Chapter 1

General introduction

Many traditional foods manufactured with dairy products are considered primary sources of nutrition. The main ingredient of dairy products is milk, which is an excellent food for a balanced mammlian diet (Hattori 1994). Fermented milk is produced by fermentation by yeast or lactic acid bacteria (LAB) and has been the focus of health-related research since the days of Mechnikoff (Jelen & Lutz 1998). Mechnikoff studied longevity resulting from consumption of fermented milk, a concept is still widely responsible for the popularity of fermented milk as a consumer product with a healthy image. The concept of probiotics, however, is more recent. Probiotics are known in some of LAB and bifidobacteria strains, described as living microorganisms that confer a health benefit to the host when administered in adequate amounts (World Health Organization 2001). Therefore, fermented milk including probiotics is expected to not only have a rich nutritional value but also to confer physiological effects. Also, the study of probiotics is important to the fields of both health care and dairy food science. Many researchers have isolated original probiotics from samples including human or animal's feces and worldwide fermented foods, especially dairy products. Probiotics has been used to develop functional dairy products, and applied to alternative healthcare.

Mongolia is one of the traditional dairy countries in the world. Mongolian food culture, especially for dairy products, is different from Western cultures. The unique characteristics of Mongolian dairy products are the production of alcoholic milk and butter oil, and not using rennet to curd milk. As a nomadic people, Mongolians have historically consumed large amounts of dairy products from their cattle, and have processed milk using natural tools and ingredients. Many of their dairy products contain several types of milk, because their domestic animals include cows, sheep, goats, yaks and horses. Because Mongolian dairy products might contain many kinds of LAB and other microorganisms, they are interesting to researchers of LAB microbiota and should yield probiotic LAB candidates strains. Therefore, this study was focused on Mongolian dairy products as a bioresource of probiotics.

According to recent LAB studies, probiotics affect the regulation of the host intestine and immunity; prevent allergy, cancer, and infection from microorganisms; and improve cholesterol metabolism, among other effects (Hamilton-Miller 2003; Fujiwara *et al*. 2004; Kimoto *et al*. 2004; Parvez *et al*. 2006; Kumar *et al*. 2010; Ooi & Liong 2010). Even people who are considered to be healthy are often in a state of chronic stress, lack exercise, and complain of intestinal disorders such as constipation and diarrhea (Ohya & Yoneda 1995). Therefore, maintaining good bowel movements and conditioning the intestinal environment are thought to be a requirement for staying healthy (Matsumoto *et al*. 2006). Also, because some LABs were reported to stimulate the host immunity by their immunomodulatory activities, daily intake of the LAB seems to provides a positive effect on host immunity (Gill *et al*. 2001; Takeda & Okumura 2007). Moreover, we are exposed to the threat of influenza virus (IFV) infection, as seen in the worldwide spread of the IFV H1N1. Since the appearance of an IFV more virulent than the pandemic H1N1 is predicted, making protection from IFV infection is even more important (Sawamura *et al*. 2010).

Here, the attempts to find an LAB strain from Mongolian dairy products with effects on human bowel habits and the intestinal environment were reported. I also attempted to find an LAB strain effective not only on the host immunity but also against IFV infection through immunomodulatory activity.

Mongolian dairy products

In Mongolia, lives of nomadic people greatly depend on the domestic livestock such as horses, cows, yaks, goats, ewes and camels. They manufacture many kinds of dairy products from their milks. Mongolian food culture, especially for dairy products, is different from Western one. Historically, Mongolian nomadic people have consumed large amounts of dairy products from their cattle, and process milk using natural tools and ingredients. Many of their dairy products contain several types of milk from cows, sheep, goats, yaks and horses. Previous reports describe in detail, from a cultural anthropological point of view, the milk processing system of the dairy products of Mongolian nomads (Ishii 2001; Hirata 2002). They manufacture airag, an alcoholic fermented milk from horse milk; and tarag, a yogurt-like fermented milk from the mixed milk of cows, ewes or goats. In addition, isgelen tarag and 'qoormog are extended fermention products of tarag; the former is ordinarily used as a coagulation agent for cheese making, and the latter for the production of milk liquor (distilled liquor) called simiyn arqi. During fermentation, isgelen tarag is not stirred; however, qoormog is stirred for alcoholic fermentation. They also produce cheese (sun-dried or non-dried curd cheese) called aarool, eezgii, or byaslag from cow, ewe or goat milk or mixed milk (Figure 1-1) (Uchida *et al*. 2007). Airag is made only from raw horse milk, in a process that does not include heating. In this completely natural fermentation, LABs are thought to be mainly derived from the container. In contrast, tarag is made using the remainder of the previous day's tarag as a starter, because the milk used for the tarag is heated for orom (high-fat, cream-like products). At the first production each year, preserved tarag from the previous year or a starter from the relative is used. Therefore, the fermented products of each family develop their original microbiota that originates from their own environment and is independent other microbiota (Uchida *et al*. 2007). People in Inner Mongolia, an autonomous region of China, have a culture similar to the Mongolian nomadic people, and use the same traditional techniques as Mongolian ones to prepare dairy products. The microbiota of traditional Inner Mongolian dairy products has been studied (Mitsuhashi *et al*. 1989; Naersong *et al*. 1996; Ishii *et al*. 1997; Watabe *et al*. 1998; Shuangquan *et al*. 2004, 2006), and probiotic LAB strains isolated from the products (Tsuda *et al*. 2007). Although probiotic LAB from traditional Mongolian dairy products are of interest, few reports have focused on products from Mongolia.

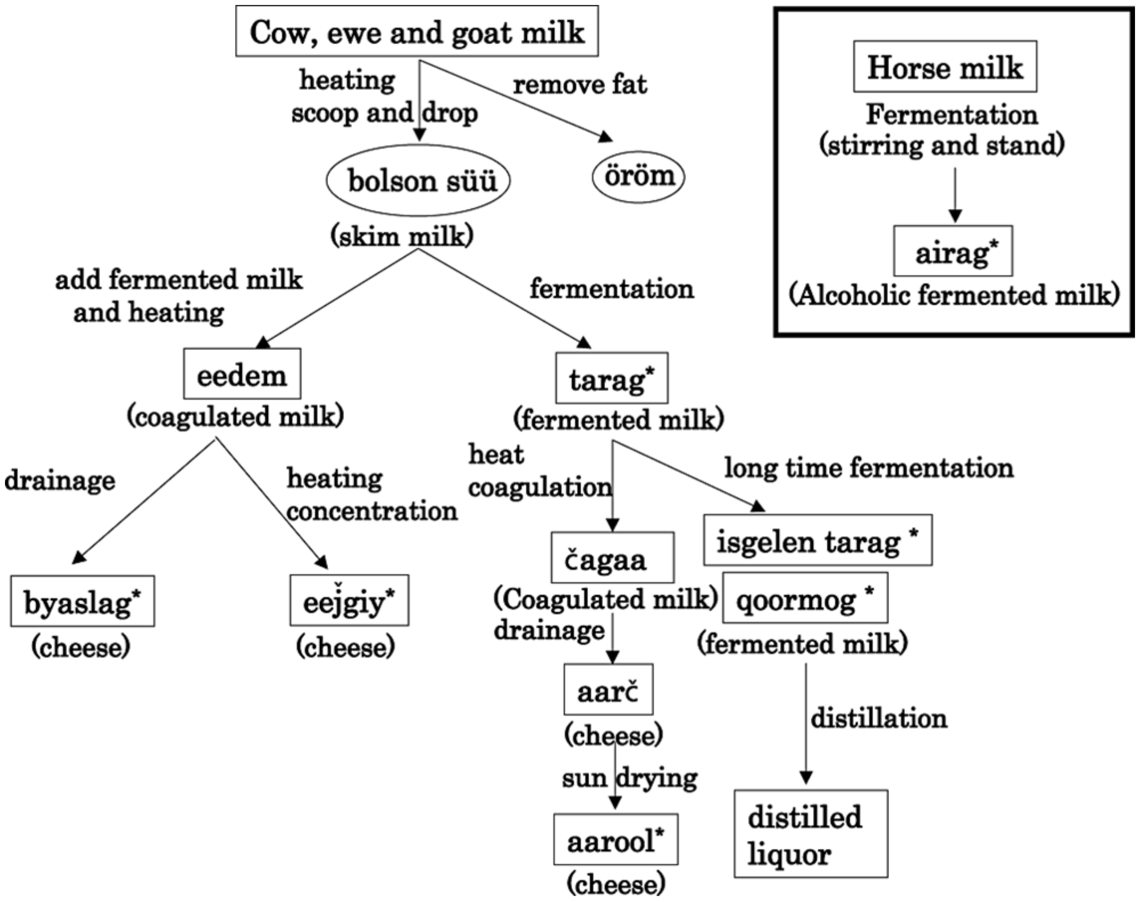

Figure 1-1 Manufacturing process of traditional Mongolian dairy products.

(Uchida *et al*. 2007)

Terms used for the dairy products in Mongolia and their basic manufacturing process are shown.

The effect of probiotics on host intestinal environment

According to Fuller (1989), probiotics are live microbial food supplements that beneficially affect the host by improving the intestinal microbial balance. The improvement of the intestinal microbial balance is the first effect of probiotics. The number of viable bacteria residing in the human intestine is more than one trillion per gram of intestinal contents, and consist of more than 100 species (Benno & Mitsuoka 1992). These form the intestinal flora and maintain either symbiotic or antagonistic relationships with each other. These intestinal bacteria are classified into three categories, namely, beneficial, harmful or neutral bacteria in relation to human health (Ogata *et al*. 1997). Because probiotic strains mainly belong to the beneficial bacterial categories such as Bifidobacteria and Lactobacilli, the number of beneficial bacteria increases and the number of harmful bacteria decreases after ingesting probiotics. This favors regulation of the human bowel. Previous reports have demonstrated these effects on the intestine by investigating improvements in defecation, frequency, and fecal characteristics, and intestinal microbiota (Ogata *et al*. 1997; Matsumoto *et al*. 2006; Olivares *et al*. 2006; Yamano *et al*. 2006; Verdenelli *et al*. 2011). In those reports, Bifidobacteria and Lactobacilli in the feces were suggested to be involved in the favorable intestinal regulation.

Also, LABs are known to release enzymes and vitamins into the intestine that exert synergistic effects on digestion, alleviate intestinal malabsorption symptoms, and produce lactic acid, which lowers the intestinal content pH and helps inhibit the development of invasive pathogens such as *Salmonella* subspecies or strains of *Escherichia coli* (Mallett *et al*. 1989; Mack *et al*. 1999). Bacterial enzymatic hydrolysis might enhance the bioavailability of proteins and fat (Fernandes *et al*. 1987) and increase the production of free amino acids, short chain fatty acids (SCFA), and lactic acid, propionic acid and butyric acid, which are also produced by probiotics. When absorbed, these SCFAs contribute to the available energy pool of the host (Rolfe 2000) and might protect against pathological changes in the colonic mucosa (Leopold & Eileler 2000). The SCFA concentration helps to maintain an appropriate pH in the intestine, which is critical for the expression of many bacterial enzymes and foreign compounds, and for carcinogen metabolism in the gut (Mallett *et al*. 1989).

Effect of lactic acid bacteria on host immunomodulation

Germfree mice have a malfunctioning immune system compared to normal mice because of immature immune tissue. However, germfree mice that are given probiotics develop a normal immune tissue and system, but mice given pathogenic bacteria do not. This suggests that probiotics are important for the development of the host immune system and have the potential for immunomodulation through the intestinal immunity (Yaeshima 2006). According to a review by Gill (1998), immunomodulation by probiotics includes enhanced effects through augmenting both nonspecific (for example, phagocyte function and natural killer (NK) cell activity) and specific (for example, antibody production, cytokine production, lymphocyte prolification, delayed-type hypersensitivity) host immune responses. For example, the activation of NK cell by probiotics was reported as a nonspecific host immune response (Takeda *et al*. 2006; Koizumi *et al*. 2008). Moreover, according to a recent report, the mechanism for the enhancement of NK cell activity by probiotics was elucidated (Rizzello *et al*. 2011). Briefly, phagocyte cells activated by probiotics produce cytokines that activate NK cell cytotoxicity and T-helper (Th) 1 responses. Type I allergic diseases are generally characterized by an elevation in serum immunoglobulin (Ig) E levels (Dreborg 2002). The production of IgE is thought to be caused by a skewed Th1/Th2 cell balance (Shirakawa *et al*. 1997; Prescott *et al*. 1999; Hopkin 2002). Several studies demonstrated that Th1 cytokine-inducing probiotics reduce allergic symptoms by shifting the Th1/Th2 balance from a Th2-dominant state to a Th1-dominant state (Fujiwara *et al*. 2004; Iliev *et al*. 2008;

Segawa *et al*. 2008). In addition, as specific host immune responses, some probiotics have reported to produce some types of immunoglobulins or antigen-specific immunoglobulins in order to prevent adherence of pathogens (Perdigon *et al*. 1990; Yasui *et al*. 1991). Moreover, focus has been on the effects of probiotics on regulatory T-cells (Treg) and Th17 cells. Shida *et al*. (2011) suggested that the immunomodulation of probiotics was classified into two categories: immunostimulation and immunoregulation (Figure 1-2). Thus, the function of immunomodulation by probiotics is under investigation and getting to be popular one.

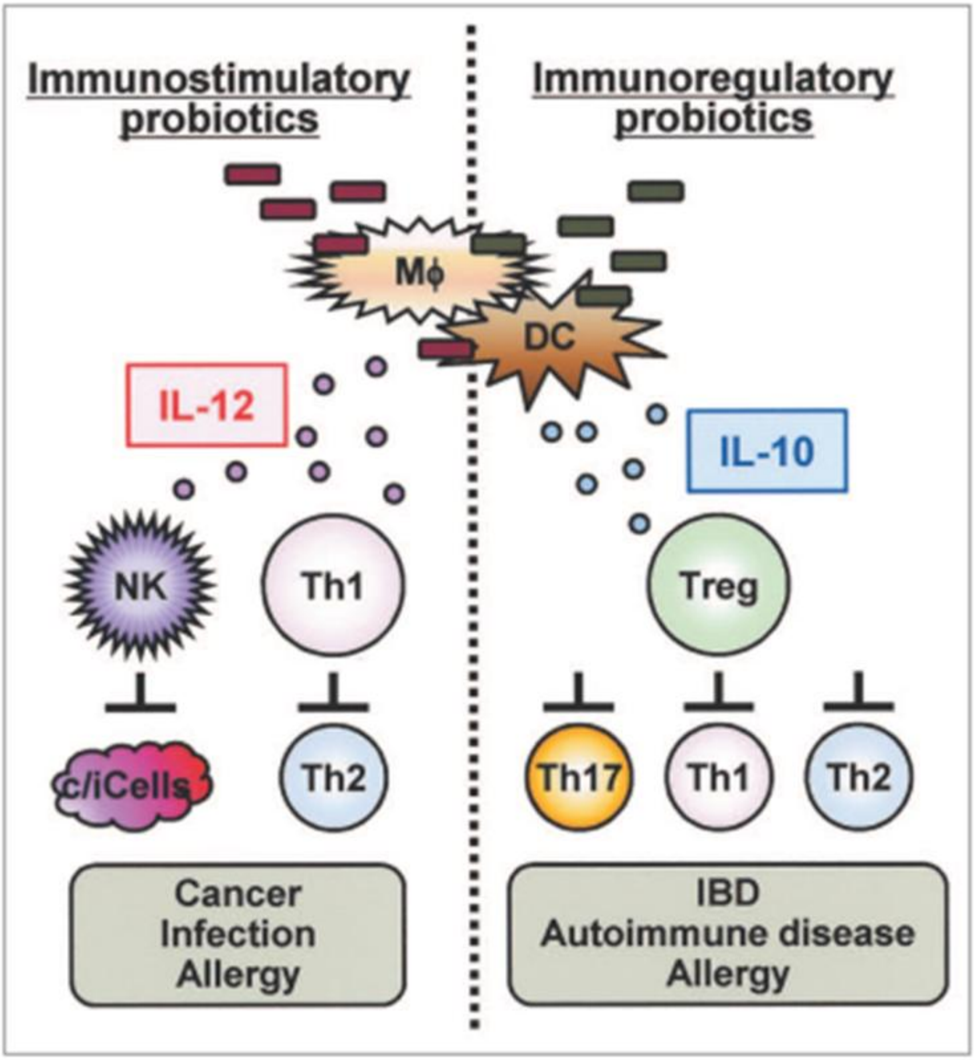

Figure 1-2 Effects of probiotics on host health through immunomodulation.

(Shida *et al*. 2011)

Protection against influenza virus by host immunomodulatory activity

IFV infects the respiratory tract of humans and causes a variety of symptoms including fever, nasal secretions, cough, headache, muscle pain, and pneumonia. These clinical symptoms often become severe, especially in high-risk groups such as the elderly and infants, who may be immunologically incompetent (Nicholson *et al*. 2000; Thompson *et al*. 2003). In IFV infection, cytokines are produced locally and systemically. Of the cytokines, the interleukin (IL) group is produced from leukocytes and acts on them, the interferon (IFN) group has antiviral effects and the tumor necrosis factor (TNF) group induces apoptosis (Kimura 2006). Figure 1-3 shows the cytokine network in innate immunity and Figure 1-4 shows the cytokine network in the adaptive immunity for host defense against viral infection. The production of cytokines is important in promoting host-immune defense and alleviating symptoms, especially in the early stages of infection (Van Reeth 2000; Kaiser *et al*. 2001; Kash *et al*. 2006). In these stages, macrophages activated by IFN-α/β from infected cells phagocytose other infected cells. This stage also produces many kinds of cytokines. TNF-α induces apoptosis of infected cells. IL-1 and IL-6 are endogenous pyrogens, producing fever with antiviral effects. IL-12 and IL-18 activate NK cell activity and promote the cell to produce IFN-γ, which activates NK cells to attack infected cells. IL-8, monocyte chemotactic protein (MCP) -1, and regulated on activation normal T-cell expressed and secreted (RANTES) promote chemotaxis of neutrophils and monocytes to infected cells. In a murine IFV infection model, cytokines such as IL-12 and IL-18 are Th1 immune-response mediators; IFN-γ is a Th1 cytokine; IL-4 and IL-10 are Th2 cytokines; IL-1α, IL-1β, IL-6, and TNF-α are proinflammatory cytokines; and IFN-α and -β are produced in the respiratory tract (Hennet *et al*. 1992; Kurokawa *et al*. 1996; Tsurita *et al*. 2001; Kurokawa *et al*. 2002, 2010). Recently, proinflammatory cytokines have been reported to be markedly elevated in human and mice cells during infection with the highly pathogenic H5N1 IFV (Cheung *et al*. 2002;

Xu *et al*. 2006; Szretter *et al*. 2007). Host immunity including cytokine production has been suggested to contribute to influenza symptoms.

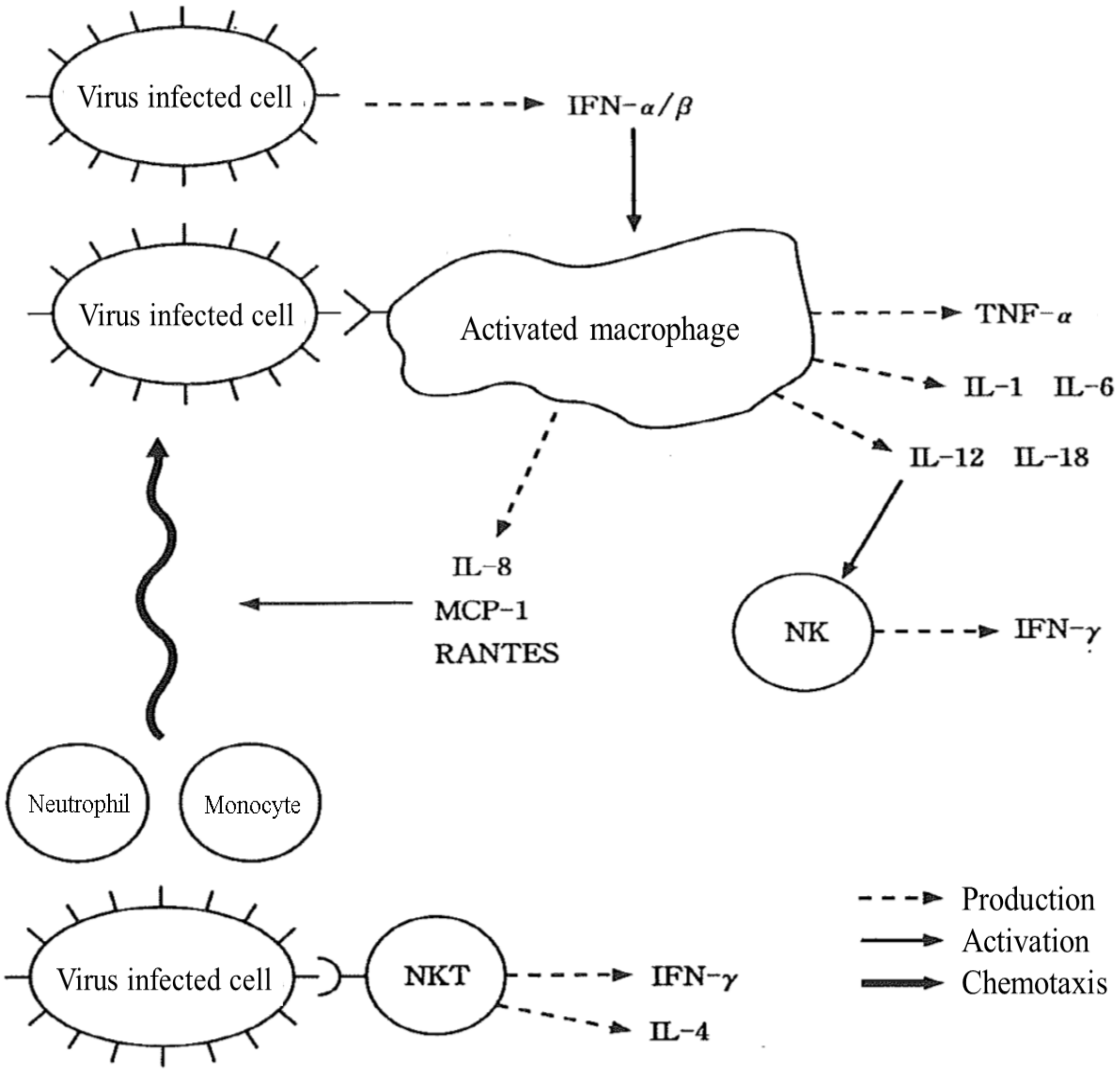

Figure 1-3 Cytokine network in innate immunity in influenza virus infection.

(Kimura 2006)

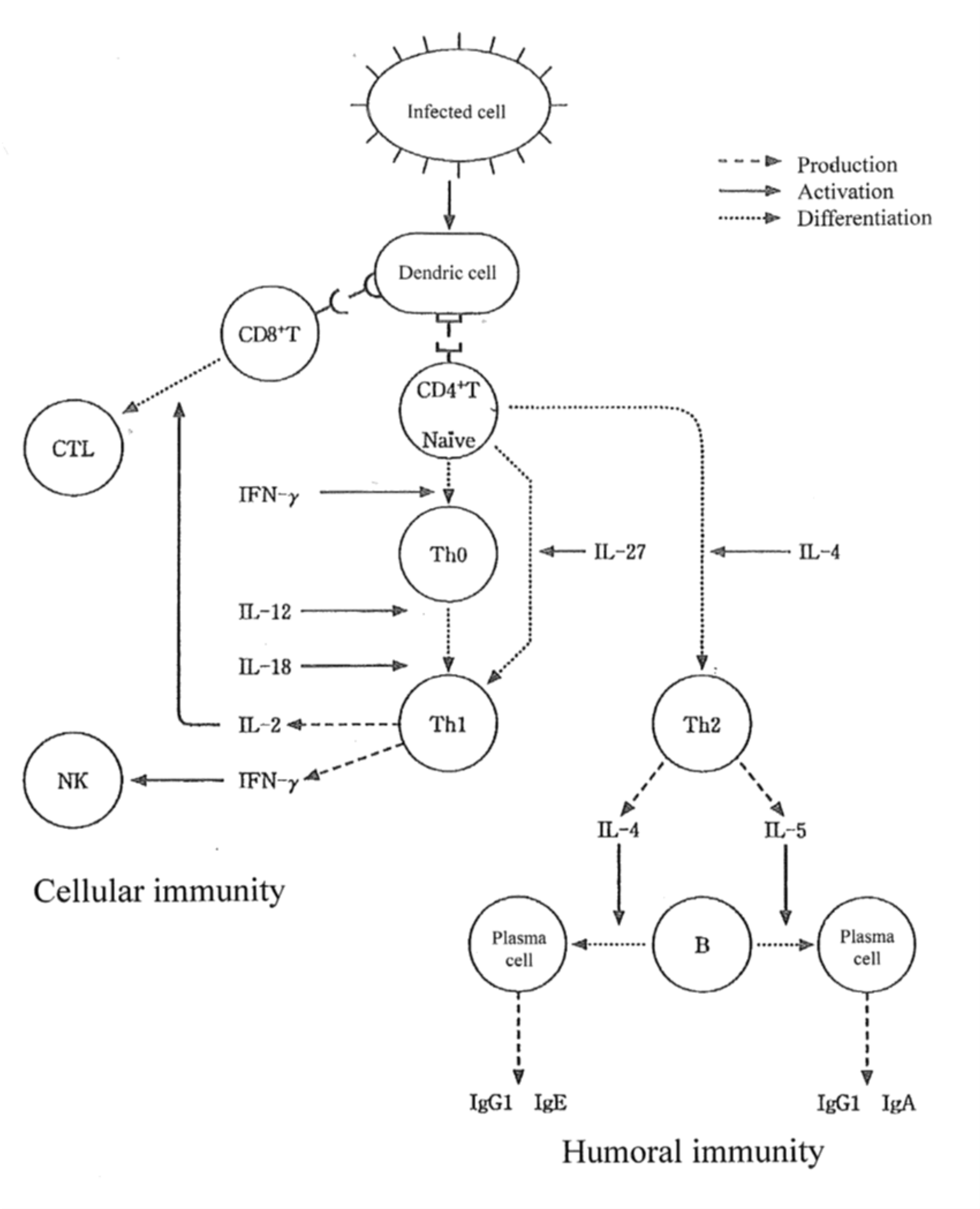

Figure 1-4 Cytokine network in adaptive immunity in influenza virus infection.

(Kimura 2006)

Objectives

The ultimate objective of the research described here is selecting LAB strains that confer health benefits to the host as probiotics from traditional Mongolian dairy products. Chapter 2 describes the diversity of LAB isolated from traditional Mongolian dairy products and the LABs were assessed for probiotic potential. Chapter 3 describes the effect of the *Lactobacillus* (*L.*) *paracasei paracasei* 06TCa19 strain indicated as a probiotics in Chapter 2 on the human bowel habit and intestinal environment. Chapter 4 describes the effect of LAB indicated as probiotics in Chapter 2 on the enhancement of Th1 immune response and NK cell activity. Finally, Chapter 5 describes the efficacy of the *L. plantarum* 06CC2 strain indicated as probiotics in Chapter 2 against IFV infection through immunomodulatory activity in mice.