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Abstract of Thesis/Dissertation

Applicant

Doctoral Program in Animal and Food Hygiene

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Title : Biochemical studies on potential probiotic strains *of Lactobacillus rhamnosus* isolated from fermented Sumbawa mare milk

(スンバワ島産発酵馬乳から単離した *Lactobacillus rhamnosus* プロバイオティクス候補菌株の生化学的研究)

Abstract

A fermented mare's milk produced in Sumbawa Island of Indonesia has been believed as medicine, because of its therapeutic properties in addition to nutritional characteristics. Furthermore, lactic acid bacteria in the fermented mare's milk are expected to confer health beneficial effects to consumers. In my laboratory, *Lactobacillus rhamnosus* strains FSMM15, FSMM22 and FSMM26 were isolated previously from the fermented Sumbawa mare's milk, and a number of probiotic properties, such as tolerances against low pH and bile salts, high survival rate on a treatment of artificial digestive fluids, and capability of adherence to a gastric mucin and extracellular matrix proteins, e.g., laminin and fibronectin, were clarified. Taking over the research background, the purpose of this study is (i) further investigation on probiotic functions of the three FSMM strains and (ii) *in vitro* safety assessment of them.

In Chapter 1, issues about mare's milk, the genus *Lactobacillus*, probiotics, and human gastrointestinal tract (GIT) were described as general research background. The research purpose of this study was also declared.

In Chapter 2, a typical probiotic function, an antimicrobial activity, was investigated to estimate suppressive effects of the FSMM strains on several pathogenic bacteria in human GIT. For this purpose, a disc diffusion assay was applied for six pathogenic bacteria, *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes*, methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA), *Shigella sonnei*, and *Escherichia coli* O157, using cell-free culture supernatants (CFCSs) of the FSMM

strains as antimicrobial agents. As a result, CFCSs of FSMM22 and FSMM26 inhibited growths of all the six pathogens in a comparable level to ampicillin and nisin used as positive controls. On the other hand, CFCS of FSMM15 showed growth inhibition activities except for MRSA and MSSA. It was most likely due to lower organic acid production of FSMM15 than others. None of the FSMM strains co-aggregate with *S.* Typhimurium, suggesting that they are not capable of preventing colonization of the pathogen in human GIT. Taken together, it was revealed that the FSMM strains possessed a wide spectrum of antimicrobial activities regardless of Gram-stainability of the target pathogens, suggesting that the FSMM strains are highly potential as new biopreservatives.

In Chapter 3, a multifaceted *in vitro* safety assessment was conducted on the three FSMM strains. An increasing requirement of safer probiotic lactic acid bacteria is obvious in recent years, because several cases of infection have been reported on *L. rhamnosus* GG ATCC53103, one of the most studied probiotic lactic acid bacteria, even though those occurred under uncommon conditions. Therefore, spectra of antibiotic resistance, bioconversion abilities of bile salts, hemolytic activities, mucin degradation activities, enzymatic activities, and plasminogen binding abilities were investigated towards the three FSMM strains. As a result, all the three FSMM strains were resistant to erythromycin and clindamycin. None of them showed bioconversion activities of bile salts, hemolytic activities, and mucin degradation activities. On the other hand, in the enzymatic activity test, α-chymotrypsin and β-glucosidase activities, which are known to be harmful to human health, were detected for all the three FSMM strains. However, they lacked *N*-acetyl-β-glucosaminidase and α-galactosidase, which are usually incorporated with α-chymotrypsin, suggesting its little effect on human health. Furthermore, activity levels of β-glucosidase in FSMM strains seemed to be negligible to those in Clostridia and *Bacteroides*, which are known to exhibit colon cancer promoting effect. There was no plasminogen binding ability in FSMM15, whereas FSMM22 and FSMM26 showed significantly higher plasminogen binding abilities. In general, some pathogenic bacteria utilize host-derived plasminogen by binding them on the cell surface in order to degrade extra cellular matrix of the host. Therefore, in this context, FSMM15 was considered to be safer than other FSMM strains.

In Chapter 4, an exhibition mechanism of 30S ribosomal protein S19 (RpsS), a protein extracted from the cell surface of FSMM22 was investigated. RpsS is a subunit protein that constitutes ribosomes, and hence it localizes normally in cytoplasm. In the previous study, it has been revealed that RpsS was extracted from the cell surface of FSMM22 by a treatment of 1 M LiCl, but not from FSMM15. Hence, expression levels of mRNA and protein of RpsS in FSMM15 and FSMM22 were investigated by real-time PCR and by Western blot analyses using an anti-RpsS polyclonal antibody, respectively. As a result, there was no significant difference in RpsS mRNA expression levels of the two FSMM strains. By Western blotting, RNA polymerase, which is a common marker protein of cytoplasmic localization, was found in the 1 M LiCl extract of FSMM22 but not in FSMM15,

indicating that 1 M LiCl treatment could lead temporal increase of cell wall permeability in FSMM22, resulting in a release of cytoplasmic protein to outside of the bacterial cells. Time-course of viable cell counts revealed that the number of viable cells were 10-fold less in FSMM22 than FSMM15 at the stationary phase. Therefore, there was a possibility that RpsS released from the dead cells were translocated to the cell surface of viable cells. On the other hand, there was no apparent difference found in the extracted RpsS levels at different growth stages, implying possibilities of another translocation pathway such as releasing RpsS from traumatized cells. Taken together, RpsS was likely to be released from the dead cells and traumatized cells whose cell wall permeability was increased by some stresses, and then to bind on the cell surface.

In Chapter 5, a general discussion of this study was described. To summarize, the data obtained in this study suggested that FSMM15 should be potential as a safe probiotic lactic acid bacteria for the application use in food industries, owing to the lack of plasminogen binding ability, despite of the narrower antimicrobial spectrum and the lower host adhesive ability targeting to the host's extracellular matrix proteins than FSMM22 and FSMM26.