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Investigation of Medium Chain Fatty Acid Feed Supplementation for Reducing *Salmonella* Typhimurium Colonization in Turkey Poults

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Abstract

Studies indicate that persistent *Salmonella* colonization occurs in poultry that are infected early in life, leading to both food safety and public health concerns. Development of improved preharvest *Salmonella* management strategies is needed to reduce poultry product contamination. The objective of this study was to evaluate the efficacy of a product containing medium chain fatty acids (MCFA) for reducing early *Salmonella* colonization in turkey poults. Day-of-hatch turkeys were provided a standard starter diet supplemented with MCFA at 0 (negative and positive controls), 1.5, 3, 4.5, or 6 lbs/ton of feed. Positive control and MCFA treated birds were also crop-gavaged with 10^8 colony forming units (CFU) of bioluminescent *Salmonella* Typhimurium. Gastrointestinal tissue samples were collected at 3 days postinoculation for bioluminescence imaging (Meckel's diverticulum to the cloaca) and selective enumeration (cecal contents). Quantification of bioluminescence indicated that the 4.5 and 6 lbs/ton MCFA groups had significantly less colonization than the positive control group ($p = 0.0412$ and $p < 0.0001$, respectively). Similarly, significantly lower numbers (1-$\log_{10}$ CFU/g reduction) of *Salmonella* were observed in the ceca of the 6 lbs/ton MCFA group compared to the positive control group ($p = 0.0153$). These findings indicate that incorporation of MCFA in turkey diets can significantly reduce early *Salmonella* colonization. In addition, this study highlights the utility of bioluminescence imaging as a screening methodology for assessing the efficacy of treatments that may reduce *Salmonella* in poultry.

Keywords: medium chain fatty acids, *Salmonella*, turkey, bioluminescence, food safety

Introduction

In the United States, an estimated 1.2 million human salmonellosis cases occur annually and are predominately associated with the consumption of contaminated food products (Scallan *et al.*, 2011; Jackson *et al.*, 2013). Foodborne illness surveillance has linked poultry products to multiple *Salmonella* outbreaks in humans (Gould *et al.* 2013). Although contamination of poultry meat with *Salmonella* can be attributed to a number of factors, there remains a strong relationship between the gastrointestinal colonization of poultry and product contamination risk (Rasschaert *et al.*, 2008; Evans *et al.*, 2015).

Poultry are susceptible to developing persistent *Salmonella* gastrointestinal colonization if they become infected early in life (Gast and Beard, 1989; Gast and Holt, 1998; Van

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Immerseel *et al.*, 2004b). These birds remain colonized for the duration of the production cycle and provide a reservoir for the continued spread of *Salmonella* throughout the flock (Gast and Beard, 1989; Gast and Holt, 1998; Van Immerseel *et al.*, 2004b). The stress associated with transport to the processing plant can then induce fecal shedding and lead to raw product contamination (Mulder, 1995; Marin and Lainez, 2009; Evans *et al.*, 2015). Therefore, treatments that reduce early *Salmonella* colonization may have an impact on overall flock carriage levels and may help reduce product contamination.

Medium chain fatty acids (MCFA; C6-C12) have recently been investigated as a preharvest treatment for controlling *Salmonella* during poultry production. These molecules are more bactericidal than short chain fatty acids (≤C4) which are routinely used to combat *Salmonella* (Kwon and Ricke, 1998; Nakai and Siebert, 2003; Van Immerseel *et al.*, 2003, 2006; Skrivanova *et al.*, 2006). Interestingly, MCFA also suppress the expression of the invasion genes, *hilA* and *hilD*, in *Salmonella* and decrease intestinal epithelial cell invasion (Van Immerseel *et al.*, 2004a; Kollanoor-Johny *et al.*, 2012). Studies in both layers and broilers show that MCFA can reduce *Salmonella* colonization in the gastrointestinal tract and in other tissues (Van Immerseel *et al.*, 2004a; Kollanoor-Johny *et al.*, 2009, 2012; Upadhyaya *et al.*, 2015). Potentially, preharvest MCFA treatment could have an impact on product contamination by reducing *Salmonella* colonization during production. However, additional studies are needed to further evaluate the efficacy of MCFA for combating *Salmonella* in not only chickens but also in turkeys.

Our laboratory has developed new methods for evaluating *Salmonella* gastrointestinal colonization. By inoculating birds with a bioluminescent *Salmonella* Typhimurium LT2 isolate and imaging with the *In Vivo* Imaging System (IVIS®; PerkinElmer), we can view colonization in the gastrointestinal tract and investigate various treatment strategies. One advantage of the IVIS is the ability to quantify bioluminescence which is directly related to the levels of the target bacteria present (Karsi *et al.*, 2008; Foucault *et al.*, 2009). The objective of this study was to evaluate the efficacy of a product containing a combination of MCFA (C6-C12) for reducing early *Salmonella* colonization in turkey poults.

Materials and Methods

Animals

All animal experiments were approved by the Virginia Tech Institutional Animal Care and Use Committee. Upon arrival, 36 day-of-hatch female turkey poults (Aviagen Turkeys, Inc., Lewisburg, WV) were randomly assigned into six groups and placed into individual isolator cages ($n = 5$–7, with 340 cm^2/bird). To ensure birds were *Salmonella* negative at the start of the study, pooled cecal droppings were collected from each group and tested with the DNAble Molecular Detection Kit for *Salmonella* (DF-026) using Extraction Set 1 (EnviroLogix, Inc., Portland, ME) as described previously (Evans *et al.*, 2015).

Bioluminescent Salmonella *culture and inoculum preparation*

A bioluminescent *Salmonella enterica* serovar Typhimurium LT2 isolate was used for this study. The isolate contains the expression vector pNSTrcD with bioluminescent luciferase cassette (CDABE) and chloramphenicol resistance gene (Seleem *et al.*, 2007a, b; Seleem *et al.*, 2008a, b). The transformed isolate was streaked onto tryptic soy agar (TSA; BD Difco™, Sparks, MD) and incubated overnight at 37°C. For preparation of frozen inoculum stocks, a single bright colony was selected from a TSA plate and used to inoculate 20 mL of tryptic soy broth (TSB; BD Difco) in 50 mL conical tubes (Fisher Scientific, Hampton, NH). Tubes were then placed in a shaking (175 rpm) incubator overnight at 37°C. Cultures were pelleted by centrifugation at 2000 × *g* for 15 min. Pellets were washed twice in sterile phosphate-buffered saline (PBS; Fisher Scientific), pooled, and frozen at −80°C with a final glycerol concentration of 20% v/v. One week later, an aliquot of the frozen stock was thawed and streaked onto TSA and TSA +5% sheep blood (Remel™, San Diego, CA). Plates were incubated overnight at 37°C to confirm purity. A plate count was performed to determine the concentration of *Salmonella* in the prepared stocks. In addition, a minimum inhibitory concentration (MIC) assay (Sensititre™; Thermo Scientific, Waltham, MA) was carried out by the Clinical Bacteriology Lab at the Virginia-Maryland College of Veterinary Medicine. The isolate was found to be resistant to 0.25 μg/mL of ceftiofur (chloramphenicol was not included in the panel). Chloramphenicol resistance was confirmed by growing the isolate overnight at 37°C on TSA plates supplemented with 30 μg/mL chloramphenicol (Fisher Scientific).

Salmonella *challenge*

Before challenge, each group of birds (day-of-hatch) was provided access to 82.5 g of HydroGel™ (ClearH_2O,

Table 1. Diet Composition of the Standard Starter Diet Fed to Turkey Poults

Ingredient	*(%) Composition*
Corn	44.23
Soybean meal (48%)	43.86
Poultry byproduct meal	5.00
Soy oil	0.99
Salt (NaCl)	0.19
Sodium bicarbonate	0.20
DL-Methionine	0.30
Lysine•HCl	0.26
Limestone	1.09
Dicalcium phosphate	3.02
Choline chloride (60%)	0.10
Vitamin/mineral premix[a]	0.75
Calculated composition	
ME (kcal/kg)	2850
Crude protein (%)	28.12
Calcium (%)	1.40
Available P (%)	0.75
Dig Lys (%)	1.62
Dig Met (%)	0.66
Dig Thr (%)	0.97
Dig Met+Cys (%)	1.05

[a]Provided the following per kg vitamin/mineral premix: cobalt 34 ppm; copper 540 ppm; iodine 134 ppm; iron 6750 ppm; manganese 8580 ppm; zinc 6500 ppm; vitamin A 881,848 IU/kg; vitamin D3 295,419 ICU/kg; vitamin E 220 IU/kg; vitamin B12 0.9 mg/kg; menadione 154 mg/kg; riboflavin 551 mg/kg; D-pantothenic acid 811 mg/kg; niacin 2646 mg/kg; and choline 51,030 mg/kg.

P, Phosphorus; Lys, lysine; Met, methionine; Thr, threonine; Met+Cys, methionine and cystine.

Westbrook, ME) mixed with 18 g of standard starter diet (Table 1) containing a MCFA product (Nuscience Group, Ghent, Belgium) at 0 (negative and positive controls), 1.5, 3, 4.5, or 6 lbs/ton of feed (0, 0.75, 1.5, 2.25, 3 g/kg) for 1 h. Birds were then fasted for 2 h during which time a frozen stock of bioluminescent *Salmonella* was thawed on ice and diluted in sterile PBS. Birds were either crop-gavaged with 1 mL of 1×10^8 colony forming units (CFU)/mL of bioluminescent *Salmonella* (positive control and MCFA treated groups) or 1 mL of sterile PBS (negative control). Birds were then provided a standard starter feed (Table 1) containing the corresponding MCFA treatment as described above and water *ad libitum* for the duration of the study.

Imaging of gastrointestinal samples

Turkeys were humanely euthanized with CO_2 at 3 days postinoculation (PI) for bioluminescence imaging. Gastrointestinal tissue samples were collected from Meckel's diverticulum to the cloaca. Samples were placed into containers premoistened with sterile PBS to prevent drying and kept warm in a 37°C incubator. The samples were opened longitudinally just before imaging and placed onto a black low-background Lexan™ sheet (PerkinElmer, Waltham, MA). An IVIS SpectrumCT (PerkinElmer) was used to quantify bioluminescence. After a 1-min incubation on the imaging stage at 37°C, images were taken with a specimen height of 1.5 cm. Exposure and binning were set accordingly to keep the bioluminescence values within 600–60,000 counts (relative luminescence units) as specified by the manufacturer. Regions of interest (ROI) were overlaid (22 × 22 cm) on each image, and total flux (photons/s) was recorded for each sample.

Salmonella *enumeration*

Salmonella levels in the ceca were enumerated for each bird using a modified plate count procedure and then compared by treatment (Evans *et al.*, 2015). Briefly, cecal contents were collected and weighed after imaging. Samples were diluted 1:10 w/v in sterile buffered peptone water (BPW; BD Difco) and then serially diluted (1:10) in PBS. From each dilution, 10 μL drops were plated in quadruplicate onto TSA plates supplemented with 30 μg/mL chloramphenicol and 0.25 μg/mL ceftiofur (Fisher Scientific). Plates were incubated for 10 h (colony diameter: 1–2 mm) at 41°C

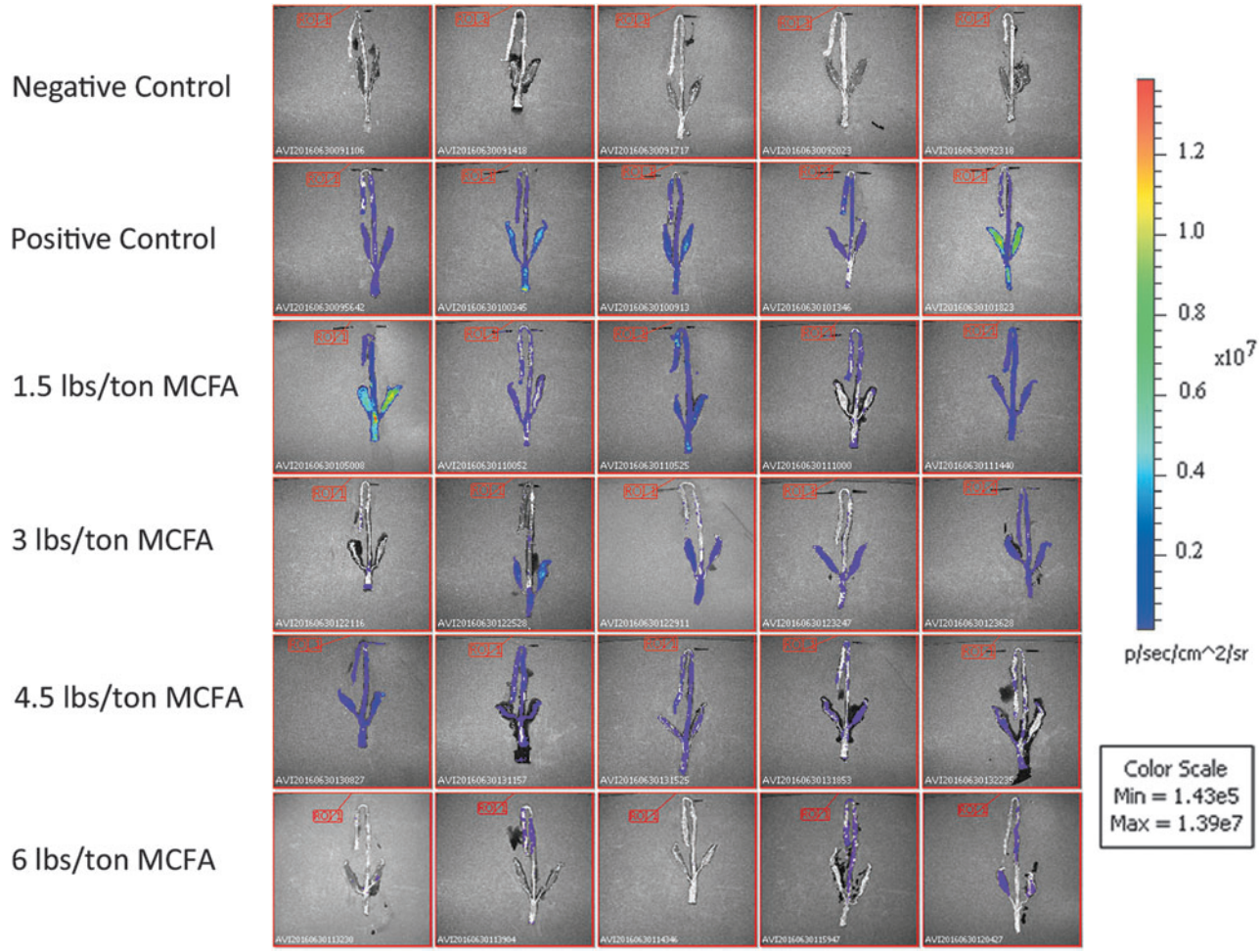

FIG. 1. Images of gastrointestinal samples (Meckel's diverticulum to cloaca) with pseudocolor overlays representing the amount of light produced by bioluminescent *Salmonella* Typhimurium (3 days PI). The color bar indicates the amount of bioluminescence (surface radiance: p/s/cm^2/sr) detected during imaging. ROI were overlaid on each image to quantify the total flux (photons/s) for each sample. MCFA, medium chain fatty acids; PI, postinoculation; ROI, regions of interest.

to approximate turkey body temperature (40.6–41.5°C). Following incubation, all plates were inspected with the IVIS SpectrumCT to confirm that only bioluminescent colonies were present, and CFU/g were calculated for each sample.

Statistical analysis

Analysis of both bioluminescence and enumeration data was conducted using JMP Pro 11 (SAS Institute, Inc., Cary, NC). Bioluminescence (photons/s) and enumeration (CFU/g) data were reported as the $\log_{10}$ mean for each treatment ± standard error of the $\log_{10}$ mean. Data were analyzed as a one-way analysis of variance (ANOVA). When the model was significant ($p \leq 0.05$), a *post-hoc* analysis (Least Squares [LS] Means Student's *t*-test) was performed.

Results

Bioluminescent Salmonella *imaging*

Imaging of bioluminescent *Salmonella* was conducted on intestinal tissues from Meckel's diverticulum to the cloaca at 3 days PI. A pseudocolor overlay representing the bioluminescent light produced by *Salmonella* colonizing the intestinal tract is shown in Figure 1. Visually, bioluminescence appeared to be lowest in the negative control group and highest in the positive control group. Light levels also appeared to be progressively reduced as the MCFA concentration increased. The highest levels of bioluminescence were typically observed in the ceca of infected birds, but bioluminescence was also present throughout the gastrointestinal samples. Total flux (photons/s) was quantified for each sample ROI and compared by treatment group (Fig. 2). The ANOVA analysis was significant ($p < 0.0001$), and the *post-hoc* LS Means Student's *t*-test indicated that both the 4.5 lbs/ton (7.9 $\log_{10}$ photons/s ± 0.10) and 6 lbs/ton (7.5 $\log_{10}$ photons/s ± 0.11) MCFA groups had significantly less ($p = 0.0412$ and $p < 0.0001$, respectively) bioluminescence than the positive control group (8.4 $\log_{10}$ photons/s ± 0.15). The 6 lbs/ton MCFA group was also significantly lower ($p = 0.0276$) than the 4 lbs/ton MCFA group. There were no significant differences among the 1.5, 3, and 4 lbs/ton MCFA groups.

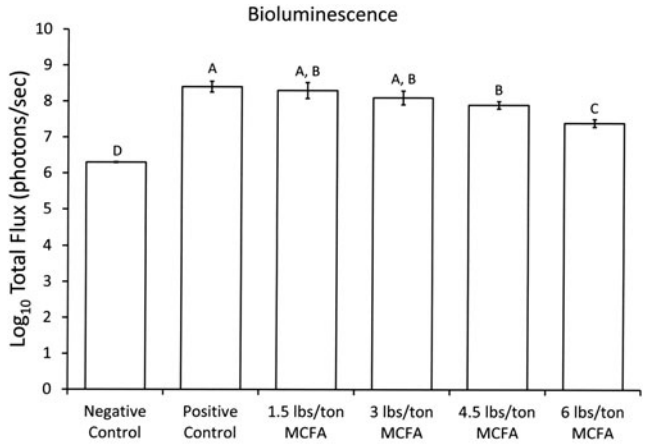

FIG. 2. *Salmonella* bioluminescence in gastrointestinal samples (Meckel's diverticulum to cloaca) from control and MCFA treated poults (3 days PI). Error bars represent standard error of the $\log_{10}$ mean for each treatment group. Treatments not connected by the same letter are significantly different ($p \leq 0.05$). MCFA, medium chain fatty acids; PI, postinoculation.

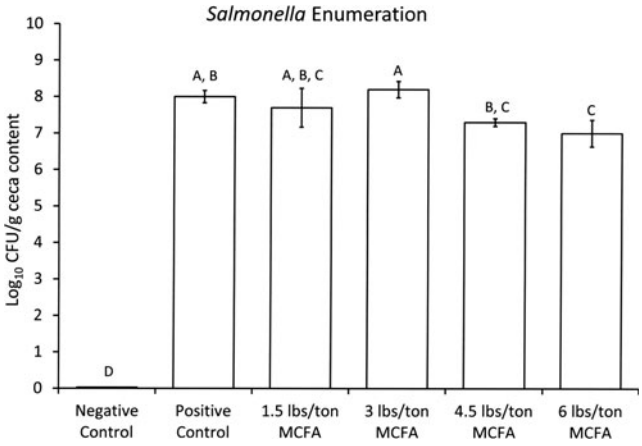

FIG. 3. *Salmonella* enumeration of cecal contents from control and MCFA treated poults (3 days PI). Error bars represent standard error of the $\log_{10}$ mean for each treatment group. Treatments not connected by the same letter are significantly different ($p \leq 0.05$). MCFA, medium chain fatty acids; PI, postinoculation.

Salmonella *enumeration*

Samples collected from the ceca were quantified by plate count (Fig. 3). As expected, no *Salmonella* was recovered from birds in the negative control group. However, *Salmonella* was recovered from all other birds and ranged between 6.0 and 9.3 $\log_{10}$ CFU/g. The ANOVA analysis was significant ($p < 0.0001$), and the *post-hoc* test (LS Means Student's *t*-test) indicated that the 6 lbs/ton MCFA (7.0 $\log_{10}$ CFU/g ± 0.37) group had a significant reduction ($p = 0.0153$) in *Salmonella* (1-$\log_{10}$ CFU/g reduction) compared to the positive control group (8.0 $\log_{10}$ CFU/g ± 0.17).

Discussion

Poultry are susceptible to *Salmonella* infections during the first few days of life and begin developing greater resistance thereafter (Gast and Beard, 1989). Birds infected immediately after hatch have more persistent colonization and higher levels of fecal shedding than birds infected at 1 week post-hatch (Gast and Beard, 1989; Van Immerseel *et al.*, 2004b). Preharvest management strategies that effectively decrease early carriage levels may reduce the spread of *Salmonella* within a flock (Gast and Beard, 1989). Presumably, this would also help reduce product contamination. Therefore, the objective of this study was to evaluate the efficacy of a MCFA product for reducing early *Salmonella* colonization in turkey poults.

A novel bioluminescent *Salmonella* infection model was used to assess MCFA efficacy. One benefit of this model is the ability to screen treatments by comparing both visual and quantitative changes. The images from the gastrointestinal samples suggest that *Salmonella* colonization was reduced as the MCFA concentration increased. Quantification of bioluminescence (photons/s) revealed a significant decrease for both the 4.5 and 6 lbs/ton MCFA groups compared to the positive control group. In addition, the 6 lbs/ton MCFA group

had significantly lower bioluminescence than the 4.5 lbs/ton MCFA group, but there were no differences among the 1.5, 3, and 4.5 lbs/ton MCFA groups. These data indicate that there was a dose-dependent decrease in *Salmonella* (Meckel's diverticulum to the cloaca) resulting from MCFA supplementation. Furthermore, when *Salmonella* was enumerated in the ceca, there was a significant decrease in *Salmonella* numbers for the 6 lbs/ton MCFA group (1-$\log_{10}$ CFU/g reduction) compared to the positive control group. This study shows that MCFA can reduce early *Salmonella* colonization in turkeys and highlights the utility of bioluminescence imaging for evaluating treatment efficacy.

Several studies have evaluated the effect of MCFA on *Salmonella* colonization in poultry. Broilers fed diets containing caprylic acid (C8) at 0.7% (14 lbs/ton) and 1% (20 lbs/ton) had reduced *Salmonella* numbers in the gastrointestinal tract, liver, and spleen at 5–10 days PI (Kollanoor-Johny *et al.*, 2009, 2012). Similarly, layers supplemented with caproic acid (C6) at 3 g/kg of feed (6 lbs/ton) had lower *Salmonella* numbers in the ceca, liver, and spleen at 3 days PI (Van Immerseel *et al.*, 2004a). In this study, we showed that turkeys provided a combination of MCFA (C6-C12) at 6 lbs/ton of feed had reduced *Salmonella* colonization in the gastrointestinal tract at 3 days PI. All of the above treatments resulted in a 1-$\log_{10}$ CFU/g, or greater, reduction of *Salmonella* in the ceca. Collectively, these results indicate that MCFA decrease *Salmonella* colonization in poultry. Feed inclusion rates as low as 6 lbs/ton appear to be effective and may further enhance the practicality of using such products in commercial poultry production.

Conclusion

For this study a bioluminescent *Salmonella* infection model was used for *in vivo* screening of a MCFA product in turkeys. Early colonization was evaluated because previous studies have indicated that treatment during this time may be particularly effective for controlling *Salmonella* within a flock. Birds were infected with bioluminescent *Salmonella* Typhimurium on day-of-hatch and provided feed containing MCFA ranging from 0 to 6 lbs/ton. Significant decreases in both bioluminescence and *Salmonella* numbers (1-log10 CFU/g reduction) were detected in the 6 lbs/ton MCFA treated poults at 3 days PI. This was the first study to evaluate the effect of MCFA on *Salmonella* in turkeys and these results indicate that MCFA supplementation can reduce early colonization. Further studies are needed to determine what impact MCFA treatments have on *Salmonella* colonization throughout the production cycle.

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Disclosure Statement

The MCFA product used in these studies is distributed in the United States by PMI Nutritional Additives™. No other competing financial interests exist.

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