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Original Article

Selection of potential *Enterococcus faecium* isolated from Thai native chicken for probiotic use according to the *in vitro* properties

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Abstracts

Sixty strains of *E. faecium* were isolated from 30 samples of native chickens' gastrointestinal tracts. All strains were tested on acid and bile tolerance. Fifteen strains passed the acid tolerance test. The best five strains were EFMC 17, 21 and 24; EFMD 25; EFMI 47 and 49. Only four strains, EFMC 21; EFMD 30; EFMI 47, and 49, survived 4 hours of bile exposure. Fifteen strains that passed the acid tolerance test were tested for their ability of intestinal mucus attachment. The results indicated that all strains were able to attach to intestinal mucus. For the ability of pathogenic bacteria inhibition test, the result found seven strains (EFMC 17, 21 and 24; EFMD 29 and 30; EFMI 46 and 49) showed better performance than strain EFC. All seven strains were acid producer, but only four strains (EFMC 21; EFMD 25; EFMI 47 and 49) were able to release bacteriocin. Based on proper probiotic properties two strains (EFMI 47 and 49) of *E. faecium* isolated from Thai native chicks in this study have a potential use as probiotics. Antimicrobial susceptibility test of these two strains have been also performed; they were susceptible to amoxicillin/clavulanic, ciprofloxacin, gentamycin, trimethoprime/sulphamethoxazole, vancomycin, and trimethoprim. On the other hand, they were resistant to cefotaxime, erythromycin, and tetracycline. The DNA-DNA hybridization percentage of DNA-DNA homology to *E. faecium* NRIC 1145 of EFMI 47 and EFMI 49 were 82.36 and 78.63%, respectively.

Keywords: probiotics, *Enterococcus faecium*, native chicken

1. Introduction

Antimicrobial residue and antimicrobial resistance are considered not only a public health problem but also an economic concern related to trade barriers. Therefore, the use of antimicrobials as growth promoters in farm animal has been prohibited in many countries. The impending crisis is the driving factor to search for alternative agents that are able to replace the using of antimicrobials additive in animal feed.

One of the most interesting replacements are probiotic. Probiotics are living microorganisms, which confer a health benefit for the host after consuming in adequate amounts (Fuller, 1989). Bacteria that have been chosen and successfully used as probiotics belong to the lactic acid bacteria (LAB), especially *Lactobacillus* sp. Another LAB, such as *Enterococcus faecium* has been proved as potential probiotic. The *E. faecium* strain (SF68) has been proved to be effective in prevention of antimicrobial associated diarrhea (Wunderlich *et al*., 1989) and infantile diarrhea heated (Bellomo *et al*., 1980). However, most of the commercial strains of *E. faecium* are imported from overseas, which are quite expensive and may not suitable for animals in Thailand,

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since it may be related with adhesion of probiotic to host intestinal mucus. Rinkinen *et al.* (2003) reported the species specific of *E. faecium* SF 68 isolated from human was able to adhere to human intestinal mucosa better than dog intestinal mucosa. Therefore, host specificity is one of the properties of probiotic bacteria and has been recommended as one of the selection criteria (Saarela *et al.,* 2000). The objective of this study was to isolate and select the potential probiotic strains of *E. faecium* from gastrointestinal tract of native chicken.

2. Materials and Methods

2.1 Native chickens, bacterial culture media and reagents

Thirty healthy-native chicken were randomly selected from Nan Province in the Northern Thailand. Kenner faecal (KF) agar was used as selective medium for enterococci, supplied by Scharlau (Barcelona, Spain). De Man, Rogosa and Sharpe (MRS) and bile salt were supplied by Himedia (Mumbai, India). Mueller Hinton Agar (MHA) and Brain Heart Infusion (BHI) broth was from Britania (Buenos Aires, Argentina).

2.2 Bacteria strains

The enterococci strains were isolated from gastrointestinal tracts (crop, duodenum, and ileum) of 30 healthy-native chicken. Twenty-five grams of each sample was diluted in peptone diluting saline (PDS) to obtain the dilutions of 10^{-1}-10^{-7} and 0.1 ml of each dilution was spread on KF agar (Jin *et al.,* 2000). The plates were incubated for 24 hrs at 37°C. Isolates were preliminary grouped based on their morphology, catalase production, and growth at 45°C in 6.5% NaCl. The second step was identification of enterococci species by using their fermentation properties on sucrose, mannitol, arabinose, raffinose, sorbitol, and lactose. All isolates were stored at -70°C in Tryptic Soya (TS) broth containing 20% glycerol.

2.3 Acid and bile tolerance

The modified method of Agus (2003) was used to determine acid and bile tolerance test. The bacteria was exposure to acid condition (BHI broth, pH 2) for 1 hour at 37°C after that added 1.2 ml of 10% bile salt solution and 1.5 ml of bicarbonate buffer incubated for 1, 2, 3 and 4 hours at 37°C. The number of colonies on KF agar was calculated to be compared with the initial bacterial concentration.

2.4 *In vitro* intestinal mucosal adhesion assay

The *in vitro* intestinal mucosal adhesion assay was described by Ehrmann *et al*. (2002). Intestinal mucosal samples were collected by scraping intestinal mucosal surface of healthy-native chicken with a rubber spatula. The mucosal cells were placed in 100 ml of PBS (4°C) and centrifuged twice at 6,000 rpm for 10 min and 13,000 rpm for 20 min at 4°C to remove other particulates. After that, they were lyophilized and stored at -20°C until use.

Intestinal mucosal solution was prepared by dissolving 0.01 g of lyophilized mucosal sample in 5 ml of 50 mM Na_2CO_3 buffer (pH 9.7). One hundred microliters of the mucosal solution was immobilized on Eppendorf tube (E-tube) by incubation for 24 hours at 4°C. The E-tube was washed twice with 200 µl of PBS to remove excess mucus. One hundred microliters of bacterial solution ($2x10^8$ CFU/ml) was added into the E-tube. After incubating for 1 hour at 37°C, the E-tubes were washed twice with 200 ìl PBS (0.05% of tween 20) to remove unbound bacteria. The bound bacteria were subject to be diluted and counted on selective media.

2.5 Determination of antimicrobial activity

The antimicrobial activity of selected strains was compared with *E. faecium* isolated from commercial probiotic (EFC) strain. The method was performed as described by Schillinger and Lücke (1989). The tested bacterial strains were cultured in BHI broth and incubated at 37°C for 18 hours. Ten milliliters of the culture suspension were spotted on MRS agar and incubated at 37°C for 24 hours. The tested MRS culture plate was overlaid with MRS soft agar that contains $1.5x10^6$ CFU/ml of indicator bacteria (*Salmonella* Enteritidis, *S.* Typhimurium and *E. coli*) and incubated at 37°C for 24 hours. Antimicrobial activity of tested strains was determined by investigating the inhibiting clear zone.

2.6 Screening for antimicrobial substance (acid, H_2O_2, and bacteriocin) production

The method for antimicrobial substance screening was performed as described previously by Ketkeaw *et al.* (2005). After the tested strains were growth in BHI broth for 24 hour at 37°C, bacterial cells were removed by centrifugation (13,000 rpm for 10 min, 4°C). The cell-free supernatant, prepared by filtering through 0.2 µm pore-size filters, was used for screening of antimicrobial substances. The cell-free supernatant for screening of acid production was prepared by adding 1 mg of protease and catalase/ml. For screening of H_2O_2 and bacteriocin production, cell-free supernatant was added with 1 mg of catalase and protease/ml, respectively, and adjusted pH to 7.0 with 1 N NaOH. These antimicrobial substances were screened by using well diffusion assay. Well diffusion assay was performed by boring 3 mm in diameter wells on MRS agar plate contained *S.* Typhimurium ($1.5x10^6$ CFU/ml). After 30 µl of the tested supernatants were placed into the wells and incubated at 37°C for 24 hours, antimicrobial substances of tested strains were determined by investigating the inhibiting clear zone.

Table 1. Oligodeoxynucleotide Primer, Kariyama *et al*. (2000).

Primer specificity	Size of PCR product (bp)	Primer pair sequences (5' to 3')
E. faecium	638	+TGAGGCAGACCAGATTGACG -TATGACAGCGACTCCGATTCC
rrs (16S rRNA)	320	+GGATTAGATACCCTGGTAGTCC -TCGTTGCGGGACTTAACCCAAC

2.7 Antimicrobial susceptibility

Antimicrobial susceptibility was performed by disk diffusion method as described in CLSI (2007). Antimicrobial agents used in this study were amoxicillin+clavulanic acid (20 μg + 10 μg), ampicillin (10 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), gentamycin (10 μg), erythromycin (15 μg), trimethoprim+sulphamethoxazole (1.25 μg + 23.75 μg), tetracycline (30 μg), vancomycin (30 μg), and trimethoprim (5 μg).

2.8 Species-specific PCR and DNA-DNA hybridization

E. faecium was confirmed by PCR technique modified from Kariyama *et al*. (2000) by using specific oligonucleotide primers (as shown in Table 1). DNA amplification was carried out in a thermal cycler programmed as stepping by an initial denaturizing step of 94°C for 5 min, 30 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 10 min. Final PCR products were analyzed by electrophoresis on 1.5% agarose gel contained ethidium bromide. DNA-DNA hybridization was also used to confirm *E. faecium*. Photobiotin labeling DNA-DNA homologies were carried out in 2X SSC (saline-trisodium citrate) and 50% formamide solution at 50°C as reported by Ezaki *et al.* (1989) and Tanasupawat *et al*. (1992a).

3. Results and Discussion

3.1 Isolation of enterococci from gastrointestinal tracts of healthy native chicken

Enterococci were isolated from gastrointestinal tracts (crop, duodenum, and ileum) of 30 healthy native chickens by using selective media. The total viable count of enterococci was in the range of 7-8 $\log_{10}$ CFU/ml (data not shown). The results of classification of *Enterococcus* sp., which based on their morphologies and phenotypes, were shown in Table 2.

3.2 Acid and bile tolerance

Sixty strains of *E.faecium* were tested for acid and bile tolerance which are important properties of probiotic. The results from four repetitive tests found 15 strains (25%) were able to survive at pH 2.0 for 1 hour, with 9 strains (60%) showing weak tolerance and 6 strains (40%) moderate tolerance to low pH (Table 3). The study of Strompfová and Lauková (2007) found that *E. faecium* can survive at pH 3.0 for 3 hours. The reason of using pH 2.0 condition in our study was to imitate the low pH of chicken's gizzard (Sturkie, 1976). It is not surprising that *E. faecium* can survive in strong acid environment since one of natural habitats of enterococci is gastrointestinal tracts of human and animals. Other factor of acid tolerance might relate to ATPase expression, which is found in lactic acid bacteria, *L. acidophilus*, and has been reported by Lorca1 and Valdez1 (2001). Kobayashi *et al.* (1986) reported earlier of finding an increase in amount and activity of the ATPase in *Enterococcus hirae*. Therefore, ATPase mechanism of enterococci should be further investigated.

The results from four repetitive tests of bile tolerance test found that all tested *E. faecium* strains were able to survive in bile condition at 1 hour. The survival strains were decreased to six strains (40%) after 2 hour-exposure and only 4 strains (26.7 %) left after 3 and 4 hour-exposure (Table 4). Previous study by Strompfova (2004) reported *E. faecium* isolated from dogs can tolerance to 1% bile for 24 hours. Bile tolerance is an important characteristic of bacteria to survive in small intestine. Hydrolyzation of bile salt by enzyme hydrolases (BSHs) had been explained by Tanaka *et al*. (2000), which can be found in *Lactobacillus* (De *et al.,* 1995) *and Enterococcus* (Agus. 2003).

3.3 *In vitro* intestinal mucosal adhesion assay

Fifteen acid tolerance strains of *E. faecium* were tested for their intestinal mucosal adhesion ability. The results show

Table 2. Total *Enterococcus* sp. isolated form native chickens' gastrointestinal tracts.

Enterococcus sp.	Isolation from			
	Crop	Duodenum	Ileum	Total
E. faecium	24	17	19	60
E. faecalis	20	13	15	48
E. gallinarum	23	13	10	36
E. durans	5	11	8	24
E. avium	2	-	2	4
				172

Table 3. Tolerance of tested *Enterococcus faecium* strains in pH 2.0 condition.

Strains	Acid tolerance [1]			
	Negative	Weak	Moderate	Strong
EFMC	7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 19, 20, 23	2, 22	17, 21, 24	-
EFMD	26, 27, 28, 31, 33, 34, 35, 36, 37, 38, 39, 40, 41	29, 30, 32	25	-
EFMI	43, 45, 48, 50, 51, 52, 53, 55, 56, 57, 58, 59, 60	42, 44, 46, 54	47, 49	-

[1] Negative = Survival rate < 50 % Weak = Survival rate 50-75 %
Moderate = Survival rate 75-90 % Strong = Survival rate >90 %

Table 4. Tolerance of tested *Enterococcus faecium* strains in bile condition.

Contact time	Bile tolerance
1 hour	EFMC 2, 17, 21, 22, 24, EFMD 25, 29, 30, 32, EFMI 42, 44, 46, 47, 49, 54
2 hours	EFMC 17, 21, 24, EFMD 30, EFMI 47, 49
3 hours	EFMC 21, EFMD 30, EFMI 47, 49
4 hours	EFMC 21, EFMD 30, EFMI 47, 49

that all strains were able to attach intestinal mucosa (Figure 1, Table 5). The adhesion ability of probiotic microorganism to intestinal mucosa is one of the most important criteria of selection. Since the better adhesion gives the longer colonizing on mucosa and preventing the attachment of pathogens. Jin *et al.* (2000) found that *E. faecium* 18C23 strain was able to inhibit the attachment of *Escherichia coli* K88 on porcine small intestinal mucosa.

3.4 Determination of antimicrobial activity and antimicrobial substance producing

Seven strains (EFMC 17, 21 and 24; EFMD 29 and 30; EFMI 46 and 49) were selected, according to their abilities of acid and bile tolerance and intestinal mucosal adhesion, for studying of antimicrobial activity and antimicrobial substance producing. All tested strains showed better performance than strain EFC. The inhibition activities of enterococci could be an effect of acid and/or bacteriocins (Franz *et al.,* 1999). Laukova *et al*. (2003) found in their study that *E. faecium* EK

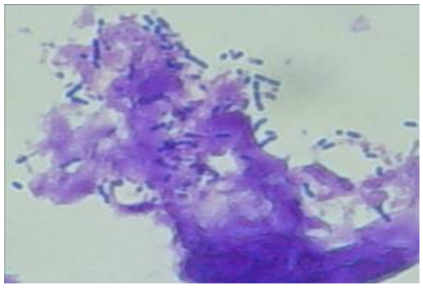

Figure 1. Microscopy illustration; showing adherence of *E. faecium* (EFMI 49) with chick intestinal mucosa.

Table 5. Ability of adherence of *E. faecium* isolated from native chicken.

Strains	Ability of adherence (%)
EFMC 2	39.0 (+0.08)
EFMC 17	40.0 (+0.53)
EFMC 21	49.9(+0.70)
EFMC 22	41.3(+0.50)
EFMC 24	42.2(+0.33)
EFMD 25	43.4(+0.64)
EFMD 29	48.4(+0.04)
EFMD 30	41.9(+0.32)
EFMD 32	50.4(+0.32)
EFMD 42	42.7(+0.57)
EFMI 44	42.9(+0.31)
EFMI 46	51.6(+0.53)
EFMI 47	59.6(+0.38)
EFMI 49	55.4(+0.11)
EFMI 54	54.6(+0.29)
EFC	55.3(+0.20)
Salmonella Enteritidis*	41.5(+0.07)
*Bacillus subtillis***	-

* Positive control, ** Negative control

13 strain produced bacteriocin A against *Salmonella* spp. All seven strains of our study were acid producer but only four strains (EFMC 21; EFMD 25; EFMI 47, and 49) were able to produce substances that acts as a bacteriocins, which should be further study for identifying bacteriocin types. However, all seven strains showed antibacterial activity against *S.* Typhimurium (Figure 2).

3.6 Antimicrobial susceptibility

Based on all of tested criteria for the selection of *E. faeium* isolated from native chicken as a potential for probiotic, EFMI 47 and 49 were found having the best performance and subjected for study in an antimicrobial susceptibility test. The results found them susceptible to amoxicillin + clavulanic, ciprofloxacin, gentamycin, trimethoprime + sulphamethoxazole, vancomycin and trimethoprim, hile they were resistant to cefotaxime, erythromycin and tetracycline.

3.7 Species-specific PCR and DNA-DNA hybridization

The *E. faecium* (EFMI 47 and 49, including EFMC 21 and EFMD 30) genotypes were confirmed by using PCR. All four isolates showed PCR product at 320 bp of *E. faecium* gene and 638 bp of 16S rRNA gene (Figure 3). The results of DNA-DNA hybridization found all four isolates (EFMC 21 EFMD 30 EFMI 47 and EFMI 49) exhibited a high degree of homology to *E. faecium* NRIC 1145 as 81.02, 78.08, 82.36, and 78.63%, respectively.

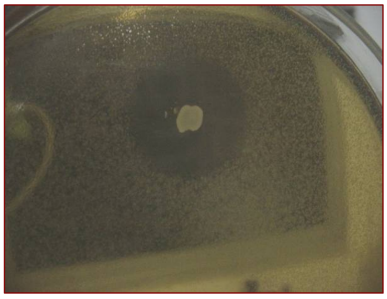

Figure 2. Inhibition zone of *S. typhimurium* from antimicrobial activity of *E. faecium* isolated from native chicken.

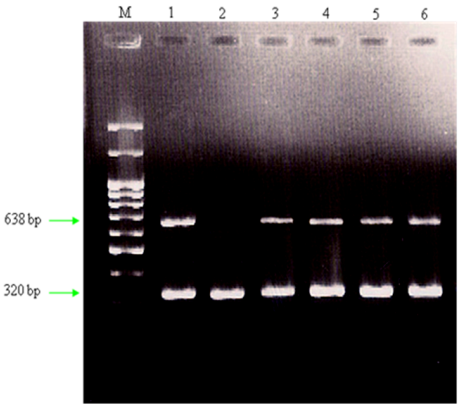

Figure 3. PCR product from DNA of *E. faecium* isolated from native chicken. Lane 1, *E. faecium* (positive control), Lane 2, *E. faecalis* (negative control). Lane 3, EFMC 21, Lane 4, EFMD 30, Lane 5, EFMI 47, Lane 6, EFMI 49.

4. Conclusions

Sixty isolates of *E. faecium* were isolated from Thai native chickens, two strains (EFMI 47 and EFMI 49) revealed the potential use as a probiotic, as they are showing the better advantage of the tested performances, acid and bile tolerance, intestinal mucus attachment, pathogenic bacterial inhibition ability, and bacteriocin producing. The genotypes of both *E. faecium* isolates were confirmed by using PCR and DNA-DNA hybridization.

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