CHAPTER 4

EFFECT OF PROBIOTIC ADDED GOAT AND COW MILK YOGHURT CONSUMPTION ON IMMUNOGLOBULIN A (IgA) PRODUCTION IN HEALTHY ADOLESCENTS

INTRODUCTION

The immune system consists of organs and several cell types. Antigen interaction with these cells induces a cellular immune response mediated by activated cells and a humoral immune response mediated by antibodies. The cellular interactions are enhanced by adhesion molecules, and the activated cells release different cytokines. These complex cellular interactions induce a systemic immune response. If the antigen penetrates by the oral route, a secretory immune response is obtained, which is mediated by secretory IgA. The determination of the number of T or B cells, the quantitative or qualitative measure of the cytokines, antibody levels, or the study of cellular function such as phagocytic activity is used to evaluate the state of the immune system. The effects of lactic acid bacteria on the systemic immune response and on the secretory immune system have been described by Phipps and Roper (1991). Potential health benefits of lactic acid bacteria include protection against enteric infections, use as an oral adjuvant, the immunopotentiator in malnutrition, and the prevention of chemically induced tumors. The results showed that *Lactobacillus casei* could prevent enteric infections and stimulate secretory IgA in malnourished animals, but could produce bacteria translocation. Yoghurt could inhibit the growth of intestinal carcinoma through increased activity of IgA, T cells, and macrophages (Meydani and Ha, 2000).

Secretory immunoglobulin A (IgA) plays a central role in local immunity and has a significant function in creating a barrier against infections by pathogenic bacteria or viruses (Underdown, 1986; Ogra and Karzon, 1970). Breast milk contains IgA, which passively helps in preventing infections in breast-fed infants and results in a lower incidence of infectious disease in breast-fed infants compared to formula-fed infants (Howie *et al*., 1990). The relatively high incidence of infectious diarrhea in

formula-fed infants can be reduced by feeding infant formulas containing viable bifidobacteria (Saavedra *et al*., 1994) or the other lactic acid bacteria (Brunser *et al.,* 1989; Gonzalez *et al.,* 1990). IgA is also actively produced in the intestine, and contributes to the elimination of infectious pathogens from the gastrointestinal tract (Underdown, 1986; Ogra and Karzon, 1970).

This section aimed to investigate the effect of long-term consumption of the goat and cow milk yoghurt powder containing probiotic bacteria on IgA production in healthy adolescents.

4.1 LITERATURE REVIEW

4.1.1 Human immune system function

The main functions of the immune system are to eliminate invading viruses and foreign microorganisms, to rid the body of damaged tissue and to destroy neoplasms in the body. Healthy humans have 2 immune mechanisms, i.e., acquired (specific) immunity which responds to specific stimuli (antigens) and is enhanced by repeated exposure; and innate (nonspecific) immunity which does not require stimulation and is not enhanced by repeated exposure. Innate immune mechanisms consist of physical barriers such as mucous membranes, and the phagocytic and cytotoxic function of neutrophils, monocytes, macrophages and lymphatic cell (NK cells). Acquired immunity can be classified into 2 types on the basis of the components of the immune system that mediate the response, i.e., humoral immunity and cell-mediated immunity. Humoral immunity is mediated by immunoglobulin produced by bone marrow-derived lymphocytes (B lymphocytes) and is responsible for specific recognition and elimination of extracellular antigens. Cell-mediated immunity is mediated by cell of the immune system, particularly, thymus-derived lymphocytes (T lymphocytes). Cell-mediated immunity is responsible for delayed-type hypersensitivity (DTH) reaction, foreign rejection, resistance to many pathogenic microorganisms, and tumor immunosurveillance. In addition to their involvement in nonspecific immunity, macrophages are important in cell-mediated immunity as antigen-presenting cells and through the production of regulatory mediators such as cytokines and eicosanoids. Several in vitro and in vivo tests were developed to assess the function of immune cells. Although the study of immune response in animals and

humans is based on similar principles, the methods used to separate cells, the types of stimuli used in vitro, and the antigen used for in vivo challenge vary. In addition, the type of antibody used to measure different mediators or to determine cell-surface proteins is species-specific **(**Simin and Woel, 2000).

4.1.1.1 Phagocytic activity

Phagocytic activity is the ability to perform phagocytosis and kill microbes, including bacterial pathogens, and is a major effector function of macrophages. These properties of macrophages are particularly important for host defense against facultative intracellular organisms which can replicate within macrophages. The pathogenesis of facultative intracellular bacteria is determined by their ability to survive within macrophages. Several organisms were used previously as targets to determine macrophage killing. These include *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Listeria monocytogenes* and *Candida albicans* (Coligan *et al*., 1994).

Bacteria bind to complement components and the bacterium complement complexes bind complement receptors on the surface of macrophages. Phagocytosis may also be mediated by specific antibodies that function as opsonins, which bind to particles, rendering them susceptible to phagocytosis. The bacterium-antibody complex then binds the macrophages via the Fc receptor and phagocytosis begins. Measurement of phagocytic activity of macrophages was among the earliest techniques for evaluating the immunologic effects of Lactic Acid Bacteria (LAB). This assay measures the ability of macrophages to bind, internalize and phagocytose bacteria. Monocytes or macrophages isolated from human peripheral blood mononuclear cell (PBMCs) or from the peritoneal cavity of animals are mixed with bacteria in suspension and incubated at 37ºC. Extracellular bacteria are then removed through washing and centrifugation or through washing only over sucrose. The degree of phagocytosis is determined by examining stained cell under oil immersion microscopy and quantifying the number of internalized bacteria in each cell. This method takes into account not only the percentage of phagocytic cell but also the strength of the phagocytic ability of these cells, i.e., how many bacteria are internalized by each cell (Coligan *et al*., 1994).

4.1.1.2 Lymphocyte proliferation assay

Wayne *et al*. (1990) reported that the measurement of the proliferative response of lymphocytes is the most commonly used technique for evaluating cell-mediated immune response. Quantitative analysis of proliferative response involves measuring the number of cells in culture in the presence and absence of stimulatory agent such as an antigen or a mitogen. The most common polyclonal mitogens used to test the proliferation of lymphocytes are concanavalin A (ConA), phytohemagglutinin, lipopolysaccharide (LPS) and pokeweed mitogen. T and B lymphocytes are stimulated by different polyclonal mitogens. ConA and phytohemagglutinin stimulate T cells, LPS stimulates B cells while pokeweed mitogen stimulates both T and B cells. When mitogens are used, prior exposure of the host to the mitogens is not necessary. However, to measure antigen-specific proliferation, the host should be exposed to the antigen before the cells are stimulated with that antigen in vitro. Lymphocytes normally exist as resting cells in the G_0 phase of the cell cycle.

When stimulated with polyclonal mitogens, lymphocytes rapidly enter the G_1 phase and progress through the cell cycle. Measuring incorporation of [^{3}H] thymidine into DNA is the most commonly used method for estimating changes in the number of cell. The proliferative assay is used to assess the overall immunologic competence of lymphocytes, as manifested by the ability of lymphocytes to respond to proliferation signals. Decreased proliferation, observed in chronic diseases such as cancer and HIV infection and in the aging process, may indicate impaired cell-mediated immune function (Coligan *et al*., 1994).

4.1.1.3 Cytokine production

Cytokines, which are protein mediators produced by immune cell, are involved in the regulation of cell activation, growth and differentiation, inflammation and immunity. Measurement of cytokine production, as determined by techniques such as bioassay, radioimmunoassay and enzyme-linked immunosorbent assay, has been used to examine various immune functions. Details of cytokine measurement were published previously (Coligan *et al*., 1994).

Interleukin 2 (IL-2) is a T cell growth factor produced by T helper (T_H) 1 and NK cells. As an autocrine and paracrine growth factor, IL-2 induces proliferation and differentiation of T and B cells. IL-2 is responsible for the progress of T lymphocytes from the G_1 to the S phase in the cell cycle and also for stimulation of B cells for antibody synthesis. IL-2 stimulates the growth of NK cells and enhances the cytolytic function of these cells, producing lymphokine-activated killer (LAK) cells. IL-2 can also induce interferon (IFN)-γ which is an important macrophage-activating lymphokine. IL-2 secreted in culture media or biological fluids can be measured by immunoassay or bioassay, the most common of which uses the IL-2-dependent cytotoxic T lymphocyte line cells to reflect IL-2 activity. IL-2activity in samples can be calculated according to a standard curve generated by adding varying concentrations of recombinant IL-2. Enzyme-linked immunosorbent assay is also used to measure IL-2. Although this assay is more specific than is the cytotoxic T lymphocyte line in measuring IL-2 protein concentrations, it does not differentiate between biologically-active and nonactive proteins. Under most conditions, changes in IL-2 production are associated with the change in lymphocyte proliferation, although sometimes these changes do not correlate with one another (Coligan *et al*., 1994; Bocci *et al*., 1998).

4.1.2 Probiotic bacteria and human immune system

The gastrointestinal tract constitutes the interface between the environment and the host, and as such has the important dual role of excluding potential pathogens while facilitating the passage of nutrients. Intestinal epithelial cells, which are joined by tight junctions that prevent passage between cells, and the mucus they produce form a physical barrier against pathogens. Increasing evidence from studies with genotobiotic animals suggest that initial colonization with commensal bacteria constitutes a vital stimulus for the synthesis of substances that fortify the mucosal barrier and decreases intestinal permeability. In addition, commensal bacteria play a vital role in colonization resistance. This term was originally introduced only in reference to the ability of the indigenous microflora to inhibit colonization by exogenous potentially-pathogenic microorganisms. It has since been broadened to include the prevention of overgrowth of indigenous

microorganisms that have the potential to become pathogenic. Anatomical and physiological factors such as an intact mucosa, salivation, swallowing, normal gastrointestinal motility, production of gastric acids and secretion of IgA contribute to colonization resistance, but are ineffective in the absence of a normal undisturbed flora (Del Piano *et al.,* 2006).

In addition to the non immunological barrier of the intestinal mucosa, the GALT plays a vital role in immune exclusion. GALT is the largest lymphoid tissue of the human body and its hallmark is the production of secretory IgA which, in contrast to serum IgA, is dimeric or polymeric and resistant to proteolysis in the intestinal lumen and does not activate inflammatory responses. Colonization with commensal bacteria is essential for the development of a fully-functional and balanced immune system, including not only the development and maturation of IgA plasmocytes and IgA production, but also the development of tolerance towards food antigens and intestinal microbes. Interactions between the intestinal microflora and GALT are thought to be vital for maintaining the intestinal immune system in the state of permanent low level activation, or physiological inflammation, that is considered to be important in host defense against pathogens. Probiotics such as *L. acidophilus* and *B. bifidum* have been shown to influence selective aspects of immune function. Such altered function can involve one or several components of an immune response, e.g., humoral, cellular or nonspecific immunity. Although several in vitro and in vivo studies on probiotic effects on immunity have been reported, the specific mechanisms of the observed changes remain unclear. Reports of probiotic-induced alteration are not limited to the localized mucosal immune systems; effects on systemic immune responses have also been reported. Macrophages represent one of the first lines of nonspecific defense against bacterial invasion and tumors. Macrophages kill bacteria and tumor cells through several effector mechanisms, including the production of soluble factors such as nitric oxide, hydrogen peroxide and superoxide. Macrophages can also use receptor-mediated attachments to kill tumor cells through direct cell-to-cell contact. During activation, macrophages acquire the capacity to bind unopsonized tumor cells as well. Macrophage responses to bacterial products are processed by a mechanism similar to that of tumoricidal

activity. It was suggested that the antitumor effect of LAB is due to enhancement of macrophage activity (Elahi *et al*., 2008; Fioramonti *et al*., 2003; Klein *et al*., 1998).

Other studies in which reconstituted lyophilized LAB were administered orally or intraperitoneally showed that enhancement of macrophage activation by lactic acid bacteria *L. acidophilus* induced production of IFN-α and IFN-β in murine peritoneal macrophage cell culture. If an antigen overcomes the nonspecific host-defense system, both the humoral and the cell-mediated immune responses are activated. Orally administered LAB may pass through the GI lumen to reach the local lymphatic organs in the gut. Subsequently, translocation of LAB can lead to the activation of the local immune system in the gut, which results, in turn, in mucosal antibody production, especially of sIgA from PP cells. Generally, sIgA is induced very poorly after intramuscular or subcutaneous immunization but can be induced vigorously by oral immunization. sIgA inhibits colonization of pathogenic microorganisms (enteric infection) and penetration of dangerous luminal antigens (Kedia *et al*., 2007; Pennacchia *et al.*, 2006; Klein *et al*.,1998;).

A study for the influence of a yoghurt-supplemented diet on the immunocompetence and survival of animals subsequently infected with *S. typhimurium* was reported by Kourkoutas *et al*. (2006). They suggested that feeding a diet supplemented with yoghurt containing live LAB for 4 weeks increased the rate of survival of young mice against *S. typhimurium* infection. These authors attributed the effect to the ability of live LAB to enhance local and systemic immune response. Interestingly, yoghurt supplemented with heat-killed bacteria was not effective. These researchers reported that the mitogenic response to ConA and phytohemagglutinin was significantly higher in mice fed a yoghurt diet than in mice fed a milk diet. It was suggested that the immunostimulatory function seen with oral administration of LAB was partially mediated by increased secretion of IFN-γ from PP cells in gut-associated lymphoid tissue. IFN-γ was shown to enhance expression of the secretory component, thus playing an important role in increasing external transport of dimeric IgA (Kourkoutas *et al*., 2006; Doleyres and Lacroix, 2005; Picot and Lacroix, 2004).

Human studies examining the immunostimulatory effects of probiotic focused primarily on the effect of yoghurt consumption in vivo indicators of immune response, such as cytokine production, phagocytic activity, specific humoral immune

response, T lymphocyte ($CD4^+$ and $CD8^+$) function and NK cell activity (De Simone *et al*., 1986). Zanini *et al*. (2007), who studied of the effects of fermented milks with simple and complex probiotic mixtures on the intestinal microbiota and immune response of healthy adults and children**,** compared the effect of two functional fermented milks with simple and complex probiotic mixtures on the microbiota and some immune system function of healthy adults and children. The complex yoghurt, containing *L. casei* and other strains of lactobacilli and bifidobacteria, and the simple yoghurt, containing only *L. casei*, were administered daily to healthy volunteers (41 adults and 36 children) for a period of 4 weeks. Microbiological and molecular analyses of faecal samples indicated that the complex product induced a larger increase in the lactic acid bacteria number, compared with the simple product, without significant alterations of the autochthonous species composition. Of the probiotic strains, *L. casei* showed the best survival rate in faeces. The influences of both products on the immune system were similar, with increase of NK activity and proliferative response to *C. albicans* in adults and secretory-IgA in children saliva, indicating that immunomodulating properties were probably supported by common traits in the two products.

The composition of the microflora in the intestines of healthy adults is known to be stable, and exerts resistance against colonization by exogenous pathogenic bacteria. In this study, fecal bifidobacteria in children aged one to three years were the second-most predominant bacteria following bacteroidaceae, indicating that the composition of the intestinal microflora in these children was closer to that of adults rather than infants. During intake of NAN BF, the number of bifidobacteria slightly increased. However, the population of the other bacteria, especially bacteroidaceae, did not change greatly during intake of NAN BF, and bifidobacteria did not become predominant as in breast-fed infants. These results suggest that the intestinal microflora of young healthy children seems to be as stable as that of adults, and the intake of probiotics does not drastically modify the microflora (Yoichi *et al.*, 1998).

One of the beneficial effects of probiotics is to prevent infectious diseases. Probiotic starter formula, which contains the same *Bifidobacterium* strain as that in this study, reduced the incidence of acute diarrhea in hospitalized infants.

Probiotics also enhance humoral immune responses by increasing Immunoglobulin A (IgA)- producing cells and stimulate antibody responses to some specific antigens. In this study, they found that the levels of total fecal IgA and anti-poliovirus IgA increased significantly during intake of the probiotic formula in healthy children. This suggests that ingestion of the formula containing *Bifidobacterium* stimulated the production of IgA in the gastrointestinal tract of the children. The role of intestinal IgA is to eliminate invading pathogens from the gastrointestinal tract. Anti-poliovirus IgA antibody prevents poliovirus multiplication in the gastrointestinal tract by inhibiting attachment to the mucosal surface (Krasaekoopt *et al.*, 2003).

Colonization of bacteria in the gastrointestinal tract is an important characteristic of probiotics which results in beneficial effects for the host. During intake of NAN BF, *B. lactis* Bb-12 was found in the feces of most of the children. In some subjects, the number of *B. lactis* Bb-12 detected in the feces during intake was more than 10^9 per g, which was sometimes more than the daily administration of *B. lactis* Bb-12 from NAN BF. This indicates that *B. lactis* Bb-12 could have reached the intestines and proliferated there. The population of *B. lactis* Bb-12 in the fecal samples was at most 27% of total bifidobacteria, which indicates that most bifidobacterial strains in the gastrointestinal tract, even during intake of the formula, were resident bifidobacteria. Some bifidobacteria strains were reported to stimulate IgA production in vitro, implying the possibility that the stimulation of IgA production was elicited by resident bifidobacteria (Yasui *et al*., 1992).

4.1.3 Yoghurt and immune related disease

The health benefits of yoghurt are due primarily to the ability of LAB to survive in the human GI tract. LAB commonly used for yoghurt production were shown to survive in the stomach and were found in the feces, although survival rates are not known for all strains of LAB. Some strains of LAB show a survival rates of 0.001-2.0%, live LAB were shown to have several prophylactic effects (Kaila *et al*., 1995; Kawai *et al*., 1980; Marteau *et al*., 1993).

4.1.3.1 Cancer

Van't Veer *et al.* (1991) reported that yoghurt containing LAB can inhibit the growth of transplantable and chemically-induced tumors in animals.

However, results from epidemiologic studies on the incidence of cancer are not consistent. Although high consumption of fermented milk products (yoghurt, buttermilk and Gouda cheese) may protect against breast cancer, yoghurt consumption was shown to be correlated with a higher incidence of ovarian cancer.

Animal studies show that LAB exerts anticarcinogenic effects. Diet-induced microfloral alteration may retard the development of colon cancer. Some indigenous LAB, such as *L. acidophilus*, *B. longum*, *Lactobacillus* GG and components of LAB (e.g., insoluble fraction of sonicated cells of *L. bulgaricus*), were shown to exert tumor-suppressing effects (Reddy and Rivenson, 1993; Goldin *et al*., 1996).

Although the mechanisms by which LAB exert antitumor and anticarcinogenic effects are not fully understood, preliminary findings suggest that the potential mechanisms can be classified into 3 categories. One potential mechanism involves the changes in fecal enzymes thought to be involved in colon carcinogenesis. Nitrate was shown to be metabolized by nitrate reductase, an intestinal bacterial enzyme, to nitrite and may be metabolized further to nitrogen or ammonia. Nitrite may also be an important intermediary in the formation of N-nitroso compound, which has been found to be highly carcinogenic in animals. Yoghurt bacteria were shown to have nitrate reductase activity. Thus, these yoghurt bacteria can reduce nitrite concentration, there by eliminating nitrate and nitrosamines (Goldin *et al.,* 1996; Dodds and Collins-Thompson, 1984).

A second possible mechanism involves LAB cellular uptake of mutagenic compounds, such as nitrite, in the gastrointestinal tract, thereby reducing the compounds' potential conversion to carcinogenic compounds, nitrosamines. The third potential mechanism involves suppression of tumors by enhancement of immune response. Although animal studies showed that LAB may inhibit tumorigensis, no evidence in this regard is available for human. *L. acidophilus,* however, was shown to reduce fecal enzyme activity of β-glu-curonidase, nitroreductase and azoreductase (Fernandes *et al*., 1987).

4.1.3.2 Gastrointestinal disorders

Yoghurt's microorganisms may prevent infections of the GI tract by influencing its microbial ecosystem. However, LAB that are colonized in the

human intestine, *L. acidophilus* species, are more resistant to gastric acid than are LAB conventionally used for yoghurt fermentation (*L. bulgaricus* and *S. thermophilus*) (Fernandes and Shahani, 1989).

The inhibitory mechanisms of LAB against disease-causing bacteria are due primarily to 2 metabolites of lactic acid fermentation, i.e., organic acids and bacteriocin (Fernandes and Shahani, 1989). It was also shown that prevention of and recovery from infection from pathogenic bacteria or viral infection in children with acute rotavirus-associated diarrhea can be enhanced through augmentation of the local immune defense, particularly by increasing the number of immunoglobulin-secreting cells. In addition, oral microbial therapy with LAB can be effective in preventing antibiotic-induced GI disorders and in recovery from diarrhea. Colombel *et al.* (1987) reported that the simultaneous intake of *B. longu*m-containing yoghurt with erythromycin reduced the frequency of GI disorders in human subjects who were taking erythromycin and a yoghurt placebo. Thus, consumption of yoghurt with LAB can reduce antibiotic-induced alterations of the intestinal microflora. Several animal studies also showed beneficial effects of yoghurt consumption in building up resistance to GI pathogens.

4.1.3.3 Immunoglobulin E-mediated hypersensitivity

LAB in yoghurt are known to enhance concentrations of IFN-γ, which is produced mainly from T_H1 cells. IgE-mediated hypersensitivity (type 1 allergy) is triggered by antigens cross-linking with preformed IgE antibodies that are bound to antibody receptors (FcεR1) on mast cell surfaces. The T_H2 cytokine, IL-4, upregulates isotype switching of IgM to IgE but IFN-γ produced by T_H1 cells inhibits isotope switching (Herich and Levkut, 2002).

In human studies, it was shown that long-term consumption of large quantities of yoghurt (450 g/d) can increase production of IFN-γ by lymphocytes (De Simone *et al*., 1986), isolated T cells and PBMCs. Shida *et al.* (1998) reported that *L. casei* added in vitro to splenocytes from ovalbumin-primed BALB/c mice induced IFN-γ production but inhibited IL-4 and IL-5 secretion and markedly suppressed total and antigen-specific IgE secretion by ovalbumin-stimulated lymphocytes. Treatment of *L. casei* with pepsin at low pH for 3 h had no effect on the

ability of *L. casei* to reduce IgE. This implies that oral consumption of *L. casei* might also be effective in reducing IgE production. These results showed that yoghurt might be effective in reducing IgE-mediated pathologies, such as asthma. Human studies, however, produced inconsistent results. Trapp *et al*. (1993) reported that consumption of yoghurt (200 g/d) with live active cultures reduced allergic symptoms in young subjects but had no effect on IFN-γ, total IgE or specific IgH concentrations. Older subjects who consumed yoghurt containing live bacteria, however, had lower IgE concentrations than a control group. Wheeler *et al*. (1997) found no effect of yoghurt consumption on asthma-related symptoms and pulmonary function in a group of patients with asthma.

4.2 EXPERIMENTAL

4.2.1 Subject

Twenty healthy adolescent volunteers (10 males, 10f emales) within the age range of 18-25 years were enrolled in 8-week dietary supplementation trial. Subjects were drawn initially in response to an advertisement for volunteers.

Suitable candidates were then selected following informed consent and consultation with a doctor. Inclusion criteria included general good health and mobility, and an agreement to conform with the trial guidelines or provide notification of compliance.

Exclusion criteria included any recent history of acute or chronic debilitating illness, any record of milk-product intolerance, and an agreement to avoid potentially-conflicting nutritional or vitamin supplements during the eight-week duration of the trial.

The subjects were made aware that some of the milks which they would consume might contain health-benefiting microorganisms, but in all other aspects were blinded with respect to trial design or diet.

4.2.2 Trial design

The subjects received a pack of freeze dried probiotic-added goat and cow milk yoghurt powder (10 g) to be reconstituted with 100 ml fresh water and

consumed every day in the morning for 8 weeks. They were instructed to avoid the consumption of other dairy products during the period of the study.

The trials were a pre-post design, blood samples were obtained by venipuncture from the subjects at eight time points throughout the trial. Blood draws for measurement of serum IgA levels were performed at weeks -1, 0, 3, 5, 8, +1 and +2. Weeks -1 (7 days before day 0) and 0 (day 0) served as baseline collections performed before ingestion of probiotic supplements, week +1 (7 days after stoppage of intake of food sample) and week +2 (14 days after stoppage of intake of food sample). Probiotic ingestion began after specimen collection on day 0.

Volunteers were interviewed at each subsequent visit about consistency of nutritional habits, exercise routine, stress levels and ingestion of immune supplements or fermented foods. Volunteers were also asked to report any changes in general health during these visits.

The control group (20 healthy adolescent volunteers) received a pack of plain freeze dried probiotic-added goat and cow milk yoghurt powder and reconstituted with 100 ml fresh water, to be consumed as placebo for the same period. They were instructed to avoid the consumption of other fermented dairy products during the period of the study.

4.2.3 IgA analysis

The total serum IgA level was determined by sandwich-type enzyme-linked immunosorbent assay (ELISA), in which anti-human IgA antibody was coated on an ELISA plate and detected with peroxidase-labeled anti-human IgA (Yoichi *et al.,* 1998).

4.2.4 Statistical Methods

Data was analyzed by a SPSS program (SPSS version 11, SPSS Inc., Chicago, USA). If the significant differences between means were found, the mean comparation with Duncan's multiple range test would be applied. The predetermined acceptable level of probability was 5% ($p<0.05$) for all the comparisons (Montgomery, 2001).

4.3 RESULT AND DISCUSSION

4.3.1 Subject's demographic data

The data in Table 27 show that the subject's age ranged from 18 – 22 years, including 10 men and 10 women (nonpregnant and nonlactating). All subjects were considered to be in good general health, reported no recent history of using immune- modulating supplements and ingesting fermented food, and had no history of difficulty donating blood. All participants were willing to maintain their current pattern of diet, exercise and fermented food avoidance for the remainder of the experiment.

During the study, only 1 subject reported to have diarrhea and over gas production in the gastrointestinal tract and he got vomiting to occur once in a while, particularly in the first day of the study. Thirty-nine subjects reported that this situation did not occur during the intervention periods. No subjects withdrew during the study, the data are shown in Table 28.

Table 27 Demographic data of the subjects in the Immunoglobulin A (IgA) study

Demographic data	Treatment group	Control group
Total subjects	2 0	20
Average age (y)	18	18
Gender (male/female)	10/10	10/10
Place of residence		
University dormitory	12	10
Private dormitory	6	8
Private home	2	2
Pregnancy history	0	0
Withdrawal	0	0

Table 28 Prevalence of subject symptoms during the Immunoglobulin A (IgA) study

Symptoms	Treatment group		Control group	
	Male	Female	Male	Female
Vomiting only	0	0	1	0
Diarrhea only	1	0	0	0
Abdominal pain only	0	0	0	0
Diarrhea and abdominal pain	0	0	0	0
Vomiting and diarrhea	0	0	0	0
Other symptom	1	0	0	0

4.3.2 The change of IgA level during probiotic-added goat and cow milk yoghurt consumption

The intake of probiotic-added goat and cow milk yoghurt significantly stimulated the production of serum IgA in healthy adolescents. The variation was significant over the 8-week ingestion ($p<0.05$). IgA number did not vary significantly in the control group during the same period of the study.

The levels of total serum IgA are shown in Table 29. The serum IgA level was 274 – 275 mg/dl before intake of probiotic-added goat and cow milk yoghurt sample. This level was not different with the control group before study. During intake, the level of serum IgA was significantly increased, with the highest level occurring at 312 and 309 mg/dl on week 8, in male and female groups, respectively, and the IgA level was about 1.13 folds higher than the initial value. After termination of probiotic-added goat and cow milk yoghurt intake on week +2 (14 days after stoping intake of sample), the level of IgA decreased and were closed to the initial level. The levels of IgA in male and female were not significantly different in both treatment and control group. At the final stage (2 weeks after stopping supplement), the subjects' immune responses (IgA levels) returned to baseline levels that were not significantly different from the pre-trial values (one week before supplementation). Katia *et al.* (2007) suggested that changes in the immunes response at the end of ingestion of probiotic period, could be due to the action on the immune system that was strictly correlated with the presence of the probiotic bacteria in the intestine. The immunity returned to baseline value following cessation indicated that immune modulation can be carefully controlled by regulating dietary intake.

Table 29 Serum IgA levels (mg/dl) of subjects before, during and after consumption of probiotic-added goat and cow milk yoghurt

Periods of blood draws*	Treatment group		Control group	
	Male	Female	Male	Female
-1	275±2.4 ^{aA}	274±2.0 ^{aA}	275±2.5 ^{aA}	275±3.8 ^{aA}
0	276±2.7 ^{aA}	274±1.4 ^{aA}	274±2.1 ^{aA}	275±1.1 ^{aA}
3	286±3.2 ^{aB}	287±4.5 ^{aB}	276±1.9 ^{bA}	276±1.6 ^{bA}
5	297±1.3 ^{aC}	295±1.2 ^{aC}	276±2.3 ^{bA}	275±2.4 ^{bA}
8	312±4.3 ^{aD}	309±1.7 ^{aD}	276±2.1 ^{bA}	275±1.3 ^{bA}
+1	302±3.6 ^{aE}	298±2.3 ^{aE}	275±3.0 ^{bA}	274±4.1 ^{bA}
+2	278±1.8 ^{aAF}	280±3.4 ^{aAF}	276±3.9 ^{aA}	276±1.7 ^{aA}

Means in the same row with different small letter superscripts for the difference between treatments are significantly different; means in the same column for the different periods with different capital letter superscripts are significantly different.

* Blood draws were performed at weeks -1, 0, 3, 5, 8, +1 and +2. Weeks -1 (7 days before day 0) and 0 (day 0) serve as baseline collections performed before ingestion of probiotic supplements, week +1 (7 days after stopping of intake of food sample) and week +2 (14 days after stopping of intake of food sample).

Similar results have been observed with Yoichi *et al.* (1998) that was performed to elucidate the influence of a probiotic formula on intestinal microflora and local immunity in healthy children. Yoichi *et al.* (1998) reported that a follow up formula containing viable bifidobacteria was given to seven healthy Japanese children for 21 days. During intake of the formula, the administered strain was detected in feces from five subjects (71%) and fecal bifidobacteria slightly increased. The levels of total IgA and anti-poliovirus IgA during intake of the formula were significantly higher than those before intake. The increase in local IgA levels resulting from the ingestion of probiotic formula may contribute to the enhancement of the mucosal resistance against gastrointestinal infection. Since secretary IgA plays a central role in local immunity and has a significant function in creating a barrier against pathogenic bacteria or viruses, such increase may contribute to reinforce the mucosal resistance to infections (Cong *et al.,* 2003).

Yasui *et al.* (1992) indicated that bifidobacteria stimulated IgA production in vitro, implying the possibility that the stimulation of IgA production was elicited by resident bifidobacteria. The ingestion of the probiotic formula containing some strains of bifidobacteria such as *B. lactis* Bb12 and colonization by the strain could trigger IgA production by the host.

Zanini *et al.* (2007) studied the complex yoghurt, containing *L. casei* and other strains of lactobacilli and bifidobacteria, and the simple yoghurt, containing only *L. casei* that were administered daily to 77 healthy volunteers (41 adults and 36 children) for a period of 4 weeks. Microbiological and molecular analyses of faecal samples indicated that the complex product induced a larger increase in the lactic acid bacteria number, compared with the simple product, without significant alterations of the autochthonous species composition. These results suggest that the probiotic products may trigger the immune response. The action on the immune system was strictly correlated to the presence of the probiotic bacteria in the intestine. The use of probiotic treatment was investigated to achieve higher immune production in healthy adolescent. This suggests that ingestion of the formula containing probiotic stimulated the production of IgA in the gastrointestinal tract of adolescent and IgA is to eliminate invading pathogens from the gastrointestinal tract. Perdigon *et al.* (1994) reported that probiotic bacteria could be used as adjuvant in oral vaccine, *L. casei* stimulated IgA and IgM synthesis in malnourished mice and could inhibit the growth of intestinal carcinoma through increased activity IgA, T cells and macrophages (Yoichi *et al.*, 1998).

The mechanisms that lactic acid bacteria use to affect the immune system and produce immunostimulative effects are unknown. Probably, LAB alone or their products are absorbed by M-cells and transported to deeper lying lymphatic follicles where they are checked by immunocompetent cells. Eventually, LAB and

their products are transported for immune analysis to systemic lymphatic tissues-mesenteric lymph nodes or the spleen (Herich and Levkut, 2002). The adherence and ingestion of orally-applied lactobacilli by M-cell in mouse Peyer's patches. LAB were found in Peyer's patches after 6-12 hours and in mesenteric lymph nodes 48 hours after ingestion (Herich and Levkut, 2002). The interaction of probiotic with the immune cells associated with the intestinal tissue was studies by Perdigon *et al*. (2000). They observed that this interaction was different for each bacteria strain. Some bacteria antigens were only associated with immune cells in Peyer's patches of small intestine, whereas others interacted with cells of lamina propria of the small intestine and large intestine (Perdigon *et al.,* 2000). Different mechanisms could influence the composition of the probotic that colonise the digestive tract. The two important are antagonism among bacteria and local immunity. Disturbances in the ecological balance in the gut lead to the growth of harmful bacteria and to their possible translocation to internal organ, which induces disease (Herich and Levkut, 2002).