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Running title: Safety and probiotic properties of LAB

Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2-1. Reagents, bacterial strains, and culture conditions

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

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

2-2. Antibiotic susceptibility test and MIC determination

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

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

2-3. Bile acid bioconversion tests

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

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

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 10^8 colony forming unit (CFU) mL^{-1} 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

brown or greenish was considered as α-hemolytic activity. Strains without any change were taken as lacking hemolytic activity (γ-hemolysis).

2-4-2. Test tube assay

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

2-5. Enzymatic activity test

Enzymatic activities were determined using an API ZYM kit (bioMérieux, Marcy I'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 x 10^8 CFU mL^{-1}. 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 $K_2HPO_4 \cdot 3H_2O$, 0.5 g KH_2PO_4,
151 0.5 g $MgSO_4 \cdot 7H_2O$, 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-μL 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 (hPlg, 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 10^9 CFU mL^{-1}, and incubated for 15 min at 37 °C with 40 μg mL^{-1} of hPlg dissolved

176 in PBS. After that, the reaction mixture was washed twice with PBS to remove unbound hPlg, then
177 the bacterial cells with hPlg were suspended in 250 μL of 50 mM Tris-HCl (pH 7.5). Subsequently,
178 100-μL aliquots of the bacterial suspension were dispensed in each well of a 96-well microtitre plate.
179 The hPlg 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 μL of 0.54 mM D-valyl-leucyl-
182 lycine-*p*-nitroanilide dihydrochloride (S-2251, Merck KGaA) as a substrate. Absorbance at 405 nm
183 (A_{405nm}) 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_{405nm} 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 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 x *g* at 4 °C. The CFCS was
192 sterilized using 0.2 μm-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 µL 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^{-1}), 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^{-1} of ampicillin and 10000 IU mL^{-1} 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 $$\%\text{Acidity} = (V_{\text{NaOH}} \text{ x } M_{\text{NaOH}} \text{ x } E)/(V_{\text{sample}}) \text{ x } 100,$$

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 mL^{-1}. Four milliliter aliquots of the bacterial cell suspensions were mixed thoroughly
220 for 10 s, then incubated without agitation at 37 °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 °C without agitation. The ratio of auto-aggregation was expressed as

225 $$1 - (A_t/A_0) \text{ x } 100,$$

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

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

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

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

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

2-11. Statistical analysis

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

3. Results

3-1. Antibiotic susceptibilities of the FSMM strains

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

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

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

3-3. Hemolytic activities of the FSMM strains

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

3-4. Enzymatic activities of the FSMM strains

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

3-5. Mucin degradation activities of the FSMM strains

An apparent clear zone was observed in the positive control (Fig. S5A, Table 4). On the other hand, clear zone was not obvious in strain FSMM15 and LGG; however, very weak clear zones were seen in strains FSMM22 and FSMM26. When the tested strains were grown on the agar plates containing mucin and glucose, clear zone formation was not seen in all the tested strains (Fig. S5B, Table 4). When mucin was supplemented to the basal medium, only HFB could proliferate significantly (1.3-fold higher in OD_{600nm}). Simultaneous supplementation of mucin and glucose did not promote the cell growth compared to the solo glucose supplementation, hence the three FSMM strains were not capable of utilizing mucin as a carbon source (Data not shown). Fragmentation of mucin protein was observed only when the HFB was cultured in the basal medium supplemented with 0.3% HGM Type III (Figs. 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 hPlg to plasmin, indicating their
294 high binding abilities to hPlg (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 ± 0.02, 0.07 ± 0.00%Acidity), FSMM22 (pH 3.95
304 ± 0.02, 0.13 ± 0.00%Acidity), FSMM26 (pH 3.97 ± 0.02, 0.11 ± 0.01%Acidity), and LGG (pH 3.99
305 ± 0.01, 0.12 ± 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 μg 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 μg mL^{-1}), which was presumed to stem from a transition
325 mutation (A^{2058} to G^{2058}) 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 (Waśko *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 α-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; Kõll *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 β-glucosaminidase and α-galactosidase, which were likely to incorporate with α-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-β-glucosaminidase and α-galactosidase activities, hence risks of the α-
345 chymotrypsin activities in the FSMM strains are little. Clostridia and *Bacteroides*, which have high
346 levels of β-glucosidase activities, are the major causative bacteria for colon cancer, and most of
347 lactobacilli are known to produce much lower β-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 hPlg (Hurmalainen *et al.*
360 2007). Conflicting results were found in LGG in this report and the previous one (Hurmalainen *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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532 gastric mucin in vitro. *International Journal of Food Microbiology* **63**, 81–90.

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 hPlg binding activity was evaluated by measuring the

536 conversion of bound hPlg 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 hPlg

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 ± SD of three replications. Asterisks represent significantly

541 different with the negative control and pre-treated bacteria with hPlg 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 (μg 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 (μg 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
S. Typhimurium LT-2	12.2 ± 1.1^{Aa}	15.8 ± 2.8^{Aab}	18.4 ± 1.3^{Ab}	14.4 ± 1.3^{Aa}	15.2 ± 0.3^{Aab}	0^{Ac}
L. monocytogenes No. 154	10.1 ± 0.2^{Ba}	14.3 ± 0.6^{Ab}	13.9 ± 0.2^{Bb}	$13.0 \pm 0B^{bc}$	27.3 ± 0.6^{Bd}	9.0 ± 0^{Be}
MRSA No. 29	0^{Ca}	9.7 ± 0.6^{Bb}	8.2 ± 0.4^{Cc}	9.7 ± 0.6^{Cb}	0^{Ca}	9.0 ± 0^{Bbc}
MSSA No. 18	0^{Ca}	11.7 ± 0.3^{Cb}	11.7 ± 0.4^{Db}	10.1 ± 0.3^{Cc}	30.0 ± 0^{Dd}	15.3 ± 0.6^{Ce}
E. coli O157 No. S-12	8.6 ± 0.6^{Da}	10.6 ± 0.1^{CBb}	10.7 ± 0.3^{Db}	10.0 ± 0^{Cb}	8.0 ± 0^{Ea}	0^{Ac}
S. sonnei No. 134	10.3 ± 0.5^{Ba}	15.3 ± 0.4^{Ab}	15.8 ± 0.9^{Eb}	13.3 ± 0.4^{Bc}	11.0 ± 0^{Fa}	0^{Ac}

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

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	α-Chy	α-Gal	β-Glc	β-Glu	NA-β-Gluc	Agar plate assay	Test tube assay
	TCA	GCA	TDCA	GDCA	TCDCA	GCDCA										
FSMM15	–	–	–	–	–	–	–	α	–	+	–	–	+	–	–	–
FSMM22	–	–	–	–	–	–	–	α	–	+	–	–	+	–	–	–
FSMM26	–	–	–	–	–	–	–	α	–	+	–	–	+	–	–	–
LGG	–	–	–	–	–	–	–	α	–	+	–	–	+	–	–	–
L. reuteri ATCC8287	ND	ND	ND	ND	ND	ND	ND	γ	–	ND	ND	ND	ND	ND	ND	ND
E. faecalis 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; TCDCA, taurochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid; α, alpha type haemolysis; γ, gamma type haemolysis; α-Chy, α-chymotrypsin; α-Gal, α-galactosidase; β-Glc, β-glucuronidase; β-Glu, β-glucosidase; NA-β-Gluc, N-acetyl-β-glucosamindase.

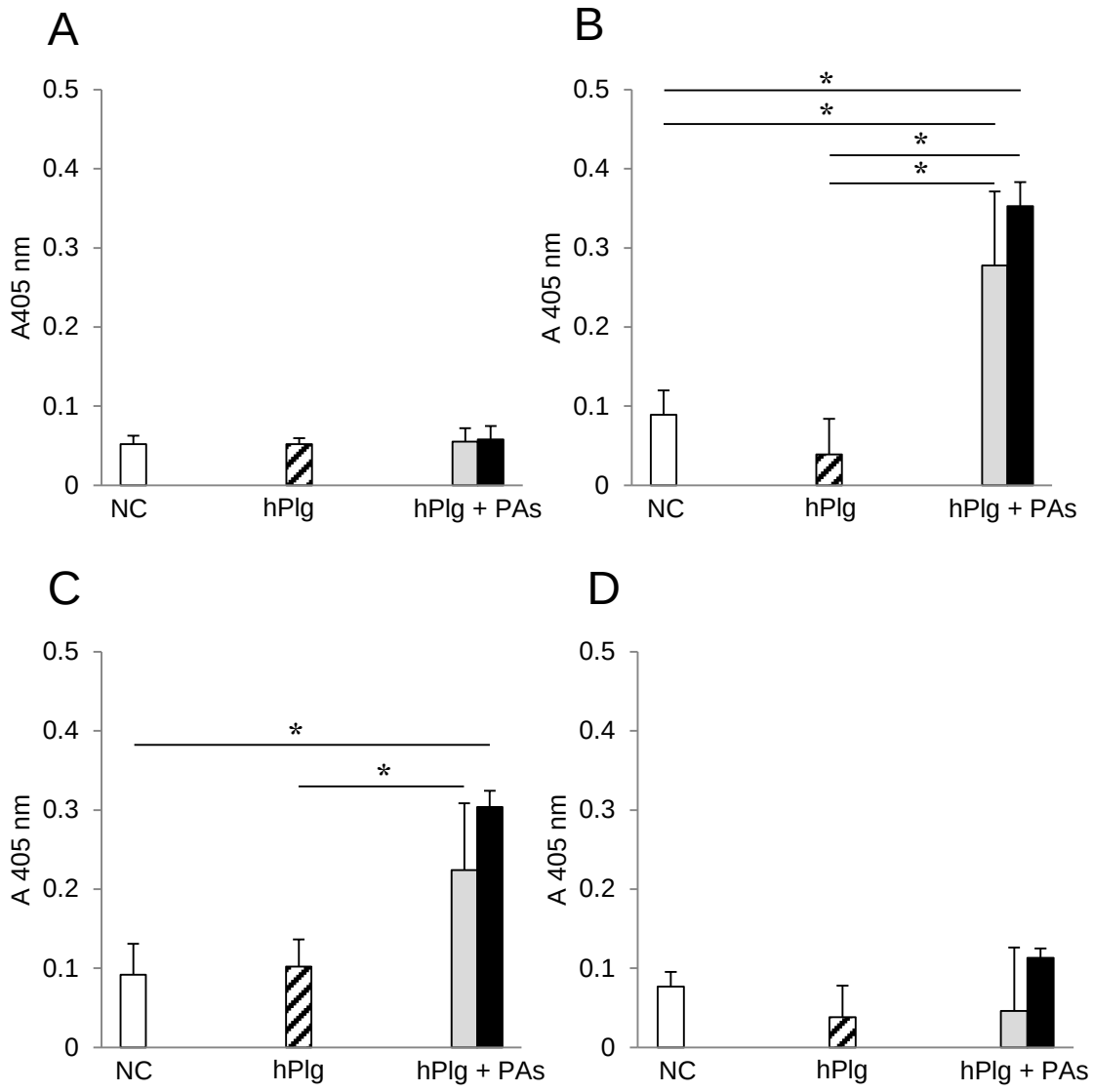

Fig. 1. Aryantini et al.