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[6-1130-P] Functional/Wellness Foods &Nutrition (6th)
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[6-1130-P-05] Probiotic characterization of thermotolerant *Lactobacillus johnsonii* isolated from broiler intestine

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Keywords: Lactobacillus johnsonii, probiotic, broiler gastrointestinal tract, feed supplement

Bacterial community in human and animal gastrointestinal tract (GI) are diverse. In GI tract of healthy hosts, lactic acid bacteria (LAB) can be found as dominant flora. Some strains of LAB have been accepted as probiotic due to the fact that they contribute many health benefits to host. Several probiotics are isolated and applied in functional food and feed products for the specific consumers, including human and animal. Nowadays, thermotolerant probiotics are of interest to industrial application, because of their high heat-resistant ability in food and feed manufacturing. The aims of this study were to isolate thermotolerant LAB from broiler intestine and evaluate their probiotic characteristics for monogastric feed application. Two promising isolates, CK3 and VCF29 were selected and identified by 16S rRNA gene sequencing. Both of them were identified to *Lactobacillus johnsonii* with 100% similarity. *L. johnsonii* CK3 and *L. johnsonii* VCF29 were not haemolytic strains and their percentages of auto-aggregation value were 18.37±5.30 and 9.19±0.71, respectively. Resistibility to acidity at pH 2.5 and 0.3% bile acid of *L. johnsonii* VCF29 (94.68 and 94.73%) were greater than those of *L. johnsonii* CK3 (62.48 and 87.34%). Both strains were susceptible to cefoxitin, chloramphenicol, vancomycin, ampicillin and ceftriaxone. In addition, they exhibited antibacterial activity against pathogenic *Staphylococcus aureus*, *Proteus vulgaris*, *Salmonella* Typhimurium and *Escherichia coli*. It might be indicated that *L. johnsonii* CK3 and VCF29 could be good probiotic candidates applied as functional feed supplement for monogastric animal, especially broiler.

Probiotic characterization of thermotolerant *Lactobacillus johnsonii* isolated from broiler intestine

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ABSTRACT

Bacterial community in human and animal gastrointestinal tract (GI) are diverse. In GI tract of healthy hosts, lactic acid bacteria (LAB) can be found as dominant flora. Some strains of LAB have been accepted as probiotic due to the fact that they contribute many health benefits to host. Several probiotics are isolated and applied in functional food and feed products for the specific consumers, including human and animal. Nowadays, thermotolerant probiotics are of interest to industrial application, because of their high heat-resistant ability in food and feed manufacturing. The aims of this study were to isolate thermotolerant LAB from broiler intestine and evaluate their probiotic characteristics for monogastric feed application. Two promising isolates, CK3 and VCF29 were selected and identified by 16S rRNA gene sequencing. Both of them were identified to *Lactobacillus johnsonii* with 100% similarity. *L. johnsonii* CK3 and *L. johnsonii* VCF29 were not haemolytic strains and their percentages of auto-aggregation value were 18.37±5.30 and 9.19±0.71, respectively. Resistibility to acidity at pH 2.5 and 0.3% bile acid of *L. johnsonii* VCF29 (94.68 and 94.73%) were greater than those of *L. johnsonii* CK3 (62.48 and 87.34%). Both strains were susceptible to cefoxitin, chloramphenicol, vancomycin, ampicillin and ceftriaxone. In addition, they exhibited antibacterial activity against pathogenic *Staphylococcus aureus*, *Proteus vulgaris*, *Salmonella* Typhimurium and *Escherichia coli*. It might be indicated that *L. johnsonii* CK3 and VCF29 could be good probiotic candidates applied as functional feed supplement for monogastric animal, especially broiler.

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1. INTRODUCTION

The microbial community of animal gastrointestinal (GI) tracts are complex and diverse, especially in the large intestine. They involve not only in nutritional digestion, but also the synthesis of vitamins, bioconversion of toxic compounds to non-toxic compounds, stimulation of immune system, maintenance of gut peristalsis and intestinal mucosal integrity and prevention of pathogen colonization (Ahasan et al., 2015). Therefore, the strategy in manipulation of microbial ecosystem in GI tract to enhance animal health, productivity and welfare has been introduced by many researcher, meanwhile, study of diversity and role of gut microbiota on animal health have being intensively investigated.

Many species of lactic acid bacteria (LAB) are accepted as probiotic and applied as feed additive in livestock production, since they play important roles on animal health, especially contribute the balance of gut microbiota. FAO and WHO (2001) defined the term "probiotic" as "live microorganisms which when administered in adequate amounts confer a health benefit to the host". By this definition, probiotics have to be able to tolerate to acid in gastric juice and bile in upper small intestine, susceptible to antibiotics, adhere to epithelial surfaces, exhibit antagonistic activity against pathogens (such as *Helicobacter pylori*, *Salmonella* sp., *Listeria monocytogenes*, *Clostridium difficile*), anti-mutagenic and anti-carcinogenic properties, and so on (Kumar and Kumar, 2015; García-Hernández et al., 2016). Lactobacilli seem to be the most well-known probiotic potentially used in livestock production. *L. reuteri*, *L. acidophilus*, *L. animalis*, *L. fermentum*, *L. salivarius* and *L. johnsonii* are commonly applied in livestock production. Supplementation of these probiotic to swine and poultry feed gain many benefits. In swine, improvement of colostrum and milk quality, feed conversion ratio, diet digestibility and meat quality, increasing of piglet weight, reducing a risk of diarrhea and limiting constipation were obtained, while the increasing of body weight gain, carcass quality and bone quality and reducing of mortality were found in poultry production.

Nowadays, promising probiotic strains for feed supplement industry have to be considered also about survivability during manufacturing and stability in the product during storage. By this context, thermotolerant probiotic are of interesting. Thermotolerant LAB have been widely used as starter cultures in many food industries such as fermented milk, alcoholic beverages and sourdough, because of their higher heat-resistant ability during manufacturing. Moreover, the strain origin of probiotic must be another criterion for selection prior use. Those isolated from the same animal as the intended use have higher possibility of survival (Gibson and Fuller, 2000).

Previously, diversity of LAB in broiler GI tract was investigated in our Lab and we found some of them were thermotolerant LAB. Promising isolates were subsequently isolated and studied for their possibility applying as probiotic additive for monogastric animal production, especially broiler. The aims of this study were then to isolate and identify thermotolerant LAB from broiler intestine and evaluate their probiotic characteristics for monogastric feed application.

2. MATERIALS AND METHODS

2.1 Isolation of thermotolerant LAB and identification.

Thermotolerant LAB were isolated from broiler feces as previously described (Niamsup et al., 2003). Briefly, the fecal samples were inoculated into glucose/peptone/yeast extract (GPY) broth, incubated anaerobically at either 40, 45 or 50°C for 24 h and spread onto De Man Rogosa and Sharpe agar (MRS). Colonies were selected and maintained on MRS agar. The genomic DNA of the isolates was extracted and purified using a genomic DNA extraction kit (TIANamp Bacteria DNA Kit, China) and used as a template to amplify and sequence 16S rDNA, resulting in species identification.

2.2 Characterization of probiotic properties

Probiotic properties of thermotolerant LAB isolated from previous experiment were characterized as follow.

2.2.1 Hemolytic activity

Hemolytic activity of the isolates was tested by inoculation on blood agar (7% (v/v) sheep blood) and incubation at 37°C for 24 h (Pieniza et al., 2014). The isolates which did not exhibit lyse zone around their colonies were considered as non-hemolysis (γ-hemolysis). In case of hemolytic isolate, there were considered and classified into 2 types, green-hued zone (α-hemolysis) and clear lysed zone (β-hemolysis) production.

2.2.2 Acid and bile tolerant ability

The test of resistance under acid condition was carried out *in vitro* according to Rajam et al. (2012). Simulated gastric juice was prepared by 0.5% (w/v) pepsin in phosphate-buffered saline (PBS), pH 2.5. One ml of cell suspension (10^8 CFU) was transferred into 9 ml of simulated gastric juice, mixed well and incubated anaerobically at 37 ºC for 3 h. The interval sampling during incubation for viable cell enumeration on MRS agar was done. The bile tolerance assay was tested according to Yamano et al. (2006) with modifications. One ml of cell suspension (10^8 CFU) were transferred to MRS broth supplemented by 0.3% (w/v) oxgall bile (Sigma) and subsequently incubated anaerobically at 37 °C for 3 h. Cell suspension was taken interval and enumerated for the survival cells on MRS agar.

2.2.3 Autoaggregation Assay

The isolates were grown anaerobically in MRS broth for 24 h at 37 °C. Cells were harvested by centrifuge at 4500 rpm for 10 min, washed twice and re-suspended in phosphate-buffered saline (PBS) at pH 7.2 to obtain approximately 10^8 CFU/ml. Bacterial cell suspensions and PBS (1:1 mL) were mixed by vortexing for 10 s and incubated at room temperature for 2 h. The optical density of the upper layer was measured at 600 nm (PBS was used as a blank). The auto-aggregation percentage expressed as

$$(1- [A_t/A_0]) \times 100$$

where A_t represents the absorbance at time t = 2 h and A_0 the absorbance at t= 0 h. (Tarep et al., 2013)

2.2.4 Antibiotic susceptibility

Antibiotic susceptibility of the isolates was tested by the agar diffusion disk method (Gheziel et al., 2019). The commercial antibiotic disc used in this study were of cefoxitin (30 µg), tetracycline (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), clindamycin (2 µg), vancomycin (30 µg), ampicillin (10 µg) and ceftriaxone (30 µg). The 24 h-old inoculum of isolated LAB was spread on MRS agar. Then each antibiotic discs were immediately placed on the surface of agar and incubated at 37 °C for 24 h. The inhibition zone diameters were measured, and susceptibility was expressed in terms of resistant (R) and susceptible (S).

2.2.5 Antimicrobial activity

Some pathogens to monogastric animal were used as tested organism in this study, including *Staphylococcus aureus*, *Proteus vulgaris*, *Salmonella* Typhimurium and *Escherichia coli*. The antimicrobial activity test against these pathogens were evaluated using the agar spot test described by Shokryazdan et al. (2014) with modifications. Briefly, two µl of 24 h-old inoculum of each isolated LAB (10^8 CFU/ml) was spotted on MRS agar plates, dried for 30 min at room temperature and then incubated anaerobically at 37 °C for 24 h. After colony development, the agar were overlaid with 10 ml of mixture between 0.7% (w/v) agar and the 24 h-old inoculum of pathogen (adjusted to 10^8 CFU/ml) and incubated aerobically at 37 °C. Inhibition zones around LAB colonies were measured after 18 h of incubation (outer edge of the colony to the outer edge of the clear zone).

3. RESULTS AND DISCUSSION

3.1 Identification of thermotolerant LAB

We found two isolates (CK3 and VCF29) from broiler feces, which could tolerate to 50 °C. They were Gram-positive, rod shape, non-spore forming and catalase negative bacteria. According to 16S rDNA sequencing, CK3 and VCF29 were identified to *Lactobacillus johnsonii* with 100% similarity. In a neighbour-joining dendrogram created based on the sequence of CK3, VCF29 and sequences from the GenBank database, the phylogenetic position of CK3 and VCF29 was determined. The phylogenetic tree showed that the strains form an evolutionary lineage within the radiation of a cluster comprising *Lactobacillus* species and is phylogenetically most closely related to *L. johnsonii.* (Figure 1)

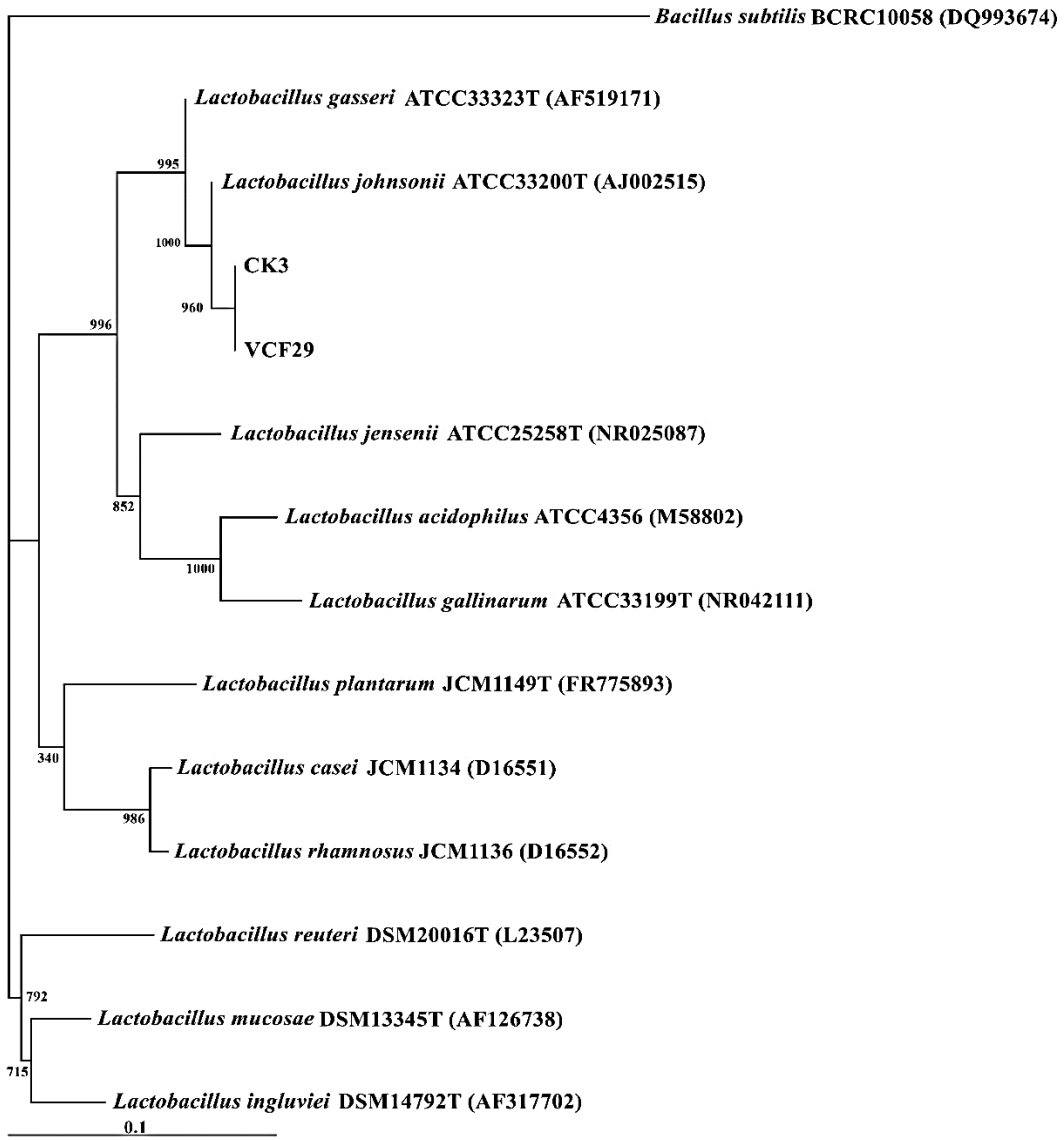

Figure 1. Phylogenetic trees constructed using the neighbor-joining method with the full-length 16S rRNA gene sequences from the isolated thermotolerant LAB strains

3.2 Characterization of probiotic properties

Two promising isolates, *L. johnsonii* CK3 and L. johnsonii VCF29 were selected and characterized for their probiotic properties. Moreover, two standard strains of *L. johnsonii* from the Japan Collection of Microorganisms, RIKEN BioResource Center, Japan, i.e. *L. johnsonii* JCM1022 and *L. johnsonii* JCM8791, were studied for comparison.

We found that all isolates including reference strains were non-hemolytic bacteria (γ-hemolysis), since they did not exhibit any effect on blood agar plates after 48 h of incubation. It might be indicated that they were not harmful strains or rarely cause illness. Probiotics must survive from the extreme conditions in GI tract of animals, especially high acidity in stomach and bile in the upper small intestine. Figure 2 and 3 show the survival ability of the selected thermotolerant isolates. After 3 h incubation in simulated gastric juice (0.5% pepsin, pH 2.5), we found that VCF29 could survive in this condition similar to the reference strain of JCM1022. Even the survivability of other two strains were lower, but about 65-80% of their survival rate were obtained (Figure 2). CK3 was the lowest survivability strain under gastric condition. This strain also

exhibited lower survivability in the simulated bile (0.3% bile acid) but only slightly lower than other strains (Figure 3). Interestingly, they could resist to bile with the survival rate higher than 90%. Effective probiotics must possess these characteristic to guarantee the number of viable probiotic cells reach to the colon. Survival of probiotic along the GI tract depends on not only these particular characteristics, but also because of the feed matrix (composition of feed ingested) and competition of microbiota in the intestine. The results from this experiment were consistent to the study of Aiba et al. (2015) and Yamano et al. (2006). A commercial *L. johnsonii* could survive when incubated at pH 1.0, 1.5 or 2.0 at 37 °C up to 120 min. Moreover, *L. johnsonii* La1 showed the great survivability after 15 h incubation in 0.1% bile acids and the simulated gastric juice among all tested bacteria. In case of bile resistance of Lactobacilli and Bifidobacteria, multi mechanisms involve in detoxification of bile; i.e. bile salt hydrolase production, active efflux of bile acids/salts and changing in the composition of cell membrane and cell wall (Ruiz et al., 2013).

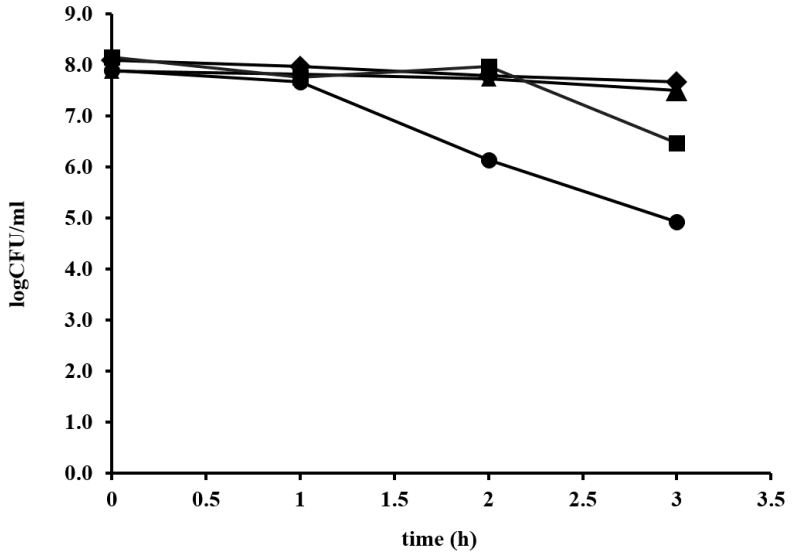

Figure 2. Survivability of thermotolerant *L. johnsonii* CK3 (●) and *L. johnsonii* VCF29 (♦) in simulated gastric juice comparing to the reference strains of *L. johnsonii* JCM1022 (▲) and *L. johnsonii* JCM8791 (■)

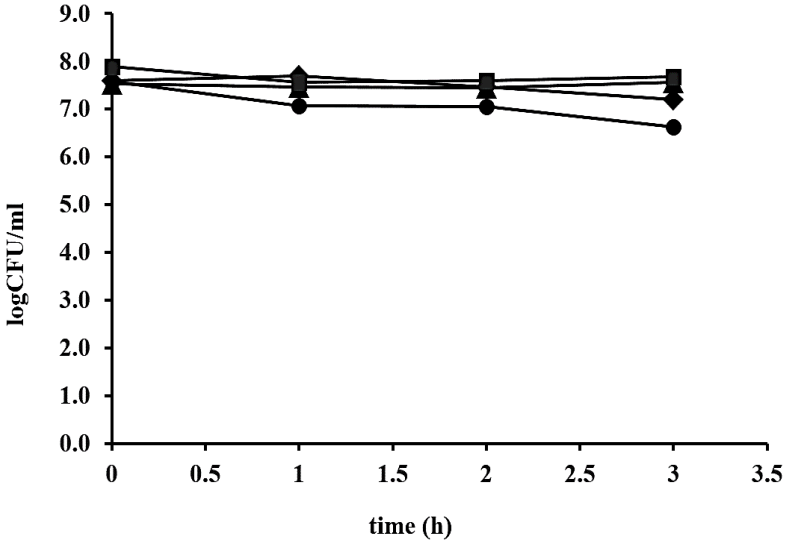

Figure 3. Survivability of thermotolerant *L. johnsonii* CK3 (●) and *L. johnsonii* VCF29 (♦) in bile comparing to the reference strains of *L. johnsonii* JCM1022 (▲) and *L. johnsonii* JCM8791 (■)

The ability in adhere to intestinal epithelial cells of these isolates were indirectly tested by the autoaggregation assay. It was found that the auttoaggregation percentage values ranged between 9.2% and 24.8% after 2 h incubation (Figure 4). Among the thermotolerant LAB isolates tested, CK3 showed significantly high autoaggregation comparing to VCF29 ($p<0.05$) and not significantly different to a reference strain of JCM1022, even that of JCM1022 was higher ($p>0.05$). Autoaggregation of probiotics was considered to be necessary for adhesion to intestinal epithelial cells, then form a barrier preventing a colonization by pathogenic microorganisms. The colonized probiotic cells may also reduce the number of pathogens by reducing the pH of the gut, causing direct antagonism against pathogen (Vesterlund et al., 2005). Thus, CK3 and JCM1022 exhibited higher potential for this purpose. Interestingly, autoaggregation ability of CK3 was slightly higher comparing to other probiotic strains and markedly higher than that of some enteric pathogens in the study of Tareb et al. (2013), although only 2 h incubation was applied in our study. Therefore, CK3 could be accepted as one of the effective competitor in colon colonization.

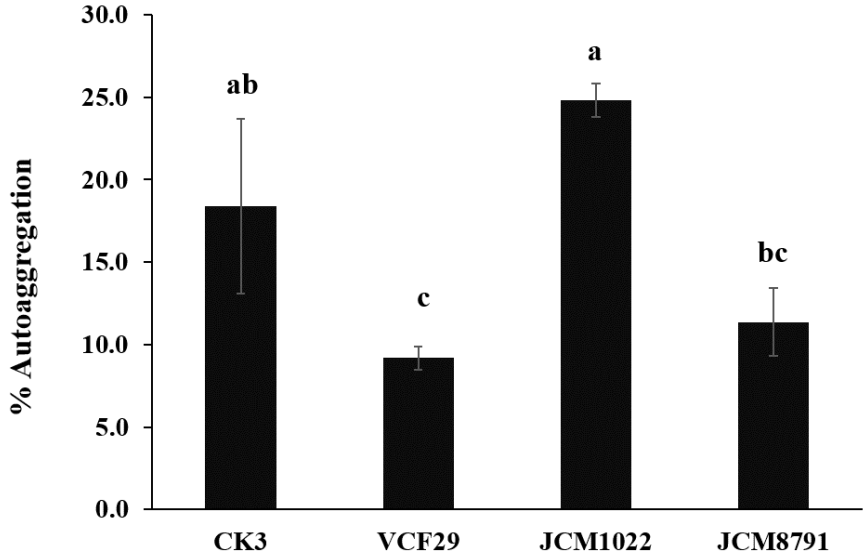

Figure 4. Autoaggregation percentages of the isolated thermotolerant LAB comparing to reference strains of *L. johnsonii*

*Different letters represent significant difference ($p<0.05$). Duncan's multiple range test

The antibiotic resistances of the isolated thermotolerant LAB against eight common antibiotics were determined by the agar diffusion method as shown in Table 1. CK3, VCF29 and JCM8791 were susceptible to almost antibiotics, but only JCM1022 was susceptible to all tested antibiotics. Our thermotolerant CK3 and VCF29 could resist to erythromycin and tetracycline, respectively, while the reference strain of JCM8791 was resistant to tetracycline and clindamycin. The obtained results were in accordance with previously reported data for Lactobacilli and Bifidobacteria. They are generally sensitive to antibiotic erythromycin, tetracycline, chloramphenicol and ampicillin (Georgievaa et al., 2015). Actually, the transferring of antibiotic resistance genes from probiotic to enteric pathogens, either in food matrix or in GI tract, has been concerned as a global issue (Sharma et al., 2017). Thus, non-antibiotic resistance probiotics are of interest for applying in feeds and foods. However, there is an argument on this ability by some researcher. The advantage of antibiotic resistibility of probiotics was introduced, for example they could survive in host GI tract during the treatment by antibiotic in the case of some diseases.

Table 1. Antibiotic resistances of thermotolerant LAB depending upon various antibiotic

Antibiotics disc	CK3	VCF29	JCM1022	JCM8791
Cefoxitin	S	S	S	S
Tetracycline	S	R	S	R
Chloramphenicol	S	S	S	S
Erythromycin	R	S	S	S
Clindamycin	S	S	S	R
Vancomycin	S	S	S	S
Ampicillin	S	S	S	S
Ceftriaxone	S	S	S	S

* The inhibition zone diameters were measured, and susceptibility was expressed in terms of resistant (R) and susceptible (S).

Finally, the antibacterial activity of the isolates against those common pathogenic bacteria was studied and the results shown in Table 2. Every *L. johnsonii* could inhibit growth of all tested organisms but different level. Antibacterial activity of CK3 and VCF29 were almost similar, exception with the test of *S. aureus*. VCF29 exhibited strong antibacterial activity against *S. aureus* (zone of inhibition > 6 mm). However, both reference strains showed strong antibacterial ability against all tested pathogens. The antibacterial activity to other enteric bacteria was also reported according to Aiba et al. (2015). They found that *L. johnsonii* No. 1088 inhibited the growth of *H. pylori*, *E. coli* O-157 and *C. difficile*. The possibility of antagonistic activity of probiotics mostly attribute to the production of antimicrobial substances or metabolites such as organic acids, hydrogen peroxide and so on (Pridmore et al., 2008).

Table 2. Antimicrobial activity of the LAB from thermotolerant LAB from broiler intestine

Strains	clear zone (mm)			
	S. aureus	*P. vulgaris*	*S.* Typhimurium	*E. coli*
CK3	4.0±0.0	6.0±0.0	5.0±0.0	4.0±0.0
VCF29	10.3±0.6	6.0±0.0	5.0±0.0	5.0±0.0
JCM1022	6.7±1.2	7.0±0.0	7.0±0.0	6.3±2.3
JCM8791	10.0±1.7	7.0±0.0	7.0±0.0	6.7±0.6

4. CONCLUSION

Two isolates of thermotolerant LAB, CK3 and VCF29, from broiler feces were identified by 16S rRNA gene sequencing to *L. johnsonii* with 100% similarity. *L. johnsonii* CK3 and *L. johnsonii* VCF29 were not hemolytic strains and able to tolerate in acidic condition of stomach and bile of upper small intestine. According to their percentages of autoaggregation values, there was possibility that both strains could colonize on colon epithelial cells. Both strains were susceptible to common antibiotics (cefoxitin, chloramphenicol, vancomycin, ampicillin and ceftriaxone). In addition, they exhibited antibacterial activity against entheric pathogenic *S. aureus*, *P. vulgaris, S.* Typhimurium and *E. coli*. Therefore, the thermotolerant *L. johnsonii* CK3 and *L. johnsonii* VCF29 isolated from broilers could be interesting probiotic candidates applied as functional feed supplement for monogastric animal, especially broiler.

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