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# ORIGINAL ARTICLE

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

Ni Putu Desy ARYANTINI,<sup>1</sup> Eiki YAMASAKI,<sup>1</sup> Hisao KURAZONO,<sup>1</sup> I Nengah SUJAYA,<sup>2</sup> Tadasu URASHIMA<sup>1</sup> and Kenji FUKUDA<sup>1</sup>

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### ABSTRACT

Safety and probiotic characteristics such as antimicrobial activities of three *Lactobacillus rhamnosus* strains, FSMM15, FSMM22 and FSMM26, previously isolated as potential probiotics from fermented mare's milk were investigated. The three FSMM strains were susceptible to ampicillin, gentamycin, kanamycin, streptomycin, tetracycline and chloramphenicol, whereas they were resistant to erythromycin (minimal inhibitory concentration (MIC) =  $4-8 \mu g/mL$ ) and clindamycin (MIC =  $4 \mu g/mL$ ); bioconversion of bile salts, hemolytic activity and mucin degradation activity were negative; enzymatic activities of  $\alpha$ -chymotrypsin and  $\beta$ glucosidase were detected, but those of  $\alpha$ -galactosidase,  $\beta$ -glucuronidase and *N*-acetyl- $\beta$ -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.

#### 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 from 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 FSMM15, FSMM22 and 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 hemolysis 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

Correspondence: Kenji Fukuda, Department of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan. (Email: fuku@obihiro.ac.jp) Received 24 February 2016; accepted for publication 23 May 2016. 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, hemolytic 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.

## MATERIALS AND METHODS 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 precultured for 18h 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 from a 30-year-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 18h at 37°C with agitation (200 rpm for 1 min) at biosafety level 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 (European Food Safety Authority (EFSA) 2012).

### Bile acid bioconversion tests

### **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 mmol/L of taurocholic acid (TCA), taurochenodeo-xycholic acid (TCDCA), taurodeoxycholic acid (TDCA), glycocholic acid (GCDCA) and glycodeoxycholic acid (GDCA) were pre-incubated anaerobically at 37°C for 48 h, then each 10  $\mu$ L of overnight MRS culture broths of the three FSMM strains and LGG were 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). *Enterocccus faecalis* ATCC 19433 was use as a positive control.

#### 2. Biotransformation of cholic acid into deoxycholic acid

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

### Hemolytic activity tests

### **1.** Agar plate assay

The 18-h culture broths of the tested strains were washed twice with phosphate-buffered saline (PBS) and the cell population was adjusted to  $10^8$  colony forming units (CFU)/mL in PBS. About 10 µL of the cell suspensions were inoculated onto 5% sheep blood agar plates (Eiken Chemical Co., Ltd., Tokyo, Japan) and incubated at 37°C for 48 h under anaerobic conditions. *Lactobacillus brevis* ATCC 8287 was used as a  $\gamma$ -hemolysis control strain. Clear zone formation around the colonies was judged as  $\beta$ -hemolytic activity (true hemolysis). Color change of the media around the colonies into shaded brown or greenish was considered as  $\alpha$ -

hemolytic activity. Strains without any change were taken as lacking hemolytic activity ( $\gamma$ -hemolysis).

**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 buffer coat and plasma layer by centrifugation at  $1500 \times q$  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  $\mu$ L of PBS. Equal volume of the bacterial cells (10<sup>8</sup>) 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  $\times q$ 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.

## **Enzymatic activity test**

Enzymatic activities were determined using an API ZYM kit (bioMérieux, Marcy I'Etoile, France) according to the manufacturer's instructions. The tested strains were grown anaerobically in MRS broth at  $37^{\circ}$ C until the late exponential phase. Bacterial cells were harvested, then the cell population was adjusted to  $3 \times 10^{8}$  CFU/mL. Enzymatic activities were evaluated by comparison with the API ZYM color chart (bioMérieux).

## Mucin degradation activity test

## **1.** Agar plate assay

Hog gastric mucin was purified from 10 g of crude powder (HGM Type III, Merck KGaA) according to the previous report (Zhou et al. 2001). To check mucin degradation activities, 10 µL of 18-h main culture broths of each FSMM strains were inoculated onto agar plates (7.5 g tryptone, 7.5 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>, 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 L of deionized water, pH 7.2) that contained 0.5% (w/v) purified HGM Type III, then anaerobically incubated at 37°C for 72 h. Clear zones formed as a result of mucin degradation were visualized by staining with 0.1% (w/v) amido black dissolved in 3.5 mol/L acetic acid for 30 min at RT, followed by washing with 1.2 mol/L acetic acid until clear zones emerged. HFB grown in BHI broth and heatinactivated HFB culture were used as positive and negative controls, respectively.

## **2.** *Test tube assay*

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

## Plasminogen binding and activation tests

Presence of human plasminogen (hPlg, Merck KGaA) bound on bacterial cell surface was evaluated by plasmin activity assay as previously described (Bergmann et al. 2005). The tested strains at stationary phase were harvested by centrifugation (5000  $\times$  *g* for 5 min at RT), washed twice with PBS, adjusted to 10<sup>9</sup> CFU/mL and incubated for 15 min at 37°C with 40 µg/mL of hPlg dissolved in PBS. After that, the reaction mixture was washed twice with PBS to remove unbound hPlg, then the bacterial cells with hPlg were suspended in 250 µL of 50 mmol/L Tris-HCl (pH 7.5). Subsequently, 100-µL aliquots of the bacterial suspension were dispensed in each well of a 96-well microtiter plate. The hPlg was activated by addition of 0.24 kallikrein inhibitor unit (KIU) tissue plasminogen activator (tPA, Merck KGaA) or 0.06 KIU urokinase plasminogen activator (uPA, Merck KGaA), then the peptide cleavage reaction was performed at 37°C for 1 h using 30 µL of 0.54 mmol/L D-valyl-leucyl-lycine-*p*-nitroanilide dihydrochloride (S-2251, Merck KGaA) as a substrate. Absorbance at 405 nm (A<sub>405nm</sub>) was measured immediately after addition of S-2251 (time point =  $t_0$ ) and after 1-h incubation at 37°C (time point =  $t_1$ ). Plasmin activity was evaluated by subtracting  $A_{405nm}$  values at  $t_0$  from those at  $t_1$ .

# Antimicrobial activity against enteropathogenic bacteria

## 1. Cell-free culture supernatant preparation

MRS broth (100 mL) was inoculated with each overnight pre-culture of the three FSMM strains at  $10^5$  to  $10^6$  CFU/mL, then incubated anaerobically at 37°C for 24 h. Cell-free culture supernatant (CFCS) was obtained by centrifugation for 30 min at  $10\,000 \times g$  at 4°C. The CFCS was sterilized using 0.2 µm pore-size filters (Advantec, Tokyo, Japan). Four milliliter aliquots of neutralized (pH 7.0) and non-neutralized CFCSs were lyophilized, then reconstituted with sterilized 20 mmol/L sodium phosphate buffer (pH 6.0) to achieve 20-fold concentrated CFCSs. MRS broth was treated by the same procedure and used as a negative control.

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

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

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

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

# Auto- and co-aggregation properties and bacterial cell surface hydrophobicity

The three FSMM strains and LGG were cultured in MRS broth and harvested at the end of the exponential growth phase. The harvested cells were washed twice with PBS and re-suspended in PBS to be 10<sup>8</sup> cells/mL. Four milliliter aliquots of the bacterial cell suspensions were mixed thoroughly for 10 s, then incubated without agitation at 37°C. Auto-aggregation was monitored by measuring the absorbance of the culture supernatant at 600 nm. Co-aggregation was clarified only with *S*. Typhimurium LT-2, because all the tested strains showed inhibitory activities against the pathogen. Equal volumes (2 mL) of the FSMM strains and the pathogenic bacterial cells were mixed and incubated at 37°C without agitation. The ratio of auto-aggregation was expressed as

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

where  $A_t$  and  $A_0$  represented the values of absorbance 600 nm at the several time points (3, 6 and 24 h) and the initial time point (0 h), respectively. The ratio of co-aggregation was calculated at 24 h, according to Handley *et al.* (1987) as follows:

$$\left\{ \left( A_{pathog} + A_{lacto} \right) / 2 - \left( A_{mix} \right) / \left( A_{pathog} + A_{lacto} \right) / 2 \right\} \times 100,$$

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<sup>8</sup> CFU/mL 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:

% 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.

### **Statistical analysis**

Numeric data except antibiotic susceptibility and enzymatic profile were expressed as means  $\pm$  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.

### RESULTS

# Antibiotic susceptibilities of the FSMM strains

All the tested strains including LGG were susceptible to ampicillin (MIC =  $0.25 \ \mu g/mL$ ), gentamycin (MIC =  $2-4 \ \mu g/mL$ ), kanamycin (MIC =  $64 \ \mu g/mL$ ), streptomycin (MIC =  $8-16 \ \mu g/mL$ ), tetracycline (MIC =  $0.5-4 \ \mu g/mL$ ) and chloramphenicol (MIC =  $4 \ \mu g/mL$ ), whereas they were resistant to erythromycin (MIC =  $4-8 \ \mu g/mL$ ) and clindamycin (MIC =  $4 \ \mu g/mL$ ) (Table 1).

# 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 (Table 2), 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 (Table 2).

| method  |        |         |         |              |        |        |        |        |
|---------|--------|---------|---------|--------------|--------|--------|--------|--------|
| Strains |        |         |         | MIC values ( | ug/mL) |        |        |        |
|         | 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      |

 Table 1
 MIC values of Lactobacillus rhamnosus FSMM strains and LGG toward eight antibiotics determined by the broth microdilution method

Am, ampicillin; Cl, Clindamycin; Cm, Chloramphenicol; Em, Erythromycin; Gm, gentamycin; Km, kanamycin; LGG, *L. rhamnosus* GG; MIC, minimal inhibitory concentration; Sm, streptomycin; Tc, tetracycline. The microbial break points for the eight antibiotics are indicated in parentheses (μg/mL). MIC values surpassing the microbiological breakpoint proposed by the EFSA Panel on Additives and Products or Substances used in Animal Feed are shaded in gray (European Food Safety Authority (EFSA) 2012).

## Hemolytic activities of the FSMM strains

Brownish color observed in the surrounding areas of the colonies of the FSMM strains and LGG indicated their  $\alpha$ -hemolytic activities (Table 2). Because  $\alpha$ -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 (Table 2).

## Enzymatic activities of the FSMM strains

The three FSMM strains showed very similar patterns in the enzymatic activities with LGG (Table 2). In respect to potentially harmful enzymatic activities,  $\alpha$ -galactosidase,  $\beta$ -glucuronidase and *N*-acetyl- $\beta$ -glucosaminidase activities were undetectable, whereas  $\alpha$ -chymotrypsin and  $\beta$ glucosidase activities were found in all the tested strains.

# Mucin degradation activities of the FSMM strains

An apparent clear zone was observed in the positive control (Table 2). On the other hand, a 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 (Table 2). When mucin was supplemented to the basal medium, only HFB could proliferate significantly (1.3-fold higher in OD<sub>600nm</sub>). 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 (Table 2). Degradation of the carbohydrate moieties of mucin by the HFB was also confirmed by a decrease of stained area in the high molecular mass region (more than 150 kDa) on the SDS-PAGE gel (Table 2). The three FSMM strains and LGG showed less than 20% degradation of protein and carbohydrate moieties (data not shown), indicating that they apparently lacked mucin degradation activities according to the criteria suggested by Miller and Hoskins (1981).

# Plasminogen activation capabilities of the FSMM strains

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

# Antimicrobial activities of the FSMM strains

Strains FSMM22 and FSMM26 showed antimicrobial activities for all the six enteropathogenic bacteria as comparable levels to LGG, while strain FSMM15 lacked inhibitory activities against MRSA and MSSA (Table 3). When MRS medium adjusted to different pH was subjected to the disc diffusion assay, MRSA and MSSA formed haloes at pH 2, but other pathogens formed at pH 3 or 4, indicated higher acid tolerance of MRSA and MSSA (data not shown). Judging from the pH and acidity values of the CFCSs of strains FSMM15 (pH  $4.23 \pm 0.02$ , 0.07 ± 0.00% acidity), FSMM22 (pH 3.95 ± 0.02, 0.13 ± 0.00% acidity), FSMM26 (pH 3.97 ± 0.02, 0.11 ± 0.01% acidity), and LGG (pH  $3.99 \pm 0.01$ ,  $0.12 \pm 0.01\%$  acidity), the lack of inhibitory activities of strain FSMM15 against MRSA and MSSA were likely due to the lower acid production than other strains.

# Auto- and co-aggregation properties of the FSMM strains

Auto-aggregation of the tested strains progressed in a time-dependent manner (Table 4). Strains FSMM22 and FSMM26 showed significantly higher auto-aggregation properties compared to strain FSMM15 and LGG. None of the tested strains co-aggregated with *S*. Typhimurium LT-2, hence these strains are unlikely to exclude *S*. Typhimurium LT-2 from the host's gastro-intestinal tract by the co-aggregation mechanism.

| Strains                                       |   |   | Bile                                       | salt bioco                                   | nversion a                                    | ctivity                      |  | Hemolysi   | s activity                              | Ū                              | ndesirabl               | e enzym                 | atic activi               | ity                        | Muci<br>degrada<br>activit          | n<br>tion<br>y        |
|---|---|---|--|--|---|------------------------------|--|--|---|--------------------------------|-------------------------|-------------------------|---------------------------|----------------------------|-------------------------------------|-----------------------|
|   |   |   | Bile salt                                  | t deconjug                                   | ation   |                              | Conversion<br>from CA<br>to DCA                            | Agar plate<br>assay                                    | Test tube<br>assay                      | α-Chy                          | α-Gal                   | β-Glc                   | β-Glu                     | NA-β-<br>Gluc              | Agar plate<br>assay                 | Test<br>tube<br>assay |
|   | TCA   | GCA                                     | TDCA                                       | GDCA   | TCDCA   | GCDCA                        |  |  |   |                                |                         |                         |                           |                            |                                     |                       |
| FSMM15  | Ι   | Ι                                       | Ι  | Ι  | Ι   | I                            | I  | α  | I                                       | +                              | I                       | Ι                       | +                         | Ι                          | I                                   | Ι                     |
| FSMM22  | Ι   | I                                       | I  | I  | Ι   | I                            | Ι  | α  | I                                       | +                              | I                       | Ι                       | +                         | I                          | I                                   | Ι                     |
| FSMM26  | Ι   | I                                       | Ι  | I  | Ι   | I                            | I  | α  | I                                       | +                              | I                       | Ι                       | +                         | I                          | Ι                                   | Ι                     |
| DDT   | Ι   | I                                       | I  | Ι  | I   | I                            | Ι  | α  | Ι                                       | +                              | Ι                       | I                       | +                         | I                          | Ι                                   | I                     |
| L. reuteri                                    | ND  | ND                                      | ND   | QN   | QN  | ND                           | ND   | λ  | I                                       | QN                             | QN                      | QN                      | ND                        | ND                         | ND                                  | ΟN                    |
| ATCC8287                                      |   |   |  |  |   |                              |  |  |   |                                |                         |                         |                           |                            |                                     |                       |
| E. faecalis                                   | I   | Ι                                       | +  | +  | I   | I                            | ND   | ΟN   | ΠŊ                                      | ND                             | ND                      | ND                      | ND                        | ŊŊ                         | ND                                  | ŊŊ                    |
| HFB   | ND  | ND                                      | ND   | ND   | ND  | ND                           | ND   | ND   | ND                                      | ND                             | ND                      | ND                      | ND                        | ND                         | +                                   | +                     |
| Plus and min<br>glycodeoxych<br>motrvosin: α- | us indicate ]<br>olic acid; TC<br>7al. a-galact | positive al<br>DCA, taur<br>tosidase: B | nd negative<br>rochenodec<br>+-Glc. B-glue | e results, re<br>oxycholic ac<br>curonidase: | spectively. N<br>id; TCDCA, t<br>ß-Ghu, B-ohu | VD, not deter<br>aurochenode | mined; HFB, hui<br>:oxycholic acid; C<br>-R-Gluc, N-acetvl | man fecal bacte<br>A, cholic acid; I<br>-6-glucosamind | ria; TCA, taur<br>DCA, deoxychc<br>Jase | ocholic acio<br>dic acid; α, a | d; GCA, g<br>alpha typo | lycocholic<br>e haemoly | acid; TDC<br>sis; γ, gamı | 2A, taurode<br>ma type hae | eoxycholic acid;<br>emolysis; α-Chy | : GDCA,<br>⁄, α-chy-  |

### DISCUSSION

As mentioned by Bernardeau et al. (2008), clarification of antibiotic susceptibility patterns is considered to be the primary requirement for the safety assessment of Lactobacillus genus, and we found in this study all the three FSMM strains, as well as LGG, showed resistances against erythromycin and clindamycin with similarly moderate MIC values (4-8 µg/mL). Acquisition mechanism of resistance to macrolides (e.g. erythromycin) and lincosamides (e.g. clindamycin) are similar among pathogenic bacteria, and the major cause is considered as modifications of ribosomal genes, whereas efflux and inactivation of those antibiotics are less effective (Leclercq 2002). This seems to be the same for Lactobacillus; for example, human vaginal isolates of *L. rhamnosus* had very high erythromycin resistance (MIC =  $2048 \ \mu g/mL$ ), which was presumed to stem from a transition mutation  $(A^{2058} \text{ to } G^{2058})$  occurred in 23S rRNA (Begovic *et al.* 2009). Presence of *ermA*, *ermB* and *ermC* and the single mutation in 23S rRNA were determined as plausible causes of erythromycin resistance in L. rhamnosus Pen (Waśko et al. 2012). Further studies are needed to avoid the risk of disseminating antibiotic resistance genes from the FSMM strains to other bacteria by horizontal gene transfer.

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

Weak clear zones observed around the colonies of strains FSMM22 and FSMM26 on the mucin containing agar plates seemed to be an experimental artifact, because abrasions were observed in the edges of bacterial spots of strains FSMM22 and FSMM26 during the amido black destaining step.

It has been reported that most bacterial isolates from patients with endocarditis produced *N*-acetyl-βglucosaminidase and  $\alpha$ -galactosidase, which were likely to incorporate with  $\alpha$ -chymotrypsin to enhance hydrolysis of glycoproteins of the host, and therefore these enzymes lead tissues into being damaged during development of endocarditis (Oakey *et al.* 1995), whereas the three FSMM strains showed no detectable *N*-acetyl- $\beta$ -glucosaminidase and  $\alpha$ -galactosidase activities, hence risks of the  $\alpha$ -chymotrypsin activities in the FSMM strains are little. Clostridia and *Bacteroides*, which have high levels of  $\beta$ -glucosidase activities, are the major causative bacteria for colon cancer, and most lactobacilli are known to



Figure 1 Plasminogen binding and activation activities of strain FSMM15 (A), strain FSMM22 (B), strain FSMM26 (C) and LGG (D). The human plasminogen (hPlg) binding activity was evaluated by measuring the conversion of bound hPlg on the cell surface of tested strains into plasmin by addition of plasmin substrate (S-2251) in the presence of tissue plasminogen activator (tPA) (gray bars) and urokinase plasminogen activator (uPA) (filled bars). Bacterial cells incubated only with S-2251 were used as a negative control (NC, open bars). Bacterial cells pretreated with hPlg were incubated with S-2251 in the absence of PAs (hatched bars) to clarify presence of endogenous PA activities. The data represent means  $\pm$  SD of three replications. Asterisks represent significant differences with the NC and pre-treated bacteria with hPlg in the absence of PAs (P < 0.05)

Table 3 Antimicrobial activity of the cell-free culture supernatant of FSMM strains and *Lactobacillus rhamnosus* GG (LGG) against six enteropathogenic bacteria

| Enteropathogenic  |  | Ar   | ntimicrobial activit   | y (diameter in m  | m)   |  |
|---|--|--|--|---|--|--|
| bacteria  | FSMM15   | FSMM22   | FSMM26   | LGG   | Ampicillin   | Nisin  |
| S. Typhimurium LT-2<br>L. monocytogenes No. 154<br>MRSA No. 29<br>MSSA No. 18<br>E. coli O157 No. S-12<br>S. sonnei No. 134 | $\begin{array}{c} 12.2 \pm 1.1^{Aa} \\ 10.1 \pm 0.2^{Ba} \\ 0^{Ca} \\ 8.6 \pm 0.6^{Da} \\ 10.3 \pm 0.5^{Ba} \end{array}$ | $\begin{array}{c} 15.8 \pm 2.8^{Aab} \\ 14.3 \pm 0.6^{Ab} \\ 9.\ 7 \pm 0.6^{Bb} \\ 11.7 \pm 0.3^{Cb} \\ 10.6 \pm 0.1^{CBb} \\ 15.3 \pm 0.4^{Ab} \end{array}$ | $18.4 \pm 1.3^{Ab}$ $13.9 \pm 0.2^{Bb}$ $8.2 \pm 0.4^{Cc}$ $11.7 \pm 0.4^{Db}$ $10.7 \pm 0.3^{Db}$ $15.8 \pm 0.9^{Eb}$ | $14.4 \pm 1.3^{Aa} \\ 13.0 \pm 0^{Bbc} \\ 9.7 \pm 0.6^{Cb} \\ 10.1 \pm 0.3^{Cc} \\ 10.0 \pm 0^{Cb} \\ 13.3 \pm 0.4^{Bc} \\ 13.3 \pm 0.4^{Bc} \\ 10.0 \pm 0^{Cb} \\ 10$ | $15.2 \pm 0.3^{Aab}$ $27.3 \pm 0.6^{Bd}$ $0^{Ca}$ $30.0 \pm 0^{Dd}$ $8.0 \pm 0^{Ea}$ $11.0 \pm 0^{Fa}$ | $0^{Ac} \\ 9.0 \pm 0^{Be} \\ 9.0 \pm 0^{Bbc} \\ 15.3 \pm 0.6^{C} \\ 0^{Ac} $ |

The antimicrobial activities were evaluated by measuring the diameters of growth inhibition zones around the discs as mean  $\pm$  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 4 | Auto-aggregation, co-aggregation and cell-surface hydrophobicity of the FSMM strains and Lactobacillus rhamnosus GG |
|---------|---|
| (LGG)   |   |

| Strains |                     | Auto-aggregation      |                     | Co-aggregation with Salmonella | %                      |
|---------|---------------------|-----------------------|---------------------|--------------------------------|------------------------|
|         | 3 h                 | 6 h                   | 24 h                | Typhimurium LT-2               | hydrophobicity         |
| FSMM15  | $6.2 \pm 5.2^{Aa}$  | $24.4\pm6.4^{\rm Ab}$ | $51.6 \pm 5.6^{Ac}$ | $-0.3 \pm 0.2$                 | $90.8 \pm 2.1^{A}$     |
| FSMM22  | $78.7 \pm 2.7^{Ba}$ | $96.3 \pm 2.3^{Bb}$   | $99.7 \pm 0.6^{Bc}$ | $-2.1 \pm 1.6$                 | $99.7 \pm 0.1^{B}$     |
| FSMM26  | $71.8 \pm 8.6^{Ba}$ | $86.5 \pm 2.9^{Bb}$   | $97.1 \pm 1.3^{Bb}$ | $-0.4 \pm 0.2$                 | $99.5 \pm 0.2^{B}$     |
| LGG     | $11.6 \pm 3.7^{Aa}$ | $30.1 \pm 4.0^{Ab}$   | $48.5 \pm 4.7^{Ac}$ | $0.0 \pm 0.1$                  | $61.3 \pm 4.5^{\circ}$ |

Data are presented as mean  $\pm$  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 were negative, co-aggregation activities with *S*. Typhimurium LT-2 were not included in the statistical analysis.

produce much lower  $\beta$ -glucosidase activities than such major causative pathogens (Wollowski *et al.* 2001).

Some pathogenic bacteria including *Helicobacter pylori* are known to capture the host-producing plasminogen via lysine residues of their cell surface proteins and to utilize it as their own tool to degrade the host's extracellular matrix (ECM) proteins (Lähteenmäki et al. 2005). Plasminogen is activated by tPA and uPA as well as by prokaryotic activators such as staphylokinase and streptokinase, resulting in a formation of a proteolytic enzyme, plasmin (Lähteenmäki et al. 2001). Strain FSMM15 had no remarkable plasminogen binding ability as well as LGG, while strains FSMM22 and FSMM26 showed significantly high capacity of plasminogen binding, indicating that plasminogen binding ability should be strain-dependent. This observation corresponded to a previous report in which cell surface associated proteins extractable with PBS from several probiotic and non-probiotic strains, including L. gallinarum T-50, L. johnsonii F133, L. amylovorus JCM 5807, L. gasseri JCM 1130/ATCC 19992, and LGG, demonstrated different binding abilities to hPlg (Hurmalainen et al. 2007). Conflicting results were found in LGG in this report and a previous one (Hurmalainen et al. 2007), but this is likely to stem from differences in experimental conditions such as incubation time with substrates. Ishibashi and Yamazaki (2001) mentioned that cell surface proteins of bacteria were associated with their aggregation abilities, which can be evaluated by measuring the cell surface hydrophobicity. It has been revealed that the amount of cell surface proteins of strain FSMM22 extracted with 1 mol/L LiCl was apparently higher than strain FSMM15 (unpublished data). Therefore, we speculated that a larger amount of cell surface proteins in strain FSMM22 reinforced the co-aggregation and the binding of plasminogen on the cell surface using hydrophobic interactions as the major driving force.

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

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

### Conclusions

Safety assessments and probiotic characteristics such as antimicrobial activities were investigated on the three potential probiotic strains isolated from fermented mare's milk in vitro. As far as the safety and probiotic characteristics investigated in this study, strain FSMM15 was similar to LGG, while strains FSMM22 and FSMM26 were very alike. Moreover, it was apparent that these characteristics were entirely strain-dependent. Despite broader antipathogenic spectrum of strains FSMM22 and FSMM26, FSMM15 was taken as the best probiotic candidate due mainly to the lack of plasminogen binding ability. However, risks of strain FSMM15 for human health remains latent, as being mentioned for LGG as a causative agent of opportunistic infection. To ensure this, in vivo experiments should be further performed. It is also important to elucidate presence of pili, biogenic amine and D-lactate productions, and molecular mechanism of horizontal transfer of antibiotic resistant genes in strain FSMM15.

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