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Original Article

Effects of gallic acid on capsular polysaccharide biosynthesis in *Klebsiella pneumoniae*

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KEYWORDS
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Gallic acid;
Capsular polysaccharide;
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Abstract *Background: Klebsiella pneumoniae* is a gram-negative opportunistic pathogen that causes diseases mostly in immunocompromised individuals. Recently, hypervirulent *K. pneumoniae* strains also cause severe disease in healthy individuals. Capsular polysaccharide (CPS) is the major virulence determinant in hypervirulent *K. pneumoniae* and protects the cell against the bactericidal activity of the immune system. Gallic acid (GA), a natural phenolic compound, is known to exhibit wide spectrum antibacterial activity; however, its effect on hypervirulent *K. pneumoniae* remains largely unresolved. We aimed to identify the effects of GA on CPS biosynthesis in hypervirulent *K. pneumoniae*.
Methods: Antibacterial activity of GA was evaluated by counting colonies. CPS amount was determined by glucuronic acid content. The transcriptions of *cps* gene cluster were measured by quantitative real time PCR (qRT-PCR) and the β-galactosidase activity. The effect of GA on the resistance of *K. pneumoniae* to streptonigrin (SNG), an iron-activated antibiotic, was evaluated. The effect of GA on the resistance of *K. pneumoniae* to serum killing and phagocytosis by macrophages was observed.
Results: GA inhibited the growth and CPS biosynthesis in *K. pneumoniae*. GA may affect the

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iron availability in *K. pneumoniae*, thus possibly repressing the *cps* transcription. In addition, GA reduced the resistance of *K. pneumoniae* to serum killing and enhanced its susceptibility to phagocytosis.
Conclusion: GA possesses bactericidal activity and inhibits the CPS biosynthesis in hypervirulent *K. pneumoniae*, thereby facilitating pathogen clearance by the host immune system. Therefore, GA may represent a promising strategy for the prevention or treatment of patients with hypervirulent *K. pneumoniae* infections.

Introduction

Klebsiella pneumoniae is a gram-negative bacterium, and a member of the normal flora of the mouth, skin, and intestines. It causes urinary tract infection and nosocomial pneumonia [1] Hypervirulent *K. pneumoniae* strains have been steadily increasing in Asia.[2] While diabetes mellitus is considered as one of the risk factors,[3] hypervirulent *K. pneumoniae* also causes severe disease in healthy individuals. In addition, carbapenem-resistant hypervirulent *K. pneumoniae* strains have been reported at two Chinese hospitals.[4,5] These strains are highly transmissible and cause severe fatal infections. Due to the serious threat to human health, there is an urgent need to develop more new drugs to combat *K. pneumoniae* infection.[2,6,7]

K. pneumoniae produces many virulence factors such as capsular polysaccharide (CPS), lipopolysaccharide, siderophores, and fimbriae.[8] CPS is probably considered the critical factor in *K. pneumoniae* pathogenesis to protect *K. pneumoniae* from killing by phagocytosis or serum factors.[9,10] In hypervirulent *K. pneumoniae* isolates, a high prevalence of the K1 and K2 serotypes was documented in *Klebsiella* liver abscess,[11] although the exact mechanism of the tight association remains unclear. However, the CPS biosynthesis needs to be tightly regulated in response to different environmental stimuli for successful infection. In past, we have demonstrated that depletion of iron increases the amount of CPS production, thus enhancing the bacterial serum resistance.[12] Under glucose-depleted conditions, CPS biosynthesis is activated, which subsequently inhibits the phagocytosis of *K. pneumoniae*.[13] Therefore, the CPS production in *K. pneumoniae* is highly affected by dynamic environmental cues.

Gallic acid (GA), a natural phenolic compound present in many fruits and plants, exerts various protective effects and has anti-inflammatory, antioxidant, anticancer, and antimicrobial properties.[14,15] It also exhibits a broad range of antibacterial activity.[14,16] For example, it inhibits the growth and biofilm formation of *Escherichia coli* and *Streptococcus mutans*.[17] Furthermore, GA modulates the sensitivity of *Pseudomonas aeruginosa* to antibiotics.[18] However, the effect of GA on *K. pneumoniae* remains largely unknown. In the current study, we show that GA inhibits the growth of hypervirulent *K. pneumoniae* and CPS biosynthesis by affecting *cps* gene transcription, and it reduced the resistance of *K. pneumoniae* to serum killing and enhanced the bacterial susceptibility to phagocytosis. These findings might be considered in the context of nutritional supplements, as well as the prevention and treatment of infections caused by hypervirulent *K. pneumoniae*.

Methods

Bacteria strains, plasmids, and reagent

Table 1 summarizes the *K. pneumoniae* strains and plasmids used in this study. GA (G7384, Sigma—Aldrich, China) was dissolved in deionized water. Unless otherwise indicated, bacteria were grown in Luria—Bertani (LB) broth at 37 °C under aerobic conditions.

Antibacterial activity and minimal inhibitory concentration (MIC) of GA

LB broth without or with 2.5, 5, and 10 mM GA was initially inoculated with 0.1 % overnight cultures of *K. pneumoniae*

Table 1 Bacterial strains and plasmids used in this study.

Strains or plasmids	Descriptions	Reference
K. pneumoniae		
CG43S3	CG43 Sm^r	39
CG43S3Δ*lacZ*	CG43S3Δ*lacZ*	23
Plasmids		
placZ15	Cm^r, promoter selection vector, $lacZ^+$	23
pOrf12	Cm^r, 500-bp fragment containing the region upstream of *Klebsiella K2 cps orf1-orf2* cloned into placZ15	23
pOrf315	Cm^r, 900-bp fragment containing the region upstream of *Klebsiella K2 cps orf3-orf15* cloned into placZ15	23
pOrf1617	Cm^r, 300-bp fragment containing the region upstream of *Klebsiella K2 cps orf16-orf17* cloned into placZ15	23

CG43S3 and incubated at 37 °C for 3 h under aerobic or anaerobic (10 % CO_2 and 90 % N_2) conditions. The cultures were serially diluted, spread on LB agar, and incubated at 37 °C for 16 h. Viable bacteria were determined by counting colonies. MIC of GA against *K. pneumoniae* CG43S3 was determined in Mueller-Hinton broth by broth dilution method in 96-well microplates as previously described.[19] In addition, *E. coli* ATCC25922 was used as a quality control strain. Experiments were carried out in triplicates.

CPS production in *K. pneumoniae*

Uronic acid that reflects the content of *K. pneumoniae* CPS was extracted from *K. pneumoniae* CG43S3 cultured overnight in LB broth without or with the indicated concentrations of GA and quantified as described.[20] Uronic acid content was determined from a standard curve of glucuronic acid (G5269, Sigma—Aldrich, Swziterland) and normalized as the numbers of colony-forming units (cfu) of bacteria to obtain the CPS concentration. The amount of CPS is expressed as the relative ratio (%) of bacterial CPS produced in LB broth containing the indicated concentrations of GA to that produced in LB broth without GA, multiplied by 100.

Quantitative reverse-transcription PCR (qRT-PCR)

LB broth without or with 5 or 10 mM GA was initially inoculated with 0.1 % *K. pneumoniae* CG43S3 that had been cultured overnight and incubated at 37 °C for 3 h. Total RNA was extracted, cDNA was synthesized by qRT-PCR, and data were analyzed as described.[21] Table 2 shows the primers and probes for selected target sequences designed using Universal ProbeLibrary Assay Design Center (Roche Applied Science, Penzberg, Germany). Relative gene expression was quantified using the comparative threshold cycle $2^{-\Delta\Delta CT}$ method with *rpoD* as the endogenous reference.[22] Values were generated from three experimental replicates that were each analyzed three times.

Measurement of promoter activity

The promoter-reporter plasmids, pOrf12, pOrf315, and pOrf1617 were individually transferred into *K. pneumoniae* CG43S3Δ*lacZ* by electroporation. The β-galactosidase activity of bacteria when cultured in LB medium under the indicated condition was measured as previously described.[23]

Table 2 Primers used for qRT-PCR.

Primer	Sequence (5'→3')	TaqMan probe[a]	Target
RT03	CGTCATCCAGACCAAAGAGC	83	*galF*
RT04	CCGGTTTTTCAATAAACTCGAC	(GGTGGCTG)	
RT05	CGATGACCGGCTTTTTAATG	83	*wzi*
RT06	CTAGCGGAGATTTGGTACTGC	(GGTGGCTG)	
RT07	CAGTCCACCTTTATTCCGATTG	67	*manC*
RT08	AGGTACGACCCCGACTGG	(CTCCAGCA)	
RT295	GAGAAGCTGCTGGAAGTGAAA	84	*rpoD*
RT296	TTTAACCTGCTCGATGGTCA	(GCAGCAGA)	

[a] The numbers and sequences of TaqMan probe in Universal ProbeLibrary Set (Roche) were shown.

Streptonigrin (SNG) sensitivity assay

Overnight cultures of *K. pneumoniae* CG43S3 were diluted 1:10 in LB broth without or with various concentrations of GA to determine bacterial susceptibility to the iron-activated antibiotic, SNG (S1014, Sigma—Aldrich, Israel). Bacteria cultures were incubated without or with SNG (2 μg/mL) at 37 °C with agitation for 2 h. Then, 5-μL portions were serially diluted 10-fold in LB broth, spotted onto LB agar, incubated at 37 °C overnight, and photographed.

Bacterial survival in serum

LB broth with no, or with 5 or 10 mM GA was initially inoculated with 0.1 % overnight culture of *K. pneumoniae* CG43S3, and incubated at 37 °C for 3 h. Then, bacterial survival in the human serum (H4522, Sigma—Aldrich, USA) was determined as previously described.[21] The survival rate was expressed as the number of viable bacteria treated with the human serum compared with the number of untreated bacteria. The assay was performed in triplicate, with three technical replicates.

Bacterial phagocytosis by macrophages

LB broth with no, or with 5 or 10 mM GA was initially inoculated with 0.1 % overnight culture of *K. pneumoniae* CG43S3, and incubated at 37 °C for 3 h. Then, phagocytosis of *K. pneumoniae* CG43S3 by mouse RAW264.7 cells was investigated as previously described.[21] Phagocytosis rate (%) are expressed as the numbers of viable bacteria incubated with RAW264.7 cells compared with the number of viable bacteria from the pretreatment and multiplied by 100.

Statistical analyses

Data are shown as the means and standard deviations of triplicate assays. For multiple comparisons, we used a one-way analysis of variance (ANOVA) with Tukey's post hoc test. A P-value < 0.01 was considered significant in all cases.

Results

GA inhibits *K. pneumoniae* growth

To determine whether GA affects *K. pneumoniae* growth, the hypervirulent *K. pneumoniae* CG43S3 was aerobically and anaerobically cultured in LB with different amounts of GA, and the subsequent colony formation was evaluated by plate counting. Fig. 1A shows that the numbers of colony-forming units were decreased when *K. pneumoniae* CG43S3 was aerobically grown in LB broth containing 5 and 10 mM GA, but

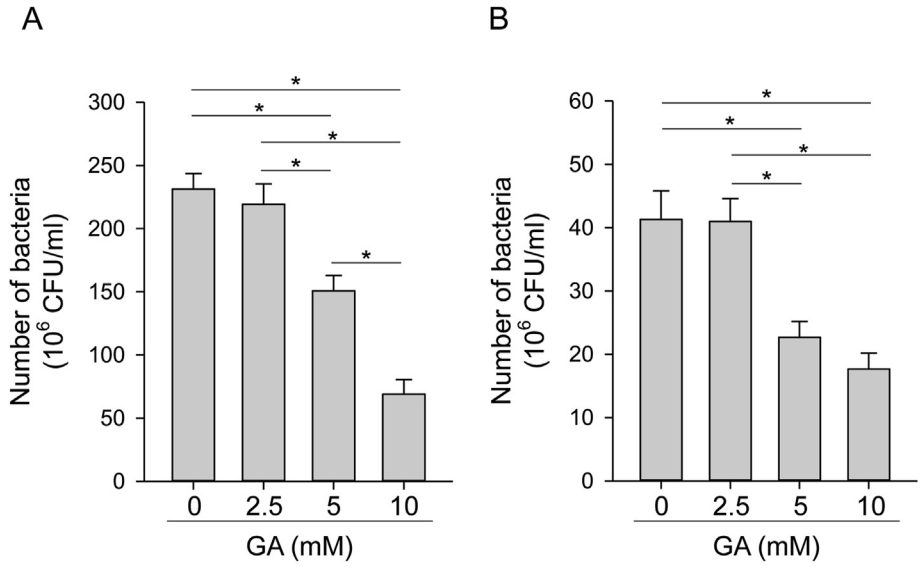

Figure 1. GA inhibits *K. pneumoniae* growth. Colony-forming units of *K. pneumoniae* in LB broth without or with indicated concentrations of GA under aerobic (A) and anaerobic condition (B) were counted. *$P < 0.01$ compared with the indicated group.

not 2.5 mM GA. Further, 10 mM GA had a stronger antibacterial activity than that of 5 mM GA. A similar antibacterial activity of GA against *K. pneumoniae* CG43S3 was found under anaerobic growth conditions (Fig. 1B). In addition, the MIC of GA against *K. pneumoniae* CG43S3 and *E. coli* ATCC25922 was 20 mM. Therefore, these demonstrated the antibacterial activity of GA against *K. pneumoniae*.

GA inhibits CPS biosynthesis and transcription

To investigate whether GA affects the CPS production in *K. pneumoniae*, the CPS amount of *K. pneumoniae* grown in LB broth containing different amounts of GA was determined. As shown in Fig. 2A, the addition of 5 and 10 mM GA obviously reduced CPS production by *K. pneumoniae* CG43S3; however, no apparent effect of 2.5 mM GA was observed. In addition, the CPS amount produced in bacteria treated with 10 mM GA was lower than that in bacteria treated with 5 mM GA. These observations indicated that GA inhibits the CPS biosynthesis in *K. pneumoniae*.

In *K. pneumoniae* CG43S3, the K2 *cps* gene cluster contains three transcriptional units, *orf* 1—2, *orf* 3—15, and *orf* 16—17.[23] To understand whether the effect of GA on CPS production was associated with changes at the transcriptional level, the *orf1* (*galF*), *orf3* (*wzi*), and *orf16*

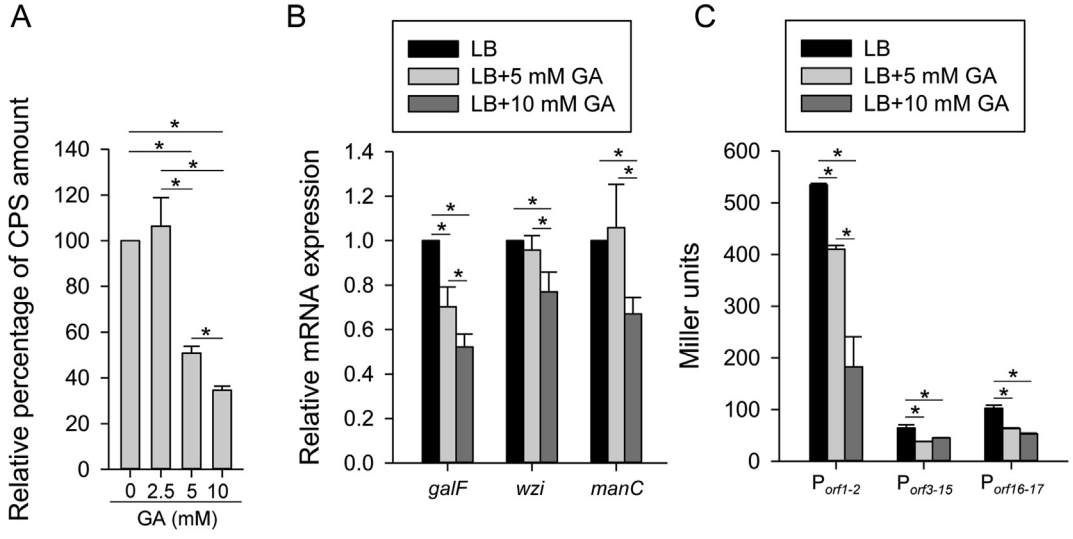

Figure 2. GA reduces the K2 CPS production at transcriptional level (A) Amounts of CPS and (B) mRNA levels of *galF*, *wzi*, and *manC* in *K. pneumoniae* CG43S3 cultured in LB broth without or with 5 and 10 mM GA (C) Activities of β-galactosidase in *K. pneumoniae* CG43S3Δ*lacZ* strain carrying reporter plasmids pOrf12 (P_{orf1-2}:*lacZ)*, pOrf315 ($P_{orf3-15}$:*lacZ*), or pOrf1617 ($P_{orf16-17}$:*lacZ)* in overnight cultures grown in LB without or with 5 and 10 mM GA. Error bars indicate standard deviations. *$P < 0.01$ compared with the indicated group.

(*manC*) mRNA levels in *K. pneumoniae* CG43S3 grown in LB broth without or with 5 and 10 mM GA were measured. The mRNA levels of *galF* were reduced in LB broth containing 5 mM and 10 mM GA, and those of *wzi* and *manC* were decreased in LB broth containing 10 mM GA (Fig. 2B). Furthermore, the effect of GA on the promoter activities of P_{orf1-2}, $P_{orf3-15}$, and $P_{orf16-17}$, which fused with *lacZ* reporter gene, were also evaluated. Compared with the bacteria grown without GA, the addition of 5 or 10 mM GA significantly decreased the activities of the analyzed promoters (Fig. 2C). Therefore, GA affected CPS production at the transcriptional level.

GA increases the SNG susceptibility of *K. pneumoniae*

Previously, we found that *cps* transcription in *K. pneumoniae* CG43S3 is closely related to intracellular iron levels.[12] To evaluate whether GA affects intracellular iron availability, *K. pneumoniae* CG43S3 cells were treated with GA, and bacterial survival in the presence of the iron-activated antibiotic, SNG, was determined. SNG requires iron to exert bactericidal activity (DNA degradation).[24] The SNG susceptibility of *K. pneumoniae* increased in LB broth supplemented with 5 and 10 mM GA compared with that in LB broth alone (Fig. 3). This suggested that GA may affect the intracellular iron availability in *K. pneumoniae* CG43S3 to further increase SNG susceptibility.

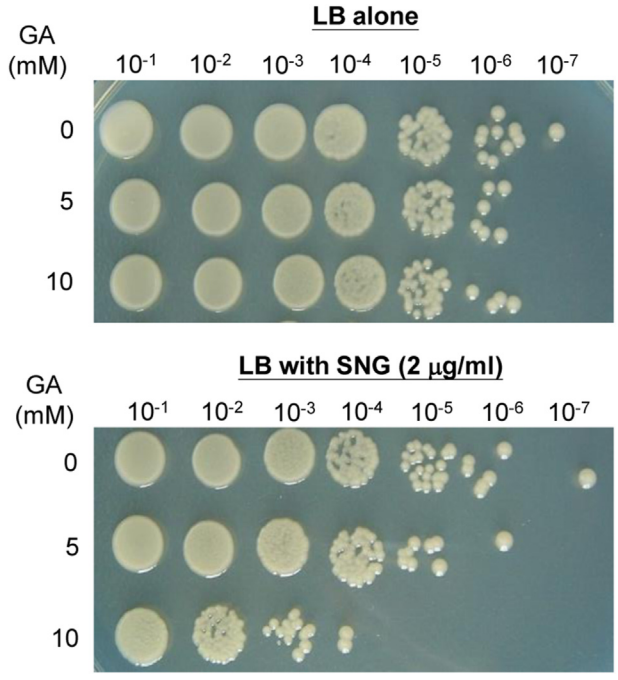

Figure 3. GA increases *K. pneumoniae* susceptibility to SNG. We cultured *K. pneumoniae* CG43S3 with indicated concentrations of GA in LB broth supplemented without or with 2 μg/mL of SNG. Ten-fold serial dilutions of cultures were then spotted onto LB agar and colony formation was observed.

GA reduces the resistance of *K. pneumoniae* to serum killing

Since GA inhibited CPS production by *K. pneumoniae* CG43S3, its effect on bacterial serum resistance was next analyzed. As shown in Fig. 4, the addition of 5 and 10 mM GA obviously decreased the survival of *K. pneumoniae* in the serum. In addition, the survival of *K. pneumoniae* in the presence of 10 mM GA was lower than that in the presence of 5 mM GA. This indicated that GA decreases the resistance of *K. pneumoniae* to serum killing.

GA decreases the *K. pneumoniae* resistance to phagocytosis by macrophages

To investigate whether GA influenced bacterial susceptibility to phagocytosis, the survival of *K. pneumoniae* CG43S3 treated with the indicated amounts of GA within a phagosome was measured. Treatment of *K. pneumoniae* CG43S3 with 5 and 10 mM GA resulted in an significant increase in the phagocytosis rate compared with that of the non-treated *K. pneumoniae* CG43S3 (Fig. 5). The effect was dose-dependent. This indicated that GA decreases the resistance of *K. pneumoniae* to phagocytosis by macrophages.

Discussion

The treatment of hypervirulent and multiple-antibiotic–resistant *K. pneumoniae* is limited and associated

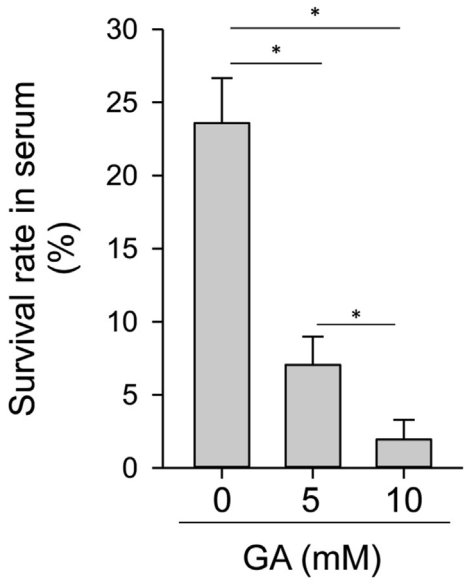

Figure 4. GA reduces the resistance of *K. pneumoniae* to serum killing. *K. pneumoniae* CG43S3 was grown in LB broth containing 0, 5, and 10 mM GA, respectively, and then susceptibility to normal human serum was determined. The survival rate in serum was quantified as described in Methods. *$P < 0.01$ compared with the indicated group.

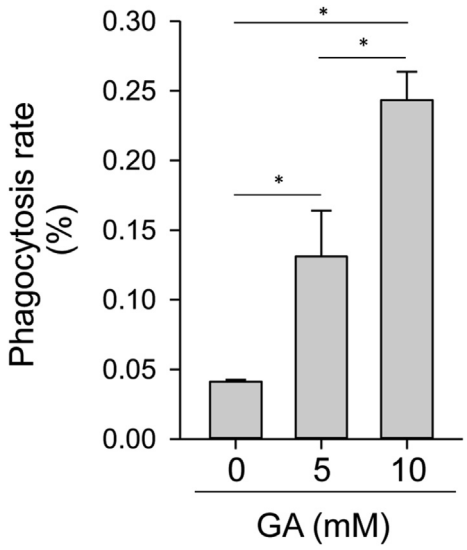

Figure 5. GA increases the susceptibility of *K. pneumoniae* to phagocytosis. The susceptibility of *K. pneumoniae* CG43S3 grown overnight in LB broth with the indicated amounts of GA to phagocytosis by RAW264.7 cells was determined. The phagocytosis rate was quantified as described in Methods. *$P < 0.01$ compared with the indicated group.

with high mortality. A need exists for new antibacterials to increase treatment efficiency. As shown previously, GA exerts an antibacterial activity against multiple pathogenic bacteria.[16–19] However, the effect of GA on *K. pneumoniae* is yet to be verified. In the current study, we demonstrated that GA represses the growth of a hypervirulent *K. pneumoniae* strain, and also reduces CPS production, to impair bacterial resistance to serum killing and macrophage phagocytosis. GA may therefore be a promising candidate molecule for treating *K. pneumoniae* infections.

In the current study, we showed that GA exerted an antibacterial effect on the hypervirulent *K. pneumoniae*. Like *E. coli* and *Salmonella*,[19] the MIC against hypervirulent *K. pneumoniae* was also 20 mM. Further, LB broth containing 5 mM GA (pH 5.24) apparently inhibited the growth of *K. pneumoniae* compared with LB broth with the pH adjusted (to pH 5.3) using HCl. This indicates that the antibacterial activity of GA is not a simple effect of its acidic characteristics. The growth-inhibiting mechanism of GA against *K. pneumoniae* remains unknown, although previous studies on the effects of GA against other bacteria provided some useful information. According to the study of Borges et al.,[16] GA inhibits the growth of *E. coli*, *P. aeruginosa*, *Staphylococcus aureus*, and *Listeria monocytogenes* by interfering with the membrane potential. Therefore, we suggest that GA inhibits the growth of *K. pneumoniae* by disrupting membrane integrity. However, this possibility needs to be investigated further.

We demonstrated that GA strongly reduces the amount of CPS produced by *K. pneumoniae*. Similar, GA decreases the polysaccharide slime production during biofilm formation by *E. coli*.[25] CPS protects the hypervirulent *K. pneumoniae* from phagocytosis and serum factor killing.[9,10] We here showed that GA decreases the CPS production to further impact the susceptibility of *K. pneumoniae* to serum killing and phagocytosis. In addition, the interplay of CPS and type 3 fimbriae expression has been demonstrated in *K. pneumoniae*.[26] Whether GA also affects the type 3 fimbria expression needs to be further investigated.

In *K. pneumoniae*, CPS biosynthesis is affected by environmental concentration and the redox status of iron.[12] Indeed, Fur protein binds to ferrous iron to repress the *cps* transcription.[12] In addition, redox status of the protein regulators containing the Fe–S cluster, FNR and IscR, affects the CPS biosynthesis.[21,27] GA is a phenolic compound with antioxidant and pro-oxidant properties.[28] In addition, GA could increase the SNG sensitivity in *K. pneumoniae*. One possibility is that GA affected the level and/or redox status of intracellular iron, which remains to be clarified. Apart from CPS, the regulation of type 3 fimbriae and biofilm formation in *K. pneumoniae* are also affected in response to iron availability.[29] Type 3 fimbria is a main virulence factor for biofilm formation in this bacterium.[29] Furthermore, GA inhibits biofilm formation in several bacteria.[17,25] This implies that GA might affect the type 3 fimbriae and biofilm formation in *K. pneumoniae*. However, the details of how GA affects iron availability and the effect of GA on type 3 fimbriae and biofilm formation in *K. pneumoniae* require further investigation.

Gallotannins are important hydrolysable tannins widely distributed in plant-based foods and medicinal plants.[30] Gallotannins can be hydrolyzed to GA by microbial enzymes and acid conditions, and GA can be further decarboxylated to pyrogallol by gallate decarboxylase in some bacteria.[31] In the gut microbiota of healthy humans, genes encoding putative gallate decarboxylases can be found in the phyla of *Proteobacteria*, *Firmicutes*, and *Actinobacteria*, but not in *Bacteroidetes*, which suggested that GA can be metabolized by gut bacteria.[31] GA has also been reported to alleviate imbalanced gut microbiota of rats with experimental colitis.[32] In contrast, pyrogallol has adverse effects on the gut microbiota and host animals.[33] These findings reveal complex interactions between GA and the gut microbiota. On the other hand, previous studies have shown that *K. pneumoniae* has gallate decarboxylase activity,[31,34] and a gene (*lpdC*; locus tag: D364_06035) encoding putative gallate decarboxylase can be found in the genome of *K. pneumoniae* CG43, suggesting that GA can be decarboxylated to pyrogallol in this strain, which requires further investigations.

Toxicity studies have shown that GA, at high concentrations, has cytotoxic effects on several cell lines.[15] GA has also been reported to interfere with digestive and absorptive functions of mammalian intestinal tracts via inhibiting the activity of brush border disaccharidases and peptidases.[35,36] In contrast, *in vivo* experiment had shown that GA, at a dosage of 5 g/kg, had no toxic effect on mice.[37] The acute toxicity of GA in mice showed that the LD_{50} of GA was greater than 2000 mg/kg, and a 28-day repeated dose sub-acute toxicity study showed that GA, at a dosage of 900 mg/kg, had no adverse effect on mice.[38] Therefore, the toxicity of GA *in vivo* is relatively faint.

However, the clinical efficacy, safety, and metabolism of GA in the treatment of infectious diseases remain largely unknown and should be studied to inform future clinical development.

The study has several potential limitations. First, no positive control group was used in this study. To clarify the effect of GA more clearly, future studies should include active control groups such as antibiotics and other phenolic acid. Second, the antibacterial mechanism of GA against *K. pneumoniae* has yet been identified. GA has been demonstrated to kill *E. coli* via disrupting the cell membrane[16]; however, whether GA causes similar effect in *K. pneumoniae* remains to be investigated. Third, whether GA could be metabolized in *K. pneumoniae* CG43S3 awaits to be verified. Finally, the strategy for the treatment of patients with *K. pneumoniae* infections by GA and the potential side effect is not presented here. Therefore, more studies are warranted to further clarify the efficacy and safety of GA in the treatment of *K. pneumoniae* infections.

Taken together, we here demonstrated that GA inhibits the growth of hypervirulent *K. pneumoniae*, and also repress CPS production in the bacterium, by affecting the iron availability to suppress the *cps* transcription. This impairs the bacterial resistance to serum killing and phagocytosis. Hence, GA might reduce the virulence of *K. pneumoniae* and thus constitute a promising new antimicrobial agent for the treatment or prevention of infections caused by hypervirulent *K. pneumoniae*.

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Authors' contributions

Conceived and designed the experiments: THL, CCW, CYT, and CTL. Performed the experiments: THL, CYT, and JHF. Analyzed and interpreted the data: THL, CCW, CYT, and CTL. Wrote the paper: THL, CCW, and CTL. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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