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Selection of Potential Probiotic *Lactobacillus* with Inhibitory Activity Against *Salmonella* and Fecal Coliform Bacteria

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Abstract Three hundred and sixty presumptive lactic acid bacteria (LAB) isolated from pregnant sows, newborn, suckling, and weaned piglets were preliminarily screened for anti-*Salmonella* activity. Fifty-eight isolates consisting of *Lactobacillus reuteri* (n = 32), *Lactobacillus salivarius* (n = 10), *Lactobacillus mucosae* (n = 8), *Lactobacillus johnsonii* (n = 5), and *Lactobacillus crispatus* (n = 3) were selected and further characterized for probiotic properties including production of antimicrobial substances, acid and bile tolerance, and cell adherence to Caco-2 cells. Eight isolates including *Lact. johnsonii* LJ202 and *Lact. reuteri* LR108 were identified as potential probiotics. LJ202 was selected for further use in co-culture studies of two-bacterial and multiple-bacterial species to examine its inhibitory activity against *Salmonella enterica* serovar Enteritidis DMST7106 (SE7106). Co-culture of LJ202 and SE7106 showed that LJ202 could completely inhibit the growth of SE7106 in 10 h of co-culture. In co-culture of multiple-bacterial species, culturable fecal bacteria from pig feces were used as representative of multiple-bacterial species. The study was performed to examine whether interactions among multiple-bacterial species would influence antagonistic activity of LJ202 against SE7106 and fecal coliform bacteria. Co-culture of SE7106 with different combinations of fecal bacteria and probiotic (LJ202 and LR108) or non-probiotic (*Lact. mucosae* LM303) strains revealed that the growth of SE7106 was completely inhibited either in the presence or in the absence of probiotic strains. Intriguingly, LJ202 exhibited notable inhibitory activity against fecal coliform bacteria while LR108 did not. Taken together, the results of co-culture studies suggested that LJ202 is a good probiotic candidate for further study its inhibitory effects against pathogen infections in pigs.

Keywords Pig probiotic · Anti-*Salmonella* activity · *Lact. johnsonii* · *Lact. reuteri* · Fecal coliform bacteria

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Introduction

Salmonellosis, an infection with bacteria called *Salmonella*, is a leading cause of foodborne illness affecting humans and animals. Although animals, like pigs, infected with *Salmonella* do not normally show clinical sign of symptoms, the carcasses and meats are main reservoirs of the pathogen [1]. Salmonellosis outbreaks have been annually reported in the USA and European Union. Antibiotics have been used for prevention and treatment of pathogenic diseases. However, the extensive use of antibiotics in livestock has linked to the emergence of antibiotic-resistant bacteria found in humans and animals [2]. As a result, the European Union has banned the use of several antibiotics in farm animals (Regulation 1831/2003/EC) [3]. Since then, the use of probiotics has received great attention as an alternative to antibiotics.

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit to the host [4]. Probiotic *Lactobacillus* has been used for promoting and improvement of animal health [5–8]. The use of probiotics to combat *Salmonella* infection has been widely studied in poultry [9–11]. However, little attention has been paid to find probiotics with protective activity against *Salmonella* infection in pigs. The following are few examples of the studies of pig probiotics with protective activity against *Salmonella*

infection. *Lactobacillus acidophilus* LAP5 isolated from swine was shown to inhibit the growth of *Salmonella* Typhimurium in vitro and reduce invasion of *Salmonella* Choleraesuis to human Caco-2 cell line [12, 13]. Casey et al. showed that feeding pigs with mix strains of probiotic *Lactobacillus* and *Pediococcus* bacteria reduced incidence, severity, and duration of diarrhea in pigs challenged with *Salmonella* Typhimurium [14].

In this study, it aimed to search for probiotic *Lactobacillus* with protective effects against *Salmonella* infection in pigs. To do this, *Lactobacillus* isolated from pigs of four ages, pregnant sows, newborn, suckling, and weaned piglets, were preliminarily screened for anti-*Salmonella* activity. The strains exhibiting anti-*Salmonella* activity were selected and further characterized for probiotic properties such as acid and bile salt tolerance, adhesion to Caco-2 cells, and production of antimicrobial substances. Eight potential probiotic strains were obtained. A potential probiotic strain, *Lactobacillus johnsonii* LJ202, was chosen for co-culture studies of two-bacterial species and multiple-bacterial species to gain insight into its inhibitory effects on the growth of *Salmonella* Enteritidis DMST7106 and fecal coliform bacteria.

Materials and Methods

Isolation of Lactic Acid Bacteria

Fecal samples were collected from three pigs of each age group comprising pregnant sows (90 days of pregnancy),newborn piglets (3 days of age), suckling piglets (10 days of age), and weaned piglets (30 days of age, weaning at 28 days of age).Twenty-five grams of each fecal sample were mixed with 225 ml of 0.1% (*w*/*v*) peptone-buffered water. The fecal samples were beaten in a stomacher (IUL instruments, Barcelona, Spain) at 2000 rpm with 3 cycles of 1 min beating and 1 min pause. The fecal slurry was filtered through gauze. A hundred microliters of the filtrate was diluted in a decimal series, and 100 μl of appropriate dilution were spread on de Man Rogosa, Sharpe (MRS) agar (BD, MD, USA) supplemented with 0.5% $CaCO_3$.The plates were incubated at 37 °C for 24–48 h in anaerobic jars using the Gas Pak system (AnaeroPack®_Anaero, MCG, Japan).The colonies surrounding with clear zone were selected from each fecal sample and tested for catalase activity. The isolates which did not exhibit catalase activity were selected for further analysis.

Anti-*Salmonella* Activity

The presumptive lactic acid bacteria (LAB) were preliminarily screened for the ability to suppress the growth of *Salmonella* spp. using agar spot test [15]. Briefly, three microliters of LAB cultures were individually spotted on bottom agar containing 1.5% MRS. Then, the molten top agar containing 0.7% tryptic soy agar (TSA) was mixed with 10 μl of indicator culture (10^9 CFU/ml) and poured on top of the bottom agar. The indicator strains used in this experiment were *Salmonella* Choleraesuis DMST5880, *Salmonella* Choleraesuis DMST8014, *Salmonella* Enteritidis DMST7106, and *Salmonella* Typhimurium DMST562. The plates were incubated at 37 °C for 18–24 h. The inhibition zones were recorded.

16S rRNA Sequence Analysis

Bacterial DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, MD, USA). The primers 27F (5′–AGAGTTTGATCMTGGCTCAG–3′) and 1492R (5′–TACGGYTACCTTGTTACGACTT3–′) were used for PCR amplification of 16S ribosomal RNA (rRNA) region. The PCR products were subjected to nucleotide sequencing (Macrogen, Seoul, Korea). BLAST nucleotide algorithm was used to search for similar sequences in the NCBI database.

Antimicrobial Activity of Cell-Free Culture Supernatant

Well diffusion was performed to investigate whether the CFS of *Lactobacillus* spp. contained antimicrobial substances which could inhibit the growth of *Salmonella* spp. [16]. Briefly, 1.5% TSA was melted, mixed with an indicator culture, and poured into 90 mm Petri dish plates. Then 7-mm wells were made, and 80 μl of neutralized and non-neutralized CFS of a *Lactobacillus* culture were added into each well. For the control, 80 μl of MRS pH 6.8 were added into wells. The indicator strains used in this experiment were *Salmonella* Choleraesuis DMST5880, *Salmonella* Choleraesuis DMST8014, *Salmonella* Enteritidis DMST7106, and *Salmonella* Typhimurium DMST562. Inhibition zones were recorded. Three replicates were performed for each sample.

Acid and Bile Salt Tolerance

The *Lactobacillus* isolates were anaerobically grown in MRS for 18 h at 37 °C. The cells were collected by centrifugation, washed once with PBS, and suspended in MRS. The initial cell number was determined by plate count. One hundred microliters of cell suspension were inoculated into 10 ml of MRS adjusted to pH 2.5 with 1 M HCl. The cell suspensions were incubated at 37 °C for 4 h. After incubation, 1 ml of the cell suspensions was used for determination of viable cells by plate count. The remaining was centrifuged, washed, and suspended in 9 ml of MRS containing 0.5% oxgall (Sigma-Aldrich; MD, USA). The cell suspensions were incubated at 37 °C for 24 h, and viable cell counts were determined. Three

replicates were performed for each strain. The ability of isolates to tolerate to acid or bile salts was indicated as percentage of survival which was calculated as follows.

% survival (after incubation in acid) = $(\log_{10} N_1/\log_{10} N_0) \times 100$

% survival (after incubation in bile) = $(\log_{10} N_2/\log_{10} N_1) \times 100$

Where $\log_{10} N_0$ is the initial cell count (number of bacterial cells before incubation in acid), and $\log_{10} N_1$ and $\log_{10} N_2$ are viable cells after incubation in acid and bile salts, respectively.

Adhesion Assay

Cell adhesion assay was followed the method described by Chauvière et al. [17]. Briefly, Caco-2 cells (ATCCHTB-37) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM, 25 mM glucose, 4 mM L-glutamine, 1 mM sodium pyruvate) (Gibco, Life Technologies, NY, USA), supplemented with 10% (*v*/*v*) fetal bovine serum (Gibco, Life Technologies, NY, USA). The cells were seeded at 10^4 cells/well in a 24-well plate (Corning Incorporated, NY, USA) and incubated until a confluent monolayer formed. Prior to performing the assay, Caco-2 monolayers were washed twice with phosphate-buffered saline (PBS). Overnight culture of LAB isolates were centrifuged, washed twice with PBS, and suspended in DMEM. The initial bacterial cell counts were determined by plate count. Five hundred microliters of the cell suspensions were added into three replicate wells. The plates were incubated at 37 °C. After 90 min of incubation, the bacterial cell suspension was removed and the Caco-2 cells were washed three times with PBS to remove unbound bacteria. The bound bacteria were removed from Caco-2 cells by addition of 500 μl of 0.1% Triton X-100, and the number of adherent cells was determined by plate count method. Three replicates were performed for each strain.

Hemolytic Activity

LAB cultures were streaked in triplicates on sheep blood agar (Department of Medical Science, National Institute of Health, Nonthaburi, Thailand). The plates were incubated at 37 °C for 24–48 h and examined for hemolytic patterns. Three types of hemolysis can occur and are classified as β-hemolysis (clear zones around colonies), α-hemolysis (green-hued zones around colonies), and γ-hemolysis (no zones around colonies).

Antagonistic Activity Test

The potential probiotics were examined for their antagonistic activity against bacteria isolated from pigs, such as *Streptococcus gallolyticus*, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus villorum*, and *Lactobacillus* spp., using agar spot test [15]. In brief, 3 μl of overnight cultures of probiotic strains were spotted on the bottom agar containing 1.5% MRS. Then, the molten top agar containing 0.7% TSA was mixed with 10 μl of an indicator culture (10^9 CFU/ml) and poured on top of the bottom agar. Reciprocally, *Streptococcus*, *Enterococcus*, and *Lactobacillus* isolated from pigs or other probiotic strains were spotted on appropriate bottom agar. Then, overnight culture of a probiotic strain was used to make bacterial lawn. Antagonistic activity was recorded by measuring the radii of inhibition zones. Two replicates were performed for each strain.

Co-Culture of Potential Probiotic and *Salmonella*

Overnight culture of probiotic *Lact. johnsonii* LJ202 (10^6 CFU/ml) and *Salmonella* Enteritidis DMST7106 (10^3 CFU/ml) were inoculated into 10 ml of co-culture medium containing an equal volume of double strength MRS and double strength tryptic soy broth (TSB), (MRS/TSB). The medium was incubated at 37 °C, without shaking. The co-culture medium containing only LJ202 or SE7106 was used as controls. Viable cells of LJ202 and SE7106 were monitored at 0, 2, 4, 6, 8, 10, 12, 14, and 24 h by plate count on MRS and xylose lysine deoxycholate (XLD) agar plates (BD, MD, USA), respectively. The pH values of the culture medium were determined at 0, 2, 4, 6, 8, 10, 12, 14, and 24 h. Three replicates were performed for each sample.

Co-Culture of Probiotic with *Salmonella* and Fecal Bacteria

Ten grams of pig feces were collected from a healthy suckling piglet (age 25 days). The fecal sample was determined as *Salmonella* free. The fecal sample was mixed with 90 ml of 0.1% buffered peptone water, and beaten using a stomacher (IUL instruments, Barcelona, Spain) at 200 rpm per minute for 2 min. The fecal slurry was filtered through gauze, and the filtrate was collected. One milliliter of the supernatant was inoculated into 9 ml of co-culture medium (MRS/TSB) and incubated at 37 °C for 18 h. The culturable fecal culture was used as a representative of mixed-bacterial species from pigs. *Lact. johnsonii* LJ202, *Lactobacillus reuteri* LR108, and *Lactobacillus mucosae* LM303 were individually grown in MRS/TSB at 37 °C for 18 h. SE7106 was grown in MRS/TSB at 37 °C for 18 h. To evaluate the inhibitory activity of probiotic and non-probiotic strains on the growth of fecal coliform bacteria and SE7106, four types of bacterial cultures were made: (i) fecal culture (1% (*v*/*v*) inoculum) was inoculated into 10 ml of MRS/TSB and served as the control; (ii) fecal culture (1% (*v*/*v*) inoculum) and SE7106 (10^6 CFU/ml) were inoculated into 10 ml of MRS/TSB; (iii) overnight culture of LJ202, LR108, and LM303 were individually inoculated into 10 ml of MRS/TSB at 10^6 CFU/ml, followed by 1%

(*v*/*v*) of fecal culture; and (iv) overnight culture of LJ202, LR108, and LM303 were individually inoculated into 10 ml (10^6 CFU/ml) of MRS/TSB followed by fecal culture (1% (*v*/*v*) inoculum) and SE7106 at 10^6 CFU/ml. The culture samples were incubated at 37 °C for 24 h without shaking. Cell numbers of presumptive lactic acid bacteria (LAB), *Salmonella*, and fecal coliforms were monitored at 0 and 24 h by plate counts on MRS containing 0.5% $CaCO_3$, XLD, and MacConkey plates (BD, MD, USA), respectively. Three replicates were performed for each sample. The mean cell counts of presumptive LAB and fecal coliforms of each culture sample were compared with those of fecal culture alone using paired *t* test.

Results

Preliminary Screening for Lactic Acid Bacteria with Anti-*Salmonella* Activity and Strain Identification

A total of three hundred and sixty isolates which showed clear zone on MRS containing 0.5% $CaCO_3$ and exhibited no catalase activity were collected from fecal samples of pregnant sows, newborn, suckling, and weaned piglets. The isolates were preliminarily tested for inhibitory activity against *Salmonella* Choleraesuis DMST5880, *Salmonella* Choleraesuis DMST8014, *Salmonella* Enteritidis DMST7106, and *Salmonella* Typhimurium DMST562 by agar spot test. Sixty isolates which inhibited the growth of *Salmonella* spp. were selected.

The sixty isolates were subjected to16S rRNA sequencing. The analysis revealed that the sixty isolates comprised of five *Lactobacillus* species and two *Enterococcus* species which were *Lact. reuteri* (*n* = 32, 53.3%), *Lactobacillus salivarius* (*n* = 10, 17.6%), *Lact. mucosae* (*n* = 8, 13.3%), *Lact. johnsonii* (*n* = 5, 8.3%), *Lactobacillus crispatus* (*n* = 3, 5%), *Ent. faecalis* (*n* = 1, 1.6%), and *Ent. faecium* (*n* = 1, 1.6%). *Lact. reuteri* was the most abundant species among the isolates. All *Lactobacillus* isolates were further evaluated for their probiotic properties.

Evaluation of Probiotic Properties

CFSs of the fifty-eight *Lactobacillus* isolates were investigated for their abilities to produce antimicrobial substances by agar well diffusion assay. The assay showed that the non-neutralized CFSs of 17 isolates of *Lact. reuteri*, 10 isolates of *Lact. salivarius*, 2 isolates of *Lact. johnsonii*, and 3 isolates of *Lact. mucosae* could suppress the growth of one of the four *Salmonella* serovars used in this study (data not shown; the strains which their CFS showed inhibitory activity against *Salmonella* spp. were marked with asterisks (Fig. 1)). However, antimicrobial activity of the CFSs was drastically reduced when the CFSs were neutralized to pH 6.8.

The abilities of *Lactobacillus* isolates to tolerate to acid and bile salts were further examined. The result showed that most of the isolates were able to survive at low pH (pH 2.5) after incubation for 4 h in acid medium (Fig. 1), except for the three isolates of *Lact. crispatus* which were highly sensitive to acidic environment. After exposure to acid, the cells were subsequently incubated with 0.5% oxgall for 24 h. Incubation with bile salts caused substantial cell death in most of *Lact. salivarius* and *Lact. mucosae* isolates (survival rate <60%). In contrast, most of *Lact. reuteri* and *Lact. johnsonii* isolates showed high tolerance to bile salts (survival rate >70%).

The *Lactobacillus* isolates which tolerated to acid and bile salts and had the abilities to produce antimicrobial substances were selected for cell adhesion assay. The isolates showed variable adhesion abilities. Most of *Lact. reuteri*, *Lact. johnsonii*, and *Lact. salivarius* isolates had higher adherence capability (8 $\log_{10}$–11 $\log_{10}$ CFU/ml) than *Lact. mucosae* and *Lact. crispatus* isolates(6 $\log_{10}$–9 $\log_{10}$ CFU/ml) (Fig. 2).

Selection of Probiotic Candidates

Three criteria were used for selection of potential probiotics: (i) the isolates must have survival rate higher than 80% after exposure to acid and bile salts, (ii) they could inhibit the growth of *Salmonella* spp.(the four serovars used), and (iii) the number of bacterial cells adhering to Caco-2 cells should be higher than 7 $\log_{10}$ CFU/ml. Based on the selection criteria, six isolates of *Lact. reuteri* (LR105, LR108, LR111, LR310, LR311, LR401), one isolate of *Lact. johnsonii* (LJ202), and one isolate of *Lact. salivarius* (LS404) were selected.

Potential Probiotic Possessed No Hemolytic Activity

For safety use in pigs, the probiotic strains were tested for hemolytic activity on sheep blood agar. The test showed that none of the probiotic strains exhibited β-hemolysis. Most of the isolates exhibited γ-hemolysis (LR105, LR108, LR111, LR310, LR311, LR401) while a few isolates displayed α-hemolysis (LJ202, LS404). Therefore, all probiotic strains were considered to be safe for use in pigs.

Antagonistic Activity Among Probiotics and Some Bacteria Isolated from Pigs

The potential probiotics were tested for antagonistic activity among them and against some of the bacteria isolated from pigs. The result revealed that probiotic *Lact. reuteri*, *Lact. johnsonii*, and *Lact. salivarius* strains had strong antagonistic activities against *Strep. gallolyticus*, *Ent. faecium*, *Ent. faecalis*, and *Ent. villorum* and a weak antagonistic effect on some *Lactobacillus* isolates, but not *Lact. mucosae* strains

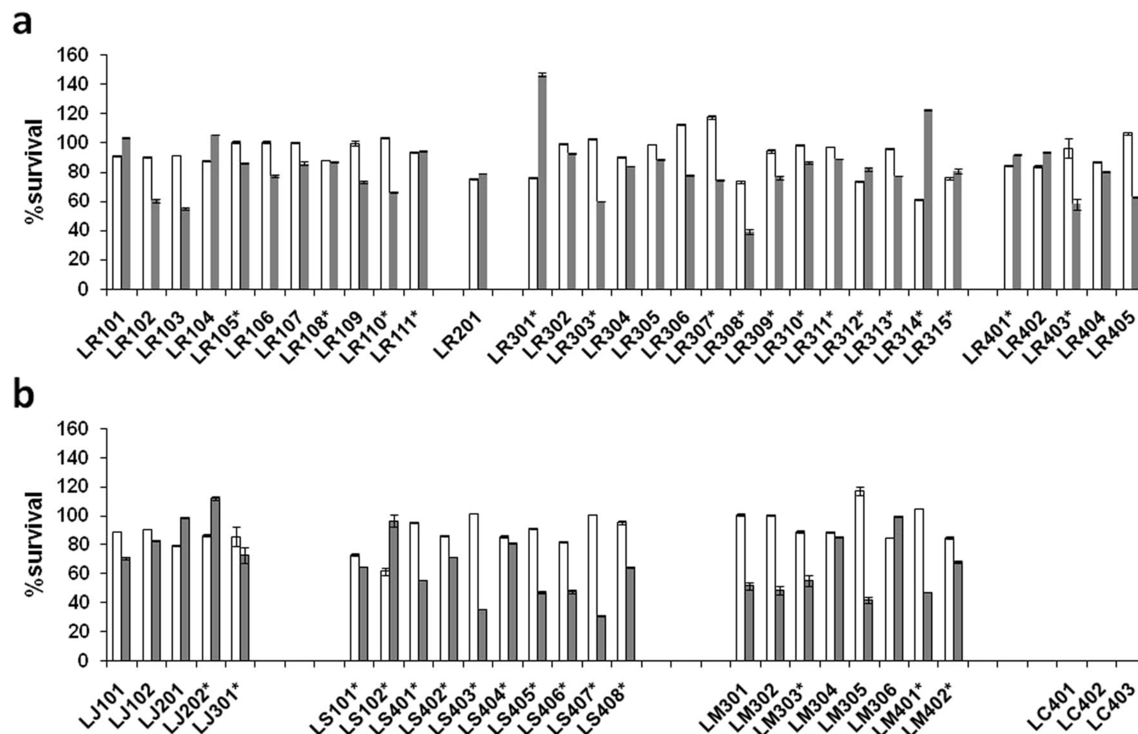

Fig. 1 The ability of *Lactobacillus* isolates to tolerate to acid and bile salts. **a**, **b** Survival rate of the *Lactobacillus* isolates after sequential incubation in acidic medium (pH 2.5) for 4 h (*white bars*) and 0.5% oxgall for 24 h (*grey bars*). *LR Lactobacillus reuteri*, *LJ Lactobacillus johnsonii*, *LS Lactobacillus salivarius*, *LM Lactobacillus mucosae*, *LC Lactobacillus crispatus*. The *numbers* 1xx, 2xx, 3xx, and 4xx behind the *Lactobacillus* species names referred to pregnant sows, newborn, suckling, and weaned piglets, respectively. *Asterisks* marked the isolates whose cell-free supernatant inhibited the growth of *Salmonella* spp., as determined by well diffusion assay

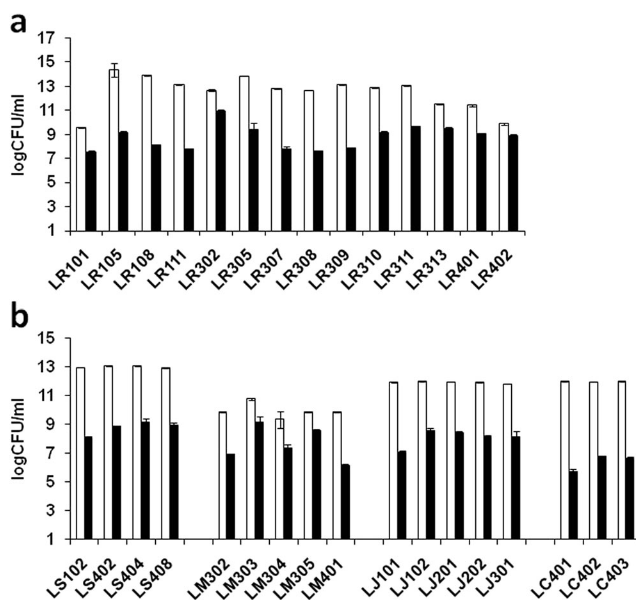

Fig. 2 The ability of *Lactobacillus* isolates to adhere to Caco-2 cells. **a** Adherence capabilities of *Lactobacillus reuteri* isolates. **b** Adhesion abilities of *Lactobacillus salivarius*, *Lactobacillus mucosae*, *Lactobacillus johnsonii*, and *Lactobacillus crispatus*. The initial bacterial cell counts used for cell adhesion assay are depicted as *white bars*. The *black bars* represent the number of bacterial cells adhered to Caco-2 cells

(Fig. 3). None of *Streptococcus* and *Enterococcus* used as indicator bacteria had antagonistic activity against the probiotic strains. However, it was found that most of probiotic *Lact. reuteri* strains and *Lact. johnsonii* LJ202 as well as some *Lact. salivarius* isolates could inhibit the growth of *Lact. salivarius* LS404 (data not shown).

Lact. johnsonii LJ202 Inhibited the Growth of *Salmonella* Enteritidis DMST7106 in the Co-Culture Study

Since all of the probiotic candidates showed the ability to inhibit *Salmonella* spp. in agar spot test and well diffusion assay, it was of interest to gain insight into how the probiotic strains inhibit the growth of *Salmonella* spp. Probiotic *Lact. johnsonii* LJ202 whose CFS showed notable inhibitory activity against *Salmonella* spp. was chosen for co-culture study with SE7106. Prior to co-culture study, LJ202 and SE7106 were preliminarily tested for their ability to grow on the co-culture medium, MRS/TSB. The result showed that both bacteria grew very well in MRS/TSB (Fig. 4a). Co-culture of LJ202 and SE7106 revealed that the number of SE7106 increased from 3 $\log_{10}$ to 5 $\log_{10}$ CFU/ml in 2 h. After that, the SE7106 count slightly decreased to 4 $\log_{10}$ CFU/ml at 4 h of co-culture and remained almost constant until 8 h (Fig. 4a). In contrast to SE7106, the number of LJ202in the co-culture

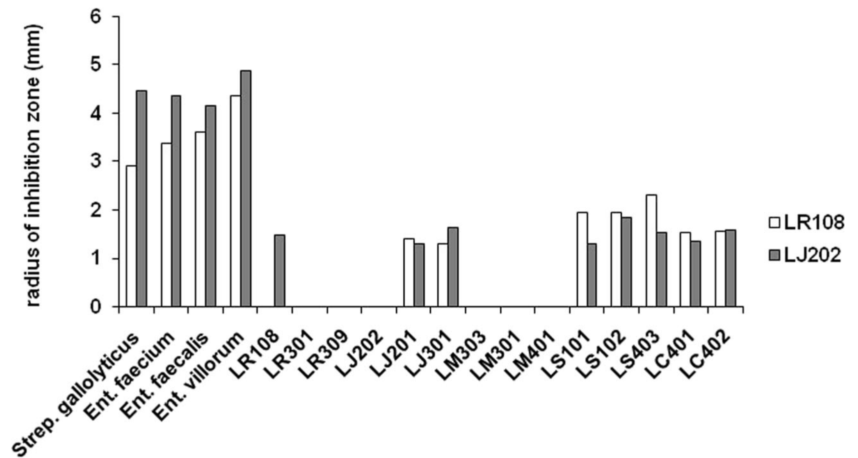

Fig. 3 Antagonistic effects of potential probiotic strains against *Streptococcus*, *Enterococcus*, and *Lactobacillus* isolated from pig feces. The figure shows a part of the data obtained from antagonistic activity test. Antagonistic activity of probiotic strains against the bacteria isolated from pig feces was determined by measuring the radii of inhibition zones. *White and grey bars* show the radii of inhibition zones produced by LR108 and LJ202 against the indicator strains

showed a steady increase until 8 h. At 10 h of co-culture, the number of LJ202 decreased by 2.5 $\log_{10}$ CFU/ml while the number of SE7106 sharply decreased to undetectable level (<10 CFU/ml) through the end of the experiment. After 10 h of co-culture, the number of LJ202 slowly increased and reached the maximum cell count at 24 h of co-culture. The maximum counts of LJ202 in mono- and co-culture were similar. Monitoring pH of the mono- and co-culture media of LJ202 revealed that the pH gradually decreased from 6.8 to approximately 4.0 at 8 h of co-culture and remained at pH 4.0 through the end of the experiment (Fig. 4b).

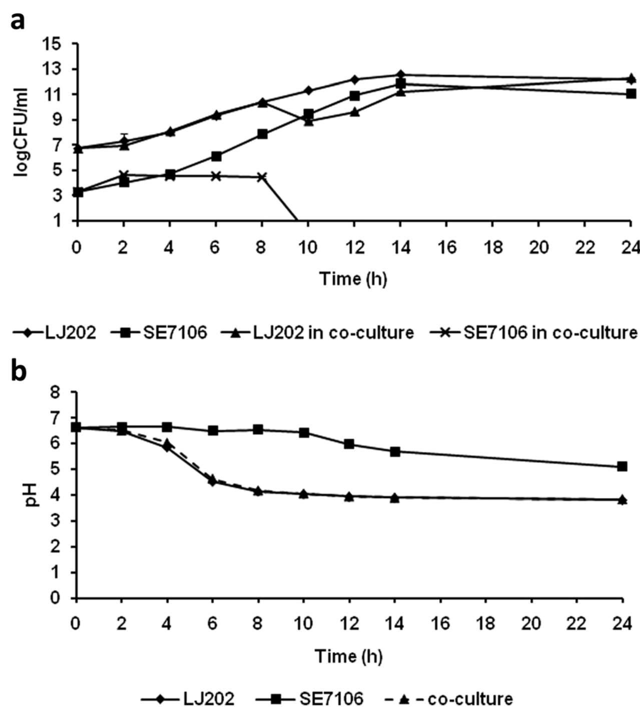

Fig. 4 Co-culture of *Lactobacillus johnsonii* LJ202 and SE7106. **a** Mono- and co-culture of LJ202 and SE7106. **b** pH values of the mono and co-culture media

Antagonistic Activity of LJ202 Against Fecal Coliform Bacteria and SE7106 in a Mixed Bacterial Community

In the gastrointestinal (GI) tract of pigs, there are many species of bacteria that interactions with one another occur. Interactions among bacterial species may modulate the diversity, behaviors, and activities of the individual species existing in the complex communities [18]. Therefore, it was of interest to investigate whether interactions among multiple-bacterial species might influence antimicrobial activity of LJ202 against SE7106. To do this, co-culture of probiotic strains with multiple-bacterial species and SE7106 was performed. The culturable fecal bacteria were used as representative of multiple-bacterial species from pigs. Probiotic LJ202 and *Lact. reuteri* LR108were used to compare their inhibitory activities. The non-probiotic *Lact. mucosae* LM303, which possessed antimicrobial activity against *Salmonella* Typhimurium DMST562 but not *Salmonella* Enteritidis DMST7106, was included in the experiment. In the co-culture study, total presumptive LAB, *Salmonella*, and fecal coliform counts were monitored at 0 and 24 h of incubation. At 0 h, the number of presumptive LAB in culture samples containing only fecal bacteria (F) or fecal bacteria spiked with SE7106 (F + SE) was 6.3 $\log_{10}$ CFU/ml. Inoculation of LJ202 or LR108 into fecal culture(F + LJ or F + LR) caused a slight increase of total counts of presumptive LAB (Table 1). After 24 h of co-culture, total numbers of presumptive LAB in F and F + SE increased by 7.6 $\log_{10}$ CFU/ml. Co-culture of probiotic or non-probiotic strains with fecal bacteria resulted in the reduction in cell numbers of presumptive LAB in F + LR, F + LJ, and F + LM compared with those in F and F + SE (about 1.3–3 $\log_{10}$ CFU/ml reduction). Addition of SE7106 into fecal culture containing LR108 or LJ202 (F + SE + LR and F + SE + LJ) did not cause significant change in the number of presumptive LAB. But, in F + SE + LM, the number of presumptive LAB decreased by 2.3 $\log_{10}$ CFU/ml compared with that of F + LM. Monitoring pH of the culture media after 24 h of co-culturing revealed that the pH of culture media of F and F + SE was 4.14. In F + LJ and F + SE +

Table 1 Co-culture of multiple-bacterial species

Cultures	pH[a]	Cell counts (log CFU/ml)[b]					
		MRS + 0.5% $CaCO_3$		XLD		MacConkey	
		0 h	24 h[c]	0 h	24 h	0 h	24 h[c]
F	4.14	6.31 ± 0.01	13.93 ± 0.06	nd	nd	5.72 ± 0.02	2.99 ± 0.13
F + SE	4.14	6.35 ± 0.03	13.99 ± 0.02	6.05 ± 0.07	nd	5.74 ± 0.04	3.30 ± 0.14
F + LR	4.27	7.26 ± 0.01	12.56 ± 0.01**	nd	nd	5.72 ± 0.02	3.26 ± 0.06
F + LJ	4.02	7.02 ± 0.03	10.90 ± 0.04**	nd	nd	5.74 ± 0.02	nd
F + LM	4.21	6.43 ± 0.00	12.61 ± 0.04**	nd	nd	5.66 ± 0.06	1.43 ± 0.23**
F + SE + LR	4.29	6.90 ± 0.03	12.82 ± 0.07**	6.19 ± 0.08	nd	5.62 ± 0.09	4.68 ± 0.07**
F + SE + LJ	4.02	6.83 ± 0.02	10.86 ± 0.03**	6.36 ± 0.09	nd	5.60 ± 0.19	nd
F + SE + LM	4.23	6.49 ± 0.01	10.25 ± 0.09**	6.31 ± 0.02	nd	5.70 ± 0.04	4.21 ± 0.18**

F culturable fecal bacteria; *SE Salmonella* Enteritidis DMST7106, *LR Lactobacillus reuteri* LR108, *LJ Lactobacillus johnsonii* LJ202, *LM Lactobacillus mucosae* LM303, *nd* not detected

[a] pH of the culture medium after incubation for 24 h

[b] The number of cells below 10 CFU/ml was not detected by plate counts

[c] Double asterisks indicate statistically significant difference ($p < 0.01$)

LJ, the pH of the culture media was lowered to 4.02 which was the lowest pH among the culture samples. The pH values of the culture media of F + LR, F + SE + LR, F + LM, and F + SE + LM were in a range of 4.21–4.29 which was higher than those of F and F + SE.

Determination of *Salmonella* numbers at 0 h revealed that *Salmonella* counts in most of the culture samples were below the detection limit (detection limit ≥10 CFU/ml), except for those spiked with SE7106 (F + SE, F + SE + LR, F + SE + LJ, F + SE + LM) where *Salmonella* counts were about 6 $\log_{10}$ CFU/ml. After 24 h of co-culture, the viable cells of *Salmonella* were not detected in all culture samples. Determination of fecal coliforms in the culture samples revealed that the number of fecal coliforms at 0 h was in a range of 5.6–5.7 log CFU/ml. After 24 h of co-culture, the decrease in fecal coliformcounts was observed in all culture samples. Significant reduction of fecal coliform counts was observed in F + LJ and F + SE + LJ where the viable cells were not detected (<10 CFU/ml). In F + LR, the number of fecal coliforms was comparable to that of F + SE; however, fecal coliform count significantly increased in F + SE + LR. In F + LM, the number of fecal coliforms was less than those of F, F + SE, and F + LR. But, addition of SE7106 (F + SE + LM) into the co-culture sample caused a significant increase in fecal coliform count.

Discussion

With the three selection criteria, eight potential probiotic strains which possessed the abilities to produce antimicrobial substances to inhibit the growth of *Salmonella*, to resist to acid and bile salts, and to adhere to Caco-2 cells were selected. The eight probiotic strains comprised of six strains of *Lact. reuteri* (LR105, LR108, LR111, LR310, LR311, LR401) and one strain each of *Lact. johnsonii* (LJ202) and *Lact. salivarius* (LS404). The three *Lactobacillus* species are known as inhabitants of pig GI tracts [19]. This would facilitate the probiotic strains to establish in GI niches and provide beneficial effects to pigs. Beneficial effects of probiotic *Lact. reuteri*, *Lact. johnsonii*, and *Lact. salivarius* on pigs have been reported. Daily feeding of probiotic *Lact. reuteri* I5007 was shown to protect newborn piglets from bacterial infections, particularly through the expression of tight junction protein [20]. Administration of *Lact. johnsonii* XS4 to sows during the end of pregnancy and during lactation increased litter weight at birth and weaning, and enhanced survival rate of newborn piglets [6]. Oral administration of *Lactobacillus salivarius* B1 to neonatal piglets showed modulatory effects on piglets by increasing the amount of intra-epithelial lymphocyte cells and IgA-producing cells in the intestinal tracts [21].

As we were interested in finding *Lactobacillus* with protective effect against *Salmonella* infection, we thus conducted further experiment to gain insight into how the potential probiotics affect the growth of *Salmonella*. By employing an in vitro co-culture model, it was found that the growth of SE7106 was retarded at the early hours of co-culturing with LJ202. At 4 h of co-culture, reduction of *Salmonella* counts occurred simultaneously with the decrease in pH of the culture medium. It has been shown that low pH environment affected the growth of *Salmonella* spp. [22, 23]. In our study, the effect of pH lowering seemed not strong enough to completely inhibit the growth of SE7106 even pH of the co-culture medium was lowered to 4.0. At 10 h of co-culture, decrease in LJ202 numbers and loss of

viable cells of SE7106 was observed, suggesting that competition for limiting nutrients might occur at this hour. After 10 h, only LJ202 could resume its growth, indicating that LJ202 had the ability to compete for the remaining nutrients but SE7106 did not. Thereby, the growth of SE7106 was then completely inhibited. Competition for nutrients was shown to affect the growth of pathogen in a co-culture study. By using transcriptome and biochemical analyses, Nouaille et al. revealed that competition for consumption of glucose between *Lactococcus lactis* and *Staphylococcus aureus* occurred when the nutrients were limited and growth rates of both bacteria were concomitantly retarded [24]. Competition for nutrients is considered as one of the mechanisms by which a bacterium used for survival in the gut [25].

The co-culture of two-bacterial species clearly showed that the probiotic LJ202 exhibited inhibitory effects on the growth of SE7106. In the subsequent co-culture study, it was interesting to investigate whether the presence of multiple-bacterial species where interactions among bacteria occurred may influence the antagonistic activity of LJ202. The use of culturable fecal bacteria as representative of multiple-bacterial species from pigs allowed us to investigate the inhibitory effect of LJ202 on fecal coliforms, apart from *Salmonella*. Also, the number of presumptive LAB was monitored since the previous antagonistic activity test showed that LJ202 displayed inhibitory activity against some lactic acid bacteria. Determination of *Salmonella* counts revealed that the number of *Salmonella* in all culture samples were below the detection limit (<10 CFU/ml) after 24 h of co-culture. The absence of *Salmonella* even in F + SE sample indicated that the presence of only culturable fecal bacteria could inhibit growth of SE7106. The ability of fecal bacteria to inhibit SE7106 can be explained as follows. (i) After 24 h of co-culture, the fecal culture contained presumptive LAB as high as 13 $\log_{10}$ CFU/ml. These LAB produced acids which lowered pH of the culture media to 4.14. The effect of pH lowering is considered as one factor that affected the growth of SE7106. (ii) Competition for nutrients, particularly among multiple-bacterial species, must be vigorous; thereby, SE7106 may lose the ability to compete for the limiting nutrients. (iii) Antimicrobial substances (like bacteriocins) produced by presumptive LAB may involve in growth inhibition of SE7106 [26].

Although inhibitory effect of LJ202 and LR108 on the growth of SE7106 was not clearly seen in the co-culture of multiple-bacterial species, it was clearly shown that LJ202 had inhibitory effect on the growth of fecal coliforms. Fecal coliforms in pig feces are known as potential pathogens (such as *Escherichia coli*) which can cause diarrhea in pigs [27]. In many studies, feeding pigs and chickens with probiotics could reduce coliform counts and reduced the incidence of diarrhea [28–33]. The presence of LJ202 in the co-culture with fecal bacteria alone(F + LJ) or fecal bacteria with SE7106 (F + SE + LJ) completely inhibited the growth of fecal coliforms. The result suggested that LJ202 exhibited antagonistic activity against fecal coliforms, and the activity was not interfered with the interactions among multiple-bacterial species in the co-culture. Inhibitory effect of LJ202 against fecal coliforms would be due to acids which lowered the pH of co-culture medium to 4.02. However, a study of Haberbecket al. showed that 75% of the 188 different *E. coli* strains used in their study had high capability to survive under low pH conditions (a pH range of 3.8–4.8) [34]. Therefore, pH lowering might not be the sole factor that inhibited the growth of fecal coliforms. It is possible that other antimicrobial substances like bacteriocin(s) produced by LJ202 might participate in inhibiting the growth of fecal coliforms [6, 35]. In the complex community, LR108 showed no inhibitory activity against fecal coliforms while LM303 displayed the activity only if SE7106 was absent. Inhibitory activity of LM303 might not be due to acids or pH lowering, since the pH values of co-culture media of F + LM and F + SE + LM were similar. It is possible that other antimicrobial substances, like bactericoins, may play a role in inhibiting the growth of SE7106. LM303 may be able to produce bacteriocins like *Lact. mucosae* strain Marseille. Draft genome sequencing of *Lact. mucosae* strain Marseille revealed that the bacterium has 12 different genes which possibly encode bacteriocins ranging from 38 to 67 amino acids [36]. Loss of inhibitory activity of LM303 against fecal coliforms in F + SE + LM was surprised. One possible explanation is that the presence of SE7106 and fecal coliforms may initiate synergistic interactions between the bacteria. The interactions may lead to stabilize or increase population of fecal coliform bacteria [37, 38].

Monitoring the number of presumptive LAB in the co-culture study revealed that LR108, LJ202, and LM303 caused significant reduction of presumptive LAB counts when they were co-cultured with fecal bacteria (F + LR, F + LJ, F + LM), particularly LJ202. In a more complex system where SE7106 was added, the number of presumptive LAB further decreased in F + SE + LM while those in F + SE + LR or F + SE + LJ did not change. From the antagonistic activity test, it was found that LR108, LJ202, and LM303 showed the capability to inhibit the growth of *Streptococcus* and *Enterococcus* but had little effect on *Lactobacillus*. Therefore, it assumed that the decrease in presumptive LAB counts was the result of the decrease in the number of *Streptococcus* and *Enterococcus* rather than *Lactobacillus*. Some species of *Streptococcus* and *Enterococcus* are opportunistic pathogens, feeding pigs with probiotics which exhibit inhibitory activity against *Streptococcus* and *Enterococcus* would benefit to pig health [39].

In conclusion, probiotic characterization and co-culture studies provided evidence to suggest that LJ202 is a good probiotic which exhibits inhibitory activity against *Salmonella* spp. and fecal coliform bacteria. Therefore, LJ202 is a suitable candidate for further study its protective effects against pathogen infections in pigs.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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