



# Environmentally relevant concentrations of triclosan exposure promote the horizontal transfer of antibiotic resistance genes mediated by *Edwardsiella piscicida*

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## Abstract

Aquaculture pathogen and antibiotic resistance genes (ARGs) co-occur in the aquatic environment. Accumulated evidence suggests that aquaculture pathogens can facilitate the horizontal transfer of plasmid-mediated ARGs. However, the role of *Edwardsiella piscicida* (*E. piscicida*) in ARG dissemination is still not fully understood. In addition, the potential impact of triclosan (TCS) on the spread of ARGs mediated by *E. piscicida* is still unknown, so a mating model system was established to investigate the transfer process of ARGs. The results showed that *E. piscicida* disseminated ARGs on RP4 by horizontal gene transfer (HGT). Furthermore, TCS exposure promoted this process. The conjugative transfer frequencies were enhanced approximately 1.2–1.4-fold by TCS at concentrations from 2 to 20 µg/L, when compared with the control. TCS promoted the HGT of ARGs by stimulating reactive oxygen species (ROS) production, increasing cell membrane permeability, and altering expressions of conjugative transfer-associated genes. Together, the results suggested that aquaculture pathogens spread ARGs and that the emerging contaminant TCS enhanced the transfer of ARGs between bacteria.

**Keywords** *Edwardsiella piscicida* · Antibiotic resistance genes · Horizontal gene transfer · Triclosan · Environmental concentration · Conjugative transfer

## Introduction

Horizontal transfer of multidrug-resistant plasmids between unrelated bacteria plays an important role in the dissemination of the ARGs and development of multi-antibiotic-resistant bacteria (ARBs). Previous studies showed that *Escherichia coli*, *Salmonella*, *Pseudomonas putida*, and some other bacteria spread ARGs via conjugation (Qiu et al. 2012; Card et al. 2017; Lu et al. 2018b). Increasing ARGs and ARBs are

becoming a serious worldwide public health problem. More than 35,000 deaths every year are caused by infection of ARBs in the USA (Atlanta, GA: U.S. Department of Health and Human Services, CDC, 2019). It is also predicted that antimicrobial-resistant infections will kill at least 700,000 people each year worldwide (O’Neill 2014). “One Health” viewpoint is therefore urgently needed to combat antibiotic resistance (McEwen and Collignon 2018).

Aquaculture is currently recognized as an important but previously neglected environmental gateway for disseminating ARGs and developing ARBs around the world (Cabello et al. 2016; Preena et al. 2020). During the rapidly expanded period of aquaculture, large amounts of antibiotics were used to control the outbreak of diseases or promote growth. It was estimated that 10,259 tonnes of antimicrobials were consumed by aquaculture in 2017 and the amount might increase to 13,600 tonnes by 2030 (Lulijwa et al. 2020; Schar et al. 2020). Abuse of antibiotics induces highly selective pressure on bacteria and promotes antibiotic resistance. Aquaculture has therefore become a reservoir of ARGs and ARBs (Cabello et al. 2016; Xiong et al. 2015). Many ARGs, such as genes encoding resistance to

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kanamycin (Kan), ampicillin (Amp), and tetracycline (Tet), have been frequently detected in different aquaculture environments (Miller and Harbottle 2018). Several aquaculture pathogenic bacteria also possess multi-antibiotic-resistant genes and have been ARBs (Abdelhamed et al. 2019; Lo et al. 2014; Sun et al. 2009). They may move through water flow or the supply chain and spread ARGs in the world, resulting in a crisis to public health (Luneberg et al. 2018; Shen et al. 2019).

*E. piscicida* (previously called *Edwardsiella tarda*) is a typical Gram-negative pathogen and is abundant in aquaculture environments. It can infect more than 20 species of fish (e.g., turbot, flounder, and salmon) and leads to high mortality (Leung et al. 2019; Park et al. 2012; Xu and Zhang 2014). *E. piscicida* infection accounts for heavy economic losses in aquaculture (Leung et al. 2019; Xu and Zhang 2014). However, there is no effective commercial vaccine available, and antimicrobial-medicated feeds are still a commonly used strategy to control edwardsiellosis (Leung et al. 2019). The occurrence of multiple antibiotic-resistant *E. piscicida* is presently very common (Lo et al. 2014; Niu et al. 2019). For example, its resistance to oxytetracycline and ampicillin ranges from 21 to 90% and 73.3 to 100.0%, respectively (Lo et al. 2014; Niu et al. 2019). Approximately 70% of isolates from red tilapia in northern Thailand were resistant to at least seven antibiotics (Niu et al. 2019). Moreover, several isolates contained transferrable antibiotic-resistant plasmids (Sun et al. 2009; Abdelhamed et al. 2019; Lo et al. 2014). *E. piscicida* is thought to transfer ARGs to bacteria in water and plays an important role in disseminating ARGs (Fu et al. 2017; Leung et al. 2019). However, few studies have focused on this phenomenon and the knowledge gap regarding the potential role of aquaculture pathogens in the dissemination of ARGs.

Triclosan, (TCS, 5-chloro-2-(2,4-dichlorophenoxy) phenol), is a synthetic, broad-spectrum antimicrobial disinfectant, which is widely used as an additive to many consumer products, including toothpaste, soaps, skin cleansers, and catheters (Singer et al. 2002; Dhillon et al. 2015; Milanovic et al. 2021). Global TCS production is estimated about 1500 tonnes per year (Alfhili and Lee 2019; Dhillon et al. 2015; Milanovic et al. 2021). The half-life of TCS is up to 100 days in aquatic environments, and it has been recognized as an emerging and persistent contaminant (Huang et al. 2014). High residual TCS levels have been detected in the environment and animal bodies (Dhillon et al. 2015; Milanovic et al. 2021). The common concentration of TCS ranged from  $1.4 \times 10^{-3}$ –40  $\mu\text{g/L}$  in surface water (Dhillon et al. 2015). Previous studies documented that TCS was associated with antibiotic resistance and other side effects (Karmakar et al. 2019; Lu et al. 2018a, 2018b). The USA has banned its use in some health care products since 2016, and the European Union also

banned its use in food contact materials. However, TCS is still widely used in China and many other countries (Alfhili and Lee 2019; Dhillon et al. 2015; Milanovic et al. 2021). More than 90% of consumed TCS has been discharged into the water environment, and its concentration ranged from 0.01 to 1.023  $\mu\text{g/L}$  in surface waters in China (Liu et al. 2020; Ma et al. 2018). Environmental TCS might have a long-term co-occurrence with many aquaculture pathogenic bacteria. However, the impact of TCS on the dissemination of ARGs mediated by aquatic pathogens has not been thoroughly studied.

To elucidate the role of *E. piscicida* in spreading ARGs and the effects of TCS on this process, a conjugative transfer model was established. First, a multidrug-resistant conjugative plasmid RP4 was introduced into *E. piscicida*. Then, the *E. piscicida* strain with the RP4 plasmid and *E. coli* JM109 were used as the donor and recipient, respectively. Third, both the donor and recipient strains were exposed to TCS concentrations ranging from 0.02 to 2,000  $\mu\text{g/L}$  for 8 h in the mating system, and then, the effect of TCS on transfer frequency of ARGs was assessed. Lastly, ROS production and cell membrane permeability and expression levels of conjugative associated genes were quantified to analyze the potential mechanism.

## Materials and methods

### Bacterial strains and culture media

The bacterial strains and plasmids used in this study are described in Supplementary Table S1. *E. piscicida* (PPD130/91) and *E. coli* JM109 are resistant to colistin (Col) and nalidixic acid (NAA), respectively. Self-transmissible RP4 plasmid contains 3 ARGs and encodes tetracycline resistance ( $\text{Tet}^r$ ), ampicillin resistance ( $\text{Amp}^r$ ), and kanamycin resistance ( $\text{Km}^r$ ) (Pansegrau et al. 1994) and was widely used to mimic endogenous multi-drug-resistant plasmids. The donor strain was *E. piscicida* (PPD130/91) containing the RP4 plasmid, and the recipient strain was *E. coli* JM109. *E. piscicida* strains were incubated in Tryptic Soy Broth (TSB, BD Biosciences, San Jose, CA, USA) containing Col (12.5 mg/L) at 28 °C for 16 h without shaking. *E. coli* JM109 was cultured in Luria–Bertani (LB) medium (yeast extract, 5 g/L; tryptone, 10 g/L; and NaCl, 10 g/L) containing NAA (80 mg/L) at 37 °C for approximately 12 h with shaking at 220 rpm. Bacterial cultures were washed with phosphate-buffered saline (PBS, pH = 7.2) and prepared for conjugation, detection of intracellular ROS, evaluation of cell membrane permeability, and quantitation of the expression levels of conjugative transfer-related genes.

## Antimicrobial resistance assays

According to the Clinical and Laboratory Standard Institute (2020), the minimum inhibitory concentrations (MICs) of *E. piscicida* and *E. coli* JM109 were first determined against Col and NAA by using broth micro-dilution. Briefly, *E. piscicida* was grown overnight in liquid TSB at 28 °C, and *E. coli* JM109 was grown in liquid LB at 37 °C. Then, overnight cultures of each strain were diluted to an initial cell density ( $\approx 5 \times 10^6$  CFU/mL) and seeded in 96-well microplates (96-well clear microplates; Costar®; Corning, NY, USA) with a twofold serial dilution of antibiotic concentrations ranging from 0 to 1024 mg/L. After incubation for 18–20 h at 30 °C, the optical density at 540 or 600 nm ( $OD_{540}/OD_{600}$ ) was measured using a microplate spectrophotometer (Varioskan Flash Spectral Scanning Multimode Reader; Thermo Fisher Scientific, Waltham, MA, USA). MICs were calculated as the minimal concentration showing no visible growth of bacterial cells. Each test was performed at least in triplicate. MICs of the donor, recipient, and transconjugant against tetracycline (Tet), ampicillin (Amp), and kanamycin (Kan) were determined similarly.

## Conjugative transfer experiments

To determine the role of *E. piscicida* in the spread of ARGs, we established an inter-genus conjugative transfer model following reference methods with modifications (Zhang et al. 2017). First, the multi-antibiotic-resistant plasmid RP4 was transformed into *E. coli* HB101 competent cells and then transferred into *E. piscicida* by conjugation as our previous study described (Lu et al. 2016). Positive colonies were screened by selective plates and PCR. Second, an *E. piscicida* colony with the RP4 plasmid was then used as a donor and *E. coli* JM109 was used as a recipient based on its antibiotic resistant spectrum (Table 1). To measure the effects of TCS on the conjugative transfer of RP4 plasmid mediated by *E. piscicida*, we selected a range (from 0.02 to 2000 µg/L) of TCS concentrations, based on MICs determined in this study. Overnight cultures of the donor and recipient bacteria were collected by centrifuging for 5 min at  $10,000 \times g$  at 25 °C. Following centrifugation, the supernatants were discarded, and the cells were resuspended in different PBS

volumes to obtain a bacterial concentration of approximately  $OD_{540} = 0.5$  ( $\approx 10^8$  CFU/mL). Then, *E. piscicida* with the RP4 plasmid and *E. coli* JM109 were mixed at a ratio of 1:1 and exposed to different sub-MIC concentrations of TCS, ranging from 0.02 to 2000 µg/L for 8 h at 25 °C without shaking. A total of 50 µL of the mixtures of bacteria cells were then inoculated on LB agar selection plates supplemented with 50 mg/L Amp, 25 mg/L Kan, 80 mg/L NAA, and 10 mg/L Tet for 24 h at 37 °C. The conjugative transfer frequency was calculated according to the formula: conjugative transfer frequency = the number of transconjugants (CFU/mL)/the number of recipients (CFU/mL). At least three independent conjugation experiments were performed.

## PCR and gel electrophoresis

The presence of the RP4 plasmid in transconjugants was confirmed by the polymerase chain reaction (PCR). At least three transconjugant colonies were randomly selected and cultured in LB broth overnight. Plasmid was extracted using a Quick Plasmid Miniprep Kit (Takara, Dalian, China). Partial fragments of two specific genes on the RP4 plasmid were amplified with 100 ng of the DNA template, 10 pmol of each primer, and  $1 \times$  Taq mixture (Takara) using the following conditions: initial denaturation at 95 °C for 5 min, followed by 31 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The last extension was at 72 °C for 10 min. The primers used in this study were RP4-for and RP4-rev, *trfAp*-for and *trfAp*-rev (Qiu et al. 2012). PCR products (104 bp and 183 bp of RP4 and *trfAp* genes, respectively) were analyzed using 2% agarose gel electrophoresis.

## Analyses of ROS and cell membrane permeability changes induced by TCS

The fluorescent reporter dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Beyotime, Shanghai, China) was used to evaluate intracellular ROS concentration as previously described (Zhang et al. 2017). Briefly, donor *E. piscicida* and recipient *E. coli* JM109 cell suspensions (in PBS at approximately  $10^6$  CFU/mL) were incubated individually with 15 µM DCFH-DA for 30 min at 28 °C, in the dark with 120 rpm shaking. The bacterial cells were then washed two times with PBS to remove the extracellular DCFH-DA probe, then treated with TCS at different concentrations (0, 0.02, 0.2, 2, 20, 200, and 2000 µg/L) for 2 h at room temperature without light. The samples were then transferred to a 96-well plate (200 µL per well) and scanned using a microplate reader (Spectra Max M5; Molecular Devices, San Jose, CA, USA) to measure the fluorescence intensity (excitation at 488 nm/emission at 525 nm). All tests were conducted with biological triplicate samples.

**Table 1** MICs of different bacteria strains

	MIC (mg/L)				
	Col	Kan	Tet	Amp	NAA
<i>E. piscicida</i>	64	8	0.5	2	32
<i>E. coli</i> JM109	0.06	8	2	2	512
<i>E. piscicida</i> (RP4)	ND	1024	256	$\geq 2048$	ND
<i>E. coli</i> JM109 (RP4)	ND	1024	128	$\geq 2048$	ND

The effect of TCS on cell membrane permeabilities of donor and recipient strains were investigated by propidium iodide (PI) staining as previously described (Riccardi and Nicoletti 2006; Zhang et al. 2017). As a popular DNA staining dye, PI could not pass through the intact cell membranes, but only stains cells with compromised membranes. Therefore, the permeability degree of bacterial cell membrane could be characterized by the intensity of the generated fluorescence (Riccardi and Nicoletti 2006; Zhang et al. 2017). *E. piscicida* and *E. coli* JM109 cells were exposed to TCS for 2 h, and then washed with PBS buffer. Then, bacterial cells were resuspended at a density of  $10^6$  CFU/mL in PBS buffer and incubated with 20  $\mu$ M PI at room temperature for 30 min in the dark with shaking at 100 rpm. The fluorescence of 200  $\mu$ L stained cells from each group was quantitated using a microplate reader (Spectra Max M5; Molecular Devices) (excitation at 488 nm/emission above 630 nm). Untreated cells were used as negative controls. All tests were conducted with biological triplicate samples.

### Qualitative real-time polymerase chain reaction (qRT-PCR)

A qRT-PCR assay was used to quantify the transcriptional levels of genes involved in HGT during conjugative processes. Total RNA of bacterial samples exposed to TCS for 5 h was isolated using a Bacterial RNA Kit (Omega Bio-Tek, Norcross, GA, USA). According to the manufacturer's instructions, the elimination of genomic DNA and reverse transcription were performed using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara). Transfer-associated genes were selected, including three global regulator genes (*korA*, *korB*, and *trbA*), one plasmid DNA transfer and replication system gene (*trfAp*), one mating pair formation system gene (*trbBp*) on RP4 plasmid, two outer membrane protein genes (*ompA* and *ompC*), and three oxidative stress genes (*rpoS*, *sodB*, and *katG*) of *E. piscicida*, two outer membrane protein genes (*ompA* and *ompC*), and one oxidative stress gene (*rpoS*) of *E. coli* JM109. The 16S rDNA was chosen as an internal control. The Power SYBR®Green PCR master mixture (Life Technologies, Carlsbad, CA, USA) was used as a dye for the qRT-PCR reaction by following the manufacturer's instructions. All PCR reactions occurred in a 96-well or 384-well ABI plate format in ViiA7 (Life Technologies, Carlsbad, CA, USA). The amplification process was initiated at 50 °C for 2 min and a secondary step at 95 °C for 10 min, followed by 40 cycles of PCR reactions (95 °C for 10 s and 60 °C for 1 min). Dissociative curves were used to confirm the reaction specificity. The  $2^{-\Delta\Delta C_t}$  method was used to quantitate the reaction (Livak and Schmittgen 2001). All PCR primers are listed in Supplementary Table S2.

### Statistical analysis

Each experiment was conducted independently at least three times. SPSS statistical software for Windows, version 18.0 (SPSS, Chicago, IL, USA), was used for all data analyses. All data are presented as the mean  $\pm$  SD. Student's *t*-test was used for comparing differences between two groups. In experiments with more than two groups, one-way analysis of variance was performed, followed by Tukey's post hoc test for comparisons among groups. A value of  $p < 0.05$  was considered to be significant, and  $p < 0.01$  was considered to be highly significant.

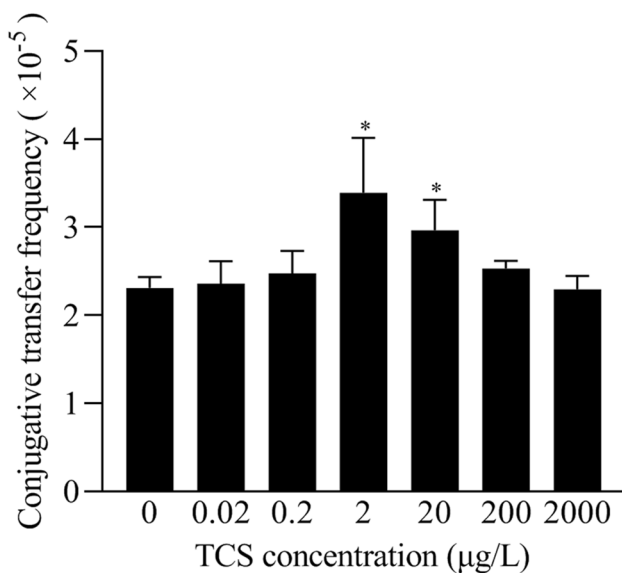
## Results

### Evidence of RP4 plasmid transfer by PCR detection and antibiotic MIC characteristics

To establish an inter-genus conjugative transfer model, the antibiotic resistance spectra of *E. piscicida*, *E. piscicida* (RP4), and *E. coli* JM109 were determined. Table 1 shows that *E. piscicida* was resistant to Col (MIC: 64 mg/L) and NAA (MIC: 32 mg/L), while it was sensitive to Kan (MIC: 8 mg/L), Amp (MIC: 2 mg/L), and Tet (MIC: 0.5 mg/L). The candidate donor strain (*E. piscicida* with RP4) was resistant to Col, Kan (MIC: 1,024 mg/L), Amp (MIC: 2,048 mg/L), and Tet (MIC: 256 mg/L). In contrast, *E. coli* JM109 was susceptible to Col (MIC: 0.06 mg/L), Kan (MIC: 8 mg/L), Amp (MIC: 2 mg/L), and Tet (MIC: 2 mg/L) but exhibited high MIC against NAA (MIC: 512 mg/L). For their different antibiotic resistance spectra, *E. piscicida* (RP4) and *E. coli* JM109 were used as donor and recipient strains, respectively, for conjugation experiments. The transfer of ARGs was conducted as previously described and transconjugants were selected on plates with Amp (50 mg/L), Kan (25 mg/L), Tet (10 mg/L), and NAA (80 mg/L), and then verified by PCR. As expected, all transconjugants were positive with the specific PCR product bands (Supplement Fig. S1) and showed high resistance against Tet (MIC: 256 mg/L), Kan (MIC: 1,024 mg/L), Amp (MIC  $\geq$  2,048 mg/L), and NAA (Table 1). No transconjugant grew on *E. piscicida* selective agar plates containing 12.5 mg/L Col, indicating that these colonies were *E. coli* species with the RP4 plasmid rather than the donor *E. piscicida*. The ARGs transfer frequency from *E. piscicida* to *E. coli* JM109 was approximately  $2.3 \pm 0.12 \times 10^{-5}$  per recipient cell.

### TCS significantly enhances the conjugative transfer frequency of ARGs mediated by *E. piscicida*

Figure 1 showed that the horizontal transfer frequencies of ARGs were significantly promoted after 8 h treatment

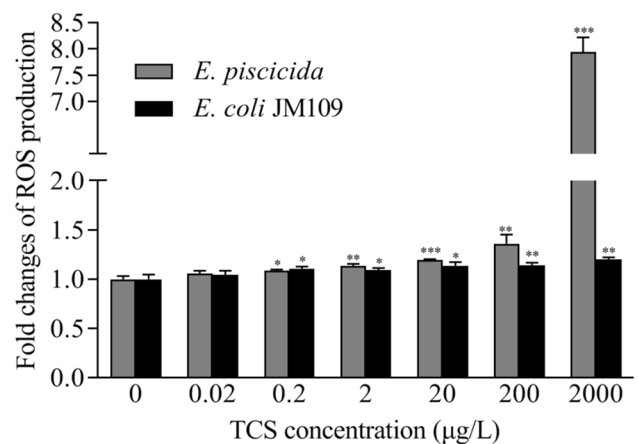


**Fig. 1** The effect of different concentrations of TCS on the conjugative transfer frequencies of the RP4 plasmid from *E. piscicida* to *E. coli* JM109 after 8 h at 25 °C. Significant differences between individual TCS-treated groups and the untreated control (0 µg/L of TCS) were shown with \* $p < 0.05$

of TCS (2.0 and 20 µg/L) ( $p < 0.05$ ) and the corresponding frequency was  $3.39 \pm 0.63 \times 10^{-5}$  and  $2.96 \pm 0.35 \times 10^{-5}$  per recipient cell. The transfer frequency reached a peak at 2 µg/L and was 1.4-fold higher than that of the untreated control group. However, the transfer frequencies were not significantly elevated under low concentrations of TCS (0.02 and 0.2 µg/L), when compared with the control group. When increasing exposure concentration to 200 or 2000 µg/L, the transfer frequency was about  $2.53 \pm 0.09 \times 10^{-5}$  and  $2.29 \pm 0.16 \times 10^{-5}$  per recipient cell, respectively. Transfer of ARGs was obviously inhibited by higher concentrations (200 and 2000 µg/L), when compared with the 2 µg/L-treated group. The exact frequencies were similar to that of the untreated control group (Fig. 1). The numbers of the donors, recipients, and transconjugants are shown in Fig. S3.

### TCS promotes ROS generation and induces oxidative stress

As shown in Fig. 2, the amounts of ROS produced in the donor *E. piscicida* and the recipient *E. coli* JM109 were significantly promoted by TCS treatment (0.2–2000 µg/L) ( $p < 0.05$ ), when compared with the untreated group. These results suggested that TCS stimulated *E. piscicida* and *E. coli* JM109 to produce ROS. The production peaked at 2000 µg/L in *E. piscicida* and was approximately 8.5 times higher than the untreated group (Fig. 2,  $p < 0.01$ ), while there was no obvious peak for the intra-ROS level in *E. coli* JM109 under all TCS dosages, even at 2000 µg/L (Fig. 2).

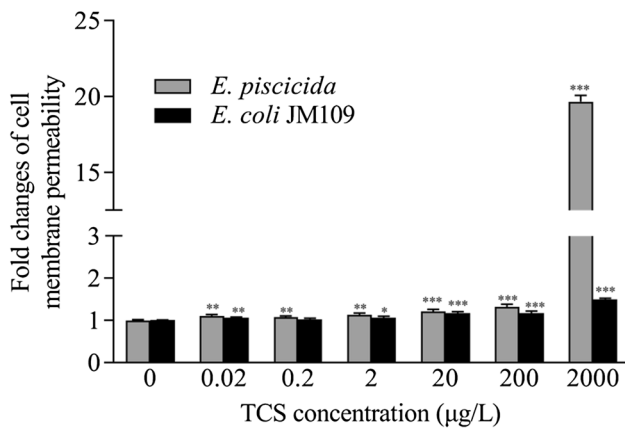


**Fig. 2** Relative ROS levels in donor (*E. piscicida*) and recipient (*E. coli* JM109) bacteria upon exposure to TCS at different concentrations. The results represent the mean ± SD of three replicate samples. The gap in the y-axis shows results with high variations in the same bar diagram. Significant differences between and within groups were marked with \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

The effect of TCS on bacterial oxidative stress response at the transcriptional level was also investigated. The results showed that TCS induced the expression of the stress-response sigma factor RpoS, which promoted survival under various stress conditions (Gutierrez et al. 2013; Xiao et al. 2009; Zhang et al. 2017). Environmental level (2 µg/L) of TCS significantly upregulated the expression of *rpoS* about 25.8% in *E. piscicida* (Fig. 4a) and approximately 15.3% in *E. coli* JM109 (Fig. 5). Moreover, the antioxidant-related genes, *sodB* and *katG* (Xiao et al. 2009; Yin et al. 2017) of *E. piscicida*, were induced and increased 21–29% and 17–22% when exposed to TCS (2–20 µg/L) (Fig. 4b), respectively. TCS induced oxidative stress in both strains.

### TCS increases the cell membrane permeability

PI staining was used to assess the membrane permeability of the donor and recipient strains (Zhang et al. 2017). The results indicated that TCS exposure disrupted the membrane integrity and significantly increased membrane permeability of *E. piscicida*, when compared with the untreated group (Fig. 3,  $p < 0.05$ ). Remarkably, exposure to 2000 µg/L TCS increased the membrane permeability of *E. piscicida* by 19-fold and reached a peak. The membrane permeability of *E. coli* JM109 was also significantly elevated at all TCS concentrations, except 0.2 mg/L (Fig. 3), although no obvious peak was observed. OmpA and OmpC are classical membrane porin proteins and play vital roles in the regulation of membrane permeability (Qiu et al. 2012; Wang et al. 2015). Figure 4c shows that environmental concentrations of TCS (2–20 µg/L) increased the expressions of *ompA* and *ompC* by approximately 12–27% in *E. piscicida* ( $p < 0.05$ ), and



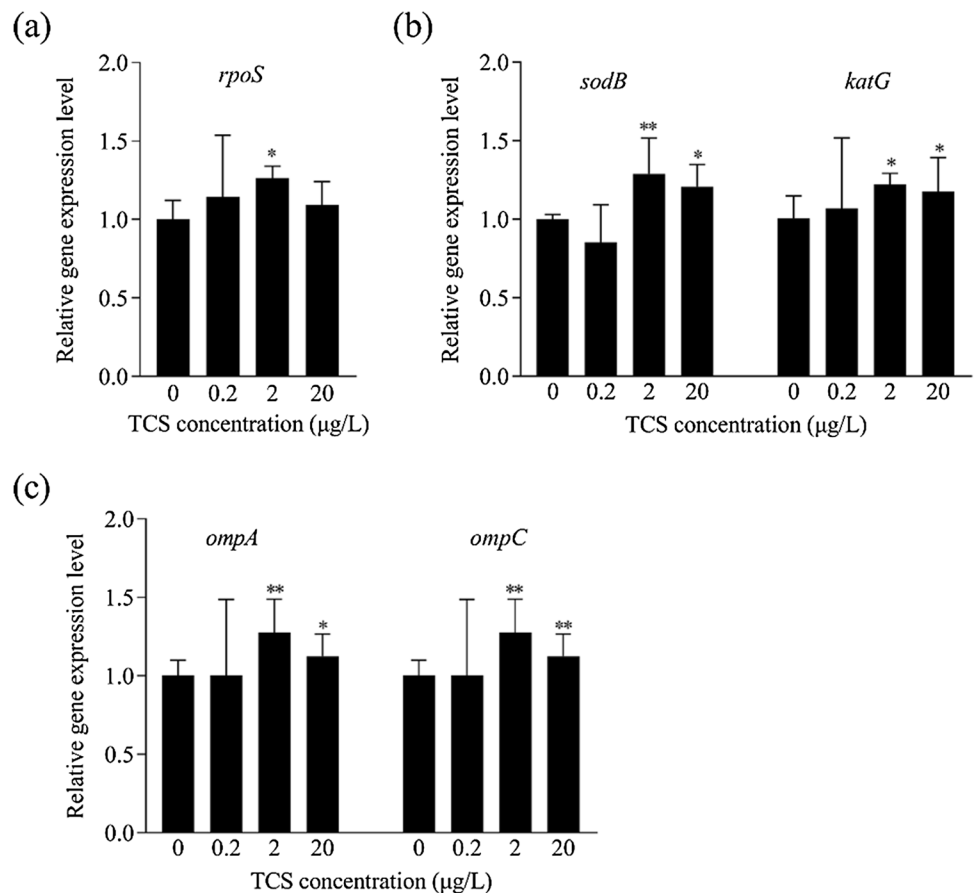
**Fig. 3** Fold changes of cell membrane permeability in donor (*E. piscicida*) and recipient (*E. coli* JM109) bacteria upon exposure to TCS at different concentrations. The results represent the mean ± SD of three replicate samples. The gap in the y-axis shows results with high variations in the same bar diagram. Significant differences between and within groups were marked with \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

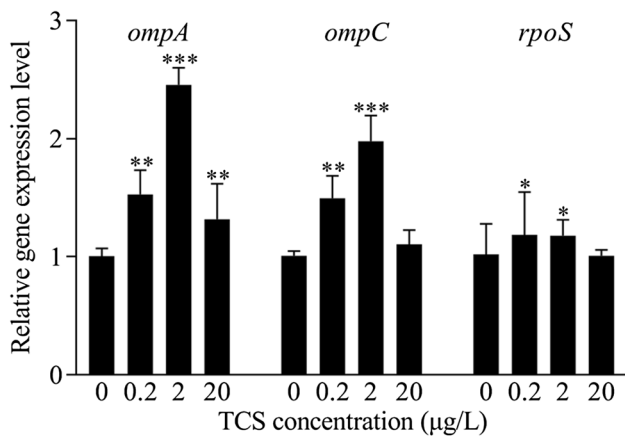
up-regulated *ompA* and *ompC* by approximately 9.8–145% in *E. coli* JM109 (Fig. 5,  $p < 0.01$ ). Notably, all the peak expressions were reached at 2.0 µg/L of TCS.

### Effects of TCS on the expression of conjugative transfer-related genes

In the present study, *E. piscicida* with the RP4 plasmid was used as the donor strain. The RP4 encodes several important conjugative transfer-related proteins, including negative global regulators (*KorA*, *KorB* and *TrbA*), mating pair formation protein (*TrbBp*), and plasmid transfer and replication protein (*TrfAp*) (Qiu et al. 2012; Schroder and Lanka 2005; Zhang et al. 2017). These proteins control the formation of the conjugation bridges and facilitate DNA transfer between the recipient and donor strains (Qiu et al. 2012; Schroder and Lanka 2005; Zhang et al. 2017). Figure 6 shows that TCS exposure regulated their expressions during conjugation, and *korA* and *korB* changed slightly at 0.2 µg/L (Fig. 6a,  $p > 0.05$ ), but *trbA* down-regulated 27% (Fig. 6b,  $p < 0.01$ ). When exposed to higher concentrations (2 and 20 µg/L), *korA*, *korB*, and *trbA* were synchronously repressed (Fig. 6a). Their expression levels were down-regulated by 51%, 26%, and 46% at 2 µg/L ( $p < 0.01$ ), respectively, when compared with the control group. Reduction of these negative global regulators subsequently activated the downstream *trfAp* and *trbBp* genes (Fig. 6b). The qRT-PCR results indicated that *trbBp* was up-regulated approximately

**Fig. 4** Relative mRNA expression levels of the oxidative stress-related genes *rpoS* (a); *sodB*, *katG* (b); and outer membrane protein genes, *ompA* and *ompC* (c) in *E. piscicida* stimulated by TCS. Significant differences between TCS-treated groups and the control group (0 µg/L of TCS) were shown with \* $p < 0.05$ ; \*\* $p < 0.01$





**Fig. 5** Relative mRNA expression levels of the outer membrane protein genes *ompA* and *ompC*, and oxidative stress related genes *rpoS* in *E. coli* stimulated by TCS. Significant differences between TCS-treated groups and the control group (0 µg/L of TCS) were shown with \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$

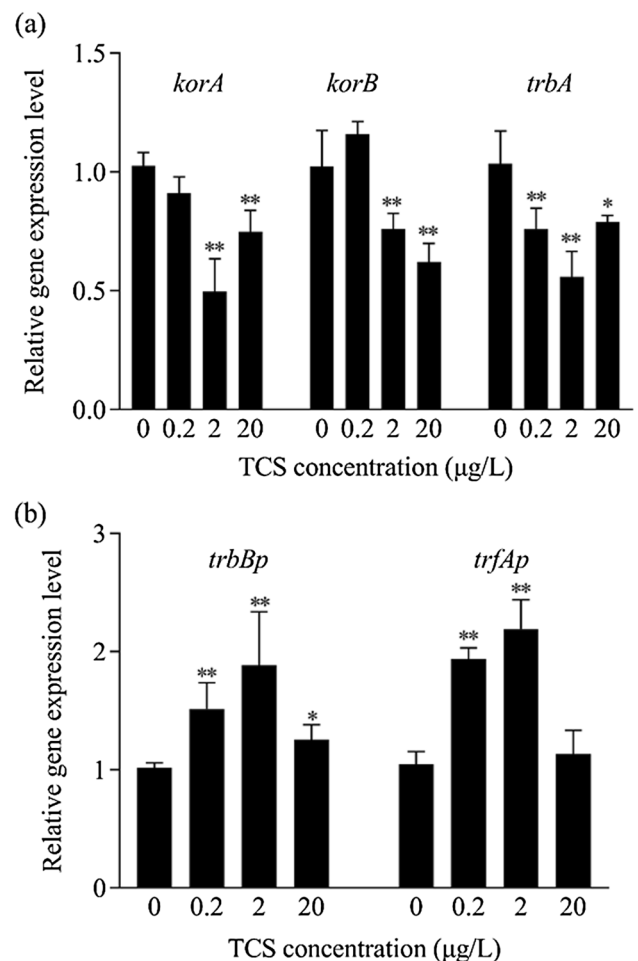
52–85% ( $p < 0.05$ ) at concentrations of 0.2–2.0 µg/L, and increased by 85–110% ( $p < 0.01$ ) for the *trfAp* gene (Fig. 6b), when compared with the control group. However, *trbBp* and *trfAp* were significantly down-regulated at 20 µg/L, when compared with the 2 µg/L-treated group, although the *trbBp* transcriptional level was still 23% higher than that of the untreated group.

## Discussion

### E. piscicida disseminates ARGs

To meet the increasing consumption of aquaculture products, the industry is rapidly expanding and veterinary pharmaceuticals (including different antibiotics) are widely over-used (Schar et al. 2020; Xiong et al. 2015). Previous reports showed that tetracyclines, sulfonamides, and quinolones are regularly used by stakeholders and also frequently detected in aquaculture systems (Xiong et al. 2015; Zhou et al. 2020). The resistance of these antibiotics could be encoded by corresponding resistant genes. For example, *tet* (*M*, *O*, *W*, *Q*, and *X*) genes encode tetracycline resistance, *sul* (*1*, *2*, and *3*) genes encode sulfonamide resistance, and *qnr* (*A*, *B*, *C*, *D*, *S* and *VC*), *oqxAB*, *qepA*, and *aac(6′)-Ib* genes encode quinolone resistance (Miller and Harbottle 2018). Moreover, these ARGs could locate on mobile genetic elements (MGEs), such as the transferable plasmid, class 1 integron, and transposon in aquaculture pathogens (Miller and Harbottle 2018).

*E. piscicida* is one of the most frequently isolated pathogenic bacteria in aquaculture surveys (Leung et al. 2019; Miller and Harbottle 2018; Niu et al. 2019). In addition,



**Fig. 6** Relative mRNA expression levels of global regulatory genes (*korA*, *korB* and *trbA*) (a) and conjugation related genes (*trbBp* and *trfAp*) on the RP4 plasmid (b) stimulated by TCS at various concentrations. Significant differences between individual TCS-treated groups and the control (0 µg/L of TCS) were shown with \* $p < 0.05$  and \*\* $p < 0.01$

many isolates are multi-resistant, even containing endogenous antibiotic genes on MGEs (Abdelhamed et al. 2019; Leung et al. 2019; Miller and Harbottle 2018; Sun et al. 2009). Therefore, the widely used conjugative plasmid RP4 was employed to investigate the transfer of ARGs between bacteria (Lu et al. 2018b; Qiu et al. 2012; Wang et al. 2015; Zhang et al. 2017). Moreover, RP4 encodes several conjugative related genes, which facilitate self-transmission (Qiu et al. 2012; Schroder and Lanka 2005; Zhang et al. 2017). This study found that RP4 transferred from *E. piscicida* to *E. coli* JM109 and other *E. coli* strains (Fig. S2) by conjugation. These transconjugants showed high resistance against Tet, Kan, Amp, and NAA (Table 1) and became ARBs. Considering that there are many pathogens with MGEs in aquaculture, the ecological risk of cascading spread of ARGs and the threat to public health should be seriously considered.

## Environmentally relevant concentrations of TCS promote transfer of ARGs

It has been reported that many emerging environmental pollutants, including TCS, can facilitate ARG dissemination via HGT between bacteria. During the COVID-19 pandemic, the usage of TCS might significantly increase (Milanovic et al. 2021). Used TCS eventually persists in aquatic environments and results in chronic exposure of aquaculture pathogens and might promote the conjugative transfer of ARGs. In the present study, we found that the MIC of *E. piscicida* against TCS was 32,000 µg/L and was much higher than that of *E. coli* JM109 (512 µg/L). Environmentally relevant concentrations of TCS (2 µg/L and 20 µg/L) still promoted transfer of ARGs from *E. piscicida* to *E. coli* (Fig. 1). However, a high concentration of TCS (200 or 2000 µg/L) inhibited ARGs transfer, perhaps because it was harmful to the *E. coli* recipient and resulted in bacterial death. Similar effects have been reported (Lu et al. 2018b). It is worth noting that some other severe fish pathogens also show high resistance to TCS. The MIC of *Saprolegnia parasitica* to TCS was 4000 µg/L and 25,000 µg/L for *Aeromonas hydrophila* ATCC® 49,140 (Karmakar et al. 2019; Kumar et al. 2020). Therefore, high concentrations of TCS might still promote ARGs transfer between these resistant pathogenic strains.

When compared to other studies (Lu et al. 2018b; Qiu et al. 2012), the transfer rates of ARGs for *E. piscicida* and *E. coli* increased only a small amount (1.4-fold) by TCS exposure. However, it should be noted that this is a serious trend and the real ARGs transfer rate is likely to be underestimated, because (1) the pure culture bacterial system under laboratory conditions of this study could only partially mimic ARGs dissemination in the real aquaculture environment. There are many aquatic bacteria that could act as donors or recipients (including some uncultured species), and ARGs may be transferred with high efficiency among indigenous bacterial species in natural biofilms of aquatic communities, but we could not detect those transconjugants by traditional selective plates; (2) in contaminated aquatic ecosystems, the donors and recipients were exposed to TCS much longer than the acute exposure time (8 h) of this study. Qiu et al. (2012) found that prolonging the exposure time could also promote the transfer of ARGs between bacteria and might induce an accumulative effect. Collectively, the underlying ecological risk of environmental TCS on ARGs spread should not be neglected and new molecular methods, such as fluorescence-activated cell sorting, high-throughput sequencing and metagenomics analysis, should be introduced to enhance future research.

## Mechanism of TCS promoting conjugative transfer

There are three key factors affecting ARGs conjugative transfer: generation of ROS, increased cell membrane permeability, and plasmid conjugation activity (Qiu et al. 2012; Zhang et al. 2017; Lu et al. 2018b). To elucidate the potential mechanisms of TCS on the transfer of ARGs, the level of ROS was characterized. The results showed that environmentally relevant concentration of TCS exposure stimulated ROS generation in *E. piscicida* and *E. coli* and the levels of intra-ROS slightly increased in the range of 0.2–200 µg/L. (Fig. 2). Growing studies reported that the bacterial cell membrane was an important permeable barrier for ARGs transfer between different bacteria, but oxidative stress damaged its integrity and increased permeability to help the transfer of ARGs (Qiu et al. 2012; Zhang et al. 2017; Lu et al. 2018b). These results were consistent with previous studies.

The ROS production pattern of *E. piscicida* was totally different from that of *E. coli* JM109 at 2000 µg/L, the former reached a peak and was up-regulated eightfold, while the later was stable without an obvious change (Fig. 2). Notably, the reaction pattern of membrane permeability was highly similar to that of ROS generation. Environmentally relevant concentrations (0.2–200 µg/L) of TCS exposure just slightly increased membrane permeability, except for 2000 µg/L in *E. piscicida*. The results may be due to their high MICs against TCS. *E. piscicida* (MIC: 32,000 µg/L), and *E. coli* JM109 (MIC: 512 µg/L) both showed poor sensitivities to TCS at 200 µg/L which led to limited ROS and membrane permeability (Figs. 2 and 3). Similar results were observed in an inter-genera transfer between *E. coli* K-12 LE392 and *Pseudomonas putida* KT2440 (Lu et al. 2018b). Even the highest concentration (2000 µg/L) was subinhibitory to *E. piscicida*, but it was completely beyond the tolerance of *E. coli* JM109. ROS and membrane permeability were therefore significantly increased at 2000 µg/L in *E. piscicida* and *E. coli*, while the transfer rates of ARGs were still inhibited.

The qRT-PCR results further revealed transcriptional responses of the oxidative stress-related pathways in *E. piscicida* and *E. coli* JM109. Both strains up-regulated the expressions of antioxidant genes to defend against TCS-induced oxidative stress, such as *rpoS* in *E. coli* (Fig. 5), and *rpoS*, *sodB*, *kat* in *E. piscicida* (Fig. 4). Additionally, environmentally relevant concentrations of TCS increased the expressions of *ompA* and *ompC* to elevate membrane permeability. In *E. piscicida*, *ompA* and *ompC* up-regulated at 2 µg/L and 20 µg/L (Fig. 4c), but they were significantly up-regulated at 0.2 µg/L and 2 µg/L in *E. coli* (Fig. 5). Notably, a lower concentration (0.2 µg/L) was needed to significantly induce *rpoS*, *ompA* and *ompC* in *E. coli* (Fig. 5), and a higher dose (at least 2.0 µg/L) was needed in *E. piscicida* (Fig. 4). Comparatively, *E. piscicida* and *E. coli* shared the



same optimally treated concentration (2.0 µg/L of TCS) (Fig. 4 and 5) which was an environmentally relevant concentration with the highest transfer rate in this study. It is therefore reasonable to assume that TCS could cause a more serious spread of ARGs in the real environment.

Besides increased ROS production and cell membrane permeability, TCS also regulated expression levels of conjugative transfer related genes on RP4 (Zhang et al. 2017; Lu et al. 2018b). In this study, we found that environmental concentrations of TCS significantly inhibited the expressions of the globally regulatory genes, *korA*, *korB*, and *trbA* (Fig. 6). Down-regulation of *korA* and *korB* significantly increased the expression of plasmid transfer and replication (Dtr) system gene, *trfA*, up to 2.2-fold (Fig. 6b), while repression of *KorB* and *TrbA* up-regulated the expression of the Mpf gene, *trbBp*, up to 1.8-fold (Fig. 6), which was associated with the production of mating pilus for transfer of plasmid DNA. It was reported that the expressions of Dtr and Mpf system genes were positively correlated to conjugative transfer (Wang et al. 2015; Zhang et al. 2017). The moderate increase of the *trfAp* and *trbBp* genes shown in our study might partly explain why the intra-genera conjugative transfer was not as high as in other studies (Qiu et al. 2012; Wang et al. 2015; Zhang et al. 2017; Lu et al. 2018b).

## Conclusion

In this study, the impact of TCS on the HGT of ARGs in a pure culture bacterial mating system was investigated. The results demonstrated that environmentally relevant concentrations (2–20 µg/L) of TCS significantly promoted the dissemination of ARGs mediated by the aquaculture pathogen, *E. piscicida*, when compared with the untreated group. The underlying mechanism may be that TCS exposure stimulated the generation of ROS, increased cell membrane permeability, and regulated a series of conjugative related genes to facilitate the transfer of ARGs. These results improved our understanding of the potential risks of aquaculture pathogens and residual TCS in the aquatic environment. However, the synergistic ecological effects of aquaculture pathogens and emerging contaminants on ARGs transfer are complex and need further investigation.

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**Author contribution** LJF, ZH, and PLL designed and conducted experiments, analyzed data, and wrote the manuscript. GWC analyzed data and revised the manuscript. LJF and LYL conceived the project, provided funding, and revised the manuscript. All authors read and approved the final manuscript.

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**Data availability** All data generated or analyzed during this study are included in this published article and its supplementary information files.

## Declarations

**Ethics approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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