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Research Article
Detection of Lactic Acid Bacteria (LAB) from Local Breed Chicken Gut as Probiotic Agent in Livestock

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Abstract

Background and Objective: Farmers are still improving growth and feed efficiency using feed-additive antibiotics in livestock although it has been banned due to food safety. The urgent need to search for probiotics as a beneficial microbial feed supplement to replace antibiotics is therefore crucial. This study was conducted to identify and characterize the potential probiotic features of lactic acid bacteria(LAB) from the gut of local breed chickens. **Materials and Methods:** Twenty-five samples were collected from the crop, gizzard, small intestine, large intestine, caecum of the chickens from different regions of Borneo, Malaysia. The bacteria were identified phenotypically (Gram staining, biochemical tests) and genotypically by $(GTG)_5$ PCR fingerprinting. The probiotic characteristics of LAB were studied using fermentation (1% glucose) and bile tolerance (0.2, 1.0, 2.0 and 3.0%). **Results:** About one hundred presumptive LAB with Gram-positive, catalase-negative, non-motile properties were obtained from 25 samples. Genotypic $(GTG)_5$ fingerprinting indicated three main clusters as shown in the dendrogram. However, only 7 isolates showed high survival rate and able to ferment glucose under gut condition, indicating their potential as probiotics agents in poultry husbandry with no adverse health consequences. **Conclusion:** Our study proposed the use of these LAB isolates from the gut of local chicken breed as potential probiotics agents to enhance the immunity and growth in the chicken.

Key words: Chicken gut, potential probiotics, lactic acid bacteria, bacterial isolates, poultry husbandry

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The increase in global population has led to higher food requirements especially protein from meat sources. According to Skarp *et al.*[1], poultry such as turkey, duck, laying hens and chicken are the most common domesticated birds and major meat producer in the world, with 70-80% of global meat production. Poultry can be considered as preferred protein sources by the population of the world because it is inexpensive and easy to obtain. The major broiler-producing countries in the world in terms of volume are United States, European Union (EU15), China, Brazil, India, Mexico, Japan, Thailand and Canada while Canada, United States, Brazil, China, EU 15, Mexico, Japan, India, Russia, Saudi Arabia are the dominant consumers[2]. The development of a safe and stable infrastructures specialized for poultry industry in most developing countries, has enabled entrepreneurs to enhance their poultry businesses to highly modernized, commercialized and efficient production systems.

Antibiotics are one of the remarkable medical disclosure of the 20th century. Antibiotics have long been used globally in animal industry since 1940s for treating or preventing zoonotic diseases[3] and as growth promoter[4,5]. However, misuse of antibiotic has led to an increase in antibiotic-resistant microbes. Chicken meat can act as a medium for the transmission of multi drug resistant bacteria to consumers[6,7]. Consequently, this has led to the failure of antibiotic treatment[5,6]. About 25,000 patients suffered from the contaminations caused by drug-resistant bacteriaand died each year. Therefore, the adoption of antibiotic growth promoter in livestock feed has been banned by several countries including South Korea[6]. Withdrawal of antibiotics was a great challenge forthe poultry industry to maintain production performance, as the feed costs has increased and the prohibition of antimicrobial use in feeds causeda high rate of mortality in poorly maintained flocks[8]. Hence, an effective approach to overcome this problem is to replace antibiotic with probiotic.

Probiotic is defined as a single culture or combination of a few cultures of live microorganisms that are added in animal feed purposely to improve the host's health by givingthem in an adequate amounts[9]. A balanced poultry diet supplemented with probiotics can balance the ecological microflora within the intestine, minimize the pathogen growth[10] and enhance the production performance[11] without inducing antibacterial resistance reaction. Lactic acid bacteria (LAB) is a common bacterial group generally applied as probiotics. LAB was found to have capacityto enhance animal wellbeing by modulation of immune system and protection against pathogens[12]. Thus, the intention of the current study was to characterize the potential probiotic features of LAB found in the gut of local breed chickens.

MATERIALS AND METHODS

Collection and cultivation of samples: Twenty-five gut samples (crop, gizzard, small intestine, large intestine, caecum) of healthy local breed chickens (20 days old) were collected from different region of Borneo, Malaysia. The samplewas collected from January-December 2017. The gut samples were serially diluted in PBS (0.1 M, pH 7.2) and spread on to the sterilized media such as Nutrient agar (NA) (pH 7 and pH 2.5) (Merck, Germany), Eosin Methylene Blue agar (EMBA) (pH 7) (Merck, Germany) and de Man, Rogosa and Sharpe agar (MRSA) (pH 2.5) (Merck, Germany) plates.All the agar plates were incubated in aerobic condition at 37°C for 24 h except for MRSA plates whichwere incubated anaerobically. The single producing colonies were selected for further characterization.

Total bacterial count: All colony forming units (CFU) that grew on the plates within range from 30-300 colonies were counted and recorded[13].

Phenotypic and genotypic screening of LAB isolates: The isolates were subjected to test of Gram reaction, catalase and motility. The gram positive, catalase-negative and non-motile isolates were suspected as LAB.Bacterial DNA of the presumptive LAB was obtained using the boiling centrifugation method as described by Sien *et al.*[14]. A volume of 1.5 mL of bacteria culture kept for 24 h was then transferred into a 2.0 mL microcentrifuge tube and centrifuged at 10,000 rpm for 5 min. The above supernatant was discarded. After that, 500 µL of sterile distilled water was added and vortexed to re-suspend the cell pellet. The microcentrifuge tube was then boiled for 10 min and immediately transferred into ice for 5 min. Lastly, the sample was centrifuged at 10,000 rpm for 10 min and the supernatant was collected. $(GTG)_5$ PCR fingerprinting technique was performed as described by Sien *et al.*[15] with slight modification. A total of 25 µL PCR mix was prepared by mixing sterile distilled water (dH_2O), 5X Taq Green Buffer (Promega, USA), 25 mM $MgCl_2$, 25 mM deoxyribonucleotide phosphate (dNTPs), 25 µM $(GTG)_5$ primer (5'- GTGGTGGTGGTGGTG-3'), Taq DNA polymerase and DNA template.The protocol of DNA amplification is as follows: pre-denaturation (7 min, 95°C), followed by four cycles involving denaturation, annealing and extension for 2 min

(95°C), 2 min (36°C) and 2 min (72°C), respectively. Next, denaturation with 30 cycles was performed at 95°C for 1 min, then annealing at 50°C for 1 min and elongation at 72°C for 1 min. Lastly, final elongation was conducted for 5 min at 72°C. Product was viewed on 1.5% (w/v) agarose gel at 100 V for 1 h and 30 min. Scoring was accomplished for assembly of dendrogram. The presence of bands were recorded as "1", while absence of band was recorded as "0". All the information was keyed in to the RAPDistance system to generate the phylogenetic tree.

Probiotic characterization of isolated bacterial strains

Glucose fermentation: The glucose fermentation test was conducted using the modified method of Shakoor *et al.*[16]. Overnight cultures of 13 LAB isolates were inoculated (1% v/v) in MRS medium 1.0% (w/v) glucose. The phenol red and Durham tube were added in MRS medium as fermentation reaction and gas indicator, respectively in the test. The media were further incubated at 37°C for 24 h. Colour changes and gas formation were recorded.

Bile salt tolerance: The bile salt tolerance test was conducted using the modified method of Shakoor *et al.*[16]. Overnight cultures of LAB isolates were inoculated (1% v/v) into MRS medium supplemented with 0.2, 1.0, 2.0 and 3.0% (w/v) Oxgall. The survival counts were determined after the incubation for 3 h at 37°C using the following equation.

$$\text{Survival rate (\%)} = \frac{\text{Final-initial (log CFU mL}^{-1})}{\text{Initial (log CFU mL}^{-1})} \times 100$$

Statistical analysis: Data were analyzed using the two-way analysis of variance (ANOVA). All means were separated using Tukey's Multiple Comparison Test in all assays and difference were considered statistically significant if $p<0.05$.

RESULTS

Total bacterial count: Table 1 shows the mean total bacteria colony count ($\log_{10}$ CFU mL^{-1}) isolated from the local breed chicken gut cultivated with multiple media and pH condition. Result indicated that large intestine hosted the highest bacterial population ($\log_{10}$ 7.05±0.05 CFU mL^{-1} and $\log_{10}$ 5.10±0.00 CFU mL^{-1}) when cultivated at pH 2.5. The caecum and crop hosted the highest bacterial population ($\log_{10}$ 6.95±0.15 CFU mL^{-1} and $\log_{10}$ 6.25±0.15 CFU mL^{-1}) when cultivated on NA and EMBA at pH 7. However, the lowest bacterial population was shown in gizzard disregarding the media type and condition. The mean bacterial colony count isolated from caecum cultivated on NA was significantly different from the other samples while gizzard or small intestine was significantly different from the other samples cultivated on EMBA at pH 7. Besides, the mean bacterial colony count isolated from all samples were significantly differ from each other when cultivated at pH 2.5.

Identification of LAB: A total of 500 isolates were isolated from the gut of local breed chickens. A total of 420 bacteria were Gram positive, 425 bacteria were catalase negative and 150 bacteria were non-motile (Table 2). Out of the total isolates, 100 were gram positive, catalase-negative and

Table 1: The means bacteria count ($\log_{10}$ CFU mL^{-1}) for bacteria isolated from local breed chicken gut cultivated on NA, EMBA and MRSA media and pH condition

	Mean bacteria colony count ($\log_{10}$ CFU mL^{-1})			
Sample sources	NA (pH 7)	NA (pH 2.5)	EMBA (pH 7)	MRSA (pH 2.5)
Crop	6.20±0.20^{b}	4.90±0.10^{d}	6.25±0.15^{a}	3.90±0.20^{bc}
Gizzard	4.80±0.00^{c}	4.15±0.05^{e}	4.40±0.20^{c}	3.05±0.05^{d}
Small intestine	5.10±0.10^{c}	6.15±0.05^{c}	5.25±0.05^{b}	4.20±0.00^{b}
Large intestine	6.25±0.05^{b}	7.05±0.05^{a}	6.05±0.05^{a}	5.10±0.00^{a}
Cecum	6.95±0.15^{a}	6.45±0.05^{b}	5.85±0.05^{a}	3.80±0.10^{c}

*Means values within a column with different superscript differ significantly at $p<0.05$. NA: Nutrient agar, EMBA: Eosin Methylene Blue agar, MRSA: de Man, Rogosa and Sharpe agar

Table 2: The phenotypic characterization of total LAB isolates (n) on Gram stain, catalase and motility test

Sample sources	Gram positive (n)	Catalase test (n)	Motility test (n)
Crop	80	80	25
Gizzard	95	85	10
Small intestine	100	90	25
Large intestine	55	80	45
Cecum	90	90	45
Total	420	425	150

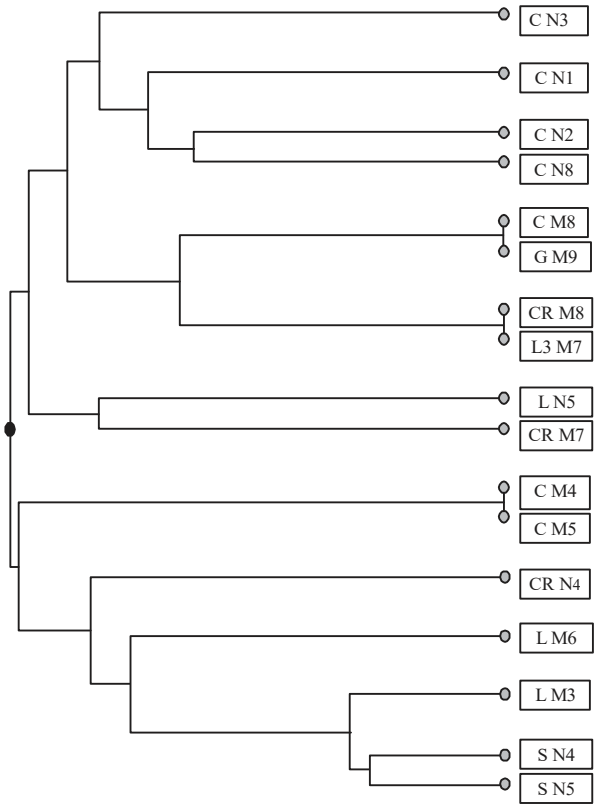

Fig. 1: Dendrogram showing the potential probiotic LAB distribution isolated from gastrointestinal tracts of local breed chicken gut

non-motile as compared to positive control (*Lactobacillus plantarum* ATCC 8014). Genotypic $(GTG)_5$ fingerprinting indicated three main clusters as shown in the dendrogram (Fig. 1).

Glucose fermentation: Among the 100 LAB, only 9 isolate species showed the ability to utilise glucose as source of energy. Energy production is a dissimilative metabolism that was shown by the change in colour and presence of gas after growth in MRS medium having 1.0 % glucose (Table 3).

Bile salt tolerance: Table 4 shows the results of bile salt tolerance analysis of LAB. Figure 2 shows the survival rate of the 13 isolates in MRS medium supplemented with 0.2, 1.0, 2.0 and 3.0% bile salt after 3 h incubation. The *Lactobacillus plantarum* ATCC 8014 was incorporated as control in the test. Among the 100 LAB, only 13 isolates showed the resistance to various concentrations of bile salt. Nonetheless, the growth rate of the isolates declined with the raised concentration of bile salt. Eleven isolates showed the maximal viability in 3.0% bile salt conditions.

Table 3: Glucose fermentation analysis of the 9 lactic acid bacteria isolates in MRS medium supplemented with1.0% glucose

	Glucose fermentation	
Bacteria isolates	Colour change*	Gas formation#
Control§	+	-
CR N4	-	+
CR M9	+	-
G M1	+	-
S N4	+	-
L M7	+	-
C N2	+	-
C N3	+	-
C M4	+	-
C M6	+	-

*Colour change: -: Red, +: Yellow, #Gas formation: -: No bubble; +: Bubble, §Control: *Lactobacillus plantarum* ATCC 8014

Table 4: Bile salt tolerance test analysis on the 13 lactic acid bacteria isolates in MRS medium supplemented with 0.2, 1.0, 2.0 and 3.0% bile salt after 3 h incubation

	Bile salt concentration* (%)			
Bacteria isolates	0.2	1.0	2.0	3.0
Control§	+	+	+	+
CR M7	+	+	+	+
CR M9	+	+	+	+
G M1	+	+	+	+
G M10	+	+	+	+
S N4	+	+	+	+
L M3	+	+	+	+
L M6	+	+	+	+
L M7	+	+	+	+
C N1	+	+	-	-
C N3	+	+	-	-
C M4	+	+	+	+
C M6	+	+	+	+
C M8	+	+	+	+

*Bile salt tolerance test: -: Less than 30 colonies, +: More than 300 colonies, §Control: *Lactobacillus plantarum* ATCC 8014

Probiotic properties analysis: Based on the overall results, only seven isolates (CR M9, G M1, S N4, L M7, C N3, C M4, C M6) displayed high survival rate and fermentation ability under gastrointestinal condition (>80%), expressing their potential to be utilized as probiotic agents.

DISCUSSION

Gastrointestinal tract is the part of organ that plays an important role in digesting food and absorbing nutrients for chicken growth. It is also act as a reservoir of microbiota which are diverse in density and diversity throughout the tract. The distribution of these microflora varied in each part of the gastrointestinal tract. These can be seen through the mean population of isolates that grew on the non-selective and selective media agar. Bacterial population isolated from large intestine was found highest when cultivated in acidity

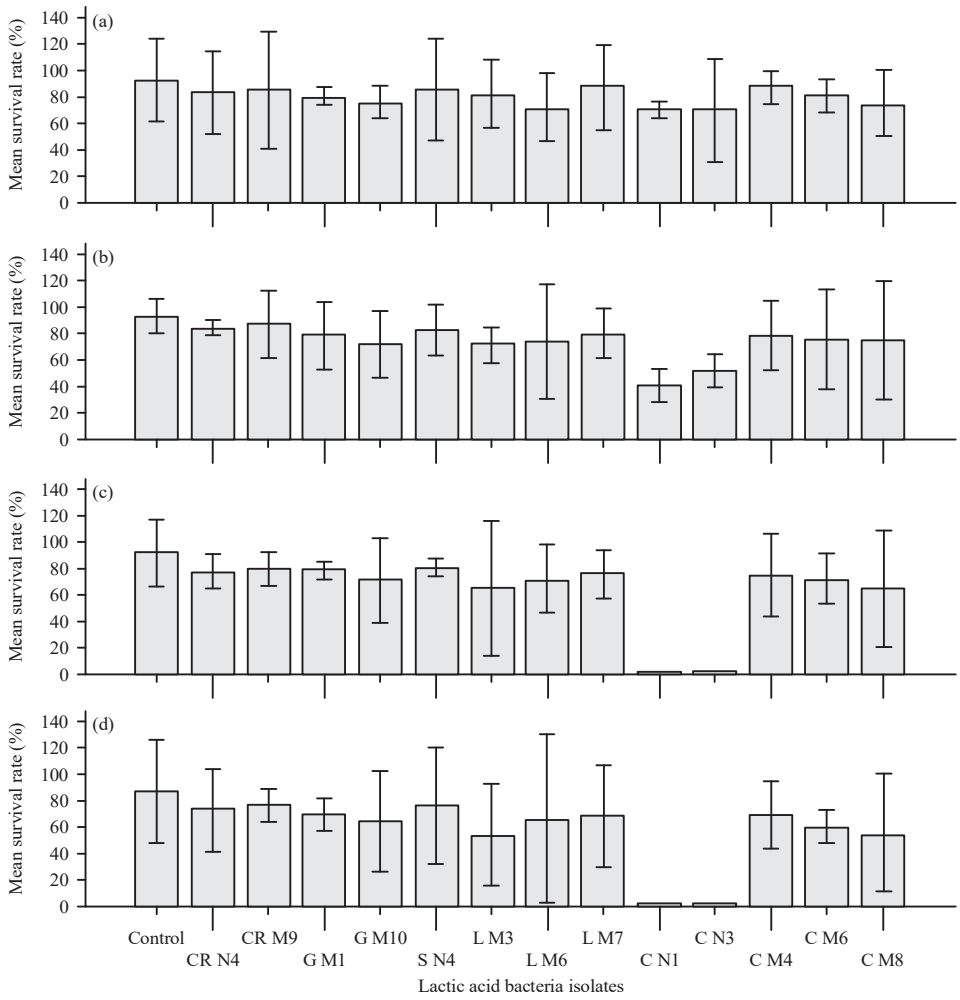

Fig. 2(a-d): Survival rate of 13 potential probiotic LAB isolates in MRS medium supplemented with 0.2, 1.0, 2.0 and 3.0% bile salt after 3 h incubation. Control: *Lactobacillus plantarum* ATCC 8014

condition. However, bacterial population isolated from caecum was found highest when cultivated in neutral condition. Both large intestine and cecum are located at the distal of poultry gut. Meanwhile, gizzard which is located at the proximal of poultry gut contain the least mean population of microflora for both culture condition. This showed that microflora population densities tend to increase from the gizzard to the cecum and microflora population densities tend to incline from the proximal to distal gastrointestinal tract[17]. Small and large intestine has a short retention time period although much simple form of nutrient are available in small intestine. The implication of environmental stress on the proventriculus, such as low pH and high bile salt content, may suppress the colonisation of bacteria in that compartment and influence the bacterial colonisation in the small intestine. This was further supported by Rinttilä *et al.*[18] who stated that low pH and rapid passage of intestinal content caused a decrease in bacterial colonisation in duodenum and jejunum but the bacterial count increased up to 10^8 cells mL^{-1} in the distal ileum. The highest content of gut microbiota in cecum and large intestine might be due to the rich content of undigested nutrients in those parts. According to Walter[19], bacteria in the distal gut obtained their carbon and energy requirements from the complex carbohydrates, fats and proteins that were not digested in small bowel and also from the mucins and sloughed epithelial cells. Therefore, the LAB was suspected to have *Lactobacillus* species including *L. plantarum*, *L.acidophilus* and *L. paracasei* which successfully inhabit in the cecum. These species were able to utilize the remaining complex carbohydrate that were undigested by enzyme secreted in the proximal gut[20]. The selective medium EMBA was used to isolate the presumptive Gram negative bacteria. Presumptive Gram negative bacteria isolated from small intestine and gizzard were significantly different. The highest

number of gram-negative bacteria were found in crop. A previous study conducted by Azad *et al.*[21] reported that Gram negative bacteria resided largely in crop.

The phenotypic characterization of bacteria isolates was successfully conducted to isolate 100 presumptive LAB with Gram positive, catalase negative and non-motile characteristic. Table 2 shows that large intestine and cecum colonized with most presumptive LAB. Dendographic analysis on the $(GTG)_5$ banding profiles was performed which showed that the constructed dendrogram tree is inferred with three main clusters. Three major clusters namely Bacteroidetes (*Porphyromonas*, *Prevotella*), Firmicutes (*Ruminococcus*, *Clostridium* and *Eubacteria*) and Actinobacteria (*Bifidobacterium*) are the most common bacterial species found in the gut microbiome[22]. Thus, the three major clusters as shown in dendrogram in the present study may come from the above mentioned bacteria family.

The ability to survive under high bile salt concentration and low pH in gastrointestinal tract[2,23] is an essential characteristic of probiotic bacteria and they are also able to utilize glucose as source of energy[8]. In this study, 100 LAB isolates were obtained from healthy local breed chicken gut and were grouped according to their low pH, survival ability under the counterfeit conditions and glucose fermentation ability. Among them, 9 isolates showed the ability to hydrolyse 1.0% glucose as source of energy and 13 isolates showed more than 80% survival rate at the 1.0 % bile concentration. The fermentation ability of LAB can help improve the feed digestibility of poultry[8]. The bile salt-resistant LAB can hydrolyse bile salts effectively, indicating their ability to endure and survive within the digestive systems. A previous study reported that bile tolerance ability might reduce the cholesterol level in serum of poultry[16]. The survival rate was reported much higher than the previous studies conducted on *Lactobacillus rhamnosus* and *Lactobacillus casei* at low pH condition[24-27]. In the current study, 11 isolates were able to survive at the bile concentration up to 3.0%. The survival rate in the present study was reported to be higher than some *Lactobacilli* found in the ducks' intestinal tract in 2% oxgall[28]. The endurance of LAB is believed to be pertinent due to its capacity to de-conjugate the bile acids and the cell layers comprising of lipids and fatty acids.

CONCLUSION

Large intestine of local breed chickens hosted the highest bacterial population which was a good source for LAB isolation. Only seven tested LAB isolates (CR M9, G M1, S N4, L M7, C N3, C M4 and C M6) showed high survival rate and ability to ferment glucose under gut condition, indicating their potential as probiotic agents in poultry husbandry with no adverse health consequences. They are able to ferment glucose (1%) and survive in bile salt concentration up to 3.0%.

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