Disinfection properties of the tea polyphenol epigallocatechin gallate in the presence of calcium ions
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ABSTRACT

The antibacterial properties of tea polyphenols make them suitable for drinking water disinfection, but it is necessary to clarify the influence of metal ions on the sterilization efficacy. Epigallocatechin gallate (EGCG) was selected as a representative tea polyphenol, and the effects of calcium ion (Ca^{2+}) concentration on its disinfection properties in *Escherichia coli* cultures were investigated. Changes in bacterial growth and structures were detected. The presence of low concentration Ca^{2+} (1–5 mM) inhibited the disinfection effect of EGCG but higher concentrations (6–10 mM) enhanced its effects. As Ca^{2+} concentration increased, the proportion of damaged bacteria also increased (15–43%). The presence of Ca^{2+} lessened the damaging effects of EGCG on the surface structures of *E. coli* but may have facilitated the entry of EGCG into the bacteria, enhancing its antibacterial efficacy. Tea polyphenols may be useful additives for the disinfection of groundwater and other raw waters containing calcium ions.

Key words | bacteriostasis, calcium ions, concentration, disinfection, EGCG, tea polyphenols

HIGHLIGHTS

- Low concentration calcium ions inhibit the disinfection effect of EGCG, but high concentrations enhance disinfection.
- Rate of EGCG-induced *E. coli* damage rises, as calcium ion concentration increases.
- Calcium ions inhibit EGCG-induced damage to cell walls and membranes but promote internal structural damage.
- Tea polyphenols may be useful for processing raw water with high calcium ion concentrations.

INTRODUCTION

Tea polyphenols is a general term for the class of polyhydroxy phenolic compounds found in tea, composed of flavanols (catechins), anthocyanins, flavonoids, condensation acids and depside acids (Yang *et al.* 2005). Tea polyphenols have biological activities, including antioxidation, antibacterial, anticancer, antivirus and antiradiation effects and have been widely used in food preservation, biomedicine and other fields (Yang *et al.* 2015; Niu *et al.* 2019). Studies in China and other countries have revealed that tea polyphenols can exert significant effects on the bacterial cell wall and membrane, cell growth and metabolism, and intracellular...
proteins and genetic material, which explains their antibacterial properties (Hossain et al. 2002; Chinnam et al. 2010; Zhao et al. 2015; Galindo-Murillo & Cheatham 2018; Wu 2018; Chanphai & Tajmir-Riahi 2019; Tze-chen et al. 2020). Tea polyphenols have, therefore, been applied to drinking water disinfection, and it has been confirmed that they have a powerful disinfection effect, killing intestinal pathogens in the pipe network (Liu et al. 2019).

The concentrations of metal elements, such as Mg\(^{2+}\), Ca\(^{2+}\), Zn\(^{2+}\), A\(^{3+}\), Fe\(^{2+}\), Fe\(^{3+}\), Mn\(^{2+}\) and Cu\(^{2+}\), are often elevated in drinking water due to groundwater recharge, water source pollution, use of coagulants and corrosion of metal pipes. For example, calcium content can be 100–200 mg/L and iron up to 10 mg/L (Liu et al. 2012; Liu 2014). Calcium ions act as the second messenger of cell function regulation and are closely related to cell activity, growth and metabolism (Zhang & Zhang 2019). Since cell proliferation is related to a constant concentration of extracellular calcium ions, changes in this concentration will affect bacterial growth and even impact the efficacy of bacteriostats. Studies have shown that low concentrations of Ca\(^{2+}\) (0.6 M) and normal human serum Ca\(^{2+}\) concentrations can promote the growth of *Escherichia coli*, while high Ca\(^{2+}\) concentrations under pathological conditions can inhibit *E. coli* growth (Huang & Liu 2010). It has also been shown that divalent ions such as Ca\(^{2+}\) changed the spatial arrangement of lipopolysaccharides on the outer layer of the cell wall. This promoted the compactness of the outer membrane of gram-negative bacteria and prevented antimicrobial peptides from approaching the outer membrane due to charge repulsion, thereby inhibiting the activity of the peptides and protecting the bacteria (Herrmann et al. 2015). Another study found that, in the presence of a certain concentration of Ca\(^{2+}\), poly-\(\beta\)-hydroxybutyric acid on the cell membrane of *E. coli* combined with Ca\(^{2+}\) and phosphoric acid (Pi) to form a complex, activating Ca\(^{2+}\) channels and helping the bacterium take in foreign DNA. Natural genetic transformation, through horizontal gene transfer, may lead to drug resistance problems (Li 2004). Tea polyphenols contain multiple ortho-phenolic hydroxyl groups which can complex with metal ions, affecting their antibacterial capacity. In addition, some metals also have antibacterial properties which may enhance the antibacterial effect of tea polyphenols (Liao et al. 2015). High concentrations of metal ions in drinking water may, therefore, have a significant impact on the disinfection efficacy of tea polyphenols. However, this effect is not yet clear and further research is required.

Some advances have been made with the disinfection properties of tea polyphenols, but no work has been done on practical applications in the complicated drinking water environment, particularly on the influence of high Ca\(^{2+}\) concentrations on the disinfection efficacy of these polyphenols. Epigallocatechin gallate (EGCG) is the most concentrated catechin in tea polyphenols and their main antibacterial component (Li et al. 2020). EGCG was, therefore, used in this study as a representative compound in tea polyphenols, the influence of Ca\(^{2+}\) concentration on its disinfection efficacy was examined, and its mechanism explored, to provide a theoretical basis for the practical application of tea polyphenols in disinfection.

**MATERIALS AND METHODS**

**Test materials and instrumentation**

EGCG (purity >98%) was purchased from Nanjing GuangRun Biotechnology Co. Ltd. Freeze-dried *E. coli* was obtained from the China Industrial Culture Collection Center and resuscitated in nutrient broth medium (1.8 g nutrient broth powder in 100 mL of sterile distilled water). Test kits from Shanghai Enzyme-Linked Biotechnology Co. Ltd and a BioTek microplate reader were used to determine the alkaline phosphatase (AKP) content of *E. coli*. A DR6000 UV spectrophotometer was used to monitor the growth of *E. coli* and an EM-1200EX transmission electron microscope to observe the morphology of the cells.

**Experimental methods**

**E. coli growth inhibition**

The diameter of the growth inhibition zone and the minimum inhibitory concentration (MIC) of EGCG were determined to explore its disinfection effect in the presence of Ca\(^{2+}\). Resuscitated *E. coli* suspension was diluted and spread on nutrient agar medium and a sterilized Oxford...
cup placed vertically on the plate. EGCG (100 μL, 2 mM) and CaCl₂ (100 μL, 0–10 mM) were mixed and added to the Oxford cup. CaCl₂ was omitted from the blank group, and EGCG was omitted from the control group. After incubating at 37 °C for 24 h, the diameter of the inhibition zone was measured. Bacterial suspension (50 μL) was also placed in test tubes containing 5 mL nutrient broth medium. Various concentrations of EGCG (0.5, 1, 1.5, 2, 2.5, 3, 4 and 5 mM) and CaCl₂ (0–10 mM) were mixed and 50 μL added to the suspension and incubated at 37 °C for 24 h. Sterile water was used as a blank control. The lowest EGCG concentration in a culture that did not become turbid was recorded as the MIC.

Inactivation effect of EGCG

The inactivation effect of EGCG on *E. coli* in the presence of Ca²⁺ was determined via inactivation rate and damage rate. A cultured bacterial suspension was diluted to 1 × 10⁵ CFU/mL, and EGCG was added at the MIC before CaCl₂ solutions were added (2, 4, 6, 8 and 10 mM). After mixing for 4 h, the suspensions were spread on nutrient agar medium and eosin methylene blue medium and cultured at 37 °C for 24 h. The blank control group omitted CaCl₂ and EGCG. Inactivation rate (Equation (1)) and damage rate (Equation (2)) were calculated as follows:

\[
\text{Inactivation rate} = \frac{\text{Number of bacteria in blank group} - \text{Number of bacteria in experimental group}}{\text{Number of bacteria in blank group}} \times 100\% \quad (1)
\]

\[
\text{Damage rate} = \frac{\text{Number of bacteria in nutrient agar medium} - \text{Bacteria count in eosin blue medium}}{\text{Number of bacteria in nutrient agar medium}} \times 100\% \quad (2)
\]

Monitoring of *E. coli* growth characteristics

The effect of EGCG on cell growth in the presence of Ca²⁺ was determined by monitoring the growth cycle of *E. coli* and detecting changes in cell shape. The control bacterial concentration was 1 × 10⁶ CFU/mL, EGCG was added to the medium at the MIC, and selected Ca²⁺ concentrations were 1 and 10 mM. The blank control group omitted Ca²⁺ and EGCG. Bacteria were cultured in a 37 °C shaker at 120 rpm for 0, 1, 2, 4, 6, 8, 10, 12, 14, 24 and 48 h. Broth was sampled (3 mL), the absorption at 500 nm measured with a UV spectrophotometer, and *E. coli* growth curves plotted. After culturing, bacterial suspensions were inoculated into four flasks containing 100 mL media. One flask was supplemented with 2 mM EGCG and 1 mM CaCl₂, one with 2 mM EGCG and 10 mM CaCl₂, and one with 2 mM EGCG. Flasks were incubated at 37 °C for 6 h, before removing 1.5 mL for centrifugation at 10,000–12,000 rpm for 10 min. The supernatant was mixed with 1 mL of 2.5% glutaraldehyde fixative solution and stored at 4 °C for 2 h (Hu et al. 2016). After staining, the shapes of the *E. coli* cells were examined by transmission electron microscopy.

Cell structure changes indicated by AKP and soluble proteins

AKP and soluble protein concentrations reflect the damaging effects of EGCG on the cell wall and membrane in the presence of Ca²⁺. *E. coli* suspensions were treated with EGCG, or EGCG with low concentration Ca²⁺, or EGCG with high concentration Ca²⁺. All were cultured at 37 °C and sampled after 2, 4, 6 and 8 h. Sterile water was used as a blank control. Absorbance was measured at 450 nm, and AKP concentration was determined from a standard curve. Following the Coomassie Brilliant Blue G250 colorimetric method, *E. coli* samples were cultured for 0.5, 1, 12 and 24 h, centrifuged at 4,500 rpm and the absorbance of the supernatant used to calculate the soluble protein content against a protein standard curve (Jiao 2016).
RESULTS AND DISCUSSION

Effect of calcium ions on disinfection properties of EGCG

The inhibition zone refers to the transparent circle formed by the spread of a bacteriostatic agent on a plate of bacteria, showing the inhibition of bacterial growth. The size of the inhibition zone directly illustrates the sensitivity of the bacteria to the bacteriostatic agent. Zone diameters can be categorized as 15–20 mm, bacteria are highly sensitive to the bacteriostatic agent; 10–14 mm, moderately sensitive; 7–9 mm, low sensitivity; less than 6 mm, insensitive or the bacteria have developed antibacterial resistance (Liu 2017).

A previous study showed that the MIC of EGCG, tested in the range 0.2–1.0 g/L, was 1.0 g/L (2 mM) (Jin 2018). Under this MIC, the diameter of the EGCG inhibition zone measured by the Oxford cup method was 10.50 mm, indicating that E. coli was moderately sensitive to EGCG (Figure 1).

The inhibition zone was approximately 8 mm in diameter in the presence of 1–2 mM Ca$^{2+}$ alone (Figure 1). When Ca$^{2+}$ was in the range 3–5 mM, there was no inhibition of bacterial growth, but with 6–10 mM Ca$^{2+}$, the diameter of the zone became 10–12 mm. When EGCG was present, inhibition of E. coli did not change across the Ca$^{2+}$ concentration range 1–5 mM. When Ca$^{2+}$ concentrations rose to 6–10 mM, E. coli became moderately sensitive to EGCG/Ca$^{2+}$. Inhibition of E. coli growth was greatest when EGCG was present along with 10 mM Ca$^{2+}$; however, this was similar to the inhibition observed with 10 mM Ca$^{2+}$ in the absence of EGCG. In summary, low concentrations of Ca$^{2+}$ may reduce the bacteriostatic effects of EGCG, while high concentrations enhance this bacteriostasis. High concentrations of Ca$^{2+}$ have no synergistic effect on EGCG bacteriostasis. The reason why Ca$^{2+}$ shows ‘low inhibition and high promotion’ of EGCG disinfection activity may be due to low concentration Ca$^{2+}$ protecting E. coli cells, while high concentration Ca$^{2+}$ having an intrinsic inhibitory effect (Huang & Liu 2010).

The MIC of EGCG in the presence of low and high concentrations of Ca$^{2+}$ is shown in Table 1 (MIC in the absence of Ca$^{2+}$ was 2 mM). The presence of low concentration Ca$^{2+}$ did not affect EGCG MIC. In the presence of high concentration Ca$^{2+}$, EGCG MIC became 1.5 mM, indicating that high concentrations of Ca$^{2+}$ can enhance the disinfection effect of EGCG. Thus, the dose of EGCG could be reduced while exerting the same disinfection effect.

Table 1 | MICs of EGCG in the presence of low/high Ca$^{2+}$

| MIC of EGCG with low Ca$^{2+}$ | EGCG concentration (mM) | 1 | 2 | 3 | 4 | 5 |
| E. coli | + | - | - | - | - |

| MIC of EGCG with high Ca$^{2+}$ | EGCG concentration (mM) | 0.5 | 1 | 1.5 | 2 | 2.5 |
| E. coli | + | + | - | - | - |

Note: ‘+’ indicates significant colony growth; ‘-’ indicates no colony growth.

Figure 1 | E. coli growth inhibition zone in the presence of EGCG and calcium ions.
Inactivation effect of EGCG on *E. coli* in the presence of calcium ions

Figure 2 shows the inactivation and damage rates of EGCG on *E. coli* in the presence of Ca\(^{2+}\). When Ca\(^{2+}\) concentration was zero and EGCG concentration was 2 mM, the inactivation rate of *E. coli* was 85\% and the damage rate was 15\%. As Ca\(^{2+}\) concentration increased, the inactivation rate initially decreased, then increased, but the damage rate steadily increased. Low and medium concentrations of Ca\(^{2+}\) (1–5 mM) weakened the inactivation of *E. coli* by EGCG but increased the damage rate. High concentrations of Ca\(^{2+}\) (6–10 mM) enhanced the inactivation of *E. coli* by EGCG, while the proportion of damaged bacteria also increased significantly. This is consistent with the inhibition zone diameter results. EGCG also exhibited the ‘low inhibition, high promotion’ effect in the inactivation of *E. coli* in the presence of Ca\(^{2+}\). Given that calcium ions themselves exhibit disinfection properties at high concentrations (Huang & Liu 2010), the influence of Ca\(^{2+}\) on the disinfection capacity of EGCG needs further research.

Changes in *E. coli* growth characteristics

Selecting 1 mM as a representative low Ca\(^{2+}\) concentration and 10 mM as a representative high concentration, the effects of EGCG on the growth of *E. coli* were monitored and plotted (Figure 3).

Compared with the control group (*E. coli* in Figure 3), there was no change in growth pattern in the presence of low or high concentrations of Ca\(^{2+}\). *E. coli* growth reached the logarithmic phase after 2 h and reached its maximum at 12 h, after which it plateaued. Adding EGCG to the bacterial suspension (*E. coli* + EGCG) delayed logarithmic growth onset to 4 h, the growth rate was lowered, and it plateaued after 8 h, giving an overall bacterial density significantly lower than the control group. Due to the superior growth conditions established in this study, the inhibitory effect of Ca\(^{2+}\) on EGCG was not obvious (*E. coli* + low calcium + EGCG group), resulting in growth similar to the *E. coli* + EGCG group. Growth of *E. coli* in the presence of a high concentration Ca\(^{2+}\) and EGCG was poor. It entered a decay phase after 6 h and a large number of bacteria died. This shows that high Ca\(^{2+}\) concentrations enhanced the disinfection effect of EGCG. However, after 12 h, the bacteria began to proliferate again before stabilizing. This pattern may be related to the increase in bacterial damage rate due to high Ca\(^{2+}\) concentration seen in Figure 2. EGCG caused sub-lethal damage to some cells, changed the physiological structure of the bacteria and formed competent cells. Calcium ions have previously been shown to promote the transformation and growth of competent cells (Liu et al. 2004; Wang et al. 2016).
To explore the sterilization mechanism of EGCG in the presence of Ca\(^{2+}\), transmission electron microscopy was used to observe changes in the shape of *E. coli* (Figure 4). In the control group, (blank) cells were rod-shaped under normal growth conditions, the cell wall and membrane were undamaged, and the cytoplasm was intact and evenly distributed. However, the cell wall and membrane edges became blurred, or even disappeared, after addition of EGCG (*E. coli* + EGCG). Cell morphology was deformed, large vacuoles formed in the cytoplasm, and large amounts of contents leaked from the cells. Asahi et al. (2014) also observed that EGCG damaged the ultrastructure of bacterial cells and caused cytoplasmic leakage. In the presence of low concentrations of Ca\(^{2+}\) (*E. coli* + EGCG + low calcium concentration), cells treated with EGCG were more complete, but they changed from the normal rod-shape to elliptical. Uneven distribution, aggregation and contraction of the inner cytoplasm were clearly evident, with a large amount of sediment in the cell. A change in cell shape is an adaptive response of a bacterium to an external pressure (Storia et al. 2011). With high Ca\(^{2+}\) concentration (*E. coli* + EGCG + high calcium concentration), cavities gradually appeared in the cells and there was less sediment inside the cells, while considerable sediment with indefinite shapes appeared on the cell surfaces, indicating that Ca\(^{2+}\) was thoroughly adsorbed onto the bacteria. The higher the Ca\(^{2+}\) concentration, the more obvious was this adsorption property. This may be a prerequisite for the disinfection effect of metal ions (Zheng et al. 2011). In addition, high Ca\(^{2+}\) concentrations caused some cell walls to rupture. Based on the above analyses, Ca\(^{2+}\) may limit the damaging effects of EGCG on bacterial cell walls but may promote the transport of EGCG into the cell, leading to the destruction of DNA, proteins and other intracellular substances, and destroying the cell shape from the inside out.

### Cell structure changes indicated by AKP and soluble proteins

AKP is present between the cell wall and membrane, and leaks out when the wall ruptures (Guo et al. 2018). The extent of cell wall damage can be inferred from the AKP content of a bacterial suspension. Changes in AKP content of *E. coli* treated with Ca\(^{2+}\) and EGCG at the MIC are shown in Figure 5.

The AKP content of the control *E. coli* increased only slightly after 8 h but increased rapidly after adding EGCG because of its bacteriostatic effect which damaged the cell wall. The rate of AKP increase slowed after 2 h, for two possible reasons. Firstly, the destruction of the cell wall caused some bacteria to die, affecting overall growth and reproduction, and reducing the amount of AKP produced. Secondly, the site where EGCG’s disinfection effect is exerted is not only the cell wall but also intracellular protein and DNA. Some EGCG proceeded to enter the cells and destroy these components, leaving the cell walls and membranes intact. There was an increase in AKP content of the *E. coli* exposed to low and high concentration of Ca\(^{2+}\), but values were consistently lower than the group treated with only EGCG, indicating that the presence of Ca\(^{2+}\) reduced the
Figure 4 | Transmission electron micrographs of changes in E. coli cell morphology in the presence of EGCG and calcium ions (magnification: 20,000×, 40,000× and 100,000× in micrographs labelled 1#, 2# and 3#, respectively).
damage to cell walls. This was consistent with the results of transmission electron microscopy. In addition, AKP in the group exposed to EGCG and high Ca\(^{2+}\) decreased slightly after 6 h. This may have been due to the stability of the lipid membrane changing further, eliminating barriers to the penetration of outer membrane lipids, while causing only local damage to the cell wall such that the wall was still relatively complete. Some bacteria may have adapted to these conditions, re-ingested AKP and resumed normal growth.

The leakage of soluble proteins also increases as cell membrane permeability increases. Soluble protein content can, therefore, be used to assess damage to cell membrane. Results of colorimetric analysis of leakage of soluble protein from \textit{E. coli} (Feng 2019) are shown in Figure 6.

Soluble protein arising from the growth of control \textit{E. coli} bacteria was low but increased significantly in the three treatment groups after 1 h. Of these treatments, EGCG and high Ca\(^{2+}\) led to the fastest growth rate, while EGCG with low Ca\(^{2+}\) gave the slowest growth. This indicates that while EGCG clearly damages the cell membrane causing a large amount of soluble protein to leak out, low concentrations of Ca\(^{2+}\) may slow down this damage. Conversely, high Ca\(^{2+}\) concentrations can promote such cell membrane damage by opening ion channels in the membrane and increasing its permeability (Chang et al. 2016).

**CONCLUSIONS**

Calcium ions at low concentrations (1–5 mM) can inhibit the disinfection effects of EGCG, but can enhance these effects at high concentrations (6–10 mM). By adjusting
Ca\(^{2+}\) concentration, the MIC of EGCG was reduced to 1.5 mM, thereby improving its disinfection efficiency. Ca\(^{2+}\) at low concentrations can also reduce the inactivation rate of EGCG against *E. coli*, but the ability of EGCG to kill *E. coli* is increased at high Ca\(^{2+}\) concentrations. However, while high Ca\(^{2+}\) concentrations cause more damaged bacteria to be produced, some may recover and pose a further safety risk.

The cell morphology, cell wall and cell membrane of *E. coli* treated with the MIC of EGCG were damaged and the bacteria died. With the addition of Ca\(^{2+}\), cells treated with EGCG showed only partial cell wall disruption, the cell membrane structure gradually disappeared, and intracellular proteins leaked out. This shows that Ca\(^{2+}\) lessened the damaging effects of EGCG on the microstructure of *E. coli* but facilitated EGCG to enter the bacteria and exert its disinfection effects.

EGCG has a greater disinfection effect in the presence of calcium ions. This suggests that tea polyphenols will be more efficient disinfectants in groundwater and other water bodies which contain high concentrations of Ca\(^{2+}\), and these tea polyphenols may find applications in the field of drinking water disinfection.

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**CONFLICT OF INTEREST**

We declare that we have no financial and personal conflicts of interest to this work.

**AUTHOR CONTRIBUTIONS**

Z. X. is a postgraduate candidate, majoring in Drinking Water Disinfection and Disinfection By-products and C. F. is a professor, majoring in Theory and Technology of Water Treatment.

**DATA AVAILABILITY STATEMENT**

All relevant data are included in the paper or its Supplementary Information.

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