



Function Characterization of Endogenous Plasmids in *Cronobacter sakazakii* and Identification of *p*-Coumaric Acid as Plasmid-Curing Agent

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Specialty section:

This article was submitted to
Microbiotechnology,
a section of the journal
Frontiers in Microbiology

Received: 29 March 2021

Accepted: 25 May 2021

Published: 25 June 2021

Citation:

Ji X, Lu P, Hu Y, Xue J, Wu J,
Zhang B, Zhang Y, Dong L, Lv H and
Wang S (2021) Function
Characterization of Endogenous
Plasmids in *Cronobacter sakazakii*
and Identification of *p*-Coumaric Acid
as Plasmid-Curing Agent.
Front. Microbiol. 12:687243.
doi: 10.3389/fmicb.2021.687243

Virulence traits and antibiotic resistance are frequently provided by genes located on plasmids. However, experimental verification of the functions of these genes is often lacking due to a lack of related experimental technology. In the present study, an integrated suicide vector was used to efficiently and specifically delete a bacterial endogenous plasmid in *Cronobacter sakazakii*. The pESA3 plasmid was removed from *C. sakazakii* BAA-894, and we confirmed that this plasmid contributes to the invasion and virulence of this strain. In addition, the pGW1 plasmid was expunged from *C. sakazakii* GZcsf-1, and we confirmed that this plasmid confers multidrug resistance. We further screened plasmid-curing agents and found that *p*-coumaric acid had a remarkable effect on the curing of pESA3 and pGW1 at sub-inhibitory concentrations. Our study investigated the contribution of endogenous plasmids pESA3 and pGW1 by constructing plasmid-cured strains using suicide vectors and suggested that *p*-coumaric acid can be a safe and effective plasmid-curing agent for *C. sakazakii*.

Keywords: plasmid curing, virulence, antibiotic resistance, *Cronobacter sakazakii*, *p*-coumaric acid

INTRODUCTION

Previously developed strategies for the treatment of systemic infections caused by pathogenic bacteria still rely on the application of various antibiotics. However, the frequent and extensive utilization of antibiotics has dramatically increased the possibility of severe infections related to bacteria with multidrug resistance, posing a more aggressive threat to public health and necessitating the development of novel antimicrobials and bacterial vaccines. Moreover, prospective research on the underlying mechanism of multidrug resistance could facilitate the modification of currently employed antibiotics and the development of novel reagents. To date, multidrug resistance has been observed in a variety of pathogenic bacteria, including *Neisseria gonorrhoeae* (Yuan et al., 2019; Chen et al., 2020; Seong et al., 2020), *Pseudomonas aeruginosa* (Horcajada et al., 2019; Kumarage et al., 2019; Tada et al., 2019), and *Acinetobacter baumannii* (da Silva et al., 2018; Salehi et al., 2018).

Related studies have unveiled the inseparable correlation between bacterial antibiotic resistance and plasmids, which could be verified by the frequent distribution of virulence determinants and antibiotic resistance genes in plasmids (Stratev and Odeyemi, 2016; Kopotsa et al., 2019). Importantly, the abuse of antibiotics promoted the spread of antibiotic-resistant plasmids that have the potential to increase the occurrence of virulence or drug resistance genes, posing an increased threat to society (Fraise, 2002; Zhang et al., 2011; Mangat et al., 2017).

Bacterial plasmids can significantly vary in size from 2 to 2,000 kbp (Canteros, 1990). Large plasmids can potentially harbor more transcriptomic information and perform relatively important functions that contribute to host survival.

Recently, *Cronobacter sakazakii*, an emerging pathogenic bacterium belonging to the family Enterobacteriaceae, has attracted a great deal of attention due to its multidrug resistance and ability to cause disease with a high mortality rate (Parra-Flores et al., 2018; Odeyemi and Abdullah Sani, 2019). *C. sakazakii* is a foodborne pathogen associated with life-threatening sepsis (Hassan and Naser, 2018), meningitis (Chaves et al., 2018; Zeng et al., 2018), and necrotizing enterocolitis (Fan et al., 2019) in premature and full-term infants. *C. sakazakii* has been widely isolated and identified from powder infant formula (PIF) (Li et al., 2019), fruit powders, vegetables, tea leaves, herbs, cereals, and spices (Shi et al., 2016).

The pathogenicity and multidrug resistance of *C. sakazakii* are believed to be associated with two large plasmids that it harbors, pESA3 and pGW1, with sizes of 134 and 340 kbp, respectively. A variety of virulence-related genes have been identified in pESA3 (Aly et al., 2019), and pGW1 has been reported to carry a number of drug resistance genes (Zeng et al., 2018).

The highly conserved replicon of RepFIB in pESA3 and its frequent identification in almost 97% of *Cronobacter* isolates has been previously described (Singh et al., 2017). pESA3 encodes two iron uptake systems (*lucABCD/lutA* and *EitCBAD*) that may facilitate host survival and pathogenesis. In 2018, one multidrug-resistant strain of *C. sakazakii*, GZcsf-1, was reported to be responsible for a case of meningitis in a neonate in China (Zeng et al., 2018). Two internalized plasmids, namely, pGW1 (340 kbp) and pGW2 (135 kbp), were identified, with 17 predicted drug resistance genes encoded by pGW1 and a high similarity observed for pGW2 to pESA3 (Zeng et al., 2018). Moreover, an *mcr-1*-harboring plasmid identified in *C. sakazakii* was confirmed to be correlated with colistin resistance (Liu et al., 2017; Nang et al., 2019), which is widely used as an antibiotic of last-resort against bacterial infections. Considering the ubiquitous presence of *C. sakazakii* in nature, as well as its excellent capacity for plasmid internalization, it may be associated with an increased risk of potential infections and an increased threat to public health, especially in infants.

Given the frequent observation of plasmid-mediated bacterial drug resistance, functional characterization of these plasmids is still needed. Currently, such functional studies primarily rely on comparative genomic analysis without further experimental verification, which may be attributed to the difficulty of transforming plasmids into *Escherichia coli* to further study their functions, especially large plasmids (Tu et al., 2016;

Chung et al., 2017). Moreover, the common presence of multiple replicons in large plasmids impedes the elucidation of their function through replicon incompatibility-mediated plasmid curing (Medaney et al., 2016; Douarre et al., 2020).

In this study, we used an integrated suicide vector to efficiently and specifically delete an endogenous plasmid of choice in *C. sakazakii* and analyzed the contributions of endogenous plasmids pESA3 and pGW1. The plasmid-cured strains were used to screen plasmid-curing agents and showed that *p*-coumaric acid had a remarkable effect on the plasmid curing, suggesting that *p*-coumaric acid can be a safe and effective plasmid-curing agent for *C. sakazakii*.

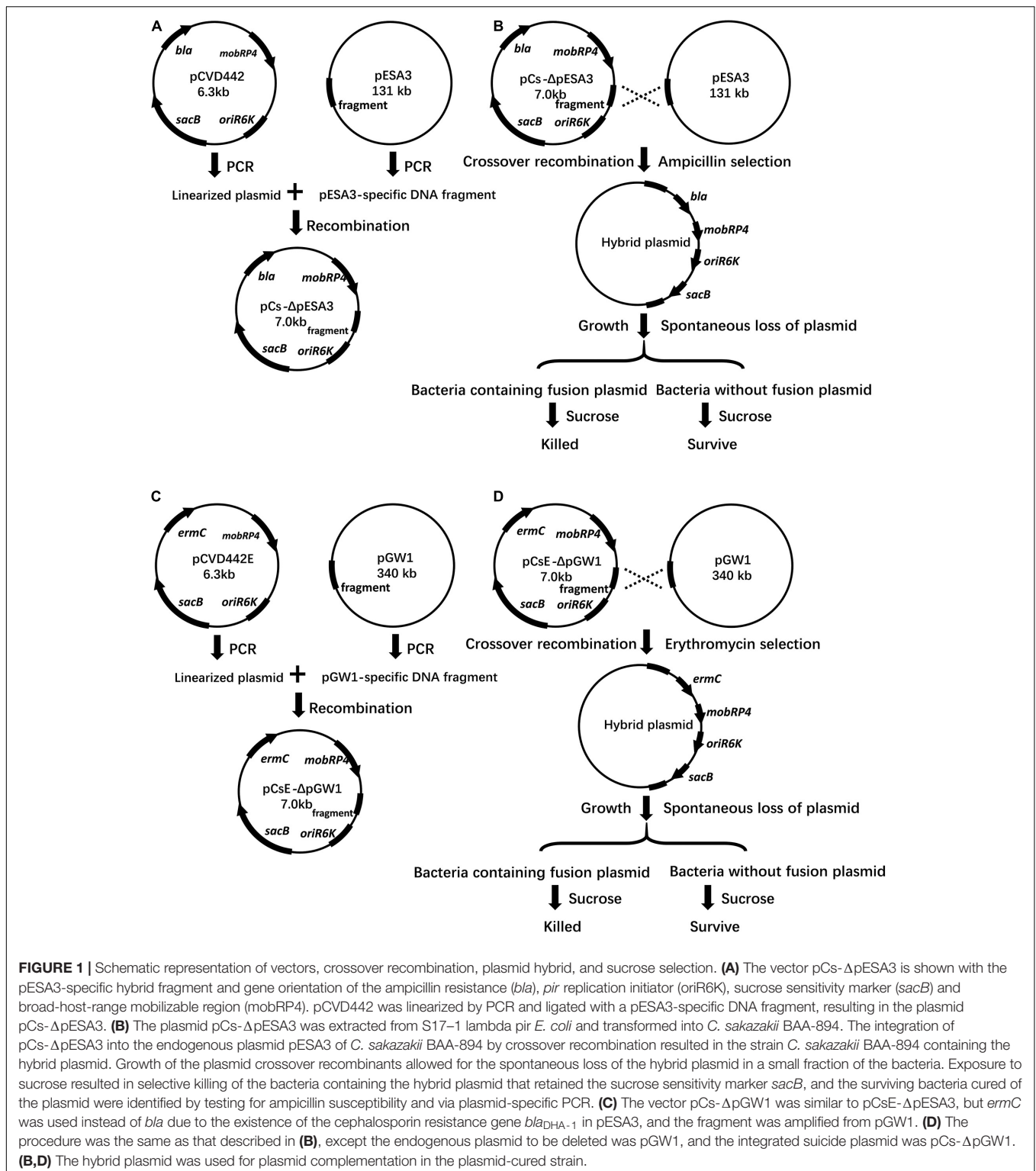
RESULTS

Strategy of Plasmid Curing in *C. sakazakii*

A strategy based on suicide vector pCVD442 was developed for plasmid curing in the current study. Generally, the plasmid-specific fragment of the suicide vector was taken from an endogenous plasmid (Figure 1), integration of the hybrid plasmid could then be specifically selected using the antibiotic corresponding to the resistance marker on the suicide vector. Then, the suicide plasmid was spontaneously removed in culture without antibiotic stress, leaving a suspension of two types of bacteria with or without the hybrid plasmid. Finally, based on the sensitivity to sucrose, the bacteria with the hybrid plasmid were eliminated by sucrose supplementation. The loss of the plasmid was verified by plasmid-specific PCR. 58 out of randomly selected 60 colonies growing on LB plates containing sucrose in pESA3 and pGW1 curing assays were verified to be plasmid curing strains, indicating a high frequency of loss of the plasmid. One hundred single colonies were selected in the sucrose selection step and verified to be plasmid-cured isolates. Compared to the traditional molecular method based on plasmid incompatibility, where 92% (92/100) of *C. sakazakii* transformant colonies retained pESA3 after 5 rounds of culturing in the presence of ampicillin, the method developed in the present study resulted in 100% (100/100) of transformant colonies being verified as pESA3-cured isolates (Figure 2). These results clearly showed that this method is efficient for expunging plasmids in *C. sakazakii*.

pESA3 Contributes to Invasion and Virulence

Many of the genes distributed on the pESA3 plasmid are predicted to be involved in the adhesion and infection of the host pathogens (Aly et al., 2019). To verify the role of pESA3 in pathogenicity, the *C. sakazakii* strain BAA-894 encapsulated with or without pESA3 was used to assess the contribution of pESA3 to the adhesion, invasion and virulence of the host. In addition, the hybrid plasmid was used for complementation pESA3 in the pESA3-cured strain (Figures 1C,D). *In vitro* analysis indicated that pESA3-cured bacteria were significantly attenuated in their ability to invade Caco-2 cells than the



wild-type strain (Figure 3B), but no difference was observed with respect to adhesion (Figure 3A). In addition, the results of an *in vivo* study revealed that *C. sakazakii* BAA-894 lacking pESA3 had notably lower lethality than wild-type bacteria after intragastric administration to 3-day-old rats (Figure 3C). The

survival rate of rats infected by wild-type bacteria was 40% at 120 h postinfection, while 80% survival was observed for those infected with pESA3-cured bacteria. Furthermore, the organs of young rats exhibited a significantly lower distribution of pESA3-cured bacteria 24 h postinfection compared to

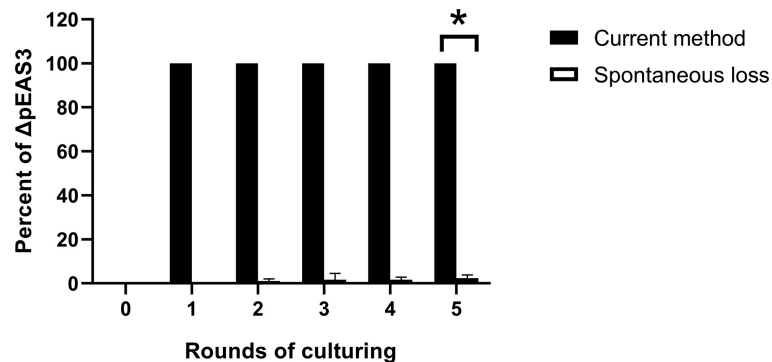


FIGURE 2 | Percentage of pESA3 plasmid-cured isolates after continuous culturing. The method described in the present study represents a strategy for plasmid curing in *C. sakazakii*, and the spontaneous loss of an endogenous plasmid represents the traditional molecular method based on plasmid incompatibility using another plasmid with the same replicon. The loss of the plasmid was verified by plasmid-specific PCR using primers listed in **Table 3**. At the beginning of the experiment, 10^5 CFU/mL bacteria harboring the integrated plasmid pCs- Δ pESA3 (**Figure 1B**) and bacteria harboring the incompatible plasmid pUC57-RepFIB were prepared and subsequently continuously cultured in LB containing sucrose and ampicillin, respectively. The cultures were shaken overnight at 37°C and then inoculated into 20 mL of fresh sterile LB medium at a 1:100 dilution the next day. After each round of culturing, bacterial samples were serially diluted and plated on agar to obtain signal colonies. Finally, 100 single colonies were selected, and their genotypes were determined by PCR using plasmid-specific primers. Each data point represents the average and standard deviation of three biological repeats. Student's t-test; * $P < 0.05$.

those infected with the wild-type or complemented strains (**Figures 3D–F**).

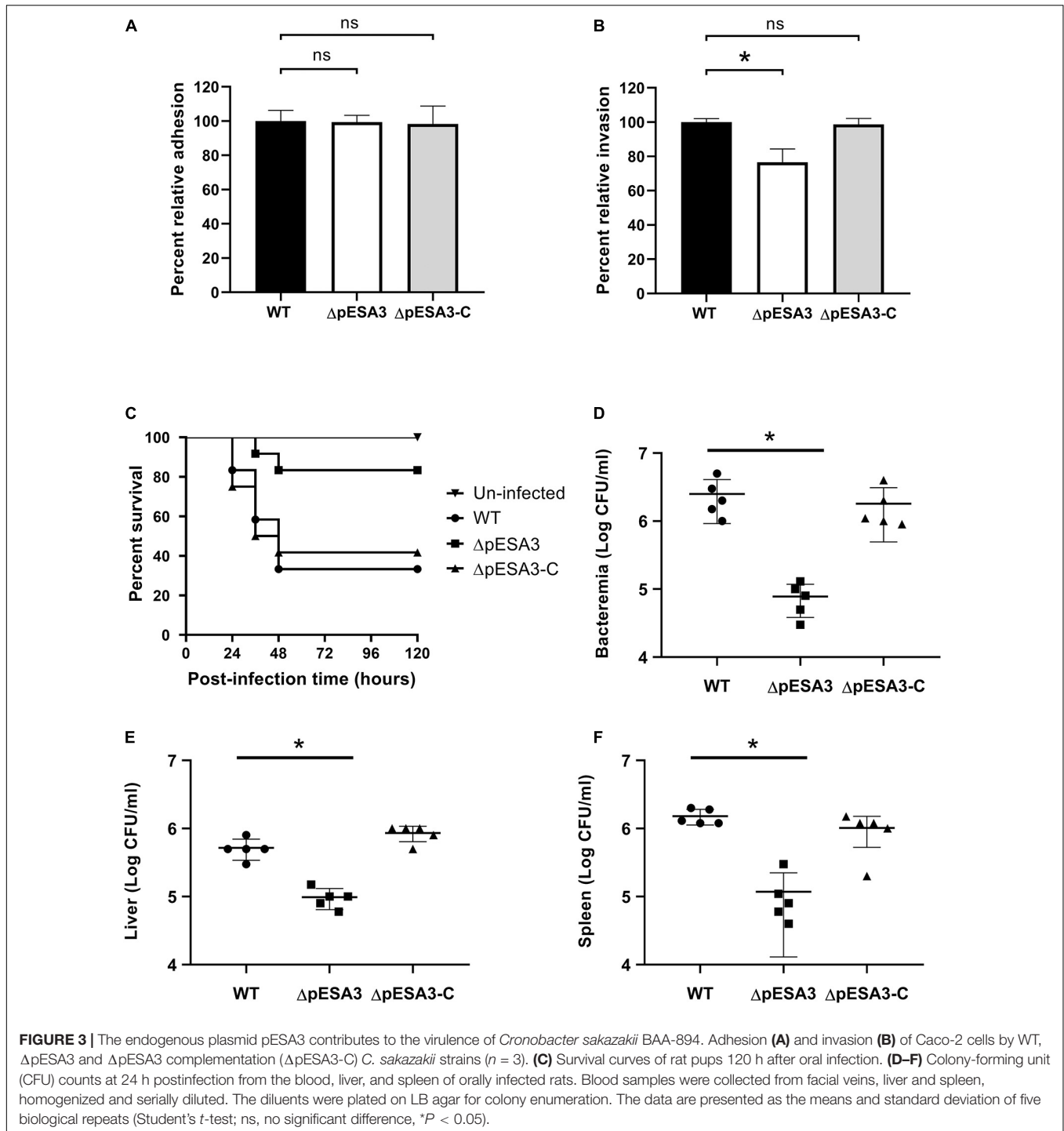
pGW1 Is Involved in Multidrug Resistance

C. sakazakii GZcsf-1 was initially isolated from the brain abscess fluid of an infant meningitis patient, and multidrug resistance to various antibiotics has been reported to be potentially linked with the endogenous plasmid pGW1 (Zeng et al., 2018). However, the dominant role of pGW1 in multidrug resistance remains obscure and needs to be investigated. Therefore, in the present study, the antibiotic sensitivity of wild-type *C. sakazakii* GZcsf-1 and *C. sakazakii* GZcsf-1 without pGW1 was determined (**Table 1**). The results of a minimum inhibitory concentration (MIC) analysis demonstrated the resistance of wild-type *C. sakazakii* GZcsf-1 to multiple antibiotics, namely, ampicillin, ceftriaxone, cefazolin, chloramphenicol, azithromycin, gentamicin, trimethoprim, aztreonam, and tetracycline, while the cured pGW1 variant was susceptible to all these antibiotics. In addition, both *C. sakazakii* GZcsf-1 strains harboring or lacking pGW1 exhibited resistance to spectinomycin and streptomycin. These results clearly demonstrated the association of pGW1 with multidrug resistance in *C. sakazakii* GZcsf-1, although not against spectinomycin and streptomycin, which is probably dependent on genes present in the genome. Indeed, the presence of aminoglycoside adenyltransferase (*aadA1*), a gene that confers antibiotic resistance to spectinomycin and streptomycin, has been confirmed in the genome of *C. sakazakii* GZcsf-1 (Zeng et al., 2018), which could potentially explain the obtained results.

Plasmid Curing of *C. sakazakii* With *p*-Coumaric Acid

It has been found that plant-derived chemicals have the effect of plasmid elimination (39). Considering that polyphenols are

naturally existing substances in food and are relatively safe, we next selected plasmid elimination drugs from polyphenols. The plasmid knockout strains obtained in our project were used to screen plasmid elimination drugs by comparing the growth rates of plasmid knockout strains and wild-type strains at sub-inhibitory concentrations of drugs. The minimal inhibitory concentration (MIC) of 12 polyphenols, including daidzin, trans-chalcone, apigenin, quercetin, flavanone, polydatin, trihydroxyflavone, caffeic acid, 5,7-Dihydroxyflavone, *p*-coumaric acid, isoferulic acid and phenethyl cinnamate, were determined using broth dilution methods. However, the MICs of 13 of these polyphenols were above 1,000 mg/L, and only *p*-coumaric acid had a MIC of 500 mg/L for both *C. sakazakii* GZcsf-1 and *C. sakazakii* BAA-894, irrespective of bacterial antibiotic resistance behavior. Thus, the plasmid-curing effects of these polyphenols at 250 mg/L were tested by comparing the growth rates between plasmid knockout strains and wild-type strains. In LB, wild-type *C. sakazakii* BAA-894 showed a similar growth rate as *C. sakazakii* BAA-894 Δ pESA3, and wild-type *C. sakazakii* GZcsf-1 showed a similar growth rate as *C. sakazakii* GZcsf-1 Δ pGW1 (**Figure 4A**). However, in LB complemented with 250 mg/L *p*-coumaric acid, wild-type *C. sakazakii* BAA-894 showed a significant reduction in optical density at 600 nm (OD600) compared to that of the *C. sakazakii* BAA-894 Δ pESA3 strain (**Figure 4B**). Similar results were also observed in *C. sakazakii* GZcsf-1 and its pGW1 curing mutant, indicating an *in vitro* fitness cost of the endogenous plasmids imposed on *C. sakazakii* with the existence of *p*-coumaric acid. Subsequently, the plasmid curing effect of *p*-coumaric acid was directly examined by continuous culture of wild-type *C. sakazakii* in LB or LB with *p*-coumaric acid. We found that with the increase of culture cycles, the proportion of plasmid-eliminating bacteria of *C. sakazakii* in the medium supplemented with *p*-coumaric acid increased gradually, while no plasmid-cured bacteria were detected in the medium without *p*-coumaric acid



(Figure 4C). These results revealed that *p*-coumaric acid exhibits plasmid-curing effect on *C. sakazakii* strains.

DISCUSSION

C. sakazakii is an opportunistic pathogen that is widely distributed in foods (Zeng et al., 2020), the gut

(Chandrasekaran et al., 2018) and various environments, increasing its chance of coming into contact with a variety of antibiotics, which may account for the accumulation of drug resistance genes in this bacterium, especially multidrug resistance genes in plasmids or the genome. In particular, the ability of *C. sakazakii* to harbor plasmids larger than 300 kbp would further increase its propensity to acquire resistance (Zeng et al., 2018). The resistance plasmids harbored by

TABLE 1 | Antimicrobial drug susceptibility profiles.

Antibiotics	MIC, mg/L antimicrobial susceptibility*	
	<i>C. Sakazakii</i> GZcsf-1	<i>C. Sakazakii</i> GZcsf-1 ΔpGW1
Ampicillin	32/R	1/S
Ceftriaxone	≥64/R	≤0.13/S
Cefazolin	32/R	2/S
Chloramphenicol	≥32/R	4/S
Azithromycin	≥ 32/R	8/S
Gentamicin	≥32/R	≤0.13/S
Trimethoprim	≥128/R	1/S
Aztreonam	≥32/R	0.13/S
Tetracycline	≥32/R	1/S
Streptomycin	≥512/R	≥256/R
Spectinomycin	≥512/R	≥512/R

*The interpretation of the minimum inhibitory concentration (MIC) on bacterial resistance (sensitive, S; intermediate, I; and resistant, R) was performed using the *Enterobacteriaceae* data from the Clinical and Laboratory Standards Institute (CLSI).

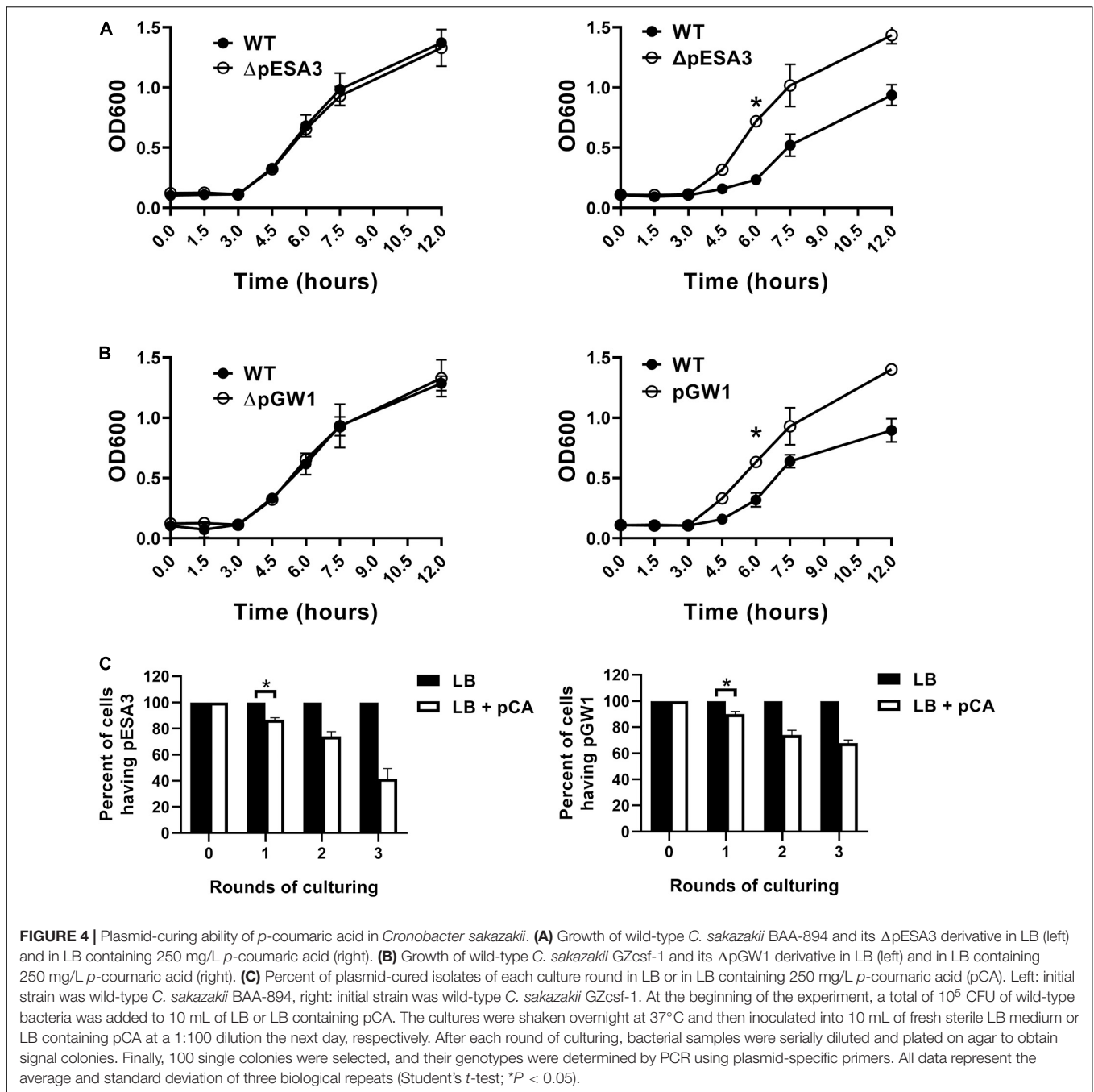
C. sakazakii could potentially serve as reservoirs for and as the origin for resistance in other strains through conjugation, transduction and transformation. The investigated fragment in pGW1 from *C. sakazakii* is also conserved in plasmids from *S. enterica*, *S. flexneri*, *C. freundii*, and *K. pneumoniae*, which could explain the dissemination of drug resistance plasmids in different bacteria.

The conventional strategy used to study the function of plasmids primarily depends on the removal of endogenous plasmids or their exogenous transformation into *E. coli* for functionality analysis. The exogenous transformation of plasmids of interest can promote a better understanding of their relationship to resistance to specific antibiotics but cannot provide sufficient insight into their roles in bacterial pathogenicity. In addition, the lower transformation efficiency of large plasmids or their restricted replication in *E. coli* can further restrict the application of the traditional strategy used to evaluate plasmids. The typical approaches used to cure endogenous plasmids primarily include chemical-based methods and molecular biological methods. Common chemical methods depend on the use of a high concentration of SDS, EDTA or ethidium bromide to interfere with plasmid replication, which has certain disadvantages, including non-specific removal, increased tendency for genomic mutation, and high toxicity (Buckner et al., 2018). Alternatively, molecular biology methods based on plasmid incompatibility involve the construction of plasmids containing replicons to cure endogenous plasmids, which primarily rely on the accurate cloning of the replicon from endogenous plasmids (Buckner et al., 2018). However, this method is subject to the prediction of plasmid incompatibility, and the multiple replicons that are frequently present in endogenous plasmids can further restrict the applicability of the plasmid incompatibility-based method (Citterio et al., 2020). Another traditional approach uses transposons harboring the *sacB* or *rpsL* genes to cure various plasmids that were established

decades ago. However, transposons have insertion sites, and the frequency of the occurrence of hybridization of the plasmid to be cured and transposon is low, resulting in a time-consuming colony screening process (Hynes et al., 1989; Stojiljković et al., 1991; Brom et al., 1992). A method based on the CRISPR/Cas9 system and homing endonuclease shows the capability of plasmid curing in certain species (Cao et al., 2017; Lauritsen et al., 2017; Tang et al., 2018; Wang et al., 2019). However, the off-target effect of the system leads to the concern over unwanted cleavages and mutations in chromosomal DNA. Furthermore, intracellular DNA ligase could repair the CRISPR/Cas9 system/endonuclease-induced DNA double-strand breaks, and an additional procedure to inhibit activity of DNA ligase has been proposed (Weber et al., 2015), casting a shadow over the application of this plasmid-curing method. Recently, suicide vectors were used in specific plasmid curing in *Agrobacterium* and *Salmonella* strains (Yamamoto et al., 2018; Yin et al., 2018), suggesting that this method has the advantages of plasmid specificity and convenience in plasmid elimination, but its broad-spectrum application needs more verification.

The current research described the integrated suicide vector pCVD442 to efficiently and specifically delete an endogenous plasmid of choice in *C. sakazakii* and analyzed the contribution of endogenous plasmids pESA3 and pGW1. The plasmid elimination method provided in the current study could facilitate the generation of plasmid knockout strains and could be applied for evaluation of selected plasmid elimination drugs by comparisons of their growth rates with those of wild-type strains under fixed pressure. Furthermore, the lower toxicity of the plasmid knockout strains could contribute to the development of attenuated vaccines after detailed investigation.

Plant-derived chemicals have many functions in the field of microorganisms, including antibacterial activity, bactericidal activity, regulation of quorum sensing and so on. The plasmid-cured strains obtained in our study were used to screen plasmid-curing agents from plant polyphenols and found that *p*-coumaric acid had a remarkable effect on the plasmid curing. Previous studies have found that *p*-coumaric acid can compete with ethidium bromide in binding to DNA (Lou et al., 2012), suggesting that *p*-coumaric acid can intercalate into the DNA base pairs. In fact, many DNA intercalating agents including ethidium bromide, such as methyl orange and acriflavine, have been found to eliminate plasmids from various strains (Buckner et al., 2018). Therefore, the plasmid-curing activity of *p*-coumaric acid in *C. sakazakii* may due to intercalation of *p*-coumaric into plasmid DNA, blocking the replication of plasmid. Previous studies have also found some polyphenols with plasmid elimination activity for specific bacteria. It seems that the plasmid elimination activity of plant polyphenols is not broad-spectrum, which may be due to the different tolerances of different bacteria to polyphenols. Our study found that *p*-coumaric acid inhibited the growth of *C. sakazakii* at high concentrations and had plasmid elimination effects at low concentrations. This is the first report on the antibacterial effect of *p*-coumaric acid on *C. sakazakii*. Considering the safety of *p*-coumaric acid derived from food, it may be a potential food additive for the inhibition of *C. sakazakii*. In addition,



this is the first report in which *p*-coumaric acid can inhibit the plasmid-carrying capacity of *C. sakazakii* at sub-inhibitory concentrations. Most importantly, we found that the antibiotic resistance of *C. sakazakii* was not related to the elimination and growth inhibition of its plasmid by *p*-coumaric acid. Therefore, *p*-coumaric acid may solve the multiple drug resistance problem of *C. sakazakii*.

Overall, the current research analyzed the contributions of the endogenous plasmids pESA3 and pGW1 by constructing plasmid-cured strains using an integrated suicide vector. The plasmid-cured strains were used to screen plasmid-curing agents

and showed that *p*-coumaric acid derived from plants had a remarkable effect on the plasmid curing, suggesting that *p*-coumaric acid can be a safe and effective plasmid-curing agent for *C. sakazakii*.

MATERIALS AND METHODS

Strains and Plasmids

All strains and plasmids used in the present study are listed in Table 1, and primers are listed in Table 2. Bacteria

TABLE 2 | Bacterial strains and plasmids used in this study.

Strains, plasmids	Description	References, sources
<i>Cronobacter sakazakii</i>		
<i>C. sakazakii</i> BAA-894	International reference strain	ATCC
<i>C. sakazakii</i> BAA-894 ΔpESA3	pESA3 curing variant	This study
<i>C. sakazakii</i> GZcsf-1	Clinical strain	Zeng et al., 2018
<i>C. sakazakii</i> GZcsf-1 ΔpGW1	pGW1 curing variant	This study
<i>Escherichia coli</i>		
S17-1 lambda pir	Strain constructed that harbors the lambda pir gene	de Lorenzo et al., 1993
Plasmids		
pUC57-ermC	pUC57 vector harboring the Erm ^s gene	Ji et al., 2019
pCVD442	Suicide plasmid	Donnenberg and Kaper, 1991
pCs-ΔpESA3	pESA3 integrated plasmid that originated from pCVD442	This study
pCVD442E	Erm ^s plasmid that originated from pCVD442	This study
pCsE-ΔpGW1	pGW1 integrated plasmid that originated from pCVD442E	This study
pUC57-RepFIB	pUC57 vector harboring the RepFIB replicon cloned in AatII site using RepFIB-F-AatII and RepFIB-R-AatII primer pair	This study

TABLE 3 | Primers used in this study.

Primer	Sequence (5'–3')
pCVD442-F	GGCTGTCAGACCAAGTTTACTCATATATACT TTAGATTG
pCVD442-R	GCAGATACTCTTCTTTTCAATATTATTGAAGCATTTA TCAGGGTTATTG
ΔpESA3-F	GAAAAAGGAAGAGTATCTGCGGTACGGTACGGCC ATACTGTTCCG
ΔpESA3-R	GCGATTAACCCATCTAAACGTCTCCACTAAA AAATCGTCATC
RepFIB-F-AatII	GCGACGTCTGAGCAAACATCCACTGTGG
RepFIB-R-AatII	GCGACGTCTGAGCAAACATCCACTGTGG
Check1-pESA3-F	GTCAACGGCACGATGGATCT
Check1-pESA3-R	CAGCGCCGATCGCCTGGCGC
Check2-pESA3-F	GAGCGGCAGTGTTGCCTGGC
Check2-pESA3-R	TCCAGCGTTGCGCTTTTCA
ermC-F	GCTAGCCTTGACAATTAATCATCGGCTCGTATA ATGCTAG
ermC-R	GTAAACTTGGTCTGACAGCCCTAACTACTATTATA AATAATTTATAGC
pCVD442E-F1	GGCTGTCAGACCAAGTTTACTCATATATACT TTAGATTG
pCVD442E-R1	GCAGATACTCTTCTTTTCAATATTATTGAAGCATTTA TCAGGGTTATTG
ΔpGW1-F	GAAAAAGGAAGAGTATCTGCGGTTTACCGATCA GCGTTACC
ΔpGW1-R	TGTTAGCAGGCAGTTCCAGGCCTATGTCG CCTTTATCC
Check1-pGW1-F	CCGATCAGCGTTACCGGTGC
Check1-pGW1-R	TGGACGTTCTGCGTTTTATC
Check2-pGW1-F	ATTTGATCGGGTTTTATCGT
Check2-pGW1-R	TCTTGTGAAAAGCCAGATTCA

were stored in LB medium (Solarbio, China) containing 15% glycerol (Solarbio, China) at -80°C. To initiate all experiments, strains were cultured overnight in LB. If necessary, antibiotics (ampicillin or kanamycin) were added at a final

concentration of 100 µg/mL (Solarbio, China), and *E. coli* S17-1 lambda pir (Weidi, China) was used to prepare the pCVD442 suicide vector (Miaoling, China). The gene cloning and transformation of *C. sakazakii* were performed using standard techniques.

Curing of Endogenous Plasmids

To remove pESA3 from the *C. sakazakii* strain BAA-894, the specific fragment amplified from the plasmid to be cured using the primer pair ΔpESA3-F and ΔpESA3-R was mixed with the linearized plasmid pCVD442 (Miaoling, China) using the primer pair pCVD442-F and pCVD442-R, and plasmid cyclization was achieved by homogenous recombination using a recombination kit according with protocols recommended by the manufacturer (Vazyme, China). The hybrid plasmid was selected by transformation into S17-1 lambda pir competent cells (Weidi, China) and selective medium. Single colonies growing on LB agar were selected. Subsequently, the plasmid was extracted from the prepared S17-1 lambda pir-Δplasmid, and *C. sakazakii* BAA-894 was transformed by electroporation as previously described (Kim and Loessner, 2008). A colony was cultured in LB overnight and then transferred to LB agar containing 20% sucrose. Strains harboring the integrated suicide plasmid will be killed by sucrose due to harboring *sacB* in the plasmid, while strains that lost the plasmid will survive. The genotype of colonies growing on sucrose LB agar was further verified by PCR and sequencing using dual pESA3 plasmid-specific primer pairs (see primer pairs, Table 3).

To cure pGW1 from the strain *C. sakazakii* GZcsf-1, the specific fragment that was amplified from the plasmid to be removed using the primer pair ΔpGW1-F and ΔpGW1-R was mixed with plasmid pCVD442 that was linearized using the primer pair pCVD442-F1 and pCVD442-R1 and the *ermC* fragment amplified from pUC57-ermC (Ji et al., 2019) using the primer pair *ermC*-F and *ermC*-R. The following procedure was identical as the protocols described for the removal of

pESA3, but 300 mg/L erythromycin was used to select strains harboring the hybrid plasmid. The genotype of colonies growing on sucrose agar was further verified by PCR and sequencing using dual pGW1 plasmid-specific primer pairs (see primer pairs, Table 3).

Generation of the Plasmid Complemented Strains

Hybrid plasmid was used for plasmid complementation in the corresponding plasmid-cured strain (Figures 1C,D). Specifically, the hybrid plasmid was extracted from S17-1 lambda pir-Δplasmid, and plasmid-cured *C. sakazakii* BAA-894 was transformed by electroporation as previously described (Kim and Loessner, 2008). The plasmid complemented strains were selected by plating the bacteria on LB agar containing corresponding antibiotics. The genotype of colonies growing on selective medium was further verified by PCR and sequencing using dual plasmid-specific primer pairs (see primer pairs, Table 3).

Susceptibility Assays

The antimicrobial sensitivity of *C. sakazakii* strains was tested by the agar dilution method according to Performance Standards for Antimicrobial Disk Susceptibility Tests, 13th Edition¹. All antibiotics were purchased from Beijing Solarbio Science and Technology Co., Ltd., China. All polyphenols were purchased from Shanghai yuanye Bio-Technology Co., Ltd., China. Briefly, bacteria cultured overnight were diluted in LB media to 10⁷ CFU/mL, and 10 μL of liquid was aliquoted on LB agar plates containing a two-fold dilution series of antimicrobials. The MICs were determined as the lowest concentration at which growth could not be observed when the plates were incubated at 37°C for 24–48 h.

Adhesion Assay

Bacterial adhesion assays were performed as described previously (Choi et al., 2015). Briefly, Caco-2 cells were washed in PBS (Solarbio, China) and then resuspended in fresh RPMI 1640 medium. *C. sakazakii* was prepared and added to a Caco-2 cell monolayer at a multiplicity of infection (MOI) of 100. After incubating for 45 min, the cells were washed with PBS three times and lysed in 1% Triton X-100. Then, the suspension was serially diluted and plated on LB agar to enumerate the CFU.

Invasion Assays

Bacterial invasion assays were performed as described previously (Choi et al., 2015). In short, Caco-2 cells were washed in PBS, and fresh RPMI 1640 media was added. Then, *C. sakazakii* cells were prepared and added to a Caco-2 cell monolayer at an MOI of 100. After incubating for 90 min, the cells were washed with PBS three times and then incubated with 100 μg/mL gentamycin for 1 h to kill extracellular bacteria. Subsequently, the wells were washed with PBS three times and lysed in 1% Triton X-100. Then,

the suspension was serially diluted and plated on LB agar to enumerate the CFU.

In vivo Rat Pup Virulence Assay

Bacterial cells were washed and resuspended in PBS (Solarbio, China), and a mixed inoculum of 5 × 10⁹ CFU of bacteria was orally administered to 3-days-old female Sprague-Dawley rat pups (4 rats/group). Where appropriate, chemicals were orally administered 1 h after bacterial administration. To analyze the colonization of bacteria in organs, after 24 h of infection, the bacterial load in the blood was determined by diluting facial vein blood and plating the diluents on LB agar. Then, the rats were sacrificed, and the liver and spleen were aseptically removed. The organs were homogenized in ice-cold PBS and serially diluted. The diluent was plated on LB agar to determine the bacterial loads.

Statistical Analysis

Statistical significance was analyzed using the GraphPad Prism (version 8.4) with the unpaired *t*-test. The data are represented as the mean and standard deviation. A *P*-value of < 0.05 was considered to indicate a significant difference.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Nankai University.

AUTHOR CONTRIBUTIONS

XJ and PL conceived, designed the research, conducted the experiments, and analyzed the data. YH and XJ contributed new reagents or analytical tools. JW, BZ, YZ, LD, and HL provided useful suggestions. SW systematically reviewed the literature and wrote the manuscript. All authors contributed to the editing of the manuscript.

FUNDING

This work was funded by grants from the National Key R&D Program of China (2019YFC1605005).

ACKNOWLEDGMENTS

We are grateful to Ping Li (Tianjin University of Science and Technology, China) for her technical assistance.

¹<https://clsi.org/standards/products/microbiology/documents/>

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Conflict of Interest: The authors have filed a China patent application (No. 202010973714.6) based on the results reported in this manuscript.

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