Sendai, 981-8555, Japan

Abstract

Recent interest has focused on the importance of intestinal immunity for the host defense, but to date, not much has been known about the underlying mechanisms. Toll-like receptor (TLR) family plays an important role in the defense through recognizing bacterial pathogen associated molecular patterns (PAMPs). Our research on the bioregulatory function of food products has investigated the immunoregulatory effects of lactic acid bacteria (LAB) via TLRs. Studies in swine, which is expected as a human model, have been examined intestinal immunoregulation by the LAB. Our research has now demonstrated modulation of intestinal immunity mediated by TLRs in Peyer's patches and the mesenteric lymph nodes. On the basis of our study, efforts have also been made to develop an immunoassay system for immunobiotic LAB DNA and cell wall components to evaluate immunoregulation by the LAB via TLRs. The findings in our research activities may provide important clues at the molecular level on TLR signal transduction pathways and recognition mechanisms. They also provide impetus to further delineate the activation mechanism of the innate immune response. In addition, identification of biofactors from LAB with immunoactivity, and better understanding of cytokine induction and intestinal immune regulation hold promise in basic research and development of "immunobiotic foods" to prevent specific diseases.

Introduction

The induction of the biological defense system begins with the recognition of pathogens. To date, 14 types of TLRs, which function as "pathogen sensors," have been identified (Takeda and Akira, 2005). The TLR family is important in all biological defense mechanisms and recognition of bacterial pathogen associated molecular patterns (PAMPs) which induce cytokine production and modulate the immune response in hosts. TLR2 and TLR9 recognize various cell wall components and cytosine-guanine sequences (CpG DNA) in bacterial genomes respectively (Takeuchi *et al.*, 1999; Hemmi *et al.*, 2000), triggers nuclear translocation of nuclear factor-kB (NF-kB) via the adapter molecule MyD88, induces production of inflammatory cytokines (e.g., TNF-α, IL-6, IL-12, IFN-α, and IFN-γ), and induces expression of cell surface costimulatory molecules (Fig.1). The development of vaccines containing this functional cell wall components and DNA shows promise in the prevention and treatment of infectious diseases, inflammatory diseases, cancer, and allergies (Shirakawa *et al.*, 1997; Klinman, 2004a; Ulevitch, 2004; Ahmed *et al.*, 2005; Prud'homme, 2005).

"Probiotics" refers to a category of microorganisms that provide beneficial health effects in the host by improving the intestinal flora. Research on probiotics has generally focused on controlling so-called "harmful bacteria" and increasing "beneficial bacteria" in the intestinal flora to improve "gut health." In addition to their intestinal regulatory effects, however, current research has also aimed at elucidating the effects of probiotics on intestinal mucosal immunity (Vaarala, 2003; Brown and Valiere, 2004; Cunningham-Rundles, 2004; Gill and Guarner. 2004). In 2003, Clancy proposed the concept of "immunobiotics" with reference to microorganisms that stimulate activation of mucosal immunity. This has prompted an interest in research and development of novel "immunobiotic foods" using the immunobiotics.

Our research on the bioregulatory function of food products, particularly in maintaining biological homeostasis via intestinal immunity, has investigated the immunostimulatory effects of LAB genomic DNA and DNA motifs and led to the identification of specific activation sequences (Kitazawa e*t al.*, 2001, 2003; Iliev *et al.*, 2005; Shimosato *et al.*, 2005a, 2006). Furthermore, in line with other

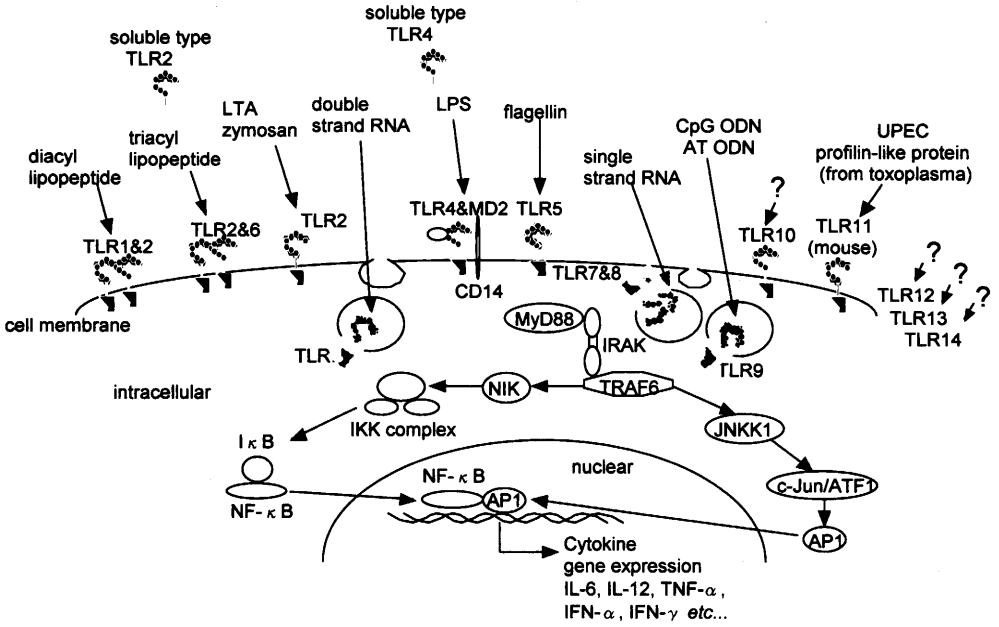

Figure 1. TLR family ligands and TLR9 mediated cell signaling. PGN(peptidoglycan), LTA(lipoteichoic acid), LPS (lipopolysaccharide), UPEC(uropathogenic. E. coli), MyD88(myeloid differentiation primary response gene 88), IRAK (IL-1 receptor-activated kinase), IKK(nuclear factor κ B (NF- κ B) kinase), JNKK1(c-JUN N-terminal kinase (JNK) kinase 1), ATF1(activating transcription factor 1), NIK(NF-kappa B-inducing kinase), AP1(activating protein 1).

recent publications, our previous study showed that structural, chemical, and conformational differences in cell surface constituents occurs even in genetically-related LAB strains containing functional cell wall components, affecting the differences of immunostimulatory effects among genetically-related LAB strains (Takeda *et al.*, 1997; Schar-Zammaretti and Ubbink. 2003).

Studies in swine, which is often used as a model for organ transplantation in humans, have examined intestinal immune activation by the LAB in food products. Efforts have also been made to develop an immunoassay system for immunobiotic LAB DNA and cell wall components to evaluate immunoactivation by the LAB, with the objective of designing functional food products. In this review, we present some of our current research on swine intestinal immunity as a human model and the potential applications of immunobiotic LAB with immunoactivity mediated via TLRs particularly TLR2 and TLR9.

1. Expression of TLRs in swine intestine

Several studies have analyzed expression of the molecules of TLR family in various organs. Strong expression in the spleen has been used as a positive control to analyze TLR molecule expression in other tissues. We assayed TLR2 and TLR9 expression from 5 sites in the intestine in newborn and adult swine (duodenum, jejunum, ileum, ileal Peyer's patches, and mesenteric lymph nodes) to examine immune system development during growth and elucidate the mechanism of TLR2- and TLR9-mediated intestinal immunity (Fig. 2). In newborn swine, we found very strong expression of TLR2 in the thymus and TLR9 in the mesenteric lymph node, at least 5 to 7 times higher than in other tissues (Fig. 2A,C). In adult swine, in the ileal Peyer's patches and mesenteric lymph nodes, which play a major role in intestinal immunity, TLR2 and 9 expression was at least 3 times higher than in the spleen (Fig. 2B,D) (Shimosato *et al.*, 2003, 2005b; Tohno *et al.*, 2005a,b).In the analysis of TLR2, TLR2 mRNA was strongly expressed in the ileal Peyer's patches and mesenteric lymph nodes, and the expression of TLR2 in these two tissues is more than 8-fold higher than in other lymphoid tissues, including spleen and thymus (Fig. 2a) (Tohno *et al.*, 2005a). Further analysis of TLR2 or TLR9 positive cells by immunohistochemistry using anti-swine TLR2 antibody or anti-swine TLR9 antibody showed the presence of TLR2 or TLR9 positive cells in the follicles of mesenteric lymph node and between the lymphoid follicles of Peyer's

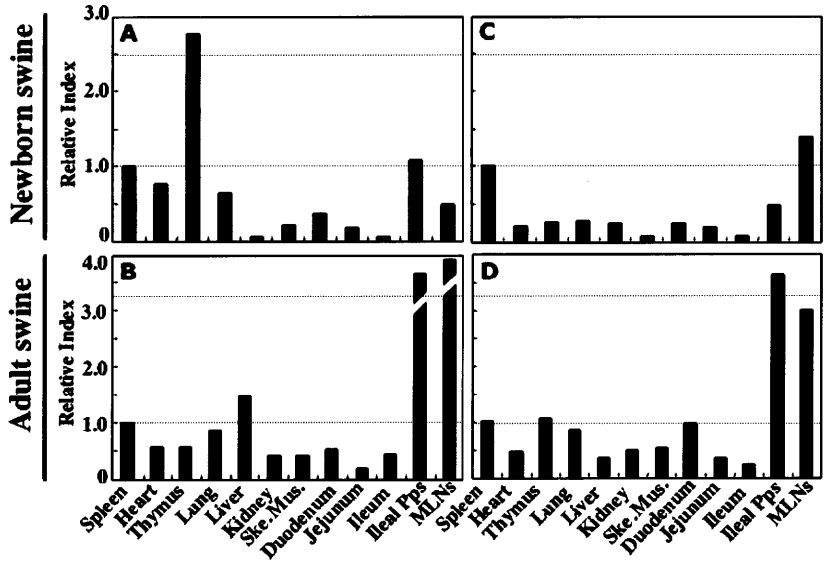

Figure 2. Real-time quantitative PCR analysis of sTLR2 mRNA in newborn (A) and adult (B) swine tissues and sTLR9 mRNA in newborn (C) and adult (D) swine tissues. The sTLR2 and sTLR9 mRNA levels were expressed as a relative index normalized against swine β-actin by the following equation: Relative index= sTLR2 or sTLR9 mRNA level of tissues/sβ-actin mRNA level of tissues. The results are presented relative to sTLR2 or sTLR9 mRNA levels in the spleen (1.0). Pps; Peyer's patches, MLNs; mesenteric lymph nodes.

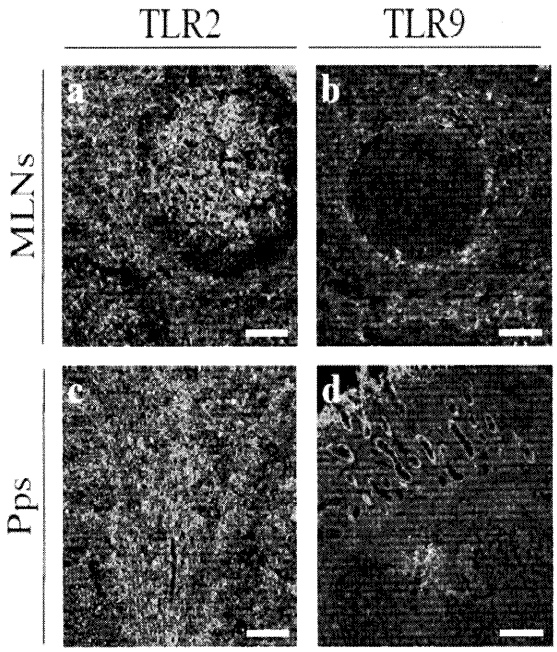

Figure 3. Immunofluorescent localization of TLR2 and TLR9 in longitudinal sections of swine MLNs and Pps. Frozen sections of MLNs and Pps were incubated with an anti-swine TLR2 or TLR9 polyclonal antibody. A panoramic view of MLNs, which was stained by anti-swine TLR2 polyclonal antibody (a) or anti-swine TLR9 polyclonal antibody (b). A panoramic view of Pps, which was stained by anti-swine TLR2 polyclonal antibody (c) or anti-swine TLR9 polyclonal antibody (d). Swine TLR2- or sTLR9-positive cells existed in and between the follicles of MLNs and Pps. Nuclei in panels a-d were stained with SYTOX orange. Original magnification = 200x. Scale bars = 100 μm. Pps; Peyer's patches, MLNs; mesenteric lymph nodes.

patchs (Fig. 3) (Shimosato *et al.*, 2003, 2005b; Tohno *et al.*, 2005a). During this research, we discovered a new finding of the strong TLR2 and TLR9 expression membranous (M) cells scattered in the follicular-associated epithelium (FAE) in the Peyer's patch dome epithelium (Shimosato *et al.*, 2003, 2005b; Tohno *et al.*, 2005a)

Recent interest has focused on the importance of intestinal immunity in medicine and immunology, but to date, not much has been known about the underlying mechanisms. Elucidation of this immune mechanisms would indeed be promising. Our research has now demonstrated modulation of intestinal immunity mediated by TLR2 and TLR9 in M cells in Peyer's patches and the mesenteric lymph nodes. Future studies will investigate in more detail the immunostimulating effects of cell wall components and LAB DNA on cells in each tissue. In addition, evaluation of the immune effects of LAB cell wall components and DNA using cell lines that express TLR2 or TLR9 will be important in elucidating the function of immunobiotic LAB and suggesting potential clinical applications.

2. Establishment of an immunoassay system for LAB DNA and cell wall components

We have shown that immunobiotic LAB cell wall components and DNA motifs can induce immunoactivation of intestinal lymphoid tissues. In addition, TLR2 and TLR9 are strongly expressed in this gut-associated lymphoid tissue (GALT). These findings demonstrate that TLR2 and TLR9 are able to recognize both pathogenic bacterial cell wall components and DNA, and dietary LAB cell wall components and DNA, thereby contributing to immunoactivation. Establishing immunoassay systems for these various components will enable researchers to evaluate both the "harmful" effects of pathogenic bacteria and the "beneficial" effects of dietary LAB. This will be an important tool in the development of functional food products. Studies must ultimately be conducted in human subjects, but basic research using animal cells and experimental animals is also essential. Therefore, to develop an immunoassay system for immunoactivity of functional cell wall components and DNA motifs from LAB, we constructed a transfectant of swine TLR2 and TLR9 with mammalian cells by the transfection of the swine TLR2 and TLR9 gene (Shimosato *et al.*, 2004, 2005a; Tohno *et al.*, 2005b).

The assay system for immunostimulatory cell wall components and DNA that we developed is a 3-step process for screening the TLR2- and TLR9-mediated immunoactivity of various DNA motifs and cell wall components by evaluating uptake, transcription activity of the intracellular signaling molecule NF-kB and cytokine induction. The cytokine assay combines both real-time quantitative PCR and ELISA to provide accurate evaluation of functional activity. This enables screening for motifs with potent immunoactivity from various DNA sequences and cell wall components in immunobiotic LAB. Elucidation of the TLR9- and TLR2-mediated immune response mechanism to LAB DNA and cell wall components are essential to future development of vaccines using normal flora and dietary LAB.

3. Future trends in immunobiotic LAB

LAB are technologically and commercially important and have various beneficial effects on the improvement of gut health through the control of intestinal ecosystem (Adolfsson *et al.*, 2004). In many studies, whole cells, including live and heat-killed cells, cell wall and cytoplasmic fractions of LAB have been shown to have various biological functions. Especially, the surface cell wall properties of LAB are important in fermentation technology (Boonaert and Rouxhet, 2000) but they are also thought to play an important role in immunomodulation of the host.

In addiontion to the cell wall components, DNA in the cytoplasmic fractions, has been shown to be a major immunomodulatory substance. An initial report in 1984 by Tokunaga *et al.* on the antitumor effects of *Mycobacterium bovis* BCG DNA, and a later report in 1995 by Krieg *et al.* on stimulation of B lymphocytes by CpG DNA motifs, has prompted keen interest in immunoactivation by microbial DNA sequences. Further understanding of the effects of CpG DNA on the immune system came with identification in 2000 of TLR9 as the receptor molecule for CpG DNA (Hemmi *et al.*, 2000). The immune effects of synthetic oligondeoxynucleotides (ODNs) containing various sequences of the 4 types of bases have been evaluated in experimental models using human PBMC and mice spleen cells. Sequences with potent immunoactivity have been identified in humans and mice. However, CpG ODNs, which are potent immunoactivators in primates (eg,

humans, rhesus monkeys, chimpanzees, orangutans), exert low immunostimulation in rodents such as mice (Klinman, 2004b; Shimosato *et al.*, 2004). This mandates the use of animal models other than mice to evaluate the immune effects of ODNs for use in humans.

Functional food factors are thought to modulate intestinal immunity by contact and stimulation of immunocompetent cells in the gastrointestinal tract and induction of cytokine production. In this "new world" of food immunology, however, much remains unknown about the underlying mechanisms of intestinal mucosal immunity. Accordingly, many details remain unclear about the effects of food product components on intestinal immune responses. The findings in our research activities may provide important clues at the molecular level on TLR2 and TLR9 signal transduction pathways and recognition mechanisms. They also provide impetus to further delineate the activation mechanism of the innate immune response. In addition, identification of LAB cell wall components and DNA with immunoactivity, and better understanding of cytokine induction and intestinal immune regulation hold promise in basic research and development of "immunobiotic foods" to prevent allergies, infectious and inflammatory diseases. Our research results can enable the design of functional food products that contribute greatly to disease prevention. This can benefit mankind by offering immunobiotic foods as a safer alternative to conventional drug therapy (Kitazawa *et al.*, 2005).

Acknowledgements

These studies were supported by a Grant-in-Aid for Scientific Research (C)(2) (No. 11660265, 13660265), (B)(2) (No. 13556018, 15380183) from the Japan Society for the Promotion of Science (JSPS) and the Uehara Memorial Foundation to Dr. H. Kitazawa.

References

Adolfsson, O., S.N. Meydani and R.M. Russell, 2004. Yogurt and gut function. Am. J. Clin. Nutr. 80: 245-256.

Ahmed, R.K., G. Biberfeld and R. Thorstensson, 2005. Innate immunity in experimental SIV infection and vaccination. Mol. Immunol. 42: 251-258.

Boonaert, C.J. and P.G. Rouxhet, 2000. Surface of lactic acid bacteria: relationships between chemical composition and physicochemical properties. Appl. Environ. Microbiol. 66: 2548-2554.

Brown, A.C. and A. Valiere, 2004. Probiotics and medical nutrition therapy. J. Nutr. Clin. Care 7: 56-68.

Clancy, R., 2003. Immunobiotics and the probiotic evolution. FEMS Immunol. Med. Microbiol. 38: 9-12.

Cunningham-Rundles, S.J., 2004. The effect of aging on mucosal host defense. J. Nutr. Health. Aging. 8: 20-25.

Gill, H.S. and F. Guarner, 2004. Probiotics and human health: a clinical perspective. Postgrad. Med. J. 80: 516-526.

Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda and S. Akira, 2000. A Toll-like receptor recognizes bacterial DNA. Nature 408: 740-745.

Iliev, I.D., H. Kitazawa, T. Shimosato, S. Atoh, H. Morita, F. He, M. Hosoda and T. Saito, 2005. Strong immunostimulation in murine immune cells by Lactobacillus rhamnosus GG DNA containing novel oligodeoxynucleotide pattern. Cell. Microbiol. 7: 403-414.

Kitazawa, H., S. Ueha, S. Itoh, H. Watanabe, K. Konno, Y. Kawai, T. Saito, T. Itoh and T. Yamaguchi, 2001. AT oligonucleotides inducing B lymphocyte activation exist in probiotic Lactobacillus gasseri. Int. J. Food Microbiol. 65: 149-162.

Kitazawa, H., H. Watanabe, T. Shimosato, Y. Kawai, T. Itoh and T. Saito, 2003. Immunostimulatory oligonucleotide, CpG-like motif exists in Lactobacillus delbrueckii ssp. bulgaricus NIAI B6. Int. J. Food Microbiol. 85: 11-21.

Kitazawa, H. T. Shimosato, M. Tohno and T. Saito, 2005. Immunostimulatory activities of Lactic acid bacteria via toll-like receptors. Jpn. J. Lactic Acid Bacteria 16: 11-20.

Klinman, D.M., 2004a. Use of CpG oligodeoxynucleotides as immunoprotective agents. Expert. Opin. Biol. Ther. 4: 937-946.

Klinman, D. M., 2004b. Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat. Rev.Immunol. 4, 249-258.

Krieg, A. M., A.K. Yi, S. Matson, T.J. Waldschmidt, G.A. Bishop, R. Teasdale, G.A. Koretzky and D.M. Klinman, 1995. CpG motifs in bacterial

DNA trigger direct B-cell activation. Nature 374: 546-549.

Prud'homme G.J., 2005. DNA vaccination against tumors. J. Gene. Med. 7: 3-17.

Schar-Zammaretti, P. and J. Ubbink, 2003. The cell wall of lactic acid bacteria: surface constituents and macromolecular conformations. Biophys. J. 85: 4076-4092.

Shimosato, T., H. Kitazawa, S. Katoh, Y. Tomioka, R. Karima, S. Ueha, Y. Kawai, T. Hishinuma, K. Matsushima and T. Saito, 2003. Swine Toll-like receptor 9 recognizes CpG motifs of human cell stimulant. Biochim. Biophys. Acta. 1627: 56-61.

Shimosato, T., H. Kitazawa, M. Tohno, S. Katoh, Y. Kawai and T. Saito, 2004. Development of immune assay system for both CpG and non-CpG DNA from lactic acid bacteria using a transfectant of swine Toll-like receptor 9. Anim. Sci. J. 75: 377-382.

Shimosato, T., H. Kitazawa, S. Katoh, M. Tohno, I.D. Iliev, C. Nagasawa, T. Kimura, Y. Kawai and T. Saito, 2005a. Augmentation of TH-1 type response by immunoactive AT oligonucleotide from lactic acid bacteria via Toll-like receptor 9 signaling. Biochem. Biophys. Res. Commun. 326: 782-787.

Shimosato, T., M. Tohno, H. Kitazawa, S. Katoh, K. Watanabe, Y. Kawai, H. Aso, T. Yamaguchi and T. Saito, 2005b. Toll-like receptor 9 is expressed on follicle-associated epithelia containing M cells in swine Peyer's patches. Immunol. Lett. 98: 83-89.

Shimosato, T., T. Kimura, M. Tohno, I.D. Iliev, S. Katoh, Y. Ito, Y. Kawai, T. Sasaki, T. Saito and H. Kitazawa. 2006. Strong immunostimulatory activity of AToligodeoxynucleotide requires a six-base loop with a self-stabilized 5'-C...G-3' stem structure. Cell. Microbiol. 8: 485-495.

Shirakawa, T., T. Enomoto, S. Shimazu and J.M. Hopkin, 1997. The inverse association between tuberculin responses and atopic disorder. Science 275: 77-79.

Takeda, K. and S. Akira, 2005. Toll-like receptors in innate immunity. Int. Immunol. 17: 1-14.

Takeda, K., T. Saito, H. Kitazawa, J. Uemura and T. Itoh, 1997. Mitogenic activity of whole cells and cell wall components of Lactobacillus acidophilus group lactic acid bacteria on murine spleen and Peyer's patch cells. Milchwissenshaft 52: 21-25.

Takeuchi, O., K. Hoshin, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda and S. Akira, 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11: 443-451.

Tohno, M., T. Shimosato, H. Kitazawa, S. Katoh, I.D. Iliev, T. Kimura, Y. Kawai, K. Watanabe, H. Aso, T. Yamaguchi and T. Saito, 2005a. Toll-like receptor 2 is expressed on the intestinal M cells in swine. Biochem. Biophys. Res. Commun. 330: 547-554.

Tohno, M., H. Kitazawa, T. Shimosato, M. Matsumoto, S. Katoh, Y. Kawai and T. Saito, 2005b. A swine toll-like receptor 2-expressing transfectant as a potential primary screening system for immunobiotic microorganisms. FEMS Immunol. Med. Microbiol. 44: 283-288.

Tokunaga, T., H. Yamamoto, S. Shimada, H. Abe, T. Fukuda, Y. Fujisawa, Y. Furutani, O. Yano T. Kataoka and T. Sudo, 1984. Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis BCG. I. Isolation, physicochemical characterization, and antitumor activity. J. Natl. Cancer. Inst. 72: 955-962.

Ulevitch, R.J., 2004. Therapeutics targeting the innate immune system. Nat. Rev. Immunol. 4: 512-520.

Vaarala, O., 2003. Immunological effects of probiotics with special reference to lactobacilli. Clin. Exp. Allergy. 33: 1634-1640.