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RESEARCH ARTICLE

Antibacterial Activities of Culture-dependent Bacteria Isolated from *Apis nigrocincta* Gut

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Abstract:

Introduction:

Apis nigrocincta is a honeybee endemic to Mindanao island (the Philippines), Sangihe island (North Sulawesi, Indonesia) and Sulawesi mainland (Indonesia). The genus *Apis* is well known to have symbiont in their guts, which helps balance the microbiome in the gut and host health.

Objective:

The objective of this study was to determine whether the bacteria isolated from the gut of honeybee *Apis nigrocincta* produce metabolites with potential growth inhibition against *Staphylococcus aureus* and *Escerichia coli*, the bacteria which are important pathogens in humans and animals.

Methods:

Bacteria isolated from honeybee gut were cultured in MRSA and several isolates were purified for testing. The antibacterial activity test method used in this study was well diffusion agar. Pure isolates were grown on NB. The treatments given were heating and also neutralizing the supernatant from each isolate.

Results:

Five bacterial isolates were successfully isolated from honeybee gut and purified. The five isolates showed antibacterial activity against pathogenic bacterial strain indicators. The results of molecular identification showed that four of these isolates were *Bacillus cereus* and the other one was *Staphylococcus arlettae*. Neutralized supernatant showed strong activity on both indicator strains. The five isolates showed higher inhibition activity against *S. aureus* compared to *E. coli*.

Conclusion:

The finding of this research concluded that two bacterial strains, *B. cereus* and *S. arlettae* isolated from *A. nigrocincta* gut can be investigated further as agents which produce bioactive compounds that have potential as an antibacterial.

Keywords: *Apis nigrocincta*, Antimicrobial peptide, Gut, Honeybee, Organic acids, *S. arlettae*.

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1. INTRODUCTION

Honeybee (genus: *Apis*) is a social insect rich in benefits. Everything produced by honeybees is known to have health benefits. One of the *Apis* species is *A. nigrocincta,* which is endemic to Mindanao island (the Philippines), Sangihe island (North Sulawesi, Indonesia) and Sulawesi mainland (Indonesia). This species is a medium-sized generalist and lodged in cavities such as caves and holes in the trunk [1, 2]. They live in groups and rarely move from one place to another.

Like other insects, honeybees have symbiotic and pathogenic interactions with microbes in their digestive tracts [3, 4], which are assumed to be influenced by the environment where they find food. The adult intestine of this insect is divided into four main organs (crop, midgut, ileum, and

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rectum), which provide different functions in catabolism and absorption of food and also different environments for symbiotic bacteria [5]. Honeybee intestinal microbiota are distributed throughout the digestive tract, where midgut holds about 1-4% and ileum/rectum more than 90% of the most dominant bacteria found in honeybees [6]. In addition, this intestinal symbiont has been shown to influence insect feeding behavior [7].

Honeybee microbiota have been investigated to play a role in balancing host nutrition, weight gain, endocrine signaling, immune function, and pathogenic resistance, while microbiota disruption can lead to reduce host fitness [8], most likely because they express antimicrobial peptides [9] and organic acids such as lactic acid and acetic acid, which are produced by lactic acid bacteria and acetic acid bacteria [10].

Most research on microbiomes in the intestine of honeybees have emphasized the lactic acid bacteria, which are known to have antimicrobial activity [11, 12]. In this study, successfully cultured bacteria were used as isolates to observe their ability to produce antimicrobial activity against *S. aureus* and *E. coli*.

2. METHODS

2.1. Isolation and Purification of Bacteria from Honeybee Gut

The honeybees were surface sterilized by following the procedure from Lombogia *et al*. [13]. The gut was removed aseptically and placed on a petri dish, cut into small pieces, then put into Eppendorf tube containing 0.9% sterile NaCl, then crushed using micropestel. The tube was centrifuged at 6000 rpm to precipitate intestinal debris. One hundred microliters of the supernatant were taken and spread on de Man, Rogosa and Sharpe Agar (MRSA) supplemented with $CaCO_3$, then incubated for 2x24 hours at 37°C. The large colonies that appeared different were separated and purified. The bacteria were then stored on Nutrient Agar (NA) slant for subsequent use.

2.2. Antibacterial Activity Test against Indicator Pathogenic Strains

Two pathogenic strains, *S. aureus* and *E. coli*, were used as indicator bacteria to determine the ability of antimicrobial activity of bacteria isolated from honeybee gut. The procedure for the antibacterial test was carried out by following Tallei *et al*. [14, 15] with modification. Pure bacterial isolates were grown on Nutrient Broth (NB) for 24 hours at 37°C in an Eppendorf tube. Isolates that grew were killed at 80°C for 2 hours and centrifuged at 10,000 rpm for 10 minutes to prepare cell-free culture supernatants (CFSs) and to inactivate antimicrobial peptides that might present in the supernatant. In addition, other supernatants that were not heated were neutralized using NaOH to reach pH 6.0. This was intended to neutralize organic acids and to predict the antimicrobial peptides that were likely produced by isolates.

Indicator bacteria were grown respectively on NB for 24 hours at 37°C then poured on each NA medium, which had wells with a diameter of 5 mm. The media were then incubated for 2 hours at 37°C. One hundred microliters of each CFFs were poured into wells. Likewise, the supernatant that had been neutralized from each isolate was poured as much as 100 µl into other empty wells. The NA media were then incubated at 37°C for 48 hours. The diameter of the inhibition zone produced was measured, which was indicated by the presence of a clear zone in the vicinity of the well. Five µg/ml of antibiotics (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic) was used as positive control and sterile dH_20 as negative control.

2.3. Molecular Identification of Bacterial Isolates

Purified bacterial isolates that exhibited antibacterial activities were identified molecularly using the 16S rRNA marker gene following the procedure carried out by Tallei *et al*. [14]. The 16S rRNA sequences from each bacterial isolate were searched for similarities in the Ez-Taxon database portal (https://www.ezbiocloud.net/) [16].

3. RESULTS

The antibacterial activities of bacteria isolated from honeybee gut were tested against pathogenic bacteria *S. aureus* and *E. coli*. The antibacterial test results from the supernatant of each isolate that was heated for 2 hours at 80°C (treatment 1) against bacterial indicators are presented in Table **1**. Table **2** shows the antibacterial test of the supernatant of each isolate, which was neutralized to pH 6 (treatment 2). The classification of inhibition according to Zare Mirzaei *et al*. [17] is as follows: <11 mm (negative -), 11-16 mm (mild +), 17–22 mm (strong ++), and >23 mm (very strong +++). In treatments 1 and 2, it can be seen that the five isolates had higher inhibitory activities against *S. aureus* than *E. coli*. Treatment 2 appeared to have a higher activity than treatment 1, both for *S. aureus* and *E. coli*. In treatment 1, isolate Lp.ov showed the highest activity against both indicator strains. In treatment 2, isolate L.pt.2 showed the highest inhibitory activity against *S. aureus* and Lp.ov showed the highest inhibitory activity against *E. coli*.

As all isolates showed antibacterial activity, all were identified using molecular markers of the 16S rRNA gene. The results of searching for the appropriate sequences performed on the Ez-Taxon database platform are shown in Table **3**. Isolates Lo.Pt 1, L.pt.2, L.10, and L.10.pt were identified as *Bacillus cereus*, while isolate Lp.ov was identified as *S. arlettae.* Of all treatments, *B. cereus* strain Lo.Pt 1, L.pt.2, and L.10 showed very strong activity in treatment 2 against *S. aureus*, and their inhibitory activities exceeded control antibiotics. It is suspected that *B. cereus* has a high AMP content.

4. DISCUSSION

The present study showed that there were five isolates that exhibited antibacterial activity against pathogenic strains *S. aureus* and *E. coli*. All isolates were identified molecularly, and 4 of them (Lo.Pt 1, L.pt.2, L.10, and L.10.pt) were identified as *B. cereus*, while Lp.ov was identified as *S. arlettae.*

Table 1. The antibacterial test of the supernatant heated at 80°C for 2 hours (treatment 1).

Isolate Codes	*S. aureus*	Activity (%)*	*E. coli*	Activity (%)*
Lo.Pt 1	14.83 ± 0.58	64,96	11.73 ± 0.40	62,73
L.pt.2	16.83 ± 0.58	73,72	11.40 ± 0.30	60,96
Lp.ov	19.73 ± 0.40	86,42	14.40 ± 0.27	77,01
L.10	17.23 ± 0.46	75,47	13.03 ± 0.46	69,68
L .10.pt	14.83 ± 0.58	64,96	12.63 ± 0.06	67,54
Positive control	22.83 ± 0.29	-	18.70 ± 0.20	-
Negative control	0	-	0	-

Table 2. The antibacterial test of supernatant which has been neutralized up to pH 6 (treatment 2).

Isolate Codes	*S. aureus*	Activity (%)*	*E. coli*	Activity (%)*
Lo.Pt 1	26.0 ± 0,5	113,89	16.3 ± 0.29	81,23
L.pt.2	24.17 ± 0.58	105,87	18.3 ± 0.29	91,18
Lp.ov	22 ± 0.5	96,36	20.4 ± 0.17	101,64
L.10	23.83 ± 0.58	104,38	19.5 ± 0.00	97,16
L.10.pt	15.67 ± 0.29	68,65	12.5 ± 0.00	62,28
Positive control	22.83 ± 0.29	-	20.07 ± 0.06	-
Negative control	0	-	0	-

*percentage activity was based on the ratio of the results of inhibition of treatment compared with positive control

Table 3. Results of identification of bacterial isolates using the 16S rRNA gene.

Isolate Code	Species	% Identity
Lo.Pt 1	*Bacillus cereus* ATCC 14579	100
L.pt.2	*B. cereus* ATCC 14579	100
Lp.ov	*S. arlettae* ATCC 43957	99.88
L.10	*B. cereus* ATCC 14579	100
L .10.pt	*B. cereus* ATCC 14579	100

The supernatant in treatment 1 was heated, so it is assumed that if there is Antimicrobial Peptide (AMP), it will become inactive so that bioactive compounds that may have a role in inhibition include organic acids such lactic acid, acetate acid, and formic acid, benzoic acid, as well as hydrogen peroxide (H_2O_2) and alcohol. As reported by Adam and Hall [18], organic acids reduced the pH of the media and inhibited the growth of pathogenic organisms. The supernatant in treatment 2 was neutralized so that the pH reached 6, assuming if there are organic acids, it will be neutralized, so that bioactive compounds such as AMPs and fatty acids play a role in antibacterial activity.

Baindara *et al*. [19] reported that halotolerant *B. cereus* isolated from a rhizosphere soil sample produced two AMPs that were active against Gram-positive bacteria. Some AMPs produced by *Bacillus* sp. include broad-spectrum bacteriocin, which has a bactericidal or bacteriostatic effect [20 - 22], surface-active biosurfactants like lipopeptides, glycopeptides and nonribosomally synthesized cyclic peptides [23, 24], and Caseicin A and B [25].

The *Bacillus* group is the dominant bacterium in the honey bee gut [26] and 67% of the bacteria isolated from honey are the *Bacillus* group [27]. Most intestinal bacteria of *A. mellifera* in the North-west region of Pakistan belong to the genus *Staphylococcus* and *Bacillus,* which are tolerant of the acidic environment caused by fermented sugars. These bacteria are thought to be beneficial microbes that are involved in maintaining the health of honey bees. *Staphylococcus* was estimated to reach 29% of the total intestinal microbial samples analyzed [28]. In this current study, *S. arlettae* showed strong inhibition against *S. aureus* for treatment 1 or 2. Staphylococcal strains are rarely discussed in the literature available in relation to microbiota bee gut [28]. On another occasion, Wu *et al*. [29] stated that most culture-dependent gut bacteria from Japanese honey bee belonged to genera *Bacillus*, *Staphylococcus*, and *Pantoea*. Gabriel [30] reported that symbiont gut in honey bees that were exposed to agrochemical stresses constitutes 10% of *Staphylococcus* from the total population. Although showing antimicrobial activity against indicator bacteria, their effectiveness varies from strain to strain. This may be because even though the species is the same, the number of metabolites produced also varies depending on the strain.

Understanding the symbiotic relationship between honey bees and their bacterial community can inspire ideas about how to exploit these microflorae to protect the health of the host [11]. The bacteria that produce dominant metabolites and are available in the digestive tracts of insects, can balance the

natural conditions.

CONCLUSION

The results concluded that culture-dependent bacteria that had been successfully isolated from the intestines of *A. nigrocincta* were *B. cereus* and *S. arlettae*. Both of these bacterial strains had strong antibacterial activity against the indicator bacteria *S. aureus* and *E. coli*. Both strains are more potent against *S. aureus* as compared to *E. coli*.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, [TET], upon reasonable request.

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None.

CONFLICTS OF INTEREST

The author declares no conflict of interest, financial or otherwise.

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