Effects of in ovo probiotic administration on the incidence of avian pathogenic *Escherichia coli* in broilers and an evaluation on its virulence and antimicrobial resistance properties

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ABSTRACT Avian pathogenic *Escherichia coli* (**APEC**) causes colibacillosis in poultry, which has been traditionally controlled by the prophylactic in-feed supplementation of antibiotics. However, antibiotics are being removed from poultry diets owing to the emergence of multidrug-resistant (**MDR**) bacteria. Therefore, alternatives to control APEC are required. This study aimed to evaluate the effects of in ovo inoculation of probiotics on the incidence of APEC in broilers and evaluate the virulence and antimicrobial resistance properties of the APEC isolates. On embryonic day 18, 4 in ovo treatments (**T**) were applied: T1 (Marek's vaccine [**MV**]), T2 (MV and *Lactobacillus animalis*), T3 (MV and *Lactobacillus reuteri*), and T4 (MV and *Lactobacillus rhamnosus*). A total of 180 male broilers per treatment were randomly placed in 10 pens. The heart, liver, spleen, and yolk sac were collected on day 0, 14, 28, and 42. Presumptive *E. coli* isolates were confirmed by real-time PCR. The positive isolates were screened for the APEC-related genes (*iroN*, *ompT*, *hlyF*, *iss*, and *iutA*), and *E. coli* isolates containing one or more of these genes were identified as APEC-like strains. A total of 144 APEC-like isolates were isolated from 548 organ samples. No differences ($P > 0.05$) among treatments were observed for the incidence of APEC-like strains in all organs when averaged over sampling days. However, when averaged over treatments, the incidence in the heart, liver, and yolk sac was different among sampling days; a significant increase was observed in these organs on day 14 compared with day 0. Twenty-five antimicrobial resistance genes were evaluated for all APEC-like isolates, and 92.4% of the isolates carried at least one antimicrobial resistance gene. Thirty-seven isolates were then selected for antimicrobial susceptibility testing; MDR strains accounted for 37.8% of the isolates. In conclusion, the in ovo inoculation of a single probiotic strain did not confer protection against APEC strains in broilers. The high prevalence of MDR isolates indicates that further research on antibiotic alternatives is required to prevent APEC infections in broilers.

Key words: in ovo inoculation, *Lactobacillus*, multidrug-resistant, real-time PCR detection, virulence gene

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INTRODUCTION

Escherichia coli is a normal inhabitant in the avian gastrointestinal tract, respiratory tract, and the surrounding environment. However, only pathogenic *E. coli* strains cause disease in birds, and these strains are known as avian pathogenic *E. coli* (**APEC**). Avian pathogenic *E. coli* causes colibacillosis and related extraintestinal illness in poultry worldwide, which are typified by septicemia, air sacculitis, and pericarditis (Ewers et al., 2003). The possible transmission of APEC can occur by vertical transmission from breeders and horizontal transmission by food, air, litter, feces, and other birds (Nakazato et al., 2009). The gastrointestinal tract and respiratory tract are significant routes of APEC infection, and APEC infection in poultry results in a high mortality rate and severe economic burden for the poultry industry (Cortés et al., 2010). Besides, APEC strains are potential zoonotic pathogens and possess a part of the same virulence genes with human extraintestinal pathogenic *E. coli*, which has been verified to cause disease in mammalian infection models (Johnson et al., 2008b; Manges and Johnson, 2012).

As a therapy, antibiotics have been widely used for disease control in the modern poultry industry against APEC. However, these antibiotics have increased the emergence and dissemination of antibiotic-resistant

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E. coli strains (Van den Bogaard et al., 2001; Subedi et al., 2018), and these resistance genes are frequently located in transferable plasmids of APEC strains (Johnson et al., 2006). The emergence of multidrug-resistant APEC not only poses difficulties to prevention and control of APEC infection but also brings many challenges in creating the potential for spread of resistance to other pathogens and commensals through mobile plasmids. Therefore, it is essential to find an alternative approach to control APEC infection for the poultry industry as a long-term strategy.

Probiotics are live microorganisms or preparations of microorganisms, which alter the host's microflora, and they are intended to have health benefits for host animals (Schrezenmeir and de Vrese, 2001). They are promising treatments against intestinal pathogens because they modulate the intestinal microbiota of broiler chickens (Tarabees et al., 2019). Probiotics provide a protective effect against pathogens by competitive exclusion of pathogens in the gastrointestinal tract, by neutralizing enterotoxins, and by stimulating the immune system (Jin et al., 1997). *Lactobacillus* is a probiotic that produces lactic acid, which was reported to have an inhibitory effect against pathogenic *E. coli* in chickens (Watkins et al., 1982). Therefore, the application of *Lactobacillus* in poultry is a promising approach to reduce APEC colonization in the gastrointestinal tract. In-feed probiotics are commonly used in poultry; however, they cannot produce protection in the hatchery at such an early life stage of broilers before the feed is provided. Oral gavage is also a possible way to deliver probiotics, but the low efficiency and high labor cost make oral gavage challenging to operate. To overcome these disadvantages, in ovo inoculation is very efficient, which is also easy to operate for administrators. In ovo administration of probiotics could help chickens establish a protective microbial community at an early stage of intestinal development (Pedroso et al., 2016) and therefore protect against APEC infection even before hatching (De Oliveira et al., 2014).

Many studies have focused on dietary probiotic supplements and in ovo nutrients; however, limited information is available on the protective effects of in ovo administration of probiotics against APEC prevalence in broilers. Therefore, the objectives of the present study were 1) to determine the effects of in ovo administration of *Lactobacillus* probiotics on the incidence of APEC in broilers and 2) to determine the virulence and antibiotic resistance properties of APEC isolates recovered from broiler organ samples. The antimicrobial and virulence properties of the APEC isolates can serve as a baseline for future surveillance and prevention studies in this region.

MATERIALS AND METHODS

Probiotic Strains

Lactobacillus animalis (ATCC 35046), *Lactobacillus reuteri* (ATCC 2837), and *Lactobacillus rhamnosus* (ATCC 23272) were used in this study. De Man, Rogosa and Sharpe (**MRS**) broth and MRS agar (BD Difco, Franklin Lakes, NJ) were used for culturing probiotics at 37°C for 48 h anaerobically (Anoxomat; Spiral Biotech, Norwood, MA; 1535 incubator; VWR International, Cornelius, OR). These strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Treatments and Bird Management

Ross 708 fertilized eggs were purchased from a commercial breeder farm and stored in a cooler set at 18 °C for 3 d. All eggs were labeled based on treatment, flat, and egg number. Before setting, eggs were acclimated to avoid moisture on their surface. The flats of eggs were set into 2 previously sterilized NatureForm incubators (18 flats per incubator and 1,080 eggs in total, model NMC-1080; Jacksonville, FL), with each incubator carrying 18 flats of eggs. Dry and wet bulb temperatures were set at 37.5 °C ± 0.1 and 28.9 °C ± 0.1, respectively, in both incubators. After 12 d of incubation, eggs were candled to discard those that presented as infertile, cracked, contaminated, or early dead embryos.

On day 18 of incubation, the treatments were applied as follows: treatment 1, Marek's disease vaccine alone (**MV**); treatment 2, MV and *Lactobacillus animals* ($\sim 10^6$ cfu/50 μL per egg); treatment 3, MV and *L. reuteri* ($\sim 10^6$ cfu/50 μL per egg); and treatment 4, MV and *L. rhamnosus* ($\sim 10^6$ cfu/50 μL per egg). On day 18 of incubation, the 4 treatments were applied using commercial Inovoject equipment (Zoetis, Parsippany, NJ). The eggs were then transferred into hatching baskets (18 baskets per treatment, 6 baskets per hatcher) distributed among 3 Georgia Quail Farm hatcher units (3 for each treatment, 12 in total, 1502 Digital Sportsman incubator; GQF MFG, Savannah, GA) until day 21 of incubation (day of hatch). The hatcher's dry and wet bulb temperatures were set at 36.9 °C ± 0.1 and 30 °C ± 0.1, respectively. On day 21, no differences were detected in hatch of fertile among the inoculated groups. The chicks were sexed by feather discrimination, and 18 male chicks were placed in each pen based on treatment (10 pens per treatment) in a grow-out research facility, where they were raised through a 42-d grow-out cycle.

Sample Collection

Heart, liver, and spleen samples were collected on day 0, 14, 28, and 42. Yolk sac samples were collected on day 0 and 14 because after day 14, and the yolk is generally absorbed through the yolk stalk duct in most birds (Buhr et al., 2006). On each sampling day, one bird was randomly picked from each pen for a total of 10 birds from each treatment. Organ samples were transferred aseptically to prelabeled sterilized Whirl-Pak filter bags (Nasco, Madison, WI) and kept on ice until further processing.

Table 1. Primers used for amplification.

Primer	Sequence (5′–3′) Forward	Reverse	Size (bp)	Reference
Minimal predictors of APEC virulence genes				
iroN	AATCCGGCAAAGAGACGAACCGCCT	GTTCGGGCAACCCCTGCTTTGACTTT	553	Jonhson et al., 2008
ompT	TCATCCCGGAAGCCTCCCTCACTACTAT	TAGCGTTTGCTGCACTGGCTTCTGATAC	496	Jonhson et al., 2008
hlyF	GGCCACAGTCGTTTAGGGTGCTTACC	GGCGGTTTAGGCATTCCGATACTCAG	450	Jonhson et al., 2008
iss	CAGCAACCCGAACCACTTGATG	AGCATTGCCAGAGCGGCAGAA	323	Jonhson et al., 2008
iutA	GGCTGGACATCATGGGAACTGG	CGTCGGGAACGGGTAGAATCG	302	Jonhson et al., 2008
Antimicrobial resistance genes				
blaTEM	ATGTGCGCGGAACCCCTATTTGTTTA	AAAAAGCGGTTAGCTCCTTCGGTCCT	558	Logue et al., 2017
aac3vIa	GGCACCCGCGACGCCCTGGTCCAAAAG	GGGCCCGGCGCCGATCGACAGGATTT	502	Logue et al., 2017
aac3vIb	GGGCAAGCGCCGCGTCACTTATT	CGCGGCGTTGTTTCGGCTTCA	302	Johnson et al., 2006
aph3IA	TCGGGCAATCAGGTGCGACAATCTA	TGCCAGCGCATCAACAATATTTTCACC	378	Logue et al., 2017
aadA	TAACGGCGCAGTGGCGGTTTTCA	AAGCTCGCCGCGTTGTTTCATCAAG	365	Johnson et al., 2006
tetA	CGGGGCGACTGGGGCGGTAGC	CAAAGCGCGGCCGGCACCTGT	372	Johnson et al., 2012
tetB	AACGCGTGAAGTGGTTCGGTTGGT	TTCGCCCCATTTAGTGGCTATTCTTC	446	Logue et al., 2017
dfr1	ATCGGGAATGGCCCTGATA	CTTCCGGCTCGATGTCTATTGTAG	328	Logue et al., 2017
dfr17	ATATCCCGTGGTCAGTAAAAGGTG	GACCCCCGCCAGAGACATA	342	Logue et al., 2017
qacEΔ	CCCTTCCGCCGTTGTCATAATCG	TACAAAGCCCCACGCATCAAGC	175	This study
qnr	TCGCCGCTGCCGCTTTTATCAGT	GCCAACAGTCGCGGGAGAAGGTG	440	Logue et al., 2017
sul1	CGCCGCTCTTAGACGCCCTGTCC	CGCCGCTCTTAGACGCCCTGTCC	462	Johnson et al., 2012
intl1	CACTCCGGCACCGCCAACTTTC	GAACGGGCATGCGGATCAGTGAG	545	Johnson et al., 2012
Heavy metal resistance genes				
terD	CCACTGCGCGGAATTTCCACTCACCAT	ACGCCGTCCCGTCTGATGTTGACAAG	231	Johnson et al., 2006
terX	ATGCGCCGCCTGCCTGTTTACCTTGTTA	CGCGCTTGTGCTGCCGGAAGACA	576	Johnson et al., 2006
terF	CCGACAAACTTCCAGAAGATGGGGTAGT	GAGGCAGCGGTTGCATTTGTACTTGACG	428	Johnson et al., 2006
terY3	CCTGGGGCCGTCAGCGGACCTG	TCCTTGCTGGTGGCCGTTCATACTTCAT	302	Johnson et al., 2006
pcoA	ATCCGGAAGGTCAGCACCGTCCATAGAC	GACCTCGCGGATGTCAGTGGCTACACCT	507	Johnson et al., 2006
pcoD	GGCGCCCAGAATGATAATCGCAACA	GGGCGTGGCGCTGGCTACACTT	502	Johnson et al., 2006
pcoE	GTGGGGCAGCTTTTGCTCAGTCCAGTGA	CGAAGCTTTCTTGCCTGCGTCTGATGTG	385	Johnson et al., 2006
arsC	ACCCGCTTCATCAACCACTT	TGCCGATATGGGGATTTCCG	268	This study
silP	ACACCCCGGCCTGGGCTCCTT	TGCGGGCACGGGAACAAACCTC	603	Johnson et al., 2006
merA	GATCCGCGCCGCCCATATCGCCCATCTG	CACGCGCTCGCCGCCGTCGTTGAGTTG	250	Johnson et al., 2012

Abbreviation: APEC, avian pathogenic *Escherichia coli*.

Table 2. Virulence profile of APEC isolates.

Virulence profile	Number of virulence factors present	Frequency (n = 548)	Percentage
iroN, iss, ompT, hlyF, iutA	5	15	2.7%
iroN, iss, ompT, hlyF	4	72	13.1%
iss, ompT, hlyF, iutA	4	2	0.4%
ompT, hlyF, iutA	3	53	9.7%
ompT, hlyF	2	2	0.4%
Total		144	26.3%

Abbreviation: APEC, avian pathogenic *Escherichia coli*.

All animals in this trial were treated in compliance with the Guide for the Care and Uses of Agriculture Animals in Research and Teaching (Federation of Animal Science Societies, 2010) and the Mississippi State University Institutional Animal Care and Use Committee (IACUC Animal Welfare Assurance #A3160-01).

Bacterial Isolation

Homogenized samples were first enriched in 20 mL of tryptic soy broth (BD Bacto, Franklin Lakes, NJ) aerobically for 18–24 h at 37°C. After incubation, a loopful of the enrichment broth was streaked onto MacConkey agar (BD Difco, Franklin Lakes, NJ) and incubated at 37°C for 18 to 24 h. A single suspect colony on MacConkey agar was selected to be suspended in Luria–Bertani broth (BD Difco, Franklin Lakes, NJ) and aerobically incubated in a shaker incubator at 150 rpm and 37°C for 18 h. All organisms were stored at −80°C in Luria–Bertani broth with 20% (vol/vol) glycerol until use.

Bacterial DNA Preparation

DNA was prepared using boiled lysates as previously described by Mohamed et al., 2018. In brief, 200 μL of overnight bacteria culture was transferred into a PCR tube and centrifuged to obtain the bacteria pellet. The supernatant was discarded, and 150 μL of nuclease-free water was added and mixed well using a vortex. The bacteria culture was boiled at 95 °C for 5 min. The resulting solution was centrifuged, and the supernatant was transferred into a new tube and served as the DNA template. The DNA template was stored at −20°C until further use.

Identification of E. coli

A real-time PCR assay was performed for *E. coli* to detect core genomic gene encoding *ybbW* (Walker et al., 2017). Each reaction contained 5 μL of PowerUp SYBR Green Master Mix (Life Technologies, Carlsbad, CA), 3.5 μL of nuclease-free water, and 0.25 μL each of the forward and reverse primer (10 μM), and 1 μL of template DNA. Primers are listed in Table 1. Real-time PCR was performed using a QuantStudio 3 (Applied Biosystems, Foster City, CA) under the following conditions: the initial denaturation step was carried out at 95 °C for 20 s and 40 cycles were carried out at 95 °C for 3 s and 60 °C for 20 s. Melting curve analysis was performed in the range of 60 °C to 95 °C at 0.5 °C per 5-s increments to analyze the specificity of the primers.

Screening of APEC Virulence Genes by Multiplex PCR

The confirmed *E. coli* isolates were then analyzed using pentaplex PCR as described by Johnson et al. (2008a) for the minimal predictors of APEC virulence: *iroN*, *ompT*, *hlyF*, *iss,* and *iutA* genes. Primers used for pentaplex PCR are listed in Table 1. To eliminate the inhibition factor of the PCR, 0.1% of BSA was added in the master mix of the amplification system (Kreader,

Table 3. Prevalence of APEC isolated from different organs in each treatment.

	Sampling organ (%)							
Treatment	Heart	SEM	Liver	SEM	Spleen	SEM	Yolk sac	SEM
Marek's vaccine (MV)	27.5	7.10	22.5	6.31	27.5	15.10	33.3	11.97
MV and *L. animalis*	27.5	7.10	32.5	8.05	22.5	11.21	38.5	15.80
MV and *L. reuteri*	32.5	7.88	30.0	7.67	22.5	11.21	11.1	6.81
MV and *L. rhamnosus*	22.5	6.21	20.0	5.80	22.5	11.21	21.1	9.20
P	0.757		0.448		0.926		0.164	
Day								
0	5.0^{c}	3.39	5.0^{c}	3.31	0	0	15.0^{b}	5.37
14	52.5^{a}	7.94	57.5^{a}	7.91	35.0	7.76	39.3^{a}	10.50
28	$35.0^{a,b}$	7.57	$20.0^{b,c}$	6.28	35.0	7.76		
42	$17.5^{b,c}$	5.98	22.5^{b}	6.58	25.0	6.85		
P	0.0003		<0.0001		0.747		0.015	

[a–c]Means in a column not sharing a common superscript were different ($P < 0.05$).
Abbreviation: APEC, avian pathogenic *Escherichia coli*.

Table 4. Results of antimicrobial resistance gene detection.

Resistance genes	Resistance profile	Number of resistant isolates (%)	Number of resistance isolates in each treatment (%)								
			Marek's vaccine (MV)	SEM	MV and *Lactobacillus animal*	SEM	MV and *Lactobacillus reuter*	SEM	MV and *Lactobacillus rhamnosu*	SEM	*P*
blaTEM	β-Lactamase	16 (11.1)	7 (18.9)	0.065	1 (2.4)	0.024	5 (13.9)	0.058	3 (10)	0.054	0.212
aac(3)-VIa	Aminoglycoside	24 (16.7)	5 (13.5)	0.057	9 (22.0)	0.065	9 (25.0)	0.073	1 (3.3)	0.033	0.149
aac(3)-VIb		21 (14.6)	3 (8.1)	0.045	11 (26.8)	0.070	6 (16.7)	0.063	1 (3.3)	0.033	0.052
aph(3′)-Ia		19 (13.2)	3 (8.1)	0.045	2 (4.9)	0.034	7 (19.4)	0.067	7 (23.3)	0.079	0.095
aadA		21 (14.6)	4 (10.8)	0.052	8 (19.5)	0.063	8 (22.2)	0.070	1 (3.3)	0.033	0.178
tetA	Tetracycline	55 (38.2)	13 (35.1)	0.080	20 (48.8)	0.079	15 (41.7)	0.083	7 (23.3)	0.079	0.178
tetB		11 (7.6)	1 (2.7)	0.027	4 (9.8)	0.047	2 (5.6)	0.014	4 (2.8)	0.063	0.428
dfr1	Trimethoprim	8 (5.6)	0	0	7 (17.1)	0.059	1 (2.8)	0.028	0	0	0.354
dfr7		51 (35.4)	19 (51.4)	0.083	12 (29.3)	0.072	14 (38.9)	0.082	6 (20.0)	0.074	0.052
dfr17		0	0	0	0	0	0	0	0	0	1
qacEΔ	Quaternary ammonium compounds	68 (47.2)	12 (32.4)[b]	0.078	27 (65.9)[a]	0.075	21 (58.3)[a]	0.083	8 (26.7)[b]	0.082	0.002
qnr	Quinolone	47 (32.6)	19 (51.4)	0.083	16 (39.0)	0.079	12 (33.3)	0.080	0	0	0.467
sul1	Sulfa	21 (14.6)	3 (8.1)	0.045	9 (22.0)	0.065	8 (22.2)	0.070	1 (3.3)	0.033	0.093
intl1	Integrase	29 (20.1)	2 (5.4)[b]	0.038	17 (41.5)[a]	0.078	9 (25.0)[a]	0.073	1 (3.3)[b]	0.033	0.001
arsC	Arsenic	137 (95.1)	36 (97.3%)	0.027	38 (92.7)	0.041	35 (97.2)	0.028	28 (93.3)	0.046	0.716
merA	Mercury	29 (20.1)	7 (18.9)	0.065	8 (19.5)	0.063	9 (25.0)	0.073	5 (16.7)	0.069	0.851
terD	Tellurite	5 (3.5)	2 (5.4)	0.038	2 (4.9)	0.034	0	0	1 (3.3)	0.033	0.982
terF		4 (2.8)	2 (5.4)	0.038	2 (4.9)	0.034	0	0	0	0	0.999
terX		3 (2.1)	2 (5.4)	0.038	1 (2.4)	0.024	0	0	0	0	0.930
terY3		4 (2.8)	2 (5.4)	0.038	2 (4.9)	0.034	0	0	0	0	0.999
pcoA	Copper	2 (5.4)	2 (1.4)	0.038	0	0	0	0	0	0	0.999
pcoD		0	0	0	0	0	0	0	0	0	1
pcoE		3 (2.1)	0	0	0	0	0	0	3 (10.0)	0.056	0.999
silE	Silver	0	0	0	0	0	0	0	0	0	1
silP		1 (0.7)	0	0	0	0	0	0	1 (3.3)	0.033	0.999

[a–c]Means in a column not sharing a common superscript were different ($P < 0.05$).

Table 5. Results of antimicrobial susceptibility testing.

Antibiotic agents tested	Antimicrobial class	Number of resistant strains, n = 37
Amoxicillin/clavulanic acid (AUG2)	β-Lactam combination agents	8 (21.6%)
Ampicillin (AMP)	Penicillins	16 (43.2%)
Azithromycin (AZI)	Macrolides	0
Cefoxitin (FOX)	Cephems	8 (21.6%)
Ceftriaxone (AXO)	Cephems	8 (21.6%)
Chloramphenicol (CHL)	Phenicols	0
Ciprofloxacin (CIP)	Quinolones	0
Gentamicin (GEN)	Aminoglycosides	17 (45.9%)
Meropenem (MERO)	Penems	0
Nalidixic Acid (NAL)	Quinolones	0
Streptomycin (STR)	Aminoglycosides	11 (29.7%)
Sulfisoxazole (FIS)	Folate pathway antagonists	11 (29.7%)
Tetracycline (TET)	Tetracyclines	23 (62.2%)
Trimethoprim/sulfamethoxazole (SXT)	Folate pathway antagonists	6 (16.2%)

1996). The initial denaturation step was carried out at 95 °C for 2 min; cycling parameters were as follows: 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 68 °C for 3 min; and an extension step was carried out at 72 °C for 10 min.

All PCR products were analyzed by gel electrophoresis using 2% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA). The results were visualized under UV light using the Kodak Gel Logic 200 Imaging System (Eastman Kodak Co., Rochester, NY).

PCR Screening and DNA Sequencing for Resistance Genes

The APEC-like positive isolates were examined by PCR for 14 antimicrobial resistance genes conferring resistance to β-lactamase (*blaTEM*), aminoglycoside (*aac(3)-VIa, aac(3)-VIb*, *aph(3′)-Ia*, and *aadA*), tetracycline (*tetA*, *tetB*), trimethoprim (*dfr1*, *dfr7*, *and dfr17*), quaternary ammonium compounds (**QAC**) (*qacEΔ*), quinolone antibiotics (*qnr*), sulfonamide (*sul1*), and integrase (*intl1*) as described previously by Kim et al., 2020. In addition, 11 heavy metal resistance genes for conferring resistance to arsenic (*arsC*), mercury (*merA*), tellurite (*terD*, *terF*, *terX*, and *terY3*), copper (*pcoA*, *pcoD*, and *pcoE*), and silver (*silE*, *silP*) were tested. Primers are listed in Table 1. The PCR products were sequenced by Eurofins Genomics (Eurofins Scientific, Louisville, KY). The DNA sequences obtained were compared with the information in the GenBank database using the Basic Local Alignment Search Tool program available in the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/BLAST).

Antimicrobial Susceptibility Testing

A total of 37 representative APEC-like isolates obtained on different days, from different treatments, and from different organs were selected and subjected to antimicrobial susceptibility testing using the broth microdilution assay and the National Antimicrobial Resistance Monitoring System (**NARMS**) panels (Thermo Scientific Sensititre NARMS Gram Negative CMV4AGNF AST Plate, Sensititre; Trek Diagnostics, Cleveland, OH) (Kim et al., 2020). Antimicrobial susceptibility testing was performed for 14 antimicrobials, including amoxicillin and clavulanic acid (**AUG2**), ampicillin (**AMP**), azithromycin (**AZI**), cefoxitin (**FOX**), ceftriaxone (**AXO**), chloramphenicol (**CHL**), ciprofloxacin (**CIP**), gentamicin (**GEN**), meropenem (**MERO**), nalidixic acid (**NAL**), streptomycin (**STR**), sulfisoxazole

Table 6. Antimicrobial resistance patterns of the tested APEC isolates against 14 antimicrobial agents.

Resistance pattern	Antimicrobial class	No. of antimicrobial class	No. of resistant isolates	Percentage
AUG2, AMP, FOX, AXO, GEN, STR, FIS, TET	β-Lactam combination agents, penicillins, cephems, aminoglycosides, folate pathway antagonists, tetracyclines	6	8	21.6%
AMP, STR, TET, FIS, SXT	Penicillins, aminoglycosides, tetracyclines, folate pathway antagonists	4	2	5.4%
GEN, TET, SXT	Aminoglycosides, tetracyclines, folate pathway antagonists	3	4	10.8%
AMP, TET	Penicillins, tetracyclines	2	4	10.8%
AMP, GEN	Penicillins, aminoglycosides	2	2	5.4%
GEN, TET	Aminoglycosides, tetracyclines	2	2	5.4%
GEN, STR, FIS	Aminoglycosides, folate pathway antagonists	2	1	2.7%
TET	Tetracyclines	1	3	8.1%
None	None	0	11	29.7%

Abbreviations: AMP, ampicillin; APEC, avian pathogenic *Escherichia coli*; AUG2, amoxicillin/clavulanic acid; AXO, ceftriaxone; FIS, sulfisoxazole; FOX, cefoxitin; GEN, gentamicin; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline.

(**FIS**), tetracycline (**TET**), and trimethoprim and sulfamethoxazole (**SXT**). A periodic quality control check of the colony count was performed as per the Sensititre protocol. In addition, a plate inoculated with *E. coli* ATCC 25922 was used for quality assurance and incubated with every batch of the minimum inhibitory concentration (**MIC**) plates. The MIC plates were read automatically on an ARIS 2X (TREK diagnostic system, Cleveland, OH). The resulting MIC were compared with inhibition breakpoints specified by the Clinical and Laboratory Standards Institute and NARMS to determine resistance or susceptibility to antimicrobials (Logue et al., 2012).

Breakpoints used in this study were AUG2 ≥32/16 μg/mL, AMP ≥32 μg/mL, AZI ≥32 μg/mL, FOX ≥32 μg/mL, AXO ≥4 μg/mL, CHL ≥32 μg/mL, CIP ≥4 μg/mL, GEN ≥16 μg/mL, MERO ≥4 μg/mL, NAL ≥32 μg/mL, STR ≥64 μg/mL, FIS ≥512 μg/mL, TET ≥16 μg/mL, and SXT ≥4/76 μg/mL (Logue et al., 2012; Whitehouse et al., 2018).

Experiment Design and Data Analysis

The incidence of APEC-like strains was determined based on the number of samples considered positive after PCR confirmation. The prevalence of antimicrobial resistance phenotypes was calculated based on the number of isolates exhibiting resistance to the antimicrobial in proportion to the total number of APEC-like isolates. The incidence of APEC-like strains was analyzed as a split plot in time design, with the probiotic treatment as the main plot and sampling time as the subplot. Data were analyzed using the PROC GLIMMIX procedure of SAS 9.4 software (Institute Inc., Cary, NC), with the LSMEANS statement. Differences were considered significant when the *P*-value was less than 0.05.

RESULTS

Effects of Probiotic Administration on the Incidence of APEC in Broilers

Growth of *E. coli* on MacConkey agar plates as confirmed by real-time PCR identification was observed in 39.0% (214 of 548) of the samples. Using the criteria described by Johnson et al. (2008b), we differentiated 144 APEC-like isolates (26.3%) from 548 samples, and 70 *E. coli* isolates (12.8%) carried none of the tested virulence traits. The virulence genes identified by pentaplex PCR were *iroN* (87 isolates, 15.9%), *iss* (89 isolates, 16.2%), *ompT* (144 isolates, 26.3%), *hlyF* (144 isolates, 26.3%), and *iutA* (70 isolates, 12.8%). Table 2 shows that fifteen isolates (2.7%) were positive for 5 genes, 72 isolates (13.1%) were positive for 4 genes (*iutA, hlyF, iroN, iss,* and *ompT*), and 2 isolates (0.4%) were positive for 4 genes with another combination (*iss, ompT, hlyF, iutA*). The *ompT, hlyF,* and *iutA* combination was present in 53 isolates (9.7%). The *ompT* and *hlyF* combination was present in 2 isolates (0.4%).

In ovo administration of different individual probiotic species did not significantly affect the incidence of APEC-like strains in broiler chickens. Table 3 shows that there were no differences ($P > 0.05$) among the 4 treatments for the presence of APEC-like strains in any of the organs when averaged over sampling days. When averaged over treatments, however, the incidence was different among sampling days for the heart ($P = 0.0003$), liver ($P < 0.0001$), and yolk sac ($P = 0.015$). In the heart, the incidence of APEC was the highest on day 14 when compared with day 0 and 42. Avian pathogenic *E. coli* incidence on day 28 was also found to be higher than that on day 0 but was not different than that on day 42; however, the APEC incidence number did decrease from day 14 to day 42. In the spleen, 35% of APEC-like strains were detected on day 14 and day 28, but none was detected on day 0. On day 42, the incidence slightly decreased to 25% but was not significantly different ($P = 0.747$). In the liver, the incidence of APEC-like strains on day 14 (57.5%) was significantly higher than on day 0 (5.0%), day 28 (20.0%), and day 42 (22.5%) ($P < 0.0001$). In the yolk sac, incidence of APEC-like–positive strains was detected in 39.3% of birds sampled on day 14, which was greater ($P = 0.015$) than that on day 0 (15.0%).

Antimicrobial Resistance Gene Detection

Table 4 shows the results of antimicrobial resistance genes identified by PCR screening and DNA sequencing of all the APEC-like isolates. The β-lactamase–encoding gene *blaTEM* was detected in 16 (11.1%) APEC-like isolates, which confers resistance to β-lactam antibiotics such as ampicillin, cephalothin, amoxicillin, and clavulanic acid. Four types of plasmid-encoded aminoglycoside–modifying enzyme genes were examined, and *aac(3)-VIa* (24 isolates, 16.7%) was the most prevalent one, followed by *aac(3)-VIb* (21 isolates, 14.6%), *aadA* (21 isolates, 14.6%), and *aph(3′)-Ia* (19 isolates, 13.2%), among which, the gene *aac(3)-VIb* showed a lower trend ($P = 0.052$) distributed in the isolates from treatment 1 (8.1%) and treatment 4 (3.3%) than in those from treatment 2 (26.8%). Tetracycline efflux genes were detected in 66 (45.8%) APEC-like isolates as follows: *tetA* (55 isolates, 38.2%) and *tetB* (11 isolates, 7.6%). The trimethoprim resistance genes were detected in 59 isolates (41.0%); *dfr7* was the most common type as it was detected in 51 isolates (35.4%), followed by *dfr1* in 8 isolates (5.6%). The distribution of the gene *dfr7* in the isolates showed a lower trend ($P = 0.052$) in treatment 2 (29.3%) and treatment 4 (20.0%) than in treatment 1 (51.4%). And all isolates were negative for *dfr17*. The QAC resistance gene *qacEΔ* was found in 68 isolates (47.2%); this gene carried by the isolates from treatment 2 (65.9%) and treatment 3 (58.3%) was significantly greater ($P = 0.002$) than the isolates from treatment 1 (32.4%) and treatment 4 (26.7%). The plasmid-borne quinolone resistance gene *qnr* was detected in 47 isolates (32.6%). The sulfonamide resistance gene *sul1* was detected in 21 isolates (14.6%).

Of all the tested heavy metal resistance genes, *arsC* and *merA* genes were the most abundant in APEC-like isolates, as shown in Table 4. In particular, the *arsC* gene (coding for the resistance mechanism to arsenic) was detected in 137 isolates (95.1%). The mercury resistance gene *merA* was found in 29 isolates (20.1%). The tellurite resistance genes *terD*, *terF*, *terX*, and *terY3* were detected in 5 isolates (3.5%), 4 isolates (2.8%), 3 isolates (2.1%), and 4 isolates (2.8%), respectively. Three types of copper resistance genes were examined: *pcoE* was detected in 3 isolates (2.1%), *pcoA* was found in 2 isolates (1.4%), and all isolates were negative for *pcoD*. Two types of silver resistance genes were tested, and only 1 isolate (0.7%) was positive for *silP*. However, the gene *intl1* was found to be distributed significantly different among the treatment groups. The *intl1* gene, coding for integrase resistance, was detected from 2 of 37 (5.4%), 17 of 41 (41.5%), 9 of 36 (25.0%), and 1 of 30 (3.3%) APEC-like isolates from treatments 1, 2, 3, and 4, respectively. Therefore, significantly higher ($P = 0.001$) levels were found in treatments 2 and 3 compared with treatments 1 and 4.

Antimicrobial Susceptibility

A total of 37 APEC-like isolates were subjected to antimicrobial susceptibility testing using the broth microdilution assay. The prevalence of isolates resistant to each antimicrobial shown in Table 5 is as follows: TET, 62.2% (23/37); GEN, 45.9% (17/37); AMP, 43.2% (16/37); STR, 29.7% (11/37); FIS, 29.7% (11/37); AUG2, FOX, and AXO, 21.6% (8/37) each; and SXT, 16.2% (6/37). All tested isolates were susceptible to the remaining 5 antimicrobials including AZI, CHL, CIP, MERO, and NAL.

The antimicrobial resistance patterns of the tested APEC-like strains are shown in Table 6. Based on the broth microdilution results, 11 (29.7%) of the isolates were susceptible to all 14 antimicrobials tested. Twenty-six (70.2%) of the isolates showed resistance to at least one antimicrobial, whereas 14 (37.8%) of the isolates demonstrated multidrug resistance to 3 or more different classes of antimicrobial agents (Magiorakos et al., 2012; Basak et al., 2016). Eight isolates (21.6%) were resistant to 8 antimicrobial agents in 6 antimicrobial classes (β-lactam combination agents, penicillins, cephems, aminoglycosides, folate pathway antagonists, tetracyclines). Two isolates (5.4%) were resistant to 4 antimicrobial agents in 4 antimicrobial classes (penicillins, aminoglycosides, tetracyclines, folate pathway antagonists). Four isolates (10.8%) were resistant to 3 antimicrobial agents in 3 classes (aminoglycosides, tetracyclines, folate pathway antagonists). Nine isolates (24.3%) and 3 isolates (8.1%) were resistant to 2 and 1 tested antimicrobial agents, respectively.

DISCUSSION

In this study, one of the main purposes was to evaluate the effects of in ovo inoculation of probiotic bacteria on the incidence of APEC in broilers. *L. animalis*, *L. reuteri*, and *L. rhamnosus* are 3 probiotics that have performed well in reducing *E. coli* incidence in animals. *L. animalis* NP51 as a direct-fed antimicrobial was suggested as effective at reducing the recovery rate of *E. coli* O157: H7 from cattle feces (Ayala et al., 2018). In addition, Spinler et al. (2008) suggested *L. reuteri* was able to produce the bacteriocin reuterin and inhibit the growth of different enteric pathogens in vitro, including enterohemorrhagic *E. coli* and enterotoxigenic *E. coli*. *L. rhamnosus* is also a probiotic that was proven to be capable of lowering the translocation rates of *E. coli* O157: H7 in a mice model (Shu and Gill, 2002).

From the present study, no differences were observed in the incidence of APEC-like strains among the probiotic-administered groups and control groups. This finding was unexpected and suggested that the in ovo inoculation of *L. animalis*, *L. reuteri*, and *L. rhamnosus* individually cannot reduce the incidence of APEC-like strains. A possible reason is that the in ovo inoculation is a one-time administration. Although it may effectively protect the birds at hatch, probiotic in ovo inoculation may not prevent APEC as chicks grow. Feed or water supplementation provides continual probiotic administration (Tortuero, 1973; Watkins and Kratzer, 1984). Dietary supplement probiotic mixes have been reported to have the capability to lower *E. coli* recovery rates from the broiler liver and spleen, such as *Bacillus subtilis*, *Clostridium butyricum*, and *Lactobacillus plantarum* (Tarabees et al., 2019); however, there is limited research that evaluates the mixes of *L. animalis*, *L. reuteri*, and *L. rhamnosus* on reducing APEC incidence. Another possible explanation for the results might be that single-strain administration is not as effective as a probiotic mixture of strains. Future work is needed to investigate the effects of the *Lactobacillus* mixes on reducing APEC incidence. Furthermore, exploration of the combination of in ovo inoculation and feed supplementation in reducing APEC incidence should be investigated in broilers.

The purpose of probiotic administration by in ovo inoculation is to affect intestinal flora at an early stage of intestinal development. The amniotic fluid is swallowed by the chicken embryo at the end of incubation (Tona et al., 2010). It has previously been observed that in ovo inoculation of probiotics was capable of reducing *Salmonella* colonization from day 1 to day 7 (Hashemzadeh et al., 2010). When comparing the effect on APEC-like strains in this study, a significantly higher prevalence was observed in the heart, liver, spleen, and yolk sac on day 14 than on day 0 in all treatments. This result suggested that probiotics did not work well to protect against APEC-like strains during the first 2 wk of the chicken's life. When mimicking the natural mode of APEC infection to broilers, Abd El Tawab et al. (2016) found that the initial APEC infection usually occurs at 4–9 wk. This can be explained by Antao et al. (2008) in which young broiler chickens were easily infected by the intratracheal pathway owing to the immature bronchus-associated lymphoid tissue in the

lung within the first 2 mo of their life. This also suggested that APEC infection is not only from intestinal microflora. The in ovo inoculation in this study was a one-time administration; although it effectively protects the birds at hatch, it may not last for 14 d after hatch. Another possible reason for the lack of a treatment effect in the present study is the virulence gene transmission among different bacteria. As birds are exposed to numerous pathogens in the environment, horizontal transfer of some virulence genes among *E. coli* and other bacteria may result in the specialization of APEC (Mora et al., 2013). In addition, the variation of the environmental *E. coli* strain also increases the infection risk for broilers.

One interesting finding is that on day 0, most of the organs were detected with the presence of APEC-like strains except for the spleen. Normally, when chicks are newly hatched, the gastrointestinal tract is expected to be sterile (Juricova et al., 2013). However, APEC strains have been reported to enter and colonize inside the egg through vertical transmission from breeder hens during egg development or by horizontal transmission by penetrating the outer surface of the eggshell after egg formation (Giovanardi et al., 2005; Petersen et al., 2006). Earlier colonization of APEC inside the embryo, well before in ovo administration of probiotics, might be a possible reason for failure of probiotic treatments in preventing APEC incidence. In addition, we found that the presence of APEC did not result in the death of embryos. In challenge model studies, the lethality of chicken embryos required 3 to 6 log 10 cfu of virulent *E. coli* (Wooley et al., 2000; Skyberg et al., 2008; Graham et al., 2019). Although, in this study, APEC was not quantified in the organs sampled, it is expected that the bacteria load was lower than that required to be lethal.

The other aim of this study was to characterize the virulence and antimicrobial properties of the APEC-like isolates. The virulence genes *iroN*, *ompT*, *hlyF*, *iss*, and *iutA* are the minimal predictors to characterize APEC (Johnson et al., 2008a). The main functions of these genes are iron acquisition (*iroN*), adhesion (*ompT*), hemolysis (*hlyF*), serum resistance (*iss*), and ferric aerobactin receptor (*iutA*) (Mohamed et al., 2018). Adhesion and hemolysis are prerequisites for colonization and invasion inside the host. In this study, *ompT* and *hlyF* were the most prevalent virulence genes in the APEC-like isolates with a 100% frequency. Owing to differences in APEC sampling origin, there is no standard range of prevalence for each virulence gene. When comparing with other research for reference, the frequency of the *ompT* gene in APEC strains from poultry samples averaged from 78.6 to 94.1% and *hlyF* accounted for 59.3 to 80.4% (Jeong et al., 2012; Ahmed et al., 2013). This indicates that the APEC-like isolates of this study may have a higher potential adhesion and hemolysis capacity. Prevalence of the gene *iutA* (46.5%) was lower than the data from related research (Ahmed et al., 2013; Mohamed et al., 2018), showing that the APEC-like isolates from these samples possibly produce less toxins and siderophores than others (Sarowska et al., 2019).

It is reported that APEC shares some common virulence factors with other extraintestinal pathogenic *E. coli*, such as human uropathogenic *E. coli* (**UPEC**) (Rodriguez-Siek et al., 2005). The serum resistance gene *iss* is associated with ColV plasmids that exist in both APEC and human UPEC, and this *iss* gene is more prevalent in APEC than in UPEC strains (Ewers et al., 2007). These findings support the idea that APEC may serve as a reservoir of virulence genes for UPEC strains, and there is a potential zoonotic risk of APEC to spread to human beings and that human UPEC should be considered.

Another promising prospect in virulence gene exploration is that virulence gene combinations may help distinguish APEC from other pathogenic *E. coli*, such as avian fecal *E. coli*, as described by Mohamed et al. (2018). From this study, 5 different combination patterns of the virulence genes were detected among the APEC-like isolates. The virulence profile combinations of *iroN, iss, ompT, hlyF* (13.1%) and *ompT, hlyF, iutA* (9.7%) were the most common in this study. These results are consistent with those of Meguenni et al. (2019) and Li et al. (2015), who found that these 2 combinations were of similar proportion in their study, which accounted for 13.9 and 4.6%, respectively. Similarly, Saviolli et al. (2016) also reported that the virulence profile combination of *iroN, iss, ompT, hlyF* was the most prevalent pattern detected in 13.9% of strains. Our results also indicate that only 2.7% of APEC-like strains possessed all 5 genes (*iroN, iss, ompT, hlyF,* and *iutA*), which is lower than those previously reported (Ahmed et al., 2013; Li et al., 2015; Meguenni et al., 2019). Li et al. (2015) obtained a higher prevalence for all 5 genes from APEC strains isolated from broilers with colibacillosis in China, with a prevalence of 4.6%. In Algeria, 13.9% of the APEC strains isolated from visceral organs (liver, lungs, heart, and spleen) of diseased broilers were positive for all 5 APEC-related genes (Meguenni et al., 2019). In Egypt, 71.4% of the APEC strains isolated from septicemic broilers carried 5 APEC virulence genes (Ahmed et al., 2013). Taken together, these studies support that the prevalence of virulence genes may vary depending on the isolation source and geographic origin of the samples.

A surprising and new finding from this research showed that the *L. rhamnosus* may affect gene prevalence, which reduces the resistance to aminoglycoside enzymes, trimethoprim, and integrase of the APEC-like isolates. Currently, there is no sufficient evidence to support the viewpoint that probiotics protect the poultry against APEC by reducing the antimicrobial resistance genes; therefore, this might be a new perspective on controlling APEC due to antimicrobial resistance. Moreover, the emerging antimicrobial resistance among different APEC-like isolates has stimulated our interest in exploring the frequencies of genetic determinants.

From all of the metal resistance genes detected in this study, the arsenic resistant gene *arsC* was the most

prevalent of the 9 metal resistance genes, and it was widely distributed in 95.1% of the tested APEC-like isolates. The resistance to arsenic has been linked to the use of arsenic-based antimicrobials in the feed of poultry for growth promotion and pathogen control since 1994 (Chapman and Johnson, 2002; Liu et al., 2016). In addition, the frequent dietary use of arsenic in poultry results in accumulation of arsenic in humans by consuming poultry products (Jones, 2007; Nachman et al., 2013; Nigra et al., 2017). Therefore, use of arsenic in feed was banned in many countries around the world. The European Union was the first to cease the use of arsenicals as feed additives in 1999 (Liu et al., 2016). In the United States, roxarsone, arsanilic acid, nitarsone, and carbarsone were banned in food-producing animals in 2013 by the Food and Drug Administration. In China, the use of phenylarsonic feed additives was officially banned in 2019 (Hu et al., 2019). Even so, the arsenic resistance gene remains in the genome of these APEC isolates, which indicates the "memory" of arsenic resistance is enduring. The high level of arsenic resistance genes in APEC isolates may affect medical treatment when arsenic is a needed drug therapy, such as the treatment for coccidiosis (Nachman et al., 2013).

The resistance gene *qacEΔ* to QAC was the second most prevalent in this study, which existed in 47.2% of the detected APEC-like isolates. Quaternary ammonium compounds are cationic surface-active detergents, usually used for control of environmental bacteria or disinfection of facility surfaces on the farm (Ioannou et al., 2007). The gene *qacED1* is a mutant version of the gene *qacE* (Kazama et al., 1999), which was found in 70.6% of *E. coli* isolates in chickens with colibacillosis in Egypt (Ibrahim et al., 2019). The possible reason for the high prevalence of the QAC resistance gene is that the QAC are of low toxicity, nonirritating to poultry, and of good efficiency; thus, unlimited, longtime use has caused bacteria to develop resistance (Hegstad et al., 2010). Although, in this study, we have found QAC resistance genes to be widespread, they are not entirely associated with antimicrobial resistance phenotypes (Jaglic and Cervinkova, 2012). Therefore, more exploration of the gene together with resistance mechanisms should be conducted in future research.

The *tetA* and *tetB* genes encode efflux mechanisms and are the most common tetracycline resistance determinant in *E. coli* (Roberts, 1994; Van et al., 2008). The prevalence of *tetA* and *tetB* in our isolates was quite high (44.4%), which is consistent with that found by other authors (Younis et al., 2017; Sgariglia et al., 2019). These results were further supported by a study from Ibrahim et al. (2019) carried out in Jordan and Egypt, which reported that more than 90% of the APEC isolates from broilers carried at least one of these genes. Tetracyclines are commonly used antimicrobials in poultry and in other animal species to treat infections caused by microorganisms (Agyare et al., 2019), which could explain the high prevalence of the *tetA* and *tetB* genes in our APEC-like isolates. When we explored the phenotype for tetracycline resistance of these strains, the antimicrobial susceptibility results showed that up to 62.2% of the APEC-like isolates were resistant to tetracycline. One possible reason is that there are not only these 2 genes directing tetracycline resistance (Subedi et al., 2018). For example, some gram-negative bacteria carry multiple *tet* genes, such as *E. coli* O157: H7, which has at least 6 known *tet* genes: *tetA, tetB, tetC, tetD, tetE*, and *tetG* genes. Therefore, more related genes should be examined to explore the mechanism and relationship between the tetracycline resistance phenotype and genotype.

From the antimicrobial susceptibility testing results, we found that these APEC-like isolates were highly resistant to TET, GEN, AMP, STR, AUG2, FOX, AXO, and SXT. These results are in agreement with Merchant et al. (2012), which showed high resistance rates of TET, AMP, and AUG2 in pathogenic *E. coli* isolates from poultry litter. In eastern China, Xu et al. (2019) isolated APEC from infected chickens with typical lesions that had high resistance rates to TET, AMP, cefotaxime, CHL, GEN, kanamycin, STR, SXT, and NAL. The emergence and dissemination of multidrug-resistant bacteria have reduced the effectiveness of antimicrobials and may pose substantial risks for human health (Mellata, 2013). The presence of strains resistant to antimicrobials in our study should be an alert with regard to the proper use of these antimicrobials.

In conclusion, inoculating *L. animalis*, *L. reuteri*, and *L. rhamnosus* individually using commercial in ovo technology indicated no protection to decrease the incidence of APEC-like strains in broilers. Under the conditions of this study, a high level of virulence genes and antimicrobial resistance genes were detected from the isolated APEC-like strains, which is a potential threat of virulence gene transmission by plasmids among bacteria in the poultry farm. In addition, there is a high possibility for these strains to develop complications during treatment. The results obtained in this study provide valuable information to better understand the phenotypic and the molecular basis of antimicrobial resistance in APEC, which is essential for developing effective intervention methods to control antimicrobial resistance of APEC.

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DISCLOSURES

The authors have declared no conflict of interest.

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