Decreasing the abundance of tetracycline-resistant *Escherichia coli* in pig feces during nursery using flavophospholipol as a pig feed additive

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ABSTRACT

Tetracyclines (TCs) are widely used for livestock, and the high prevalence of TC-resistant Escherichia coli in livestock has become a serious concern worldwide. In Japan, the National Action Plan on Antimicrobial Resistance in 2016 aimed to reduce the TC resistance rate in E. coli derived from livestock. Flavophospholipol (FPL), used as a feed additive, has an inhibitory effect on the spread of plasmid-mediated antimicrobial resistance. The number of TC-resistant E. coli was determined in pigs administered TCs and/or FPL to clarify the effect of FPL on reducing the number of TC-resistant E. coli in pigs. TC-resistant E. coli and their plasmids were then analyzed. The pigs were divided into four groups: control, doxycycline (DOXY; a TC), FPL, and a DOXY-FPL combination. Their feces were collected from the nursing period to the day before being transported to the slaughterhouse, followed by estimation of TC-resistant E. coli (colony-forming units [CFU]/g). The number of TC-resistant E. coli increased with the use of DOXY, suggesting that DOXY administration provides a selective pressure for TC-resistant E. coli. Supplementation with FPL as a feed additive significantly suppressed the increase in the number of TC-resistant E. coli, especially during the DOXY administration period. Transfer and growth inhibition analyses were performed for TC-resistant isolates. FPL inhibited the conjugational transfer and growth of a few TC-resistant E. coli isolates. These results suggest that FPL is effective against the spread of TC-resistant E. coli.

1. Introduction

The emergence and spread of antimicrobial-resistant bacteria (ARB) and antimicrobial resistance genes (ARGs) associated with the use of antimicrobials have become a global public health concern (Munita & Arias, 2016). All antimicrobial use in any sector enhances the development and dissemination of antimicrobial-resistant bacteria and their genes (Medina & Pieper, 2016). The use of antimicrobials as veterinary medicines and feed additives has become a selective pressure for ARB/ARGs. In addition, there is a concern that ARB/ARGs may spread to the environment and humans through food, manure, and direct animal contact (Bengtsson & Greko, 2014; Ferri, Ranucci, Romagnoli, & Giaccone, 2017).

Tetracyclines (TCs) are classified as "highly important" in the "Critically Important Antimicrobials for Human Medicine" by the World Health Organization (WHO, 2017). TCs are used worldwide for livestock, including pigs (FDA, 2020; MAFF, 2012). The high TC resistance rate of *E. coli* derived from livestock, especially pigs, is a global issue. The TC resistance rate of *E. coli* derived from healthy pigs was 55% in 2018 in the Japanese Veterinary Antimicrobial Resistance Monitoring System (JVARM, 2019).

Doxycycline (DOXY), a semi-synthetic TC, is a common orally administered medicine for pigs in Japan (MAFF, 2012). However, excessive use of DOXY in weaning and nursery pigs for bacterial disease prevention can exert selective pressure on TC-resistant bacteria. Most TC resistance-related genes are encoded on mobility factors such as plasmids and transposons and are thus easily spread by horizontal gene transfer (HGT) (Roberts, 2005). HGT is an important mechanism

underlying the spread of ARB (von Wintersdorff et al., 2016). Therefore, it is supposed that the high TC resistance rate of *E. coli* is maintained by HGT, and therefore, it is necessary to take countermeasures against HGT.

Flavophospholipol (FPL) is used as a feed additive in some countries, including Japan (Butaye, Devriese, & Haesebrouck, 2003). FPL has conjugational transfer-inhibitory and plasmid-curing effects on multidrug-resistant plasmids, including TC resistance *in vitro* (Kudo et al., 2019; Poole et al., 2006). *In vivo* studies have also reported that FPL reduces the number of antimicrobial-resistant *E. coli* isolated from pig feces (Saranya, Abdolvahab, Terri, & Robert, 2018; van den Bogaard, Hazen, Hoyer, Oostenbach, & Stobberingh, 2002). In our hypotheses, FPL could reduce the TC resistance rate of livestock-derived *E. coli*.

In this study, we aimed to clarify the effect of DOXY and FPL oral administration in pig feeds on the abundance of TC-resistant *E. coli* and the spread of resistance genes. In addition, the effect of FPL on the transfer of plasmids encoding TC resistance genes was investigated.

2. Materials and methods

2.1. Animals and ethical approval

Newborn pigs (three-way crossbred pig) were obtained from Rakuno Gakuen University farm, Ebetsu City, Hokkaido, Japan. The Field Education and Research Center of Rakuno Gakuen University approved all animal experiments. Ten pigs born in February 2019 (total number of litters was ten) were equally divided into a control group and a DOXY administration group (DOXY group). Ten other pigs born in April 2019 (total number of litters was ten) were equally divided into the FPL administration group (FPL group) and DOXY and FPL combined administration group (DOXY-FPL group). The number of animals per group was set at five for justification. These pigs were siblings. These pigs were maintained under the same conditions, such as feed (except for the tested antibiotics), temperature, and humidity, as much as possible in Rakuno Gakuen University farm. Individual pigs can give birth to a limited number of piglets at one time. Therefore, the experiments were divided into two parts (each with two groups).

2.2. Experimental design

The pigs in all four groups were raised from birth to the day before transport to the slaughterhouse at 150 days of age. Avilamycin and morantel, which were preliminary included in the feed, were administered to all four groups from day D3–D60 after birth. For the DOXY group, DOXY 10% powder (Kyoritsu Seiyaku, Tokyo, Japan) was mixed with the feed. The mixed feed was provided to the pigs for 7 days (D61–D67 after birth) at 200 g (titer)/1-ton feed (Table S1). For the FPL group, Flavomycin 80 (Miyarisan Pharmaceutical, Tokyo, Japan) was mixed with the feed and provided at 500 g (titer)/1-ton feed to the weaning pigs from D36 to D60 and at 250 g (titer)/1-ton feed from D61 to D120. For the DOXY-FPL group, DOXY 10% powder and Flavomycin 80 were administered at the same concentrations and times as for the DOXY and FPL groups (DOXY 200 g (titer)/1-ton feed was administered for 7 days (D61–67), and FPL500 g (titer)/1-ton feed was administered from D36 to D60 and at 250 g (titer)/1-ton feed from D61 to D120). Each pair of group (control and DOXY; FPL and DOXY-FPL) was maintained together for 55 days from birth. They were then moved to separate groups with five pigs in each pen. Each pen was located in a different part of the same holding. The concentrations of drugs used in this study are the same as those approved for treatment drugs or feed additives in pigs in Japan.

2.3. Sample collection

Five pen-level fecal samples were taken from each experimental pen

nine times between D55 and D150 (D55, D61, D64, D68, D71, D81, D101, D121, and D150) to estimate the abundance of TC-resistant bacteria. The samples were not pooled.

2.4. Microbiological analysis

Serial (10-fold) dilutions of fecal samples suspended in phosphate-buffered saline were plated on deoxycholate hydrogen sulfide lactose (DHL) agar medium (Nissui Pharmaceutical, Tokyo, Japan) containing 16 μg/mL TC (DHL-TC; Sigma Aldrich, St. Louis, MO, USA), which specifically allows the growth of TC-resistant bacteria, to estimate the number of TC-resistant *E. coli* (colony-forming units [CFU]/g). The plates were incubated at 37 °C for 14–18 h. Pink colonies on the plates were identified as *E. coli,* and further, two to three colonies/plate were confirmed as *E. coli* using the MALDI Biotyper system (Bruker Daltonics, Bremen, Germany).

2.5. Plasmid analysis

2.5.1. Tetracycline resistance genes, replicon typing, and plasmid profiling

Forty-one TC-resistant *E. coli* strains were randomly selected from each tested group (11, 10, 10, and 10 from the Control, DOXY, FPL, and DOXY-FPL groups, respectively) for further analysis (Table 1). DNA (including chromosome and plasmid) was extracted using a Cica Geneus DNA extraction kit (Kanto Chemical, Tokyo, Japan). The presence of resistance genes encoding *tetA, tetB,* and *tetC* was detected by multiplex PCR, as previously described (Jun et al., 2004).

Plasmid incompatibility (Inc) groups were determined using multiplex PCR (Johnson et al., 2007), the extracted DNA (including chromosome and plasmid) was used as a template. Plasmid profiling was performed as described previously (Kado & Liu, 1981).

2.5.2. Nanopore sequencing

The nucleotide sequence of the three representative plasmids (Table 2) was determined by nanopore sequencing, using MinION (Oxford Nanopore Technologies, Oxford, UK). Bacterial genomic DNA was extracted using the Genomic-tip 20/G and Genomic DNA Buffer Set (QIAGEN, Hilden, DE). Library was prepared using the Rapid Barcoding Sequencing kit SQK-RBK004 (Oxford Nanopore Technologies), according to the manufacturer's protocol. All bead washing steps were performed using AMPure XP beads (Beckman Coulter, CA, USA). Sequencing was performed on MinION with FLO-MIN-106 R9.4 Flow cell (Oxford Nanopore Technologies) using MinKNOW software for the entire 48-h run time with no alterations to any voltage scripts. De novo assembly was performed using Flye v2.8.3 (https://pubmed.ncbi.nlm.nih.gov/30936562/) with default parameters (Kolmogorov, Yuan, Lin, & Pevzner, 2019). The assembled sequences of circulation were confirmed using Flye v2.8.3. Geneious (Tomy Digital Biology, Tokyo, Japan) was performed to determine the plasmid size using the nucleotide sequence data. Assembled sequences were determined to be a

Table 1
tet gene prevalence and replicon types of 41 tetracycline (TC)-resistant *E. coli*.

Inc	*tetA* (30 strains)	*tetB* (11 strains)
FIB	100% (30)	100% (11)
Frep	100% (30)	54.5% (6)
FIA	36.7% (11)	9.1% (1)
B/O	6.7% (2)	72.7% (8)
Y	0% (0)	72.8% (8)
P	20.0% (6)	0% (0)

Forty-one TC-resistant *E. coli* strains were randomly selected from each tested group (11, 10, 10, and 10 from the Control, DOXY, FPL, and DOXY-FPL groups, respectively). Thirty *tetA*-positive (10, 9, 6, and 5 from the Control, DOXY, FPL, and DOXY-FPL groups, respectively) and 11 *tetB*-positive (1, 1, 4, and 5 from the Control, DOXY, FPL, and DOXY-FPL groups, respectively) *E. coli* strains were selected.

Table 2

Three representative tetracycline (TC)-resistant strains and the tetracycline resistance plasmids harbored.

Strain Name	Plasmid name	Plasmid size (kbp)	Replicon type	Antimicrobial resistance genes
I66	pI66	103.5	FIB	*tetA, blaTEM-1B, mdfA, aph I b, aph(6')-Id*
P63	pP63	170	FIA	*tetA, blaTEM-1B, mdfA, aph (3')-I, sul2*
K38	pK38	110	B/O/K/Z	*tetB, blaTEM-156, blaTEM-1A, mdfA, sul2, aadA1, dfrA1*

plasmid based on the length of the circulation sequences. The plasmid Inc type and ARGs were determined using Plasmid Finder and Res Finder of the Center for Genomic Epidemiology (CGE, 2020). The nucleotide sequences of pI66, pP63, and pK38 have been submitted to the DNA Data Bank of Japan with accession numbers LC620533, LC620535, and LC620534, respectively.

2.6. Plasmid transfer

2.6.1. First-round transfer with FPL

The strains listed in Table 2 were used as donor strains and subjected to a transfer test (Kudo et al., 2019). Rifampicin (RIF)-resistant *E. coli* DH5α was used as the recipient strain. The filter mating method was used to determine the inhibitory effect of FPL on conjugative transfer. Briefly, the donor strain was incubated overnight at 37 °C in 2 mL of tryptic soy broth (TSB). Next, 100 μL of the overnight culture was added to 2 mL of TSB containing 0, 0.5, 1, 2, 4, 8, 16, 32, and 64 μg/mL FPL. The recipient strain was incubated overnight at 37 °C in 5 mL of TSB. Next, 250 μL of the donor and recipient cultures was added to 4.5 mL of TSB and mixed. In the filtration step, all cultures were collected on a membrane filter (ADVANTEC, Tokyo, Japan; pore size 0.22 μm). The filter was placed on tryptic soy agar (TSA), supplemented with FPL (concentration as described above), and incubated overnight at 37 °C. The filter was transferred into a centrifuge tube containing 0.85% saline (0.5 mL) and washed three times. This solution was serially diluted 10-fold with 0.85% saline, and 10 μL of this solution was spotted on TSA plates supplemented with selective antibiotics as follows. The donor-selective plate was supplemented with 16 μg/mL TC, the recipient-selected plate was supplemented with 50 μg/mL RIF (Sigma Aldrich), and a transconjugant (TC strain)-a selective plate was supplemented with 16 μg/mL TC and 50 μg/mL RIF. The plates were incubated overnight at 37 °C, and the colonies were counted. Transfer of the plasmid in the TC strain was verified by PCR analysis and plasmid profiling, as described above. This test was performed three times, and the transfer frequency was calculated as the number of TC strains (CFU/mL) per number of donors (CFU/mL).

2.6.2. Second-round transfer with FPL

The second-round transfer experiment was performed following the same method as the first-round transfer experiment to evaluate the effect of FPL on plasmids. The TC strains obtained from the first-round experiment were used as the donor strains. Ciprofloxacin (CPFX)-resistant *E. coli* DH5α was used as the recipient strain (Kudo et al., 2019). A donor-selective plate was supplemented with 16 μg/mL TC and 50 μg/mL RIF, a recipient-selective plate was supplemented with 1 μg/mL CPFX (Sigma Aldrich), and a transconjugant strain-selective plate was supplemented with 16 μg/mL TC and 1 μg/mL CPFX. Verification of plasmid transfer in the transconjugant strain and calculation of the transfer frequency were performed as those in the first round of the experiment.

2.7. Growth curve analysis

Three transconjugants from donor strains listed in Table 2 were subjected to growth curve analysis. *E. coli* DH5α was used as a control strain. The strain was incubated overnight in 1 mL of TSB at 37 °C. The culture was aligned with TSB, its optical density was adjusted to 0.1 at 600 nm (OD_{600}), and 200 μL of it was dispensed into 96-well plates. OD_{600} values were periodically measured using a Nivo multimode microplate reader (PerkinElmer, Waltham, MA, USA), and growth curve analysis was performed under the following conditions: temperature, 37 °C; shaking, 600 rpm; interval, 15 min; and time, 16 h.

2.8. Growth inhibition effect of FPL

Three transconjugants from donor strains listed in Table 2 were subjected for analyzing growth inhibitory effect of FPL. The strain was incubated overnight in 1 mL of TSB at 37 °C. The culture was aligned with TSB, its optical density was adjusted to 0.1 at 600 nm (OD_{600}), and 175 μL of it was dispensed into 96-well plates. Next, 25 μL of FPL solution was added, and the final concentration was adjusted to 0, 1, 2, 4, 8, 16, 32, and 64 μg/mL. The plate was incubated at 37 °C with shaking at 600 rpm. After 20 h treatment, serial (10-fold) dilutions of the treated samples suspended in phosphate-buffered saline were plated on Mueller–Hinton agar to estimate the number of *E. coli* (CFU/g). The plates were incubated at 37 °C for 14–18 h. Isolates on the plates were counted for estimating the number of *E. coli* (CFU/mL).

2.9. Plasmid-curing experiment

The strains listed in Table S2 were incubated overnight in 1 mL of TSB at 37 °C. After that, 50 μL of the culture was added to 1 mL of TSB without antibiotics and 1 mL of TSB with 16 and 32 μg/mL FPL, respectively, and incubated overnight at 37 °C. The culture was serially diluted with 0.85% saline, and 100 μL of this solution was spread on TSA without antibiotics and incubated overnight at 37 °C. A total of 384 colonies were randomly picked from the plate and transferred to four 96-well plates (AS ONE, Osaka, Japan) containing 200 μL of TSB and incubated overnight at 37 °C. Subsequently, the culture was transferred to a square plate (Eiken Chemical, Tokyo, Japan) with a transfer set (Tokyo Glass Kiki, Tokyo, Japan), TSA supplemented with 50 μg/mL RIF to grow all bacteria, and TSA supplemented with 16 μg/mL TC and 50 μg/mL RIF for screening plasmid-cured strains. Colonies that grew only in the TSA supplemented with RIF were counted as plasmid-cured strains. The colonies were subjected to plasmid profiling, as described above, to confirm the plasmid-curing effect. *E. coli* TC7–1, plasmid-cured by 8 μg/mL FPL, was used as a positive control strain (Kudo et al., 2019).

2.10. Antimicrobial susceptibility testing

The susceptibility to FPL was determined using the Broth Microdilution method. The test was performed according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI). The inoculum concentration was adjusted to 5×10^5 CFU/mL. The incubation temperature and time were 35 °C and 16 h, respectively.

2.11. Statistical analysis

CFU counts among the four groups were analyzed using Tukey's multiple comparison test. Briefly, CFU counts were transformed using the logarithm, and transformed data were examined for normal distribution using the Shapiro-Wilk test. Tukey's multiple comparison test was performed after confirming that the data followed a normal distribution. CFU counts within the group were analyzed using a two-tailed unpaired *t*-test. To analyze the effect of the presence of FPL *in vitro* on the CFU counts for transconjugants, test groups were compared with the

control using log-transformed CFU data by Dunnett test. Differences among the mean values were considered significant at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$).

3. Results

3.1. Changes in the abundance of TC-resistant E. coli in pigs administered DOXY/FPL

The abundance of TC-resistant *E. coli* from pig feces during the DOXY administration period significantly increased on D64 compared with the Control group (Fig. 1). After the DOXY administration period (D71), the abundance of tetracycline-resistant *E. coli* in pig feces decreased substantially compared with D64 (Fig. 1). The abundance of TC-resistant *E. coli* obtained from pig feces from the FPL and DOXY-FPL groups was lower than that from the DOXY group. On D64, the abundance of TC-resistant *E. coli* in the FPL administration group was significantly lower than that in control group (Fig. 1B).

We compared the abundance of TC-resistant *E. coli* within the group. In the control group, the abundance of TC-resistant *E. coli* on D64, 68, and 71 was significantly lower than that on D55. In the DOXY group, the abundance of TC-resistant *E. coli* on D61 and 74 was significantly higher than that on D55. In the FPL group, the abundance of TC-resistant *E. coli* on D61, 64, 68, 71, 81, and 101 was significantly lower than that on D55. In the DOXY-FPL group, the abundance of TC-resistant *E. coli* on D61, 64, 68, 71, and 101 was significantly lower than that on D55.

3.2. Plasmid profiling and replicon typing

Among 41 tested TC-resistant *E. coli*, 30 *tetA*-positive (10, 9, 6, and 5 from the Control, DOXY, FPL, and DOXY-FPL groups, respectively) and 11 *tetB*-positive (1, 1, 4, and 5 from the Control, DOXY, FPL, and DOXY-FPL groups, respectively) *E. coli* strains were detected (Table 1). No *tetC*-positive strains were found. All 41 TC-resistant *E. coli* strains were positive for IncFIB, as determined by replicon typing (100%). By plasmid profiling, 41 plasmids harboring *tet* were divided into three groups based on the plasmid length (approximately 100, 110, and 170 kbp). In the 100-kbp plasmids (19/41), both *tetA* and IncFIB replicon

were always detected; in the 110-kbp plasmids (8/41), both *tetB* and IncB/O replicon were always detected; and in the 170-kbp plasmids (4/41), both *tetA* and IncFIA replicon were always detected.

The nucleotide sequences of the three representative plasmids were determined by long-read sequencing (Table 2). These three plasmids harbored other antibiotic resistance genes, such as bla_{TEM} and *sul2*.

3.3. Transfer frequency of three representative TC resistance plasmids with FPL

The minimum inhibitory concentration against FPL was 256 µg/mL for each of the three representative isolates.

Fig. 2 shows the transfer frequency of the three TC-resistant *E. coli* with FPL. The transfer frequency decreased with the concentration of FPL only for K38, whereas a decreasing effect of transfer frequency by FPL was not observed in I66 and P63. The transfer frequency of I66 was lower than that of P63 and K38 without FPL. In the second round of transfer, the transfer frequency of the pK38 plasmid (TC1–10) decreased with the concentration of FPL, similar to the donor strains (K38) (Fig. S1). In contrast, the transfer frequency of the pP63 plasmid (TC1–6) was not decreased by FPL, the same as the donor strain (P63). The pI66 plasmid (from TC1–2) was not transferred to the recipient strain during the second transfer round.

The growth ability of transconjugants TC1–6 and TC1–10 was significantly decreased compared with that of the recipient strain (Fig. S2).

3.4. Growth-inhibitory effect of FPL

In the presence of FPL, for TC1–2 and TC1–6, growth inhibition was significantly observed when exposed to more than 32 µg/mL FPL compared with no exposure to FPL (Fig. 3). Growth inhibition was observed in TC1–10 at an FPL concentration of 64 µg/mL.

3.5. Plasmid-curing effect of FPL

The plasmid-curing effect of FPL in the three tested isolates was not observed in this study (Table S2). TC7–1, which was used as a positive
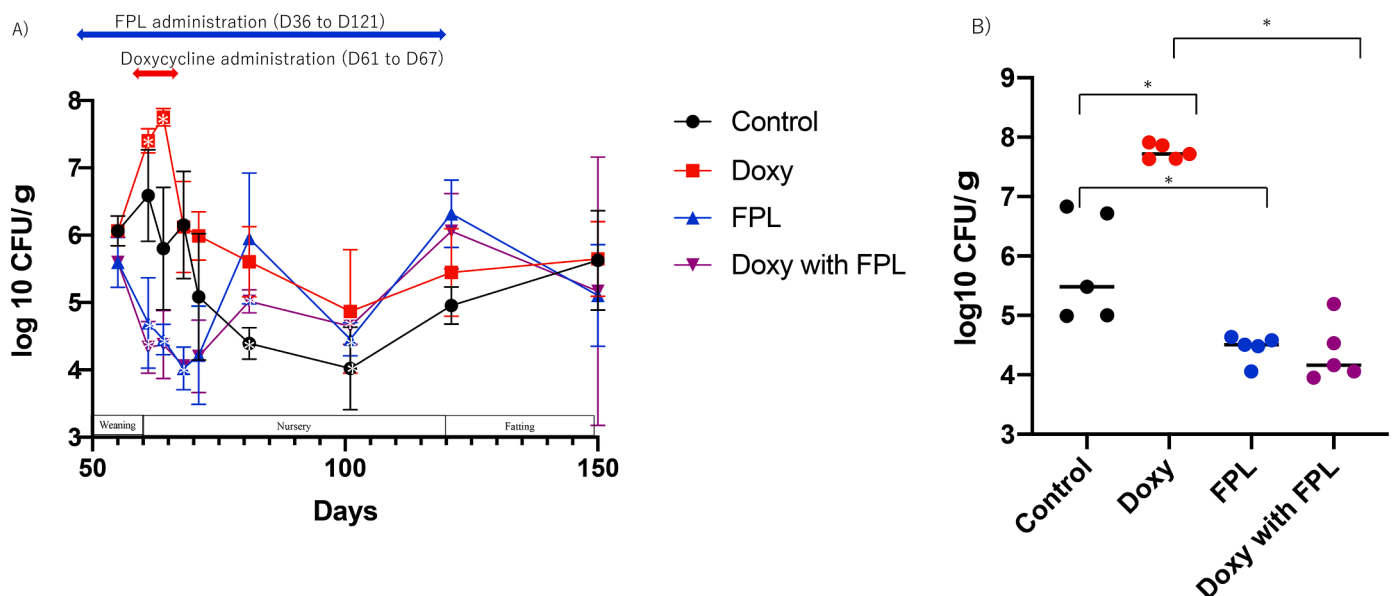

Fig. 1. (A): Number of tetracycline (TC)-resistant *E. coli* detected in the fecal samples of pigs ($n = 5$) from D55 to D150. In the doxycycline (DOXY) and DOXY with flavophospholipol (FPL) groups, the pigs were administered DOXY from D61 to D67. In the FPL and DOXY with FPL groups, the pigs were fed FPL from D36 to 121. Statistical analyses were performed using a two-tailed unpaired *t*-test compared with the abundance of TC-resistant *E. coli* on D55 within the group (*$p < 0.05$). (B): Comparison of the abundance of TC-resistant *E. coli* among the four groups during the DOXY administration period (D64). Statistical analyses were performed using Tukey's test (*$p < 0.05$, **$p < 0.01$).
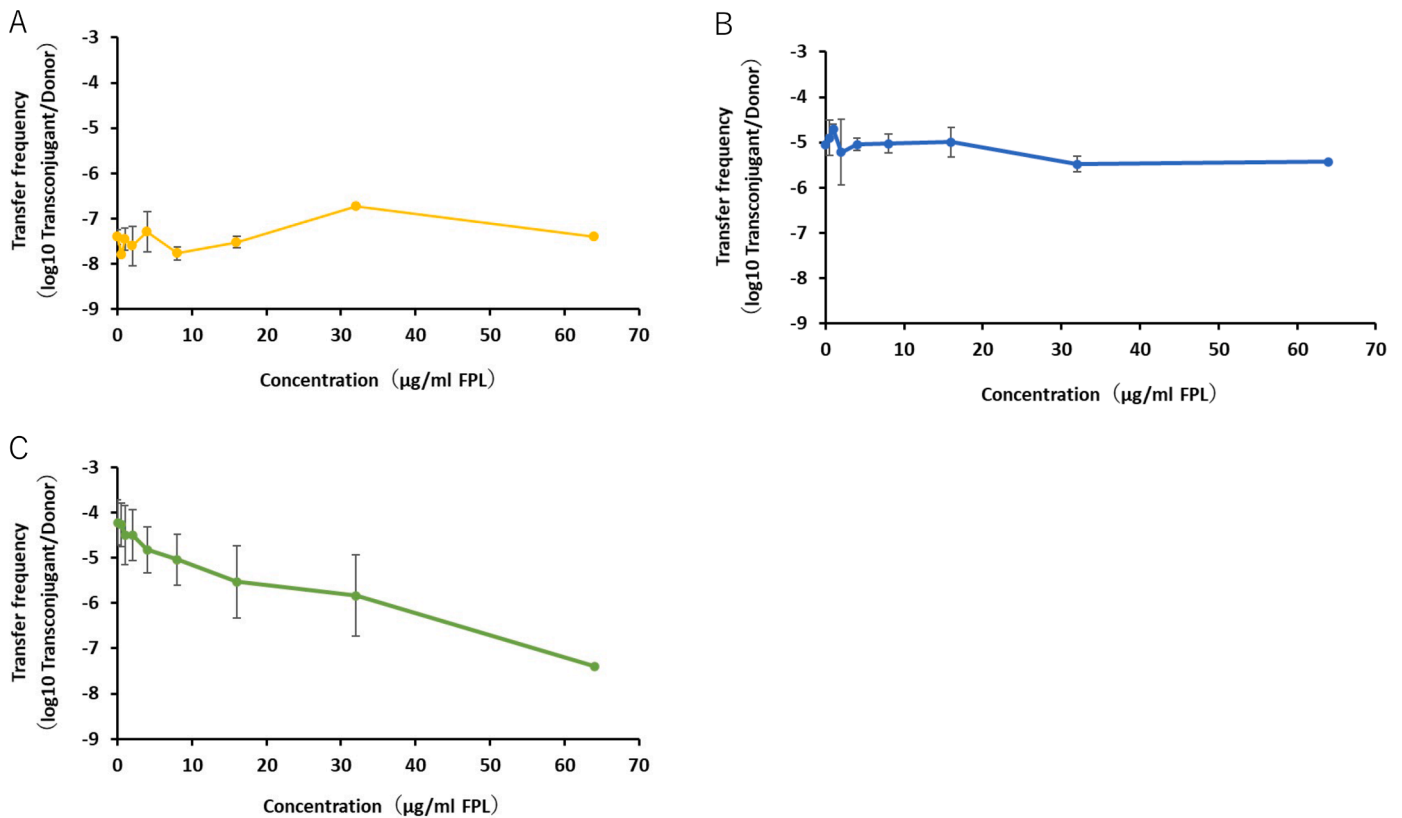
Fig. 2. Transfer frequency of three tetracycline (TC)-resistant *E. coli* in the presence of flavophospholipol (FPL). (A) Donor: I66 (*tetA*, IncFIB). (B) Donor: P63 (*tetA*, IncFIA). (C) Donor: K38 (*tetB*, IncB/O/K/Z).
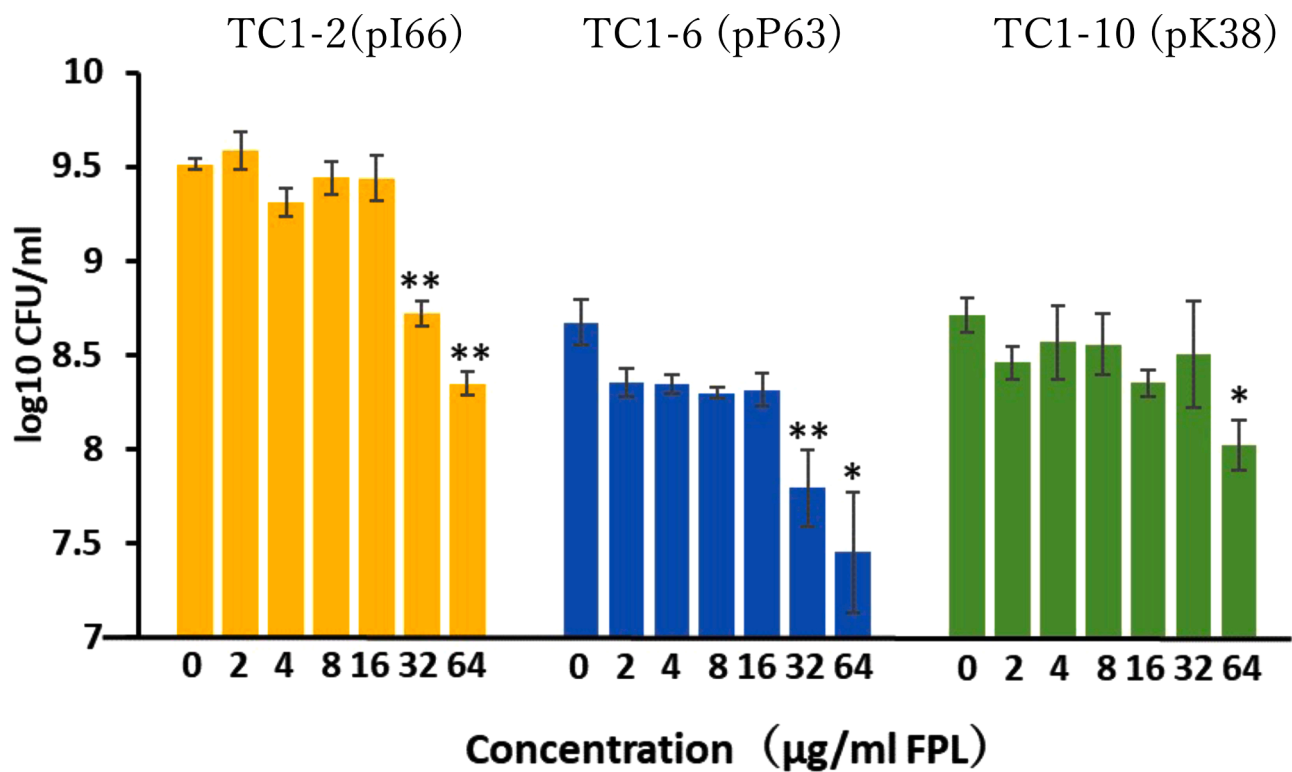

Fig. 3. Growth-inhibitory effect of flavophospholipol (FPL) on transconjugants compared with no exposure to FPL. The vertical axis indicates the number of bacteria (CFU/mL), and the horizontal axis indicates the FPL concentration (µg/mL). All data are shown as mean ± SEM. Statistical analyses were performed using Dunnett test (*$p < 0.05$, **$p < 0.01$).

control strain, was cured by 6.3% and 16.9% at FPL concentrations of 8 and 16 μg/mL, respectively (Kudo et al., 2019).

4. Discussion

This study showed that the abundance of TC-resistant E. coli decreased in FPL pigs and the DOXY-FPL group compared with that in the DOXY and the control group. FPL inhibited the conjugational transfer and growth of TC-resistant *E. coli* isolates. FPL affected the *tetA/tetB*-harboring plasmids.

The abundance of TC-resistant *E. coli* in the DOXY group was significantly higher than that in the control group during the DOXY administration period (D64). The abundance of TC-resistant *E. coli* was high even before the administration. This high abundance might be because TC-resistant *E. coli* has stabilized in the tested pig farm. DOXY administration would select the TC-resistant *E. coli.* In previous studies, a temporary increase in the number of TC-resistant bacteria was also observed in the feces of beef cattle orally administered chlortetracycline; however, no long-term effects were observed (Agga, Schmidt, & Arthur, 2016). In the growth curve analysis, acquisition of two plasmids, pP63 (*tetA*-harboring plasmid) and pK38 (*tetB*-harboring plasmid), among the three predominant plasmids decreased the growth ability of the host. It may depend on the biological cost of the maintenance of plasmids. Compensatory mutations may decrease the biological cost of maintaining plasmids in the gene or host chromosome (Hernand-Amado, Sanz-Garcia, Blanco, & Martinez, 2017). In contrast, the transferability of the remaining plasmid, pI66 (*tetA*-harboring plasmid), was notably lower than that of the other plasmids. Environmental factors could also affect the maintenance of TC-resistant plasmids. TC-resistant plasmids might be temporarily selected by DOXY administration, and then their abundance might have decreased owing to the maintenance burden, low transferability, or other selection mechanisms.

The abundance of TC-resistant bacteria was lower in the FPL and DOXY-FPL groups than in the other groups. In Japan, FPL is permitted as a feed additive at 2–10 g (titer)/ton for weaning piglets and 2 g (titer)/ton for pigs approximately 60–120-days-old (FAMIC, 1976). Because FPL is hardly absorbed from the intestine and most of it is excreted in feces, it is expected that the concentration of FPL in the intestines is close to 5–10 μg/mL (Kudo et al., 2019). In previous studies, the abundance of plasmid-mediated TC-resistant *E. coli* was significantly reduced in cattle and pigs treated with 9 mg/kg FPL (Pfaller, 2006). These results suggest that the administration of FPL to livestock can reduce the abundance of plasmid-mediated ARB.

TC resistance in this study was mostly caused by *tetA* and *tetB*, similar to that in previous studies (Koo & Woo, 2011; Usui, Shirakawa, Fukuda, & Tamura, 2015). These results suggest that *tetA*- or *tetB*-harboring plasmids are spreading in the Japanese farms. In this study, plasmids harboring three types of *tet* genes were spread on the farm throughout the study period. Therefore, to control TC resistance in farms, decreasing the abundance of these plasmids is required.

The transfer frequency of pK38 was inhibited by over 8 μg/mL FPL. In previous studies, the transfer frequency of extended-spectrum β-lactamase (ESBL)-producing cephalosporin resistance gene coding plasmid was inhibited by over 2 μg/mL FPL (Kudo et al., 2019). In addition, another study showed that 2 μg/mL FPL completely blocked the transfer of resistance genes in transfer experiments using wild strains as recipient strains (Poole et al., 2006). Although the growth-inhibitory effect was observed in two of the predominant plasmids (pI66 and pP63) in transconjugants, the transfer frequency of these plasmids was not inhibited by FPL. FPL has been reported to inhibit the growth of multidrug-resistant *E. coli*, including ESBL-producing *E. coli* and TC-resistant *E. coli* (pI66 and pP63). The plasmid-curing effect of FPL was not observed in this experiment. Many plasmids encode addiction systems to prevent plasmid loss (Tsang, 2017). Although plasmid addiction genes were not detected in the tested plasmids in this study, an unknown plasmid addiction system may prevent their loss. FPL has a plasmid-curing effect on IncN, IncFIB, and IncI1-Iγ type plasmids encoding ESBL-producing cephalosporin resistance genes (Kudo et al., 2019). Isothiocyanate and pectin-capped platinum nanoparticles have plasmid-curing effects; however, their effects are limited to several replicon-type plasmids (Bharathan et al., 2019; Kwapong, Stapleton, & Gibbons, 2019). These results showed the possibility that FPL has an inhibitory effect on conjugational transfer and growth in plasmid-carrying strains depending on the plasmid type. This would decrease the number of ARB mediated by antimicrobial resistance plasmids, including *tet* genes and ESBL-producing gene coding plasmids in the field.

The growth inhibition effect of FPL against transconjugants was observed at at least 32 μg/mL concentration in this study. This concentration is much higher than the intestinal concentrations when FPL is used according to the national instructions. These results suggest that the decrease in the number of TC-resistant *E. coli* in the field was not dependent on the growth inhibition effect of FPL.

In this study, the abundance of TC-resistant *E. coli* in the pigs of all tested groups (control, DOXY, FPL, and DOXY-FPL) before being transported to the slaughterhouse was not significantly different. However, the high abundance of TC-resistant *E. coli* in the weaning and nursery periods would play an important role as a reservoir of TC resistance in farms. Therefore, decreasing the abundance of TC-resistant *E. coli* during these periods in the farms is important for controlling TC resistance. Many pig farms in Japan currently use TCs, such as DOXY to prevent bacterial infections. Appropriate and careful use of TCs is required to decrease the abundance of TC resistance in pigs.

The study has limitations because the study period for the FPL/DOXY-FPL group and that for the other groups was different. Furthermore, the study was conducted on a non-commercial university farm. There is a limit to the number of individual pigs that can give birth at one time. Therefore, the experiments were divided into two parts (two groups each). These specific conditions may affect the results.

In conclusion, using FPL as a feed additive in pigs may reduce the abundance of TC-resistant *E. coli* in pig feces depending on the FPL concentration. Combined with the careful use of veterinary medicines, including DOXY, FPL may reduce the high TC resistance rate of *E. coli* worldwide. It is necessary to clarify the mechanism of action of FPL on ARB/ARGs to confirm these possibilities.

5. Funding

6. Availability of data and materials

The data supporting the findings are presented in the manuscript. The corresponding author can also be reached for any data inquiry.

7. Ethics approval

The study was approved by The Field Education and Research Center of Rakuno Gakuen University. All methods were carried out following relevant guidelines and regulations.

Declaration of Competing Interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.vas.2022.100236.

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