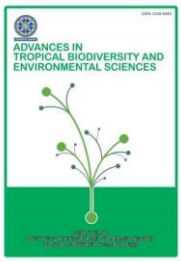

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Identification of Bacteria Associated in the Small Intestine of White-Tailed Forest Rats (*Maxomys hellwandii*) in South Minahasa Regency, North Sulawesi

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Abstract. Sulawesi Island in the Wallaceae line area has diverse endemic animals, including the white-tailed forest rats. White-tailed forest rat (*Maxomys hellwandii*) is one of the endemic animals in North Sulawesi, so this study aimed to determine the type of bacterial genus in the small intestine of *Maxomys hellwandii*. This research used a laboratory-based qualitative descriptive method using a pour plate isolation method and bacterial identification based on morphological, and physiological characters with biochemical tests. The small intestines of *Maxomys hellwandii* taken from the South Minahasa forest area, North Sulawesi were used as samples of this research. The results of the study obtained 22 isolates of bacteria that were successfully isolated and have been identified based on morphological characteristics, and physiology with biochemical tests based on the book Bergey's Manual of Systematic Bacteriology suspected 14 isolates as genus Bacillus and 8 isolates as genus Lactobacillus.

Keywords: Bacillus, Lactobacillus, *Maxomys helwandii*, rat, small intestine

I. INTRODUCTION

The white-tailed forest rat (*Maxomys hellwandii*) is one of the endemic species of North Sulawesi. White-tailed forest rat (*Maxomys hellwandii*) has a conservation status of "least concern" or a species included in the endangered category based on IUCN (The International Union For The Conservation of Nature and Natural Resources), due to hunting for sale [1]. The local people of North Sulawesi call this rat the white-tailed rat because it is white at the end of its tail. This rat also has a brownish ash color, white chest, and underbelly. This rat also has various names according to its distribution area. In Minahasa, this rat is named *Turean* (Tountemboan language) and has a small body size, when during the day it lies in the ground or holes of trees, while at night it looks for food in trees [2]. White-tailed forest rats (*Maxomys hellwandii*) consume types of fruits and leaves present in the forest such as papaya, coconut, betel fruit, and betel leaf, and consume insect species such as grasshoppers, ants, moths, cockroaches, and beetles [3].

The small intestine is one of the main locations in the digestive tract of mammals that has the function of digesting and absorbing nutrients and water before being circulated by the blood throughout the body [4]. The intestinal mucosa, which performs the main function of digestion and absorption, also serves as a barrier to toxic and harmful materials and a protector from antigens and inflammatory reactions [5]. Because the surface of the gastrointestinal tract is often exposed to various foreign substances. including pathogenic agents causing various diseases [6].

Bacteria are single-celled microorganisms (prokaryotic) that colonize, do not have a nuclear envelope but can live anywhere [7]. In the intestine, there is microflora in the form of bacteria, fungi, and yeast which if in a balanced state will help the growth of intestinal villi so that it can improve intestinal function as a defense against infection from pathogenic bacteria [8]. Bacteria can attach to epithelial cells of the intestinal mucosa. The attachment of bacteria to intestinal epithelial cells affects the number and length of intestinal villi. An increase in the length of intestinal villi can cause an increase in the area of food absorption so that the efficiency of the intestine in digesting food increases [9].

Meanwhile, research on the identification of bacteria in the small intestine of the white-tailed forest rat (*Maxomys hellwandii*) as one of the endemic animals of North Sulawesi has never been conducted. Therefore, a study was conducted to determine the type of bacteria associated in the small intestine of white-tailed forest rats (*Maxomys hellwandii*) in South Minahasa, North Sulawesi.

II. METHOD

Study Site

The research was carried out at the Microbiology Laboratory of Manado State University in March-June 2023. The population and sample in this study were small intestines from white-tailed forest rats (*Maxomys hellwandii*) obtained from the South Minahasa forest area, North Sulawesi. This study used a laboratory-based qualitative descriptive research method by identifying bacteria morphologically, and physiologically with biochemical testing to determine the type of bacteria in the small intestine organs of white-tailed forest rats (*Maxomys hellwandii*).

Materials

The tools used in this study were scales, magnetic stirrers, hot plates, autoclaves, incubators, vortexes, Petri dishes, test tubes, Erlenmeyer, ose needles, drip pipettes, micropipettes, tips, mortars, bunsen, biological microscopes, bouncing needles, tweezers, surgical scissors, preparation glass, spray bottles, aluminum foil, and gloves. The materials used in this study were Nutrient Agar (NA) media, Nutrient Broth (NB) media, TSIA media, Simmon's Citrate Agar media, aquadest, crystal violets, iodine solution, safranin, immersion oil, malachite green, sodium chloride (NaCl), hydrogen peroxide (H_2O_2), 70% alcohol, and 96% alcohol.

Research Procedure

a. Sample Preparation

Sampling of white-tailed forest rats (*Maxomys hellwandii*) by random sampling, obtained 3 mice. Furthermore, physical euthanasia was carried out by cervical dislocation method and surgery was carried out to take 1 cm of small intestine organ samples.

b. Bacterial Isolation

Small intestinal bacteria are isolated by the enrichment method. The small intestine was inserted into Erlenmeyer which contains nutrient broth (NB) and incubated at room temperature for 2 days. Furthermore, bacterial isolation was carried out using the pour plate method on nutrient agar (NA) media which began with diluted up to series 10-7, incubated for 24 hours at a temperature of 37°C. After incubation, there are bacterial colonies that can be observed morphologically such as shape, color, edges, elevation, and size. The colonies obtained were purified by quadrant streak technique on nutrient agar (NA) media and incubated for 48 hours at a temperature of 37°C. The pure isolates that have been obtained are regrown in nutrient broth (NB) media and incubated at 37°C for 24 hours. After that, pure isolates were grown on oblique agar media, to be used as bacterial stocks.

c. Gram Staining

Gram staining starts by creating a bacterial smear, taking 1 ose bacterial culture, and put in the center of the preparation glass that has been sterilized with 70% alcohol, fixed on a bunsen fire. Drip crystal violet paints over a bacterial smear and leave for 1 minute, rinse with aquadest. Drip iodine and leave for 1 minute, rinse with aquades. Drip 98% alcohol (decolorization) and leave for 30 seconds, rinse with aquadest. Drip safranin and leave for 1 minute, rinse with aquadest, Further dry by aerating. After drying, drip the immersion oil and observe it using a microscope with a magnification of 100×.

d. Endospore Staining

Taken 1 ose pure culture of bacteria, suspended with aquades in the preparation. The preparation is fixed on a bunsen fire. Drip malachite green and leave for 10 minutes in a water bath, rinse with water. Drip Safranin and leave for 30 seconds, rinse under running water, and drain. After drying, it is observed with a microscope.

e. Biochemical Testing

Observation of the physiological characteristics of bacteria is carried out by biochemical testing. Biochemical tests are carried out in the form of citrate tests, motility tests, TSIA (Triple Sugar Iron Agar) tests, and Catalase tests. The citrate test begins with the manufacture of a test medium, namely Simmon's citrate agar which is sterilized in an autoclave with a temperature of 121°C for 15 minutes. Then 1 ose bacterial culture was taken, inoculated vertically to 3/4 part of the media, and incubated for 24 hours at a temperature of 37°C. A positive citrate test result is characterized by a change in color from green to blue on the media.

The motility test was carried out by making a semi-solid textured nutrient agar (NA) test medium that was dissolved in an autoclave with a temperature of 121°C for 15 minutes. Then 1 ose bacterial culture was taken and inoculated vertically on the media, then incubated for 24 hours at a temperature of 37°C. Bacterial motility is characterized by the formation of a white color around the inocation.

The TSIA test is carried out by making TSIA (Triple Sugar Iron Agar) test medium which is sterilized in an autoclave with a temperature of 121°C for 15 minutes.

Then 1 ose bacterial culture was taken and inoculated vertically on the media, then incubated for 24 hours at a temperature of 37°C. The results of the TSIA test of bacteria carrying out carbohydrate fermentation are characterized by yellow discoloration at the base of the media and the slope of the media.

The catalase test is carried out by taking 1 ose bacterial culture, flattened in the middle of a sterile preparation glass, then dripping H_2O_2 on top of the bacteria. The presence of air bubbles or foam characterizes a positive catalase test result.

Data Analysis

Isolates of small intestine bacteria of white-tailed forest rats (*Maxomys hellwandii*) were characterized based on cell morphological, and physiological observations with biochemical tests. The data obtained are analyzed descriptively and presented in the form of tables and paragraphs. Further identified bacteria referring to the book *Bergeys's Manual of Systematic Bacteriology*.

III. RESULTS AND DISCUSSION

Isolation and Morphological Characteristics of Small Intestinal Bacteria of White-tailed Forest Rats (*Maxomys hellwandii*)

The results of bacterial isolation on nutrient agar (NA) media from the small intestine of white-tailed forest rats (*Maxomys hellwandii*) in the first sample obtained 6 isolates, the second sample obtained 7 isolates, and the third sample obtained 9 isolates, a total of 22 isolates. Nutrient agar (NA) is a non-selective medium, so all bacteria can grow. The nutritional content needed by bacteria to support growth and meet the needs of life is contained in nutrient agar (NA) media [10]. Based on morphological identification data (Table 1, Table 2, and Table 3.) of the three samples that were successfully isolated generally have morphological characteristics of circular, flat edges (entire), convex elevation (convex), and white, as in Figure 1. The characterization of isolated colonies is suspected to be a type of bacteria from the genus *Bacillus* sp. and Lactobacillus.

Table 1 shows that most isolated colony morphological characteristics indicated the colony shape of the genus Bacillus. Most colonies had round shapes (circular), entire margins, convex elevation, glistening appearance, and white pigmentation. A few colony isolates showed irregular shape; lobate, curled or undulated margins; raised or flat elevation; dull appearance; and transparent pigmentation. Cell observation showed that all colonies were positive gram, had a basil shape, and some colonies did not have an endospore. This result follows the character of *Bacillus* sp. based on a comparison of bacterial colony morphology libraries from Bergey's [11] that the morphological characteristics of colonies vary greatly from circular colony shapes to irregular colony shapes. This corresponds to isolates of *Bacillus* sp which was successfully isolated by Henoch *et al.* [12], namely round colonies, white, and some edges are clustered.

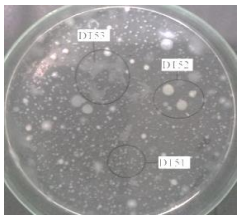

Figure 1. Results of Bacterial Isolation

Previous research obtained bacterial isolates from the genus *Bacillus* sp. have a whitish color, round colony shape, and flat edges, and the surface of the colony is convex and flat [13]. Isolating *Bacillus* sp. from the digestive tract of native chickens (*Gallus domesticus*) with the same morphological, namely, round colony shape, convex, flat edges, not too large size, and white [14]. According to Cowan *et al.* [15], Bacillus colonies in nutrient agar medium (NA) are macroscopically milky push colored and resistant to heat.

Colony morphology indicated the genus Lactobacillus in DT533A and DT53B isolates (sample 1), DT52 and IT73 isolates (sample 2), and IT51, IT62, IT71, and IT72 isolates (sample 3). They showed the morphology of the colonies was circular, with flat edges (entire), convex elevation, and transparent and shiny colony surfaces (glistening). A research isolating bacteria from the intestines of Bangkok chickens with the same macroscopic morphological characteristics, namely round colony shape, flat edges, white color, and convex surface [16]. Another research also succeeded in isolating bacteria of the genus *Lactobacillus* with morphological characteristics of round colonies, flat edges, and convex colony surfaces [17]. Furthermore, the observation of microscopic morphological characters is based on the results of gram staining and bacterial endospore staining. Most isolates show gram-positive staining results characterized by purple color on bacterial cells, rod cell shape, and endospores. This is following the test results conducted by Eka *et al.* [18] on the character of *Bacillus* sp. The stem cell form is gram-positive and has endospores. According to Holt *et al.* [19], *Bacillus* sp. Gram-positive bacteria are rod-shaped and have oval endospores. Endospores in bacteria *Bacillus* sp. have a green round shape on vegetative cells that are pink [13]. Spores are a form of bacterial self-defense from unfavorable conditions. Spored bacteria are shown in green on vegetative cells. The structure of the capsule form of *Bacillus* sp. contains a polypeptide of D-glutamic acid, which is a bacterium that has spores [20].

TABLE 1.
MORPHOLOGY OF SMALL INTESTINAL BACTERIA OF WHITE-TAILED FOREST RAT
(*Maxomys hellwandii*)

Animal	Isolate	Colony Shape Observation					Cell Shape Observation		
		Shape	Margin	Elevation	Appearance	Pigmentation	Cell shape	Gram	Endospore
Sample 1	DT533A	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	-
	DT535	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	+
	DT53B	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	-
	DT5393	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	+
	DT711	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	+
	DT713	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	+
Sample 2	DT51	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	+
	DT52	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	-
	DT53	*Irregular*	*Lobate*	*Raised*	*Dull*	Transparent	Basil	+	+
	DT62	*Circular*	*Curled*	*Convex*	*Glistening*	White	Basil	+	+
	DT72	*Circular*	*Curled*	*Convex*	*Glistening*	White	Basil	+	+
	JT72	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	+
	IT73	*Irregular*	*Undulated*	*Flat*	*Dull*	Transparent	Basil	+	-
Sample 3	DT6	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	+
	DT71	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	+
	DT72	*Circular*	*Entire*	*Convex*	*Glistening*	Transparent	Basil	+	+
	JT5	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	+
	IT51	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	-
	IT52	*Circular*	*Entire*	*Convex*	*Glistening*	White	Basil	+	+
	IT62	*Circular*	*Entire*	*Convex*	*Glistening*	Transparent	Basil	+	-
	IT71	*Circular*	*Entire*	*Convex*	*Glistening*	Transparent	Basil	+	-
	IT72	*Circular*	*Undulated*	*Flat*	*Dull*	Transparent	Basil	+	-

While isolates of endospore staining results that showed no green color were found in bacterial vegetative cells showed bacteria of the genus Lactobacillus. Lactobacillus is classified as Lactic Acid Bacteria (LAB) which has characteristics of gram-positive bacteria, does not form spores, has the form of rods (bacil), and produces lactic acid from carbohydrate fermentation [8].

Biochemical Test of Small Intestinal Bacterial Isolate of White-tailed Forest Rat (*Maxomys hellwandii*)

Bacterial colonies that have been successfully isolated and have identified morphological characters are subjected to biochemical testing to determine the physiological properties of bacteria. Biochemical tests are carried out in the form of citrate tests, motility tests, TSIA (Triple Sugar Iron Agar) tests, and catalase tests.

The citrate test is carried out to determine the use of citrate by bacteria as a carbon source, which is characterized by a change in the color of the media from green to blue. In bacterial isolates suspected to be the genus *Bacillus* sp. and Lactobacillus, each showed citrate test results that vary according to the characters contained in *Bergey's Manual of Systematic Bacteriology 2nd* (Table 2). The use of citrate by bacteria causes acid to disappear from the culture, resulting in an increase in pH and a change in media color [21].

Motility tests are carried out to determine the movement of bacteria, which is characterized by the presence of white formations around the inoculation puncture. Based on the results of the study, bacteria were obtained which are suspected to be a genus group of *Bacillus* sp. showing variation against motility tests. The bacteria *Bacillus* sp. are motile, namely *Bacillus subtilis* and *Bacillus thurgenensis* species and some are non-motile [13].

Previous research obtained isolates of *Bacillus* sp. are motile [22]. The negative motility test results of *Bacillus* sp. in this study are the same as those obtained from isolates of *Bacillus* sp. that are non-motile [23]. Those bacteria are motile because they move with the help of flagella as a means of locomotion. In isolates suspected of Lactobacillus bacteria, it is known that these isolates are non-motile.

By the statement of Holt *et al.* [19], the genus *Lactobacillus* is not motile. The TSIA test is carried out to determine the ability of bacteria to ferment carbohydrates (glucose, lactose, and sucrose) to produce acid or gas on TSIA media, which is characterized by the presence of yellow on the media. The red color indicates a basic reaction and yellow is an acid. The red agar surface indicates glucose fermentation, while the agar surface and bottom of the tube are yellow indicating lactose and sucrose fermentation [24].

Based on the TSIA test, isolates are suspected to be the genus *Bacillus* sp. and Lactobacillus each showed test results that varied according to the characters contained in *Bergey's Manual of Systematic Bacteriology 2nd* (Table 2). Test results that show positive indicate bacteria genus *Bacillus sp.* and Lactobacillus fermented lactose and sucrose, while the test results that showed negative indicated bacteria of the genus *Bacillus* sp. and Lactobacillus do not ferment glucose, lactose, or sucrose.

TABLE 2.
BIOCHEMICAL TEST OF SMALL INTESTINAL BACTERIA OF WHITE-TAILED FOREST RAT (*Maxomys hellwandii*)

Animal	Isolate	Biochemical Test				Types of Bacteria
		Citrate	Motility	TSIA	Catalase	
Sample 1	DT533A	-	-	-	-	*Lactobacillus*
	DT53B	-	-	-	+	*Bacillus* sp.
	DT535	+	-	+	-	*Lactobacillus*
	DT5393	-	-	-	+	*Bacillus* sp.
	DT711	+	-	+	+	*Bacillus* sp.
	DT713	+	-	+	+	*Bacillus* sp.
Sample 2	DT51	+	-	+	+	*Bacillus* sp.
	DT52	-	-	+	-	*Lactobacillus*
	DT53	-	+	+	+	*Bacillus* sp.
	DT62	-	+	-	+	*Bacillus* sp.
	DT72	-	+	+	+	*Bacillus* sp.
	JT72	+	-	+	+	*Bacillus* sp.
	IT73	-	-	+	-	*Lactobacillus*
Sample 3	DT6	-	+	-	+	*Bacillus* sp.
	DT71	+	-	+	+	*Bacillus* sp.
	DT72	-	+	+	+	*Bacillus* sp.
	JT5	+	+	+	+	*Bacillus* sp.
	IT51	+	-	+	-	*Lactobacillus*
	IT52	+	+	+	+	*Bacillus* sp.
	IT62	+	-	+	-	*Lactobacillus*
	IT71	-	-	+	-	*Lactobacillus*
	IT72	-	-	-	-	*Lactobacillus*

Note: Citrate test : (+) Blue ; (-) In addition to blue
Motility test : (+) There are puncture mark ; (-) No puncture mark
TSIA test : (+) Yellow (-) In addition to yellow
Catalase test : (+) Bubble (-) Non-bubbly

TABLE 3.
COMPARISON OF BACTERIAL CHARACTERS WITH THE *BERGEYS'S MANUAL OF SYSTEMATIC BACTERIOLOGY*

Bacteria	Isolate	Gram	Cell Shape	Endospore	Biochemical Test			
					Citrate	Motility	TSIA	Catalase
Bacillus sp.	DT53B	+	Basil	+	-	-	-	+
	DT5393	+	Basil	+	-	-	-	+
	DT711	+	Basil	+	+	-	+	+
	DT713	+	Basil	+	+	-	+	+
	DT51	+	Basil	+	+	-	+	+
	DT53	-	Basil	+	-	+	+	+
	DT62	+	Basil	+	-	+	-	+
	DT72	+	Basil	+	-	+	+	+
	JT72	+	Basil	+	+	-	+	+
	DT6	+	Basil	+	-	+	-	+
	DT71	+	Basil	+	+	-	+	+
	DT72	-	Basil	+	-	+	+	+
	JT5	+	Basil	+	+	+	+	+
	IT52	+	Basil	+	+	+	+	+
Lactobacillus	DT533A	+	Basil	-	-	-	-	-
	DT535	+	Basil	-	+	-	+	-
	DT52	+	Coccus	-	-	-	+	-
	IT73	+	Basil	-	-	-	+	-
	IT51	+	Basil	-	+	-	+	-
	IT62	+	Basil	-	+	-	+	-
	IT71	+	Basil	-	-	-	+	-
	IT72	+	Basil	-	-	-	-	-

A catalase test is performed to determine the ability of bacteria to produce the enzyme catalase that converts hydrogen peroxide (H_2O_2) into water and oxygen. The presence of air bubbles or foam characterizes a positive catalase test result. The results of the catalase test on bacterial isolates suspected to be the Bacillus sp group showed positive catalase, which is the presence of bubbles when dripped with H_2O_2 solution. According to Barrow *et al*., [25] and Puspita *et al*. [13] *Bacillus* sp. is an obligate aerobic bacteria or facultative aerobes and positive to the enzyme catalase. The catalase test of bacterial isolates suspected to be the *Lactobacillus* group showed negative results. This is because Lactobacillus, as lactic acid bacteria (LAB), cannot produce the enzyme catalase to convert hydrogen peroxide (H_2O_2) into water and oxygen [26].

Based on the results of this research, 22 isolates of bacteria were obtained from the small intestine of

Maxomys hellwandii. The results of identification based on morphology, and physiology with biochemical tests from 22 bacterial isolates are suspected to be 14 isolates in the genus *Bacillus* sp. and 8 isolates in the genus *Lactobacillus*. Purba in his research [27] said lactic acid bacteria (LAB) are found in human and animal organs, such as in the intestinal pathway, genital pathway, respiratory, and sewer pathway. In other research, it was stated that Lactobacillus, one of the genera of lactic acid bacteria (LAB), is found mostly in the gastrointestinal tract of humans and animals with a range of 10^6-10^7 cells/g in the small intestine and 10^{10}-10^{11} cells/g in the large intestine [28].

IV. CONCLUSION

Twenty-two isolates of bacteria were isolated from the small intestine of three white-tailed forest rats (*Maxomys hellwandii*). The character identification of morphology (shape, edge, surface, and elevation of the colony, cell shape, gram stain, and endospore) and physiology by biochemical tests (citrate test, Triple Sugar Iron Agar, Motility, and Catalase) suspected 14 isolates as genus *Bacillus* and 8 isolates as genus *Lactobacillus*.

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