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Isolation and Identification of Bacteriocin-producing Bacillus Strain Isolated from the Gastrointestinal Tract of Indonesian Native Chicken (Gallus domesticus)

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ABSTRACT

This research aimed to obtain *Bacillus* bacteria from the gastrointestinal tract (GIT) of native Indonesian chicken (*Gallus domesticus*) for bacteriocin production purposes against *Escherichia coli*. *Bacillus* bacteria were isolated from GIT (*duodenum, jejunum*, and *ileum*) on *Trypticase Soya Agar* (TSA+5% NaCl). The dilution was heated at 80°C for 20 min to kill other bacteria and induced *Bacillus* spore. Screening method based on the ability to against pathogens, then microbiological and biochemical characteristics. Inhibition test against pathogen using a well diffuse method. Thirteen *Bacillus* isolates then continued for testing against *Escherichia coli* FNCC 0091 for bacteriocin production purposes. Biochemical identification using conventional identification. The selected *Bacillus* bacteria, which had the highest inhibition zone, were isolates 2, 3B, and 11A (23.7, 18.6, and 19.9 mm). Biochemical and microbiological identification revealed that the isolate 2 isolated from *duodenum* was mixed culture (*Bacillus subtilis*, and *Bacillus* sp.), isolate 3B isolated from *jejunum* was mixed culture (*Bacillus subtilis*, *Bacillus* sp., and *Bacillus* sp.) and isolate 11A isolated from *ileum* was pure culture (*Bacillus subtilis*). All isolates were identified as rod-shaped, Gram-positive, aerobic, endospore-forming bacteria, and produced catalase.

Keywords**:** *Bacteriocin, Bacillus, Gallus domesticus, Gastrointestinal digestive tract, Isolation and Identification.*

1. INTRODUCTION

Escherichia coli is the bacteria which typically present in chicken gastrointestinal tract (GIT). This bacteria nearly always caused a septicemic bacterial infection. The pathogenic diseases caused by these bacteria mostly happen when the chickens were stressed, and its immune system was down. *Escherichia coli* could multiply and cause a systemic infection that typically resulted in mortality and gross lesions characterized by the accumulation of a fibrino-purulent exudate [1]. The use of synthetic antibiotics added in feed or water is one of many ways to prevent and control the disease caused by *Escherichia coli*. However, the continued feeding chicken with synthetic antibiotic at sub-therapeutic levels has created concerns about the extent to which usage increases the possibilities of antibiotic residue in meat, the development of antibiotic-resistant bacteria, imbalance of beneficial, healthy gut flora, and a reduction in the ability to cure bacterial infections [2]. The residues of amoxicillin as much as 16.92–152.62 µg/kg in liver and 45.38–60.55 µg/kg in breast meat in broiler chickens and layer chicken in Bangladesh [3]. Increased awareness of the potential problems associated with the use of antibiotics has stimulated research efforts to identify alternatives to their use as feed additives. Among of these alternatives, probiotics have received much attention as the most promising substitute to in-feed antibiotics and for improving animal productivity [2]. Bacteria from genus *Bacillus* are widely known as the producer of bacteriocin as antimicrobial substances. Bacteriocin produced by *Bacillus* bacteria have broad-spectrum [4] and could potentially be used in the feed industries as natural antibiotics for livestock feed [5].

In Indonesia, there are 31 types of local chickens with genotypic and phenotypic characteristics that differ from one species to another. One of these local chickens is the native chicken (*Gallus domesticus*), which is the result of domestication from partridge (*Gallus gallus*) [6]. Native chicken is maintained and fed

conventionally without in-feed antibiotics. With conventional maintaining and antibiotics-free feed, it is expected that *Bacillus* bacteria can be isolated from its GIT. Bacteriocin-producing bacteria was isolated from feces of cattle (*Bacillus subtilis* strains CB 153 and CB 189) and chickens (*B. subtilis* MSC 156 and *Bacillus parabrevis* MSC 164). Moreover, the isolates were screened after determining the inhibitory activity against the pathogenic indicator *Clostridium perfringens* KCTC 3269, and are believed to be potential probiotics in the livestock industry [2]. The purpose of this study was to isolate and identify the bacteriocin-producing *Bacillus* bacteria with antagonistic activities against *Escherichia coli* from native chicken (*Gallus domesticus*), which maintained conventionally and to develop a potential candidate for probiotic use in the chicken as an alternative natural antibiotic.

2. MATERIAL AND METHODS

2.1. Material

Bacillus bacteria were isolated from GIT (duodenum, jejunum, and ileum) and obtained from native Indonesian chicken (*Gallus domesticus*), which maintained in a conventional farm. Trypticase Soya Agar (TSA+5% NaCl) media to isolate the Bacillus bacteria and indicator organism (*Escherichia coli* FNCC 0091) were obtained from the Food and Nutrition Culture Collection (FNCC) Universitas Gadjah Mada. The tools used in this study, such as a set of tools to grow and to identify the bacteria. Spectrophotometer (Genesys 20™), centrifuge type 5810R (Eppendorf®), vortex mixer model VM-1000, 1.5 mL safe lock tube, laminar airflow, autoclave, calipers, and analytic scale.

2.2. Methods

2.2.1. Isolation of Bacillus Bacteria.

Bacillus bacteria were isolated from native chicken GIT [7] with modification in the media. Chicken intestines were cut (1 cm) according to each part (*duodenum, jejunum*, and *ileum*). Then, the sample were placed in a sterile centrifuge tube which contains NaCl physiological, then centrifuged 3000 rpm for 10 minutes to get the pellets. The pellet was taken and diluted up to 10^{-6}, then 1 ml solution was heated at a temperature of 80°C for 20 minutes to turn off the other bacteria and induce the spores of the *Bacillus*. Next, 1 ml of the solution poured onto TSA+NaCl 5%, which is already solid, then the incubation carried out for 48 hours. Each colony that grew and has a different appearance is taken and carried out for purification using the method of streaks (*streak plate*) for three times. The result of isolation is stored slant or NB broth at 4°C (refrigerator) for further testing.

2.2.2. Selection of Antimicrobial Activity Produced by Isolates Against Escherichia coli.

The antimicrobial activity assay using a well-diffused method [8] with modification in well diameter (5 mm). A total of 50 µL indicator organisms (*Escherichia coli*) dripped on to solid TSA media then swabbed evenly using a sterile cotton swab, and then 5 mm well was made using a cork borer. Each well was dripped as much 50 µL of isolate; then, the incubation period lasted for 24 hours at 37°C. The presence of a clear zone around the well indicated that the bacteria produced the bacteriocin to inhibit the indicator organism's growth. Clear zone diameter was measured by calipers. The next step used the isolates that have the broader clear zone.

2.2.3. Identification of superior isolates.

The identification of selected isolates was determined by the conventional method refers to *Bergey's Manual of Systematic Bacteriology* [9]. The identification includes microbiological tests, biochemical tests, and catalase production test.

3. RESULT AND DISCUSSION

3.1. Isolation and Selection of antimicrobial activity produced by Bacillus bacteria against Escherichia coli

The source of the bacteria *Bacillus* was from the native chicken's digestive tract, a part of the intestine (*duodenum*, *jejunum*, and *ileum*). Furthermore, the isolates were selected to obtain superior isolates, which produced the most extensive clear zone. Bacteriocin-producing bacteria namely *B. subtilis* MSC 156 and *Bacillus parabrevis* MSC 164 were isolated from chickens' feces. Then, the isolates were screened after determining the inhibitory activity against the pathogenic indicator *Clostridium perfringens* KCTC 3269 [2]. Table 1 shows the measurement of the inhibition of each isolate against *Escherichia coli*.

Table 1. The selection of superior isolates based on the inhibition of *Escherichia coli*

Codes of isolates	Clear zone diameter (mm)
1A (*duodenum)*	11.7
2 (*duodenum*)	23.7
3A (*jejenum*)	4.7
3B (*jejenum*)	18.6
3C (*jejenum*)	3.7
4A (*jejenum*)	-
4B (*jejenum*)	-
9A (*jejenum*)	4.3
9B (*jejenum*)	-
10 (*jejenum*)	5.4
11A(*ileum*)	19.9
11B(*ileum*)	8.6
12 (*ileum*)	9.7

The results showed that the broadest clear zone found on the isolates code of 2 (duodenum isolate), the 3B (jejunum isolate), and the 11A (ileum isolate). Each

isolate has an average value of the clear zone by 23.7, 18.6, and 19.9 mm. The clear zone diameter of isolate 2 against *Escherichia coli* was higher than the previous research [10], which was 21.33 ± 0.33 mm. The clear zone of isolate 3B and isolate 11A also showed good results as seen in the clear zone diameter which was close to the results of the previous research [10]. Bacteria of the genus *Bacillus* had broad antimicrobial activity spectrum against various pathogenic bacteria of Gram-positive (*Staphylococcus aureus, Streptococcus pyogenes*, and *Enterococcus faecalis*) and Gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia,* and *Proteus vulgaris*) [10] [11]. Antimicrobial compounds of bacteriocin inhibited the pathogenic bacteria cell walls forming, causing the cells would not able to develop and bring the pathogenic bacteria to the death phase [12]. Antimicrobial compounds produced by *Bacillus* bacteria were a strategy to maintain the balance of the population and reduce the population of competitors [13].

Table 2. The result of identification isolates 2, 3B and 11A

Code of bacteria	Gram Staining	Morphological form	Catalase test	Species identification
2	Gram positive	Rod	+	*Bacillus subtilis, Bacillus sp.*
3B	Gram positive	Rod	+	*Bacillus sp., Bacillus sp., Bacillus sp.*
11A	Gram positive	Rod	+	*Bacillus subtilis*

Table 3. The result of biochemical and identification of isolates 2, 3B and 11A

Substrate Type	Code of isolates		
	2	3B	11A
Glucose	+	+	+
Lactose	-	-	-
Mannitol	+	+	+
Maltose	+	+	+
Sucrose	+	+	+
Glucose 6%	+	-	+
Ammilum	+	+	+
Nitric	+	+	+
Anaerob condition	-	-	-
Fat globule	-	-	-
Lesitinase	-	-	-

[a] + : positive
[b] - : negative

3.2. Identification of superior isolates

The selected bacteria, i.e., isolates 2, 3B, and 11A, then identified by the biochemical and morphological tests. Catalase production assay was done to determine the isolates belong to the group of aerobic bacteria or anaerobic. Table 2 and 3 show the results of the identification of isolates 2, 3B, and 11A.

Bacteriocin-producing bacteria selected from chickens' feces were characterized as a Gram-positive, catalase-positive, and strict aerobic, rod-shaped bacterium. Based on comparisons of their characteristics with Bergey's manual and the results of the API test (data not shown), the isolates were classified as *B. subtilis* [2].

3.2.1. Gram Staining.

Based on the results of the Gram staining, all isolates cells showed purple color, which showed these bacteria belonged to the Gram-positive bacteria. All isolates also produced spores in the middle of the cell. Genus *Bacillus* bacteria had a rod shape, Gram-positive bacteria, and produced spores [15]. When environmental conditions did not support growth, such as lack of nutrients, high or low temperature, pH conditions of acid or alkaline, it will lead to spores growth [5]. Figure 1 presents the results of the Gram stain.

Gram-positive bacteria had a thicker layer of peptidoglycan than Gram-negative. Gram-positive protein cell wall would denature with alcohol wash. Protein will become hard and rigid, the pores shrink, less permeability so that the purple complex of iodine crystal is maintained, thus, bacteria remain purple. The lipid structure of Gram-negative bacteria will dissolve during the alcohol wash; the cell wall would be enlarged, the permeability of the cell wall became large so that the color substance which had been absorbed was quickly released and the bacterial cell became colorless [15]. The cell of Gram-positive bacteria has a thick cell wall consisting of several layers of mucopeptide and two types of teichoic acid. The walls of the molecules of teichoic acid connect onto a layer mucopeptide and molecules of the teichoic acid link to the second mucopeptide and the cytoplasmic membrane [16].

3.2.2. Catalase Production Assay.

Based on the results of the catalase production assay, all isolates showed the presence of air bubbles when spilled with hydrogen peroxide (H_2O_2). When colonies of bacteria were spilled with the hydrogen peroxide, the liberation of oxygen as gas can be seen. The air bubbles of oxygen (O_2) are formed due to the *Bacillus* bacteria enzymes which able to break down H_2O_2 [15]. The catalase enzyme converts H_2O_2 into water (H_2O) and oxygen (O_2), so that all isolates were categorized in positive catalase. Positive catalase indicated that all

isolates of bacteria were aerobic or facultative anaerobic bacteria. The type of aerobic bacteria and facultative anaerobes could convert H_2O_2 using the superoxide dismutase enzyme, then will be broken down by the catalase enzyme. Obligate anaerobes bacteria did not have superoxide dismutase and catalase enzymes so that it could not be tolerated to the presence of oxygen [16]. Catalase enzyme in *Bacillus subtilis* was produced in the stationary phase, and the vegetative phase (spores). The production of the enzyme catalase in *Bacillus* bacteria is the mechanism to protect bacteria against oxidative stress. The presence of the enzyme catalase in *Bacillu*s bacteria plays an important role, namely to protect bacteria against H_2O_2 that could damage the cells. The genes which code for the secretion of the enzyme catalase in *Bacillus subtilis* was the *KatA* [17].

3.2.3. Determining The Superior Isolates.

The species of *Bacillus* bacteria are determined by the phenotype based on the biochemical test, namely fermentation ability of different types of sugar and the substrate. All isolates were able to ferment glucose, but not able to ferment lactose. *Bacillus subtilis* could ferment glucose to produce acid but was not able to ferment lactose [18] [19]. Table 3 shows the results of the biochemical test and the identification of the species. The results showed that isolate 2 was identified as mix culture between *Bacillus subtilis* and *Bacillus sp*., isolates 3B identified as mix culture of three *Bacillus sp*. and isolate 11 was identified as the pure culture of *Bacillus subtilis*.

The isolation and identification results showed that the digestive tract of native chicken contained the bacteria of the genus *Bacillus*. *Bacillus* bacteria were microbes that exist in the normal/healthy conditions of the digestive tract [20]. The *Bacillus* bacteria found in the digestive tract of the chicken are namely *Bacillus subtilis, Bacillus pumilus, Bacillus lincheniformis*, *Bacillus clausii, Bacillus megaterium,* and *Bacillus firmus* [21]. The presence of *Bacillus* bacteria in the digestive tract comes from the soil, feed, and drinking water consumed by the chicken. It could suppress the presence of a wide variety of pathogens, such as *Salmonella typhimurium, Salmonella pullorum, Escherichia coli, Clostridium perfringens, Staphylococcus aureus*, and *Listeria monocytogenes* [22]–[24]. *Bacillus subtilis* was GRAS (generally regarded as safe) and safe for consumption [25].

4. CONCLUSION

It can be concluded from this research that there are several LAB found in A. nigrocincta, which also commonly found in other Apis species. Leuconostoc, Lactobacillus, and Bifidobacterium are proposed here as the core gut LAB of A. nigrocincta.

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