Immunomodulation effect of lactic acid bacteria isolated from fermented *Brassica rapa* L.

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ABSTRACT

Lactic acid bacteria (LAB), belonging to the genus *Lactobacillus,* are widely distributed in nature and have been used to produce fermented foods, including pickles. In addition, part of LAB strains have been used as health-promoting probiotics. They can provide immunological protection to the host through the modulation of immune responses, due to their ability to modulate the production of cytokines. Since functions of LAB, including modulation of immune reactions, are strain-specific, it is important to isolate promising LAB strains from suitable sources.

T cells and natural killer (NK) cells produce the cytokine interferon (IFN)-γ that activates dendric cells (DCs) and macrophages, which fight infections. Some LAB strains reportedly enhanced IFN-γ production via interleukin (IL)-12 dependent manner. On the other hand, DCs, macrophages, and regulatory T cells produce the anti-inflammatory cytokine IL-10. This cytokine inhibits the activation of macrophages, T cells, and NK cells and suppresses the production of proinflammatory cytokines. Enhancement of IL-10 production contributes to the anti-inflammatory effects of certain LAB strains. Allergic inflammation is characterized by the infiltration of tissues by mast cells and activated eosinophils, which release Th2 cytokines, particularly IL-4 and IL-5. IL-12 and IFN-γ suppress Th2 differentiation, and IL-10 is a potent inhibitor of inflammation through inhibition of the production of Th2 cytokines. Therefore, LABs that induce the production of IFN-γ and IL-10 may have preventive and therapeutic effects for treating allergies.

Brassica rapa L., is commonly consumed as a lactic-acid fermented food called nozawana-zuke. It has been traditionally cultivated at Nozawa-Onsen village, in Nagano for more than 240 years. *B. rapa* L. fermentation is mainly achieved by various plant-derived genera of LAB, including *Lactobacillus* and *Leuconostoc,* and special pickling flavor and longer shelf life are added by the fermentation. Previous study (Yamamoto *et al.*, 2018) reported that fermented *B. rapa* L. and the LAB isolates showed immune enhancement effects, suggesting the contribution of LAB to the immunomodulatory effect of fermented *B. rapa* L.

The main objective of this work was to clarify the immunomodulatory effects of LAB strains isolated from fermented *B. rapa* L. by *in vitro* and *in vivo* tests. We focused on induction of IFN-γ and IL-10 by LAB strains, because these cytokines play pivotal roles in suppression of allergic reactions as described above.

Firstly, we investigated the microbial community and cytokine producing activities during the fermentation of *B. rapa* L. Fresh *B. rapa* L. was fermented in 7% (w/v) NaCl at 10 °C for 28 d, and part of *B. rapa* L. was collected on 3, 7, 14, 21 and 28 d after starting fermentation for microbiota and cytokine production tests. Amplicon analysis of 16S rRNA genes revealed that the soil- and plant-derived bacteria were mainly observed on day 3, and *Lactobacillus* became the most abundant taxon in *B. rapa* L. which was fermented for 7 d or longer. *L. curvatus* was the predominant species during fermentation, followed by *L. plantarum* and *L. brevis*, and *L. sakei* was occasionally detected. Spleen cells of C57BL/6 mice were co-cultured with heat-treated vegetable samples, and IFN-γ and IL-10 levels in the supernatants were quantified by ELISA. Fermented *B. rapa* L. induced more IFN-γ and IL-10 production by mouse spleen cells compared with non-fermented vegetables. Correlation analysis showed that IFN-γ was positively correlated with the numbers of *L. curvatus* and *L. plantarum*, and IL-10 was correlated with the numbers of *L. sakei* in addition to these two species. Thus, these *Lactobacillus* are likely to contribute to the cytokine production activities of fermented *B. rapa* L.

Next, we isolated 46 strains of LAB from *B. rapa* L. which was fermented for 28 d. PCR using species-specific primers and sequencing of 16S rRNA gene revealed that 40 were *L. plantarum*, four were *L. curvatus*, and two were *L. brevis*. Those LAB strains were heat-treated and were used for cytokine production tests. Although all strains induced both IFN-γ and IL-10 from mouse spleen cells, the activities were different among the strains. *L. plantarum* Lp4 and *L. curvatus* Lc3 induced the highest levels of IFN-γ and IL-10, respectively. Therefore, they were used as starter cultures to produce fermented *B. rapa* L. Bacterial cells (1×10^{10} cfu) were inoculated into 1 kg of fresh *B. rapa* L. in a salt solution (7% w/w, NaCl), and incubated for 3 d at 10 °C. Quantification of *Lactobacillus* genus was carried out by quantitative PCR using genus-specific primers, and heat-treated fermented vegetable was used for cytokine production tests. IFN-γ and IL-10 production activities of fermented *B. rapa* L. were significantly increased by the inoculation of both Lp4 and Lc3 as starter cultures when compared to naturally fermented vegetable. The numbers of *Lactobacillus* drastically increased by the inoculation of these strains compared with naturally fermented *B. rapa* L. Thus, the addition of starter cultures induced an early increase in the number of lactobacilli, leading to enhance the production of cytokines.

Finally, we tried to optimize the culture conditions of Lp4 and Lc3 to enhance their immunomodulatory activities. The Lp4 and Lc3 were cultured in glucose or sucrose

containing-MRS at 15 °C or 30 °C. The cells in log and stationary phases were harvested and used for cytokine production tests after the heat-treatment. Although carbon source nor growth phase affected cytokine production activities of Lp4 or Lc3, both strains cultured at 15 °C induced more cytokines by mouse spleen cells, when compared to those cultured at 30 °C. Lp4 and Lc 3 cells in stationary phase were obtained by culturing in MRS-glucose at 15 °C for 96 hr or 30 °C for 24 hr, and the heat-treated cells were used for an animal experiment. Female BALB/c mice were randomly divided into 5 groups, and they were orally administered LAB samples (1mg/day) or distilled water for 28 d. The mice were immunized by intraperitoneal injection of ovalbumin (OVA) and alum on days 0 and 14. On day 28, mice were sacrificed, sera and mesenteric lymph nodes (MLNs) were harvested. Administration of Lp4 which were cultured at 30 °C tended to upregulate IFN-γ and IL-10 expressions in the MLNs, and those cultured at 15 °C significantly upregulated these cytokines, suggesting culture temperature affected immunomodulatory activities of Lp4 *in vivo*. On the other hand, significant changes in the levels of IFN-γ and IL-10 in the MLNs were not observed in the mice fed Lc3 irrespective to culture temperatures.

In conclusion, we demonstrated that the number of *Lactobacillus* increased during the fermentation of *B. rapa* L., and that *L. curvatus* and *L. plantarum* induced the productions of IFN-γ and IL-10 by spleen cells. *L. curvatus* Lc3 and *L. plantarum* Lp4 were selected among the LAB isolates from fermented *B. rapa* L. because of their high cytokine producing activities. IFN-γ and IL-10 production activities of *B. rapa* L. can be enhanced by the addition of Lc3 and Lp4 as starter cultures. Moreover, IFN-γ and IL-10 were upregulated in the MLNs of mice by the oral administration of Lp4, especially when they were cultured at low temperature. Thus, fermented *B. rapa* L. possessing immunomodulatory activities could be produced by fermentation at low temperature with the addition of Lp4 as a starter culture.

CONTENTS

LIST OF ABBREVIATION

APCs	Antigen presenting cells
cDNA	Complementary DNA
CFU	Colony Forming Unit
CLRs	C-type lectin receptors
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
FAO	Food and Agricultural Organization
GALT	Gut associated-lymphoid tissue
GIT	Gastrointestinal tract
GRAS	Generally regarded as safe
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
LA	Lactic acid
LAB	Lactic acid bacteria
LPS	Lipopolysaccharides
LS	LAB suspension
LTA	Lipoteichoic acid
MALT	Mucosa-associated lymphoid tissue
MAMPs	Microbial-associated molecular patterns
mRNA	Messenger RNA
MRS	de Man, Rogosa, Sharpe
NF-kB	Nuclear factor kappa B
NLR	NOD-like receptor
NO	Nitric oxide
NZ	Nozawana-zuke
OD	Optical density
PBS	Phosphate-buffered saline
PGN	Peptidoglycan
PRRs	Pattern recognition receptors

RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RIG	Retinoic acid-inducible
SCFA	Short chain fatty acid
Th	T helper lymphocytes
Th1	T helper cells type 1
Th2	T helper cells type 2
TJ	Tight junctions
TLR	Toll-like receptor
TLR1	Toll-like receptor type 1
TNFα	Tumor necrosis factor- α
Treg	Regulatory T cells
WHO	World Health Organization

LIST OF PUBLICATION

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CHAPTER 1. GENERAL INTRODUCTION

1.1 Fermented foods and microorganisms

Fermentation is a traditional food preservation technique that is still used today to produce fermented products such as yoghurt, wine, pickles, bread and sauerkraut. Besides preservation, fermentation imparts characteristic aroma, flavor, texture, and nutritional profile into food. The fermentation process is the anaerobic catabolism of organic compounds, generally carbohydrates, in the absence of an external electron acceptor. Many kinds of microbes contribute the fermentation, and various kinds of metabolites are produced during the fermentation. Fermentation can be classified by the primary metabolites and microorganisms involved. Yeast mainly produce alcohol and carbon dioxide. Acetobacter is known as an acetic acid producer. Lactic acid bacteria belonging to genera such as *Leuconostoc, Lactobacillus, and Streptococcus* produce lactic acid. On the other hand, propionic acid, or ammonia and fatty acids are mainly produced by *Propionibacterium freudenreichii* or *Bacillus* and fungi, respectively (Table 1.1, Edition, 2006). Fermentations may also be described based on food substrates comprising meat and fish, dairy products, vegetables, soybeans and other legumes, cereals, starchy roots, grapes and other fruits. Raw materials contain high concentrations of monosaccharides and disaccharides, or in some cases, starch, are fermented by yeasts or lactic acid bacteria (Fig. 1.1, Marco *et al.*, 2017).

Table 1.1 Foods and fermentation microorganisms

Food category	Primary fermenting microorganism
Dairy foods Cheese	*Lactococcus, Lactobacillus, Streptococcus thermophilus*
Fermented milk products Buttercream Sour cream Yoghurt	*Lactococcus, Lactobacillus, Streptococcus thermophilus*
Alcoholic beverages	*Zymomonas Saccharomyces*
Yeast bread	*Saccharomyces cerevisiae*
Meat products Dry and semidry sausages	*Pediococus, Lacrobacillus Micrococus, Staphylococus*
Vegetables Cabbage(sauerkraut) Cucumber (pickles)	*Leuconostoc, Lactobacillus*
Soy sauce	*Aspergillus, Tetragenococus halophilus,* Yeasts

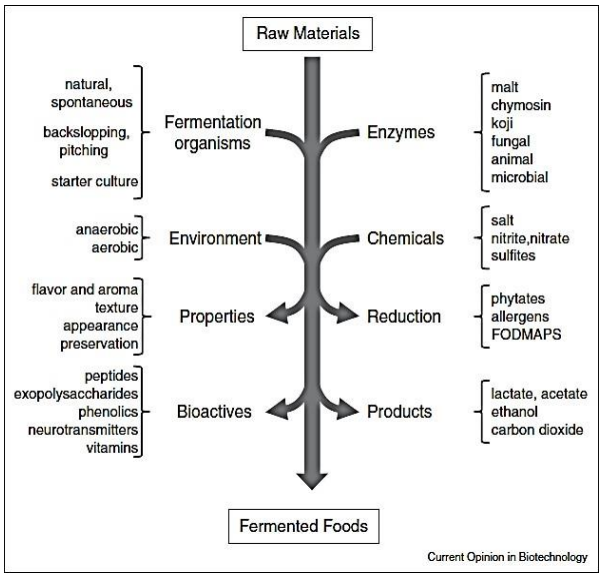

Fig.1.1. General description of the transforming nature of fermented foods.

1.1.1 Lactic acid fermentation of vegetables and fruits

Vegetables are an essential part of the human diet as they are rich in vitamins, dietary fiber and minerals. World Health Organization (WHO) / Food Agricultural Organization (FAO) recommends to consume at least 400 grams of fruits and vegetables everyday (except for potatoes and other starchy tubers) to prevent diseases such as hypertension, cardiovascular disease, cancer, diabetes, and obesity, as well as to prevent and alleviate several micronutrient deficiencies (Amine *et al.*, 2003). During lactic acid (LA) fermentation process, the cell walls of vegetables are broken down by microorganisms to make the vegetables easier to be digested. In addition, certain kinds of vitamins increase along with bacterial growth. Therefore, fermented vegetables and fruits sometimes possess more nutritious than the raw materials.

LA fermentation of vegetables can be divided into three types; dry-salted, brined and non-salted, based on contents of salt and water (Fig.1.2). LA fermentation of vegetables imparts acidic flavor, and is usually carried out with addition of salt, because growth of spoilage bacteria can be suppressed by the addition of salt (Ray, Montet and Zakhia-Rozis, 2014; Swain *et al.*, 2014). Growth and activity of LAB are affected by pH, moisture and water activity, oxygen concentration, temperature, nutrients, selected starter culture and concentration of inoculum in the fermentation of fruit and vegetables (Lee and Salminen, 1995; Ballesteros, Palop and Sánchez, 1999).

There are two types of fermentation methods, namely "spontaneous" and "controlled" fermentation. Spontaneous fermentation is natural, and it leads to variations in the sensory properties of the products, which depend on the quality of raw material, temperature and harvesting conditions (Paramithiotis, Hondrodimou and Drosinos, 2010; Wouters *et al.*, 2013). LAB which attach the surface of vegetables, such as *Lactobacillus* spp., *Leuconostoc* spp., and *Pediococcus* spp., contribute “spontaneous” LA fermentation, and the responsible bacteria differ time to time. Contributions of LAB to the production of sauerkrauts, kimchi, and pickled cucumbers have been widely studied for many years (Etchells *et al.*, 1964; Etchells *et al.*, 1964; Zabat *et al.*, 2018). On the other hand, controlled fermentation requires created condition to enhance the growth of commensal or inoculated LAB with excluding other microorganisms (Gardner *et al.*, 2001). Critical methods for controlled LA fermentation of vegetables and fruits are pursued through the use of autochthonous or allochthonous starters (Di Cagno *et al.*, 2011). Autochthonous starters are isolated and reused on the same raw matrix, apart from the geographical origin. Allochthonous starters are isolated from specific raw matrices but used to ferment different products. *Lactobacillus acidophilus, L. plantarum, L. rhannosus,* and *L. provides* are usually used as starters for the fermentation of vegetables and fruits.

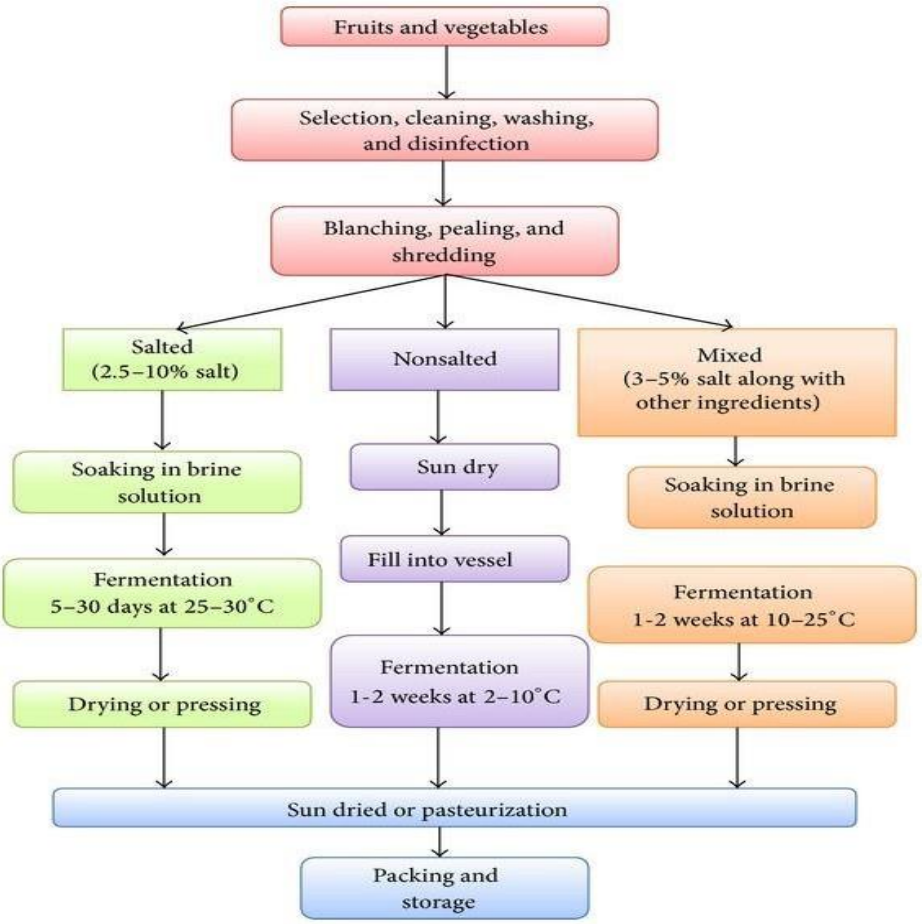

Fig.1.2. The general process of fermentation of fruits and vegetables.

1.1.2 *Brassica rapa* L. as nozawana-zuke a traditional Japanese fermented vegetable

Brassica rapa L., which belongs to *Brassica* genus, *Brassicaceae* family, is known as nozawana. This leafy vegetable is commonly consumed as a LA-fermented food called nozawana-zuke (NZ). It is pickled in salt water, sometimes also prepared by adding red pepper powder or other spicy components. *B. rapa* L. belongs to the same family as the Japanese species of common turnip, and its aerial part grows up to 60-90 cm long. It has been traditionally cultivated in the village of Nozawa-Onsen, in Nagano, for more than 240 years. Historically, between 1751-1764, it was introduced from Kyoto to Nozawa-Onsen by a master of Buddhist. Since then the plant has been grown in Nozawa-Onsen village, and the idiomatic name of this plant comes from this village (https://www.nozawaholidays.com/news/nozawana-perfect-pickle-nozawa-onsen/).

It has been reported that cruciferous vegetables, including *B. rapa* L., contain glucosinolates which have health-promoting properties, such as anticancer activity and effects on the metabolism of cholesterol (Manchali, Chidambara Murthy and Patil, 2012).

The best season for making NZ is during late fall; it is said that "the best time to pickle them is after there is frost once." There are two types of NZ; the asa-zuke type, which is brined for several days, and thus it contains very few numbers of LAB. Hon-zuke type nozawana is produced by LA fermentation by LAB, and color of the plant changes to amber during the fermentation because of degradation of chlorophyll in low pH (https://journey-of-japan.com/article/341/en).

Various plant-derived genera of LAB including *Lactobacillus* and *Leuconostoc*, contribute fermentation hon-zuke type NZ, and they give unique pickling flavor and long shelf life by the production of organic acids. *L. curvatus* is one of major species which contributes LA fermentation of commercial NZ (Kawahara and Otani, 2006b).

1.2 Lactic acid bacteria as probiotics

LAB are gram-positive, low GC content, non-breathable, non-responsive, and aerotolerant cocci or rods, which produce lactic acid as one of the main fermentation products of carbohydrate (Lebeer, Vanderleyden and De Keersmaecker, 2008). Among LAB, *Lactobacillus* is a large genus, and it includes 241 species (November 2014. http://www.bacterio.net/lactobacillus.html#r). LAB belong to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and at the genera, its core *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus,* and *Streptococcus,* as well as the more peripheral *Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Sporolactobacillus, Tetragenococcus, Vagococcus,* and *Weissella*.

Probiotics are defined as "living microorganism that confers several health benefits when administrated in adequate amount to host" by the FAO/WHO (Hill *et al.*, 2014). In healthy human intestine, *Lactobacillus (L. acidophilus, L.casei, L. salivarius and L. lactis)* and *Bifidobacterium* (*B. bifidum, B. bifidum and B. lactis*) genera are commonly found and are known as important probiotics (Azad *et al.*, 2018). LAB are considered as health-promoting microorganisms, and some strains are shown as probiotics and are present in many kinds of fermented foods as well as in the intestines of most mammalian (Ohland and MacNaughton, 2010). A significant number of potential probiotic LAB are isolated from naturally fermented foods (Swain *et al.*, 2014).

1.2.1 Probiotics: mechanism and actions on the host

The mechanisms of the beneficial functions of probiotics are not fully understood but are likely to be multifactorial. Several mechanisms related to the antagonistic effect of probiotics on various microorganism include; i) enhancement of the epithelial barrier, ii) increased adhesion to the intestinal mucosa, iii) concomitant inhibition of pathogen adhesion, iv) competitive exclusion of pathogenic microorganisms, v) production of antimicrobial substances, iv) and modulation of the immune system (Bermudez-Brito *et al.*, 2012; Hill *et al.*, 2014).

Enhancement of the epithelial barrier: The epithelium is host tissue, in permanent contact with luminal contents and lamina propria. This intestinal barrier is an important defense mechanism for maintaining epithelial integrity and to protect the organism from the external environment as a protective layer of mucus covers it. Defenses of intestinal barrier consist of the mucous layer, antimicrobial peptides, secretory IgA and the adhesion complex of the epithelial junction (Ohland and MacNaughton, 2010). When the barrier function is disturbed, bacteria and food antigens can reach the submucosa and induce inflammatory responses, which lead to intestinal disorders, such as inflammatory bowel disease (Hooper *et al.*, 2001). Consumption of non-pathogenic bacteria can contribute to the function of the intestinal barrier, and probiotic bacteria have been extensively studied for their participation in maintaining this barrier. However, how probiotics enhance or protect the intestinal barrier function is not fully understood. Several studies have reported that lactobacilli modulate the expression of genes that encode the proteins of the adhesion junction (Hummel *et al.*, 2012), initiate repair of the barrier after damage (A.A. *et al.*, 2007), and prevent cytokine-induced epithelial damage (Sartor, 2006). Mucin glycoproteins (mucins) are the main macromolecular components of epithelial mucus and have long been implicated in health and disease. Probiotics can promote mucus secretion as a mechanism to improve barrier function and the exclusion of pathogens. Several species of *Lactobacillus* increase the expression of mucin in the cell lines of the human intestine (Mattar *et al.*, 2002; Mack *et al.*, 2003).

Increased adhesion to the intestinal mucosa: The intestinal mucosal layer is an important site for the interaction between probiotic strains and the host, regarded as a prerequisite for colonization (Juntunen *et al.*, 2001). And it is also important for immune system modulation (Schiffrin *et al.*, 1997) and antagonism against pathogens (Hirano *et al.*, 2003). LAB show various surface determinants involved in their interaction with intestinal epithelial cells (IECs) and induce mucin secretion from IECs. For example, it has been reported that *L. plantarum* induces mucins MUC2 and MUC3 to inhibit adhesion of enteropathogenic *E. coli*. These observations indicate that enhanced mucous layers and glycocalyx the intestinal epithelium, as

well as the occupation of microbial binding sites by *Lactobacillus* spp. provide protection against pathogen invasion (Hirano *et al.*, 2003; Kim and Ho, 2010).

Production of antimicrobial substances: Probiotics have been reported to produce various non-viable metabolic by-products, such as bacteriocins, organic acids, acetaldehydes, diacetyl, ethanol and hydrogen peroxide, which are non-toxic, non-pathogenic and resistant to enzyme systems in mammals. Those effects noted as an alternative to antibiotics because of their biological activity and pathogenic inhibitory properties.

Symbiotic actions of probiotics: LAB fermentable non-digestible food are known as prebiotics, and they exert elite properties including anti-pathogenicity, anti-obesity, and diabetic, antidiabetic, anti-inflammatory, anticancer, and angiogenic activities and efficacy on the brain and central nervous system (George Kerry *et al.*, 2018). According to Borja. S *et al.*, probiotic interacts with the host at three different cites of the lower part of the human GIT; mucous layer, epithelial layer and gut-associated lymphoid tissue (GLAT) (Fig.1.3, Sánchez *et al.*, 2017).

The following section will introduce the immune system, the gut-microbiota interaction, and the important effect of probiotics that stimulate the host's immune system through cytokine production.

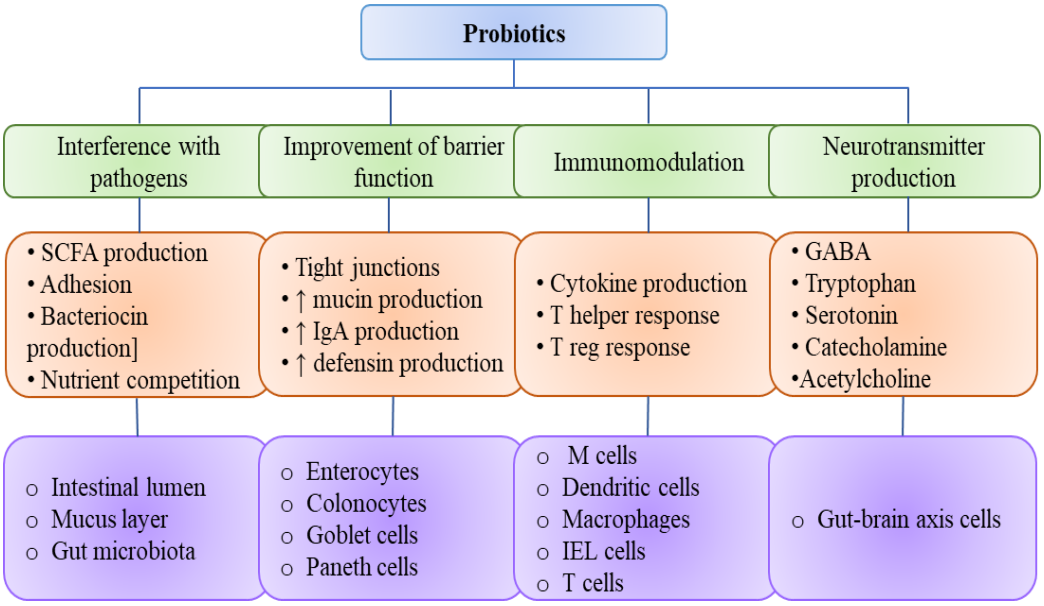

Fig. 1.3. Diagram of important mechanisms of action of probiotics. The mechanisms, biological processes and host cells responsible for the interaction are shown in color code

1.3 Immunomodulatory effects of lactic acid bacteria

The immune system and its functions are a very complex network system which protects the human body from foreign pathogens, viruses, and substances. Two main parts of the immune system called innate and adaptive are controlling infections and chronic diseases through their cytokine-producing responses. Imbalanced cytokine production patterns, over-suppression or over-expression of cytokines, cause to develop many kinds of diseases and fail to protect the body and even attack themselves, resulting in allergic or autoimmune diseases. (Fig.1.4).

Innate immune system. The innate immunity is recognized as a first-line defense system against pathogens. Phagocytic cells, including neutrophils, monocytes and macrophages, and NK cells are involved in the innate immune system which destroys pathogens to protect against ourselves from the corresponding infections. The innate immune system responds based on the recognition of pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRR). The best-studied PPRs are toll-like receptors (TLRs) (Gómez-Llorente, Muñoz and Gil, 2010). TLRs are expressed on phagocytic cells, which then activate the adaptive immune response in vertebrates through the activation of antigen-presenting cells (APCs) (Alberts *et al.*, 2019; Gourbeyre, Denery and Bodinier, 2011).

Adaptive immune system. The adaptive immune response depends on the B and T lymphocytes which are specific for antigens and provide immune protection through the development of a pathogen-specific memory.

LAB exert immunomodulatory activity through inhibition of inflammatory responses, regulation of TLRs expression, activation of DCs and NK cells in innate immunity, induction of lymphocyte proliferation, inducing IgA in the intestinal tract, and balancing Th1 / Th2 responses in adaptive immunity. Immunomodulatory effects of probiotics result in controlling of inflammatory bowel diseases, suppression of pathogens in the gut and alleviation of the symptoms of allergic reactions.

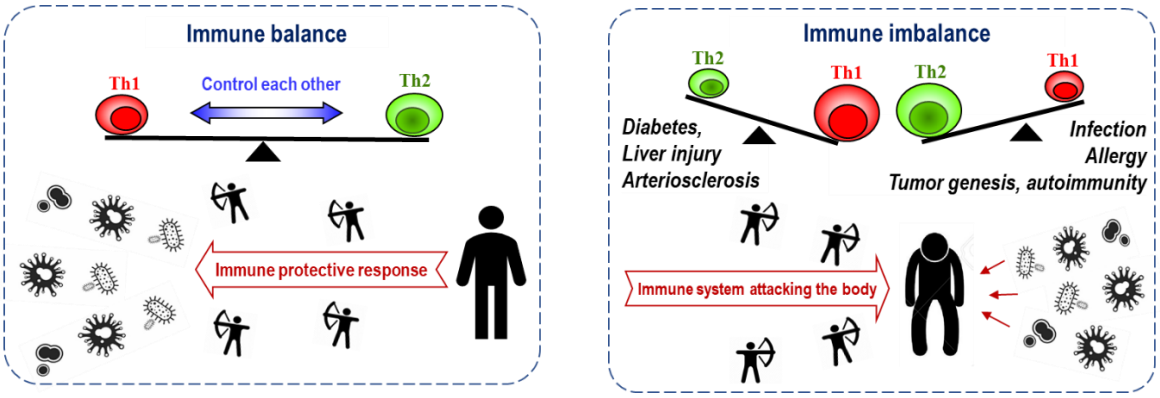

Fig. 1.4. The immune system, the balance of Th1 and Th2 cell response.

1.3.1 Effects of alteration of cytokine production by LAB

Cytokines are small, soluble secreted peptides or proteins that affect the growth or function of cells. Cytokines include ILs, IFNs, chemokines, colony-stimulating factor and many growth factors. Cytokines bind to specific surface receptors on the membrane of cells. Most commonly, cytokines are autocrine (act on the same cell that produces them) or paracrine (they act on nearby cells). Cytokines are most often secreted by immune cells and act on immune cells, thus orchestrating most aspects of the immune system. The importance of cytokines lies in binding to specific receptors on the cell membrane and in triggering cellular cascades for the induction and improvement, as well as the inhibition of gene expressions.

LAB interact with enterocytes, DCs, Th1, Th2 and Treg cells in the intestine to modulate adaptive immunity into pro- and/or anti-inflammatory cytokine-producing actions. The immunomodulatory activity of LAB is based on the release of cytokines from immune cells (lymphocytes, granulocytes, macrophages, mast cells, epithelial cells and DCs), which further regulate the innate and adaptive immune system (Savan and Sakai, 2006). Properties of LAB immunomodulatory actions can be classified as immunostimulatory and immunoregulators. (Fig. 1.5). Immunostimulatory probiotics can protect against infection and cancer cells by inducing IL-12, which activates NK cells and develops Th1 cells. These probiotics also treat allergies through a balancing Th1 and Th2 via IFN-γ production from Th1 cells. On the other hand, immunoregulatory probiotics have been characterized with production of IL-10 and induction of Treg cell, resulting in a decrease in allergy, IBD, autoimmune diseases, and inflammatory responses (Chiba *et al.*, 2010).

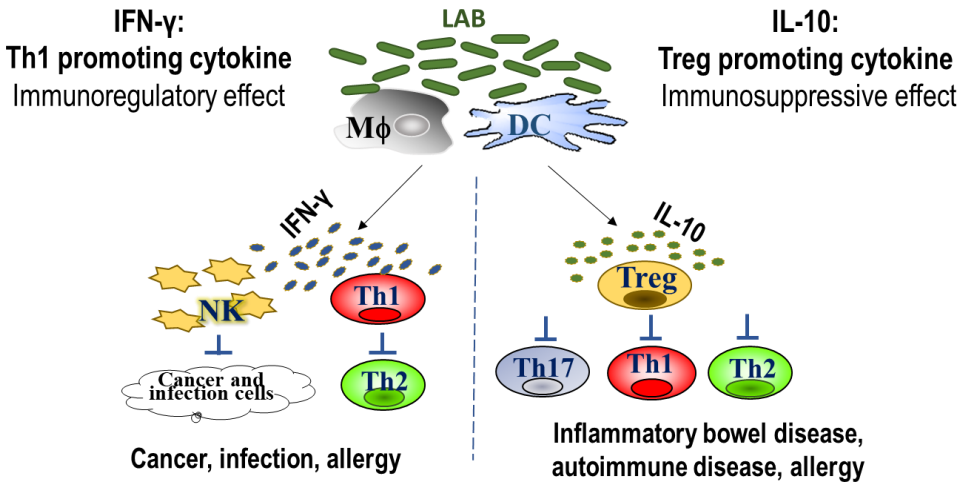

Fig. 1.5. Mechanism of immune response regulation by probiotics

For example, the onset of allergic diseases is a result of an imbalanced T helper (Th1/Th2) cells, especially pro-inflammatory Th1 cell suppression. Therefore, immunomodulatory therapeutic approaches focus on enhanced Th1 and regulatory T cell (Treg) promoting cytokines. IL-10 is a cytokine derived from Treg cells identified as a suppressor of cytokines from Th1 cells. In addition, IL-10 is one of key anti-inflammatory cytokines released from regulatory T (Treg) cells as well as dendric cells (DCs), which inhibits the activation of macrophages, T cells, and NK cells and suppresses the production of proinflammatory cytokines (Couper, Blount and Riley, 2008). IFN-γ is a product of Th1 cells and exerts inhibitory effects on Th2 cell functions, and have been implicated in the pathogenesis of asthma and allergies (Chung, 2001a).

The following cytokines have been suggested to contribute amelioration and prevention of allergic reactions:

IL-12 is a pro-inflammatory cytokine. Earlier, it was simultaneously described as CLT maturation factor (CLMF), a T cell stimulating factor (TSF) and a NK cell stimulatory factor (NKSF). Thus, the main action of IL-12 is to activate NK cells and to induce the differentiation from naïve cells to Th1 cells. IL-12 is mainly produced by antigen presenting cells, such as DC and macrophages.

IL-10 is an anti-inflammatory cytokine which is a key regulator of immune responses. It was originally described as cytokine synthesis inhibitory factor (CSIF) because of its ability to turn off cytokine production in T cells. IL-10 is produced mainly by leukocytes including T cells, B cells, monocytes, macrophages and DCs, as well as some epithelial cells. For antigen presenting cells such as DCs and macrophages, the production of IL-10 is induced by recognition of MAMPs or pathogen-associated molecular patterns (PAMPs) by cell surface of cytoplasmic PRR. Secretion of IL-10 can result in numerous effects. The main effect of IL-10 is to suppress multiple immune responses through individual actions on T cells, B cells, antigen presenting cells, and other cell types, and to skew the immune response from Th1 to Th2.

IFN-γ is a pro-inflammatory cytokine and a critical role in Th1 cell differentiation. It induces IL-12 production by antigen presenting cells (APC) as include DCs and macrophages. IFN-γ also exerts a direct inhibitory effect on cytokine IL-4 and IL-5 production by Th2 cells.

1.3.2 Anti-allergic effect of LAB

Allergic inflammation is determined by the activation of mast cells or basophils, and production of food protein-specific immunoglobulin E (IgE) contributes development of food hypersensitivity reactions. Th2-cells induced by food allergens secret IL-4, which in turn activates B cells to produce IgE antibody (Fig. 1.6). Recently, experimental and clinical

evidences supporting the efficacy of probiotic bacteria in the treatment of allergic symptoms has triggered strong interest in the identification of novel strains and characterization of biological mechanisms behind the beneficial effects.

The cytokines produced by immunocompetent cells such as APCs and T lymphocytes play a significant role in the modulation of allergy. The precise mechanism of interaction of LAB and immune cells is not fully understood. However, there is considerable evidence that the recognition of cell wall components of Gram-positive and Gram-negative bacteria by TLRs on the surface of DCs is essential for bacteria-induced DC maturation (Michelsen *et al.*, 2001). The components of the bacterial membrane, such as peptidoglycans and lipopeptides are recognized by DCs (Dziarski, 2003). Lactobacilli may deliver signals in myeloid DCs through TLR-2, thereby promoting activation of these cells (Wells, 2011; Mohamadzadeh *et al.*, 2005). Some LAB strains such as *Streptococcus thermophilus* AHU1838 and *L. paracasei* subsp. *casei* AHU1839 enhanced expression of cell surface molecules on DCs, which is activation of differentiation of naïve T cells towards a Th1 phenotype and inhibition of differentiation of naïve T cells towards a Th2 phenotype. The selected LAB enhanced the production of IL-12 which enhances Th1 cell proliferation and IFN-γ production and such LAB play a key role in modulation of allergy (Ongol *et al.*, 2008).

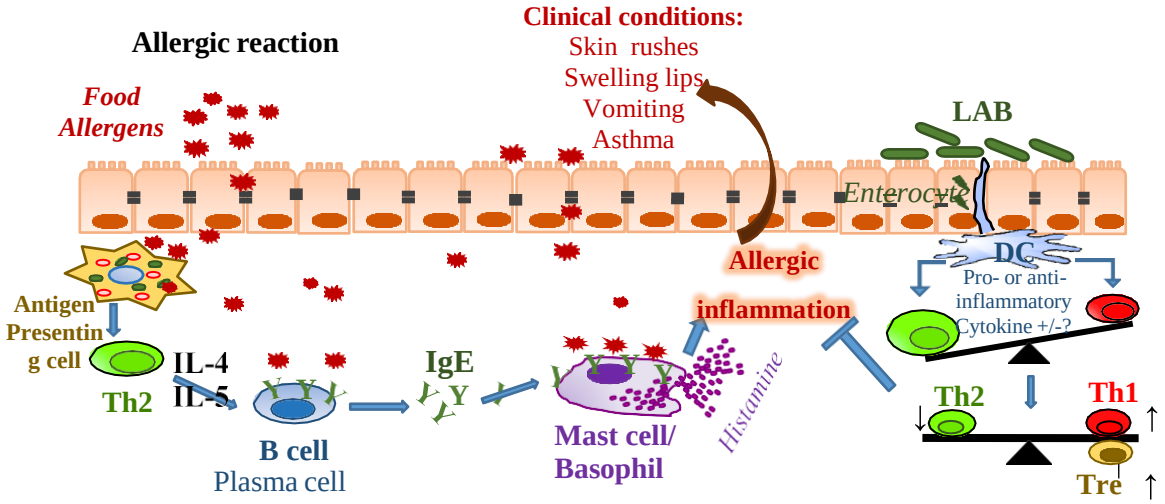

Fig.1.6. Allergic inflammation development and suppression by probiotics.

1.4 The safety issue of LAB and viable or non-viable cells

Nowadays, probiotics are widely used in nutritional supplements, foods, infant formulas, medical devices and clinical practice due to their significant potential as therapeutic options for various diseases, mainly gastrointestinal diseases in many countries, and consumers often buy

them without a prescription (Wilkins and Sequoia, 2017). However, the safety issue of the live probiotic uses leads some risks including infection trough translocation of bacteria from intestine, mainly in vulnerable patients and pediatrics; acquisition of antibiotic resistance genes; and interference with intestinal colonization in newborn (Goldenberg *et al.*, 2017). In this context, the use of non-viable (mostly heat-killed) probiotics, mainly *Lactobacillus* or *Bifidobacterium*, becomes of great interest to researchers.

The number of studies have shown that both live and heat-killed LAB can release biological responses, particularly their immunomodulation effect. For example, heat-killed cells can release bacterial components with antagonistic properties against pathogens. It has been suggested that bacterial components such as lipoteichoic acids, peptidoglycans or exopolysaccharides are involved in these properties in preparations containing heat-treated bacteria (Singh *et al.*, 2017). Furthermore, it has been shown that heat-killed bacteria have more favourable properties in animal experiments and clinical studies for different indications. For example, in neonates, without incurring the risks associated with live microorganisms and with the pharmaceutical advantages in terms of transport and storage conditions. (Table 1.2 Piqué, Berlanga and Miñana-Galbis, 2019).

Table 1.2 Advantages of heat-killed bacteria in comparison with live bacteria

Safety	**Physiological effect**	**Pharmaceutical characteristics**
No risk of the following problems: - translocation from the intestinal lumen to blood, especially in vulnerable people - acquisition and re-transfer of antibiotic resistance genes. - interference with normal colonization of the intestinal microbiota in neonates.	- Release of active molecules from inactivated disrupted cells, passing through mucus layers and stimulating epithelial cells more directly. - Loss of viability and cell lysis can produce further and more complex beneficial effects.	Easier to standardize, transport and store.

Aims of the research

The general objectives of this research are to isolate capable LAB strains from fermented *B. rapa* L., and to evaluate their functions in practical ways. To accomplish these objectives, this thesis includes three main research topics:

i. investigating changes in the immunomodulatory effect and microbiota during fermentation of *B. rapa* L. to clarify which species of LAB involved in the effect of fermented *B. rapa* L.
ii. isolation and screening of LAB strains, and production of fermented *B. rapa* L. with immunomodulation effect by using screened LAB strains
iii. evaluating the immunomodulatory effect of screened LAB strains by an animal model

This thesis consists of following 5 chapters.

In Chapter 1, background of this research, including contribution of LAB on food fermentation and possibility of immunomodulation functions of LAB is described.

In Chapter 2, microbial community and cytokine-producing activities during the fermentation of *B. rapa* L. were determined to find a suitable source of promising LAB strains possessing immunomodulatory effects. Immunomodulatory effect of fermented *B. rapa* L. was evaluated by measuring IFN-γ and IL-10 production in spleen cells of mice. In addition, correlation analyses between cytokine production and the number of each *Lactobacillus* species.

In Chapter 3, LAB strains isolated from fermented *B. rapa* L. were identified by molecular biological technics, and their immunomodulatory functions, in terms of IFN-γ and IL-10 production in spleen cells of mice, were determined. Two LAB strains were selected based on their potent IFN-γ and IL-10 induction activities. They were used as starter cultures for preparation of fermented *B. rapa* L., and immunomodulatory functions of resulting fermented *B. rapa* L. were evaluated.

In Chapter 4, screened 2 LAB strains were cultured in different conditions to enhance their immunomodulatory activities. We clarified that their immunomodulatory activities were enhanced when they were cultured at lower temperature. To evaluate their immunomodulatory activities *in vivo*, gene expressions in the mesenteric lymph nodes and serum antibody levels of mice were determined after the oral administration of bacterial cells.

All findings were discussed in chapter 5 with proposing LAB isolates from *B. rapa* L. as promising starters for the production of fermented B. *rapa* L. possessing immunomodulatory activities.

CHAPTER 2. MICROBIOTA CHANGES AND IMMUNOMODULATORY EFFECTS OF FERMENTED *BRASSICA RAPA* L.

2.1 Introduction

Essential health benefits and functional effects of plant-derived foods strongly connected with their own and attached microorganisms. Fermentation is a traditional biological method for food preservation, and the microorganisms associated with raw plants are involved in the fermentation which requires certain environmental conditions.

Lactic acid bacteria (LAB) adjoining on the surface of the raw plant become predominant during the fermentation process of vegetables (Nguyen *et al.*, 2013). LAB utilize sugars with producing lactic acid and other bioactive compounds as metabolic end-products. The health-promoting functions of LAB are resulted from the production of bioactive compounds, such as organic acid, vitamins, exopolysaccharides and bioactive peptides (Swain and Ray, 2017).

Immunomodulation property is one of the health-promoting functions of probiotic LAB. Thus, immunomodulation by naturally fermented food, including pickles, has been focused on because of their great numbers of LAB. Some LAB strains reportedly enhanced IFN-γ production via IL-12 dependent manner (Koizumi *et al.*, 2008; Kawahara and Otani, 2006a). IL-10 is a cytokine, which inhibits the activation of macrophages, T cells, and NK cells, and suppresses the production of proinflammatory cytokines. Enhancement of IL-10 production contributes to the anti-inflammatory effects of certain LAB strains (Morita *et al.*, 2002). Allergic inflammation is characterized by the infiltration of tissues by mast cells and activated eosinophils, which release Th2 cytokines, particularly IL-4 and IL-5. IL-12 and IFN-γ suppress Th2 differentiation, and IL-10 is a potent inhibitor of inflammation through inhibition of the production of Th2 cytokines. Therefore, LABs that induce the production of IFN-γ and IL-10 may have preventive and therapeutic effects for treating allergies.

Brassica rapa L., known as Nozawana, is a cruciferous vegetable, which has been cultivated mainly in Nagano area. Fermentation of *B. rapa* L is mainly achieved by various plant-derived genera of LAB, including *Lactobacillus* and *Leuconostoc*, with resulting special pickling flavour and longer shelf life (Kawahara and Otani, 2006c). Previous studies reported that *B. rapa* L. enhances natural killer activity and IFN-γ production of mouse spleen cells through an IL-12-dependent mechanism (Yamamoto *et al.*, 2018). In addition, fresh and fermented *B. rapa* L. induce changes in short-chain fatty acid production in the colon and cecum of mice, which induces immune regulatory effects (Tanaka *et al.*, 2016; 2017). Thus, increased numbers of LAB

during fermentation of *B. rapa* L. may activate immune cells to produce altered cytokine productions.

In this chapter, we determined the microbiota and immunomodulatory properties of *B. rapa* L. during fermentation. To examine the immunomodulatory properties, spleen cells from mice were stimulated with *B. rapa* L. samples, and IFN-γ and IL-10 cytokine levels were measured. The correlation analysis between microbiota community and cytokine production was conducted to clarify which bacterial groups contributed to the immunomodulatory properties of fermented *B. rapa* L.

2.2 Materials and Methods

2.2.1 Preparation of fermented *B. rapa* L.

Fresh *B. rapa* L. (approximately 5 kg), purchased from Takeuchi Nousan (Nagano, Japan), was washed with tap water and then fermented in 20-L pickle jars containing a salt solution (7% w/w, NaCl) at 10°C for 28 d. Vegetables (approximately 500 g each) were collected on days 0, 3, 7, 14, 21, and 28 after the start of fermentation. Three independent experiments were conducted using different plant materials to prepare fermented *B. rapa* L.

Fresh or fermented *B. rapa* L. was suspended in phosphate-buffered saline (PBS), and the suspension was passed through a 100-μm nylon cell strainer (BD Biosciences, San Jose, CA, USA) to eliminate large particles. Filtrates were centrifuged at 20,630×*g* for 5 min, and the pellets obtained were used as the LAB suspension (LS). LS was treated with RNAlater (Qiagen, Hilden, Germany) and stored at 4°C. In the immunological analysis, LS was heated at 65°C for 30 min to kill bacteria and then lyophilized (FDU-1200 freeze dryer; Eyela, Tokyo, Japan). Lyophilized LS was stored at –30°C.

2.2.2 Preparation of fermented *B. rapa* L.

Bacterial DNA extracted from LS was purified using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) and a ZircoPrep Mini Kit (Nippon Genetics, Tokyo, Japan) according to the manufacturers' instructions.

2.2.3 Amplicon sequencing of 16S rRNA gene fragments

DNA samples were quantified, followed by PCR-amplification of the V3 and V4 regions of 16S rRNA genes. PCR primers were 341F (5´-CCTACGGGNGGCWGCAG-3´) (Klindworth *et al.*, 2013) and 806R (5´-GGACTACHVGGGTWTCTAAT-3´) (Kennedy *et al.*, 2016) joined to the Illumina overhang adapter sequences. A second PCR was performed to add barcodes to each sample. After quantification, amplicons were pooled in equal amounts, and pair-end 2 × 300 bp sequencing was performed using a MiSeq System (Illumina, San Diego, CA, USA) and MiSeq Reagent Kit v3 (Illumina).

Sequences in the demultiplexed format were analyzed using QIIME 1.9.0 (Caporaso *et al.*, 2010). Merged paired-end reads were quality-filtered with default settings. The filtered sequences were then clustered into operational taxonomic units (OTUs) using Greengenes 13.8 as reference sequences for taxonomy assignments at 97% identity (Machiels *et al.*, 2014). A representative sequence of each OTU was selected, and chimeric sequences were identified using VSEARCH (Rognes *et al.*, 2016), with singletons filtered from the OTU table. OTUs assigned

as originating from chloroplast sequences were eliminated from further analyses. Alpha diversity was analyzed by rarefying the OTU table at a consistent sampling depth of 34,100 sequences.

Phylogenetic metrics were compared by estimating Faith's Phylogenetic Diversity (PD_whole_tree) between samples, and species richness was estimated using Chao1, the Shannon index, and number of OTUs (Faith and Baker, 2006). Representative sequences of major OTUs were compared using the BLAST algorithm to query a public-domain database (Altschul *et al.*, 1990). Ward's hierarchical clustering method using a weighted Unifrac distance matrix was performed using the APE package of R 3.4.4 (Paradis and Schliep, 2019).

2.2.4 The real-time quantitative PCR analysis of *Lactobacillus* species and the total *Lactobacillus* population

L. curvatus, L. plantarum, L. brevis, L. sakei, and total lactobacilli present in LS preparations were quantified by real-time quantitative PCR with primer pairs specific to each *Lactobacillus* species and the genus *Lactobacillus* (Berthier and Ehrlich, 1999; Torriani, Felis and Dellaglio, 2001; Oswari *et al.*, 2013; Fusco *et al.*, 2016). PCR reaction mixtures (25 μL) contained 12.5 μL KAPA SYBR Fast qPCR Master Mix (KAPA Biosystems, Wilmington, MA, USA), 100 pmol each of forward and reverse primers and 1 μL of DNA. Reaction conditions were 95°C for 30 s, followed by 40 cycles at 95°C for 10 sec, and 60°C for 30 sec. Fluorescent products were detected at the last step of each cycle. A melting curve analysis was performed after amplification to identify target amplicons. A standard curve for each *Lactobacillus* species was generated using the genomic DNA of the type-strain cultures JCM 1059, 1096, 1149, and 1157. All samples and standard genomic DNAs were analyzed in duplicate.

2.2.5 Preparation and culture of mouse spleen cells

Male C57BL/6 mice (CLEA Japan, Tokyo, Japan) were housed at 23±2°C with a 12-h light/dark cycle. All experimental procedures were conducted in accordance with the Regulations for Animal Experimentation of Shinshu University, Japan. The preparation of spleen cells involved a treatment with 0.17 M Tris-HCl buffer (pH 7.65) containing 0.83% NH_4Cl to deplete red blood cells. After centrifugation, cells were resuspended in RPMI-1640 medium containing 10% (v/v) fetal calf serum, 100 U mL^{-1} penicillin G, and 100 μg mL^{-1} streptomycin. Cells (5×10^5 cells per well) were cultured in 96-well flat-bottomed plates in the presence of LS at 37°C for 48 h in an atmosphere containing 5% CO_2.

2.2.6 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IFN-γ and IL-10 in culture supernatants were measured using ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

2.2.7 Statistical analysis

Data represent the mean ± standard deviation (SD). Statistical analyses were performed using a one-way ANOVA followed by Tukey's test for group comparisons. $P<0.05$ was considered to be significant. The relationship between cytokine levels and the number of 16S rRNA genes of each *Lactobacillus* species was analyzed using Pearson's correlation analysis. GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for data analysis.

2.3 Results

2.3.1 Changes in the microbial community during the fermentation of *B. rapa* L.

High-quality 16S rRNA gene sequences (n = 968,180) were analyzed (average, 53,788 ± 22,818 sequences per sample). After excluding chimeric sequences or singletons from the OTU table, sequences were divided into 2,979 OTUs. We further excluded 428 OTUs that were identified as chloroplast sequences.

Before and on day 3 of fermentation, most bacteria were identified as *Rhizobiales*, *Sphingomonadales*, and *Pseudomonadales*. The relative abundances of these bacteria significantly decreased on day 7 or 14 (Table 2.1). In contrast, *Lactobacillales* became the most predominant order on day 7 or later, reaching a relative abundance of ≥70 % (Table 2.1). All alpha-diversity parameters were significantly lower on day 7 than those of non-fermented vegetables (Table 2.2).

The hierarchical cluster analysis using a weighted Unifrac distance matrix indicated that the microbiotas of non-fermented and fermented *B. rapa* L. comprised two main clusters (Fig. 2.1). One of the main clusters consisted of the microbiotas of non-fermented and 3-day fermented *B. rapa* L., and the other comprised those of vegetables fermented for ≥ 7 days.

Representative sequences of the five most dominant OTUs, which were assigned to *Lactobacillales*, showed the highest sequences identities with the 16S rRNA genes of *L. curvatus*, *L. plantarum*, *Weissella confusa*, *L. sakei*, and *L. brevis*. The relative abundance of an OTU, whose representative sequence showed the highest similarity with 16S rRNA gene of *Weissella confusa*, exceeded 10% at only one time point for only 1 batch of 3 repeated preparation of fermented *B. rapa* L. In contrast, the relative abundances of other OTUs exceeded 10% at ≥ 3 sampling times for all batches of fermented *B. rapa L*. (Fig. 2.2)

Table 2.1. Changes in relative abundances of bacterial orders during fermentation of *B. rapa* L.

Taxonomy	Fermentation (day)						p value
Phylum / Order	0	3	7	14	21	28	
Bacteroidetes							
Flavobacteriales	3.05 ± 1.94[a]	3.04 ± 2.24 [a]	0.21 ± 0.15 [a]	0.12 ± 0.07 [a]	0.07 ± 0.04 [a]	0.08 ± 0.03 [a]	0.013
Firmicutes							
Lactobacillales	2.43 ± 3.85[b]	18.77 ± 26.55 [b]	78.71 ± 12.77 [a]	85.67 ± 8.88 [a]	83.80 ± 8.23 [a]	84.03 ± 9.27 [a]	< 0.001
Proteobacteria							
Rhizobiales	38.22 ± 13.72 [a]	31.75 ± 14.10 [a]	5.23 ± 3.30 [b]	3.23 ± 1.05 [b]	2.36 ± 0.17 [b]	2.07 ± 0.21 [b]	0.0002
Rickettsiales	4.40 ± 2.15 [a]	2.08 ± 0.99[ab]	0.35 ± 0.06 [b]	0.29 ± 0.10 [b]	0.46 ± 0.2 [b]	0.36 ± 0.23 [b]	0.0011
Sphingomonadales	18.43 ± 4.14 [a]	20.44 ± 7.34 [a]	3.63 ± 1.81 [b]	2.55 ± 1.21 [b]	1.55 ± 0.19 [b]	2.11 ± 1.22 [b]	< 0.001
Burkholderiales	4.44 ± 1.70[ab]	6.85 ± 4.71[a]	1.21 ± 0.81[ab]	0.63 ± 0.46 [b]	0.38 ± 0.19 [b]	0.59 ± 0.45 [b]	0.0113
Enterobacteriales	4.76 ± 6.35 [a]	3.64 ± 3.24 [a]	8.12 ± 9.99 [a]	6.21 ± 7.95 [a]	5.03 ± 5.05 [a]	5.38 ± 6.62 [a]	0.977
Oceanospirillales	0 ± 0 [a]	0.10 ± 0.16 [a]	0.04 ± 0.07 [a]	0.07 ± 0.11 [a]	1.72 ± 2.98 [a]	1.87 ± 3.23 [a]	0.599
Pseudomonadales	16.66 ± 8.80 [a]	8.29 ± 6.82[ab]	1.70 ± 1.55[b]	0.65 ± 0.32 [b]	0.29 ± 0.13 [b]	0.20 ± 0.11 [b]	0.0044
Vibrionales	0 ± 0 [a]	0.09 ± 0.16 [a]	0.04 ± 0.07 [a]	0.17 ± 0.29 [a]	4.03 ± 6.98 [a]	3.02 ± 5.24 [a]	0.586
Others	7.63 ± 1.39 [a]	4.95 ± 1.95 [a]	0.75 ± 0.32[b]	0.41 ± 0.09 [b]	0.29 ± 0.08 [b]	0.30 ± 0.14 [b]	< 0.001

Values are relative abundances (%) of each order in fermented or non-fermented *B.rapa* L. and are shown as means ± SD of percentages of triplicated fermentations. Values with different letters significantly differ ($P < 0.05$, Tukey's test). Others are composed of orders each showing a relative abundance of less than 5% of the total read in all the samples.

Table 2.2. Changes in alpha diversity parameters during fermentation of *B. rapa* L.

Parameter	Fermentation (day)						p value
	0	3	7	14	21	28	
chao1	945.2 ± 233.6[a]	806.5 ± 103.0[ab]	548.0 ± 101.3[b]	499.3 ± 119.5[b]	484.8 ± 104.8[b]	503.6± 158.1[b]	0.0075
observed_otus	626.2 ± 86.2 [a]	522.7 ± 116.1 [a]	315.8 ± 61.5 [b]	278.2 ± 54.7 [b]	252.8 ± 41.6 [b]	257.9 ± 41.1 [b]	< 0.001
PD_whole_tree	24.37 ± 7.14 [a]	19.45 ± 2.71 [ab]	12.52 ± 1.16[bc]	10.75 ± 0.34 [bc]	10.36 ± 0.67[c]	10.33 ± 0.47 [c]	< 0.001
Shannon	5.65 ± 0.39 [a]	5.35 ± 0.58 [a]	3.17 ± 1.11 [b]	2.95 ± 1.16 [b]	3.13 ± 0.39 [b]	3.12 ± 0.28 [b]	0.0012

Values are means ± SD of triplicated fermentations. Values with different letters significantly differ ($P < 0.05$, Tukey's test).

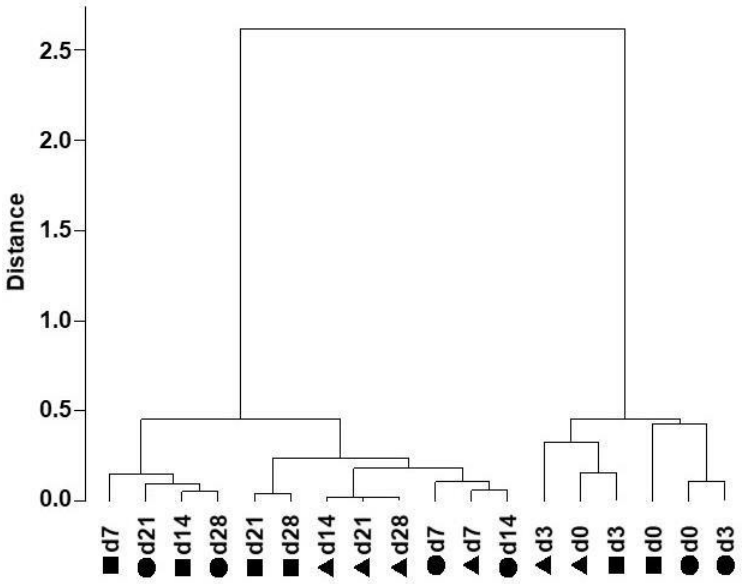

Fig. 2.1. Hierarchical clustering of the microbiotas of fermented and non-fermented *B. rapa* L.
B. rapa L. fermentation was conducted for 28 days at 10 °C, and samples were acquired 0, 3, 7, 14, 21, and 28 days after the start of fermentation. The V3-V4 regions of the 16S rRNA gene were amplified, and sequencing was performed using a MiSeq System. The sequencing data were analyzed with the QIIME pipeline, and hierarchical clustering of the microbial population of each sample was performed using a weighted Unifrac distance matrix. Circles (●), triangles (▲), and squares (■) represent batches of fermented *B. rapa* L. preparations.

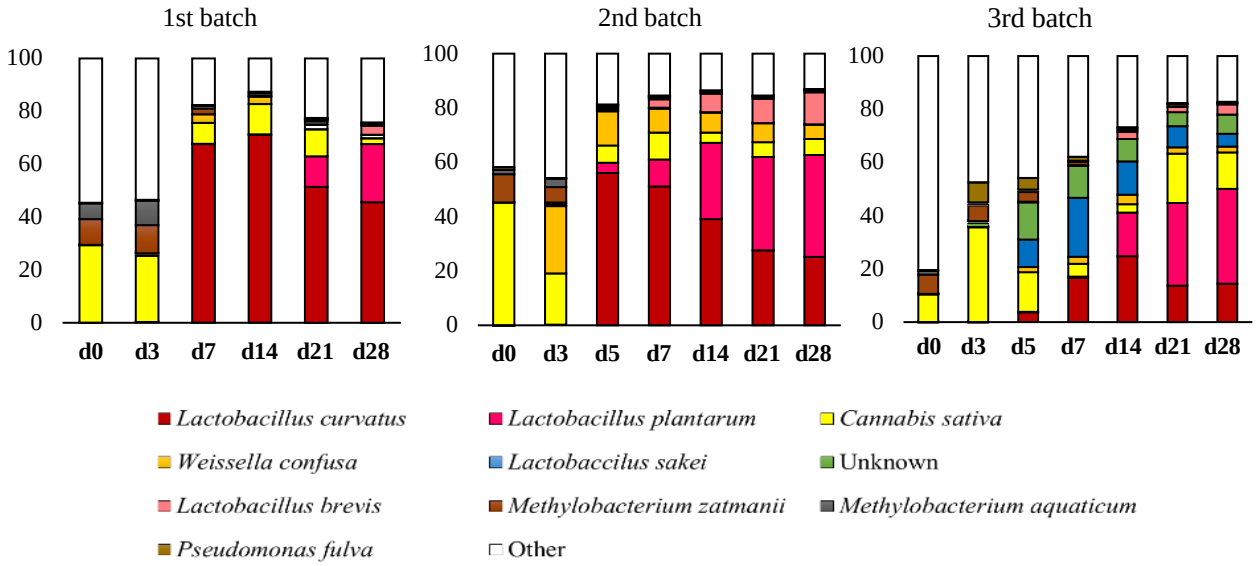

Fig. 2.2. Time-course changes of most abundant microbial community during the fermentation of *B. rapa* L. *Lactobacillus* became the most dominant genus on day 7 or later in three independent experiments. *L. curvatus* and *L. plantarum* were predominant, but *L. brevis* and *L. sakei* were observed as the minor among *Lactobacillus*.

2.3.2 Changes in the number of *Lactobacillus* species during the fermentation of *B. rapa* L.

Although *L. curvatus*, *L. plantarum*, *L. brevis*, and *L. sakei* were not detected in fresh *B. rapa* L., they were present in all batches of fermented *B. rapa* L. (Fig. 2.3). Among them, *L. curvatus* (Fig. 2.3A) was the most numerous *Lactobacillus* throughout the fermentation, reaching a maximum on day 7. *L. plantarum* (Fig. 2.3B) and *L. brevis* (Fig. 2.3C) were the second most populous *Lactobacillus* species. In contrast, the numbers of *L. sakei* (Fig. 2.3D) were much lower compared with those of other lactobacilli and varied among the batches. The populations of these *Lactobacillus* species became relatively stable on day 14 or later.

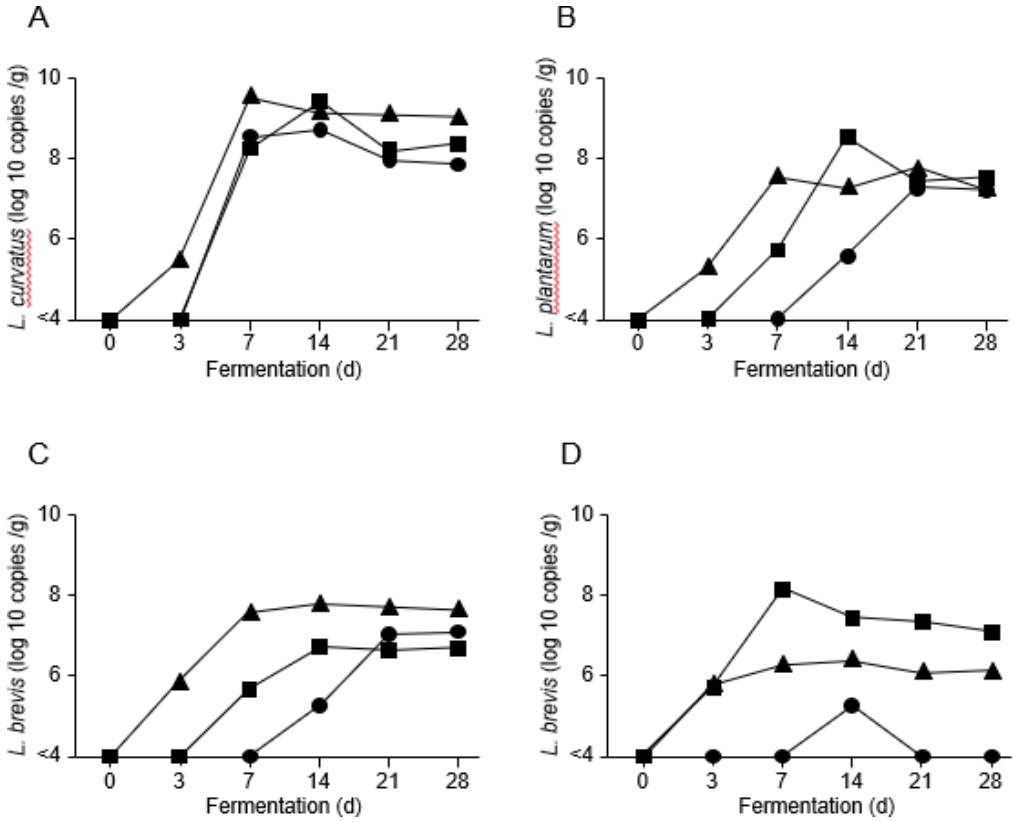

Fig. 2.3 Changes of the LAB population during the fermentation of *B. rapa* L. Fermentation was performed as described in Fig. 2.1. We used real-time PCR to quantify *L. curvatus* (A), *L. plantarum* (B), *L. brevis* (C) and *L. sakei* (D) in DNA samples of the LS. A standard curve for each *Lactobacillus* species was generated using the genomic DNA of type strain cultures. Data from three independent experiments are shown. Circles (●), triangles (▲), and squares (■) represent batches of fermented *B. rapa* L. preparations.

2.3.3 Changes in cytokine production induced by fermented *B. rapa* L.

To assess the changes in IFN-γ and IL-10 production induced by fermented *B. rapa* L., mouse spleen cells were stimulated with LS from non-fermented or fermented *B. rapa* L. for 48 h, and the concentrations of IFN-γ and IL-10 in the supernatants were analyzed. Fig. 2.4A shows the

change in IFN-γ production during fermentation of *B. rapa* L. The LS significantly increased IFN-γ production on day 7 or 14 after starting the fermentation compared to that of the non-fermented vegetables. In Fig. 2.4B, IL-10 levels induced by the LS increased on day 14 compared with non-fermented *B. rapa* L., which was less than that of the IFN-γ levels.

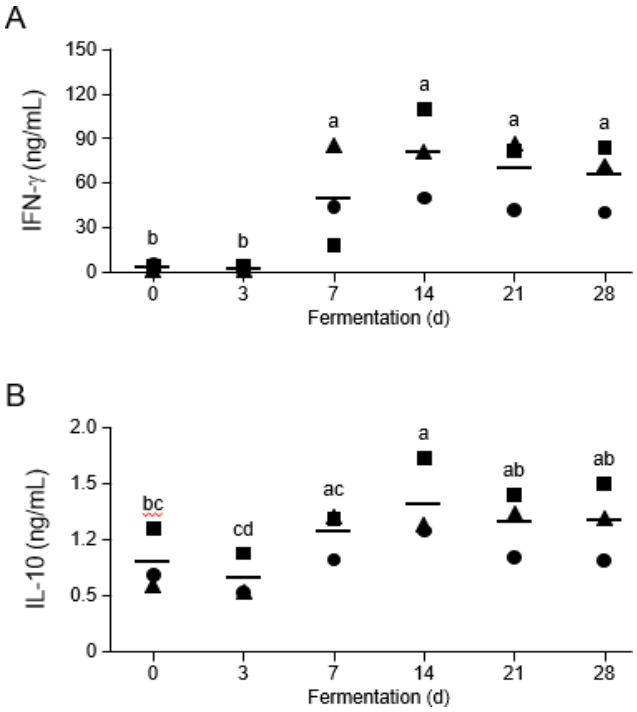

Fig. 2.4. Changes in cytokine induction activity of fermented *B. rapa* L. *B. rapa* L. fermentation was performed as described in Fig. 2.1. Mouse spleen cells (5×10^5 cells per well) were treated with 0.25 and 10 μg mL^{-1} of LS to measure IFN-γ (A) and IL-10 (B) levels, respectively. After 48 h, culture supernatants were collected, and IFN-γ and IL-10 levels were analyzed by ELISA. Bars show the mean of three independent experiments.
Circles (●), triangles (▲), and squares (■) represent different batches of fermented *B. rapa* L. preparations. Data were analyzed using a one-way ANOVA followed by Tukey's multiple comparison test. The letters indicate significant differences (P<0.05).

2.3.4 Relationship between cytokine production and the numbers of LAB

To assess the involvement of each *Lactobacillus* species in the ability of fermented *B. rapa* L. to induce cytokine production, we examined the relationship between cytokine production and the number of each *Lactobacillus* species. The levels of IFN-γ strongly correlated with the numbers of *L. plantarum* (r=0.865; P=0.0003) and *L. curvatus* (r=0.785; P=0.0015), but not with those of *L. brevis* (r=0.574; P=0.0510) and *L. sakei* (r=0.262; P=0.4367). *L. curvatus* had the highest correlation coefficient associated with IL-10 production (r=0.673; P=0.0118) (Fig. 2-5).

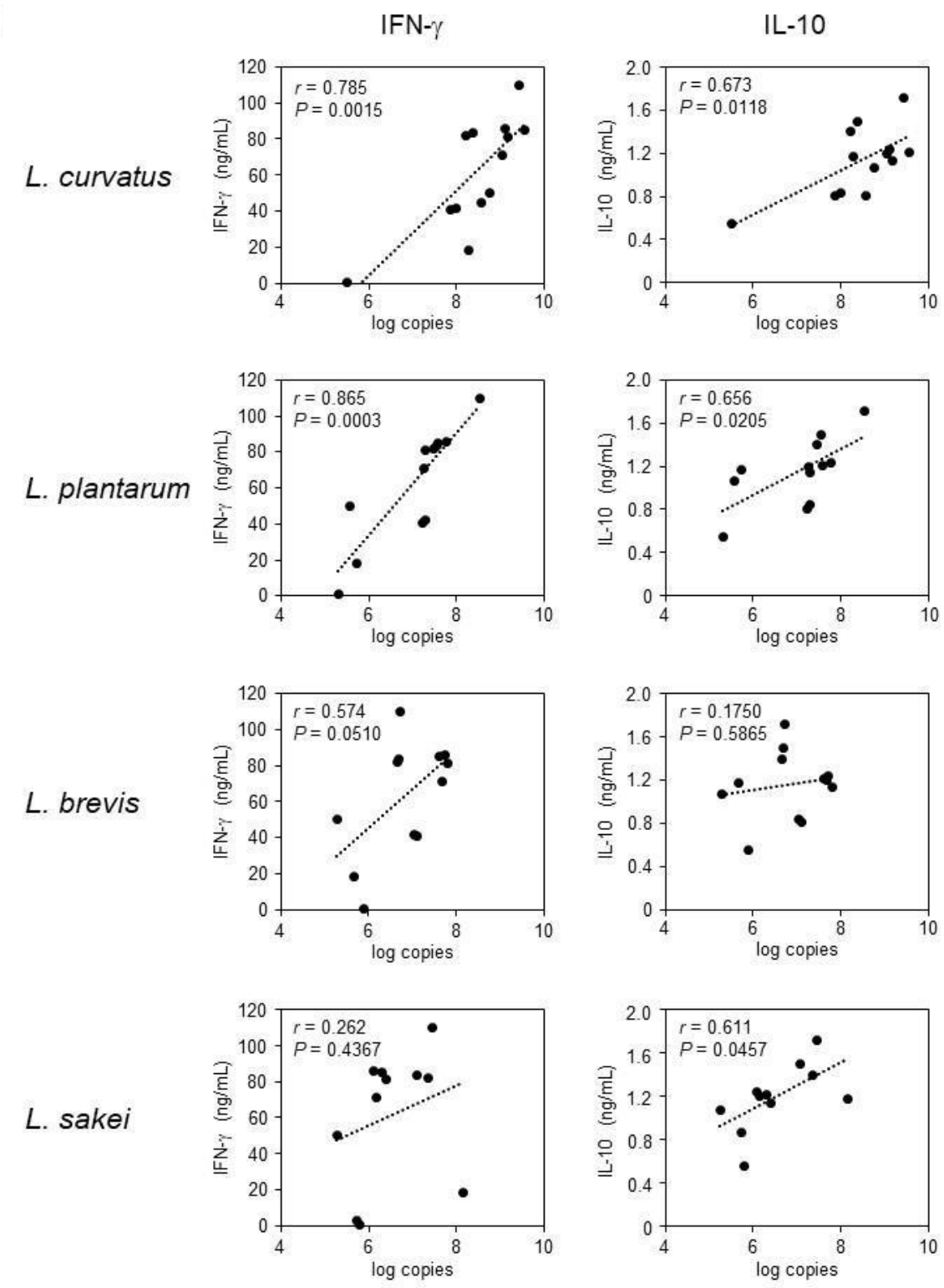

Fig. 2.5. Correlation between cytokine production and the number of LAB.

Pearson's correlation analysis was used to evaluate the relationship between cytokine production and the number of LAB. Each plot shows the number of LAB and cytokine production of all LS from three batches. The detection limits of the number of bacteria were 4 log copies g^{-1}, and data below the detection limit were eliminated.

2.4 Discussion

In this study, we herein clarified changes in the overall bacterial community during the fermentation of *B. rapa* L., and demonstrated that the number of LAB and cytokine-inducing activity increased during fermentation. The number of LAB correlated with the levels of IFN-γ and IL-10.

An amplicon analysis of the V3–V4 regions of the 16S rRNA gene revealed microbial succession during the fermentation of *B. rapa* L. *Rhizobiales*, *Sphingomonadales*, and *Pseudomonadales* were the most prominent in non-fermented or 3-d fermented vegetables (Table 2.1). The relative abundance of *Lactobacillales* markedly increased on day 7 of fermentation, and *Lactobacillales* was the most dominant when *B. rapa* L. was fermented for at least 7 d (Table 2.1). Parameters that represent the diversity of the bacterial community were lower on day 7 than those of non-fermented vegetables (Table 2.2). These results suggest that increasing the population of *Lactobacillales* produced organic acids that lowered the pH of the brine and vegetables, resulting in a decrease in the populations of other bacterial species. The pH values of 7-d or longer fermented vegetables were significantly lower than that of non-fermented vegetables (data not shown). Similar bacterial succession has been observed during kimchi fermentation, which is mainly mediated by *Lactobacillaceae* (Jeong *et al.*, 2013).

A hierarchical clustering analysis using a weighted Unifrac distance matrix indicated that non-fermented and 3-d fermented vegetables harboured similar bacterial communities, which were distinct from the bacterial communities observed in ≥7-d fermented vegetables (Fig. 2.1). This suggests that the bacterial community became robust when *B. rapa* L. was fermented for at least 7 d. Thus, the robustness of the microbiota may contribute to elongating the shelf life of pickled vegetables.

A microbial community analysis revealed that the representative sequences of the four most dominant OTUs showed the highest sequence identities with the 16S rRNA genes of *L. curvatus*, *L. plantarum*, *L. sakei*, and *L. brevis* in three independent experiments (Fig. 2.2), suggesting that these are major *Lactobacillus* species that are the primary contributors to the fermentation of *B. rapa* L.

The numbers of these *Lactobacillus* species throughout fermentations were quantified by real-time PCR using species-specific primers. Real-time PCR analyses revealed that *L. curvatus* was the most dominant lactobacillus, followed by *L. plantarum* and *L. brevis* (Fig. 2.3 A, B, C). In contrast, the numbers of *L. sakei*, which widely varied among batches, were markedly lower (Fig. 2.3D). The API50CHL test detects the LAB species *L. curvatus*, *L. plantarum*, *L. brevis*, *L. coprophilus*, *L. delbruekii*, *L. fermentum*, and *Leuconostoc mesenteroides* in fermented *B.*

rapa L (Kawahara and Otani, 2006c). Some of these species were detected here in fermented *B. rapa* L., suggesting that *B. rapa* L. induced the proliferation of specific LAB species during fermentation.

Non-fermented *B. rapa* L. induced a negligible amount of IFN-γ from mouse spleen cells (Fig. 2.4A). The levels of IFN-γ increased in fermented *B. rapa* L. after 7 or 14 d of fermentation (Fig. 2.4A), which corresponds to the times when the number of major *Lactobacillus* species increased (Fig. 2.3). Mouse spleen cells were treated with LS, which was prepared by the centrifugation of a PBS suspension of non-fermented or fermented vegetables. Thus, LS mainly comprised bacterial cells. Furthermore, the levels of IFN-γ correlated with the numbers of *L. curvatus* and *L. plantarum* (Fig. 2.4). These two LAB species promote IFN-γ production by mouse spleen cells (Song *et al.*, 2016). The cell wall components of LAB, such as peptidoglycan (PGN) and lipoteichoic acid (LTA), induce IL-12 production by murine macrophages and splenic DCs in a TLR2-dependent manner (Hirose *et al.*, 2010; Lawrence and Nauciel, 1998). Since LS consisted of not only lactobacilli, but also many types of bacteria, synergistic effects on cytokine production by *Lactobacillus* and other bacteria including *Lactobacillus* are possible.

IL-10 production, unlike that of IFN-γ, was relatively high in the LS of non-fermented *B. rapa* L. (Fig. 2.4B), suggesting that IL-10 was induced by bacteria other than LAB in non-fermented *B. rapa* L. After fermentation, the levels of IL-10 correlated with the number of LAB species, particularly those of *L. curvatus*, *L. plantarum*, and *L. sakei*. For example, *L. plantarum* strains potently induce IL-10 production by mouse peritoneal macrophages, and the IL-10-induced synthesis of teichoic acids by this strain is mediated by TLR2-dependent ERK activation (Kaji *et al.*, 2010). Therefore, the cell wall component of LAB may induce IL-10 production. The number of *L. sakei* correlated with IL-10 production, but not with IFN-γ production, suggesting that *L. sakei* in fermented *B. rapa* L. may have a stronger IL-10-producing ability than IFN-γ. However, we failed to obtain *L. sakei* isolates, possibly because of weaker dominance.

Fermented *B. rapa* L. induced greater increases in IFN-γ and IL-10 production than non-fermented vegetables, and, thus, lactobacilli appear to be involved in cytokine production. IL-12 and IFN-γ suppress the differentiation of Th2 cells, and IL-10 is a potent inhibitor of inflammation through its suppression of the synthesis of Th2 cytokines (Chung, 2001b). Allergic inflammation is characterized by the infiltration of tissues by mast cells and activated eosinophils, which release Th2 cytokines, particularly IL-4 and IL-5 (Ngoc *et al.*, 2005). Thus, fermented *B. rapa* L. may suppress allergic reactions through the production of IFN-γ and IL-10 by immune cells. A previous study reported that *L. casei* variety *rhamnosus* alleviated atopic

dermatitis, possibly by improving the Th1/Th2 balance and intestinal microbiota (Yeom *et al.*, 2015).

Although *L. curvatus*, *L. plantarum* and *L. brevis* are likely to contribute the immunomodulatory activities of fermented *B. rapa* L., immunomodulatory activities of bacteria differ among species even within the same species. Therefore, it is noteworthy to isolate and investigate LAB strains which can possess immunomodulatory properties. Furthermore, such LAB isolates could be employed for manufacturing functional food possessing anti-inflammatory or anti-allergic effects.

CHAPTER 3. IMMUNOMODULATION EFFECTS OF LACTIC ACID BACTERIA ISOLATED FROM FERMENTED *BRASSICA RAPA* L.

3.1 Introduction

Lactic acid bacteria (LAB) belong to a group of Gram-positive bacteria and are widely distributed in nature. LAB have been used for fermentation of various foods, including fermented vegetables. In addition, certain strains of LAB are well known as probiotics, which exert beneficial health promoting effects including the modulation of immune responses.

Possible beneficial effects of LAB on the immune system include preventing upper respiratory tract infections, atopic eczema, allergic rhino conjunctivitis, and asthma, as well as treating acute inflammatory bowel disease (IBD) and maintenance therapy of IBD. In animal models, LAB supplementation prevents chemically induced colitis, asthma, and allergic rhinitis by down-regulating inflammatory cytokine production or inducing anti-inflammatory cytokine production (Borchers *et al.*, 2009). Immunomodulatory properties of LAB are recognized as strain specific, even though they belong to the same species. Therefore, investigating the LAB strains possessing immunomodulation effects from fermented foods are of important to develop future functional food products.

As we discussed on Chapter 2, LAB that induce the production of IFN-γ and IL-10 may have preventive and therapeutic effects for treating allergies. *L. pentosus* strain S-PT84 induces IL-12 production by activating the Toll-like receptor (TLR) isoforms TLR2, TLR4, or both, on DCs. *L. plantarum* strain YU, which is present in fermented food, inhibits viral infections by enhancing the production of IL-12 and IFN-γ by immune cells (Kawashima *et al.*, 2011a). Furthermore, S-PT84 induces IFN-γ production by $NK1.1^+$ cells in an IL-12-dependent manner (Koizumi *et al.*, 2008). Concerning IL-10 induction by LAB, *L. plantarum* strains stimulate IL-10 production by macrophages, whereas *L. reuteri* and *L. casei* strains induce IL-10-producing Treg cells by modulating the functions of DCs (Morita *et al.*, 2002; Smits *et al.*, 2005).

In this chapter, we isolated 46 strains of LAB from *B. rapa* L. which was fermented for 28 days. Their IFN-γ and IL-10 production activities were determined by co-cultivation with spleen cells of mice. These LAB isolates were identified by molecular biological technics. Furthermore, selected strains were used as the starter cultures for the fermentation of *B. rapa* L., and their immunomodulation effects were determined.

3.2 Materials and Methods

3.2.1 Isolation of LAB

LAB were isolated from *B. rapa* L. fermented for 28 days according to a published procedure (Kawahara and Otani, 2006a) with modifications. Briefly, the sample was diluted with PBS and plated on de Man, Rogosa, Sharpe (MRS) agar (Kanto Chemical, Tokyo, Japan) containing 0.5% (w/v) $CaCO_3$. After anaerobic incubation at 29 °C for 48 h, the colonies surrounded with clear $CaCO_3$-dissolution zones were transferred to MRS broth (Difco Laboratories, Detroit, MI, USA). The isolated strains were suspended in 10% glycerol and stored at –80 °C.

3.2.2 DNA extraction from LAB

All strains were cultured at 30 °C for 24 h in MRS broth, and cells were harvested by centrifugation at 13,300 ×*g* for 5 min. Bacterial DNA was extracted and purified using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) and a ZircoPrep Mini Kit (Nippon Genetics, Tokyo, Japan) according to the manufacturers' instructions.

3.2.3 16S rRNA gene sequencing of isolated LAB strains

The 16S rRNA gene was amplified using PCR with the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3'). PCR products were separated in 1.5% agarose gels, and purified with an Isospin Agarose Gel (Nippon Gene, Tokyo, Japan). Resulting purified DNA amplicons were used as templates for sequencing reactions with a primer 926R (5'-CCGTCAATTCCTTTRAGTTT-3'). The DNA amplicons of strains *L. plantarum* - Lp4 and *L. curvatus* – Lc3 were sequenced with primers 27F, 1492R, 926R, 786F (5'-GATTAGATACCCTGGTAG-3'), 529R (5'-ACCGCGGCKGCTGGC-3') for whole genomic sequence.

The sequences were read using an ABI Prism 310 Genetic Analyzer (Thermo Fisher Scientific Japan, Japan). Sequences of 16S rRNA gene of the LAB strains were compared with those in the public database using a blastn algorithm (https://blast.ncbi.nlm.nih.gov/). Multiple alignments of 16S rRNA gene sequences of the isolates and those from public database were conducted on Clustalw (clustalw.ddbj.nig.ac.jp), and the phylogenetic tree was drawn by neighbor joining method.

3.2.4 *Lactobacillus* species-specific PCR

DNA isolated from LAB isolates was amplified by PCR using primers specific to *L. curvatus*, *L. plantarum*, *L. brevis*, and *L. sakei* (Berthier and Ehrlich, 1999; Torriani, Felis and Dellaglio, 2001; Oswari *et al.*, 2013). PCR reaction mixtures (25 μL) contained 12.5 μL KAPA SYBR Fast qPCR Master Mix (KAPA Biosystems, Wilmington, MA, USA), 100 pmol each of forward and reverse primers, and 1 μL of DNA. Reaction conditions were 95°C for 30 s, followed by 40 cycles at 95°C for 10 sec, and 60°C for 30 sec. Fluorescent products were detected at the last step of each cycle.

3.2.5 Fermentation of *B. rapa* L. with starter cultures

L. plantarum strain Lp4 and *L. curvatus* strain Lc3 were grown in MRS at 29 °C for 24 h. The cells were washed with saline and then suspended in saline. Cell suspensions (1×10^{10} cfu) were inoculated into 1 kg of fresh *B. rapa* L. in a salt solution (7% w/w, NaCl) in a pickle jar for 3 days at 10 °C. The samples were obtained 3 days after the start of fermentation. LS was prepared from each sample following the procedures described in 2.2.2.

3.2.6 Multilocus sequence typing analysis (MLST): PCR amplification, gene selection, sequences and analysis

The MLST analysis as identifying the novel strain, slightly modified methods using by Haiyan Xu (Xu *et al.*, 2015). Briefly, genomic DNA of strains Lp4 and Lc3 isolates were used as a template for PCR amplification of MLST loci using an automatic thermal cycler (TakaraBio, Otsu, Japan).

Eight housekeeping genes *pheS, clipX, groEL, murC, murE, pyrG, recA and uvrC* were used in MLST for *L.plantarum* - Lp4 isolates and seven housekeeping genes *clpX-3, pyrG-10, groL-2, fstQ-8, pepV-10, dnaK-5, recG-1* were used in MLST for *L.curvatus* - Lc3 isolates. Primers sequences for the PCR were shown at (Table 3.2). Thermal cycling conditions were: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, the appropriate temperature for the gene of interest for 1 min (Table 3.2), and 72 °C for 2 min; a final elongation step of 72 °C for 10 min. Each PCR reaction was performed in a volume of 25 μL containing 75 ng of genomic DNA, 5 mL of each dNTP, 10 mM of each gene primer and 0.625 mL KOD.Fx as enzyme. PCR products were electrophoresed in a 1.5 % agarose gel and purified by Isospin Agarose Gel kit (Nippon gene, Japan). Resulting purified DNA amplicons were used as templates for sequencing reaction with the same primers. The sequences were read by using an ABI Prism 310 Genetic Analyzer

(Thermo Fisher Scientific Japan, Japan). The sequence of each gene of LAB strains were concatenated and compared with those in the public database using a blastn algorithm (http://blast.bcbi.nlm.nih.gov/). Multiple alignment of these genes sequences of the isolates and those from public database were conducted on Clustalw (https://www.genome.jp/tools-bin/clustalw), and the phylogenetic tree was drawn by neighbor joining method.

Table 3.2 The information of primers and housekeeping genes for MLST of *L. plantarum* and *L. curvatus*

Gene	Forward (5' → 3')	Reverse (3' → 5')
Lactobacillus plantarum		
PheS	CCGTGAAGAACTGGAACA	CCTAACCCAAAGGCAAAA
PyrG	AGTGATTTAGGTTCCGACAA	TGCATTCCCAAGCAGATA
UvrC	GATCATTTATGTGGGTAAGGC	TGACACTACTGGGAACAAGC
recA	TTTTAGTTGTTGACTCGGTGGC	TTCCGCTGGTGTCGCTTT
clpX	ATCGCCAAGAAGAGTGAA	ATAATCGAGCGTAGACCC
murC	TATCGCTCCCACCAGTTA	CGGCCAAGATTTCCTTAT
groEL	CGGCTACTTATCACAATACA	GCCTTCTAAACCAGCATT
murE	ACTAATAAGGTCGCTGTTCTG	TTTAGCGGCTTCTTCACT
Lactobacillus curvatus		
clpX-3	CGTTTTGTGGGAAGTCGCAA	CGCGTGATCGAGACGTTTTC
pyrG-10	GACAGCACCAGAAGCTGAGA	TGGTTTGTTGGGGCGTGATA
groL-2	TAGGACCAAAAGGCCGGAAC	GCAACCACGTTGAATGTCCC
fstQ-8	CGCTCGATCCGCTCCAATTA	ACAGGGCTATTGCCGAGAGA
pepV-10	GCGCGACGTTGACCATAAAG	GCCGGCTTTTGGTTCTTGAG
dnaK-5	GAAGCTGTTGCTCTTGGTGC	TCGCCGTCAACAGTACCATC
recG-1	GAGAATCCATCGTTTGCGGC	TTCACGCAAAATTCGCCGTT

3.2.7 Preparation and culture of mouse spleen cells

Male C57BL/6 mice (CLEA Japan, Tokyo, Japan) were housed at 23±2°C with a 12-h light/dark cycle. All experimental procedures were conducted in accordance with the Regulations for Animal Experimentation of Shinshu University, Japan. The preparation of spleen cells involved treatment with 0.17 M Tris-HCl buffer (pH 7.65) containing 0.83% NH_4Cl to deplete red blood cells. After centrifugation, cells were resuspended in RPMI-1640 medium containing 10% (v/v) fetal calf serum, 100 U mL^{-1} penicillin G, and 100 μg mL^{-1} streptomycin. Cells (5×10^5 cells per well) were cultured in 96-well flat-bottomed plates in the presence of LS at 37°C for 48 h in an atmosphere containing 5% CO_2.

LAB isolates were cultured at 29 °C for 48 h in MRS broth. The cells were harvested by centrifugation at 20,630 × *g* for 15 min, washed with MilliQ water, and heated at 65 °C for 30 min. The heat-killed cells were lyophilized and co-cultured with spleen cells.

3.2.8 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IFN-γ and IL-10 in culture supernatants were measured using ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

3.2.9 Real-time quantitative PCR

Numbers of total bacteria and lactobacilli present in LS from fermented *B. rapa* L. with addition of starter cultures were quantified by real-time quantitative PCR with primer pairs for bacteria universal and specific to *Lactobacillus* genus (Fusco *et al.*, 2016). PCR reaction mixtures (25 μL) contained 12.5 μL KAPA SYBR Fast qPCR Master Mix (KAPA Biosystems, Wilmington, MA, USA), 100 pmol each of forward and reverse primers and 1 μL of DNA. Reaction conditions were 95°C for 30 s, followed by 40 cycles at 95°C for 10 sec, and 60°C for 30 sec. Fluorescent products were detected at the last step of each cycle. A melting curve analysis was performed after amplification to identify target amplicons. A standard curve was generated using the genomic DNA of *L. plantarum* JCM1149. All samples and standard genomic DNAs were analyzed in duplicate.

3.2.10 Statistical analysis

Data represent the mean ± standard deviation (SD). Statistical analyses were performed using a one-way ANOVA followed by Tukey's test for group comparisons. $P<0.05$ was considered to be significant.

3.3 Results

3.3.1 Identification of LAB strains isolated from fermented *B. rapa* L.

Total 46 LAB strains were isolated from *B. rapa* L. fermented for 28 days. They were identified by sequencing of 16S rRNA gene and PCR amplification using *Lactobacillus* species-specific primers. All of 46 strains belonged to genus *Lactobacillus*. Among them, 2 were *L. brevis*, 4 were *L. curvatus*, while other 40 strains were *L. plantarum* (Table. 3.1).

Table 3.1 Identification of LAB strains analyzed by 16s rRNA sequences and PCR using species-specific primers

№	Strain	Identification		№	Strain	Identification	
		16S rRNA sequence	PCR			16S rRNA sequence	PCR
1	#K4-1	*Lactobacillus brevis*		24	#K5-2	*Lactobacillus plantarum*	
2	#K4-2	*Lactobacillus plantarum*		25	#K5-3	*Lactobacillus plantarum*	
3	#K4-3	*Lactobacillus plantarum*		26	#K5-4	*Lactobacillus plantarum*	
4	#K4-5	*Lactobacillus curvatus*		27	#K5-5	*Lactobacillus plantarum*	
5	#K4-6	*Lactobacillus brevis*		28	#K5-6	*Lactobacillus plantarum*	
6	#K4-7	*Lactobacillus curvatus*		29	#K5-7	*Lactobacillus plantarum*	
7	#K4-8	*Lactobacillus curvatus*		30	#K5-8	*Lactobacillus plantarum*	
8	#K4-9	*Lactobacillus plantarum*		31	#K5-9	*Lactobacillus plantarum*	
9	#K4-10	*Lactobacillus plantarum*		32	#K5-10	*Lactobacillus plantarum*	
10	#K4-11	*Lactobacillus plantarum*		33	#K5-11	*Lactobacillus plantarum*	
11	#K4-12	*Lactobacillus curvatus*		34	#K5-12	*Lactobacillus plantarum*	
12	#K4-13	*Lactobacillus plantarum*		35	#K5-13	*Lactobacillus plantarum*	
13	#K4-14	*Lactobacillus plantarum*		36	#K5-14	*Lactobacillus plantarum*	
14	#K4-15	*Lactobacillus plantarum*		37	#K5-15	*Lactobacillus plantarum*	
15	#K4-17	*Lactobacillus plantarum*		38	#K5-16	*Lactobacillus plantarum*	
16	#K4-18	*Lactobacillus plantarum*		39	#K5-17	*Lactobacillus plantarum*	
17	#K4-19	*Lactobacillus plantarum*		40	#K5-18	*Lactobacillus plantarum*	
18	#K4-20	*Lactobacillus plantarum*		41	#K5-19	*Lactobacillus plantarum*	
19	#K4-21	*Lactobacillus plantarum*		42	#K5-20	*Lactobacillus plantarum*	
20	#K4-22	*Lactobacillus plantarum*		43	#K5-21	*Lactobacillus plantarum*	
21	#K4-23	*Lactobacillus plantarum*		44	#K5-22	*Lactobacillus plantarum*	
22	#K4-24	*Lactobacillus plantarum*		45	#K5-23	*Lactobacillus plantarum*	
23	#K5-1	*Lactobacillus plantarum*		46	#K5-24	*Lactobacillus plantarum*	

3.3.2 Cytokine production activities of LAB isolates from fermented *B. rapa* L.

Although all isolated strains induced IFN-γ and IL-10 production from mouse spleen cells, each strain showed different patterns (Fig. 3.1). In addition, in a preliminary test, we also used LPS as a positive control which induced a lower cytokine production effect compared to some of the selected strains (data was not shown). Among 46 strains, Lp4 and Lc3 induced the highest levels of IFN-γ and IL-10, respectively. Therefore, we selected these two strains as starter cultures to produce fermented *B. rapa* L.

3.3.3 Number of lactobacilli in starter-fermented *B. rapa* L.

Quantitative PCR analysis revealed that the addition of starter cultures drastically increased the number of lactobacilli in LS from 3 day-fermented *B. rapa* L.; The numbers (log) lactobacillus derived from the LS of naturally fermented (NF), Lp4-, and Lc3-fermented *B. rapa* L. were 5.7/g, 9.2/g, and 8.9/g, respectively (Fig. 3.2). Total bacterial 16S sequences in the LS were significantly increased by the addition of Lp4 and Lc3 as starter cultures.

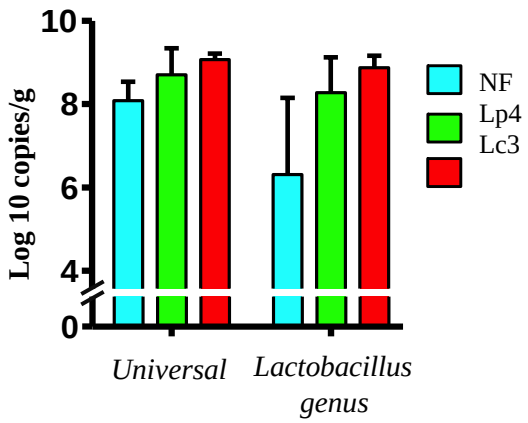

Fig. 3.2. Numbers of total bacteria and *Lactobacillus* genus in naturally fermented and starter fermented *B. rapa* L.

B. rapa L. fermentations were carried out with and without starter cultures (Lp4 or Lc3) for 3 days at 10 °C. The numbers (log) of universal and *Lactobacillus* derived from the LS were measured by quantitative PCR. Data are shown as mean ± SD. Data were analyzed using a one-way ANOVA followed by Tukey's multiple comparison test.

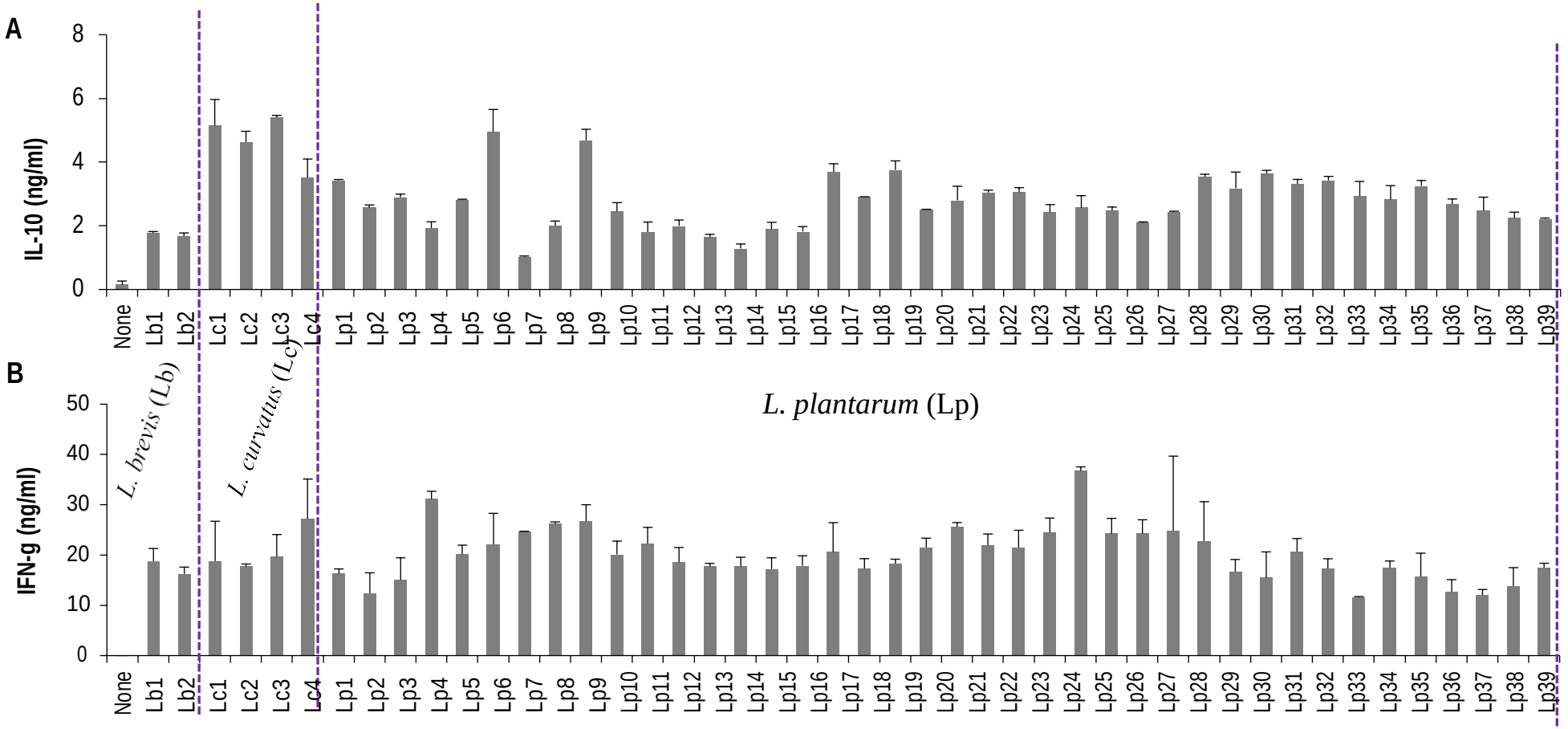

Fig. 3.1. Effects of LAB isolated from fermented *B. rapa* L. on cytokine production by mouse spleen cells.
Mouse spleen cells (5×10^5 cells/well) were treated with 1 μg/mL and 10 μg/mL of heat-killed bacteria to measure IFN-γ and IL-10 levels, respectively. After 48 h of culture, cell-free supernatants were collected, and IFN-γ and IL-10 levels were analyzed using ELISAs. Data are shown as the mean ± SD. Results represent seven independent experiments.

3.3.4 MLST analyses

The results of the phylogenetic placement of isolates Lc3 and Lp4 were shown in Fig. 3.2. These isolates were mainly clustered in 2 clusters, with comparison of isolates from the public database. Both strains Lc3 and Lp4 possessed exactly the same sequences of concatenated each of their housekeeping genes with any sequences of other strains on the public database.

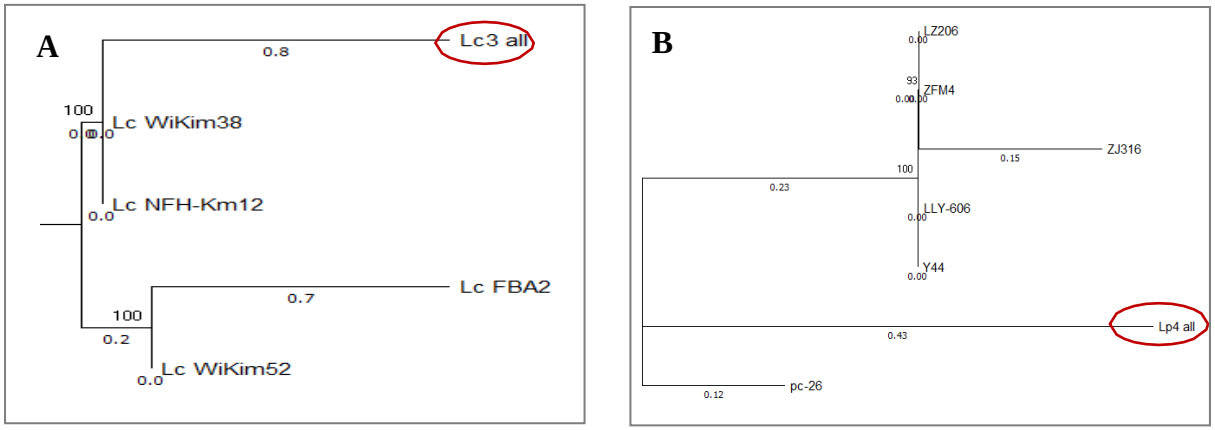

Fig 3.2 Phylogenetic tree of strains Lc3 *(L. curvatus)* and Lp4 *(L. plantarum)*

The neighbour junction tree constructed from seven chained nucleotide sequences of seven genes used in *L. curvatus* (A) and eight chained nucleotide sequences of eight genes used in *L. plantarum* (B) for MLST analysis, is compared with another highly similar genus of lactobacilli sequences.

3.3.5 Cytokine inducing activity of starter fermented *B. rapa* L.

To assess the effect on cytokine production of the addition of Lp4 and Lc3 isolates as starter cultures to the fermentation of *B. rarefepa* L., mouse spleen cell suspensions were treated with LS from 3 day-fermented *B. rapa* L., and the concentrations of IFN-γ and IL-10 in the supernatants were determined. IFN-γ was not detectably induced by LS from naturally fermented (NF) *B. rapa* L. The LS from fermented *B. rapa* L. initiated with Lp4 and Lc3 significantly increased the levels of IFN-γ (Fig. 3.2A). IL-10 production was significantly induced by Lc3-fermented *B. rapa* L. compared with naturally fermented *B. rapa* L. (Fig. 3.2B).

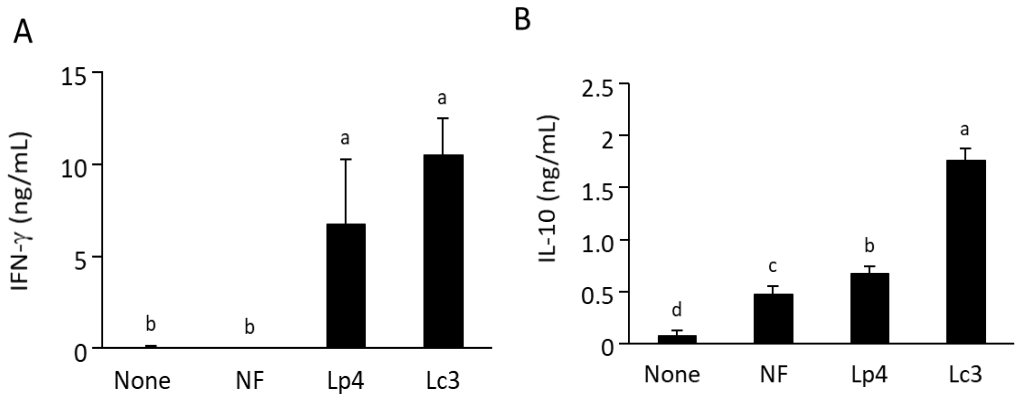

Fig. 3.3. Effects on cytokine production of *B. rapa* L. fermented using starter cultures.
B. rapa L. fermentations were performed with and without starter cultures (Lp4 or Lc3) at 10°C for 3 d. LS was prepared from each sample. Mouse spleen cells (5×105 cells per well) were stimulated with 0.25 and 10 μg mL^{-1} of LS to measure IFN-γ (A) and IL-10 (B) levels, respectively. After 48 h of culture, cell-free supernatants were collected, and IFN-γ and IL-10 levels were analyzed using ELISA. Results represent two independent experiments. Data were analyzed using a one-way ANOVA followed by Tukey's multiple comparison test. The letters indicate significant differences (P<0.05)

3.4 Discussion

We isolated 46 strains of LAB from *B. rapa* L. fermented for 28 d. Sequencing of 16S rRNA gene and PCR using *Lactobacillus* species specific primers revealed that most of LAB isolates were *L. plantarum* (Fig. 3.1). These results are consistent with a previous study demonstrating that *L. plantarum* plays a dominant role in community dynamics during the final phase of the spontaneous fermentation of cauliflower and mixed vegetables (green tomatoes, carrots, and cauliflower) (Wouters *et al.*, 2013). However, a quantitative PCR analysis using species-specific primers revealed that *L. curvatus* was the most dominant lactobacillus (Fig. 2.2). This difference may be explained by evidence indicating that viable *L. plantarum* are present during the later stages of the fermentation of *B. rapa* L., in contrast to other species. Another possibility is differences in the growth of lactobacilli on MRS agar.

IFN-γ and IL-10 production activities of LAB isolates were different, although all isolates induced IFN-γ and IL-10 production from spleen cells of mice (Fig. 3.1). Differences in cytokine induction activities among the strains would be reasonable, because immunomodulatory activities of LAB are recognized as strain specific. The cell wall components of LAB, such as peptidoglycan (PGN) and lipoteichoic acid (LTA), induce IL-12 production by murine macrophages and splenic DCs in a TLR2-dependent manner with resulting induction of IFN-γ (Hirose *et al.*, 2010; Lawrence and Nauciel, 1998). In addition, *L. plantarum* strains potently induce IL-10 production by mouse peritoneal macrophages, and the IL-10-induced synthesis of teichoic acids by this strain is mediated by TLR2-dependent ERK activation (Kaji *et al.*, 2010). Thus, difference in cytokine production activities among the LAB isolates possibly derived from different compositions of cell walls.

B. rapa L. fermentations initiated with starter cultures increased the levels of IFN-γ and IL-10 induction significantly (Fig. 3.3) and number of *Lactobacillus* genus drastically increased on day 3 comparing with naturally fermented *B. rapa* L. (Fig. 3.2). Thus, the addition of starter cultures appears to have induced an early increase in the number of lactobacilli, leading to enhanced cytokine production. The Lc3 strain was associated with higher levels of IL-10 being produced than the Lp4 strain. Like so, the cytokine-inducing activity of LAB strains may affect the levels of cytokine production in the fermentation of *B. rapa* L. initiated using starter cultures. Moreover, the use of these starter cultures may enhance the induction of cytokine production, which contributes to the manufacture of fermented *B. rapa* L.

CHAPTER 4. *IN VIVO* IMMUNOMODULATION EFFECTS OF LAB ISOLATES

4.1 Introduction

LAB are believed to have probiotic properties. It have been well recognized for their roles in preventing and treating acute gastrointestinal infections, diarrhea, allergic rennet, atopic dermatitis as well as inflammatory bowel disease (Kalliomäki *et al.*, 2001; H. Wu *et al.*, 2016). The beneficial effect of these strains on the immune system occurs through direct and indirect interactions with immune or non-immune cells (Wu *et al.*, 2016; Lebeer *et al.*, 2018; Villena and Kitazawa, 2014), by activation and proliferation of those cells, production of cytokines, secretion of IgA, synthesis of antimicrobial peptides and the improvement of tight junctions (Sánchez *et al.*, 2017). However, immunomodulation effects of LAB are based primarily on their ability to regulate the production of anti- and pro-inflammatory cytokines and Th1/Th2 balance. Several studies reported that *L. pentosus* strain S-PT84 induces the production of IL-12 and IL-10 *in vitro*. In a mouse model of OVA-induced allergy, orally administered S-PT84 decreased the concentration of serum IgE and inhibited the active cutaneous anaphylaxis reaction as well as the production of IL-4 by spleen cells. In addition, IL-10 production by the splenocytes of OVA immunized mice was increased by dietary S-PT84 (Nonaka *et al.*, 2008).

Culture conditions, such as carbon sources and culture temperature, as well as growth phase affect characteristics of bacterial cells. Toshimitsu *et al*. (2016) reported that *L. plantarum* OLL2712 cells in exponential growing phase induced more IL-10 by immune cells of mice when compare to those in stationary phase, with showing anti-inflammatory activities in obese and type2 diabetic mice. Strains Lc3 and Lp4 isolated from fermented *B. rapa* L. induced potent IFN-γ and IL-10 production by spleen cells of mice (Chapter 3). In this chapter, Lc3 and Lp4 were cultured in different conditions, and the immunomodulatory activities were compared by co-culturing bacterial cells and mouse spleen cells. In addition, to clarify immunomodulation activities of these strains *in vivo*, gene expressions in the mesenteric lymph nodes (MLNs) and antibody levels in the sera of mice were compared after the oral administration of Lc3 and Lp4.

4.2 Materials and Methods

4.2.1 Cultivation of LAB

L. curvatus Lc3 and *L. plantarum* Lp4 were cultured in MRS-glucose and MRS-sucrose at 15 and 30 °C for 5 d, and the growth was monitored by measuring turbidity at 660 nm. To obtain Lc3 cells of log or stationary phases, they were cultured in MRS-glucose at 15 °C for 35 or 96 hrs, and at 30 °C for 8 or 24 h, respectively. Lp4 was cultured at 15 °C for 45 or 96 h, and at 30 °C for 9 or 24 h, to obtain cells of log or stationary phases, respectively. Cells were collected by centrifugation at 1500×*g* for 15 min, washed with MilliQ water and heated at 65 ^{o} C for 30 min. Heat-killed cells were lyophilized, and then stored at -30°C.

4.2.2 *In vitro* immunomodulation assay

Male BALB/c mice (CLEA Japan, Tokyo, Japan) were housed at 23±2°C with a 12-h light/dark cycle. All experimental procedures were conducted in accordance with the Regulations for Animal Experimentation of Shinshu University, Japan. The preparation of spleen cells involved a treatment with 0.17 M Tris-HCl buffer (pH 7.65) containing 0.83% NH_4Cl to deplete red blood cells. After centrifugation, cells were resuspended in RPMI-1640 medium containing 10% (v/v) fetal calf serum, 100 U mL^{-1} penicillin G, and 100 μg mL^{-1} streptomycin. Cells (5×10^5 cells per well) were cultured in 96-well flat-bottomed plates in the presence of heat-treated LAB cells at 37^{o}C for 48 h in an atmosphere containing 5% CO_2. IFN-γ and IL-10 in culture supernatants were measured using ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

4.2.3 *In vivo* immunomodulation assay

Female BALB/c mice (5 wk-old) were purchased from Japan Charles River (Yokohama, Japan). They were acclimated for 1 wk by feeding an AIN93M composition diet and were randomly divided into 5 groups. The experimental procedure for immunization with OVA and administration of heat-inactivated lactobacilli strains is summarized in Fig. 4.1. On days 0 and 14, mice (n=5 to 6 per group) were injected intraperitoneally with 50 μg of OVA and 50 μL of Inject Alum (Thermo Fisher Scientific, Tokyo, Japan) in a total volume of 0.1 mL. The mice were orally administered 1 mg (3×10^6 cells) /mouse/day of heat-inactivated Lc3 and Lp4 cells which were cultured at 15 °C or 30 °C throughout the experimental period. On day 28, mice were fasted for 12 h, anaesthetized with inhalation of isoflurane, and exsanguinated by cardiac puncture. Mesenteric lymph nodes (MLNs) were harvested.

This animal experiment was approved by the Animal Use Committee of Food Research Institute, National Agriculture and Food Research Organization, and all mice were maintained in accordance with the guidelines for the care and use of laboratory animals of the research organization (approval no. H31-011).

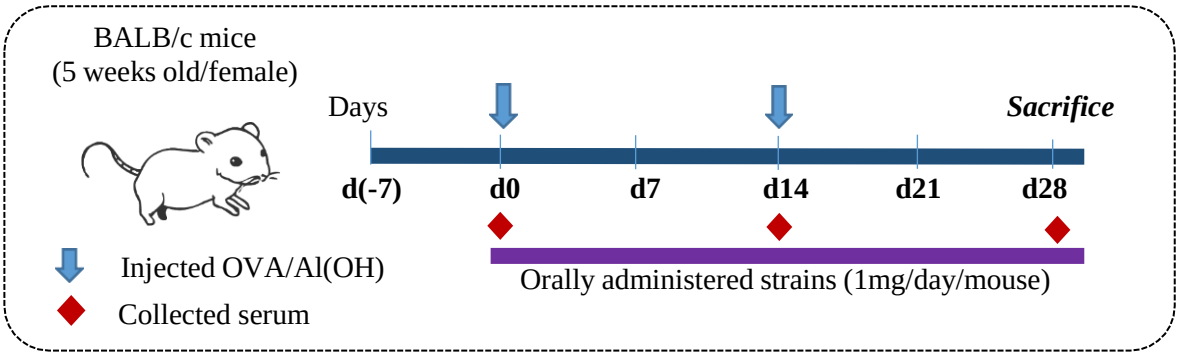

Fig. 4.1. The experimental schedule for immunization with OVA and administration of heat-inactivated Lactobacilli.
Mice were divided into 5 groups as control (1), Lc3-30ºC (2), Lp4-30ºC (3), Lc3-15ºC (4) and Lp4-15ºC (5). Mice were orally treated with bacterial cells, sensitized with OVA, and sacrificed on day 28.

4.2.4 OVA-specific antibody levels in the sera

Determination of antibody IgE, IgG1 and IgG2a levels in the sera were performed by sandwich ELISA using commercial ELISA kits (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's instructions with modifications. Sera of BALB/c mice which were immunized with OVA were kindly provided from Dr. Masao Goto at Food Research Institute, NARO, and used to draw standard curve of each subclass of OVA-specific antibodies. The results were expressed as units/mL of serum.

4.2.5 Quantitative reverse-transcription (RT) PCR

Total RNA was isolated from mouse MLNs using Tryzol reagent (Thermo Fisher Scientific) and cleaned with RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total RNA was reverse transcribed into complementary DNA using ReverTra Ace (Toyobo, Osaka, Japan) and oligo (dT) primers (TakaraBio, Otsu, Japan). The cDNA was used for quantitative PCR using a KAPA SYBR Fast qPCR Master Mix (KAPA Biosystems, Wilmington, MA, USA). The relative quantification of expression levels of IFN-γ, IL-10 and forkhead/winged-helix transcription factor box protein 3 (Foxp3) was calculated by $^{\Delta\Delta}$Ct method with normalization to ribosomal protein S18 (T. Ogita, Y. Tanii, H. Morita and S. Tanabe, *Int. J. Mol. Med.*, 2011, 28, 817–822).

4.2.6 Statistical analysis

Data are shown as mean ± standard deviation (SD). Statistical analyses were performed using a one-way ANOVA followed by Tukey's test for group comparisons. $P<0.05$ was considered to be significant.

4.3 Results

4.3.1 Growth of Lc3 and Lp4 cells

Lc3 and Lp4 were cultured in MRS-glucose or MRS-sucrose at 15°C and 30°C. Both strains grew well in both sugars, and Lc3 grew faster than Lp4 at both of the temperatures (Fig. 4.1).

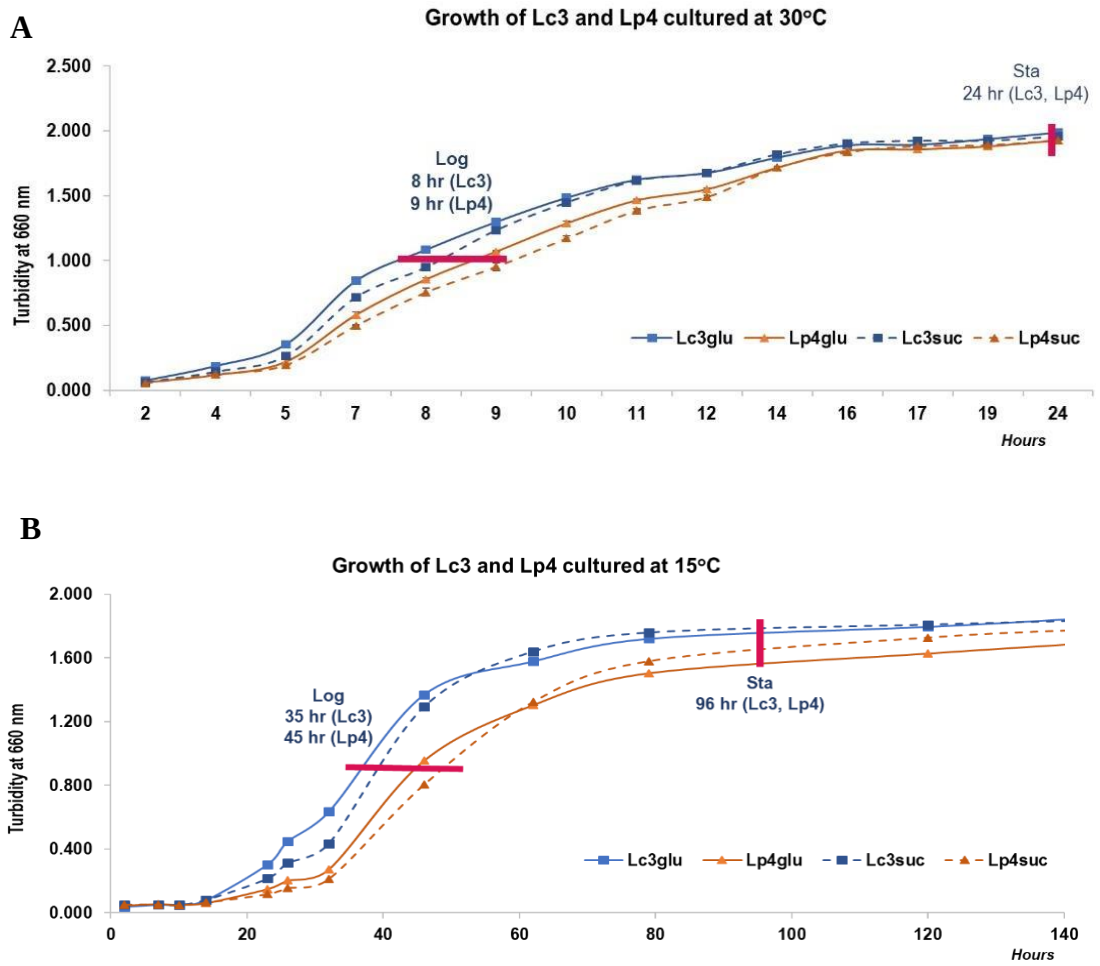

Fig 4.1. Culture condition and growth of Lc3 and Lp4 cells.
Lp4 and Lc3 were cultured in glucose or sucrose containing-MRS at 30 °C (A) or 15 °C (B). Bacteria growth was measured by measuring turbidity at 660 nm. The test was duplicated.

4.3.2 Effect of culture conditions on immunomodulatory activities of Lc3 and Lp4

We examined the effect of the culture phases, carbon sources and culture temperatures on immunomodulatory activities of Lc3 and Lp4 cells. Spleen cells of BLAB/c mice were stimulated with 2 strains of bacterial cells which were cultured at different conditions. Effects of carbon sources in the media and growth phases on IFN-γ and IL-10 productions by spleen cells were not obvious in both strain (Fig. 4.2). Contrary, both strains cultured at 15 °C induced more IFN-γ and IL-10 productions, when compared to those cultured at 30 °C.

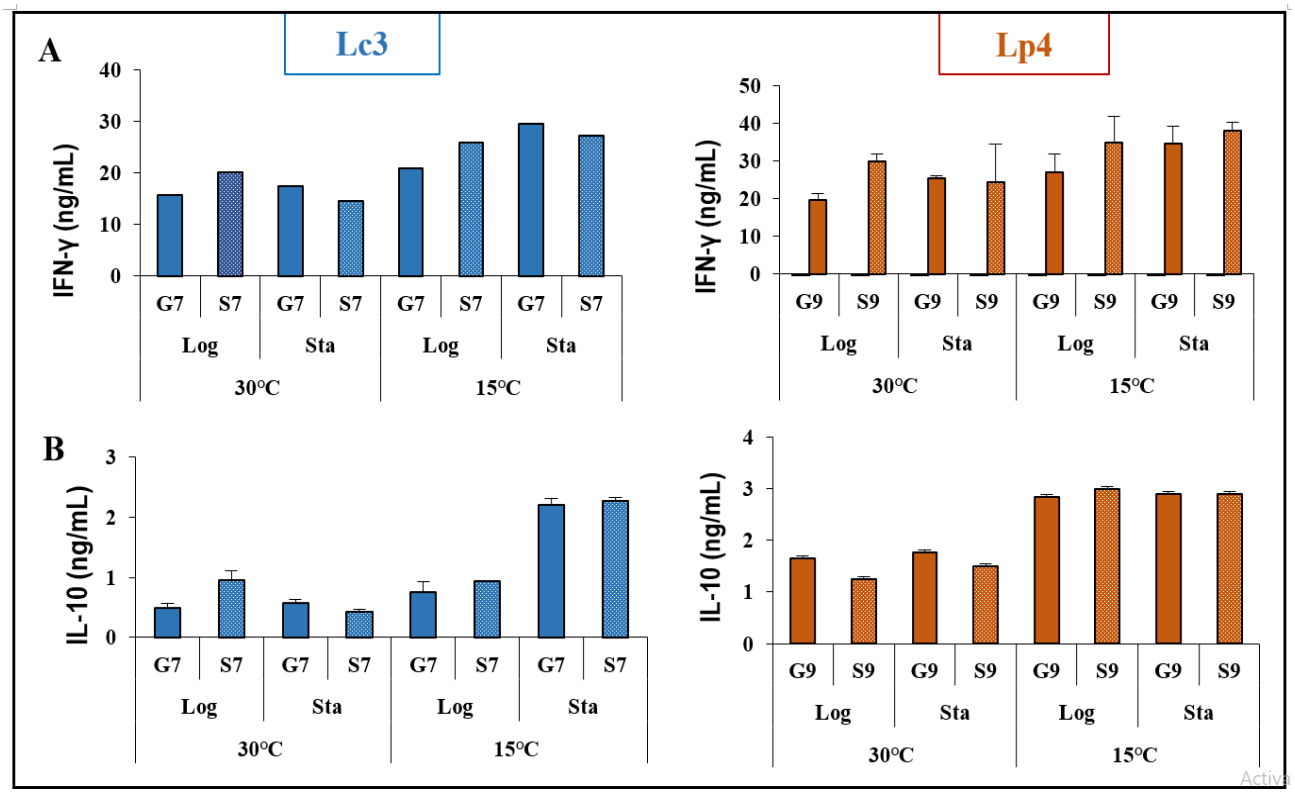

Fig.4.2. Cytokine inducing activity of Lc3 and Lp4 in different cultured condition.
Lc3 and Lp4 were cultured with glucose or sucrose and grew at 15 °C for 96 h or 30 °C for 24 h. Heat-inactivated cells (1μg/ml) were co-cultured with mouse spleen cells (5×10^5 cells per well) to measure IFN-γ (A) and IL-10 (B) levels. After 48 h, culture supernatants were collected, and IFN-γ and IL-10 levels were analyzed using ELISA. Bars show the mean of three independent experiments.

4.3.3 OVA-specific antibody levels in the sera of mice

Orally administered Lc3 and Lp4 did not show significant changes in OVA-specific IgE, IgG1 and IgG2a levels in the sera of mice compared with control group, irrespective of culture temperatures (Fig. 4.3).

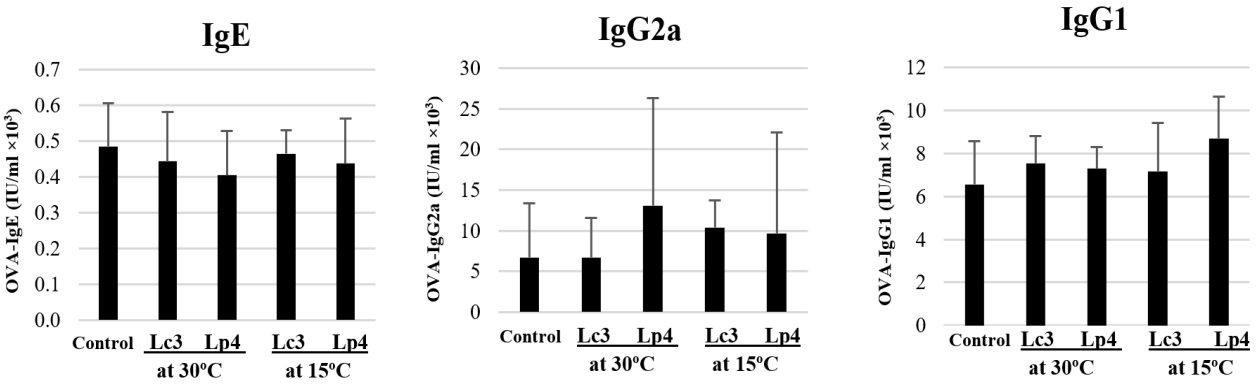

Fig.4.3. OVA-specific IgE, IgG1 and IgG2a in sera from the administrated of Lc3 and Lp4.
Mice in all groups were immunized with OVA. The control group received MilliQ water while the other groups received Lc3 and Lp4, which were cultured at 30 °C and 15 °C. Values are shown as means ± SEM (n=5-6).

4.3.4 Gene expressions in MLNs of mice

Orally administered Lp4 which was cultured at 30 °C tended to upregulate IFN-γ, IL-10 and Foxp3 expressions in the MLNs, and those cultured at 15 °C significantly upregulated these genes. On the other hand, significant changes in the levels of IFN-γ and IL-10 in the MLNs were not observed in the mice treated with Lc3 irrespective to culture temperatures (Fig. 4.4)

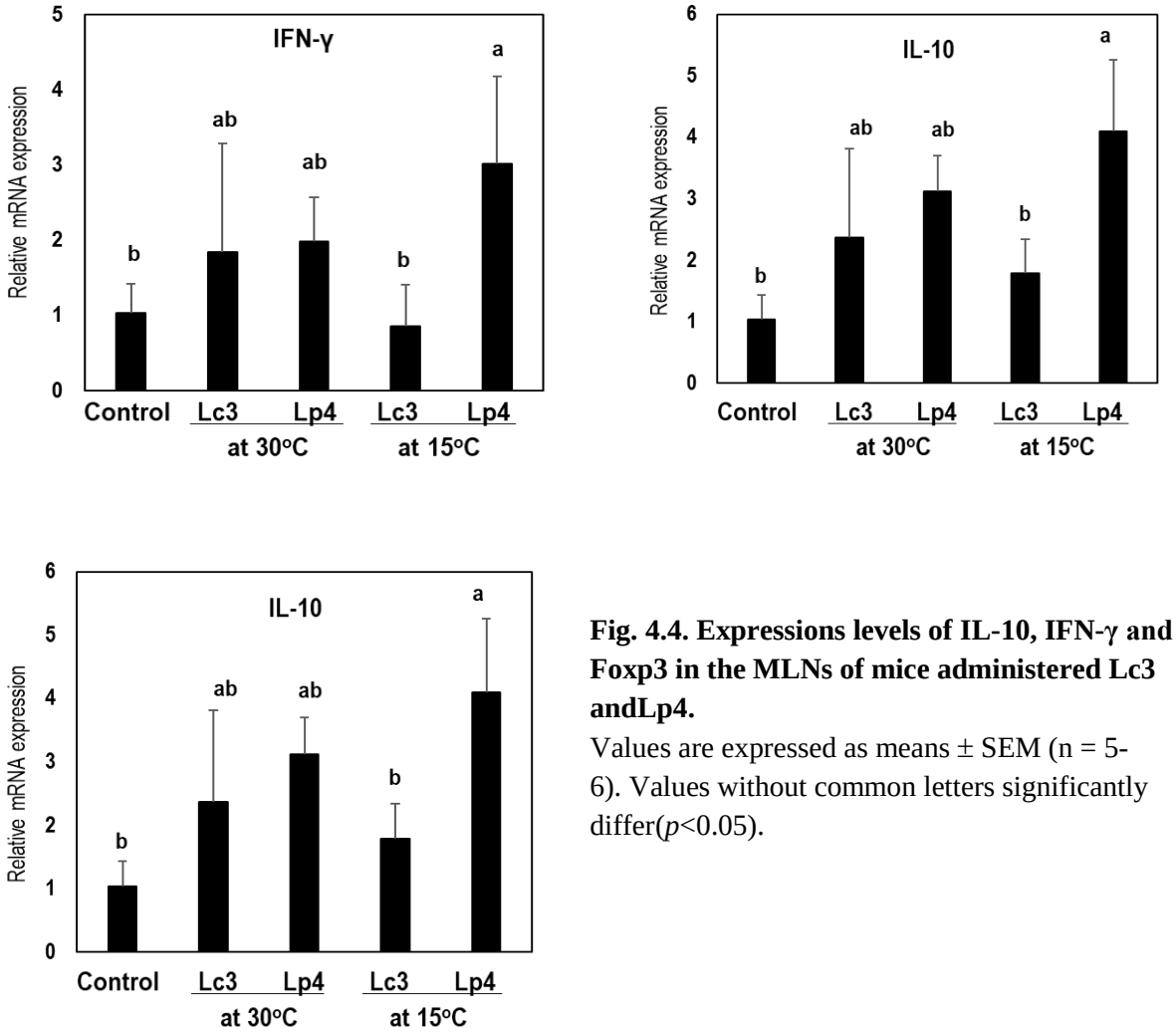

Fig. 4.4. Expressions levels of IL-10, IFN-γ and Foxp3 in the MLNs of mice administered Lc3 andLp4.

Values are expressed as means ± SEM (n = 5-6). Values without common letters significantly differ(p<0.05).

4.4 Discussion

Physiological functions, including immunomodulatory activity, of LAB are recognized strain specific. In chapter 3, *L. curvatus* Lc3 and *L. plantarum* Lp4 were selected based on their high IL-10 and IFN-γ production activities among the LAB isolates from fermented *B. rapa* L. It has been reported that immunomodulatory activities of LAB are altered by culture conditions (Sashihara *et al.*, 2007; Toshimitsu *et al.*, 2017; Haller, Bode and Hammes, 1999). Toshimitsu *et al*. (2017) showed that the logarithmic phase of *L. plantarum* strains MEP222801 and OLL2712 induced bone marrow-derived dendritic cells (BMDCs) to secrete an increased IL-10 than those in stationary phase. In contrast, Sashihara *et al*. (2007) showed that stationary phase of *L. gasseri* OLL2809 stimulated the mouse splenocytes to secrete a greater amount of IL-12 than the cells in the logarithmic phase. Maassen *et al*. (2003) reported that degradation or chemical change in the composition of pro-inflammatory substances such as peptidoglycan and lipoteichoic acid could explain the reason for these observations. From these backgrounds, we hypothesized that culture conditions alter immunomodulatory activities of Lp4 and Lc3. In addition, Lp4 and Lc3 were cultured in an optimized condition, and the heat-treated cells were administered to mice to evaluate the immunomodulatory activities *in vivo*.

It has been reported that the optimal growth temperature for *Lactobacillus* is between 30°C and 40°C, but it depends on the species. They can grow at temperatures a lower as 5°C and upper limit as 53°C. The growth rate of LAB depends on the strains (Ahmed, Kanwal and Ayub, 2006). Both Lc3 and Lp4 grew at 15°C and at 30°C and the growth of Lc3 was faster than that of Lp4 at both temperatures (Fig. 4.1). Both strains utilized glucose and sucrose, and their growths were similar between the carbon sources (Fig. 4.1). Interestingly, increased IFN-γ and IL-10 productions by mouse spleen cells were observed in Lc3 and Lp4 cells which were cultured at 15°C when compare to those cultured at 30°C (Fig. 4.2). Temperature downshift induces physiological changes in bacterial cells, such as a decrease in membrane fluidity and stabilization of the secondary structures of RNA and DNA, resulting in reduced efficiency of translation, transcription and DNA replication (Van de Guchte *et al.*, 2002). Adequate membrane fluidity is essential for the mobility and functionality of embedded proteins and lipids, the diffusion of proteins and other molecules across the membrane and the proper separation of membranes during cell division (Haddaji *et al.*, 2015). Several studies have been reported that some LAB strains grown at low temperatures exhibited a greater bacteriocin effect compared to the optimal culture condition (Mataragas *et al.*, 2003). As far as I know, there is no adequate studies which report effect of low temperature culture conditions on immunomodulatory activities. It has been

reported that bacterial cell wall components, such as peptidoglycan and lipoteichoic acid, as well as nucleotide fragments with specific motifs are responsible for immunomodulatory activities of LAB (Cleveland *et al.*, 1996; Grangette *et al.*, 2005). In addition, environmental stresses such as oxygen, temperature and pH activate stress responses and change the composition of their cell membrane in order to maintain the adequate membrane fluidity (Haddaji *et al.*, 2015). Thus, cultivation at lower temperature possibly alters the composition of bacterial cell wall with resulting increased immunomodulation activities of Lc3 and Lp4.

BALB/c inbred mice reportedly show Th2 skewed immune reactions, on the other hand, those of C57BL/6 mice are Th1-skewed (Watanabe *et al.*, 2004). We expected anti-allergic activities of Lc3 and Lp4, because those stimulated spleen cells to produce IFN-γ and IL-10 which could suppress allergic reactions (Chung, 2001c). For *in vitro* tests in this chapter, we used spleen cells from BALB/c mice, since those have been commonly used as a model for allergy. However, Lc3 and Lp4 cultured at 15°C induced increased IFN-γ and IL-10 productions by spleen cells not only from BALB/c mice but also from C57BL/6 mice (data not shown), suggesting increased immunomodulatory activities by culturing at lower temperature is not likely only on BALB/c mice.

BALB/c mice were orally administered heat inactivated Lc3 or Lp4 and then were immunized with OVA. Immunomodulatory activities of Lc3 and Lp4 *in vivo* were evaluated by measuring expression of cytokines in MLNs and OVA-specific antibody levels in the sera. MLNs are important immune organ in which antigen and intestinal microbes are captured and presented to T cells, and following activation and proliferation of antigen-specific lymphocytes, and the development of memory cells occurred (Macpherson and Smith, 2006). Lp4 tended to upregulate IFN-γ, IL-10 and Foxp3 in the MLNs of immunized mice, and this upregulation was potently observed in mice fed Lp4 cultured at 15°C (Fig. 4.4). This trend in upregulation was consistent with observations in *in vitro* tests (Fig. 4.2). Foxp3 has been considered an immunophenotype marker of T regulatory cells and suggested a potential protective role for Foxp3 and IL-10 in food allergies (Krogulska *et al.*, 2011). Ai *et al*, (2016) showed that the probiotic strains *L. plantarum* CCFM47 and *L. casei* Lc2w increased IL-10 and IFN-γ production in splenocytes from BLAB/c mice *in vitro*, and that oral administration of these LABs increased Foxp3 expression in MLNs (Ai *et al.*, 2016). Thus, upregulation of IL-10 in the MLNs are possibly induced by upregulated Foxp3. In contrast, Lc3 failed to alter expressions of IFN-γ, IL-10 and Foxp3 in the MLNs irrespective of the culture temperatures (Fig. 4.4). Although reasons for such functional differences between strains are unclear, structure of Lp4 is possibly enough resisted

to atmospheres of gastrointestinal tract, such as low pH and exposure to bile acids and enzymatic hydrolysis, to show the immunomodulatory activities in MLNs.

Antigen-specific IgE antibody has been considered as an indicator for the clinical diagnosis of type I allergic diseases (Baldacci, Omenaas and Oryszczyn, 2001). In addition, Th1 and Th2 mainly contribute productions of antigen specific IgG1 and IgG2a, respectively. *L. plantarum* strain YU reportedly induces IFN-γ and suppresses IgE production in OVA-immunized mice (Kawashima *et al.*, 2011b). However, no significant alterations in OVA-specific antibody levels in the sera were induced by the administration of Lc3 or Lp4, although Lp4 upregulated IFN-γ, IL-10 and Foxp3 in the MLNs. The reasons for these contradictory observations are unclear. Kumagai *et al*., (2013) demonstrated that *L. sakei* K244 and *L. paracasei* K122 showed greatly increased Th1 responses and suppressed Th2 responses *in vivo*, however, these were less effective on reduction of antigen-specific IgE in the sera (Kumagai *et al.*, 2013). Alleviation of food allergy by probiotic *B. longum* KACC91563 does not accompany suppressed production of IgE or Th2 cytokines, but accompanies suppressed degranulation of mast cells and increased apoptosis of mast cells (Kim *et al.*, 2016). In addition, several human studies have reported that oral administration of probiotics such as *L. salivarius* did not change antigen-specific IgE, but ameliorated allergic symptoms on nose and eyes, and medication score (Lin *et al.*, 2013). Similarly, *L. rhamnosus* GG and *L. gasseri* TMC0356 improved nasal blockage and medication score without decreasing total and antigen-specific IgE in the sera (Kawase *et al.*, 2009). These previous reports suggest that reduction of serum antigen-specific antibodies is not always indispensable for the anti-allergic effects of probiotic LAB. A well-studied probiotic like L. acidophilus - 92 as an anti-allergic strain was significantly suppressed OVA-specific serum IgE levels over a long period of time when administered with high doses compared to low doses (Torii *et al.*, 2007). Therefore, further animal experiments would be necessary to unveil the anti-allergic effect of Lp4 compared with well-studied strains as a positive control to know the effect of our isolates.

CHAPTER 5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 General discussion

LAB are considered to be health-promoting microorganisms, and some strains are known as probiotics which are found in many types of fermented foods (Swain *et al.*, 2014), as well as in the intestines of most mammals (Ohland and MacNaughton, 2010). Numerous studies have reported that ingestion of fermented foods containing specific LAB strains can prevent many kinds of diseases including chemically induced colitis, asthma and allergic rhinitis. This beneficial effect on the modulation of immune system is, at least partly, due to their ability to regulate cytokine production and control Th1/Th2 balance. However, the immunomodulatory properties of LAB differ among the strains. Therefore, it is important to investigate an effective LAB strain capable for modulating the immune response from suitable sources.

The main objectives of this study are to isolate LAB strains which have high potential immunomodulatory effects from fermented *B. rapa* L., a traditional Japanese pickle, and to clarify the immunomodulatory functions in *in vitro* and *in vivo*. To accomplish these objectives, following three aspects needed to be addressed; i) investigating immunomodulatory effects of fermented *B. rapa* L. and clarifying responsible LAB, ii) isolation of LAB strains from appropriate resources and evaluation of their immunomodulatory effects, iii) examining immunomodulatory effects of selected LAB strains using an animal model. I have focused on IFN-γ and IL-10 production by the stimulation of fermented *B. rapa* L. and isolated LBS *in vitro* and *in vivo*, because of their possible contributions to the suppression of allergic symptoms.

In chapter 2, we prepared naturally fermented *B. rapa* L., and changes in microbiota composition were determined by molecular biological technics. *Lactobacillus* became the most dominate genus on day 7 of the fermentation or later, and *L. curvatus* and *L. plantarum* were contributed to the fermentation of *B. rapa* L. Mouse spleen cells were treated with heat-treated fermented vegetable samples, and IFN-γ and IL-10 productions were measured. Increased IFN-γ and IL-10 productions were induced by the fermentation, suggesting contribution of lactobacilli to increased cytokine production activities of *B. rapa* L. The numbers of *L. curvatus* and *L. plantarum* were positively correlated with IFN-γ and IL-10 levels. These findings suggest that lactobacilli, especially *L. curvarus* and *L. plantarum*, are likely to contribute the cytokine production activities of fermented *B. rapa* L.

We isolated 46 strains of LAB from *B. rapa* L. which was fermented for 28 d, because microbiotas with increased numbers of lactobacilli were stably established in longer fermented *B. rapa* L. In chapter 3, IFN-γ and IL-10 induction activities of 46 LAB isolates were measured.

Among these, *L. plantarum* strain Lp4 induced the second-highest level of IFN-γ, and *L. curvatus* strain Lc3 induced the highest level of IL-10. Thus, we selected these strains for further research. To clarify the applicabilities of Lc3 and Lp4 on the addition of immunomodulatory functions to *B. rapa* L., these strains were used as starter cultures for the fermentation. The addition of starter cultures appears to have induced an early increase in the number of lactobacilli, leading to enhanced cytokine production of fermented *B. rapa* L.

In chapter 4, we firstly tried to enhance cytokine production activities of Lc3 and Lp4 by changing the culture conditions. Both isolates cultured at 15°C induced more cytokines by mouse spleen cells, when compared to those cultured at 30°C. We prepared naturally and starter fermented *B. rapa* L. by inoculating at 10°C (Chapters 2 and 3). In addition, hon-zuke type Nozawana is generally produced in winter to prevent growth of unfavorable bacteria. Thus, fermentation at low temperature would be advantageous to obtain fermented *B. rapa* L. possessing immunomodulatory activities.

In vivo immunomodulatory activities of Lc3 and Lp4 were also evaluated in chapter 4. Lp4, but not Lc3, tended to upregulate IFN-γ and IL-10 expressions in the MLNs of OVA-immunized mice, and the upregulation was potent in mice fed Lp4 which was cultured at 15 °C. This suggests that culture temperature affects immunomodulatory activities of Lp4 *in vivo*. Upregulation of IL-10 accompanied upregulation of Foxp3 in the MLNs, suggesting induction of Treg cells by the administration of Lp4. Neither Lc3 nor Lp4 induced alterations in the serum antibody levels in the mice. Anti-allergic effects of probiotic LAB do not necessarily accompany alterations of serum antibody levels (Kawase *et al.*, 2009; Kim *et al.*, 2016). Further animal experiments using animal models for allergy would be necessary to unveil anti-allergic effects of Lp4.

All these observations suggest that Lp4 isolate from *B. rapa* L. could modulate immune responses by inducing IFN-γ and IL-10. In addition, Lp4 can be used as a starter culture for the fermentation of *B. rapa* L. Thus, I believe that Lp4 can be applicable to produce *B rapa* L. possessing immunomodulatory activities. Although cultivation of Lp4 at low temperature contributed to increase the cytokine induction activities, the mechanisms on this enhancement is unclear. A series of studies to clarify the mechanisms would contribute to the addition of scientific knowledge on the immunomodulatory activities of LAB.

5.2 Concluding remarks and further work

This work demonstrates that the *in vitro* and *in vivo* study of *L. plantarum* strain Lp4 isolated from fermented *B. rapa* L. has the potential for immunomodulation effect.

However, we are posed with several questions unanswered which are:

1. Several strains of Lactobacillus, including *L. casei* strain Shirota, *L. plantarum* strain L-137 and *L. acidophilus* strain L-92 have been reported as probiotics that modify antigen-specific IgE levels and anti-allergic effects in vitro and in vivo test. However, those suggested strains showed anti-allergic effects depending on their dose or period. Therefore, it is necessary to test Lp4 with one of these proposed strains both in vitro and in vivo tests in order to know anti-allergic effectiveness.
2. It is also necessary to clarify mechanisms behind increased production of IL-10 and IFN-γ of LAB cultured at low-temperature.
3. It is also not clear that the component of B rapa L. may affect increasing the number of LAB, especially our isolated strain only specifically in fermented B rapa L or not.
4. It is challenging to explain the underlying mechanism by which way absorb and metabolite our strain in the digestive system also necessary to clarify.
5. There are similar effective strains like Lp4 already applied as a supplement or food additives and starter cultures. Therefore, Lp4 can be applicable to produce *B. rapa* L. possessing immunomodulatory activity.

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