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<Original Article>

Assessment of probiotic potential of *Lactobacillus reuteri* MD5-2 isolated from ceca of Muscovy ducks

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Abstract : Lactic acid bacteria (LAB) are commonly used as probiotics in poultry. The present study employed *in vitro* and *in vivo* methods to select and test LAB isolated from Muscovy duck ceca as potential probiotics. In the *in vitro* study, 50 LAB were isolated from Muscovy duck ceca and tested for growth inhibition against *Salmonella* (*S.*) Enteritidis. Eleven isolates strongly inhibited *S.* Enteritidis and only 1 isolate (MD5-2) showing the strongest inhibition was selected for identification. This isolate was called as *Lactobacillus* (*L.*) *reuteri* MD5-2. For the *in vivo* investigation, 90 1-day-old Muscovy ducks were randomly assigned into three groups of 30 animals each (group 1, control; group 2, treated with 10^8 colony-forming unit (CFU) of *L. reuteri* MD5-2 orally once on day 1; and group 3, treated with 10^8 CFU of *L. reuteri* MD5-2 orally once daily from days 1 to 5). The ducks were housed in three large cages and raised for 50 days, after which body weight, duodenal villus height and crypt depth were measured. Both villus height and villus height to crypt depth ratio were significantly greater in group 3 than in groups 1 and 2. In conclusion, further investigation of *L. reuteri* MD5-2 as a potential probiotic strain is warranted.

Keywords : *Lactobacillus reuteri*, Muscovy ducks, *Salmonella* Enteritidis, lactic acid bacteria, probiotics

Introduction

Poultry is a major source of protein foods for human worldwide, but several problems affecting consumers' health still arise from poultry production. One important problem is a concern about risks of enteric pathogens contaminated in poultry products. *Salmonella* (*S.*) Enteritidis is one of the most important enteric pathogens that can cause severe diarrhea in patients who consume contaminated poultry products [6, 19]. This results in a substantially economic loss for health care service [30]. Another important problem is a concern about potential risks of antibiotic resistance in human associated with consuming poultry products [24, 36]. In traditional poultry production, antibiotics have been used not only for treating infectious diseases, but also for preventing diseases, decreasing mortality, and increasing growth performances when used in sub-therapeutic levels as feed additives or growth promoters [5]. Because of public pressure and concerns about antibiotic resistance in human, use of antibiotics as a growth promoter in poultry feeds has been banned in European Union [5, 23]. This ban has created many difficulties for poultry industry; therefore, searching for alternative products is required. These alternative products include: probiotics [20, 25], prebiotics [13], organic acids [11, 21], and bacteriocins [15, 22].

Among alternative products, probiotics or direct-fed microbials are under extensive studies in chickens [17, 26, 33, 34]. Probiotics are defined as live microorganisms, which when administered in adequate amount confer a health benefit to the host [29]. In chickens, most probiotics studied are belonging to lactic acid bacteria (LAB), particularly in the genus *Lactobacillus* [4, 26, 33]. Unlike in chickens, probiotic studies are rare in Muscovy ducks. These ducks, although they are far less in number when compared to broilers, are globally raised and their meat is popular for consumers in some Asian countries including Thailand. In 2011, it was estimated that more than 6 millions of Muscovy ducks were raised in Thailand (data from Department of Livestock Development of Thailand). Therefore, the objectives of the present study were to screen and test indigenous LAB, isolated from Muscovy ducks' ceca, for a potential probiotic strain.

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Materials and Methods

Animals for *in vitro* study

The present study was conducted in the agreement with the Guide for Care and Use of Laboratory Animals approved by the Animal Ethics Committees of Khon Kaen University (AEKKU 38/2555). Five healthy adult Muscovy ducks (3 males and 2 females, aged about 180 days) were used. All ducks were checked for *S.* Enteritidis free. To obtain the ceca, the ducks were humanely killed by using cervical dislocation.

Bacterial isolation

After abdomen of the Muscovy duck was carefully exposed and the ceca of both sides were cut out, 1 g of cecal contents was aseptically collected. The collected cecal content was then transferred to 9 mL of Man Rogosa and Sharpe (MRS) broth, the culture medium. The medium was incubated at 37°C in microaerophilic condition with gas generating system (BD GasPak EZ; BD Diagnostics, USA) for 48 h. Then, the bacteria were spread on MRS agar plates. The plates were incubated at 37°C in microaerophilic condition for 48 h. Colonies from each agar plate were randomly selected and purified by sub-culturing in MRS broth and MRS agar for 3 times.

Characterization of LAB

Gram staining: The bacteria grown on the agar plate were spread on a glass slide. The slide was left to air dry and was passed quickly through fire for 3 times to make the bacterial cells adhere to it. Then, the slide was stained consecutively with crystal violet, Gram's iodine, and Safranin. After the slide was finally rinsed and air-dried, it was examined under a light microscope to characterize the observed bacteria. Gram-positive bacteria that are stained dark blue or violet were selected and used for the catalase test.

Catalase test: A single colony of the selected bacteria was spread on a glass slide. The slide was added with a drop of hydrogen peroxide (H_2O_2) with the intensity of 3% (w/v) and then was observed for a reaction. The result of a reaction was considered catalase-positive if bubbles presented; otherwise, it would be catalase-negative. In this step, Gram-positive and catalase-negative bacteria were selected and stored in MRS broth mixed with 20% of glycerol at −70°C for further use.

The inhibitory effects of the selected LAB against *S.* Enteritidis

Preparation of the culture supernatants: The culture supernatants of the selected LAB (50 isolates) were prepared according to the following processes. The selected bacteria stored at −70°C were left at room temperature. Then, the bacteria were sucked into test tubes containing 9 mL of MRS culture medium and incubated at 37°C in microaerophilic condition for 48 h. After that, the bacteria were shaken off at 4,500 rounds per min at 4°C for 15 min. The culture supernatants were filtered with 0.2 μm filter paper for testing the inhibitory effects on *S.* Enteritidis with agar well diffusion method.

Preparation of *S.* Enteritidis: *S.* Enteritidis from a chicken aged 3 days were grown in tryptic soy broth and incubated at 37°C for 24 h (the type of *S.* Enteritidis was proved by the National Institute of Animal Health, Thailand). The appropriate amount (10^8 colony-forming unit [CFU]/mL) of *S.* Enteritidis was prepared. The turbidity of *S.* Enteritidis must be equal or similar to McFarland nephelometer standard 0.5 (10^8 CFU/mL). Then, *S.* Enteritidis were diluted until its turbidity was equal to 10^6 CFU/mL by using 0.1% peptone water.

Agar well diffusion test: The prepared *S.* Enteritidis was sucked in the quantity of 100 μL and dropped on Mueller Hinton (MH) agar plates. Then, seven holes that six holes on the outer area and a hole at the center of the plate with diameter of 6 mm were made on each MH agar plate by using sterile techniques. Eighty mL of culture supernatants of LAB was dropped into the holes. After that, *S.* Enteritidis was incubated at 37°C for 24 h. Inhibition zones occurred around the holes were observed. The width of inhibition zones were measured by using a vernier caliper.

Acid tolerance test

LAB with inhibition zone width of 10 mm or wider from testing the inhibitory effects on *S.* Enteritidis by agar well diffusion were chosen. The chosen bacteria were grown in MRS broth and centrifuged at 4,500 rounds per min at 4°C for 15 min. Then, the bacteria were stored in sterile tubes and washed with PBS (pH 7) twice before growing the bacteria in MRS broth. The pH value was then changed to 2.5 and 3 by using 1 M hydrogen chloride. The bacteria were grown on MRS. Finally in this step, only one colony of the bacteria that had the greatest acid resistance was selected for further tests: *i.e.*, bacterial identification by 16S rDNA sequencing method, antibiotic susceptibility test, and co-culture growth curve with *S.* Enteritidis.

Bacterial identification

The selected LAB strain was sent to The National Center for Genetic Engineering and Biotechnology center, Thailand, to identify species of the bacterium by using 16S rDNA sequencing method. This method involved PCR amplification of 16S rDNA sequencing, direct sequencing of 16S rDNA, and sequence analyses. The phylogenetic tree was constructed [28].

Antibiotic susceptibility test

An antibiotic susceptibility test was determined by disc diffusion method. The selected LAB strain was grown on the Rogosa agar plate. In each plate, six wafers (each containing one kind of antibiotic) were placed on. The antibiotics tested included erythromycin, streptomycin, penicillin, gentamycin, nitrofurantoin, vancomycin, neomycin, tetracycline, bacitracin, nalidixic acid, and choramphenicol. Then, the medium

was incubated at 37°C in microaerophilic condition with gas generating system (BD GasPak EZ; BD Diagnostics) for 48 h. Inhibition zone around each wafer was observed and compared to standard value.

Co-culture growth curves

To investigate the impact of the selected LAB strain on the growth of *S.* Enteritidis, a method of Drago *et al*. [8] was modified and used. According to this method, the culture medium was prepared by mixing MRS broth with MH broth (5 : 5 mL). Then, 10^8 CFU/mL of *S.* Enteritidis and the selected LAB strain were dropped on the medium. The medium was incubated at 37°C in microaerophilic condition for 2, 4, 6, and 24 h. Then, the numbers of live cells were count at each mentioned time point. The medium was diluted by using a 10 fold-dilution method. Then, the medium was poured on MRS agar in order to observe the growth of the selected LAB strain and poured on xylose lysine deoxycholate (XLD) in order to observe the growth of *S.* Enteritidis. The MRS agar was incubated at 37°C in microaerophilic condition for 48 h, while the XLD was incubated at 37°C in microaerophilic condition for 24 h.

***In vivo* study**

The selected LAB strain (identified as *Lactobacillus* [*L.*] *reuteri* MD5-2) was tested in a small trial. In this trial, 90 of 1-day-old Muscovy ducks were randomly assigned into 3 groups of 30 animals each (group 1, control; group 2, each duck receiving 10^8 CFU of *L. reuteri* MD5-2 orally once in day 1; and group 3, each duck receiving 10^8 CFU of *L. reuteri* MD5-2 orally once daily from day 1 to day 5). The ducks were housed in 3 large cages (3×4 m^2) and fed *ad libitum* for 50 days. At the end of the trial, all ducks were weighed and then were humanely killed. The duodenal loop was removed. A sample at the mid-duodenal loop (n = 5 per group) was fixed in 10% buffered formalin for histological evaluation. Height of the duodenal villus (defined as a length from the base to the tip of a villus) and depth of the duodenal crypt were randomly measured from 3 positions per section with the aid of digital camera (AxioCam ERc 5s Camera; Cark Zeiss MicroImaging, Germany) and its software (AxioVision; Carl Zeiss Microscopy, Germany). To reduce bias in this measurement, evaluators were unaware of treatment group assignment for the ducks.

Statistical analysis

Data were checked for their normality with Shapiro-Wilk test. Analysis of variance (ANOVA) and Tukey HSD test for multiple comparisons between groups were used to analyze body weight, duodenal villus height, duodenal crypt depth, and villus height to crypt depth ratio in experimental ducks. All tests were two-tailed and a value of $p < 0.05$ was considered significant. The statistical tests were performed using SPSS (ver. 17.0; SPSS, USA).

Table 1. Survival of lactic acid bacteria (LAB) isolates at pH 2.5 and 3 for 24 h in Man Rogosa and Sharpe broth

LAB isolates	Number of survived colonies		
	pH 7	pH 3	pH 2.5
MD1-1	> 300	4	0
MD1-3	> 300	3	0
MD1-4	> 300	3	0
MD2-1	104	1	0
MD2-2	> 300	0	0
MD3-2	> 300	1	0
MD3-3	> 300	1	1
MD4-1	> 300	15	3
MD4-4	> 300	8	0
MD5-2	> 300	20	5
MD5-3	> 300	1	0

Results

Isolation and characterization of LAB

In the first step of screening, 50 LAB isolates were found and selected after sub-cultured in MRS broth and MRS agar for 3 times. All isolates had characteristics of LAB based on Gram staining and catalase test. All selected LAB isolates had round, turbid white colony with diameters approximately 0.1 to 0.3 mm. In addition, the isolates had bar-shape and motionless when observed under a light microscope (data not shown).

Inhibitory effects of the isolated LAB against *S.* Enteritidis

Of 50 LAB isolates tested against *S.* Enteritidis by using agar well diffusion method, 11 isolates strongly inhibited *S.* Enteritidis with diameter of inhibition zone of 10 mm or greater. Therefore, these 11 LAB isolates were selected for acid tolerance test (Table 1).

Acid tolerance test

Of 11 LAB isolates tested, 3 isolates survived at pH 2.5 for 24 h (Table 1). However, only 1 isolate (MD5-2) was selected, based on number of survived colonies (Table 1).

Bacterial identification

Only 1 LAB isolate survived well at pH 2.5 and 3.0 for 24 h was subjected for 16S rDNA sequence. This isolate was 99.6% identity with *L. reuteri*. With the assigned strain, this was called as *L. reuteri* MD5-2. The phylogenetic tree was provided (Fig. 1).

Co-culture growth curve

Number of *S.* Enteritidis decreased after co-incubation with *L. reuteri* MD5-2 for 2, 4, 6 and 24 h (Table 2).

Table 2. Results from co-culture test between *Lactobacillus* (*L.*) *reuteri* MD5-2 and *Salmonella* (*S.*) Enteritidis

Time (h)	Control		Co-culture of *L. reuteri* and *S.* Enteritidis	
	L. reuteri MD5-2 (CFU/mL)	*S.* Enteritidis (CFU/mL)	*L. reuteri* MD5-2 (CFU/mL)	*S.* Enteritidis (CFU/mL)
0	7.38*	8.14	7.38	8.14
2	5.61	9.48	5.60	7.48
4	6.45	7.48	6.70	7.40
6	7.40	7.48	7.42	7.44
24	7.48	9.48	7.79	7.58

*All colony-forming unit (CFU)/mL values were calculated from mean of 2 replicates.

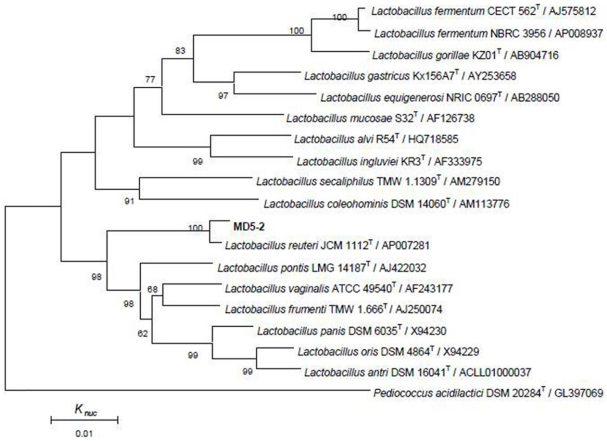

Fig. 1. Phylogenetic tree of *L. reuteri* MD5-2 isolated from Muscovy duck ceca.

Table 3. Antibiotic sensitivity test of *L. reuteri* MD5-2

Antibiotics	Diameter of clear zone (mm)		
	Replicate 1	Replicate 2	Replicate 3
Erythromycin (E10)	0	0	0
Streptomycin (S10)	0	0	0
Penicillin G (P10)*	49	35	29
Gentamicin (CN10)	17	15	17
Nitrofurantoin (F300)*	37	35	35
Vancomycin (VA30)	0	0	0
Neomycin (N30)	15	19	21
Tetracycline (TE30)	13	15	13
Bacitracin (B10)	21	21	20
Nalidixic acid (NA30)	0	0	0
Chloramphenicol (C30)	13	9	8

**L. reuteri* MD5-2 was sensitive to penicillin and nitrofurantoin.

Antibiotic sensitivity test

From the present study, the result showed that *L. reuteri* MD5-2 was sensitive to penicillin and nitrofurantoin (Table 3).

In vivo study

No ducks died and no illness was observed during experimental period. Body weight of the ducks was not signifi-

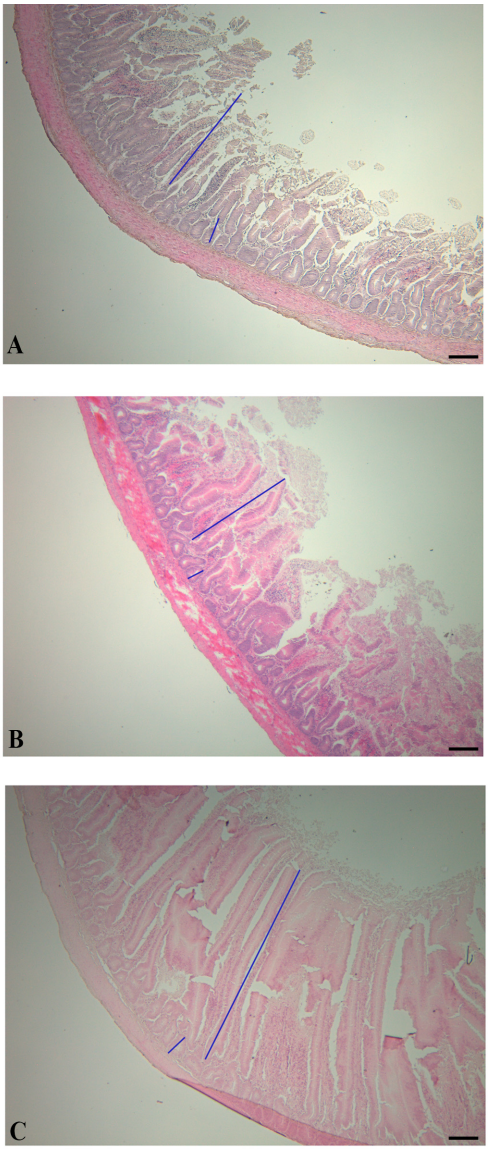

Fig. 2. Samples of histological sections from duodenum of Muscovy ducks. (A) Group 1, control group. (B) Group 2, receiving 10^8 CFU of *L. reuteri* MD5-2 orally once in day 1. (C) Group 3, receiving 10^8 CFU of *L. reuteri* MD5-2 orally once daily from day 1 to day 5. Scale bars = 200 μm. Long lines (villus heights) = 424.47 μm (A), 528.8 μm (B), 1045.6 μm (C). Short lines (crypt depths) = 50.04 μm (A), 50.65 μm (B), 65.71 μm (C).

Table 4. Body weight, villus height, crypt depth, and villus height to crypt depth ratio of the duodenum of the ducks in 3 experimental groups

Variables	Group 1	Group 2	Group 3
Body weight (kg, n = 30)	0.68 ± 0.07[a]	0.69 ± 0.06[a]	0.70 ± 0.06[a]
Villus height (μm, n = 5)	420.4 ± 68.5[a]	484.4 ± 51.5[a]	917.0 ± 151.5[b]
Crypt depth (μm, n = 5)	85.4 ± 2.9[a]	85.4 ± 7.6[a]	92.2 ± 7.9[a]
Villus height/crypt depth ratio (n = 5)	5.0 ± 0.8[a]	5.7 ± 0.9[a]	9.9 ± 1.2[b]

Values are the mean ± SD. Group 1, control; Group 2, *L. reuteri* MD5-2 orally once in day 1; Group 3, *L. reuteri* MD5-2 orally once daily from day 1 to day 5. [a,b]For each row, different superscripts indicated a significant difference in means ($p < 0.05$).

cantly different among groups (mean ± SD; 0.68 ± 0.07 kg, 0.69 ± 0.06 kg, and 0.70 ± 0.06 kg for group 1, group 2, and group 3, respectively) (Table 4). Villus height was significantly longer in group 3 (mean ± SD, 917.0 ± 151.5 μm) than in group 1 (420.4 ± 68.5 μm) and group 2 (484.4 ± 51.5 μm) (Table 4 and Fig. 2). Although crypt depth was not significantly different among groups, villus height to crypt depth ratio was significantly greater in group 3 (mean ± SD, 9.9 ± 1.2%) than in group 1 (5.0 ± 0.8%) and group 2 (5.7 ± 0.9%) (Table 4 and Fig. 2).

Discussion

In the present study, we found that *L. reuteri* MD5-2 isolated from the ceca of Muscovy ducks had probiotic potential *in vitro*. The evidence is that *L. reuteri* MD5-2 can survive in acidic environment (pH 2.5 and 3.0) for at least 24 h and it can strongly inhibited *S.* Enteritidis by both agar well diffusion test and co-culture growth curve test.

Our findings are consistent with those from previous studies in chickens that *L. reuteri* can inhibit *S.* Enteritidis *in vitro* [37] and can decrease *S.* Enteritidis colonization in intestine of chickens [26]. It was found that *L. reuteri* was the most abundant *Lactobacillus* species in gastrointestinal tract of chickens [35]. Moreover, *L. reuteri* is well-known for its probiotic potential in human and in other animal species. For examples, *L. reuteri* DSM 17938 is associated with reducing the risk of necrotic enteritis in preterm infants [1]. Encapsulated and freeze-dried *L. reuteri* CRL 1324 has potential for vaginal probiotic application to prevent or treat urogenital infections in women [16]. In pigs, probiotic *L. reuteri* has been well reviewed for its use and efficacy [14]. *L. reuteri* can reduce enterotoxigenic *Escherichia coli* colonization and modulate development of fecal microbiota in weaning pigs [38, 39].

Although exact mechanisms of *L. reuteri* for host benefits remain elusive, some of its beneficial effects have been proved. *L. reuteri* can secrete potent antimicrobial substances, reuterocyclin and reuterin, that can inhibit pathogenic bacteria [9, 31, 38]. *L. reuteri* shows competitive exclusion effect to protect human keratinocytes from *Staphylococcus aureus* [27]. In addition, *L. reuteri* exhibits immunomodulatory activity on the host [26].

Studies of probiotics in ducks, unlike in chickens, are relatively uncommon. *Lactobacillus* species are found to have probiotic potential in ducks [7, 18, 32]. The result of our study was consistent with that of the previous study by Kimprasit *et al*. [18] that *L. reuteri* isolated from ducks had probiotic potential *in vitro*. In another *in vitro* study [32], *L. salivarius* isolated from Pengging ducks had probiotic potential. For *in vivo* study, Choi et al [7] found that *L. salivarius* isolated from ducks showed immune enhancing effects. These evidences indicate that ducks provide a good source for probiotic candidates.

Antibiotic susceptibility and resistance in probiotic bacteria are now receiving more attention and have been extensively reviewed [10, 12]. Because antibiotic susceptibility and resistance may associate with consumer's health, these issues are important for medical clinicians who treat clinical infections and for industry that use lactobacilli as starter cultures for fermented foods [10]. *Lactobacillus* species are generally susceptible to cell wall-targeting penicillin, but some species are intrinsically resistant to vancomycin [10, 12]. This statement coincides with our findings that *L. reuteri* MD5-2 is susceptible to penicillin and resistance to vancomycin. Therefore, try to aware when used *L. reuteri* MD5-2 with these antibiotics. However, issues of antibiotic susceptibility and resistance in *Lactobacillus* species are still controversial especially in the standard methods for susceptibility testing [10].

Results from *in vivo* study indicated that orally administration of *L. reuteri* MD5-2 for long time may improve gut health. This indication was supported by the results that villus height and villus height to crypt depth ratio were significantly greater in ducks receiving 10^8 CFU of *L. reuteri* MD5-2 orally once daily from day 1 to day 5 than in ducks of control group or in ducks receiving 10^8 CFU of *L. reuteri* MD5-2 orally once in day 1. This finding was consistent with that of previous studies [2, 3].

The present study has some limitations. Although antimicrobial property against pathogenic bacteria and resistance to acidic environment are important criteria for *in vitro* probiotic screening, there are some additional criteria (according to the report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food), i.e., resistance to bile and ability to adhere epithelial cells of gastrointestinal tract (Caco-2 cells). These criteria were not tested in the present study due to the limitation in budget and

lab facilities. However, a good probiotic from *in vitro* test does not guarantee that it is good for use *in vivo*. Thus, the *in vivo* pilot study was conducted to overcome *in vitro* limitations.

In conclusion, the present study found that *L. reuteri* MD5-2 isolated from Muscovy duck ceca can inhibit the growth of *S.* Enteritidis *in vitro* and resist acids at pH level of 2.5 and 3. These findings indicate the potentials for further studies on using *L. reuteri* MD5-2 as a probiotic for raising Muscovy ducks and other birds in the future.

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