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Abstract of thesis

Lactic acid bacteria (LAB) 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 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 the research was to clarify the immunomodulatory effects of LAB strains isolated from fermented *B. rapa* L. by *in vitro* and *in vivo* tests. The author 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.

In chapter 2 of the thesis, the author 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, the author suggested contribution of these*Lactobacillus* to the cytokine production activities of fermented *B. rapa* L.

In chapter 3, the authorevaluatedimmunomodulatory activities of 46 strains of LAB from *B. rapa* L.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 compare to naturally fermented vegetable. The numbers of *Lactobacillus* drastically increased by the inoculation of these strains comparedwith naturally fermented *B. rapa*L. Thus, the author suggested applicability of these lactobacilli as starter cultures to produce fermented *B. rapa* L. with immunomodulatory activities.

In chapter 4, the authortried to optimize culture conditions of Lp4 and Lc3 to enhance their immunomodulatory activities, and *in vivo* evaluation was also conducted. 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 and 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. The author successfully showed immunomodulatory activity of Lp4 both *in vitro* and *in vivo*, especially when the isolate was cultured at low temperature condition.

In conclusion, the author 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. The author suggested that fermented *B. rapa*L. possessing immunomodulatory activities could be produced by fermentation at low temperature with the addition of Lp4 as starter culture.

Abstract of assessment result

【Review】

The author aimed to isolate promising LAB strains from fermented *Brassica rapa*L. with particular interest in their enhancing capability on IFN-γ and IL-10 production, expecting their anti-allergic effects. Among the dominant LAB the author found in fermented *Brassica rapa*L, *L. curvatus* and *L. plantarum* (heat treated) induced the productions of IFN-γ and IL-10 by cultured spleen cells. The author suggested contribution of these*Lactobacillus* to the cytokine production activities of fermented *B. rapa* L. The authorevaluated immunomodulatory activities of 46 strains of LAB isolated from *B. rapa* L.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. IFN-γ and IL-10 production activities of fermented *B. rapa*L. were enhanced by the addition of Lc3 and Lp4 as starter cultures. IFN-γ and IL-10 were upregulated in the MLNs of mice by the oral administration of Lp4 cultured at low temperature. From these results, the author provides practical insights into the use of these strains as novel food ingredients for anti-allergy in addition to their use for adding new values to fermented *Brassica rapa*L.

【Result】

The final examination committee conducted a meeting as a final examination on January 8th, 2020. The applicant provided an overview of dissertation, addressed questions and comments raised during Q&A session. All of the committee members reached a final decision that the applicant has passed the final examination.

【Conclusion】

Therefore, the final examination committee approved that the applicant is qualified to be awarded Doctor of Philosophy in Food Innovation.