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APPLICATION OF BACTERIOPHAGES COCKTAIL TO CONTROL *Salmonella typhimurium* IN CHICKEN MEAT

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ABSTRACT

Food safety is an increasingly important public health issue. Many efforts are intensified all over the world to improve food safety. The number of cases of several foodborne diseases has been rising continuously in many countries. Salmonellosis is one of the most common and widely distributed foodborne diseases and is caused by Salmonella. Antibiotics are usually used to tackle the problem of foodborne diseases. However, antibiotic have adverse effects such as bacterial resistance to antibiotics as well as killing the entire colony of microbes in the gut both beneficial and adverse. The use of lytic bacteriophages for the bio-control of foodborne pathogens should be a promising strategy to overcome this problem. In this study, the effectiveness of a lytic bacteriophage cocktail (Φ14) isolated from chicken intestine was determined in chicken meat experimentally contaminated with *Salmonella typhimurium*. Production of Φ14 with the concentration of 8,3 x 10^3 PFU/ml was conducted by using *Shigella flexnery* as a host. To obtain the effective application of bacteriophage, the optimization of host infection was performed. A 30 minutes infection of bacteriophage to cell target (*S. typhimurium*) was found to be an optimum time to lyze the cell target. A reduction of *S. typhimurium* (1-3 log) was obtained after infection of bacteriophage in chicken meat under 37^{o}C for 2, 4, 6 days. This result shows that the potential effectiveness of this bacteriophage cocktail as a biocontrol agent of *S. typhimurium* in food product under conditions similar to those used in their production.

Keywords: foodborne diseases, bacteriophage, bio-control, *Salmonella typhimurium*, chicken meat

INTRODUCTION

Food safety issues have become a global problem, so it received great attention in the establishment of public health policy. Diseases caused by consuming foods that contain pathogenic bacteria (foodborne disease) and incidence of food contamination events occur not only in developing countries where sanitation and hygiene conditions are generally poor but also in developed countries. An estimated one in three people resident in developed countries experienced food poisoning each year. (Secretariat General of the Food Intelligence Network, 2005). During 2004, based on reports Center / POM throughout Indonesia have occurred an outbreak (KLB), food poisoning as many as 153 events in 25 provinces (Secretariat General of the Food Intelligence Network, 2005). In Indonesia from 18 cases of food poisoning that occurred in 2003, 83.30% are caused by pathogenic bacteria.

Salmonella is a bacteria that can cause typhoid, paratyphoid, and salmonellosis. In Europe and the United States cases of illness caused by Salmonella enteritidis is transmitted through chicken meat, eggs, and dairy products (Baumler *et al*., 2000). Salmonella are bacteria that cause intestinal infections second most common in the United States. More than 7,000 cases of Salmonellosis were confirmed in 2009, but most cases are not reported (Djafaar and Rahayu, 2004). To overcome the problem of foodborne disease are usually people use antibiotics. But antibiotics have been used extensively by people had detrimental effects such as bacterial resistance to antibiotics as well as the killing of entire colonies of microbes in the gut both beneficial and adverse. As an alternative to antibiotics it is used bacteriophages. Bacteriophage has the advantage of its ability to specifically attack bacteria with high specificity without disturbing other microbiota. Bacteriophages are highly specific nature is beneficial because it makes safe when consumed by humans. Based on the United States Food and Drug Administration (2006) bacteriophages on cheese can be used to kill *Listeria monocytogenes* and classified in the GRAS (Generally Recognized As Safe) and can be used in food products.

Meat products, including chicken meat is a food product susceptible to pathogen contamination, one Salmonella typhimurium. Based on the advantages offered by bacteriophages so in the present study using bacteriophage applied to chicken meat after contaminated with *Salmonella typhimurium*. The aim of this study was to determine the optimum time of bacteriophages in lysing *Salmonella typhimurium* in chicken meat as well as knowing the amount of reduction in *Salmonella typhimurium*.

MATERIALS AND METHOD

Materials

Samples used are fresh chicken meats, bacteriophages Φ14, *Salmonella typhimurium* , *Salmonella flexnery* as a host. The chemicals used are $CaCl_2$, NaCl, distilled water, alcohol, methylated spirits, PP plastic, aluminum foil , cotton.

Bacteriophage production

Seven ml Nutrient Broth inoculated with 200 μL of bacterial host then incubated for 90 minutes under 37^{0}C. After it was added 40 μL and 250 μL $CaCl_2$ bacteriophages, incubated for four to six hours, then observed the level of turbidity. Then the liquid is centrifuged at 6000 rpm, 5ºC for 10 minutes. The filtrate formed was filtered with a sterile filter of 0.2 μm.

Optimization of *Salmonella typhimurium* infection

75 ml of sterile Nutrient Broth were inoculated with 200μl of pathogenic bacteria and then incubated for 0, 30, 60 and 90 minutes. After that 100 μL $CaCl_2$, 200 μL bacteriophage Φ14 were added. The absorbance was measured every 2 hours for the control (pathogens without infection of bacteriophage Φ 14) and pathogens with infection of bacteriophage Φ 14.

Application of bacteriophage in meat

Fresh meat was sterilized at 121^{0}C for 15 minutes, then meat was homogenized by using stomacher for 15 minutes. Fifteen ml of aliquots were taken and added with 50μl of *Salmonella typhimurium*, incubated at 37ºC for 0,30,60 minutes. One hundreds (100 μl) of 0.3 M of $CaCl_2$ and 500 μl of bacteriophages Φ 14 were further added then incubated at 37º C incubated for 0,2,4,6 days. Aliquots were diluted to a certain dilution. Then grown in SSA media using spread plate method, under 37^{0}C for 24 hours. The number of bacteria were counted in CFU / ml.

Data analysis

The data obtained were analyzed using descriptive analysis method to investigate the addition of a bacteriophage (30, 60, and 90 minutes) to lyse the growth of *Salmonella typhimurium*. The lysis can be observed by comparing the growth of Salmonella with phage infection and without phage infection.

RESULT AND DISCUSSION

Bacteriophage production

Bacteriophages Φ14 were used in this study were obtained from the isolation in previous research (Zein, 2011). Isolation of bacteriophages is intended to obtain mixture of virulent bacteriophages (phage cocktail) capable of inhibiting the growth of pathogenic bacteria. Bacteriophages isolated from the intestinal samples of fresh chicken, ground, salted vegetables, and garbage. Isolation of bacteriophages and host was done with double layer method. From the results showed that one isolate as a host number 14 was successfully isolated from the intestinal samples (U14). Bacteriophage production is done to obtain high concentration of bacteriophage which is enough to be used for application in chicken meat. Production was done by inoculating host (*Shigella flexnery*) in 7 ml NB then incubation was performed at 37^0C for 90 minutes to allow *S. flexnery* grow. Subsequently, it was added $CaCl_2$ and bacteriophages Φ14, and then incubated for 8 hours. The observation was done every half an hour until the lysis mechanism were observed. Lysis occurred when solution become clear, when compared to the control (without addition of bacteriophage). Table 1 show the degree of turbidity (lysis) of the solution.

Table 1. Turbidity of solution (lysis) during 8 hours incubation

Time (min)	Control (without Φ14 infection)	Treated sample (with Φ14 infection)
0	+	+
30	+	+
60	+	+
90	+	+
120	+	+
150	++	+
180	++	+
210	+++	++
240	+++	++
270	+++	++
300	++++	++
330	++++	++
360	++++	++
390	++++	+++
420	+++++	+++
450	+++++	++++
480	+++++	++++

Nb: + very clear; ++ clear; +++ slightly turbid; ++++ turbid; +++++ very turbid

Based on the result, it shows that the solution with bacteriophage Φ14 infection has more clearer than the control solution (without bacteriophage Φ14 infection) during 6 hours incubation. The level of solution clarity dictated the host lysis due to bacteriophage infection. To produce the phage, the liquid was centrifuged and filtered with 0.2 μm sterile filter to obtain lysate for a new stock. The lysis phenomenon can be observed visually as shown in figure 1.

a b

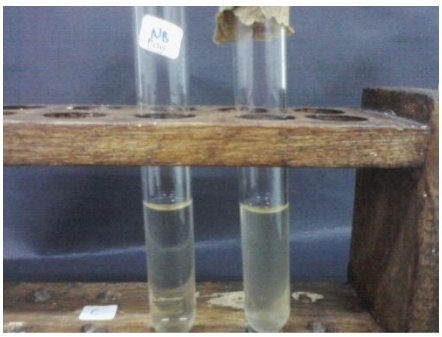

Figure 1.. Bacteriophage production. Solution with Φ14 infection (a); solution without Φ14 infection (b)

To determine the concentration of bacteriophages, double layer method was done (Chow *et al.* , 1988) . 15 ml of media hard agar NA (1.5 %) is poured dish and allowed to solidify. Then 3 ml of soft agar NA (0.7 %) was added 100 μL of 20 hours *Shigella flexnery*, 100 μL of 0.3 M $CaCl_2$, and 100 μL of bacteriophages and then poured on top of the hard agar.

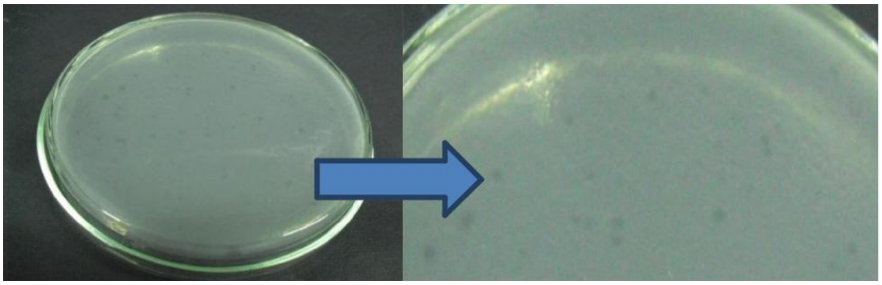

Figure 2. Plaque formation

Double layer was incubated at 37^0C for 6 hours and observed the formation of plaque (clear zone on host bacteria). The formation of plaque is presented in Figure 2. Plaque is formed then calculated by unit PFU / ml (plaque forming units / ml) as many as 8.3×10^3 PFU / ml .

Optimization of *Salmonella thypimurium* lysis

The potency of bacteriophage Φ14 as antimicrobial should be tested against pathogens, *Salmonella typhimurium*. Before the application of bacteriophages Φ14 into chicken meat, it is important to determine the optimum time of bacteriophage infection in inhibiting *Salmonella typhimurium*. The measurement of absorbance values of Salmonella growth show the infection of bacteriophages against *S. typhimurium* (Table 2).

Table 2. Growth of *Salmonella typhimurium*

Time (min)	OD 600 nm	OD 600 nm	OD 600 nm	OD 600 nm
	Control (without phage infection)	With phage infection after 30 min	With phage infection after 60 min	With phage infection after 90 min
0	0,024	0,029	0,0301	0 033
30	0,054	0,049	0,047	0,055
60	0,072	0,079	0,076	0,081
90	0,116	0,093	0,089	0,097
120	0,127	0,117	0,101	0,112
150	0,135	0,121	0,111	0,116
180	0,171	0,154	0,136	0,142
210	0,196	0,068	0,145	0,162
240	0,216	0,102	0,129	0,179
270	0,256	0,147	0,137	0,191

Table 2 shows that the bacteriophage Φ14 is able to inhibit *S. typhimurium*. Inhibition of bacteriophage is shown by the decline of the absorbance of *S. typhimurium* growth. This decrease indicates the lysis of *S. typhimurium*. Lysis of bacteria due to the bacteria receptors is compatible with bacteriophage receptors. This allowed the inserting of genetic material of bacteriophage into the bacteria and use it for bacteriophage multiplication. Bacteriophages can only enter the bacterial cell membrane receptors when the natural bacterial cell is suitable with bacteriophage receptor (Mayer, 2010). The inhibition of bacteriophage Φ14 to *S. typhimurium* is presented in figure 3.

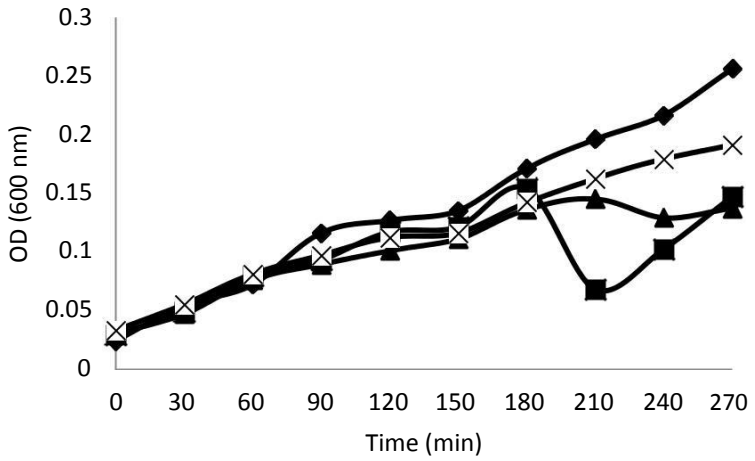

Figure 3. Inhibition of bacteriophage Φ14 to *Salmonella typhimurium*
control (without Φ14 infection)
Φ14 infection after 30 min; Φ14 infection after 60 min
Φ14 infection after 90 min

The variation of phage injection after 30 minutes, 60 minutes, and 90 minutes incubation of *S. typhimurium* is to give the time to grow of *S. typhimurium* which will be the optimum time of bacteriophage Φ14 in lysing *S. typhimurium*. Figure 3 shows that the OD values of control (without phage injection) have continued to rise indicating *S. typhimurium* has grown and multiply. In the ccase of the phage addition after 30 minutes incubation, it shows that OD value continues to rise from minute 0 to minute 180, then declined sharply until 210 minutes. This decrease indicates that there is a lot of *S. typhimurium* lysis of the bacteriophage Φ14 attack. During phage addition after 60 minutes incubation, the OD values have continued to rise and begins to decrease in minute 240, however not so great decrease was observed. At the time of the addition of phage after 90 minutes incubation, the OD value continues to increase but the increase is lower than control. This result show that the addition of phage 30 minutes is the best time for Φ14 to lyse *S. typhimurium* because the OD value was decline significantly, which means that most *S. typhimurium* lysis. According Todar (2008), the lysis of bacteria can occur every 15 minutes up to several days depending on the type of bacteria. From a bacteriophage that infects bacteria it will produce new phages 200-300 with effect become infected bacteria lyse (Ellis, 2007).

APPLICATION OF BACTERIOPHAGE IN CHICKEN MEAT

Bacteriophage Φ14 was applied to chicken meat to investigate the effectiveness of bacteriophage to inhibit or lyse *S. typhimurium*. According to Guenther et al., (2008) bacteriophage is a natural enemy of bacteria, have specific properties invade their host bacteria. Bacteriophage specific targets to eliminate

bacteria without interfering with other bacterial viability. It is desirable from an antimicrobial agent for use in food, because it can maintain the quality of products. In this study, a comparison of the growth of *S. typhimurium* on chicken meat without addition of bacteriophage and the growth of of *S. typhimurium* on chicken meat with the addition of bacteriophages. In this study, the concentration of *S. typhimurium* which is applied in chicken meat was determined before the application of bacteriophages Φ14. Total number of *S. typhimurium* was calculated in SSA media using spread plate method, and the results obtained *S. typhimurium* concentration of 5,7 x 10^4 CFU / ml. The next step of the application, chicken meat was sterilized at 121^0C for 15 minutes, to make chicken meat sterile from contaminant so that only *S. typhimurium* that will grow. Furthermore, chicken meat was homogenized for 120 seconds and the resulting aliquots were transferred to 10 ml centrifuge tube. Aliquots were then inoculated with *S. typhimurium* (5,7 x 10^4 CFU / ml) and incubated for 0, 30, 60 minutes to give the time for *S. typhimurium* growing. Furthermore, the addition of $CaCl_2$ and bacteriophage Φ14 (8,3 x 10^3 PFU / ml) and incubated for 0, 2, 4, 6 days. The next step, to apply the diluted aliquot in media SSA (Salmonella Shigella Agar) using a spread plate method then incubated at 37^0C for 24 hours. Then calculated the number of Salmonella typhimurium that grows in plate. Plating results of *S. typhimurium* in SSA media is presented in Figure 4

Figure 4. Plating of *Salmonella typhimurium* in SSA *media*

Salmonella typhimurium calculation was done at 0 , 2 , 4 , and 6 days incubation. The result of the calculation of *S. typhimurium* during 6 days incubation can be seen in Table 3.

Table 3. Number of *Salmonella typhimurium* during 6 days incubation

Sample	Number of bacteria (CFU/ml)			
	0 day	2 days	4 days	6 days
Without phage infection	$4{,}2\text{x}10^{3}$	$5{,}2\text{x}10^{7}$	$3{,}6\text{x}10^{8}$	$6{,}1\text{x}10^{9}$
With phage infection (0 min)	$3{,}5\text{x}10^{3}$	$4{,}1\text{x}10^{4}$	$7{,}3\text{x}10^{5}$	$5{,}8\text{x}10^{7}$
Without phage infection	$9{,}2\text{x}10^{3}$	$4{,}6\text{x}10^{6}$	$5{,}3\text{x}10^{8}$	$8{,}5\text{x}10^{9}$
With phage infection (30 min)	$8{,}6\text{x}10^{3}$	$5\text{ x}10^{5}$	$5{,}2\text{x}10^{5}$	$9{,}3\text{x}10^{6}$
Without phage infection	$4{,}9\text{x}10^{4}$	$6{,}7\text{x}10^{7}$	$7{,}9\text{x}10^{9}$	$3{,}1\text{x}10^{10}$
With phage infection (60 min)	$3{,}7\text{x}10^{4}$	$4{,}1\text{x}10^{5}$	$5{,}3\text{x}10^{7}$	$5{,}6\text{x}10^{8}$

Based on Table 5 the number of *S. typhimurium* were grown on chicken meat increased after for 6 days incubation, but the increase was reduced by the addition of bacteriophage Φ14. This is because the Φ14 is capable to lyze or kill *S. typhimurium* during incubation. On 2 day incubation, after addition of Φ14 (30 min), the number of *S. typhimurium* fell as much as 1.01 log, whereas the addition of phage (60 min) decreased as much as 2.21 log. Whereas, the addition of phage (0 min) shows the decrease of *S. typhimurium* reached at 3.10 log. This is because most of *S. typhimurium* has been attacked by bacteriophages Φ14. On 4 days incubation, the number of *S. typhimurium* after addition of phage (30 min) the decreased 3.01 log was observed, on the addition of phage 60 minutes the decrease at 2.17 log, whereas the addition of phage 0 minutes the decrease at 2.70. On the 6 days incubation, the number of *S. typhimurium* decreased until 2.96 log, on the addition of phage 60 minutes decrease until 1.75 log, while the addition of phage 0 minutes fell as much as 2.03 log. The lysis of pathogenic bacteria caused by the appropriate receptor of pathogens with the receptor of bacteriophage. This resulted in the suitability of a bacteriophage capable of inserting its genetic material into the bacteria and use it for multiplication. According to Mayer (2010) Bacteriophages can only enter the bacterial cell membrane where the receptor naturally bacterial cells is consistent with the receptor of bacteriophage. In addition, the bacteriophage does not cause a change in the organoleptic (color, odor, flavor, and structure) of the food product, therefore bacteriophages can be used as a controller of harmful pathogenic bacteria in food product (Hagens, 2007). Lysis curve of *S. typhimurium* attacked by bacteriophage Φ14 in chicken meat during incubation is presented in Figure 5.

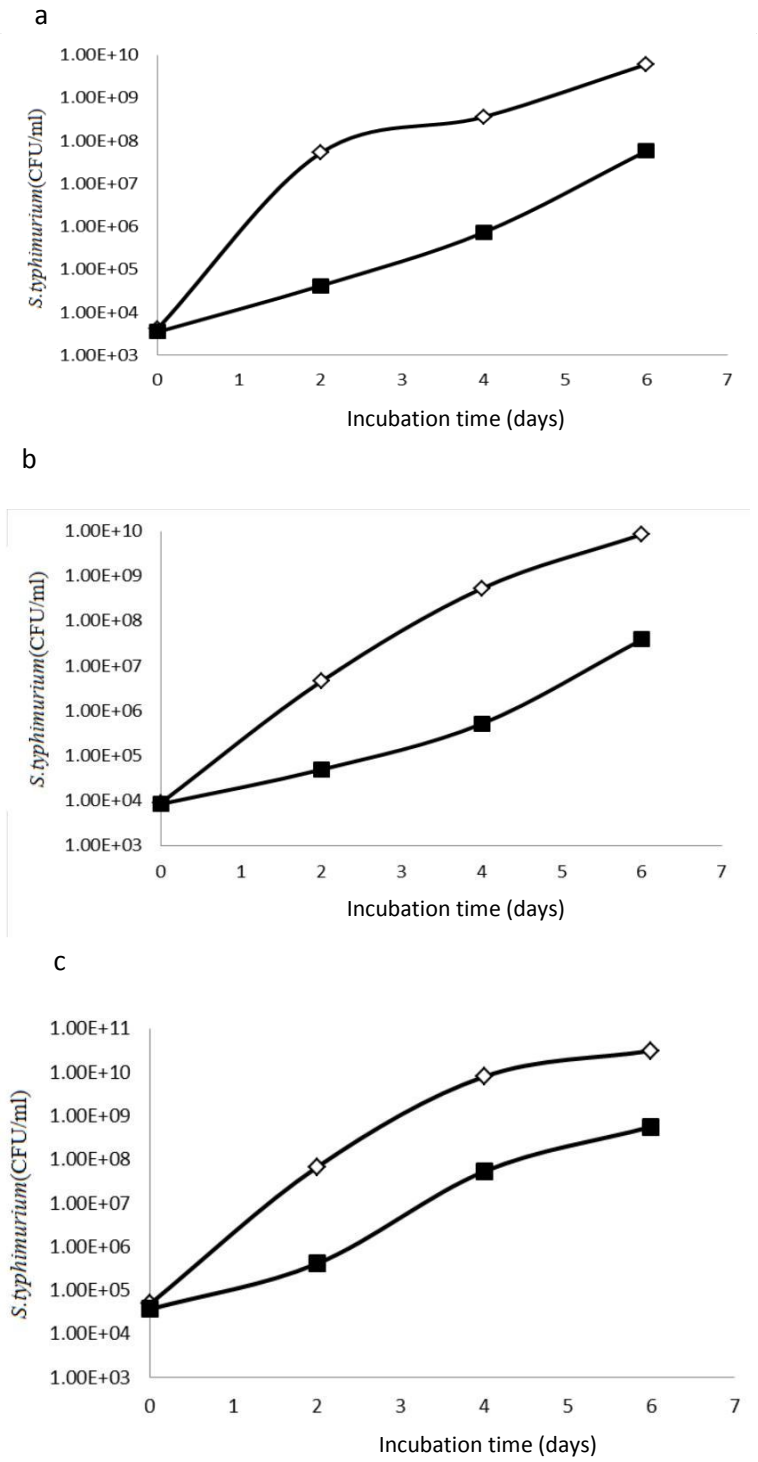

Figure 5. Lysis curve of *S. typhimurium* in chicken meat; phage addition at 0 minute (a); phage addition at 30 minutes (b); phage addition at 60 minutes (c). ◇ Control (without phage addition); ■ (with phage addition)

Based on the pictures it show that the number of *S. typhimurium* in control (without addition of bacteriophage Φ14) always higher than the number in sample with the addition of bacteriophage Φ14.

Meaning that bacteriophage Φ14 capable of inhibiting or reducing the growth of *S. typhimurium* during 6 days of incubation. After 6 days of incubation, the highest reduction of *S. typhimurium* was obatained with phage addition at 30 minutes. While on the 2nd day of incubation, the addition of phage at 0 minutes show the highest reduction of *S. typhimurium*. According to Leverentz *et al*., (2004) a high concentration of bacteriophages are important in reducing the number of bacterial targets. In addition, the addition of bacteriophage also plays an important role in reducing the amount of bacteria during storage. In the study conducted by Guenther, *et al*. (2008) the application of bacteriophage A511 ($3x10^8$ PFU / ml) in milk chocolate that has been contaminated with *Listeria monocytogenes* ($1x10^3$ CFU / ml), with a time addition of bacteriophage at 1 hour, after 6 days of storage (20^0C) the amount of *Listeria monocytogenes* is able to be reduced as much as 6.4 log units.

CONCLUSION

Bacteriophages Φ14 has the potency to be applied as bio-control for *Salmonella typhimurium* in meat product. It has the capacity to reduce the number of S. typhimurium as much as 2.96 log. The most optimum time of inhibition of bacteriophages against *Salmonella typhimurium* was observed at 30 minutes after incubation.

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