



# In vitro safety assessments and antimicrobial activities of *Lactobacillus rhamnosus* strains isolated from a fermented mare's milk

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1 ***In vitro* safety assessments and antimicrobial activities of *Lactobacillus rhamnosus* strains**  
2 **isolated from a fermented mare's milk**

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14

15 Running title: Safety and probiotic properties of LAB

16 **Abstract**

17 Safety and probiotic characteristics such as antimicrobial activities of three *Lactobacillus rhamnosus*  
18 strains, FSMM15, FSMM22, and FSMM26, previously isolated as potential probiotics from a  
19 fermented mare's milk were investigated. The three FSMM strains were susceptible to ampicillin,  
20 gentamycin, kanamycin, streptomycin, tetracycline, and chloramphenicol, whereas resistant to  
21 erythromycin (MIC = 4 – 8 µg mL<sup>-1</sup>) and clindamycin (MIC = 4 µg mL<sup>-1</sup>); bioconversion of bile salts,  
22 hemolytic activity, and mucin degradation activity were negative; enzymatic activities of α-  
23 chymotrypsin and β-glucosidase were detected, but those of α-galactosidase, β-glucuronidase, and N-  
24 acetyl-β-glucosaminidase, were undetectable. Among the strains, strain FSMM15 was chosen as a  
25 safer probiotic candidate due mainly to the lack of plasminogen binding ability. Despite lower acid  
26 production of strain FSMM15 than others, its cell-free culture supernatant inhibited growths of  
27 *Salmonella* Typhimurium LT-2, *Shigella sonnei*, *Listeria monocytogenes*, and *Escherichia coli* O157  
28 with comparable levels of ampicillin, suggesting a favorable aspect of strain FSMM15 as a probiotic  
29 strain.

30

31 Key words: antibacterial activity, fermented Sumbawa mare's milk, food safety

32 **1. Introduction**

33 Mare's milk contains lower concentrations of fat and protein and a higher concentration of lactose  
34 than cow milk and is consumed regularly by about 30 million people throughout the world either as  
35 the milk or as fermented products such as koumiss and airag (Potočnik *et al.* 2011). Fermented  
36 beverages made of mare's milk have high potential as functional foods, because certain bacteria  
37 including probiotics commonly produce health-promoting agents such as bioactive peptides and  
38 organic acids during the fermentation process (Batdorj *et al.* 2006; Chen *et al.* 2010).

39 The home-made fermented mare's milk produced by farmers on the Sumbawa Island of Indonesia  
40 tastes fairly sour, indicating the presence of strong acid producers during the fermentation process.  
41 Our previous study (Shi *et al.* 2012) revealed the predominance of *Lactobacillus rhamnosus* and of  
42 the isolated strains, strain FSMM15, strain FSMM22, and strain FSMM26 were selected as potential  
43 probiotics on a number of criteria such as resistance to acid and bile salts, survival rate on artificial  
44 gastric and intestinal fluids, and capability of binding on porcine gastric mucin and several  
45 extracellular matrix proteins.

46 Safety assessments and characterization of efficacy are prerequisite for probiotic claims. Although  
47 fairly infrequent, *L. rhamnosus* strains are potentially causative agents of opportunistic infections in  
48 severely immunocompromised patients, as was found in *L. rhamnosus* GG (LGG) (Salminen *et al.*  
49 2004). Antibiotic resistance patterns of probiotics should be clearly documented to avoid horizontal

50 transfer of the related genes (FAO/WHO, 2002) and haemolysis activity test and bile salt  
51 deconjugation are also considered to be important traits. Also, enzymatic activity, mucus degradation  
52 activity, and invasion abilities have been proposed as preliminary selection criteria (Salminen *et al.*  
53 1996). Conversely, one of the most attractive effects of probiotics is prevention of intestinal tract  
54 infection (Collado *et al.* 2008). Therefore, screening of antimicrobial activity and competitive  
55 adhesion on human cell lines to exclude pathogens are commonly characterized. Ultimately, the safety  
56 and efficacy should be confirmed by clinical studies (FAO/WHO, 2002).

57 In this study, antibiotic susceptibility, bile acid bioconversion activity, haemolytic activity,  
58 enzymatic activities, mucin degradation activity, and interactions with plasminogen were investigated  
59 to assess the safety of strains FSMM15, FSMM22, and FSMM26. In addition, antimicrobial activities  
60 against six enteropathogenic bacteria and aggregate characteristics were investigated to clarify their  
61 probiotic properties.

62

## 63 **2. Materials and methods**

### 64 **2-1. Reagents, bacterial strains, and culture conditions**

65 All chemicals used in this study were analytical grade. Strains FSMM15, FSMM22, FSMM26,  
66 and all pathogenic bacteria were from our bacterial culture collections at Obihiro University of  
67 Agriculture and Veterinary Medicine. *Lactobacillus rhamnosus* GG ATCC 53103, *Lactobacillus*

68 *brevis* ATCC 8287, and *Enterococcus faecalis* ATCC 19433 were purchased from the American Type  
69 Culture Collection (Manassas, VA, USA). LAB were propagated on de Man-Rogosa-Sharpe (MRS)  
70 agar (Oxoid, Basingstoke, UK) for 24 – 48 h at 37 °C under anaerobic conditions using Anaeropack  
71 Kenki system (Mitsubishi Gas Chemical Company, Tokyo, Japan). For liquid culture, a single colony  
72 was inoculated into MRS broth (Oxoid) and pre-cultured for 18 h at 37 °C. An adequate amount of the  
73 pre-culture broth was inoculated into a fresh MRS broth and incubated for up to 24 h at 37 °C  
74 anaerobically as a main culture. Human fecal bacteria (HFB) was obtained from a stool sample of 30  
75 years old healthy woman. The standard strain *Salmonella enterica* subsp. *enterica* serovar  
76 Typhimurium LT2 (*Salmonella* Typhimurium LT-2) and a laboratory stock of *Shigella sonnei* strain  
77 No. 134 were propagated in Luria-Bertani (LB) broth (Merck KGaA, Darmstadt, Germany).  
78 Laboratory stocks of methicillin-resistant *Staphylococcus aureus* (MRSA) strain No. 29, methicillin-  
79 sensitive *S. aureus* (MSSA) strain No. 18, *Listeria monocytogenes* strain No. 154, and *Escherichia*  
80 *coli* O157 strain No. S-12 were propagated in Brain Heart Infusion (BHI) broth (BD Biosciences,  
81 Spark, MD, USA). For antimicrobial activity and co-aggregation activity assays, all pathogenic  
82 bacteria were incubated for up to 18 h at 37 °C with agitation (200 rpm min<sup>-1</sup>) at biosafety level 2.

83

## 84 **2-2. Antibiotic susceptibility test and MIC determination**

85 All antibiotics were purchased from Merck KGaA. Antibiotic susceptibility test was performed

86 according to the guidelines of ISO 10932/IDF 223 standard (2010). Minimal inhibitory concentrations  
87 (MICs) for ampicillin, gentamycin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline,  
88 and chloramphenicol were determined by the microdilution broth method using hand-made  
89 microdilution plates. Antibiotic susceptibilities were evaluated by comparison with the MIC  
90 breakpoint values for *L. rhamnosus* recommended by the European Food Safety Authority Panel on  
91 Additives and Products or Substances used in Animal Feed (EFSA, 2012).

92

### 93 **2-3. Bile acid bioconversion tests**

#### 94 **2-3-1. Bile salt hydrolase activity test by agar plate method**

95 All bile salts were purchased from Merck KGaA. MRS agar plates containing 1.7% (w/v) agar and  
96 each 1 mM of taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCa), taurodeoxycholic acid  
97 (TDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), and glycodeoxycholic acid  
98 (GDCA) were pre-incubated anaerobically at 37 °C for 48 h, then each 10 µL of overnight MRS culture  
99 broths of the three FSMM strains and LGG was inoculated onto the plates and incubated at 37 °C for  
100 72 h anaerobically. Bile salt deconjugation was evaluated by observing the formation of precipitation  
101 zones around the emerged colonies (Begley *et al.* 2006). *Enterococcus faecalis* ATCC 19433 was use  
102 as a positive control.

103

104 **2-3-2. Biotransformation of cholic acid into deoxycholic acid**

105 Biotransformation activity of cholic acid (CA) into deoxycholic acid (DCA) was investigated as  
106 described previously (Kurdi *et al.* 2003) with modifications. Each of the tested strains was inoculated  
107 into 1/2MRS broth containing 0.15 mM sodium cholate, then the broths were incubated at 37 °C for  
108 48 h under anaerobic conditions. After pH adjustment to 2.0, bile acids were extracted from 200 µL  
109 of the culture broths with 1 mL of ethyl acetate. The extracted bile acids were separated by thin-layer  
110 chromatography (TLC) using a Silica gel 60 plate (Whatman, Maidstone, UK) with cyclohexane/ethyl  
111 acetate/acetic acid (7:23:3, v/v) as a developing solvent. Spots of bile salts were visualized by spraying  
112 5% (w/v) phosphomolybdic acid in absolute ethanol and heating.

113

114 **2-4. Hemolytic activity tests**

115 **2-4-1. Agar plate assay**

116 The 18-h culture broths of the tested strains were washed twice with phosphate-buffered saline  
117 (PBS) and the cell population was adjusted to 10<sup>8</sup> colony forming unit (CFU) mL<sup>-1</sup> in PBS. About 10  
118 µL of the cell suspensions were inoculated onto 5% sheep blood agar plates (Eiken Chemical Co., Ltd.,  
119 Tokyo, Japan) and incubated at 37 °C for 48 h under anaerobic conditions. *Lactobacillus brevis* ATCC  
120 8287 was used as a γ-hemolysis control strain. Clear zone formation around the colonies was judged  
121 as β-hemolytic activity (true hemolysis). Color change of the media around the colonies into shaded



122 brown or greenish was considered as  $\alpha$ -hemolytic activity. Strains without any change were taken as  
123 lacking hemolytic activity ( $\gamma$ -hemolysis).

124

#### 125 **2-4-2. Test tube assay**

126 Red blood cells (RBCs) prepared from defibrinated sheep blood (Nippon Biotest Laboratory,  
127 Tokyo, Japan) were used for colorimetric assay of hemolytic activities in test tubes, adapting from  
128 Sperandio *et al.* (2010). After removal of the buffy coat and plasma layer by centrifugation at 1500 x  
129 g for 2 min at room temperature (RT), pelleted RBCs were washed three times with PBS. The number  
130 of RBCs was counted using a hemocytometer, then the cell population was adjusted to  $10^8$  cells in 500  
131  $\mu$ L of PBS. Equal volume of the bacterial cells ( $10^8$  CFU in 500  $\mu$ L) was mixed gently with the RBCs  
132 suspension. A 500  $\mu$ L-aliquot of the mixture was collected after 1.5-h incubation at 37 °C, then  
133 centrifuged at 1500 x g for 10 min at RT. Hemolytic activity was monitored by measuring the  
134 absorbance at 405 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham,  
135 MA, USA). RBCs suspension was incubated with an equal volume of 1% (v/v) Triton X-100 in PBS  
136 and with an equal volume of PBS for positive and negative controls, respectively.

137

#### 138 **2-5. Enzymatic activity test**

139 Enzymatic activities were determined using an API ZYM kit (bioMérieux, Marcy l'Etoile, France)

140 according to the manufacturer's instructions. The tested strains were grown anaerobically in MRS  
141 broth at 37°C until the late exponential phase. Bacterial cells were harvested, then the cell population  
142 was adjusted to  $3 \times 10^8$  CFU mL<sup>-1</sup>. Enzymatic activities were evaluated by comparison with the API  
143 ZYM color chart (bioMérieux).

144

## 145 **2-6. Mucin degradation activity test**

### 146 **2-6-1. Agar plate assay**

147 Hog gastric mucin was purified from 10 g of crude powder (HGM Type III, Merck KGaA)  
148 according to the previous report (Zhou *et al.* 2001). To check mucin degradation activities, 10 µL of  
149 18-h main culture broths of each FSMM strains were inoculated onto agar plates (7.5 g tryptone, 7.5  
150 g casitone, 5.0 g yeast extract, 5.0 g beef extract, 5.0 g NaCl, 3.0 g K<sub>2</sub>HPO<sub>4</sub> · 3H<sub>2</sub>O, 0.5 g KH<sub>2</sub>PO<sub>4</sub>,  
151 0.5 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.5 g L-cysteine HCl, 0.002 g resazurin, 15 g agarose, and 0 or 30 g glucose per  
152 litre of deionized water, pH 7.2) that contained 0.5% (w/v) purified HGM Type III, then incubated at  
153 37 °C for 72 h anaerobically. Clear zones formed as a result of mucin degradation were visualized by  
154 staining with 0.1% (w/v) amido black dissolved in 3.5 M acetic acid for 30 min at RT, followed by  
155 washing with 1.2 M acetic acid until clear zones emerging. HFB grown in BHI broth and heat-  
156 inactivated HFB culture were used as positive and negative controls, respectively.

157

158 **2-6-2. Test tube assay**

159 Mucin degradation activities were investigated in a liquid medium using the similar composition  
160 used in the agar plate assay, but without glucose, purified HGM Type III, and agar (termed as a basal  
161 medium). A 150- $\mu$ L aliquot of the 18-h main culture broths of each strain was inoculated into 15 mL  
162 of the basal media and incubated at 37 °C for 48 h anaerobically. Growth of each strain was evaluated  
163 by measuring the pH and the optical density at 600 nm of the culture broth. After 48-h incubation, the  
164 remained mucin was recovered according to the previous report (Zhou *et al.* 2001). Mucin degradation  
165 was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using  
166 12% (w/v) polyacrylamide gels. Carbohydrate and protein contents of the remained mucin were  
167 evaluated respectively by phenol-sulfuric acid method (Dubois *et al.* 1956) using galactose as a  
168 standard and by BCA method using a Pierce BCA kit (Thermo Fisher Scientific) adapted from Miller  
169 and Hoskins (1981); the samples were pre-heated with reagent A at 70 °C for 45 min.

170

171 **2-7. Plasmiongen binding and activation tests**

172 Presence of human plasminogen (hPIg, Merck KGaA) bound on bacterial cell surface was  
173 evaluated by plasmin activity assay as described previously (Bergmann *et al.* 2005). The tested strains  
174 at stationary phase were harvested by centrifugation (5000 x *g* for 5 min at RT), washed twice with  
175 PBS, adjusted to 10<sup>9</sup> CFU mL<sup>-1</sup>, and incubated for 15 min at 37 °C with 40  $\mu$ g mL<sup>-1</sup> of hPIg dissolved

176 in PBS. After that, the reaction mixture was washed twice with PBS to remove unbound hPIg, then  
177 the bacterial cells with hPIg were suspended in 250  $\mu$ L of 50 mM Tris-HCl (pH 7.5). Subsequently,  
178 100- $\mu$ L aliquots of the bacterial suspension were dispensed in each well of a 96-well microtitre plate.  
179 The hPIg was activated by addition of 0.24 kallikrein inhibitor unit (KIU) tissue plasminogen activator  
180 (tPA, Merck KGaA) or 0.06 KIU urokinase plasminogen activator (uPA, Merck KGaA), then the  
181 peptide cleavage reaction was performed at 37 °C for 1 h using 30  $\mu$ L of 0.54 mM D-valyl-leucyl-  
182 lysine-*p*-nitroanilide dihydrochloride (S-2251, Merck KGaA) as a substrate. Absorbance at 405 nm  
183 ( $A_{405\text{nm}}$ ) was measured immediately after addition of S-2251 (time point =  $t_0$ ) and after 1-h incubation  
184 at 37°C (time point =  $t_1$ ). Plasmin activity was evaluated by subtracting  $A_{405\text{nm}}$  values at  $t_0$  from those  
185 at  $t_1$ .

186

## 187 **2-8. Antimicrobial activity against enteropathogenic bacteria**

### 188 **2-8-1. Cell-free culture supernatant preparation**

189 MRS broth (100 mL) was inoculated with each overnight pre-culture of the three FSMM strains  
190 as giving  $10^5$  to  $10^6$  CFU  $\text{mL}^{-1}$ , then incubated anaerobically at 37 °C for 24 h. Cell-free culture  
191 supernatant (CFCS) was obtained by centrifugation for 30 min at 10000  $\times$   $g$  at 4 °C. The CFCS was  
192 sterilized using 0.2  $\mu\text{m}$ -pore-size filters (Advantec, Tokyo, Japan). Four milliliter aliquots of  
193 neutralized (pH 7.0) and non-neutralized CFCSs were lyophilized, then reconstituted with sterilized

194 20 mM sodium phosphate buffer (pH 6.0) to achieve 20-fold concentrated CFCSs. MRS broth was  
195 treated by the same procedure and used as a negative control.

196

## 197 **2-8-2. Measurement of antimicrobial activities by disc diffusion assay**

198 Disc filter papers (hereafter abbreviated as discs) with 6 mm diameter (Whatman no. 1, GE  
199 Healthcare, Little Chalfont, UK) were placed into sterile Petri dishes and impregnated with 30  $\mu$ L of  
200 the various concentrations of CFCS or MRS broth prepared as describe above. The discs were allowed  
201 to dry at RT for 1 h. The 1.2% (w/v) BHI agar pre-incubated at 50 °C was thoroughly mixed with an  
202 overnight culture of each pathogenic bacteria ( $10^7 - 10^9$  CFU mL<sup>-1</sup>), including *S. Typhimurium* LT-2,  
203 *S. sonnei*, MRSA, MSSA, *L. monocytogenes*, and *E. coli* O157, poured into the Petri dishes, and  
204 incubated at RT for 30 min. The discs were then placed on the pathogen-seeded BHI agar plates. These  
205 were first incubated at 4 °C for 1 h to allow antimicrobial compounds to diffuse into the agar, then  
206 incubated at 37 °C for 24 h aerobically. Antimicrobial activities were estimated by measuring  
207 diameters of growth inhibition zones around the discs. Discs impregnated with 20 mM sodium  
208 phosphate buffer (pH 6.0) were used as negative controls. As positive controls, solutions of 0.1 mg  
209 mL<sup>-1</sup> of ampicillin and 10000 IU mL<sup>-1</sup> of nisin from *Lactococcus lactis* (Merck KGaA) were used.  
210 The acidity of CFCS was measured by acid-base titration method as described by Wakil and  
211 Osamwonyi (2012). The acidity was calculated as being equivalent to lactic acid by the following

212 equation:

$$213 \quad \% \text{Acidity} = (V_{\text{NaOH}} \times M_{\text{NaOH}} \times E) / (V_{\text{sample}}) \times 100,$$

214 where  $V$ ,  $M$ , and  $E$  indicate volume, molarity, and equivalent factor (90.08/mg), respectively.

215

## 216 **2-9. Auto- and co-aggregation properties and bacterial cell surface hydrophobicity**

217 The three FSMM strains and LGG were cultured in MRS broth and harvested at the end of the  
218 exponential growth phase. The harvested cells were washed twice with PBS and re-suspended in PBS  
219 to be  $10^8$  cells  $\text{mL}^{-1}$ . Four milliliter aliquots of the bacterial cell suspensions were mixed thoroughly  
220 for 10 s, then incubated without agitation at  $37^\circ\text{C}$ . Auto-aggregation was monitored by measuring the  
221 absorbance of the culture supernatant at 600 nm. Co-aggregation was clarified only with *S.*  
222 Typhimurium LT-2, because all the tested strains showed inhibitory activities against the pathogen.  
223 Equal volume (2 mL) of the FSMM strains and the pathogenic bacterial cells were mixed and  
224 incubated at  $37^\circ\text{C}$  without agitation. The ratio of auto-aggregation was expressed as

$$225 \quad 1 - (A_t/A_0) \times 100,$$

226 where  $A_t$  and  $A_0$  represented the values of absorbance 600 nm at the several time points (2, 6, 20, and  
227 24 h) and the initial time point (0 h), respectively. The ratio of co-aggregation was calculated at the  
228 same time points, according to Handley *et al.* (1987) as follows:

$$229 \quad \{(A_{\text{pathog}} + A_{\text{lacto}})/2 - (A_{\text{mix}}) / (A_{\text{pathog}} + A_{\text{lacto}})/2\} \times 100,$$

230 where  $A_{pathog}$ ,  $A_{lacto}$ , and  $A_{mix}$  represent the absorbance at 600 nm of the culture supernatant of the  
231 pathogenic bacteria, of the FSMM strains, and of their mixtures, respectively.

232 Cell surface hydrophobicity was evaluated according to the previous report (Collado *et al.* 2008).  
233 Equal volumes of xylene and approximately  $10^8$  CFU mL<sup>-1</sup> of the bacterial cells were mixed  
234 vigorously for 5 min. After 1-h incubation at RT, turbidity of the aqueous phase was measured at  
235 absorbance 600 nm. The cell surface hydrophobicity was calculated as follows:

$$236 \quad \% \text{Hydrophobicity} = \{(A_0 - A_1) / A_0\} \times 100,$$

237 where  $A_0$  and  $A_1$  are the absorbance at 600 nm of the aqueous phase before and after mixing with  
238 xylene, respectively.

239

## 240 **2-11. Statistical analysis**

241 Numeric data except antibiotic susceptibility and enzymatic profile were expressed as means  $\pm$   
242 standard deviation (SD) from three replications. The statistical significance was assessed by one-way  
243 analysis of variance (ANOVA) with Tukey's post-hoc test. Data were considered significant at  $P$  value  
244 less than 0.05.

245

## 246 **3. Results**

### 247 **3-1. Antibiotic susceptibilities of the FSMM strains**

248 All the tested strains including LGG were susceptible to ampicillin (MIC = 0.25  $\mu\text{g mL}^{-1}$ ),  
249 gentamycin (MIC = 2 – 4  $\mu\text{g mL}^{-1}$ ), kanamycin (MIC = 64  $\mu\text{g mL}^{-1}$ ), streptomycin (MIC = 8 – 16  $\mu\text{g}$   
250  $\text{mL}^{-1}$ ), tetracycline (MIC = 0.5 – 4  $\mu\text{g mL}^{-1}$ ), and chloramphenicol (MIC = 4  $\mu\text{g mL}^{-1}$ ), whereas  
251 resistant to erythromycin (MIC = 4 – 8  $\mu\text{g mL}^{-1}$ ) and clindamycin (MIC = 4  $\mu\text{g mL}^{-1}$ ) (Table 1).

252

### 253 **3-2. Bile acid bioconversion abilities of the FSMM strains**

254 No apparent precipitation of any bile salts was observed for the three FSMM strains and LGG in  
255 contrast to *E. faecalis* ATCC 19433 (Fig. S1, Table 4), hence none of the tested strains were capable  
256 of deconjugating primary bile salts into free CA and DCA. No metabolic ability of converting CA into  
257 DCA was also confirmed for the three FSMM strains and LGG by TLC (Fig. S2, Table 4).

258

### 259 **3-3. Hemolytic activities of the FSMM strains**

260 Brownish color observed in the surrounding areas of the colonies of the FSMM strains and LGG  
261 indicated their  $\alpha$ -hemolytic activities (Fig. S3A, Table 4). Because  $\alpha$ -hemolytic activity was  
262 considered as a partial hemolysis, disruption of sheep RBCs in aqueous phase was further tested. As a  
263 result, apparent hemolysis was not observed for all the tested strains including *L. brevis* ATCC 8287  
264 (Fig. S3B, Table 4).

265



266 **3-4. Enzymatic activities of the FSMM strains**

267 The three FSMM strains showed very similar patterns in the enzymatic activities with LGG (Fig.  
268 S4, Table 4). In respect to potentially harmful enzymatic activities,  $\alpha$ -galactosidase,  $\beta$ -glucuronidase,  
269 and *N*-acetyl- $\beta$ -glucosaminidase activities were undetectable, whereas  $\alpha$ -chymotrypsin (5 nmol of  
270 substrate hydrolyzed) and  $\beta$ -glucosidase (> 30 nmol of substrate hydrolyzed) activities were found in  
271 all the tested strains.

272

273 **3-5. Mucin degradation activities of the FSMM strains**

274 An apparent clear zone was observed in the positive control (Fig. S5A, Table 4). On the other hand,  
275 clear zone was not obvious in strain FSMM15 and LGG; however, very weak clear zones were seen  
276 in strains FSMM22 and FSMM26. When the tested strains were grown on the agar plates containing  
277 mucin and glucose, clear zone formation was not seen in all the tested strains (Fig. S5B, Table 4).  
278 When mucin was supplemented to the basal medium, only HFB could proliferate significantly (1.3-  
279 fold higher in OD<sub>600nm</sub>). Simultaneous supplementation of mucin and glucose did not promote the cell  
280 growth compared to the solo glucose supplementation, hence the three FSMM strains were not capable  
281 of utilizing mucin as a carbon source (Data not shown). Fragmentation of mucin protein was observed  
282 only when the HFB was cultured in the basal medium supplemented with 0.3% HGM Type III (Figs.  
283 S6A and S6C, Table 4). Degradation of the carbohydrate moieties of mucin by the HFB was also

284 confirmed by a decrease of stained area in the high molecular mass region (more than 150 kDa) on the  
285 SDS-PAGE gel (Figs. S6B and S6D, Table 4). The three FSMM strains and LGG showed less than  
286 20% degradation of protein and carbohydrate moieties (data not shown), indicating that they  
287 apparently lacked mucin degradation activities according to the criteria suggested by Miller and  
288 Hoskins (1981).

289

### 290 **3-6. Plasmiongen activation capabilities of the FSMM strains**

291 No significant difference was observed when the plasmin activity was evaluated in the absence of  
292 PAs, hence the three FSMM strains have no endogenous PA activity (Fig. 1). Among them, strains  
293 FSMM22 and FSMM26 showed obviously higher conversion level of hPIg to plasmin, indicating their  
294 high binding abilities to hPIg (Figs. 1B and 1C), whereas strain FSMM15 and LGG showed almost no  
295 binding ability (Figs. 1A and 1D).

296

### 297 **3-7. Antimicrobial activities of the FSMM strains**

298 Strains FSMM22 and FSMM26 showed antimicrobial activities for all the six enteropathogenic  
299 bacteria as comparable levels to LGG, while strain FSMM15 lacked inhibitory activities against  
300 MRSA and MSSA (Table 2). When MRS medium adjusted to different pH was subjected to the disc  
301 diffusion assay, MRSA and MSSA formed haloes at pH 2, but other pathogens formed at pH 3 or 4,

302 indicated higher acid tolerance of MRSA and MSSA (data not shown). Judging from the pH and acidity  
303 values of the CFCSs of strains FSMM15 (pH  $4.23 \pm 0.02$ ,  $0.07 \pm 0.00\%$  Acidity), FSMM22 (pH  $3.95$   
304  $\pm 0.02$ ,  $0.13 \pm 0.00\%$  Acidity), FSMM26 (pH  $3.97 \pm 0.02$ ,  $0.11 \pm 0.01\%$  Acidity), and LGG (pH  $3.99$   
305  $\pm 0.01$ ,  $0.12 \pm 0.01\%$  Acidity), the lack of inhibitory activities of strain FSMM15 against MRSA and  
306 MSSA was likely due to the lower acid production than other strains.

307

### 308 **3-8. Auto- and co-aggregation properties of the FSMM strains**

309 Auto-aggregation of the tested strains progressed in time-dependent manner (Table 3). Strains  
310 FSMM22 and FSMM26 showed significantly higher auto-aggregation properties comparing to strain  
311 FSMM15 and LGG. None of the tested strains co-aggregated with *S. Typhimurium* LT-2, hence these  
312 strains are unlikely to exclude *S. Typhimurium* LT-2 from the host's GIT by the co-aggregation  
313 mechanism.

314

## 315 **4. Discussion**

316 As mentioned by Bernardeau *et al.* (2008), clarification of antibiotic susceptibility patterns is  
317 considered to be the primary requirement for the safety assessment of *Lactobacillus* genus, and we  
318 found in this study all the three FSMM strains, as well as LGG, showed resistances against  
319 erythromycin and clindamycin with similarly moderate MIC values ( $4 - 8 \mu\text{g mL}^{-1}$ ). Acquisition

320 mechanism of resistance to macrolides (e.g. erythromycin) and lincosamides (e.g. clindamycin) are  
321 similar among pathogenic bacteria, and the major cause is considered as modifications of ribosomal  
322 genes, whereas efflux and inactivation of those antibiotics are less effective (Leclercq, 2002). This  
323 seems to be the same for *Lactobacillus*; for example, human vaginal isolates of *L. rhamnosus* had very  
324 high erythromycin resistance (MIC = 2048  $\mu\text{g mL}^{-1}$ ), which was presumed to stem from a transition  
325 mutation (A<sup>2058</sup> to G<sup>2058</sup>) occurred in 23S rRNA (Begovic *et al.* 2009). Presence of *ermA*, *ermB* and  
326 *ermC* and the single mutation in 23S rRNA were determined as plausible causes of erythromycin  
327 resistance in *L. rhamnosus* Pen (Waško *et al.* 2012). Further studies are needed to avoid the risk of  
328 disseminating antibiotic resistance genes from the FSMM strains to other bacteria by horizontal gene  
329 transfer.

330 Presence in high quantity of hydrogen peroxide, which is commonly produced by lactobacilli as  
331 an antibacterial substance, enhances oxidation of hemoglobin in the blood agar plate, resulting in the  
332 formation of greenish methemoglobin without complete destruction of the RBCs (Rabe & Hillier  
333 2003). Despite  $\alpha$ -hemolysis observed for the three FSMM strains, destruction of the RBCs were not  
334 confirmed by the test tube assay, therefore the FSMM strains have negligible hemolytic activities in  
335 agreement with previous reports (Maragkoudakis *et al.* 2006; Vesterlund *et al.* 2007; Köll *et al.* 2010;  
336 Rodrigues da Cunha *et al.* 2012).

337 Weak clear zones observed around the colonies of strains FSMM22 and FSMM26 on the mucin

338 containing agar plates seemed to be an experimental artifact, because abrasions were observed in the  
339 edges of bacterial spots of strains FSMM22 and FSMM26 during the amido black destaining step.

340 It has been reported that most of bacterial isolates from patients of endocarditis produced *N*-acetyl-  
341  $\beta$ -glucosaminidase and  $\alpha$ -galactosidase, which were likely to incorporate with  $\alpha$ -chymotrypsin to  
342 enhance hydrolysis of glycoproteins of the host, and therefore these enzymes lead tissues into damaged  
343 during development of endocarditis (Oakey *et al.* 1995). Whereas the three FSMM strains showed no  
344 detectable *N*-acetyl- $\beta$ -glucosaminidase and  $\alpha$ -galactosidase activities, hence risks of the  $\alpha$ -  
345 chymotrypsin activities in the FSMM strains are little. Clostridia and *Bacteroides*, which have high  
346 levels of  $\beta$ -glucosidase activities, are the major causative bacteria for colon cancer, and most of  
347 lactobacilli are known to produce much lower  $\beta$ -glucosidase activities than such major causative  
348 pathogens (Wollowski *et al.* 2001).

349 Some pathogenic bacteria including *Helicobacter pylori* are known to capture the host-producing  
350 plasminogen via lysine residues of their cell surface proteins and to utilize it as their own tool to  
351 degrade host's extracellular matrix (ECM) proteins (Lähteenmäki *et al.* 2005). Plasminogen is  
352 activated by tPA and uPA as well as by prokaryotic activators such as staphylokinase and streptokinase,  
353 resulting in a formation of a proteolytic enzyme, plasmin (Lähteenmäki *et al.* 2001). Strain FSMM15  
354 had no remarkable plasminogen binding ability as well as LGG, while strains FSMM22 and FSMM26  
355 showed significantly high capacity of plasminogen binding, indicating that plasminogen binding

356 ability should be strain-dependent. This observation was corresponding to the previous report in which  
357 cell surface associated proteins extractable with PBS from several probiotic and non-probiotic strains,  
358 including *L. gallinarum* T-50, *L. johnsonii* F133, *L. amylovorus* JCM 5807, *L. gasseri* JCM  
359 1130/ATCC 19992, and LGG, demonstrated different binding abilities to hPIg (Hurmala *et al.*  
360 2007). Conflicting results were found in LGG in this report and the previous one (Hurmala *et al.*  
361 2007), but this is likely to stem from differences in experimental conditions such as incubation time  
362 with substrates. Ishibashi and Yamasaki (2001) mentioned that cell surface proteins of bacteria were  
363 associated with their aggregation abilities, which can be evaluated by measuring the cell surface  
364 hydrophobicity. It has been revealed that the amount of cell surface proteins of strain FSMM22  
365 extracted with 1 M LiCl was apparently higher than strain FSMM15 (unpublished data). Therefore,  
366 we speculated that larger amount of cell surface proteins in strain FSMM22 reinforced the co-  
367 aggregation and the binding of plasminogen on the cell surface using hydrophobic interactions as the  
368 major driving force.

369 The anti-pathogenic effects of the FSMM strains were comparable to ampicillin and nisin,  
370 therefore they are promising anti-enteropathogenic agents as long as they could proliferate and  
371 produce sufficient amounts of organic acids in one's intestine. According to Keersmaecker *et al.*  
372 (2006), strong antimicrobial activity of LGG to *S. Typhimurium* was mediated by production of  
373 organic acids, mainly lactic acid, when cultured in MRS medium.

374 Auto-aggregation capability of LAB is considered to correlate with the host adhesion, whereas co-  
375 aggregation with pathogenic bacteria is expected to interfere the pathogenic bacterial infection to the  
376 host (Collado *et al.* 2008). Although strain FSMM15 and LGG showed similar auto-aggregation  
377 properties, a significant difference observed in their cell-surface hydrophobicities. Therefore, cell-  
378 surface hydrophobicity was not only the determinant of the strength of auto-aggregation in lactobacilli.  
379 Despite the moderate and high auto-aggregation abilities and the cell surface hydrophobicities of the  
380 FSMM strains, none of them co-aggregated with *S. Typhimurium* LT-2 in this study, hence the major  
381 driving force of the FSMM strains to co-aggregate with *S. Typhimurium* LT-2 was unlikely to  
382 hydrophobic interactions. Similar conflicting result was reported that several factors other than  
383 hydrophobicity such as passive forces, electrostatic interaction, and the presence of lipoteichoic acids,  
384 lectins, and soluble secreted proteins may responsible for aggregation abilities of bacteria (Solieri *et*  
385 *al.* 2014).

386

## 387 **5. Conclusions**

388 Safety assessments and probiotic characteristics such as antimicrobial activities were investigated  
389 on the three potential probiotic strains isolated from a fermented mare's milk *in vitro*. As far as the  
390 safety and probiotic characteristics investigated in this study, strain FSMM15 was similar to LGG,  
391 while strains FSMM22 and FSMM26 were very alike. Moreover, it was apparent that these

392 characteristics were entirely strain-dependent. Despite broader anti-pathogenic spectrum of strains  
393 FSMM22 and FSMM26, FSMM15 was taken as the best probiotic candidate due mainly to the lack  
394 of plasminogen binding ability. However, risks of strain FSMM15 for human health remains latent, as  
395 being mentioned for LGG as a causative agent of opportunistic infection. To ensure this, *in vivo*  
396 experiments should be further performed. It is also important to elucidate presence of pili, biogenic  
397 amine and D-lactate productions, and molecular mechanism of horizontal transfer of antibiotic  
398 resistant genes in strain FSMM15.

399

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533 **Figure legends**

534 **Fig. 1. Plasminogen binding and activation activities of strain FSMM15 (A), strain FSMM22 (B),**  
535 **strain FSMM26 (C), and LGG (D).** The hPIg binding activity was evaluated by measuring the  
536 conversion of bound hPIg on the cell surface of tested strains into plasmin by addition of plasmin  
537 substrate (S-2251) in the presence of tPA (gray bars) and uPA (filled bars). Bacterial cells incubated  
538 only with S-2251 was used as a negative control (NC, open bars). Bacterial cells pre-treated with hPIg  
539 were incubated with S-2251 in the absence of PAs (hatched bars) to clarify presence of endogenous  
540 PA activities. The data represent means  $\pm$  SD of three replications. Asterisks represent significantly  
541 different with the negative control and pre-treated bacteria with hPIg in the absence of PAs ( $P < 0.05$ ).

**Table 1. MIC values of *L. rhamnosus* FSMM strains and LGG towards eight antibiotics determined by the broth microdilution method.**

Strains	MIC values ( $\mu\text{g mL}^{-1}$ )							
	Am (4)	Gm (16)	Km (64)	Sm (32)	Em (1)	Cl (1)	Tc (4)	Cm (4)
FSMM15	0.25	4	64	16	8	4	4	4
FSMM22	0.25	4	64	8	8	4	1	4
FSMM26	0.25	4	64	8	4	4	1	4
LGG	0.25	2	64	16	8	4	0.5	4

Am, ampicillin; Gm, gentamycin; Km, kanamycin; Sm, streptomycin; Em, Erythromycin; Cl, Clindamycin; Tc, tetracycline; Cm, Chloramphenicol. The microbial break points for the eight antibiotics were indicated in the parentheses ( $\mu\text{g mL}^{-1}$ ). MIC values surpassing the microbiological breakpoint proposed by the EFSA Panel on Additives and Products or Substances used in Animal Feed were shaded in gray (EFSA, 2012).

**Table 2. Antimicrobial activity of the cell-free culture supernatant of FSMM strains and LGG against six enteropathogenic bacteria.**

Enteropathogenic bacteria	Antimicrobial activity (diameter in mm)					
	FSMM15	FSMM22	FSMM26	LGG	Ampicillin	Nisin
<i>S. Typhimurium</i> LT-2	12.2 ± 1.1 <sup>Aa</sup>	15.8 ± 2.8 <sup>Aab</sup>	18.4 ± 1.3 <sup>Ab</sup>	14.4 ± 1.3 <sup>Aa</sup>	15.2 ± 0.3 <sup>Aab</sup>	0 <sup>Ac</sup>
<i>L. monocytogenes</i> No. 154	10.1 ± 0.2 <sup>Ba</sup>	14.3 ± 0.6 <sup>Ab</sup>	13.9 ± 0.2 <sup>Bb</sup>	13.0 ± 0 <sup>Bbc</sup>	27.3 ± 0.6 <sup>Bd</sup>	9.0 ± 0 <sup>Bc</sup>
MRSA No. 29	0 <sup>Ca</sup>	9.7 ± 0.6 <sup>Bb</sup>	8.2 ± 0.4 <sup>Cc</sup>	9.7 ± 0.6 <sup>Cb</sup>	0 <sup>Ca</sup>	9.0 ± 0 <sup>Bbc</sup>
MSSA No. 18	0 <sup>Ca</sup>	11.7 ± 0.3 <sup>Cb</sup>	11.7 ± 0.4 <sup>Db</sup>	10.1 ± 0.3 <sup>Cc</sup>	30.0 ± 0 <sup>Dd</sup>	15.3 ± 0.6 <sup>Ce</sup>
<i>E. coli</i> O157 No. S-12	8.6 ± 0.6 <sup>Da</sup>	10.6 ± 0.1 <sup>CBb</sup>	10.7 ± 0.3 <sup>Db</sup>	10.0 ± 0 <sup>Cb</sup>	8.0 ± 0 <sup>Ea</sup>	0 <sup>Ac</sup>
<i>S. sonnei</i> No. 134	10.3 ± 0.5 <sup>Ba</sup>	15.3 ± 0.4 <sup>Ab</sup>	15.8 ± 0.9 <sup>Eb</sup>	13.3 ± 0.4 <sup>Bc</sup>	11.0 ± 0 <sup>Fa</sup>	0 <sup>Ac</sup>

The antimicrobial activities were evaluated by measuring the diameters of growth inhibition zones around the discs as mean ± SD from three replications. Different superscript lowercase letters in the same row and different superscript uppercase letters in the same column represent significant differences ( $p < 0.05$ ) of antimicrobial activity among each strain, ampicillin, and nisin to the enteropathogenic bacteria.

**Table 3. Auto-aggregation, co-aggregation, and cell-surface hydrophobicity of the FSMM strains and LGG.**

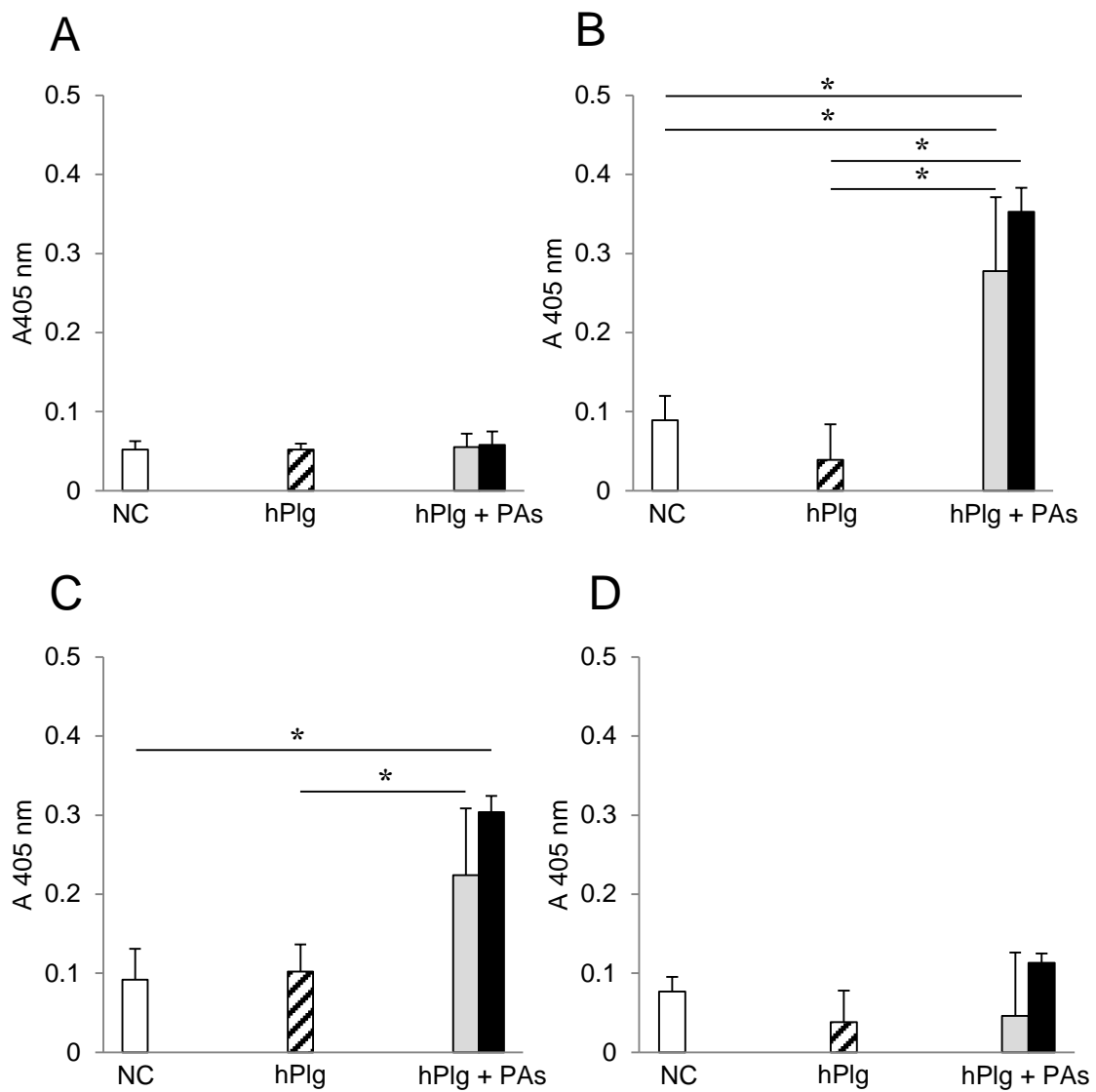
Strains	Auto-aggregation			Co-aggregation with <i>S. Typhimurium</i> LT-2	%Hydrophobicity
	3 h	6 h	24 h		
FSMM15	6.2 ± 5.2 <sup>Aa</sup>	24.4 ± 6.4 <sup>Ab</sup>	51.6 ± 5.6 <sup>Ac</sup>	-0.3 ± 0.2	90.8 ± 2.1 <sup>A</sup>
FSMM22	78.7 ± 2.7 <sup>Ba</sup>	96.3 ± 2.3 <sup>Bb</sup>	99.7 ± 0.6 <sup>Bc</sup>	-2.1 ± 1.6	99.7 ± 0.1 <sup>B</sup>
FSMM26	71.8 ± 8.6 <sup>Ba</sup>	86.5 ± 2.9 <sup>Bb</sup>	97.1 ± 1.3 <sup>Bb</sup>	-0.4 ± 0.2	99.5 ± 0.2 <sup>B</sup>
LGG	11.6 ± 3.7 <sup>Aa</sup>	30.1 ± 4.0 <sup>Ab</sup>	48.5 ± 4.7 <sup>Ac</sup>	0.0 ± 0.1	61.3 ± 4.5 <sup>C</sup>

Data were represented as mean ± SD from three replications. Different superscript lowercase letters in the same row and different superscript uppercase letters in the same column represent significant differences ( $p < 0.05$ ) of auto-aggregation activity or %Hydrophobicity among each strain. As the data was negative, co-aggregation activities with *S. Typhimurium* LT-2 were not included in the statistical analysis.

**Table 4. Bile salt bioconversion, haemolysis, undesirable enzymatic, and mucin degradation activities of the tested bacteria.**

Strains	Bile salt bioconversion activity							Haemolysis activity		Undesirable enzymatic activity					Mucin degradation activity	
	Bile salt deconjugation						Conversion from CA to DCA	Agar plate assay	Test tube assay	$\alpha$ -Chy	$\alpha$ -Gal	$\beta$ -Glc	$\beta$ -Glu	NA- $\beta$ -Gluc	Agar plate assay	Test tube assay
	TCA	GCA	TDCA	GDCA	TCDC	GDCD										
FSMM15	-	-	-	-	-	-	-	$\alpha$	-	+	-	-	+	-	-	-
FSMM22	-	-	-	-	-	-	-	$\alpha$	-	+	-	-	+	-	-	-
FSMM26	-	-	-	-	-	-	-	$\alpha$	-	+	-	-	+	-	-	-
LGG	-	-	-	-	-	-	-	$\alpha$	-	+	-	-	+	-	-	-
<i>L. reuteri</i> ATCC8287	ND	ND	ND	ND	ND	ND	ND	$\gamma$	-	ND	ND	ND	ND	ND	ND	ND
<i>E. faecalis</i> ATCC19433	-	-	+	+	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HFB	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+

Plus and minus indicate positive and negative results, respectively. ND, not determined; HFB, human fecal bacteria; TCA, taurocholic acid; GCA, glycocholic acid; TDCA, taurodeoxycholic acid; GDCA, glycodeoxycholic acid; TCDC, taurochenodeoxycholic acid; GDCD, taurochenodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid;  $\alpha$ , alpha type haemolysis;  $\gamma$ , gamma type haemolysis;  $\alpha$ -Chy,  $\alpha$ -chymotrypsin;  $\alpha$ -Gal,  $\alpha$ -galactosidase;  $\beta$ -Glc,  $\beta$ -glucuronidase;  $\beta$ -Glu,  $\beta$ -glucosidase; NA- $\beta$ -Gluc, N-acetyl- $\beta$ -glucosaminidase.



**Fig. 1. Aryantini et al.**