

The Earthworm (*Lumbricus Rubellus*) Extract Decreased Amino Transaminase Enzyme Level and Number of Bacterial Colony in Male Wistar Rats Infected with *Salmonella Typhimurium*

Anak Agung Wiradewi Lestari¹, I. Dewa Made Sukrama² and Dian Nurmansyah³

¹Clinical Pathology Department, Faculty of Medicine, Udayana University, Sanglah General Hospital, Bali, Indonesia.

²Clinical Microbiology Department, Faculty of Medicine, Udayana University, Sanglah General Hospital, Bali, Indonesia.

³Master Degree of Biomedical Science, Udayana University, Bali, Indonesia.

*Corresponding author E-mail: wiradewilestari@gmail.com

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Lumbricus rubellus earthworm is known for its antioxidant and antibacterial properties such as Polyphenol, Glycoprotein G-90, and Lumbricin I. These substances function as hepatoprotective agents in the parenchymal cell when damage by infection. This study determines the antioxidant properties of earthworm extract (*Lumbricus rubellus*) in reducing the levels of ALT, AST, and a number of the bacterial in male Wistar rats infected with *Salmonella Typhimurium* as a model of *S. Typhii* infection. Posttest-only control group method was carried out in 28 samples which were divided into 4 treatment groups. The blood samples were taken and assay for ALT and AST on day 18. The bacterial colony growth inhibition was determine by growing the bacteria in the feces with Total Plate Count (TPC). The ALT levels in T2 were decreased significantly at ($P < 0.05$; 25.9 ± 5.50 U/L), followed by T1 ($P < 0.05$; 35.6 ± 1.46 U/L). The AST levels in the T2 and T1 groups were also decreased significantly at ($P < 0.05$; 81.4 ± 13.44 U/L and 107.8 ± 9.45 U/L, respectively). The bacterial colony of *S. Typhimurium* significantly decreased in the T2 and T1 with the mean $6.89 \pm 2.06 \times 10^{-1}$ and $8.38 \pm 2.15 \times 10^{-1}$, respectively. The Kruskal-Wallis test found a significant difference between various groups ($P < 0.05$), but the Mann-Whitney test showed no significant difference between T1 and T2 group for the bacterial colonies ($P = 0.180$). The *Lumbricus rubellus* extract has hepatoprotective and antibacterial properties by significantly reducing the levels of amino transaminase (ALT and AST) and bacterial colonies of *S. Typhimurium* in male Wistar rats.

Keywords: Aminotransaminase, *S. Typhimurium*, Earthworm Extract, *Lumbricus rubellus*.

Typhoid fever is a foodborne disease caused by *Salmonella enteric* serovar *Typhi* (*S. Typhi*).¹The World Health Organization (WHO) in 2003 reported that the incidence of typhoid fever reached 17 million where 600,000 typhoid-related deaths occurred worldwide (insert reference). In Indonesia, the incidence of typhoid fever is 900,000 cases annually amounting to 20,000 deaths, this

correlates with 91% cases reported for children within the the age of 3-19 years.^{2,3}

Besides the increased in O and H antibodies in the diagnosis of typhoid fever, elevated transaminase in *S. Typhi* bacterial infections also occur as AST (Aspartate Aminotransferase) and ALT (Alanine Transaminase).⁴ Both increases in these enzymes occur at the time of *S. typhi*

adhesion on the small intestine and epithelial cells. *S. typhi* infection enters the bloodstream through the lymph vessels to the liver, and spleen. *S. typhi* will stimulate the proliferation of inflamed cells in the liver (insert ref). The damage of the liver parenchymal cells and membrane permeability may result in the production of AST and ALT. Thus a good indices for establishing the liver damage (Ref.). The measurement of AST and ALT level will increase significantly formerly.⁴

Various types of drugs such as chloramphenicol, ampicillin, and cotrimoxazole are antibiotics that have been used for typhoid fever for decades until the emergence of multidrug-resistant (MDR).⁵ The cause of the emergence of resistance an administration of the irrational drug; consumer behavior does not comply with the rules and the intrinsic changes that takes place in the microbe.⁵ The antibiotic resistance is the underlying issues for researchers to find alternative medicine such as earthworms (*Lumbricus rubellus*).

Several studies have reported that the bioactive compounds and antimicrobial properties of earthworms can inhibit pathogenic bacteria. These active substances include G-90 glycoprotein and fetidin from *Eisenia foetida*,⁶ lysozyme from *E. fetida Andrei*,⁷ as well as histidine from *Dendrobaena Veneta* and *Nereis diversicolor* earthworms.^{8,9} In addition to the inhibition of pathogenic bacteria, earthworm flour (*L. rubellus*) has a fairly high protein content of 63.06% of its dry matter.¹⁰

The effects of *Lumbricus rubellus* earthworms against pathogenic bacteria have also been widely performed *in vitro*. A study conducted by (Purwaningroom is this supposed to be a ref?) showed that *Lumbricus rubellus* earthworms were better at generating inhibition zones against *S. typhi* bacteria growth compared to *Pheretima aspergillum*.¹¹ While the study conducted by (Ratriyani this supposed to be a ref?)(found that the *Lumbricus rubellus* earthworms proved to have reduced a number of *S. typhi* bacterial colonies *in vitro*.¹²

Salmonella typhimurium bacteria was used as a model for typhoid fever by using rats to induced *Salmonella typhimurium* infection. This may represent the pathological state of typhoid fever in humans caused by *Salmonella typhi* according to (Rosenberger *et al.* *Make this a ref*).¹³

Thus, the use of *Salmonella typhimurium* bacteria can be used as a surrogate of typhoid fever in rats.

Based on the above explanation, the researchers aimed at determining the effect of earthworm (*Lumbricus rubellus*) extract in decreasing ALT, AST, and *in vivo* inhibition of *S. typhimurium* colonies.

MATERIAL AND METHODS

This study adopted posttest-only control group design beginning from December 2016 - January 2017. The test animals were male Wistar rats, of two months old, weighing 200-250 grams. The infected bacteria was *S. typhimurium*. The sample size was 28 male Wistar rats divided into 4 groups (C+, C-, T1, and T2). This study had received ethical clearance from the Ethics Committee on Animal Research, Faculty of Medicine, Udayana University.

Animal Preparation

The 28 male Wistar rats were adapted for one week, then divided into four groups of seven rats. The groups consists of negative group (only gave placebo), positive control (infected by *S. Typhimurium* bacteria), treatment group 1 (infected by *S. Typhimurium* day 1 and giving *L. rubellus* earthworm extract on the following days until day 18) and treatment group 2 (giving *L. rubellus* earthworm extract in the first week then infected by *S. Typhimurium* day 8, followed by extract until day 18). The number of mice infected by *S. Typhi* was 10⁵ and the dosage of earthworm extract was 100 mg/kg based on the previous study. All rats were fed and given drink in ad libitum way. On the 18th day, blood samples were taken for further measurement of the ALT and AST levels as well as a bacterial colony from feces.

Crude Extract Preparation of *Lumbricus rubellus* earthworm

The collected *Lumbricus rubellus* earthworms were washed with running water to remove the mucus on its surface. After the earthworms were cleaned then it dried at 40°C for 24 hours. The dried earthworms were cut into small pieces then put into a glass tube of 80% ethanol solvent for the evaporation process in obtaining the crude extract. This process took 2 days to complete the extraction.

Salmonella Typhimurium suspension preparation

S. typhimurium ATCC 14028 bacterial isolates were obtained from the Microbiology Department, Faculty of Medicine, Udayana University. Bacteria were cultured in Salmonella Shigella Agar, and incubated for 18-24 hours at 37°C. The *S. typhimurium* suspension preparation used cop count method. About 3-5 colonies were inserted into a tube containing 10 ml of 0.1% peptone; then the *S. typhimurium* bacteria suspension was diluted in multiple series of 10⁶. In addition, 0.1 ml of 0.1% peptone was planted to control contamination. The colony counting was performed if the number of colonies growing between 30-300 colonies. To determine the culture contamination, gram staining was performed and observed under a microscope for each dilution. The results of the concentration obtained will be made bacteria suspension containing 10⁵ cells/ml.

Assessment of AST and ALT levels

The AST and ALT were measurement using the IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) method. The measurement steps were as follows: 1) Prepared test tubes and other; 2) Four parts of reagent A were mixed with one part of reagent B then homogenized (monoreagen); 3) Approximately 1 mL of monoreagen was taken and inserted into the test tube, then incubated at 37°C for at least 1 hour; 4) Added serum/sample as much as 0.1 ml; 5) Incubated in a water bath at 37°C for 1 minute;

6) the sample absorbance in a spectrophotometer with a wavelength of 340 nm was read.¹⁴

Assessment of Bacterial Colony in Feces

The measurement of bacterial colonies in the feces were conducted by a modified Total Plate Count (TPC) method with the following steps: 1) The intestine specimens or rats were taken with aseptic techniques and then feces collected in sterile containers; 2) The transport medium (TSB) was used to carry samples to the laboratory; 3) 1 gram sample was put into a 10 mL sterile NaCl; 4) Dilution was carried out from 10¹ - 10⁶ and then transferred into a medium plate to Salmonella Shigella Agar (SS Agar); 5) Media plate Salmonella Shigella Agar (SS Agar) then incubated at 37°C for 1x24 hours; 6) the colony measurement was performed with a colony counter on a plate that was eligible to be calculated with colony counts of 30-300 colonies; 7) the average number calculation of bacterial colonies grown expressed by cfu/g sample unit; 8) the gram staining was carried out to ensure that the growing bacterial colony was *S. typhimurium* with red color and rod-shaped; and 9) The calculation of cfu / gram of sample was carried out by using the formula: (CFU number x dilution x 10) / sample weight (gram).^{15,16}

Statistical Analysis

Data of AST, ALT levels and abacterial colony were described by normality test using Saphiro Wilk, homogeneity, Levene's test, and comparability test using Kruskal-Wallis. Data were not distributed normally and not homogeneous of

Table 1. The results of AST level measurement in rats

Group	Upper Values	Lower Values	Mean	P-Values
Control (+)	274.4 U/L	150.4 U/L	184.7 ± 42.95 U/L	0.001
Control (-)	133.2 U/L	124.2 U/L	128.1 ± 3.89 U/L	
T1	117.9 U/L	96.3 U/L	107.8 ± 9.45 U/L	
T2	92.4 U/L	66.2 U/L	81.4 ± 13.44 U/L	

Table 2. The results of ALT level measurement with the IFCC method

Group	Upper Values	Lower Values	Mean	P-Values
Control (+)	106.1 U/L	41.9 U/L	58.6 ± 21.92 U/L	0.001
Control (-)	42.2 U/L	38.4 U/L	39.8 ± 1.46 U/L	
T1	37.7 U/L	34.2 U/L	35.6 ± 1.46 U/L	
T2	33.6 U/L	20 U/L	25.9 ± 5.50 U/L	

data variants. In addition, the Mann-Whitney test was also conducted to know the difference between each variable.

RESULTS

AST and ALT levels

Table 1 shows the upper, lower, and mean values of aspartate transaminase (AST) serum levels. Results showed that the highest mean value of AST levels was in the positive control group (C +) of 184.7 ± 42.95 U/L. While the lowest mean value of AST levels was found in the treated group 2 (T2), 81.4 ± 13.44 U/L. Based on the highest and lowest AST values suggested that the lowest values were in the T2 group (92.4 U/L and 66.2 U/L). The AST levels were known to have a P value of 0.001 (<0.05), so it can be concluded that there was a significant difference in AST levels between treatment groups.

Table 2 showed the highest, lowest, and mean value of alanine transaminase (ALT) serum levels by using the IFCC method without peroxide. The highest mean value of ALT levels was found in the positive control group (C +) which accounted for 58.6 ± 21.92 U/L. Based on the highest and lowest ALT values suggested that the lowest values were in the T2 group (33,6 U/L and 20 U/L). Besides, the ALT levels were known to have a P

value of 0.001 (<0.05), so it can be concluded there was a significant difference in ALT levels between treatment groups. (Table 2).

Bacterial colonies

The results of bacterial colonies were performed by TPC and Harrigan methods that demonstrated in Figure 1. *S. Typhimurium* bacteria that grow on SS agar media have a clear and transparent colony. Some have a black dot on the colony due to H_2S produced by *S.typhimurium*. Gram staining was carried out to see the properties and morphology of bacteria in which indicate for gram-negative due to rods shape and red appearance from Safranin Red stain (Figure 2). In the negative control group, there were no colonies eligible for 30-300 counting colony so that all results in the group could not be calculated. The results of this study also showed that the mean colony growth of *S. Typhimurium* bacteria was the lowest in the treatment group 2 (P2) about $6.89 \pm 2.06 \times 10^{-1}$. Besides, the colony counts were known to have a P value of 0.001 (<0.05), so it can be concluded there was a significant difference in the bacterial colony counts between treatment groups (Table 3).

Multivariate analysis

Mann-Whitney test was carried out to determine whether there were differences between each variable. In Figure 1, the results indicated that

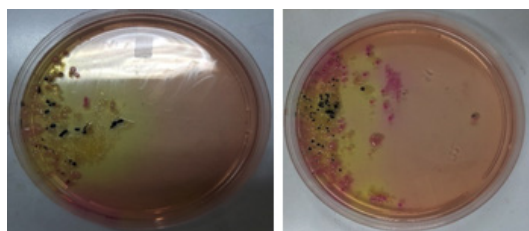


Fig. 1. *Salmonella Typhimurium* colonies on the SS agar medium

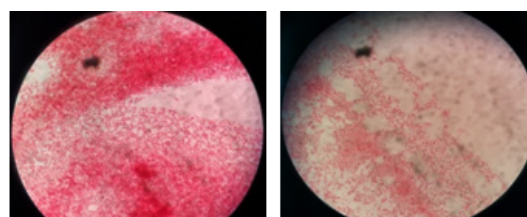


Fig. 2. Gram-staining of *Salmonella typhimurium* colonies on the SS agar medium

Table 3. Colonies count results of *S.typhimurium* with TPC method (*Total Plate Count*)

Group	Σ The Highest Colonies	Σ The Lowest Colonies	Σ Mean	P-Values
Control (+)	29.92×10^{-1}	14.15×10^{-1}	$21.28 \pm 5.64 \times 10^{-1}$	
Control (-)	0	0	0	
T1	11.74×10^{-1}	6.03×10^{-1}	$8.38 \pm 2.15 \times 10^{-1}$	0.001
T2	9.69×10^{-1}	4.34×10^{-1}	$6.89 \pm 2.06 \times 10^{-1}$	

there was a significant difference between either AST or ALT measurement groups ($P = 0.001$). However, there was only no significant difference between T1 and T2 group ($P = 0.180$) for the bacterial colonies measurement (Figure 2). Based on this result, it can be concluded the treatment

provided for T1 and T2 group to the bacterial colonies was no significant difference.

Based on Table 4, this result indicates that there is a decrease in the levels of AST, ALT, and bacterial colonies on treatment 1 and 2 compared with the positive and negative control groups. In

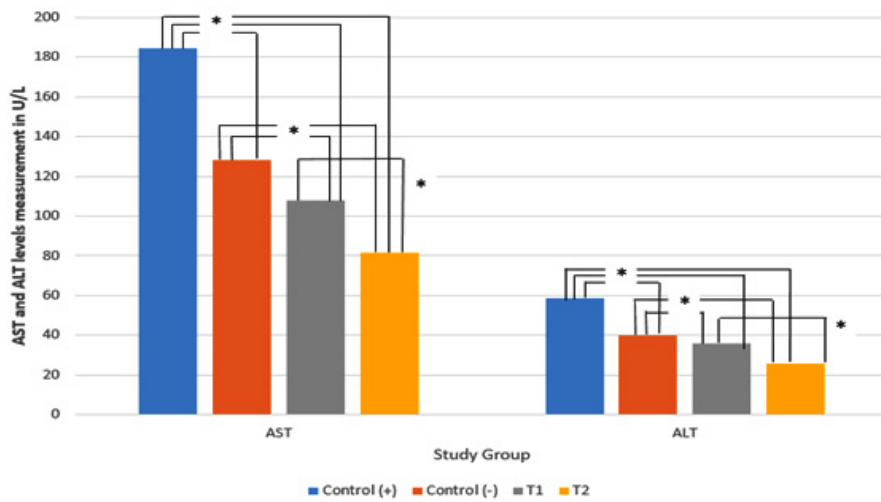


Fig. 3. Mann-Whitney test results between AST and ALT measurement

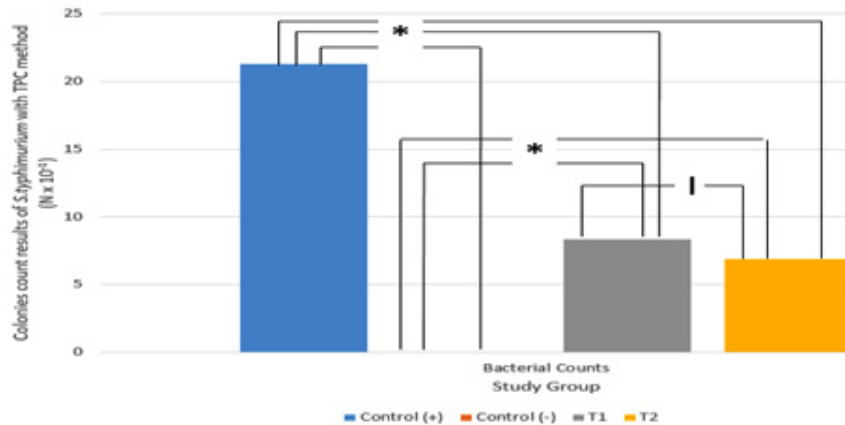


Fig. 4. Mann-Whitney test results in the bacterial counts group

Table 4. The mean comparison of AST, ALT, and number of bacterial colonies between groups

Variable	Control positive	Control negative	T1 group	T2 group	Description
AST	184.7 U/L	128.1 U/L	107.8 U/L	81.4 U/L	↓ Levels
ALT	58.6 U/L	39.8 U/L	35.6 U/L	25.9 U/L	↓ Levels
Σ Bacterial colonies	21.28 x 10 ⁻¹	0	8.38 x 10 ⁻¹	6.89 x 10 ⁻¹	↓ Σ Colonies

addition, the observed variables (AST, ALT, and bacterial colonies) also resulted in a decline in enzyme levels and colonies.

DISCUSSION

Typhoid fever is a foodborne disease caused by *Salmonella enteric* serovar *Typhi*, a genus of gram-negative bacteria. It remains one of the endemic diseases in Indonesia, based on data from the World Health Organization (2003).³ This infection triggers the release of proinflammatory cytokines from the liver parenchymal cells and membrane permeability that is resulting in the AST and ALT enzymes production. Both of them are known as a good indicator for detecting liver damage since these enzymes will increase significantly formerly.¹⁷ AST is a chemical parameter which has a 90% of sensitivity but only has a specificity of 18% on liver damage.¹⁸ AST levels will increase in the acute infection or hepatic injury as well as in a person with typhoid fever.^{19,20} In addition, ALT is similar with AST as an enzyme which is abundant in the liver cells (also present in other organs such as the kidneys, heart muscle, pancreas but in the very slight levels) despite having better sensitivity and specificity for biomarkers of liver cell damage.²¹

The regarding the hepatoprotective activity of earthworm extracts, it seems that these substances not only exert antioxidative properties but also may regulate the expression of specific genes. Earthworm (*L. rubellus*) extract is known to contain Polyphenolic which has anti-oxidation and anti-inflammatory properties, G-90 glycoprotein, and also fibrinolytic enzyme. Dewi NWS found that the ethanolic extract of *L. rubellus* powder had total phenolic content of 1016.31 mg/100 g gallic acid equivalent (GAE) and exhibited IC 50% of 12.33 mg/ml as antioxidant capacity.²² A study conducted by Popovic *et al* showed that G-90 glycoproteins had antimicrobial properties where 10 mg / mL concentrations were able to inhibit the growth of facultative pathogenic bacteria such as *S. enteritidis*, *S. aureus*, and *S. pyogenes* also exerts anticoagulative and fibrinolytic activities.⁶ Furthermore, it is also noted in the study of Tasiemski (2006) that *L. rubellus* also contained Lumbricin I as a broad-spectrum antimicrobial compound against both gram-positive and gram-

positive bacteria.⁹

The comparative analysis results using Kruskal-Wallis test of AST and ALT levels in 4 treatment groups (positive control, negative control, treatment 1 and treatment 2), showed that there were significant differences between treatment groups which receiving *L. rubellus* extract ($P = 0.001$). This study found that the treatment groups (1 and 2) had lower results of AST and ALT levels compared with positive and negative control groups. It is presumably due to the hepatoprotective effect activity of the compound found in earthworm (*L. rubellus*). A previous study conducted by Muchtaromah demonstrated that there were antimicrobial effects either *in vitro* or *in vivo* in earthworms of 60% dosages of *L. rubellus* earthworms where reducing the transaminase enzyme levels within 14 days.²³

In treatment 1 (T1) where bacterial infection and earthworm extract were given on the first day up to the 18th day of treatment, AST and ALT levels were higher than treatment 2 (T2). However, both groups were still had lower levels of AST and ALT compared with positive and negative control group. This shows that earthworm extract has a hepatoprotective effect on *S. typhimurium* infection. The hepatoprotective effect is better in treatment 2 where bacterial infection performed on the 8th day after administration of the extract on day 1-7 of treatments, followed by administration of the extract on days 10-18. This result was supported by Salzet *et al* which stated that *L. rubellus* earthworm had peptide compounds as the first defense against microbes with their own anti-microbial properties.⁷ The hepatoprotective effect was also reported by Balamurugan *et al* where a decrease in ALP, AST, ALT, and Bilirubin enzyme levels in Rats occurred in the hepatic cellular injury exposed to substances.²⁴

In typhoid fever due to *S. typhi* bacterial infection, patients who have recovered still possible to spread the *S. typhi* called as a carrier. Carrier is a person who has recovered from typhoid fever and still able to secrete *S. typhi* bacteria in feces and urine.²⁵ Patients who did not receive appropriate treatment then will have a possibility for bacteria still be remain in the organs of the reticuloendothelial system and able to do re-proliferated and re-secreted by the patient.²⁵ The appropriate treatment for this issue is by giving

specific antibiotics. Antibiotics are substances that kill or inhibit bacteria, e.g., growth, by any of several mechanisms that specifically target the bacterial cell. However, Kelanitet demonstrated that MDR was found to be resistant to 18 antibiotics for *S.typhi* infection in Jayapura, Papua, such as 8 isolates resistant to Amoxicillin 100%, 75% for Cefazolin (6 isolates), and 75% for ampicillin (6 isolates).²⁶In this regards, our study is trying to figure out the antibacterial properties belong to *L.rubellus* extract using bacterial colonies counts. The bacterial colonies measurement using the TPC (Total Plate Count) method in rat feces shown that there were significant differences between treatment groups by Kruskal-Wallis test ($P < 0.05$). The decreasing number of bacterial colonies indicates that the antibacterial properties possessed by the *L.rubellus* earthworm work against *S.typhimurium* infection. glycoprotein-90 and Lumbricin I antibacterial molecules in *L.rubellus* extract are known highly sensitive to gram-positive bacteria, but it can also work against gram-negative bacterial infections such as *S.typhimurium*.^{7,27} G-90 glycoprotein is also known to be a stimulant of proliferation, anti-inflammation, and antimicrobial.²⁸ Chauhan et al demonstrated that the earthworm extracts were able to inhibit the *Paeruginosa* bacteria growth similar to the inhibition properties produced by Streptomycin.²⁹ Purwaningroom using *L.rubellus* and *Pheretima aspergillum* flours also suggested that the *L.rubellus* earthworm flour was better and significantly different in inhibiting the *S.typhi* bacteria growth in vitro with the processing temperature of 50°C.¹¹

However, multivariate analysis using Mann-Whitney test demonstrated no significant difference only between T1 and T2 groups for a number of bacterial colonies ($P = 0.180$). In the treatment group 2 (T2), *L.rubellus* earthworm extract was administered for 7 days of treatment, then infected with *S.typhimurium* bacteria for the following days, in contrast to treatment group 1 (T1) where infection and administration of the extract were performed at the same time. These results suggested that despite *L.rubellus* extract has anti-inflammatory and hepatoprotective properties; the treatment differences show no significant impact on the number of bacterial colonies observed. Both either the *L.rubellus* extract

or infection given simultaneously or separated, its effect on the inhibition of bacterial colony count was not significantly different in rats.

CONCLUSION

The *Lumbricus rubellus* earthworm extract has hepatoprotective and antibacterial properties through its effect on decreasing levels of AST, ALT, and number of *S.typhimurium* bacterial colonies in male Wistar rats.

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