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RES-213

Dynamic Changes in Tetracycline Resistance Induced by Oxytetracycline as a Pig Feed Additive and the Modulatory Impact of *Clostridium butyricum* MIYAIRI 588

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BACKGROUND

- Antimicrobial use is the major driver for antimicrobial resistance (AMR) in humans and animals in general.
- Tetracyclines (TC) are largely used in livestock animals, especially in pigs for disease. It is reported that the percentage of isolates resistant to TC in *Escherichia coli* of pigs was 55.1% in Japan in 2022.
  National Veterinary Assay Laboratory, Japan. (**2025**). *Nippon AMR One Health Report 2024*.
- The culture supernatant of *Clostridium butyricum* inhibits the growth of ESBL-producing *Escherichia coli*, reduces its β-lactamase enzymatic activity and gene expression, and has an inhibitory effect on transmissibility of antibiotic resistance gene.
  Kunishima H, *et al. J. Infect. Chemother.* **2019**, *25*, 894-900.
- In this study, we examined the alteration of antimicrobial resistance organism (AMO) and/or antimicrobial resistance genes (ARGs) in the gastrointestinal tract during antibiotic use in fattening piglets and possible effectiveness of probiotic treatment.

MATERIALS & METHODS

Animal	LWD piglets, 8 castrated males and 11 females, 13-14 weeks old
Rearing method	Individual housing, ad libitum access to feed and water
Antibiotic	"Oxytetracycline (OTC) for animals 10% powder" (Kyoritsu Seiyaku), 400mg (potency) /kg feed
Probiotic	*C. butyricum* MIYAIRI 588 (CBM) powder (Miyarisan Pharmaceutical), 2.5×10^8 CFU/kg feed

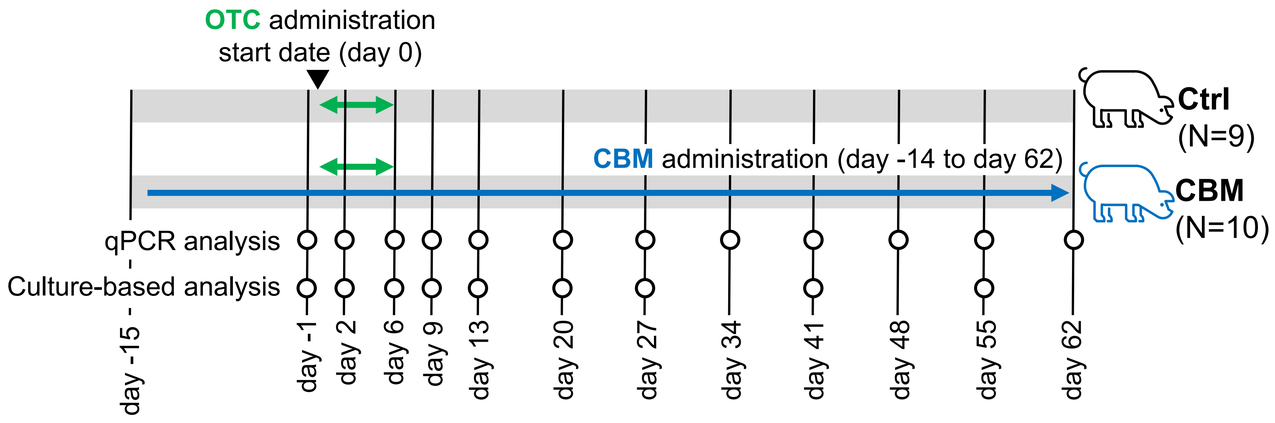


Figure 1. Dosage schedule design, sampling periods and analysis items.

Fecal samples from each pig were collected at 12 different time points and subjected to an estimation of the number of TC-resistant *E. coli* or to qPCR analysis.

Quantitative PCR (qPCR) analysis of ARGs

The fecal DNA was extracted by bead-beating method. TC resistance genes (*tetA*, *tetM*, *tetW* and *tetX*) were quantified by qPCR and normalized by the 16S rRNA gene.

Enumeration of TC-resistant *E. coli*

Dilutions of fecal suspension were plated on DHL agar medium (Shimadzu Diagnostics) containing 16 μg/mL TC to enumerate the number of TC-resistant *E. coli* (CFU/g). MALDI-TOF MS (Bruker Daltonics) is used for species identification.

In vitro growth inhibition assay

The growth of isolated TC-resistant *E. coli* was examined in BHI broth under the presence of cell free culture supernatant of *C. butyricum* MIYAIRI 588. Culture of resistant strain was sampled with time; the number of live bacteria were examined. Modified Drigalski agar (Becton Dickinson) was used for enumeration for resistant bacteria.

RESULTS

qPCR analysis of ARGs

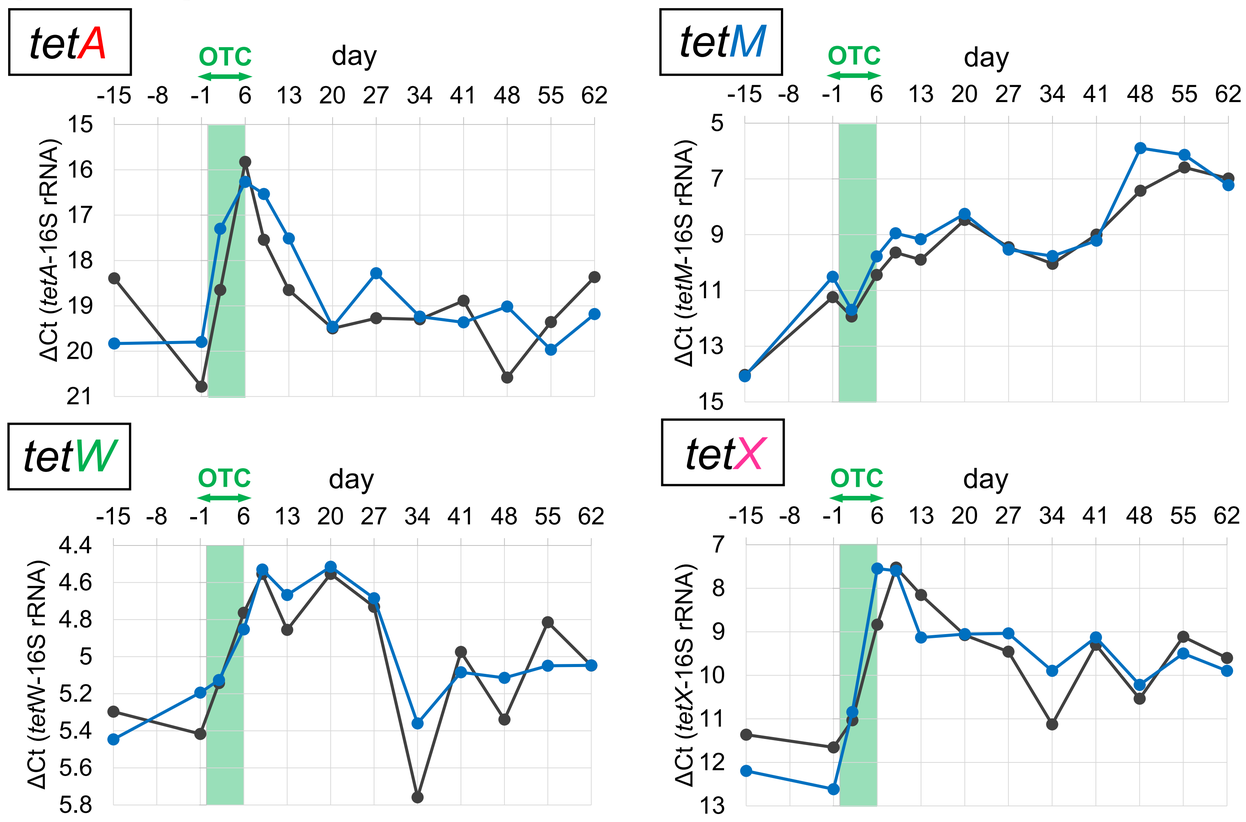
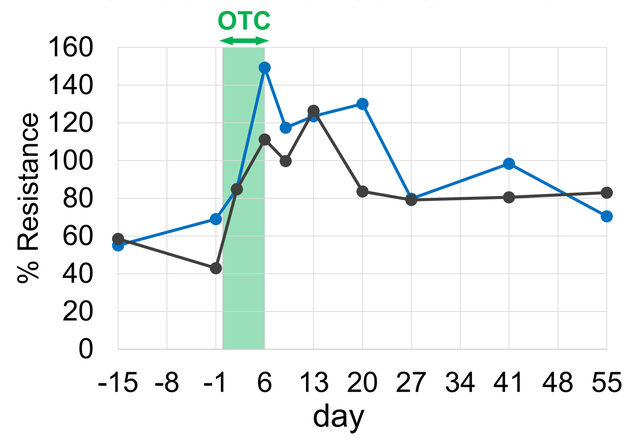


Figure 2. Changes in TC resistance-related genes levels. **Gray**: **control**; **Blue**: **CBM**

- Tetracycline resistance-related genes increased drastically after 1 week OTC treatment. After discontinuation of OTC treatment, the *tetA*, *tetW* and *tetX* genes gradually decreased but *tetM* was persistently identified in the fecal specimens.

Enumeration of TC-resistant *E. coli*



- The rate of TC resistance of isolated *E. coli* increased to 100% from 60% at baseline after 1 week OTC treatment.

$$\%\ \text{Resistance} = \frac{\text{CFU of } \textit{E. coli} \text{ grown on DHL agar } \textbf{with} \text{ 16 μg/mL TC}}{\text{CFU of } \textit{E. coli} \text{ grown on DHL agar } \textbf{without} \text{ TC}} \times 100\ (\%)$$

Figure 3. The rate of TC resistance of isolated *E. coli*. **Gray**: **control**; **Blue**: **CBM**

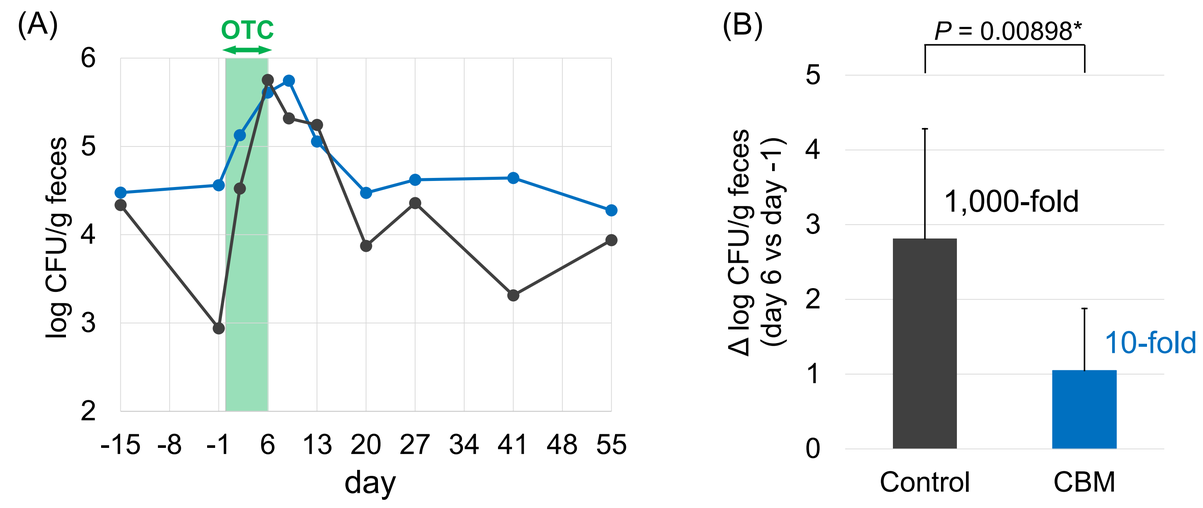


Figure 4. The number of TC-resistant *E. coli*. (A) Changes in bacterial counts between day -15 and day 55. (B) Increase in bacterial counts at day 6 compared to day -1. *Mann–Whitney U test. **Gray**: **control**; **Blue**: **CBM**

- After administration of OTC, the number of TC-resistant *E. coli* increased approximately 1,000-fold (from 2.94 to 5.75 log CFU/g feces) in the control group. On the other hand, only a 10-fold increase (from 4.56 to 5.61 log CFU/g feces) was observed in the CBM group. The increase in bacterial counts from day -1 to day 6 between two groups was significantly lower in the CBM group.

In vitro growth inhibition assay

Table 1
MICs and Types of ARGs in TC-resistant *E. coli* in this study

Strain	MIC (μg/mL)	Types of ARGs		
		tetA	*tetB*	$bla_{\text{CTX-M}}$
E. coli strain no.1	TC=64	+	−	−
E. coli strain no.2	TC=32	−	+	−
E. coli strain no.3	TC=32,CPFX=4	+	−	−
E. coli strain no.4	TC=64,CPFX=8,CTX≧256	+	−	+

TC: tetracycline; CPFX: ciprofloxacin; CTX: cefotaxime

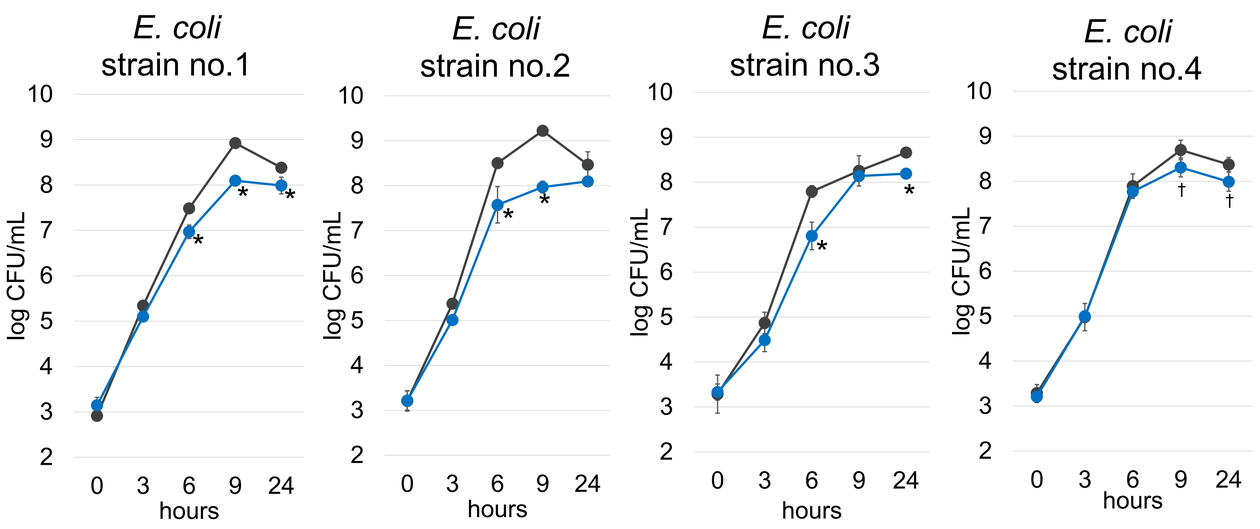


Figure 5. Effects of culture supernatant of *C. butyricum* MIYAIRI 588 on growth of TC-resistant *E. coli*. **Gray**: **control** (culture supernatant **0%**); **Blue** (culture supernatant **100%**). Data were shown as mean ± SD. *: $p < 0.05$ vs Control and †: $p < 0.1$ vs Control by Student's *t*-test.

- The growth of TC-resistant *E. coli* strains were suppressed by the culture supernatant of *C. butyricum* MIYAIRI 588.

CONCLUSIONS

- After 1 week OTC treatment, tetracycline resistance-related genes increased drastically and the rate of TC resistance of isolated *E. coli* increased to 100%.
- The number of TC-resistant *E. coli* increased approximately 1,000-fold by OTC treatment. On the other hand, only 10-fold increase was observed in the CBM treated group.
- The inhibitory effects of culture supernatant of *C. butyricum* MIYAIRI 588 strain on the growth of several TC-resistant *E. coli* strains were observed.
- These results demonstrated that *C. butyricum* MIYAIRI 588 supplementation may mitigate the increase of AMO and ARGs induced by antibiotic treatment.