

Immuno-modulation by heat-killed *Lacticaseibacillus paracasei* MCC1849 and its application to food products

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Abstract

Probiotics are microorganisms that confer health benefits to host. Well-known examples include *Bifidobacterium* and *Lactobacillus* species. In recent years, interest in promoting our health with probiotics has grown as life expectancy and health awareness has increased. However, some concerns for safety and stability exist for these live organisms. Thus, “postbiotics” and “paraprobiotics,” non-viable heat-killed microbial cells or cell fractions that retain health benefits, are increasingly favored. Unfortunately, little information on clinical efficacy and mechanisms of action is available compared with many available probiotics. *Lacticaseibacillus* (previous name *Lactobacillus*) *paracasei* MCC1849 is a commonly used lactic acid bacterial strain in Japan that displays immuno-modulatory effects in humans in non-viable heat-killed form. This review discusses health benefits of heat-killed *L. paracasei* MCC1849 immune modulation and offers a theoretical basis for its mechanisms of action. We also discuss the feasibility of using heat-killed probiotics for application in food products.

Keywords

food application, heat-killed probiotics, immuno-modulation, *Lacticaseibacillus paracasei* MCC1849

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Introduction

Gut microbiota influences essential human functions, including digestion, energy metabolism, and inflammation by modulating multiple endocrine, neural, and immune host pathways.¹ Many studies show that dysbiosis of gut microbiota is closely related to diseases, such as inflammatory bowel disease, allergy, and obesity.² Modulating gut microbiota composition through dietary intervention may thus become more popular as a way to maintain general health.³

Probiotics are defined as “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host.”⁴ Probiotic bacteria are found mainly in genera *Lactobacillus* and *Bifidobacterium*. Probiotics have been incorporated

in various products such as milk-based drink, fermented milks, infant formula, and nutritional supplements. Probiotic supplementation may provide physiological benefits to hosts such as balancing and restoration of the gut microbiota,⁵ immuno-modulation,⁶ prevention of infection,⁷ antimicrobial effects,⁸ anti-obesity influences,⁹ improvement of hypercholesterolemics,^{10,11} and improved central nervous system function.¹² Probiotic use still has

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issues despite demonstrating excellent physiological benefits. For example, probiotics have restrictions on commercialization and application because they are live strains.¹³ Furthermore, risks associated with the use of live strains, such as bacterial translocation, should be considered when administering probiotics to high-risk groups, such as elderly and acute-stage patients with reduced immunity.¹³ “Postbiotics” and “paraprobiotics” have attracted attention as possible solutions to such problems. Postbiotics are soluble factors (products or metabolic byproducts) secreted by live bacteria or released after bacterial lysis.¹⁴ Conversely, paraprobiotics are inactivated (non-viable) microbial intact cells, which when administered in sufficient amounts confer benefits to the consumer.¹⁵ *In vitro* and *in vivo* studies were reported that some postbiotics and paraprobiotics exhibit the physiological benefits.¹⁶ For example, oral intake of heat-killed *Lactobacillus gasseri* TMC0356 daily for 4 weeks by elderly subjects was reported to enhance natural defense mechanisms against pathogenic infections.¹⁷ Furthermore, inactivated *Bifidobacterium longum* BR-108 administration to obese diabetic mice was reported a significant decrease of body weight gain, adipose tissue mass, and blood glucose levels, as well as a significant reduction in blood glucose after a 5 weeks treatment.¹⁸

Various health benefits of heat-killed probiotic strains have been reported,^{17,19–23} but immuno-modulation has gained attention because it may prevent aging- or stress-related attenuation of immunity. This activity lessens the risk of infection in the elderly, leading to extended healthy life expectancy.²⁴ Accumulating studies suggest that multiple bacterial components, such as peptide glycans, nucleic acids (DNA, RNA), lipoteichoic acids, and surface proteins, act in the digestive tract and systemic immune system via pattern recognition. Possible targets include toll-like receptors to regulate the balance of T helper 1 (Th1)/Th2 cells, as well as innate and acquired immunity.¹³ However, choosing suitable strains for specific efficacy is increasingly challenging because of differences in efficacy among strains, mechanisms of action, safety profiles, and origins.²⁵ Many studies suggest that the effects of probiotics, either viable or heat-killed, are highly strain-specific and not equally safe and effective.²⁶ Regarding heat-killed probiotic strains, efficacy and safety with supporting human data and mechanisms of action are often lacking. Therefore, a detailed review of

beneficial properties of specific heat-killed probiotic strains is needed to inform appropriate heat-killed probiotic strain selection.

Heat-killed *Lacticaseibacillus paracasei* MCC 1849 (hereafter designated as heat-killed MCC1849) is a well-established heat-killed probiotic strain with immuno-modulatory properties. The strain has a long history of use in human foods and is reported as safe in single-dose and long-term repeated dose safety trials.²⁷ In this review, we summarize beneficial effects of heat-killed MCC1849 for humans mainly focusing on immuno-modulation, safety, and use in various commercial products.

***Lacticaseibacillus paracasei* MCC1849**

Origin and characteristics

L. paracasei MCC1849 is a probiotic strain isolated from the intestine of a healthy adult. It has been commercially available in Japan since 2014 and is currently used in various kinds of products including yogurt, juice, bread, and soup. MCC1849 is a non-motile, non-spore-forming, rod-shaped anaerobic Gram-positive bacterium. It was identified as *L. paracasei* based on morphological, physiological, and genetic characteristics. MCC1849 is reported to induce high levels of interleukin-12 (IL-12) in a non-viable heat-killed form.²⁸ IL-12 is a cytokine released from antigen-presenting cells that acts on naïve T cells to promote differentiation into Th1 cells²⁹ and follicular helper T (Tfh) precursor cells.³⁰ Th1 cells activate various immune cells, including natural killer (NK) cells and macrophages, that are responsible for innate immunity and removing pathogenic bacteria, viruses, and infected cells.²⁹ Tfh cells play an important role in acquired immunity by promoting B cell maturation, thereby facilitating antigen-specific antibody responses to viral, bacterial, parasite, and fungal infections.³¹ In one experiment, MCC1849 or other bacteria such as *Lactobacillus* and *Lacticaseibacillus* strains were cultured for 16 h at 37°C in Lactobacilli-MRS broth, collected via centrifugation, washed twice with phosphate-buffered saline (PBS), and then washed twice with sterile distilled water. The organisms were suspended in distilled water and were killed by heating them at 100°C for 30 min. A portion of each heated suspension was lyophilized to measure the dry weight of the bacterial cells in the suspension. The pasteurized cells were counted directly under the microscope (Figure 1). These heat-killed microorganisms (10 µg/ml) obtained thereby were

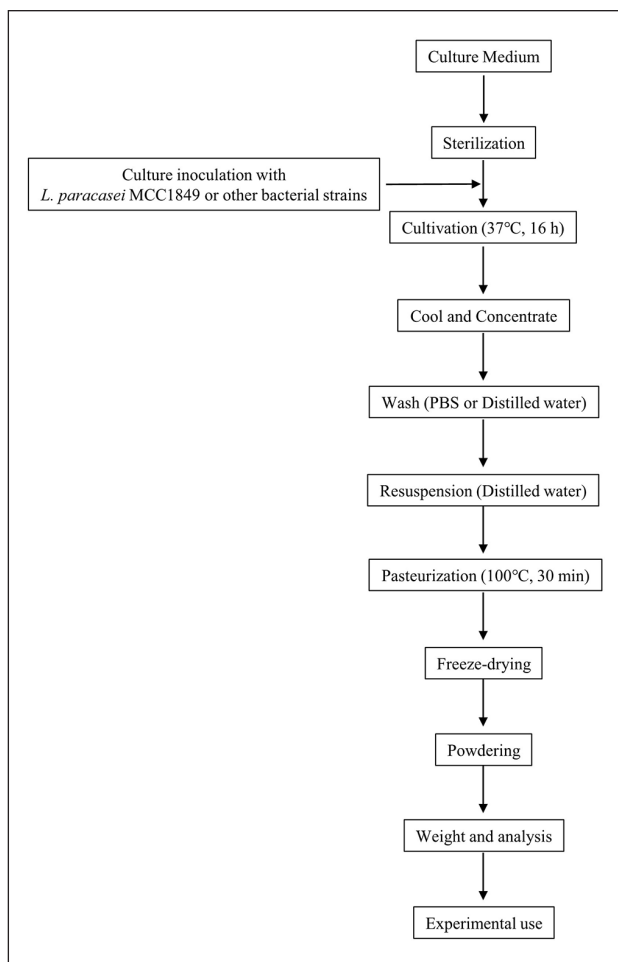


Figure 1. Flowchart of the preparation process for the heat-killed probiotic bacteria.²⁸

added to spleen cells derived from BALB/c mice. A significant increase in IL-12 concentration was observed in culture supernatant 48 h after addition of MCC1849, as compared with other strains,²⁸ indicating immuno-modulatory effects.

Safety

L. paracasei species are granted qualified presumption of safety status by the European Food Safety Authority, with the generic qualification that “the strains should not harbor any acquired antimicrobial resistance genes to clinically relevant antimicrobials,” indicating a lack of safety concerns.³² *L. paracasei* is consumed globally as a component of yogurt, other fermented foods, and various dietary supplements.³²

Studies on acute and subchronic toxicity of viable MCC1849 revealed that both single and repeated oral administration of MCC1849 did not

cause death and any symptoms of toxicity in a rat model.²⁷ In an acute toxicity study, groups of five male and female 6 week-old Crj:CD (SD) rats were orally administered a single dose of 6000 mg/kg (2.21×10^{12} CFU/kg) of MCC1849 and examined for clinical signs of acute toxicity for 14 days. No abnormalities or histopathological findings attributable were found in any organ throughout the test period.²⁷ Furthermore, in a 90 day study, oral administration of MCC1849 with repeated doses (1000 mg/kg; 3.27×10^{11} CFU/kg) to 10 male and female 6 week-old Crj:CD (SD) rats did not cause any toxicologically relevant adverse impacts.²⁷

As mentioned in the introduction, administration of live probiotics to people with weak immunity may be hazardous.³³ In such cases, non-viable heat-killed probiotics could be an alternative since no safety concerns of heat-killed probiotics are reported to date. Heat-killed bacteria might thus display safety advantages over live probiotics.³³ Taken together, non-viable heat-killed *L. paracasei* MCC1849 is likely to be safe for human consumption.

Efficacy of heat-killed *Lacticaseibacillus paracasei* MCC1849

Immuno-modulation

Several interventional clinical studies suggested that heat-killed *L. paracasei* MCC1849 could potentiate immunity or prevent infection. In a randomized, double-blind, placebo-controlled trial, intake of heat-killed MCC1849-containing jelly improved responsiveness of vaccinations in the elderly as compared with the placebo group.³⁴ The study included 45 elderly subjects aged 65 years or older. Participants were randomly divided into two groups: MCC1849 group, provided with jelly containing 1×10^{10} heat-killed MCC1849 cells, and placebo group, provided with the same jelly without MCC1849. They were instructed to consume jelly once a day for 6 weeks from autumn to winter. All participants received influenza vaccination (A/H1N1, A/H2N3, and B) 3 weeks after the start of the experiment. Efficacy of vaccination decreases in elderly populations because of reduced immune function associated with aging.³⁵ Yet, subgroup analysis of subjects over 85 (MCC1849 group: 16, placebo group: 11) showed numbers of blood vaccine antigens significantly higher in the MCC1849 group subjects. Likewise, impaired antibody responses to the A/H1N1 and B antigens were

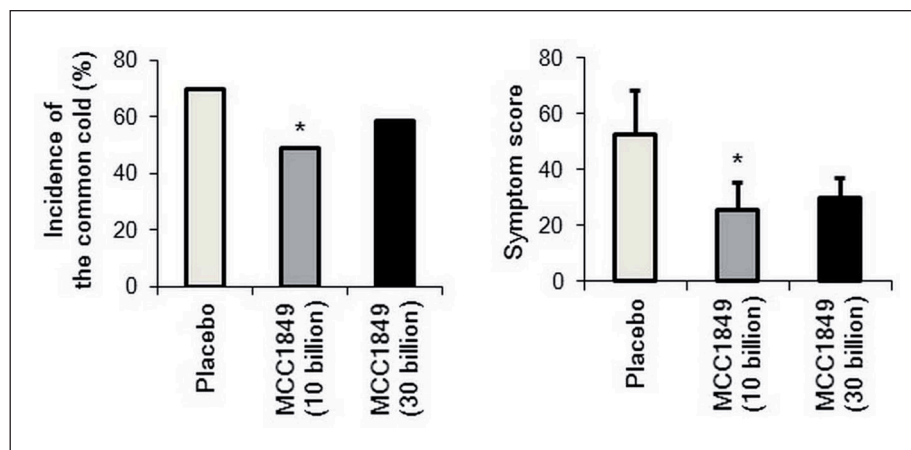


Figure 2. Changes in the common cold incidence and symptom score.³⁷ * $P < 0.05$ versus placebo group by Pearson's chi square test.

improved only in the MCC1849 group.³⁴ MCC1849 intake may affect responsiveness to vaccinations, particularly in populations with reduced immunity.

Another randomized controlled study also indicated an ability of heat-killed MCC1849 (1×10^{10} cells per day) to potentiate immunity in the elderly.³⁶ Sixty-two subjects aged 65 years or older were randomly allocated to an MCC1849 group supplemented with a oral nutritional supplement containing 1×10^{10} heat-killed MCC1849 cells per day and control group supplemented with fruit juice group without MCC1849. MCC1849 group participants exhibited significantly higher combined immunological scores from eight-point immunity subunit scores, including blood T cell number and NK cell number³⁵ after 4 weeks of treatment. Further investigation is needed, however, the nutritional supplement containing MCC1849, may show positive effects on the immune system in the elderly.

Another randomized, placebo-controlled, double-blind comparison study, including healthy young adults, reported that heat-killed MCC1849 was effective in preventing common colds.³⁷ The study involved 241 healthy women over 18 years of age, who were randomly divided into three groups (30 billion group: 3×10^{10} heat-killed MCC1849 cells, 10 billion group: 1×10^{10} heat-killed MCC1849 cells, and placebo group: dextrin) based on the occurrence of common colds in the previous year.³⁷ In the subgroup of individuals susceptible to colds in the previous year ($n = 150$), ingestion of 1×10^{10} MCC1849 cells per day resulted in a significant improvement in the

incidence of the common cold (placebo group: 69.8%, 10 billion group: 49.0%, and 30 billion group: 58.7%) and symptom score (placebo group: 52.8 ± 15.2 , 10 billion group: 25.5 ± 9.7 , and 30 billion group: 29.7 ± 7.1) after 12 weeks of intervention (Figure 2).³⁷ No adverse events associated with the consumption of heat-killed MCC1849 were reported in either study summarized above. Findings serve as a basis for the use of MCC1849 as a safe immune modulator for both the elderly and adults.

Deduced mechanisms of action for immunomodulation

Heat-killed MCC1849 induced significant IL-12, previously reported to induce differentiation from naïve T cells into Tfh precursor cells.³⁰ In Peyer's patches, key players of gut mucosal immune host response toward antigens and bacteria,³⁸ Tfh cells induce differentiation into IgA-positive B cells, and antigen-specific IgA production.^{31,39} IgA plays a major role in preventing initial infection by eliminating and neutralizing foreign pathogens at mucosal immune tissue.⁴⁰

In vivo model, oral supplementation with heat-killed MCC1849 for 5 weeks exhibited potential for enhancing differentiation of B cells into IgA-positive B cells via activation by Tfh cells in Peyer's patches. Control animals were administered with the same diet without heat-killed MCC1849. Antigen stimulation was induced by oral administration of ovalbumin and cholera toxin (Figure 3a).²⁸ Antigen-specific IgA production and intestinal immune cells were

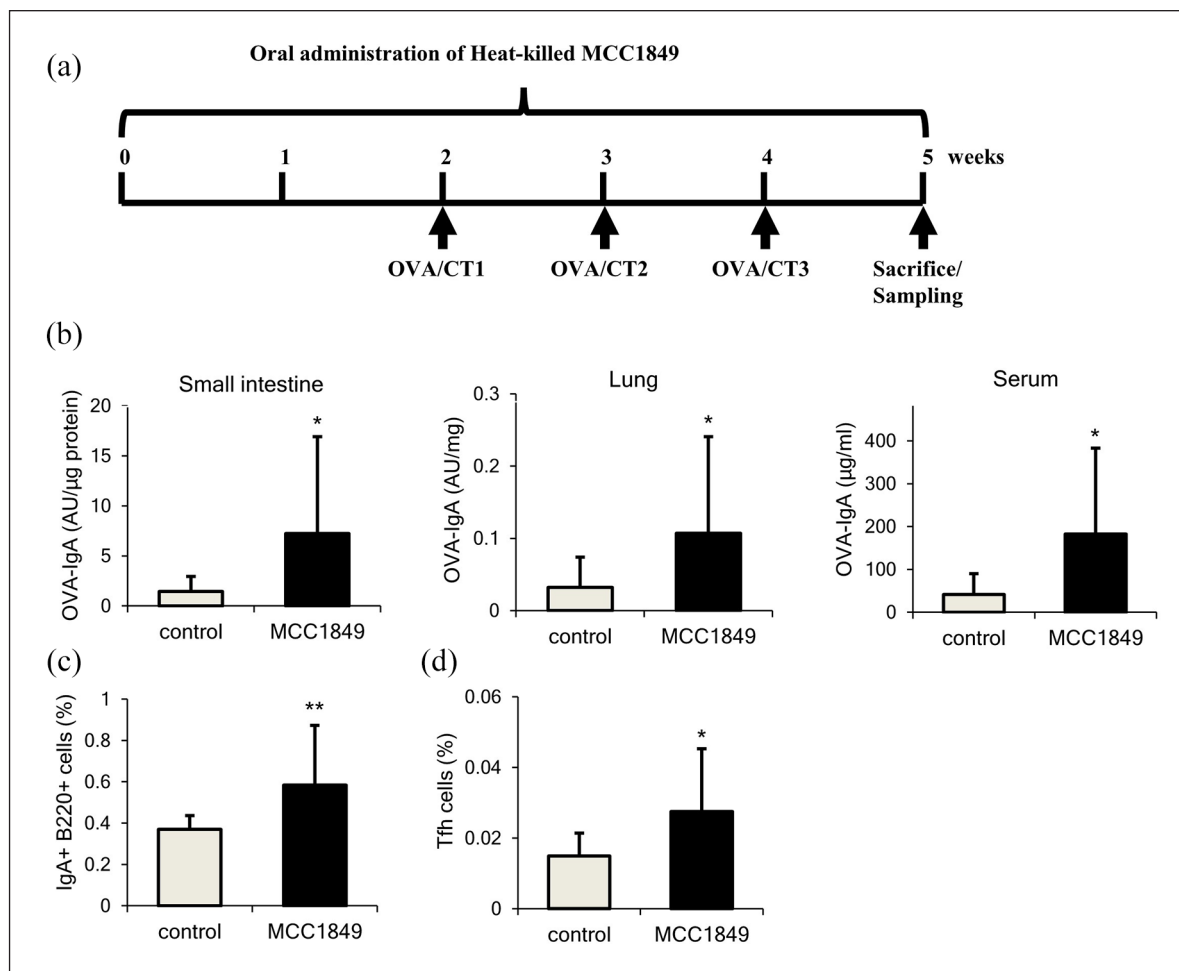


Figure 3. (a) Flowchart of the animal experiment. (b) Antigen-specific IgA amount in small intestine, lungs, and serum after MCC1849 ingestion. (c) Proportion of IgA + B cells. (d) Tfh cells in Peyer's patch after MCC1849 ingestion.²⁸ * $P < 0.05$, ** $P < 0.01$ versus control group by Student's *t*-test.

then evaluated by enzyme-linked immunosorbent assay and flow cytometry analysis. Administration of heat-killed MCC1849 significantly increased antigen-specific IgA in the small intestine, lung, and serum (Figure 3b).²⁸ Heat-killed MCC1849 ingestion also induced a significant increase of IgA-positive B cells in Peyer's patch (Figure 3c).²⁸ Also, the percentage of Tfh cells, which promote differentiation into IgA-positive B cells, was also found to increase significantly in animals receiving heat-killed MCC1849 (Figure 3d).²⁸ Moreover, significant increases in cytokine gene expression, including IL-12 and IL-21 expression, in Peyer's patch cells were observed in MCC1849 group as determined using quantitative polymerase chain reaction (PCR).²⁸ IL-21 is a cytokine secreted from Tfh cells that facilitates differentiation into IgA-positive B cells.⁴¹ Expression of Bcl-6, a transcription factor

highly expressed in Tfh cells, was also found to increase after MCC1849 ingestion.²⁸ These findings suggest that heat-killed MCC1849 intake induces differentiation of IgA-positive B cells and possibly promotes antigen-specific IgA production in the small intestine. Also, MCC1849 increased IgA levels in the serum and lungs, indicating that MCC1849 modulates not only gut mucosal immunity but also respiratory and systemic immunity.

In another *in vivo* model study, administration of heat-killed MCC1849 starting before infection with the influenza virus significantly suppresses pulmonary viral presence 6 days after infection. Thus, MCC1849 exerts an anti-viral effect against influenza in respiratory tract mucosa.²⁸

One potential mechanism underlying heat-killed MCC1849 immuno-modulatory effects is shown in Figure 4. Orally administered heat-killed MCC1849

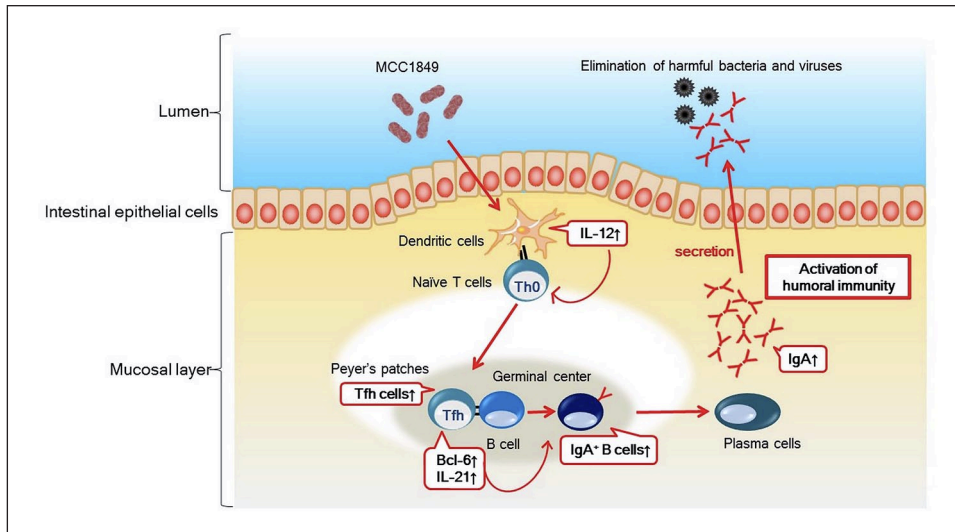


Figure 4. Estimated mechanism of action of immuno-modulatory effect by heat-killed MCC1849.

is recognized by antigen-presenting cells, such as dendritic cells, in Peyer's patches to produce IL-12. In addition to an increased expression of IL-12, expression of Bcl-6 and IL-21 induces the formation of Tfh cells from naïve T cells. Promotion of antigen-specific IgA production in intestinal tissue then occurs by facilitating differentiation into IgA-positive B cells in Peyer's patches. IgA production is likely a mechanism for immuno-modulation exhibited following heat-killed MCC1849 ingestion (Figure 4).²⁸

Impact on quality of life and mood status

Besides immuno-modulatory effects of heat-killed MCC1849, some clinical studies mentioned above showed psychological effects on humans. For example, one randomized controlled study involving elderly subjects reported significant improvement in subjective symptoms associated with quality of life (QOL) after 4 weeks of ingestion. Responses such as "laughing less than before" and "tired for no obvious reason" were significantly reduced in subjects receiving heat-killed MCC1849.³⁶ Moreover, in another randomized, placebo-controlled, double-blind comparison study including healthy young adults under stress due to an upcoming examination, POMS 2 mood profile assessment showed significantly higher scores in "Vigor-Activity" and "Friendliness" at weeks 6 and 12 in subjects receiving MCC1849 (1×10^{10} cells per day) compared with placebo.³⁷ Heat-killed MCC1849 may thus

help maintain QOL and positive mood both in the elderly and adults.

Heat-killed *Lactobacillus gasseri* CP2305, another probiotic strain, ameliorated stress-related symptoms in adults possibly by modulating autonomic nerves via the vagus nerve that connects the intestine and brain.⁴² Modulation of the vagus nerves, as well as regulation of the hypothalamo-pituitary-adrenal axis involved in stress modulation, could be affected by probiotics or their components⁴³; however, no studies that provide mechanistic insights to support psychological effects of heat-killed MCC1849 are currently available. Additional well-designed randomized controlled trials and mechanistic studies are needed to develop conclusive evidence on psychological effects of heat-killed MCC1849.

Application of heat-killed probiotics to food products

Live bacteria are highly susceptible to heat and low pH and require advanced technologies to remain stable for long periods in food products.⁴⁴ This issue places a significant constraint on product composition and manufacturing processing. Addition of live bacteria to products and processes can change the flavor and physical properties of the final products and may shorten the product lifespan.⁴⁵ Conversely, heat-killed probiotics are sterile and can be used in any product regardless of food formulation or product lines. Contamination

risk by live bacteria at the time of manufacturing is low. Furthermore, the likelihood of flavor being affected is extremely low. Therefore paraprobiotic products are more simple and convenient in regard to the industrial handling when compared to the probiotic products.⁴⁶ Heat-killed *L. paracasei* MCC1849 maintains its physiological efficacy in aluminum storage bags as well as in neutral liquid foods, such as dairy drinks and nutritional supplements, across a wide range of temperatures and after prolonged storage.⁴⁷ Heat-killed MCC1849 is used in various popular food products, such as miso soup, soups, confectioneries, tofu, and seasonings, into which live bacteria are traditionally difficult to incorporate.⁴⁷

Despite many advantages, some issues for using heat-killed bacteria remain. One is that no defined procedure for bacterial count of heat-killed probiotics. Measurement methods such as hemocytometer,⁴⁸ quantitative PCR,⁴⁹ and flow cytometry⁴⁸ are being developed. We need to choose a method that is appropriate for each type of product. Another issue is that guidelines and product standards for heat-killed probiotics have not been established. If these problems are solved, more heat-killed probiotics can be expected to be introduced into a variety of foods.

Conclusion

This paper describes heat-killed *L. paracasei* MCC1849 as an immuno-modulator that can be easily and safely consumed daily. Products containing MCC1849 may be an effective tool for maintaining health in an aging society. Heat-killed *L. paracasei* may prevent aging- or stress-related attenuation of immunity and risk of infection in the elderly and lead to extended healthy life expectancy.²⁴ Psychological effects of heat-killed MCC1849 are also reported, including improvement of mood state and QOL.^{36,37}

Despite promising results, clinical studies summarized here have limitations in insufficient sample size and target populations. Furthermore, the mechanism of action for immuno-modulatory properties of heat-killed MCC1849 may involve Tfh cell induction and the promotion of IgA production in Peyer's patches²⁷; however, mechanistic insights are not robust. Therefore, additional well-designed randomized controlled trials with larger sample size and further mechanistic studies on

active ingredients in heat-killed MCC1849 and the molecular basis of induction of Tfh differentiation are needed to understand the full potential of heat-killed MCC1849 as an immuno-modulator.

In conclusion, although further investigations are required to clarify how heat-killed MCC1849 confers health benefits to humans; still, heat-killed MCC1849 has the potential to modulate immunity and maintain desirable psychological state.

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