



Research Note: Longitudinal fecal shedding patterns and characterization of *Salmonella enterica* and *mcr*-positive *Escherichia coli* in meat-type ducks raised in an open-house system

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ABSTRACT This longitudinal study aimed to determine the fecal shedding pattern and characterize *Salmonella enterica* and *mcr*-positive *Escherichia coli* from meat-type ducks raised in an open-house system in Thailand. Fecal samples (n = 1,475) were collected from ducks over a 6-month period. Overall, the detection rate of *S. enterica* was 5.4% and the highest fecal shedding rate was noted in 4-day-old ducklings (28.8%). Then, *S. enterica* shedding decreased to 10, 8, 4.7, and 0.7% when ducks reached the ages of 10 d, 17 d, 3 wk, and 4 wk, respectively. Seventy-nine isolates were recovered and *Salmonella* Amsterdam was the predominant serovar (79.7%). With respect to colistin-resistant *E. coli*, *mcr*-positive *E. coli* (colistin MICs = 8–16 µg/mL) was noted in ducks at the ages of 16 wk (6.0%) and 24 wk (18.7%). *mcr-1* was the most common (75.7%), followed by *mcr-3* (13.5%), and *mcr-1* and *mcr-3* co-carriage

(10.8%). Most *S. enterica* isolates were susceptible to antibiotics and multidrug resistant (MDR) was found in only a single isolate. However, as many as 89.2% of *mcr*-positive *E. coli* were defined as MDR. Almost all *S. enterica* isolates (97.5–100%) carried several virulence genes involving in invasion, intracellular survival, and iron metabolism. Pulsed Field Gel Electrophoresis revealed that several *mcr*-positive *E. coli* isolates were clonally unrelated. Conjugative transfer of *mcr-1*, *mcr-3* as well as co-transfer of *mcr-1* and *mcr-3* was observed with the frequencies ranging from 10⁻⁸ to 10⁻³. All *mcr-1* resided on IncI2, while *mcr-3* was associated with IncF and IncX4 plasmids. Our study provides the evidence of fecal shedding pattern of *S. enterica* and *mcr*-positive *E. coli* from meat-type ducks, highlighting the importance of duck farming in the dissemination of pathogenic bacteria that are potentially hazardous to human.

Key words: *S. enterica*, *mcr*, colistin, *E. coli*, duck

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INTRODUCTION

Salmonella enterica (*S. enterica*) is a worldwide zoonotic pathogen that possesses a major public health concern due to its ability to disseminate between animal and human. It is mainly found in intestinal tracts of food-producing animals, including poultry, and transmitted to human by direct contact or consumption of contaminated food. Human salmonellosis is a type of gastroenteritis which is usually self-limiting and patients may recover without antibiotic treatments. However,

invasive infections with bacteremia and other extra-intestinal infections may occur, especially in the risk groups such as children and immunocompromised patients (Eng et al., 2015).

Recently, reports on the colistin-resistant *Escherichia coli* (*E. coli*) mediated by mobile colistin resistance (*mcr*) determinant is increasing rapidly. Food-producing animals, particularly poultry, are regarded as an important reservoir for *mcr* (Nang et al., 2019). Several types of *mcr* (*mcr-1* to *mcr-10*) have been discovered however, the most frequently detected gene is *mcr-1* and, to a lesser extent *mcr-3*, which are now being found in a variety of bacterial species among humans, animals, and environments.

Raising poultry in an open-house system is common in many developing regions, including Thailand and, in several cases, it is the primary source of family income.

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However, this represents a great concern in public health issues, possibly due to the poor biosecurity measures in animal handling and farm management. Direct or indirect contact with poultry could be hazardous to human because live poultry usually appear healthy despite shedding bacteria intermittently. *S. enterica* and *mcr*-positive *E. coli* are among the highest priority for public health concern. Several cases of human salmonellosis have been associated with food-producing animals, including ducks, of which the case fatalities were noted. In Thailand, human salmonellosis, both gastroenteritis and invasive infections, occurred in patients at all ages. The presence of *mcr*-positive *E. coli* in clinical specimen of Thai patients has been observed, although data on the disease severity or case fatality were not available.

Duck meat is flavorsome and contains a high amount of amino acids as well as polyunsaturated fatty acids but a low content of fat. Accordingly, the demand of duck meat is increasing and duck farming has been growing in many countries. Several studies on fecal shedding of *S. enterica* in ducks have been carried out and the results varied greatly. A recent study in central Thailand involving 694 duck fecal samples revealed that the prevalence of *S. enterica* was 12.2% (Sinwat et al., 2021). In contrast, data on the *mcr*-positive *E. coli* in ducks are limited. In this study, the fecal shedding pattern of *S. enterica* and *mcr*-positive *E. coli* in meat-type ducks, raised in an open-house system, was investigated over a prolonged period in field conditions. Phenotypic and genotypic characterization, including antibiotic-resistant pattern, virulence gene profiles, transferability of *mcr* and genetic relatedness of bacterial isolates were also carried out.

MATERIALS AND METHODS

Study Setting and Sampling

The experiment was conducted according to the animal use protocol number 2031019, which was approved by Chulalongkorn University Animal Care and Use Committee. This prospective longitudinal study on fecal shedding pattern of *S. enterica* and *mcr*-positive colistin-resistant *E. coli* from meat-type ducks (Muscovy duck) was carried out from August 2020 to January 2021 in Phitsanulok province, Lower Northern Thailand, where open poultry housing system is concentrated. The studied flock comprised approximately 1,500 birds, obtaining from the same hatchery, and raised in an open-house system with fencing. Rice straws were used as bedding materials. Ducks were housed with a small concrete water trough to freely provide an open water source for drinking. Biosecurity measures have not strictly been implemented. Ducks were fed once in the morning with standard commercial feeds without addition of any antibiotic and were managed the same way throughout the study period.

This study was conducted under normal farming conditions. Fresh feces from ducks were collected at the ages of 4 d, 10 d, 17 d, 3 wk, 4 wk, 5 wk, 7 wk, 11 wk,

16 wk and 24 wk, using sterile spoons and containers. When sampling, care was taken to avoid feces that had contacted dirt or soil. All fecal samples ($n = 1,475$) were placed in individual sterile containers and transported on ice to the laboratory. Bacterial isolations were performed within 3 h.

Isolation of *S. enterica* and Serovar Identification

One gram of each fecal sample was enriched in 9 mL buffered peptone water (BPW) and incubated overnight at 37°C. Then, 0.1 mL and 1 mL of the incubated BPW were transferred to Rappaport-Vassiliadis Soya peptone (RVS) broth and Tetrathionate (TT) broth (Oxoid Ltd, Basingstoke, UK), respectively, and incubated at 42°C (37°C for TT broth) for 24 h. The incubated RVS and TT broth were then streaked onto Xylose Lysine Deoxycholate agar and incubated overnight at 37°C. Suspect *S. enterica* colonies (red color with a black center) were identified by API-20E (bioMérieux SA, Marcy-l'Etoile, France).

S. enterica isolates were sent for serovar identification at the Center for Antimicrobial Resistance Monitoring in Foodborne Pathogens (in cooperation with WHO), Faculty of Veterinary Science, Chulalongkorn University, Thailand. One colony of each serovar in each positive sample was collected for further analysis.

Isolation of *mcr*-Positive *E. coli*

Fecal samples were enriched in 9 mL Enterobacteria Enrichment broth (Oxoid) at 37°C for 24 h. Subsequently, the culture was plated on Eosin Methylene Blue agar supplemented with 1 µg/mL colistin and incubated overnight at 37°C. Putative *E. coli* colonies (1 isolate per sample) were selected and streaked on Tryptic Soy Agar (TSA) for further studies. Bacterial identification was performed by API 20E. Individual colony was suspended in 300 µL sterile water and used as template. Screening for *mcr-1-mcr-9* was performed by multiplex PCR using primers and conditions as previously described (Lescat et al., 2018; Borowiak et al., 2020). Selected positive amplicons were purified and sent to a commercial facility for sequencing (Macrogen Inc., Korea).

Susceptibility Test

All *S. enterica* and *E. coli* isolates were tested for susceptibility to antibiotics by disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI). *E. coli* DMST4212, obtaining from the National Institute of Health, Thailand, was used as a control strain. Isolates showing intermediate results were considered as susceptible. An isolate was defined as multidrug resistance (MDR) if it was resistant to at least three or more antibiotic classes.

Colistin minimum inhibitory concentrations (MICs) were determined by broth microdilution method and the results were interpreted according to CLSI (resistant, $\geq 4 \mu\text{g/mL}$).

Screening for *Salmonella* Virulence Gene

All *S. enterica* isolates were screened for 13 virulence genes (*invA*, *sipB*, *prgH*, *spaN*, *orgA*, *sopB*, *sifA*, *pefA*, *sitC*, *spvB*, *spiA*, *pagC*, *msgA*) by multiplex PCR using primers and conditions as previously described (Skyberg et al., 2006). *Salmonella* Typhimurium DMST562 and *Salmonella* Enteritidis DMST17368 were used as positive controls.

Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) was conducted to investigate genetic relationship among *mcr*-positive *E. coli* isolates. Chromosomal DNA in agarose plugs was prepared and digested with *Xba*I. Plugs were then subjected to PFGE analysis in 1% agarose gel (Pulsed Field Certified agarose; Bio-Rad Laboratories, CA, USA) and 0.5X Tris-borate-EDTA buffer using a CHEF Mapper XA System (Bio-Rad Laboratories, CA, USA). The gels were run at 6.0 V/cm with an angle of 120 at 14° C for 20 h. PFGE profiles were visually analyzed. Isolates with indistinguishable banding patterns were considered to represent the same strain.

Transferability of *mcr-1* and *mcr-3*

Conjugation experiments were carried out by broth mating method using sodium azide-resistant *E. coli* J53 as a recipient. Cultures of donor and recipient cells were mixed and incubated at 37°C for 24 h without shaking. Transconjugants were selected on TSA supplemented with sodium azide (150 $\mu\text{g/mL}$) and colistin (2 $\mu\text{g/mL}$). Conjugation frequency was expressed as the number of transconjugants divided by the number of donor cells. Transfer of *mcr-1* and *mcr-3* was confirmed by PCR. Plasmid incompatibility groups were determined by PCR-based replicon typing (PBRT).

RESULTS AND DISCUSSION

Persistence of pathogenic bacteria, including *S. enterica* and *E. coli*, in fecal deposits of food-producing animals is a critical factor in facilitating their spread in the environment since animal manure are frequently used as fertilizers in agriculture practices. This is of concern for public health particularly *S. enterica*, which can greatly multiply in fecal materials outside a host. This concern is expanded to colistin-resistant *E. coli* because colistin is one of the last few antibiotics for the treatment of carbapenem-resistant Enterobacterales.

Our study revealed that 5.4% of fecal samples from ducks were positive for *S. enterica* (Table 1). The highest prevalence of *S. enterica* was noted in 4-day-old

ducklings (28.8%), which is probably due to the vertical transmission from breeder flock. As the ducks grew, *S. enterica* shedding decreased to 10, 8, 4.7, and 0.7% when ducks reached the ages of 10 d, 17 d, 3 wk and 4 wk, respectively. This shedding pattern of *S. enterica* in duck populations was similar to the previous study in Taiwan (Yu et al., 2008). As opposed to our study, a recent report on *S. enterica* prevalence in feces of meat-type ducks raising in open-house systems in central Thailand demonstrated a significantly higher rate of *S. enterica* shedding in older ducks (40 d- and 70 day-old) compared with that in ducklings (1-day-old) in one of the 3 studied farms (Sinwat et al., 2021). The differences in *S. enterica* shedding pattern could be attributed to the duck feeds. Only commercial feeds were used in our study whereas, in the study by Sinwat et al. (2021), ducks in that particular farm were fed with a home-mixed feed formulation, which could be contaminated with a higher prevalence of *S. enterica* compared with commercial feed.

Various *Salmonella* serovars have been reported to cause salmonellosis. The major serovars responsible for human infections include *S. Enteritidis* and *S. Typhimurium* (Eng et al., 2015). In our study, 9 different serovars were observed and interestingly, the dominant serovar was *Salmonella* Amsterdam (79.7%, 63/79) (Table 1). This result is in contrast with the recent study in a duck farm in Thailand which revealed that serovars Altona and Orion were the most frequently detected (Sinwat et al., 2021). To date, *S. Amsterdam* is a very rare serovar and its role in human salmonellosis remains unclear. However, other serovars, such as Agona, Krefeld, Stanley, Kentucky, and Typhimurium have been associated with human infections in Thai patients.

Presently, an increasing prevalence of antibiotic-resistant *S. enterica*, including those with MDR phenotype, recovered from ducks and duck-related products has been observed. Recently, it was shown that 23.2% of *S. enterica* from duck feces in Thailand were defined as MDR (Sinwat et al., 2021). In contrast, our susceptibility data showed that most *S. enterica* isolates were susceptible to antibiotics tested. The highest resistant rate was observed only 8.9% for cefazolin. All isolates were susceptible to chloramphenicol and trimethoprim/sulfamethoxazole and a low resistant rate was observed for ampicillin (7.6%). These 3 antibiotics are the traditional first-line drugs for treatment of human salmonellosis (Eng et al., 2015). Low resistant rates were also observed for cefuroxime (6.3%), doxycycline (3.8%), gentamicin (2.5%), cefotaxime (1.3%), and nitrofurantoin (1.3%). All isolates were susceptible to amoxicillin/clavulanate, imipenem and ciprofloxacin. MDR was found in only 1 isolate. All isolates were susceptible to colistin (MICs = 0.5–2 $\mu\text{g/mL}$).

All *S. enterica* isolates were screened for 13 virulence genes that are involved in adhesion (*pefA*), iron metabolism (*sitC*), invasion into macrophage and epithelial cells (*invA*, *sipB*, *prgH*, *sifA*, *spaN*, *orgA*, *sopB*), intracellular survival and replication (*spiA*, *pagC*, *msgA*) and macrophage apoptosis (*spvB*). All 79 isolates

Table 1. Fecal shedding pattern of *S. enterica* and *mcr*-positive *E. coli* in meat-type ducks.

| Age (d or w) | No. of samples | No. of <i>S. enterica</i> isolate (%) | Serovars | No. of <i>mcr</i> -positive <i>E. coli</i> (%) | <i>mcr</i> Variants |
|--------------|----------------|---------------------------------------|--|--|---|
| 4 d | 125 | 36 (28.8) | Amsterdam (n = 33) Stanley (n = 1) Stockholm (n = 1) Serogroup C (n = 1) | 0 | – |
| 10 d | 150 | 15 (10.0) | Amsterdam (n = 13) Stanley (n = 1) Agona (n = 1) | 0 | – |
| 17 d | 150 | 12 (8.0) | Amsterdam (n = 7) Krefeld (n = 2) Agona (n = 1) Typhimurium (n = 1) Bovismorbificans (n = 1) | 0 | – |
| 3 w | 150 | 7 (4.7) | Amsterdam (n = 6) Krefeld (n = 1) | 0 | – |
| 4 w | 150 | 1 (0.7) | Agona (n = 1) | 0 | – |
| 5 w | 150 | 3 (2.0) | Amsterdam (n = 2) Agona (n = 1) | 0 | – |
| 7 w | 150 | 2 (1.3) | Amsterdam (n = 2) | 0 | – |
| 11 w | 150 | 0 | – | 0 | – |
| 16 w | 150 | 0 | – | 9 (6.0) | <i>mcr-1</i> (n = 9) |
| 24 w | 150 | 3 (2.0) | Stockholm (n = 1) Kentucky (n = 1) Newport (n = 1) | 28 (18.7) | <i>mcr-1</i> (n = 19) <i>mcr-3</i> (n = 5) |
| Total | 1,475 | 79 (5.4) | Amsterdam (n = 63) Agona (n = 4) Krefeld (n = 3) Stanley (n = 2) Stockholm (n = 2) Bovismorbificans (n = 1) Kentucky (n = 1) Newport (n = 1) Typhimurium (n = 1) Serogroup C (n = 1) ^a | 37 (2.5) | <i>mcr-1</i> + <i>mcr-3</i> (n = 4) <i>mcr-1</i> (n = 28) <i>mcr-3</i> (n = 5) <i>mcr-1</i> + <i>mcr-3</i> (n = 4) |

^aSerovar identification was untypeable but agglutination with *Salmonella* antiserum revealed that this isolate belonged to serogroup C.

possessed *sipB*, *prgH*, *spaN*, *orgA*, *spiA* *pagC*, *msgA*, and *sitC* while *invA* and *sopB* were detected in 77 isolates each (97.5%). None of isolates were positive for *pefA* and *spvB*. With respect to *S. Amsterdam*, the most common serovar, *sifA* was entirely absent. Specifically, SifA is an effector protein which is required to maintain stability of *Salmonella*-containing vacuoles (Wang et al., 2020). Lacking SifA has been shown to cause a replication defect in macrophages. These results suggest that *S. Amsterdam* from meat-type ducks may be less pathogenic, consistent with the previous study showing that *S. Amsterdam* failed to cause clinical symptoms in naturally occurring infection in adult rats (Seps et al., 1987).

Detection of *mcr*-positive colistin-resistant *E. coli* from livestock farming is rising rapidly, therefore, farmers are at high risk of infection or colonization with *mcr*-carrying bacteria. The study in duck farms in several provinces in China revealed that 24.6% of duck feces yielded *mcr-1*-positive *E. coli*, however, data on duck ages were not available (Zhang et al., 2018). Our study demonstrated that 2.5% of fecal samples from ducks yielded *mcr*-positive *E. coli* (Table 1). However, shedding of *mcr*-positive *E. coli* was not observed in ducks at 4 d to 11 wk of ages. It was firstly detected in ducks at 16-wk old (6.0%) and the highest prevalence (18.7%) was found when ducks were at the age of 24 wk. The

explanation for *mcr*-positive *E. coli* shedding in adult ducks, but not in ducklings, is not completely understood. *mcr-1* was the most common (75.7%, 28/37), followed by *mcr-3* (13.5%, 5/37). Four isolates (10.8%, 4/37) simultaneously carried *mcr-1* and *mcr-3*. No other *mcr* variants were detected.

All 37 *mcr*-positive *E. coli* were resistant to colistin with MICs of 4 – > 16 $\mu\text{g}/\text{mL}$. Most isolates possessed colistin MIC of 8 $\mu\text{g}/\text{mL}$ (n = 18), followed by 16 $\mu\text{g}/\text{mL}$ (n = 13) and 4 $\mu\text{g}/\text{mL}$ (n = 6). Almost all or all *mcr*-positive *E. coli* isolates were resistant to ampicillin (100%), cefazolin (100%), chloramphenicol (81.1%), and doxycycline (89.2%). Lower resistant rates were observed for trimethoprim/sulfamethoxazole (43.2%), amoxicillin/clavulanate (37.8%), and ciprofloxacin (27.2%). Few isolates were resistant to gentamicin (8.1%), cefuroxime (5.4%), cefotaxime (5.4%), imipenem (2.7%), and nitrofurantoin (5.4%). As many as 89.2% (33/37) of isolates were defined as MDR.

All 37 *mcr*-positive *E. coli* isolates were investigated for their genetic relationship by PFGE. Representatives of PFGE profiles are presented in Figure 1. Identical profiles were observed among isolates carrying *mcr-1* (Lanes 2, 3 & 5 and Lanes 6, 10 & 12) and *mcr-1* and *mcr-3* co-carriage (Lanes 18–20). The rest of isolates including 5 *mcr-3*-carrying isolates showed different DNA profiles (Lanes 13–17). In these cases, horizontal

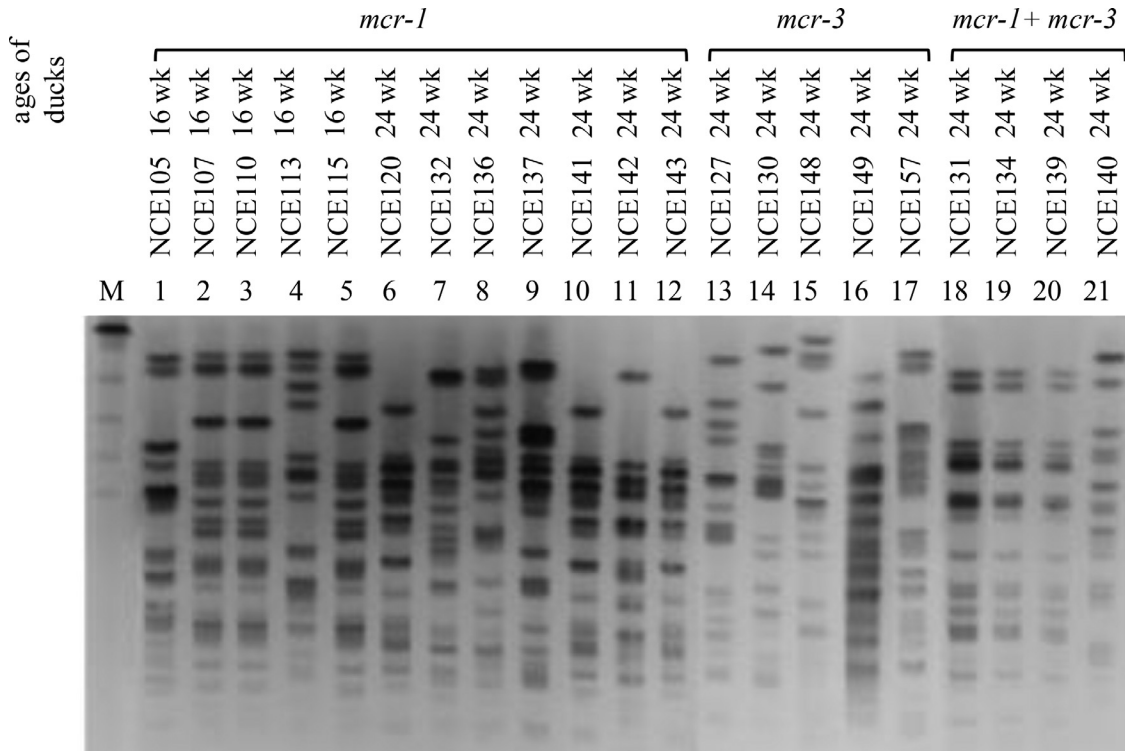


Figure 1. Representative DNA macrorestriction profiles of *mcr*-positive *E. coli* from fecal samples of meat-typed duck. Chromosomal DNA was prepared, digested with *Xba*I and subjected to pulsed-field gel electrophoresis (PFGE). *Saccharomyces cerevisiae* chromosomal DNA (Bio-Rad Laboratories) was used as a molecular size standard. PFGE profiles were visually analyzed. Isolates with indistinguishable banding patterns were considered to represent the same strain.

gene transfer may play a role in the spread of these organisms.

Broth-mating experiments were performed to examine the transferability of *mcr*. *E. coli* isolates carrying *mcr-3* ($n = 5$), *mcr-1* and *mcr-3* ($n = 4$) as well as randomly selected *mcr-1*-positive isolates ($n = 9$) were used as donors. All 18 *mcr*-positive isolates were able to transfer *mcr* to the recipient, *E. coli* J53, with the conjugation frequencies ranging from 4.8×10^{-8} to 1.2×10^{-3} . It is interesting to note that *E. coli* isolates carrying both *mcr-1* and *mcr-3* could simultaneously transferred both genes, suggesting that *mcr-1* and *mcr-3* may reside on the same plasmid or on different plasmids but may transfer together. Colistin MICs were 2 to 8 folds increase in transconjugants carrying *mcr* (1–4 $\mu\text{g}/\text{mL}$) compared with that in *E. coli* J53 (0.5 $\mu\text{g}/\text{mL}$). All *mcr-1* plasmids were typed as IncI2 while *mcr-3* was associated with IncX4 ($n = 3$) or IncF ($n = 2$) plasmids. All three plasmid types have been shown to accommodate several *mcr* variants, especially IncI2 and IncX4 which are the major types of *mcr* plasmids (Nang et al., 2019). Despite the fact that IncI2 and IncX4 are narrow-host range conjugative plasmids and IncF is limited to Enterobacterales, *mcr*-positive bacteria have been found in different origins worldwide.

In conclusion, this is the first longitudinal study demonstrating the fecal shedding pattern of *S. enterica* and *mcr*-positive *E. coli* from meat-type ducks in Thailand. With the characteristics of open-house farming, dissemination of bacterial pathogens within the farm and its environment easily occur. Control measures at the farm

level can help to limit the level of contamination in duck and duck-related products.

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DISCLOSURES

The authors declare no conflicts of interest.

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