



# Tryptanthrin Reduces *Campylobacter jejuni* Colonization in the Chicken Gut by a Bactericidal Mechanism

Taketoshi Iwata,<sup>a</sup> Ayako Watanabe-Yanai,<sup>a</sup> Yukino Tamamura-Andoh,<sup>a</sup> Nobuo Arai,<sup>a</sup> Masato Akiba,<sup>b</sup>  Masahiro Kusumoto<sup>a,c</sup>

<sup>a</sup>Division of Zoonosis Research, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan

<sup>b</sup>Laboratory of Veterinary Bacteriology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan

<sup>c</sup>Graduate School of Veterinary Science, Osaka Metropolitan University, Izumisano, Osaka, Japan

**ABSTRACT** *Campylobacter jejuni* is a leading cause of foodborne bacterial gastroenteritis worldwide, and raw or undercooked chicken meat is considered the major source of human campylobacteriosis. In this study, we identified 36 compounds that showed inhibitory effects on *C. jejuni* growth at low concentrations by screening a chemical compound library. Three of the 36 compounds were herbal compounds, including tryptanthrin (TRP), an indoloquinazoline alkaloid. TRP has been reported to have a variety of biological properties, such as antimicrobial, anti-inflammatory, and antitumor activities, but there was previously no information about its anti-*C. jejuni* activity. We further conducted *in vitro* and *in vivo* experiments to evaluate the potential of TRP for the control of *C. jejuni* in chicken farms. The MIC of TRP for *C. jejuni* was much lower than that of 13 other herbal compounds that were previously reported to have anti-*C. jejuni* activities. Time-kill assays under growing and nongrowing conditions demonstrated that TRP has bactericidal activity against *C. jejuni*. In addition, TRP showed a narrow-spectrum antimicrobial effect against *C. jejuni*, and there was little potential for the development of TRP-resistant *C. jejuni* during serially passaged culture. In chick infection experiments, the administration of TRP in drinking water significantly reduced the cecal colonization of *C. jejuni* when TRP was used either before or after *C. jejuni* infection. These data suggest that TRP is effective for the control of *C. jejuni* in chicken farms.

**IMPORTANCE** *Campylobacter* is a widespread pathogen in the food chain of chickens. Once chickens become infected, large numbers of *Campylobacter* cells are excreted in their feces. The development of an effective material for reducing the amount of *Campylobacter* in the chicken intestinal tract will make it possible to reduce the contamination of the food chain with *Campylobacter* and to produce safe and secure chicken meat. In the present study, *in vivo* experiments revealed that the use of an herbal compound, tryptanthrin, significantly reduced the number of *Campylobacter* cells in the chicken gut by a bactericidal mechanism. Furthermore, our *in vitro* experiments demonstrated that, compared with the other herbal compounds, tryptanthrin achieved antimicrobial activity against *C. jejuni* at the lowest concentration. The use of tryptanthrin may lead to the development of a novel control measure for reducing the colonization of *C. jejuni* in the food chain.

**KEYWORDS** *Campylobacter jejuni*, chemical compound library, chicken, herbal compound, tryptanthrin

**C**ampylobacter is the most common cause of bacterial foodborne disease in humans worldwide. The World Health Organization (WHO) estimated that more than 95 million cases of foodborne illness caused by *Campylobacter* species occurred worldwide in 2010 (1). In the European Union, the annual cost associated with human campylobacteriosis was estimated to be €2.4 billion (2). Contamination by *Campylobacter jejuni* and

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Address correspondence to Masahiro Kusumoto, kusu555@affrc.go.jp.

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*Campylobacter coli* throughout chicken food supply chains, from farm to commercial meats, is responsible for the frequent occurrence of this infection in humans (3–5). Of the two *Campylobacter* species, *C. jejuni* is the predominant cause of campylobacteriosis in many countries, including those in the European Union and the United States (6, 7).

Because a large amount of *C. jejuni* (up to  $10^8$  or  $10^9$  cells per gram of feces) is present in the feces of chickens infected with this bacterium (8), chicken farm environments are highly contaminated with *C. jejuni*: the corresponding infection is rapidly disseminated throughout chicken flocks within farms and is therefore easily transmitted to other chicken farms through wildlife, insects, and vendors (5, 9). It is difficult to control the *C. jejuni* contamination of carcasses from chicken intestinal contents by current treatment techniques because the chicken body surface is also highly contaminated by *C. jejuni* via feces (10, 11). Therefore, the widespread contamination of the food supply chains is attributed to the large amounts of *Campylobacter* present in the intestinal tracts of infected chickens.

Previous studies showed that an approximately 10-fold or thousandfold reduction in *C. jejuni* cells on chicken carcasses significantly reduced the incidence of campylobacteriosis in humans (12, 13). Therefore, a reduction in the number of *C. jejuni* in the chicken gut is an essential strategy for controlling campylobacteriosis in humans. To date, many studies have been conducted to develop preventive measures against *Campylobacter* for use on chicken farms, such as feed and water additives, vaccination, prebiotics and probiotics, bacteriocins, and bacteriophages (5, 12, 14). In recent years, there has been increasing interest in the use of natural substances to control *Campylobacter* on chicken farms and processing plants because of consumer demands for antibiotic-free products. Many researchers have proposed controlling *Campylobacter* in the chicken gut by using feed or water additives, including natural substances, such as plant-derived essential oils and organic acids, that are known to show antimicrobial activities *in vitro* (14, 15). However, there are few reports of *in vivo* studies demonstrating the anti-*Campylobacter* effects of natural substances in the chicken gut. Certain herbal essential oils and compounds, such as carvacrol and thymol, which are present in several herbal plants, have achieved a statistically significant reduction in *C. jejuni* in the chicken gut, but the effects were limited (16, 17).

In the present study, we screened a chemical compound library to identify novel and effective natural substances for controlling *Campylobacter* colonization in the chicken gut. We demonstrated that tryptanthrin (TRP), a known herbal compound, showed strong antimicrobial activity against *C. jejuni* at low concentrations. We further performed *in vitro* and *in vivo* experiments to evaluate the anti-*C. jejuni* activity of TRP, especially whether TRP was able to reduce the amount of *C. jejuni* in the chicken gut.

## RESULTS

**Potent anti-*C. jejuni* activity of TRP.** We identified 36 compounds that inhibited the growth of the two *C. jejuni* strains (NCTC 11168 and 81-176) at a rate of more than 99.0% at 10  $\mu$ M through the high-throughput screening of 1,926 compounds. Of the 36 compounds, 23 were not commercially available or were used as antibiotics. The remaining 13 compounds consisted of 3 herbal compounds and 10 other compounds. TRP, one of the three herbal compounds, is an indoloquinazoline alkaloid that was first isolated from natural indigo and later identified in various natural sources (18). Certain herbal compounds were reported to show antimicrobial effects against *C. jejuni*, and therefore, we compared the antimicrobial activities of TRP against two *C. jejuni* strains (NCTC 11168 and 81-176) with those of 13 herbal compounds that are known to have antimicrobial activities against *C. jejuni* (19–24). As shown in Table 1, the MICs of TRP for the *C. jejuni* NCTC 11168 and 81-176 strains were 6.25 and 3.12  $\mu$ M, respectively. Among the previously reported 13 herbal compounds, cinnamaldehyde showed the lowest MIC for both NCTC 11168 and 81-176 strains (200 and 100  $\mu$ M, respectively). However, the MIC of TRP was 32-fold lower than that of cinnamaldehyde.

**Antimicrobial spectra of TRP.** We investigated the antimicrobial spectra of TRP against three species of *Campylobacter* that are known to be pathogenetic in humans and animals (*C. jejuni*, *C. coli*, and *C. fetus*) and seven species of gut bacteria (*Escherichia*

**TABLE 1** MICs of herbal compounds against *C. jejuni*

Class <sup>a</sup>	Compound	MIC ( $\mu\text{M}$ ) of:	
		NCTC 11168	81-176
Organic heterocyclic compound	Tryptanthrin	6.25	3.12
Organochalcogen compounds	Cinnamaldehyde	200	100
	Benzyl isothiocyanate	800	800
	Allyl isothiocyanate	>800	>800
	Perillaldehyde	>800	>800
Phenols	Carvacrol	400	200
	Eugenol	800	400
	Rosmarinic acid	>800	>800
	Thymol	>800	>800
Terpenoids	Citral	800	800
	Cineole	>800	>800
Alcohol	Linalool	>800	>800
Hydrocarbons	<i>p</i> -Cymene	>800	>800
	$\alpha$ -Pinene	>800	>800

<sup>a</sup>Herbal compounds are classified according to the ChEBI database.

*coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella enterica*, *Enterococcus faecalis*, and *Clostridium perfringens*), as shown in Table 2. The MICs of TRP for 20 strains of *C. jejuni* belonging to different sequence types ranged from 0.78 to 6.25  $\mu\text{M}$ . In addition, the MICs of TRP for four strains of *C. coli* and one strain of *C. fetus* (CFF018) were 6.25 or 12.5  $\mu\text{M}$ , but those for the other three strains of *C. fetus* and all seven strains of gut bacteria were greater than 100  $\mu\text{M}$ .

**Time-kill kinetics of TRP for *C. jejuni* under growing conditions.** We cultured *C. jejuni* NCTC 11168 and 81-176 strains in Mueller-Hinton (MH) broth with various TRP concentrations (0, 0.625, 1.25, 2.5, 5, and 10  $\mu\text{M}$ ) to evaluate in depth the antimicrobial effect of TRP on *C. jejuni* under growing conditions. As shown in Fig. 1, viable cells of both the NCTC 11168 and 81-176 strains became undetectable by 24 h after inoculation with more than 5  $\mu\text{M}$  TRP. In the presence of 1.25 and 2.5  $\mu\text{M}$  TRP, the growth of the NCTC 11168 strain was observed, but viable cells of the 81-176 strain were hardly observed by 48 h after inoculation. TRP concentrations under 0.625  $\mu\text{M}$  did not show strong effects on the growth of either strain. These experiments clearly showed that TRP at low concentrations inhibited *C. jejuni* growth in a dose-dependent manner.

**Time-kill kinetics of TRP for *C. jejuni* under nongrowing conditions.** To assess the effect of TRP on *C. jejuni* under nongrowing conditions, the number of viable cells in phosphate-buffered saline (PBS) with various TRP concentrations was measured at different temperatures. First, we determined the growth of the *C. jejuni* NCTC 11168 and 81-176 strains in MH broth at different temperatures (4, 15, 25, and 37°C) under microaerobic conditions, as shown in Fig. 2A. The bacterial growth of both strains was observed at 37°C but not at 25°C (Fig. 2A). In the viability tests, the bacterial suspensions in PBS with various TRP concentrations (0, 0.1, 1, 10, and 100  $\mu\text{M}$ ) were incubated at different temperatures under aerobic conditions. There were no significant differences in cell viability among different TRP concentrations at 4, 15, and 25°C at any of the three time points for either the NCTC 11168 or 81-176 strains (Fig. 2B). In contrast, when the incubation temperature was 37°C, the cell numbers of *C. jejuni* treated with 1, 10, and 100  $\mu\text{M}$  TRP were significantly lower than those without TRP treatment at several time points for both strains ( $P < 0.05$  and  $P < 0.01$ ).

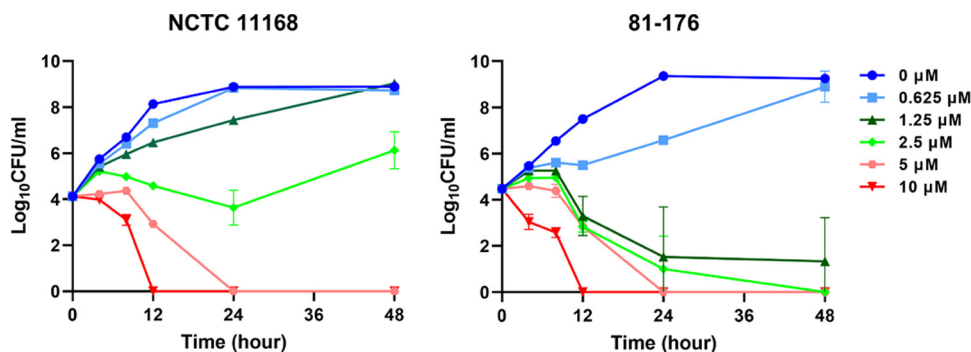
**Potential for the development of TRP resistance in *C. jejuni*.** The potential for the development of TRP resistance in the *C. jejuni* NCTC 11168 and 81-176 strains was evaluated by comparing the MICs of TRP over 20 subcultures to those of two antimicrobials, erythromycin (EM) and ciprofloxacin (CPFX). The initial MICs of TRP, EM, and CPFX for the NCTC 11168 strain were 1.56, 0.5, and 0.125  $\mu\text{g}/\text{mL}$ , respectively, and those for the 81-176 strain were 0.78, 0.25, and 0.0625  $\mu\text{g}/\text{mL}$ , respectively. In addition, the final MICs of TRP, EM, and CPFX for the NCTC 11168 strain after 20 subcultures

**TABLE 2** Antibacterial spectrum of TRP

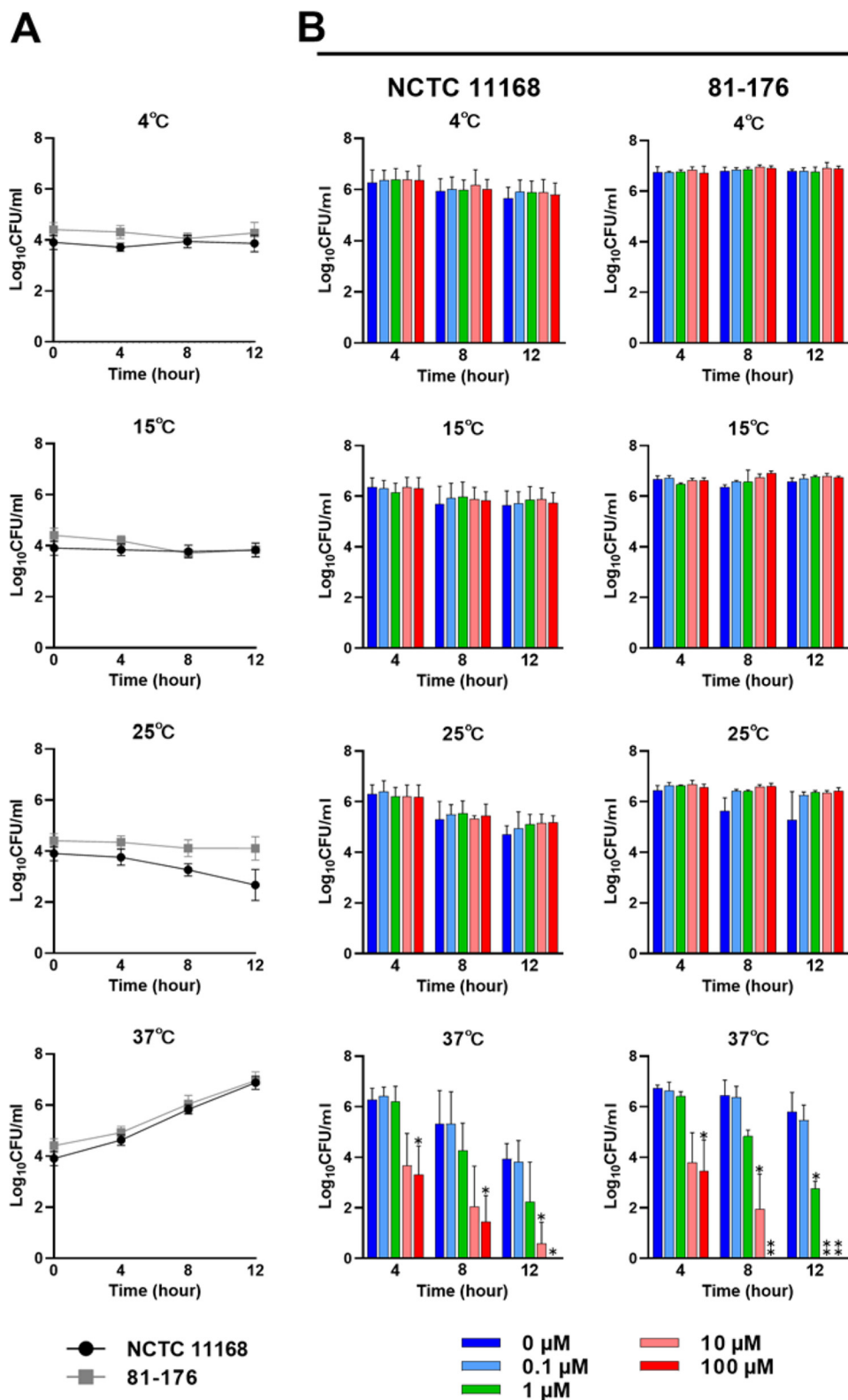
Bacterial species	Strain	Source	Sequence type (clonal complex) <sup>a</sup>	MIC ( $\mu\text{M}$ )
<i>Campylobacter jejuni</i>	NCTC 11168	Human	43 (21)	3.12
	81-176	Human	604 (42)	3.12
	ATCC 33560	Bovine	403 (403)	3.12
	11-164	Chicken	6849 (354)	3.12
	CJ069	Chicken	50 (21)	3.12
	CJ049	Chicken	4526 (21)	1.56
	CJ060	Chicken	22 (22)	3.12
	CJ046	Chicken	45 (45)	3.12
	CJ012	Chicken	3503 (48)	3.12
	CJ061	Chicken	61 (61)	3.12
	CJ067	Chicken	257 (257)	1.56
	CJ070	Chicken	4063 (283)	6.25
	CJ038	Chicken	3911 (353)	1.56
	CJ007	Chicken	4052 (353)	3.12
	CJ021	Chicken	354 (354)	0.78
	CJ019	Chicken	5402 (354)	6.25
	CJ001	Chicken	443 (443)	3.12
CJ011	Chicken	460 (460)	3.12	
CJ004	Chicken	5262 (464)	6.25	
CJ037	Chicken	607 (607)	3.12	
<i>Campylobacter coli</i>	ATCC BAA-1061	Chicken	1063 (828)	12.5
	CC003	Chicken	854 (828)	12.5
	CC001	Chicken	1767 (828)	6.25
	CC002	Chicken	4172 (828)	12.5
<i>Campylobacter fetus</i>	CFF009	Bovine	2 (not provided)	>100
	CFF045	Bovine	3 (not provided)	>100
	CFF018	Bovine	5 (not provided)	6.25
	CFF028	Bovine	6 (not provided)	>100
<i>Escherichia coli</i>	ATCC 23736	Unknown	NT	>100
<i>Enterobacter cloacae</i>	ATCC 13047	Human	NT	>100
<i>Klebsiella pneumoniae</i>	ATCC 9997	Unknown	NT	>100
<i>Proteus mirabilis</i>	ATCC 29906	Unknown	NT	>100
<i>Salmonella enterica</i>	LT2	Unknown	NT	>100
<i>Enterococcus faecalis</i>	ATCC 19433	Unknown	NT	>100
<i>Clostridium perfringens</i>	ATCC 13124	Unknown	NT	>100

<sup>a</sup>Not provided, clonal complex of *C. fetus* are not provided by PubMLST database; NT, not tested.

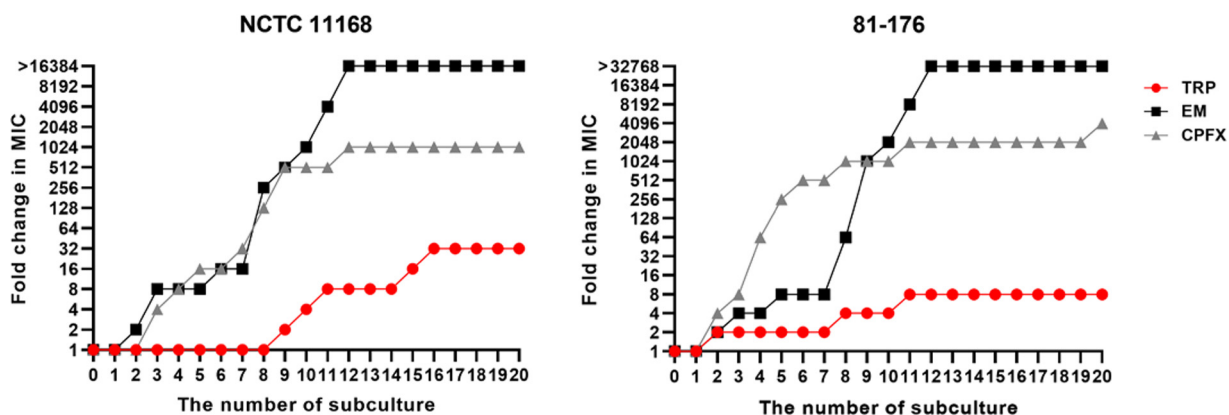
were 50, >8,192, and 128  $\mu\text{g}/\text{mL}$ , respectively, and those for the 81-176 strain were 6.25, >8,192, and 256  $\mu\text{g}/\text{mL}$ , respectively. The *C. jejuni* strains exposed to EM and CFX rapidly acquired resistance to antimicrobials within 12 subcultures. During this period, the MICs of EM for the NCTC 11168 and 81-176 strains were increased more



**FIG 1** Time-kill kinetics of TRP for *C. jejuni* under growing conditions. Time-kill curves of the *C. jejuni* NCTC 11168 and 81-176 strains in MH broth with different TRP concentrations (0, 0.625, 1.25, 2.5, 5, and 10  $\mu\text{M}$ ) under microaerophilic conditions. The data are shown as the mean values and SDs of the results from three independent measurements.



**FIG 2** Time-kill kinetics of TRP for *C. jejuni* under nongrowing conditions. (A) Growth curves of the *C. jejuni* NCTC 11168 and 81-176 strains in MH broth at different temperatures (4, 15, 25, and 37°C) under microaerophilic conditions. (B) Viable cell counts of the *C. jejuni* NCTC 11168 and 81-176 strains in PBS at different temperatures (4, 15, 25, and 37°C) under aerobic conditions. The TRP concentrations were set at 0, 0.1, 1, 10, and 100 µM. The data are shown as the means and SDs of the results from three independent experiments. Asterisks indicate statistically significant differences from the control (PBS without TRP) by Student's *t* test (\*, *P* < 0.05; \*\*, *P* < 0.01) at the same time point.



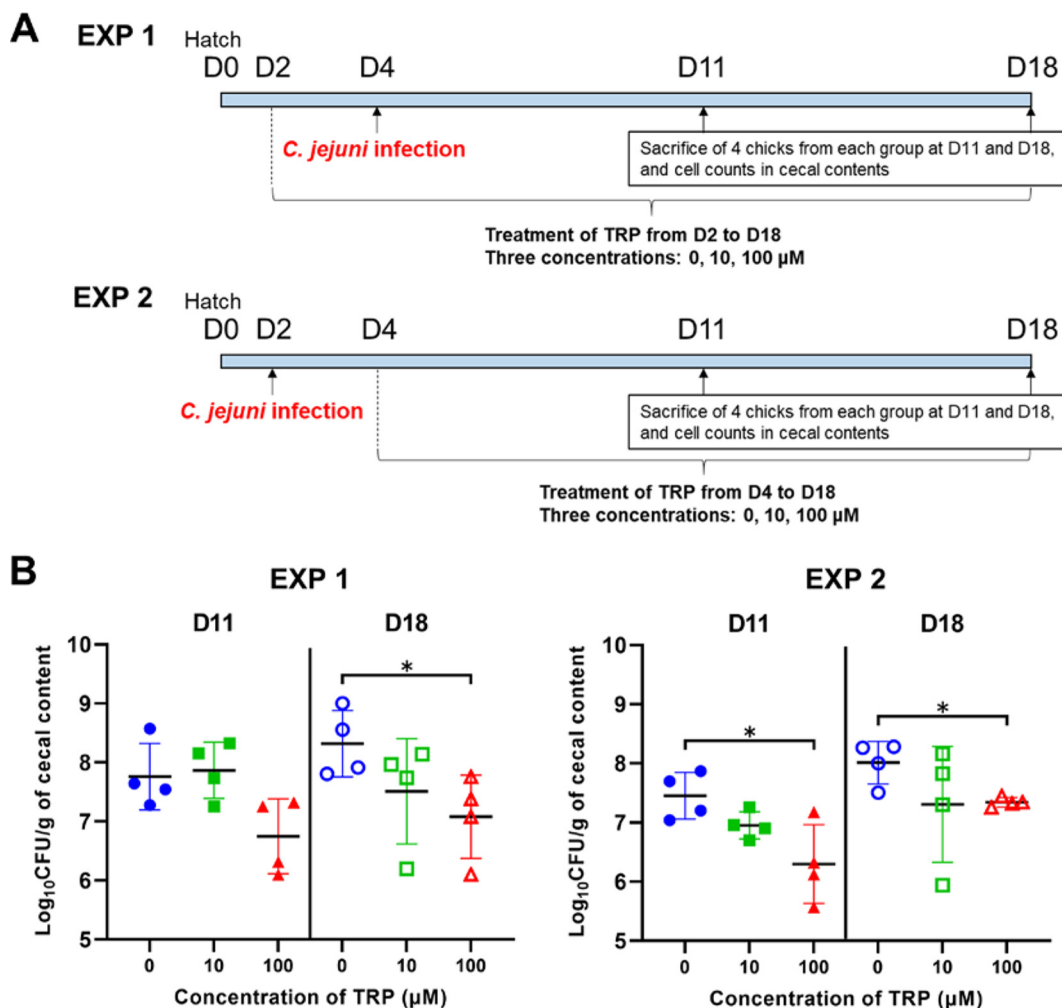
**FIG 3** The development of resistance to TRP, EM, and CPF of *C. jejuni* by serially passaged culture. The fold changes in the MICs of the NCTC 11168 and 81-176 strains were monitored up to 20 passages with sub-MICs of TRP, EM, and CPF.

than 16,384- and 32,768-fold, respectively, and those of CPF were increased 1,024- and 2,048-fold, respectively (Fig. 3). Notably, the maximum EM concentration in the determination of MICs was 8,192  $\mu\text{g}/\text{mL}$  due to its solubility, but the growth of both NCTC 11168 and 81-176 strains was still observed at this concentration after 12 subcultures. In sharp contrast to those of EM and CPF, the MICs of TRP for the NCTC 11168 and 81-176 strains were increased only 32- and 8-fold, respectively, even after 20 subcultures (Fig. 3).

**Effect of TRP on *C. jejuni* colonization in the chick gut.** Figure 4A illustrates two types of infection experiments that were carried out to determine the effect of TRP on the colonization of *C. jejuni* in the chicken gut. In both experiments, 24 newly hatched 1-day-old chicks were assigned to three groups (8 chicks/group). Two of the three groups were treated with TRP, which was added to drinking water at final concentrations of 10 and 100  $\mu\text{M}$ , and the control group was not treated with TRP. In experiment 1, TRP treatment was started 2 days before infection with the *C. jejuni* 11-164 strain. In experiment 2, TRP treatment was started 2 days after infection with the *C. jejuni* 11-164 strain. In both experiments, four chicks from each group were sacrificed at 11 and 18 days old, and the number of *C. jejuni* in their cecal contents was measured. The results of experiment 1 indicated that the numbers of *C. jejuni* in the 11- and 18-day-old chicks treated with 100  $\mu\text{M}$  TRP (6.7 and 7.1  $\log_{10}$  CFU/g) were lower than those in the control group (7.8 and 8.3  $\log_{10}$  CFU/g), respectively, and a significant difference ( $P < 0.05$ ) was observed for the 18-day-old chicks (Fig. 4B). In addition, the results of experiment 2 also showed that the numbers of *C. jejuni* in the 11- and 18-day-old chicks treated with 100  $\mu\text{M}$  TRP (6.3 and 7.3  $\log_{10}$  CFU/g) were significantly lower ( $P < 0.05$ ) than those in the control group (7.5 and 8.0  $\log_{10}$  CFU/g), respectively (Fig. 4B). When the chicks were treated with 10  $\mu\text{M}$  TRP, the viable cell numbers of *C. jejuni* in the 18-day-old chicks in experiment 1 and the 11- and 18-day-old chicks in experiment 2 (7.5, 7.0, and 7.3  $\log_{10}$  CFU/g, respectively) were lower than those in the control group, but the difference was not significant ( $P > 0.05$ ).

**DISCUSSION**

Natural herbal compounds and essential oils have been previously proposed to control *Campylobacter* in chicken farms, but their effectiveness has not been clearly defined. As shown by the method of identification of TRP in the present study, compound libraries are useful tools for discovering novel antimicrobial compounds. There have been a few reports of using chemical library screening to identify novel anti-*C. jejuni* agents: Johnson et al. identified compounds that inhibited the flagellar expression and growth of *C. jejuni* (25), and Kumar et al. identified compounds that had anti-*C. jejuni* effects in Caco-2 cells (26). Both of these screening experiments identified synthetic compounds that showed anti-*Campylobacter* effects *in vitro*, but it remained unclear whether these compounds



**FIG 4** The effect of TRP on the cecal colonization of *C. jejuni* in chicks. (A) Schedule for the animal experiments. Two types of experiments were conducted to evaluate the antibacterial effect against the *C. jejuni* 11-164 strain in the chick cecum. TRP treatments (10 and 100 μM added to drinking water) were started 2 days before infection in experiment 1 (EXP 1) and 2 days after infection in experiment 2 (EXP 2). The control groups were not treated with TRP. Four chicks from each group were sacrificed at 11 and 18 days old, and the numbers of *C. jejuni* in their cecal contents were quantified. (B) Each dot represents the amount of *C. jejuni* expressed as the log<sub>10</sub> CFU per gram in the cecal content of each individual infected chick in experiments 1 and 2. Error bars represent the SDs derived from four chicks per group. All the data were analyzed by unpaired Student's *t* test (\*, *P* < 0.05).

have the same effects in the chicken gut. In addition, consumers are generally concerned with the use of synthetic additives in animal feed and their possible inclusion in the meat that we consume (27). Therefore, we used a chemical compound library, which included natural substances, to find novel anti-*Campylobacter* agents that were expected to reduce the numbers of this pathogen in the chicken intestinal tract. TRP, which was identified as an anti-*Campylobacter* agent in the present study, is an herbal compound belonging to a unique chemical class with an indoloquinazoline structure that showed the lowest MIC values against *C. jejuni* compared to 13 plant molecules previously reported to exhibit anti-*C. jejuni* activities (Table 1). Furthermore, there have been no previous reports of the anti-*C. jejuni* and anti-*C. coli* activities of TRP, although this compound was shown to have antimicrobial activities against several bacteria, e.g., *Escherichia coli* (28), *Bacillus subtilis* (29), *Staphylococcus aureus* (30, 31), *Mycobacterium tuberculosis* (32, 33), and *Helicobacter pylori* (34). Among *Campylobacter* species, *C. rectus*, which is known as a periodontal pathogen, was the only species whose susceptibility to TRP was previously investigated (the MIC of TRP was 25 μg/mL, approximately 100 μM) (35). To our knowledge, this is the first report demonstrating the antimicrobial effect of TRP against both *C. jejuni* and *C. coli*.

TRP showed narrow-spectrum antimicrobial effects against *C. jejuni* and *C. coli*, i.e., the MICs of TRP for all strains of the two *Campylobacter* species were low (ranging from 0.78 to 12.5  $\mu\text{M}$ ), but those for most strains of *C. fetus* and all seven species of gut bacteria were greater than 100  $\mu\text{M}$  (Table 2). Bandekar et al. investigated the effects of TRP and its nine derivatives on the growth and survival of the *E. coli* AS19 strain and found that 10  $\mu\text{g}/\text{mL}$  (approximately 40  $\mu\text{M}$ ) TRP slowed growth and reduced the optical density at 650 nm ( $\text{OD}_{650}$ ) endpoint of *E. coli* by approximately 2-fold (28). However, we observed growth of the *E. coli* ATCC 23736 strain in the presence of 100  $\mu\text{M}$  TRP (Table 2). In addition, the growth inhibition assay based on  $\text{OD}_{600}$  showed no significant difference in the growth of the *E. coli* ATCC 23736 strain with and without treatment with 100  $\mu\text{M}$  TRP (data not shown). The difference in the results between these two studies may be due to the use of different strains in each experiment. Although further experiments are needed to determine the susceptibilities of various *E. coli* strains, TRP concentrations ranging from several dozens to hundreds of micromolar are considered to be required for the complete inhibition of *E. coli* growth. In contrast, TRP completely inhibited the growth of *C. jejuni* at concentrations lower than 5 or 6.25  $\mu\text{M}$  in the present study (Table 1 and Fig. 1). These results suggested that *C. jejuni* is more susceptible to TRP than conventional gut bacteria. Johnson et al. reviewed the need for anti-*Campylobacter* compounds to be safe for both humans and livestock and to have narrow-spectrum effects to reduce the impact of these compounds on normal gut microbes (27). However, there have been no synthetic or natural compounds that satisfy these requirements. Although further studies on the effects of TRP on the microbiome in the chicken gut are needed, our results suggested that TRP has bacterial species-specific antimicrobial activity against *C. jejuni* and *C. coli*, with little effect on normal gut bacteria.

Our *in vivo* experiments demonstrated a remarkable antimicrobial effect of TRP that significantly reduced the numbers of cecal *C. jejuni* cells in infected chicks. Interestingly, a significant reduction was observed not only in experiment 1 (TRP treatment before *C. jejuni* infection) but also in experiment 2 (TRP treatment after *C. jejuni* infection), as shown in Fig. 4B. Our results suggested that TRP shows a bactericidal effect against *C. jejuni* in the chicken gut, and our *in vitro* experiments confirmed that relatively high TRP concentrations reduce the number of *C. jejuni* at its growth temperature (Fig. 1; Fig. 2A and B). Bactericidal effects of TRP and indigo plant extract on several bacteria other than *Campylobacter*, such as *E. coli*, *Streptococcus mutans*, *Porphyromonas gingivaris*, and *Prevotella intermedia*, have also been reported (28, 35). Therefore, TRP is suggested to be a promising agent for reducing the colonization of *C. jejuni* in the chicken gut.

Many studies have shown that plant-derived compounds and essential oils have antimicrobial properties against *C. jejuni*, but only a few of these compounds can significantly reduce the number of bacterial cells in the chicken gut (15). Arsi et al. investigated the application of carvacrol, thymol, and their combination as feed additives to prevent the cecal colonization of *C. jejuni* in chickens (16). Significant reductions (0.6 to 2.0  $\log_{10}$  CFU/mL) in *C. jejuni* were observed with the use of 1% carvacrol, 0.25% and 2% thymol, and 0.5% carvacrol and thymol combined. However, the body weight gains of the treated chickens were significantly reduced at higher concentrations (>0.5%) of both thymol and carvacrol. In contrast, our data demonstrated that cecal *C. jejuni* was significantly reduced, with reductions ranging from 0.7 to 1.2 log CFU/g cecal content, in chicks treated with 100  $\mu\text{M}$  TRP (approximately 0.0025%). Importantly, there were no significant changes in body weight ( $P > 0.05$ ) between the TRP-treated and control groups (see Fig. S1 in the supplemental material). These results indicated that TRP is a promising candidate for the control of *C. jejuni* without health hazards in chicken farms; however, the animal experiments in this study were limited to small sizes and short feeding periods. Further studies are necessary to evaluate the impacts of slaughter age and field conditions on the antimicrobial effect of TRP.



The antimicrobial mechanism of TRP remains unclear: Tripathi et al. reported that TRP showed a high affinity for an enoyl-acyl carrier protein reductase (InhA) in *M. tuberculosis* (33), while Bandekar et al. suggested that TRP and its derivatives bound DNA by intercalation in *E. coli* (28). In the present study, we evaluated the potential of *C. jejuni* to develop resistance to TRP by monitoring the changes in the MIC of TRP through continuous-passage cultures in the presence of TRP at sub-MICs. Because it was previously reported that *C. jejuni* developed resistance to EM and CPFY through *in vitro* plating experiments (36, 37), we compared the potential for developing resistance to TRP with that of developing resistance to EM and CPFY in the same manner. The results showed that TRP produced only small changes in the MIC values for both the *C. jejuni* NCTC 11168 and 81-176 strains, whereas EM and CPFY greatly increased the MIC values by >16,384- and 1,024-fold for the NCTC 11168 strain and by >32,768- and 4,096-fold for the 81-176 strain, respectively, after a 60-day experimental period (Fig. 3). Interestingly, a two- or three-step increase in the MICs of TRP was observed for both *C. jejuni* strains, suggesting that multiple low-level resistance-causing mutations occurred in the different responsible genes. Whole-genome sequencing analyses of TRP-resistant *C. jejuni* strains are being conducted to identify the antimicrobial resistance mechanism of TRP caused by these chromosomal mutations.

Recently, herbal plants and their extracts have been commonly used as feed and water additives in chicken farms worldwide. To date, TRP has been isolated from various plants, such as *Persicaria tinctoria*, *Strobilanthes cusia*, and *Isatis tinctoria* (18). The leaves of *P. tinctoria* and *I. tinctoria* were found to contain high TRP concentrations (38, 39); therefore, their extracts also exhibited antimicrobial effects (35, 40). These plants contain several antimicrobial compounds other than TRP, such as kaempferol, 6-methoxykaempferol, and 3,5,4'-trihydroxy-6,7-methylenedioxy flavone (41). Kataoka et al. reported that the combined oral administration of TRP and kaempferol tended to cause a further decrease in *H. pylori* cells in the stomach of Mongolian gerbils compared with the single administration of each compound, although no significant differences were observed (34). TRP shows an antimicrobial effect against *C. jejuni* at a low concentration and with a single administration to chickens, as shown in the present study, and it may be possible to achieve synergistic effects by combining TRP and other herbal compounds. Future research is expected to improve TRP-containing anti-*C. jejuni* agents and to develop feed or drinking water additives that are effective for the control of *Campylobacter* in chicken farms.

## MATERIALS AND METHODS

**Screening of anti-*C. jejuni* agents using a chemical compound library.** The validated compound library (consisting of 1,926 chemical compounds) obtained from the Drug Discovery Initiative (The University of Tokyo, Tokyo, Japan) was used for the screening of anti-*C. jejuni* agents. The chemical compounds were dissolved in 0.5  $\mu$ L of dimethyl sulfoxide (DMSO) at a concentration of 2 mM in each well of a flat-bottom 96-well plate. Inhibition of the growth of *C. jejuni* was determined by a broth microdilution assay. Briefly, the *C. jejuni* 81-176 and 11-164 strains were grown in 3 mL of MH broth (Becton, Dickinson and Company, Sparks, MD, USA) overnight at 42°C under microaerophilic conditions. The bacterial culture was diluted with MH broth to a concentration of  $10^6$  CFU/well, which was set based on our previous study (42), and 99.5  $\mu$ L of the dilution was added to each well of the 96-well plate, i.e., the final concentration of each compound was 10  $\mu$ M. DMSO alone was used as a negative control, and 20  $\mu$ g/mL chloramphenicol was used as a positive control. OD<sub>600</sub> values were measured after incubating the 96-well plates for 72 h at 42°C under microaerobic conditions. The hit compounds were selected according to the screening methods of Kumar et al. (26). The growth inhibition rates (%) of *C. jejuni* strains were calculated as follows:  $100 \times (\text{OD}_{600} \text{ of the negative control} - \text{OD}_{600} \text{ of the test compound}) / (\text{OD}_{600} \text{ of the negative control} - \text{OD}_{600} \text{ of the positive control})$ . The compounds that inhibited the growth of the two *C. jejuni* strains at a rate of more than 99.0% were selected as hit compounds.

**Susceptibility testing with a liquid dilution method.** In the present study, TRP, cinnamaldehyde, perillaldehyde, carvacrol, eugenol, rosmarinic acid, thymol, citral, cineole, linalool, *p*-cymene, and  $\alpha$ -pinene were purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan), and benzyl isothiocyanate and allyl isothiocyanate were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). These 14 herbal compounds were classified into six categories according to the ChEBI database (Table 1). The stock solution of each compound was prepared at 10 mM with DMSO (Fujifilm Wako Pure Chemical Co.). The stock solutions were diluted to a concentration of 800  $\mu$ M in MH broth and serially diluted to concentrations ranging from 0.78 to 800  $\mu$ M in 96-well plates. *C. jejuni* NCTC 11168 and 81-176 strains were

inoculated into the diluted solutions at  $10^6$  CFU/well, and bacterial growth was evaluated after incubation in 96-well plates for 72 h at 42°C under microaerobic conditions.

To investigate the antibacterial spectrum of TRP, we used 28 *Campylobacter* and 7 non-*Campylobacter* strains (Table 2). The *Campylobacter* strains consisted of 20 *C. jejuni*, 4 *C. coli*, and 4 *C. fetus* strains. The non-*Campylobacter* strains consisted of gut bacteria, namely, *E. coli*, *E. cloacae*, *K. pneumoniae*, *P. mirabilis*, *S. enterica*, *E. faecalis*, and *C. perfringens*. The MIC values of TRP were determined by the standard microtiter broth dilution method. The stock solution of TRP was diluted to a concentration of 100  $\mu$ M in MH broth and serially diluted to concentrations ranging from 0.1 to 100  $\mu$ M in 96-well plates. Only the test for *C. perfringens* was conducted with dilutions of *Brucella* broth (Becton, Dickinson and Company) supplemented with 5  $\mu$ g/mL hemin (Fujifilm Wako Pure Chemical Co.). The inoculum concentration of bacterial strains was  $10^6$  CFU/well. The inoculated plates were incubated at 42°C (for *C. jejuni* and *C. coli*) or 37°C (for *C. fetus*) for 72 h under microaerophilic conditions, 37°C for 24 h under aerobic conditions (for aerobic gut bacteria), or 37°C for 48 h under anaerobic conditions (for *C. perfringens*). The experiments were repeated three times, and the results of one representative experiment for each bacterial strain are shown in Table 2.

**Time-kill assay under growing conditions.** To determine the time-kill kinetics of TRP against *C. jejuni* under growing conditions, 2-fold serial dilutions were prepared in MH broth ranging from 0.625 to 10  $\mu$ M. The *C. jejuni* NCTC 11168 and 81-176 strains were inoculated into 3 mL of the diluted solutions and MH broth (0  $\mu$ M TRP) at a concentration of  $10^4$  CFU/mL in sterile borosilicate tubes and incubated at 42°C under microaerophilic conditions. To quantify the number of viable bacteria, samples were obtained 4, 8, 12, 24, and 48 h after inoculation followed by serial dilutions, and 100  $\mu$ L of each solution was spread onto MH agar (Becton, Dickinson and Company).

**Time-kill assay under nongrowing conditions.** First, the growth of *C. jejuni* strains in MH broth at different temperatures was determined by the following procedure. The *C. jejuni* NCTC 11168 and 81-176 strains were inoculated as described in 3 mL of MH broth at a concentration of  $10^4$  CFU/mL and incubated at 4, 15, 25, and 37°C under microaerobic conditions. The number of bacteria was measured at 4, 8, and 12 h after inoculation as described above.

To determine the time-kill kinetics of TRP against *C. jejuni* under nongrowing conditions, the viable cells of the *C. jejuni* NCTC 11168 and 81-176 strains in the presence of TRP were measured at different temperatures. Tenfold serial dilutions of TRP from 0.1 to 100  $\mu$ M were prepared in PBS. *C. jejuni* cells at a concentration of  $10^6$  CFU/mL were inoculated into 3 mL of the diluted solutions in sterile borosilicate tubes and incubated at 4, 15, 25, and 37°C under aerobic conditions. The number of viable cells was counted 4, 8, and 12 h after inoculation as described above.

**Verification experiment for the development of drug resistance.** The development of the drug resistance of *C. jejuni* was verified by a previously described method (43) with some modifications. TRP, EM, and CPFX solutions were prepared in MH broth at concentrations ranging from 0.25 to 32 times the MICs of the *C. jejuni* NCTC 11168 and 81-176 strains. The ranges of the concentrations were modified depending on the results of each passage. EM and CPFX were obtained from Fujifilm Wako Pure Chemical Corporation. *C. jejuni* strains were inoculated into 500  $\mu$ L of each of the dilutions of TRP, EM, or CPFX in 48-well plates and incubated at 42°C under microaerobic conditions. After 72 h of incubation, the bacterial cells at the second-highest concentration that showed growth were used for the subsequent culture. This subculture procedure was repeated 20 times.

**Chick infection experiment.** Two types of chick infection experiments were conducted to determine the effect of TRP on the numbers of *C. jejuni* in the chicken intestinal tract. The experimental schedules are illustrated in Fig. 4A. Newly hatched 1-day-old chicks (L-M-6 strain) were obtained from Nisseiken Co., Ltd. (Tokyo, Japan). In experiment 1, TRP treatment was started 2 days before infection with the *C. jejuni* 11-164 strain. On the other hand, in experiment 2, TRP treatment was started 2 days after infection with the *C. jejuni* 11-164 strain. The treatment was performed by adding TRP to the drinking water at final concentrations of 10 and 100  $\mu$ M, and the drinking water was changed every day. The chicks were allowed to freely drink water from a bell drinker. In both experiments, chicks were orally challenged with  $10^6$  CFU of the wild-type *C. jejuni* 11-164 strain through stomach tubes. Four chicks from each group were sacrificed at 11 and 18 days old, and the numbers of *C. jejuni* in their cecal contents were quantified by spreading serial dilutions on MH agar plates containing modified Preston *Campylobacter* selective supplement (catalog no. SR0204E; Oxoid Ltd., Basingstoke, UK) and 10  $\mu$ g/mL nalidixic acid (Fujifilm Wako Pure Chemical Co.). These experiments were conducted in strict accordance with the guidelines of animal experimentation defined by the National Institute of Animal Health (NIAH), Japan. The protocol was approved by the committee on the Ethics of Animal Experiments of the NIAH (permit numbers 20-060 and 21-039).

**Statistical analysis.** Differences in the results were tested using two-tailed unpaired Student's *t* tests. A *P* value of <0.05 was considered to indicate statistical significance (see the figure legends for specific values).

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.1 MB.

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