Composition of bacteria types in the guts of *Odontotermes* sp. and *Coptotermes* sp. Preliminary study

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Composition of bacteria types in the guts of *Odontotermes* sp. and *Coptotermes* sp. Preliminary study

A Djunaid, I Yastrib, A Arif, and M Muin

Forest Products Processing and Utilization Laboratory, Forestry Faculty, Hasanuddin University, Makassar

Email: anniza.djunaid@gmail.com

Abstract Termites (Arthropoda: Isoptera) are social insects and well known as the most lignocellulose digesting insects with a great diversity of symbiont bacteria in their guts. The aim of this study was to isolate and identify bacteria types present in the termite guts of *Odontotermes* sp. and *Coptotermes* sp. These bacteria were isolated from the termite guts and characterized by using morphological observation and stain Gram test. Results showed that there were 19 colonies of bacteria isolated from *Coptotermes* sp. guts and 37 colonies of bacteria isolated from *Odontotermes* sp. In the lower termite *Coptotermes* sp., two bacterial isolates with different morphological characteristics were found, namely isolate BcCo-1 with *Coccus* form and BbCo-2 with *Bacillus* form. The bacteria present in the lower termite species consisted of 84% *Coccus* form and 16% *Bacillus* form. Both of the bacterial isolates were Gram positive. In the higher termite *Odontotermes* sp., two types of bacteria isolates were also found, namely BbOd-1 and BbOd-2 isolates. Both of the bacterial isolates were *Bacillus* forms and Gram positive. Isolate BbOd-1 has round shape, entire edges and flat elevation, meanwhile isolate BbOd-2 has a round shape, serrate edges, and convex elevation. In order to verify the species of the bacteria types, molecular diagnostic is truly needed.

1. Pendahuluan

Termites as phytophagus insects that consume organic material containing cellulose such as wood stumps, twigs and leaves that fall are widely available in the environment. In terrestrial ecosystems, termites break down the dead organic matter into the nutrients needed by plants and increase land productivity by modifying the physical and chemical properties of soil [1,2]. In the process of decomposing organic matter, termites utilize microorganisms to degrade cellulose, such as bacteria and protozoa in the digestive tract, especially in the hindgut. In lower termites, protozoa dominate compared to bacteria; whereas in the higher termites, bacteria more dominant than protozoa [3,4].

The diversity of termites reaching 3,106 species [5] is a place of life that allows the discovery of various types of new microorganisms. The existence of microorganisms in termite intestines is a form of mutually beneficial interaction known as symbosis mutualism [6]. It is well known that bacteria are present in termite intestines, both in the higher termaites and the lower termites. Report by [7] regarding the diversity of termite species and bacteria showed differences in the color characteristics of the types of bacteria found in each type of termites, such as *Macrotermes gilvus, Macrotermes insperatus, Coptotermes mohri, Schedorhinotermes javanicus, and Coptotermes curvignathus*. Isolation of bacteria in the gut of *Macrotermes* spp. conducted by [8] showed that oxidase testing was able to distinguish bacteria from genera *Neisseria* and *Pseudomonas* which have positive oxidase and *Enterobacteriaceae* which have negative oxidase.

The bacteria found in the intestines of each type of termite have different characteristics. However, reports related to the identification and isolation of bacteria in termites of *Odontotermes* sp. still limited. Study by [9] found 19 bacteria from the intestine of *Odontotermes parvidens* using isolation methods. However, information related to the types and characteristics of bacteria isolated from the intestines of termite species distributed in the Wallacea region, especially in South Sulawesi, is not widely known. This study aims to identify and isolate bacteria found in the hidgut of *Odontotermes* sp. and *Coptotermes* sp. This research is expected to contribute in the future in helping to understand how the mechanism works and the functional role of bacteria in termite gut. In addition, it is expected to be useful in developing defaunation products from termite intestinal microorganisms for the purpose of Integrated Pest Management (IPM).

2. Materals and Methods

2.1 Sample collection

Samples of *Coptotermes* sp. workers. collected from stake-baiting at Unhas Housing located in Tamalanrea; while the workers of *Odontotermes* sp. taken from soil litter around the Faculty of Forestry. Workers' caste is collected and kept alive or fresh to ensure that intestinal bacteria remain alive.

2.2 Preparation of culture media

Nutrient agar media (Merck, Germany) is a special medium for bacterial growth, but not specific to certain bacteria. Culture media is made by dissolving 8 grams of NA and 400 ml of distilled water, then heated until the color changes to clear. Furthermore, the NA solution was sterilized using an autoclave at a temperature of 120 ^{o}C at a pressure of 1 atm for 15 minutes.

2.3 Isolation of bacteria from termite digestion tract

Bacteria are isolated from the digestive tract of worker termites. The bacteria was isolated from six workers. Before the isolation process, termites were sterilized on the surface of their bodies using 70% alcohol and repeated three times. The surface sterilization process in termite bodies serves to kill or minimize the microbial contaminants that exist on the surface of the body. The termite intestine was placed into the Eppendorf tube and 1 ml of pro-injection water was added, then 1 ml of the Eppendorf tube was taken and added to the vial bottle containing 9 ml sterile water, then serial dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} dan 10^{-5} are prepared. One ml was taken from each dilution to be platting into NA media using a micropipette. Platting results were incubated at 20^{o}C for 24 to 48 hours before being continued on characterization based on morphology and Gram stain.

2.4 Karakterisasi dan Identifikasi Bakteri Simbion pada Saluran Pencernaan Rayap

Morphology: Before observing bacterial morphology, bacterial colonies that grow are purified first. The purification process was carried out using a zigzag pattern on the petridisk containing nutrient agar media. The morphological characters observed were shape, edges, elevation, and color.

Gram staining test: At the staining stage, a dense bacterial culture is needed, then placed on the glass object and flattened an area of 1 cm^2 and dried for one minute and then drops a solution of gram A (crystal violet) for 2-3 drops and then dried again. The preparations were washed with distilled water until the dye was all washed and dried. The stages of the coloring process are also carried out for solutions of Gram B (iodine), Gram C (Alcohol 70%) and Gram D (safranin) solutions respectively. At the last stage an observation is made with a light microscope.

2.5 Observation variable

For the purpose of identification of bacteria from hindgut termites, the following morphological characters were observed: (i) forms, (ii) Edges, observed the edges of bacteria on the media whether it was flat, entire, or serrate; (iii) elevation, seen through the side of the Petri dish, such as flat, raised, and convex; (iv) color, color appearance observed in the medium whether it was opaque, translucent, shiny,

and dull; (v) the composition of the type of bacteria, determined based on the ratio of the types of bacteria identified; and (vi) type of Gram stain, see the results of Gram positive (purple) or Gram negative (red).

3. Result and Discussions

The results of the observation of bacteria from the gut of *Coptotermes* sp. and *Odontotermes* sp. those bred on NA medium are described as follows:

3.1 Bacterial Isolates of Termites Digestive Tract

Bacteria are obtained by thinning the gut termites and growing them on nutrient agar media. NA media is a medium that is clinically tested to grow bacteria and produce colonies that grow larger, real and easily observed [10]. The results of bacterial isolation from the intestines of termite *Coptotermes* sp. showed that bacterial isolates can be clearly observed at 10^{-1} and 10^{-2} dilution rates after an incubation period of 24 hours. At 10-1 dilution there were 16 bacterial isolates represented by one colony, namely BcCo-1 (Figure 1a); While at 10-2 dilution there are three bacterial isolates which are also represented by one colony, namely BbCo-2 (Figure 1b). These growing bacterial isolates are purified to produce pure bacterial isolates, which will then be used in the process of characterization and identification..

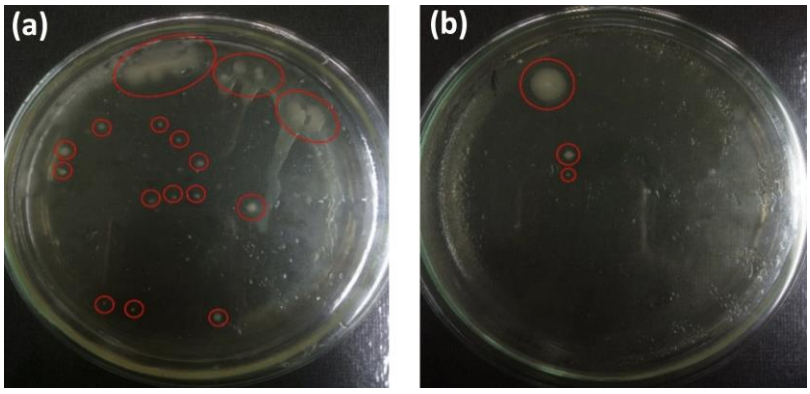

Figure 1. Growth of bacterial isolates from *Coptotermes* sp. gut: (a) a dilution rate of 10^{-1} (BcCo-1), and (b) a dilution rate of 10^{-2} (BbCo-2).

Bacteria isolated from *Odontotermes* sp. gut observed after an incubation period of 1 x 24 hours and 2 x 24 hours and showed the presence of two different bacterial colonies. The dilution process is carried out up to a 10^{-5} dilution rate. Among the five dilution levels observed by bacterial growth, the presence of bacteria was only found at the first three dilution levels, 10-1, 10-2, and 10-3 (Figure 2). Observation of bacteria was carried out 2 times. In the incubation period of 1 x 24 hours, only 1 colony of 20 bacterial isolates was known as BbOd-1, and in the incubation period of 48 hours, 1 colony was found from 17 isolates called BbOd-2.

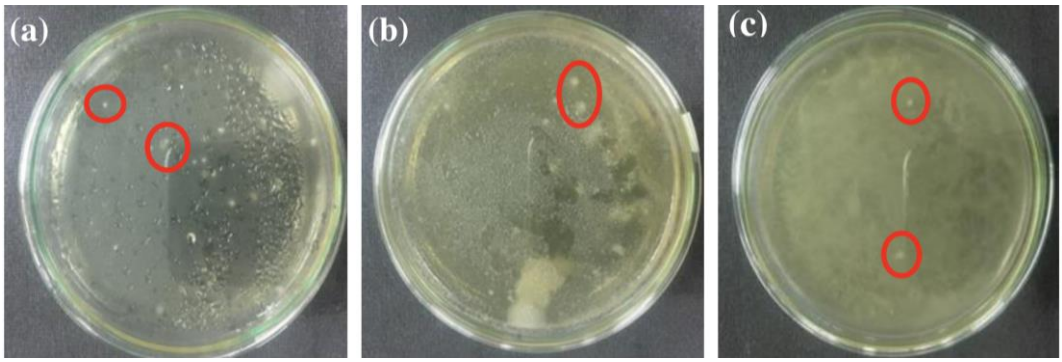

Figure 2. Appearance of bacterial colonies from *Odontotermes* sp. gut at 1x24 hour observation: (a) dilution 10^{-1}, (b) dilution 10^{-2}, dan (c) dilution 10^{-3}

3.2 Morphological characteristics

Morphological characterization of bacterial isolates was observed in the medium after incubating for 48-hour. Based on macroscopic observations, such as shape, color, edges and elevation, there are clear differences from bacterial isolates. The morphological characteristics of the bacteria can be seen in Table 1.

Table 1. Morphological characteristics of bacterial isolates

Colonies	Morphological characteristics			
	form	edge	elevation	color
Coptotermes sp.				
BcCo-1	round	flat	flat	yellowish white
BbCo-2	capsule	flat	convex	yellowish white
Odontotermes sp.				
BbOd-1	round	entire	convex	yellowish white
BbOd-2	round	serrate	flat	yellowish white

Morphological characteristics of BcCo-1 isolates from *Coptotermes* sp. gut shows round in shape, flat in edges with flat elevation, and yellowish white in color; while BbCo-2 isolates have capsule in shape, flat in edges with convex elevation, and yellowish-white in color. In *Odontotermes* sp, the two isolates found also showed different morphological characters. The BbOd-1 isolate has a round shape with entire in edges and convex elevation, while BbOd-2 isolates are round with serrate edges and have flat elevation. The color character of all bacterial isolates found only one type of color, namely yellowish white. After observing the morphology, then purification of each bacterial isolate was carried out. Purification was conducted to produce the pure culture without any contaminants from other microbes. The purification results showed that Bbod-2 isolates had more bacteria that grew than Bbod-1 isolates.

3.3 Characteristic of Gram Stain

In Gram staining, bacterial isolates must be purified first in order to determine Gram positive and Gram negative, whereas Gram positive indicates that the cell wall bacteria absorb violet color and have a thick peptidoglycan layer; whereas Gram negative indicates that the cell wall bacteria absorb red and have a thin peptidoglycan layer. The Gram staining process is conducted after passing the purification process, with the results of the purification process as shown in Figure 3 and Figure 5.

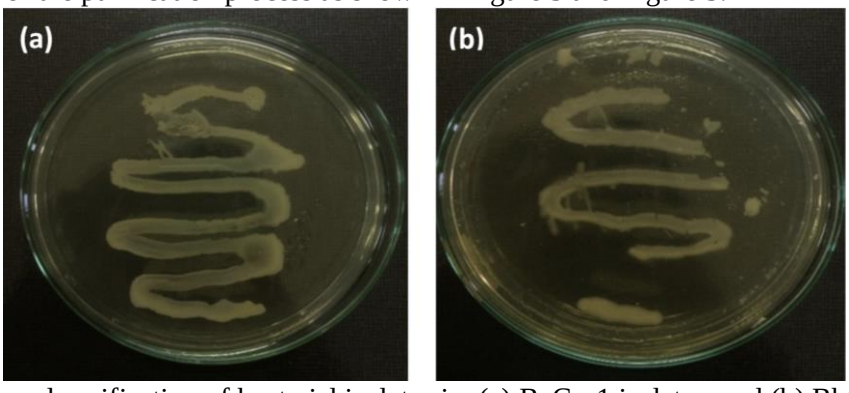

Figure 3. Second purification of bacterial isolates in: (a) BcCo-1 isolates, and (b) BbCo-2 isolates

At the third purification stage only BbCo-2 is purified again because the previous purification has not been perfectly pure. This is different from purification of BcCo-1 which only arrived at the second purification because BcCo-1 in the second purification was perfectly pure. After the macroscopic observation, microscopic stages were carried out in which the purified isolates were carried out by bacterial characterization in the form of bacteria, in BcCo-1 isolates in the form of Coccus and bacterial

forms in Bacillus BbCo-2 isolates which can be seen in Figure 4a and Figure 4b. The observation of Gram staining in both BcCo-1 and BbCo-2 isolates showed Gram positive bacteria.

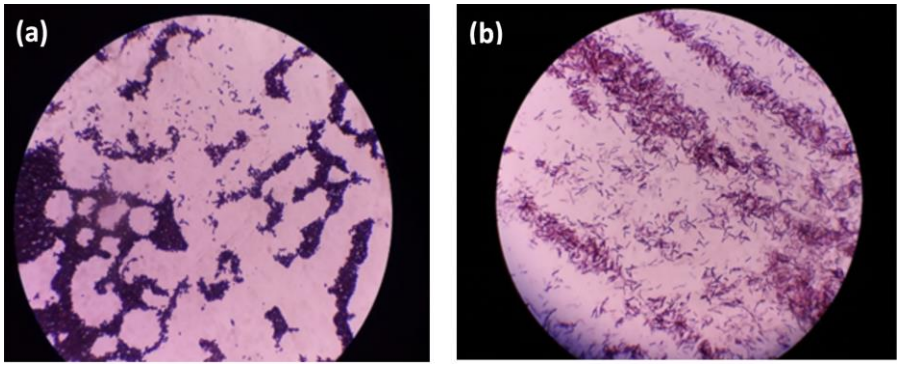

Figure 4. Characterization with Gram staining: (a) bacteria from BcCo-1 isolates in the form of Coccus, and (b) bacteria and BbCo-2 isolates in the form of Bacillus.

Based on the results of observations of the morphological characteristics of bacterial isolates then bacterial isolates from BbOd-1 and BbOd-2 purification were taken (Figure 5) to proceed to Gram stain testing.

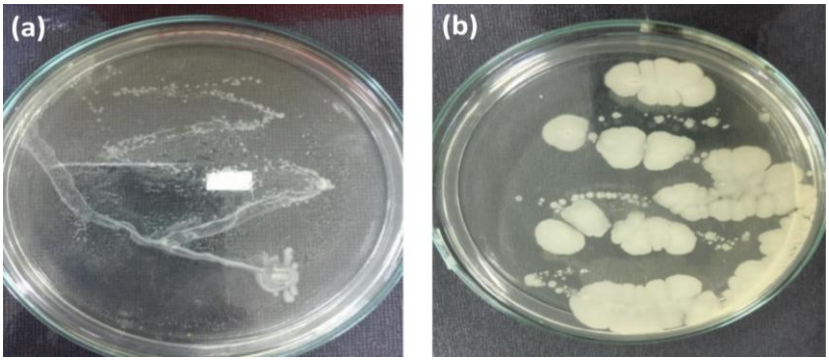

Figure 5. Purification of Isolate BbOd-1 (a) and isolate BbOd-2 (b)

The results of gram staining show that only one Gram type in the two bacterial isolates from *Odontotermes* sp. gut tested is Gram positive, as can be seen in Figure 6.

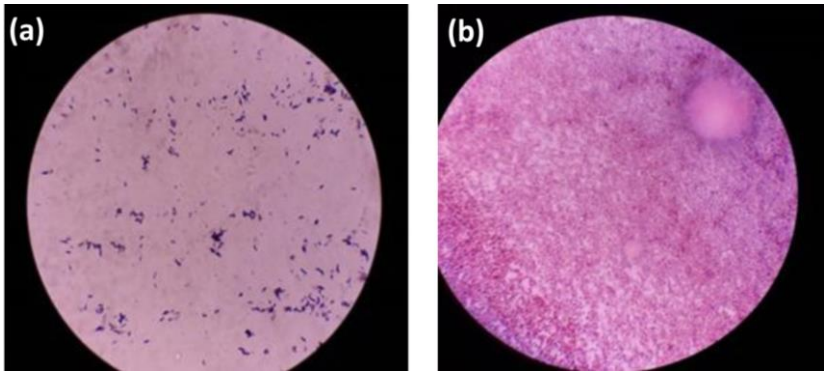

Figure 6. Characterization with Gram staining from BbOd-1 isolates (a) and BbOd-2 isolates (b).

3.4 Composition of bacterial types

The results of the calculation of the number of colonies showed that in BcCo-1 isolates that grew there were 16 colonies so that the total number in BcCo-1 was 1,600 colonies, whereas in BbCo-2 there were 3 colonies so that the total number of BbCo-2 was 300 colonies. The number of bacterial colonies is calculated based on the number of colonies in each isolate which is divided by a dilution of 10-2 then multiplied by 100 so as to get the total amount in each bacterial colony. Based on Table 2, it can be seen that in the intestines of termites *Coptotermes* sp. which is dominant in the BcCo-1 isolate category, which is 84%. So that it can be concluded that the dominant type of bacteria in the intestines of termites *Coptotermes* sp. is BcCo-1.

Tabel 2. Composition of bacterial types of gut termites

Isolates	Enumeration		Gram Stain		
	N	%	N	Gram (+) %	Gram (-) %
Coptotermes sp.					
BcCo-1	16	84	16	100	0
BbCo-2	3	16	3	100	0
Total	19	100	19	100	0
Odontotermes sp.					
BbOd-1	20	54	20	100	0
BbOd-2	17	45	17	100	0
Total	37	100	37		

After 48 hours incubation, the number of BbOd-1 isolates observed was 20 at 10-1 dilutions, so the total number of BbOd-1 was 200 colonies; while the number of BbOd-2 isolates at 10-1 dilutions was 17 colonies, so the total number of BbOd-2 was 170 colonies. So it can be concluded that the dominant type of bacteria in the intestines of Odontotermes sp. is BbOd-1 (54%) as can be seen in Table 2.

3.5 Discussion

At present, termites reach 3,106 species in nine families [5], which based on the presence of the dominant symbionts in their digestive tract can be classified into two groups, namely higher termites and lower termites. The higher termites include all members of the family Termitidae, while the lower termites include all termite families, except family Termitidae. *Coptotermes* sp. (Family Rhinotermitidae) belonging to the lower termites, which are found to be widely distributed throughout the South Sulawesi region [11,12]. The *Odontotermes* sp. (family Termitidae) belonging to the higher termites can also be widely distributed and found easily in various habitats [12]. Like other types of termites, the survival of these two types of termites is very dependent on intestinal microorganisms to obtain energy. These microorganisms play major physiological functions such as digestion of cellulose and hemicellulose, acetogenesis, hydrogenesis, metanogenesis, sulfate reduction and nitrogen fixation [13]. In addition, intestinal microbes create conditions suitable for symbionts through the production of nutrients and maintenance of pH and anaerobic conditions in the intestine [14]. In this study, the role of bacteria found cannot be ascertained because of the limited testing carried out, so that a series of other tests are needed, including biochemical test.

The number of bacterial colonies is an indicator of the spread of bacterial colonies in bacterial culture media. Calculation of bacteria is one way to find out how many bacterial colonies are found in a medium. In calculating bacterial colonies, the higher the dilution, the less the number of bacterial colonies produced. This means that the dilution rate is inversely proportional to the number of bacterial colonies that grow [15]. Furthermore, it was stated that dilution is generally done to reduce the density or spread of bacteria found in the media. Dilution can be done up to a 10-5 dilution rate. In this study, the number of bacterial colonies from the intestine of Odontotermes sp. more than Coptotermes sp. This result is

supported by the statement of Bignell and Eggleton (2000) and Breznak (2000) that bacteria are more predominantly found in higher termites than lower termites.

Morphological characterization of bacterial isolates from both types of termites (Table 1) showed a difference between one isolate and the other. The form of bacteria observed under the microscope is round and capsule. The results of thus study are different from *Odontotermes parvidens* gut bacteria observed by Kakkar, *et al*. (2015) who found three types of bacteria, namely spiral, vibrio, and basil. To observe the color character of isolates from bacterial isolation, only one type of color was found. Visual colors that are visible are yellowish white, different from the results obtained by Mubin (2013) who isolated *O. javanicus* showing shiny yellow.

In the Gram staining test, bacterial features including Gram positive were indicated by changes in bacterial cells to purple and which included Gram negative with changes in cell color to red. This is based on the thickness or thinness of the constituent cell walls of bacteria. The cell wall of Gram positive bacteria is thicker than the cell wall of Gram negative bacteria. In crystal violet staining, the primary dye will enter the cell walls of Gram positive bacteria until the final coloring process. In contrast to Gram negative bacteria, the cell wall is thinner so it will be washed away at the color degradation stage by 95% ethanol and safranin coloring process (counter dye), so that the wall of Gram negative cells will be colored to red [7]. Gram staining results showed that the bacterial isolates from both termites (Table 1) showed similarities as gram-positive bacteria. Research conducted by Mubin (2013) which also explores the presence of bacteria from lower- and higher termites gut shows all the results of gram staining carried out at high termites including gram positive.

In this study, the type of bacteria isolated from *Coptotermes* sp. and *Odontotermes* sp. gut cannot be ascertained because of the limited testing performed. Nevertheless, a number of studies have found the type of bacteria isolated from the same genus but different species. The results of the study by [16] which isolates bacteria from the hidgut of *Coptotermes formosanus* (family Rhinotermitidae) identify facultative bacteria from the species *Serratia marcescens, Enterobacter erogens, Enterobacter cloacae*, and *Citrobacter farmeri* which play critical role in breaking down cellulose, hemicellulose and nitrogen fixation. In *Coptotermes* sp., workers, the type of that is most commonly found in hidgut is the genera *Flavobacteria* and *Enterobacter* [17]. Research on the distribution of cellulotic bacteria showed that there was correlation between the main bacteria of the intestine and the termite family, which family in the lower termite had the main bacteria *Streptococcus, Staphylococcus* and *Enterobacter*, and were found in coculture that grew anaerobically. Glucose fermented by *Streptococcus lactis* produces lactate and is utilized by *Bacteroides* sp. be propionate, acetate and CO_2 [4].

4. Conclusion

Referring to the results and the discussion can be concluded several things, as follows: (i) In *Coptotermes* sp. gut found 19 isolates, whereas in *Odontotermes* sp. gut found 37 bacterial isolates; (ii) The four isolates, namely BcCo-1, BbCo-2, BbOd-1, and BbOd-2 showed differences in morphological characters; (iii) Bacterial composition of *Coptotermes* sp. (lower termites) gut consisting of 84% in coccus form (BcCo-1) and 16% in bacillus form (BbCo-2), whereas in *Odontotermes* sp. (higher termites) gut consists of BbOd-1 (54%) and BbOd-2 (45%). All isolates were Gram-positive bacteria.

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