AQUA
Water Infrastructure, Ecosystems and Society

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IWA PUBLISHING

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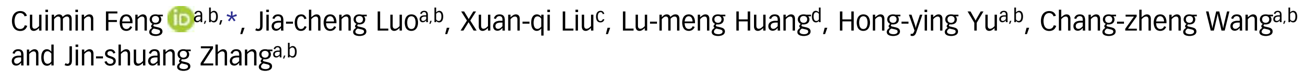
AQUA — Water Infrastructure, Ecosystems and Society Vol 72 No 3, 246 doi: 10.2166/aqua.2023.190

Mechanism of oxidative damage in *Escherichia coli* caused by epigallocatechin gallate (EGCG) in the presence of calcium ions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ABSTRACT

Tea polyphenols can be developed into new types of disinfectants for drinking water. The antibacterial effect of epigallocatechin gallate (EGCG) on *Escherichia coli (E. coli)* in the presence of Ca^{2+} is affected by the Ca^{2+} concentration. The oxidative damage mechanism and oxidative damage process of EGCG in *E. coli* under the presence of Ca^{2+} were deeply analyzed under three aspects: reactive oxygen species (ROS), antioxidant system, and oxidative stress response in *E. coli* to provide a theoretical basis for the use of EGCG as a disinfectant in drinking water disinfection. EGCG leads to excessive production of superoxide anion in *E. coli* and the presence of Ca^{2+} promotes further imbalance of superoxide anion in *E. coli*; Ca^{2+} has little effect on EGCG hindering the scavenging of hydroxyl radicals in bacteria; EGCG can hinder the effect of antioxidant enzymes in *E. coli*, and Ca^{2+} has a particular regulatory effect on antioxidant enzymes, thus hindering the oxidative damage of EGCG to *E. coli*; Ca^{2+} can cause the expression of the oxyR and DPS genes, protect bacterial DNA, and prevent EGCG from damaging bacterial DNA. In the presence of a high concentration of Ca^{2+}, it may activate the cell efflux pump through the soxS gene, resulting in *E. coli* resistance to EGCG.

Key words: bacteriostasis, Ca^{2+}, disinfection, EGCG, tea polyphenols

HIGHLIGHTS

- EGCG has potential for disinfection of drinking water.
- The antibacterial effect of EGCG on *E. coli* in the presence of Ca^{2+} is affected by the Ca^{2+} concentration.
- EGCG leads to excessive production of superoxide anion in *E. coli*.
- Ca^{2+} has an effect on EGCG hindering the scavenging of hydroxyl radicals in bacteria.
- Ca^{2+} may activate the cell efflux pump through the soxS gene, resulting in *E. coli* resistance to EGCG.

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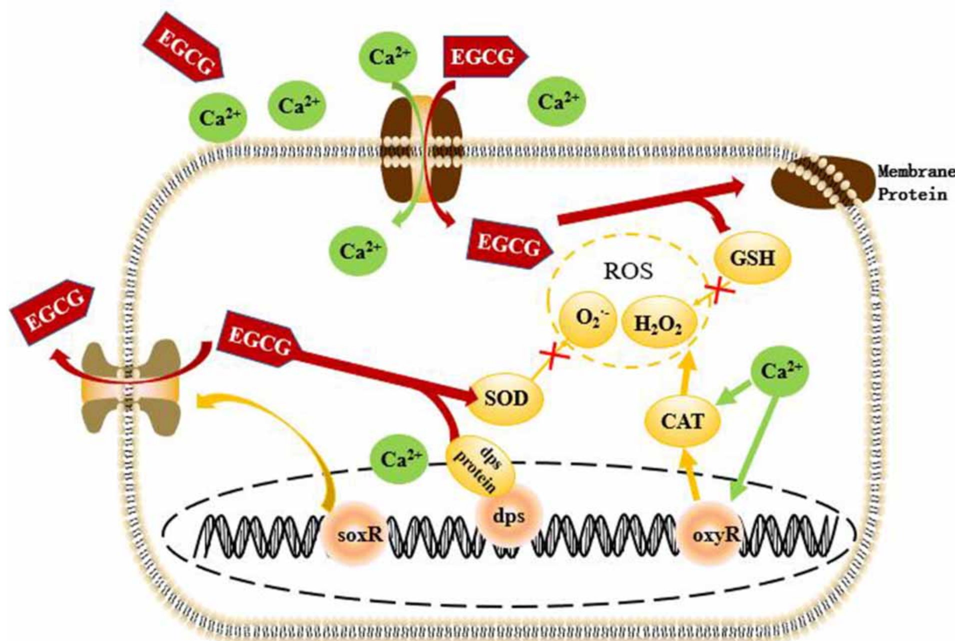
GRAPHICAL ABSTRACT



1. INTRODUCTION

Polyphenols are a class of natural biologically active polyphenols present in tea (Xiong *et al.* 2019). Catechins are the main polyphenolic compounds and include eight different monomers, namely catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), catechin gallate (CG), epicatechin gallate (ECG), gallocatechin gallate (GCG), and epigallocatechin gallate (EGCG) (Li *et al.* 2020). Tea polyphenols have antioxidant, antibacterial, anti-cancer, anti-viral, anti-radiation, and other biological activities (Xiaolin *et al.* 2019) and they have been proven to have great disinfectant effects in water disinfection processes (Xuanqi *et al.* 2019). Among all tea polyphenols, EGCG is the main component with an antibacterial effect.

Drinking water contains numerous beneficial metal elements. Specifically, calcium concentrations vary between 100 and 200 mg/L (Licai *et al.* 2012; Wei 2014). Calcium is an essential second messenger in regulating cell function, as well as affecting cell activity and metabolism (Zhang & Zhang 2019). Notably, changes in the concentration of extracellular Ca^{2+} affect cell growth and proliferation of bacteria and can even interfere with the antibacterial effects of bacteriostatic agents.

Previous studies have shown that the Ca^{2+} concentration can impact the bacteriostatic effect of EGCG. Macroscopically, in the presence of a relatively low concentration of Ca^{2+} (1–5 mM), the bacteriostatic effect of EGCG is weakened, while in the relatively high concentrations of Ca^{2+} (5–10 mM), EGCG inhibits bacterial growth by accessing the bacterial body and exerting a bacteriostatic effect (Xu *et al.* 2021).

In this manuscript, we focus on the interrelationship between Ca^{2+} and the damage generated by reactive oxygen species (ROS), antioxidant system, and the oxidative stress response within *Escherichia coli* (*E. coli)* to then rationalize the destructive effect enhanced by EGCG in the bacterial cell milieu.

2. MATERIALS AND METHODS

2.1. Materials

EGCG ($C_{22}H_{18}O_{11}$) was purchased from Nanjing Guangrun Biological Products Co., Ltd, after extraction and separation from green tea, in the form of white powder, with a purity greater than 98%. The molecular structure of EGCG is shown in Figure 1.

E. coli cells were obtained from the China Industrial Microorganism Culture Collection and Management Center, in the form of a freeze-dried powder with low activity. Resuscitation was performed by culturing in the fresh bacterial suspension. The bacterial count was approximately 10^8 CFU/mL. The contents of superoxide dismutase (SOD), catalase (CAT), and

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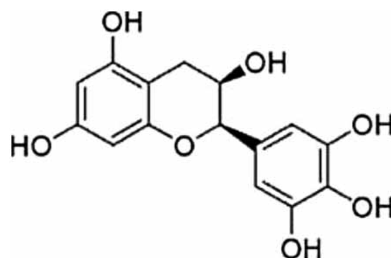

Figure 1 | Molecular structure of EGCG.

glutathione (GSH) in *E. coli* were determined using, respectively, the SOD, the CAT, and the microbially reduced GSH ELISA kits (96 T) purchased from Shanghai Enzyme Link Biotechnology Co., Ltd.

2.2. Methods

2.2.1. Activation, culture, and preservation of *E. coli*

After dissolving the lyophilized *E. coli* vial in a small amount of sterile water, the cells were inoculated in 10 mL of sterilized nutrient broth medium, sealed, and placed in a constant temperature shaker to be cultured overnight at 37 °C and 220 rpm, obtaining the first-generation bacterial culture. Second- and third-generation bacterial cultures were prepared by successive re-inoculation in 10 mL of fresh medium and overnight culture in the same experimental conditions. Eight 10-mL test tubes tilt-filled with fresh sterile agar medium were inoculated with the third-generation bacterial culture with a sterile loop. The tubes were sealed with a rubber stopper and cultured at 37 °C constant temperature in an incubator until the colony was visible, and stored at 4 °C. Throughout the study, the solid cultures were used to prepare *E. coli* suspension cultures by inoculating scraps of the colony into fresh medium and culturing in the same experimental conditions mentioned above, to an estimated bacterial count of 10^8 CFU/mL.

2.2.2. Detection of antibacterial activity

The bacteriostatic activity of EGCG was characterized by measuring the diameter of the inhibition zone, the minimum inhibitory concentration (MIC), and the growth curve of *E. coli*.

Bacterial inhibition circle assays were performed as follows: after diluting a bacterial suspension to 10^6 CFU/mL with sterile distilled water, 100 μL of the solution was applied to nutrient agar medium plates under aseptic conditions. Three sterile Oxford cups were gently placed onto the plates with forceps in a triangle arrangement and filled with 100 μL of 2 mM EGCG (blank), 100 μL of 1–10 mM $CaCl_2$ (control), or 100 μL of 2 mM EGCG and 1–10 mM $CaCl_2$. The diameter of the inhibition circle was then measured after the incubation of plates for 24 h at 37 °C in a constant temperature incubator.

The MIC of EGCG on *E. coli*, before and after the presence of $CaCl_2$, was determined by the test tube doubling-dilution method. Each experiment was repeated three times.

The count of injured bacteria was calculated as the difference between selective and non-selective media counts, i.e., colonies that were unable to grow on selective media but could grow on non-selective media without inhibitors were considered injured bacteria. After diluting a culture suspension to 10^3 CFU/mL with sterile distilled water, EGCG at the MIC concentration was added in combination with 0, 2, 4, 6, 8, and 10 mM $CaCl_2$. After a 4 h reaction, the bacterial solutions were applied on nutrient agar and eosin-methylene blue media at the same time, in three repeats per condition tested. Colonies were counted after 24 h at 37 °C. The bacterial solution without the drug was used as the blank group and the bacterial count was determined using the plate counting method. The following equations were used to calculate the bacterial inactivation rate (E) and damage rate.

$$\textit{Inactivation rate}(\mathrm{E}) = \frac{a-b}{a} \times 100\% \quad (1)$$

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where a is the number of bacteria in the blank group and b is the number of bacteria in the experimental group.

$$Damage\ rate(\mathrm{F}) = \frac{c-d}{c} \times 100\% \quad (2)$$

where c is the number of bacteria cultured on nutrient agar and d is the number of bacteria cultured in eosin-methylene blue medium

2.2.3. Determination of calcium ion concentration

In our previous experiments, the changes of the diameter of the EGCG-dependent inhibition circle in the presence of different concentrations of Ca^{2+} were explored and the Oxford cup containing only Ca^{2+} without EGCG was used as the control group. From Figure 2, it can be seen that the diameter of the inhibition circle was about 8 mm on average at 1–2 mM Ca^{2+} concentration, no inhibition circle appeared at 3–5 mM Ca^{2+}, and the inhibition circle was between 10 and 12 mm at 6–10 mM Ca^{2+}, indicating that low Ca^{2+} concentrations (1–5 mM) had no significant inhibition effect on *E. coli*, while high Ca^{2+} concentrations (6–10 mM) had some inhibitory effect on *E. coli*. Although 10 mM Ca^{2+} and EGCG induced the largest inhibition circle diameter, this was virtually identical to the inhibition circle diameter observed in the presence of 10 mM Ca^{2+} only.

From the previous experiments, it emerged that low and high Ca^{2+} concentration values exert different effects on EGCG inhibition, therefore, 1 and 10 mM Ca^{2+} were selected as representative values of, respectively, low and high Ca^{2+} concentrations, to further analyze the effects of Ca^{2+} on the inhibitory activity of EGCG. The effect of Ca^{2+} and EGCG on the growth curve of *E. coli* is shown in Figure 3.

Compared with the blank group, *E. coli* growth in the medium with Ca^{2+} only did not exhibit significant differences. *E. coli* growth in 1 and 10 mM Ca^{2+} conditions entered the logarithmic phase after 2 h (largest bacterial growth rate in the medium enriched with nutrients), while the plateau (stable phase of bacterial growth) was reached after 12 h. In contrast, in the medium added with EGCG only, *E. coli* entered the logarithmic phase only after 4 h, and the bacterial growth rate was significantly lower than that of the blank group. Moreover, the cell density of the bacteria decreased significantly, *E. coli* entered the stable phase after 8 h, and the bacterial mortality rate exceeded the proliferation rate.

As can be seen from figure 4, the decay of EGCG under different conditions in the 0–4 h time window was in the following order: EGCG + 1 mM Ca^{2+} > EGCG + 10 mM Ca^{2+} > EGCG. The reason for the slower decay of EGCG without Ca^{2+} is twofold: first, the initial bacterial content (10^2 CFU/mL) was low and EGCG had not yet entered the cells; secondly, the concentration of EGCG was relatively high, and the pH of the bacterial solution decreased due to H^+ ionization, leading to a lack of EGCG oxidation, hence slow EGCG decay.

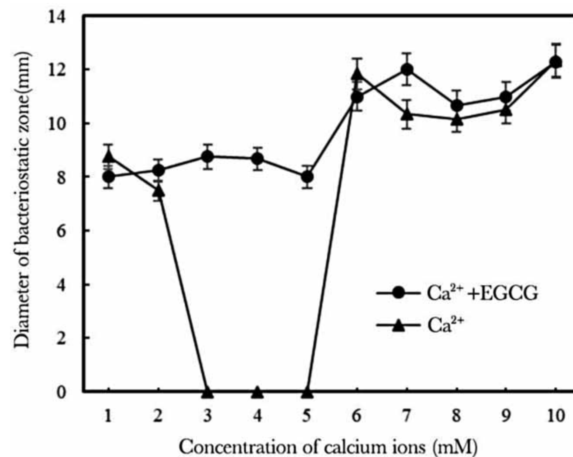

Figure 2 | Inhibitory zone diameter of Ca^{2+} and EGCG (Xu *et al.* 2021).

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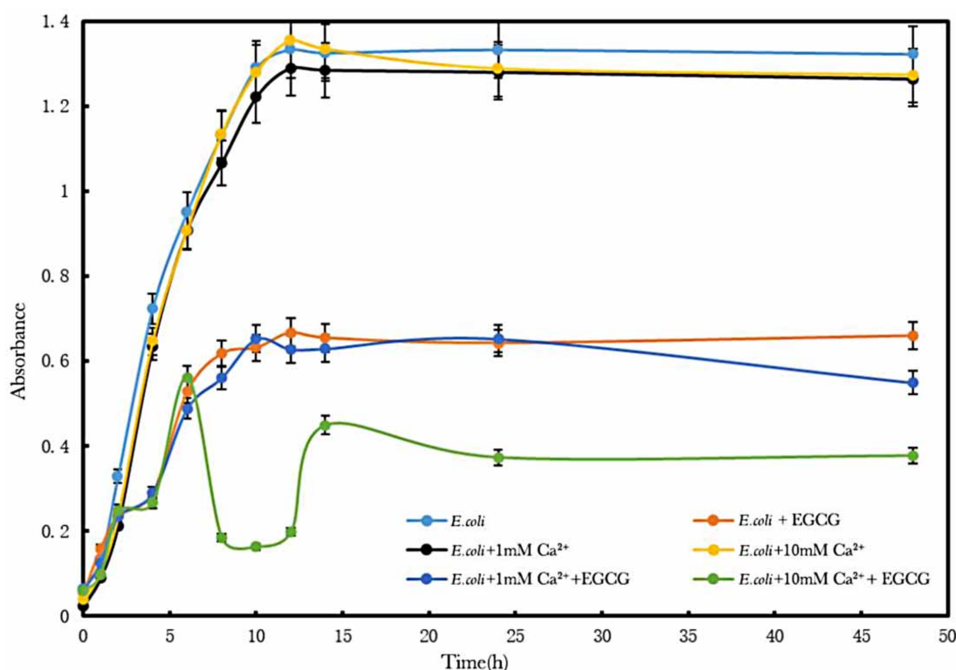

Figure 3 | Effects of Ca^{2+} and EGCG on the growth curve of *E. coli* (Xu *et al.* 2021).

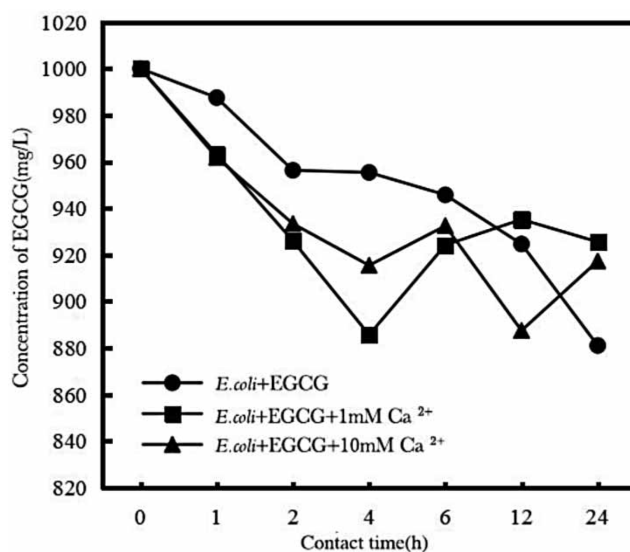

Figure 4 | Attenuation of EGCG under different conditions.

2.2.4. Detection of antibacterial functional groups

As the superoxide anion ($\cdot O_2^-$) reacts with hydroxylamine hydrochloride, it generates NO_2^-. Under the action of *p*-aminobenzenesulfonic acid and α-naphthylamine, NO_2^- forms a red azo compound, with a characteristic absorption peak at 530 nm. By detecting the absorbance at a wavelength of 530 nm with a visible light spectrophotometer, we calculated the $\cdot O_2^-$ content in each experimental group. Through the 'Fenton reaction', H_2O_2/Fe^{2+} generates hydroxyl radicals that can oxidize Fe^{2+} in phenanthroline-Fe^{2+} aqueous solution to Fe^{3+}, resulting in a decrease in the absorbance of the solution at 536 nm. The detection of changes in absorbance at 536 nm allowed us to back-calculate the scavenging rate of hydroxyl radicals.

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In this experiment, *E. coli* cells were cultured for 8 h up to 10^8 CFU/mL, to then split into three culturing subgroups: (i) 2 mM EGCG; (ii) 2 mM EGCG and 1 mM Ca^{2+}; (iii) 2 mM EGCG and 10 mM Ca^2. Cells were cultured at 37 °C for an additional 8 h, to be then diluted with Phosphate-Buffered Saline (PBS) (pH 7.2–7.4) to 10^6 CFU/mL and frozen-and-thawed repeatedly to lyse the cells and release the intracellular components. The lysates were centrifuged at 2,500 rpm for 20 min and the supernatants were collected. The bacterial lysate before the addition of ECGC was used as a blank. The enzyme kits mentioned above were used to detect the changes in SOD enzyme, CAT enzyme, and GSH content in *E. coli* (and compared to a blank group of *E. coli* cultured for 6 h in a medium without either EGCG or Ca^{2+}).

Three 1-mL *E. coli* cultures were collected after 8 h culturing up to 10^8 CFU/mL. Samples were mixed with 2 mM EGCG, or 2 mM EGCG and 1 mM Ca^{2+}, or 2 mM EGCG and 10 mM Ca^{2+}, to be then cultured further in a constant temperature incubator at 37 °C for 6 h. RNA was then extracted with the RNA extraction kit, reverse-transcribed into cDNA, then quantified by q-Polymerase Chain Reaction (PCR) with SYBR Green fluorescent dye and specifically designed primers obtained from Sangon Biotech®.

3. RESULTS AND DISCUSSION

3.1. ROS levels in *E. coli* cell milieu

ROS are one-electron reduction products obtained from oxygen participating in life processes. ROS include superoxide anion ($\cdot O_2^-$), hydrogen peroxide, hydroxyl radical (·OH), etc. and reflect the oxidation state of *E. coli* cells. In response to excessive ROS production by the cell, antioxidant systems are activated to help protect from redox damage. $\cdot O_2^-$ is initially generated in the early stages of oxygen bio-reactivity. ·OH is the most active and most reactive ROS, it is easily combined with other molecules and has great oxidative potency. In this experiment, the changes of $\cdot O_2^-$ and ·OH after *E. coli* exposure to EGCG in combination with different Ca^{2+} concentrations were detected, to explore *E. coli* levels of internal oxidation and antioxidant consequent responses.

3.1.1. Changes in superoxide anion ($\cdot O_2^-$) content

Superoxide anion ($\cdot O_2^-$) is related to several physiological activities in cells. Under normal physiological conditions, $\cdot O_2^-$ in cells is maintained at relatively balanced levels, but in the presence of external stimuli, its concentration can reach excessive amounts. $\cdot O_2^-$ can damage DNA, proteins, and other biomacromolecules, leading to apoptosis (Min *et al.* 2020).

The measurements of superoxide anion variations in *E. coli* under different conditions are shown in Figure 5.

Figure 5 shows that the content of $\cdot O_2^-$ in the blank group was low, between 0.110 and 0.115 μmol/mL, indicating that the cells were in a state of dynamic redox equilibrium.

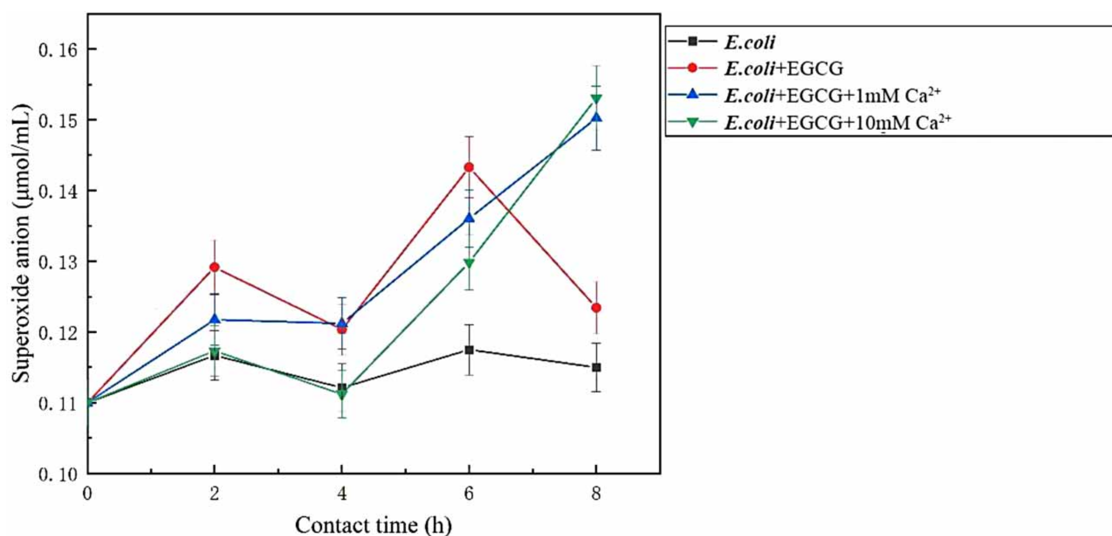

Figure 5 | Changes of superoxide anion in *E. coli* under different conditions.

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When *E. coli* cells were treated with EGCG in combination with 1–10 mM Ca^{2+}, the $\cdot O_2^-$ content increased within 2 h. This phenomenon indicated that the oxidation of EGCG would stimulate *E. coli* to produce $\cdot O_2^-$, but 10 mM Ca^{2+} inhibited EGCG oxidation.

Interestingly, when *E. coli* cells were treated with EGCG only, the $\cdot O_2^-$ levels increased at first, then decreased and increased again significantly after 2 h, reaching a maximum $\cdot O_2^-$ value at 6 h, to then finally decrease again, indicating that EGCG affected the $\cdot O_2^-$ balance in *E. coli*. Notably, even though the $\cdot O_2^-$ amounts decreased in this process, they never recovered to the initial content. The putative reasons behind this are twofold: (1) the oxidation of EGCG within *E. coli* caused oxidative stress, thus antioxidant enzymes helped to decompose some of the $\cdot O_2^-$ excess; and (2) the $\cdot O_2^-$ is further converted into a hydroxyl radical by the Fenton reaction or the Haber–Weiss reaction (Lan 2017). Generally, $\cdot O_2^-$ concentration was increased in the presence of Ca^{2+} and the increasing trend was stronger after 4 h of exposure, which indicates that the presence of Ca^{2+} could inhibit the antioxidant systems in *E. coli*. The oxidative stress response was weakened, so that the $\cdot O_2^-$ produced by EGCG oxidation was not counter-attacked by antioxidant enzymes, resulting in further oxidative damage in *E. coli*.

3.1.2. Changes in hydroxyl radicals (·OH) scavenging rate

Compared to $\cdot O_2^-$, hydroxyl radicals are more hydrophobic and more reactive to phospholipid biomolecules in cell membranes and generally, can react with almost all biological macromolecules when they enter the cell. Excessive ·OH in the organism results in DNA bases and deoxyribose decomposition and leads to cell apoptosis (Shuyan *et al.* 2021). EGCG contains multiple phenolic hydroxyl groups with strong antioxidant properties and can scavenge ·OH to prevent cells from being oxidized to detrimental extents. When the concentration of EGCG is relatively high, it promotes pro-oxidative effects and accelerates cell oxidation (Xianqiang 2013). Therefore, the effect of EGCG on ·OH in the presence of Ca^{2+} is affected by the scavenging rate of EGCG on ·OH.

Our experiments detected the ·OH content in *E. coli* at different incubation times and calculated the hydroxyl radical scavenging rate, after exposure to: (i) EGCG ; (ii) EGCG + 1 mM Ca^{2+}; and (iii) EGCG + 10 mM Ca^{2+}. The results are shown in Figure 6.

Figure 6 shows that the hydroxyl radical scavenging rate of *E. coli* in the blank group remained between 40 and 50% after culturing for 2 h and the overall change was not significant. This is due to the fact that the bacterial physiological metabolism is relatively strong under sufficient nutrient conditions and no external interference, and the excess hydroxyl free radicals generated during the growth and reproduction of bacteria can be rapidly decomposed. It is possible to maintain a relatively balanced concentration of free radicals in cells.

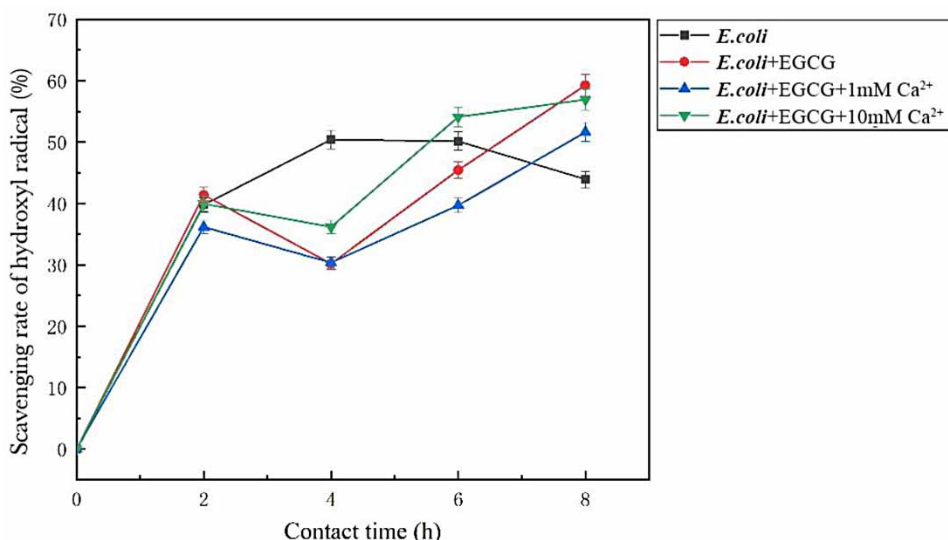

Figure 6 | Scavenging rate of hydroxyl radical in *E. coli* under different conditions.

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We observed an overall increasing trend of the hydroxyl radical scavenging rate in each group, although small differences were detected. During the 2–4 h incubation period, each experimental group's hydroxyl radical scavenging rate showed a similar downward trend (20% for cells treated with EGCG or EGCG + 1 mM Ca^{2+}; 16% for cells treated with EGCG + 10 mM Ca^{2+}). This indicates that the addition of EGCG did not enhance the free radical scavenging. However, to a certain extent, EGCG addition hinders the scavenging effect of *E. coli* antioxidant system on ·OH. Finally, our data also show that EGCG still has this hindering effect in the presence of Ca^{2+}.

After 4 h of culture, the scavenging rate of ·OH increased significantly, specifically of 59, 51, and 57% in *E. coli* cells treated with EGCG, EGCG + 1 mM Ca^{2+}, and EGCG + 10 mM Ca^{2+}, respectively, after 8 h of growth. The clearance rate was significantly higher than that of the blank group.

In conclusion, the addition of 2 mM EGCG can inhibit ·OH scavenging in bacteria. However, with prolonged exposure times, EGCG concentration is attenuated, and the scavenging effect of ·OH is enhanced. Ca^{2+} had little effect on ·OH in our experimental conditions. If EGCG is not added in an effective concentration, it will be difficult to inhibit the antioxidant reaction in bacteria.

3.2. Internal antioxidant system of *E. coli*

The *E. coli* internal antioxidant system includes antioxidant enzymes and antioxidant substances, which constitute two barriers of the antioxidant system in living cells.

3.2.1. Changes in the content of antioxidant enzymes

Antioxidase is the first barrier of the antioxidant system in the body, including, amongst others, SOD, peroxidase (POD), and CAT. SOD and CAT are most closely related and act to remove reactive oxygen radicals (Fangfang *et al.* 2019). The amounts of SOD and CAT in *E. coli* in the different experimental conditions were detected, as illustrated in Figures 7 and 8, respectively.

SOD catalyzes the decomposition of $\cdot O_2^-$ in cells to generate oxygen and hydrogen peroxide. From the data in Figure 7, it can be seen that the SOD content in each experimental condition was greater than that in the blank group at the beginning of the experiment. Combined with the data reported in Figure 5, this implies that the excess of $\cdot O_2^-$ produced by *E. coli* in the presence of EGCG increased the amount of SOD. After 2 h, the SOD content in each experimental group was lower than that of the blank group and the data in Figure 5 showed that the $\cdot O_2^-$ content was still high at this time point. The results showed that the presence of EGCG inhibited the activity of the SOD enzyme, resulting in SOD being unable to remove the excess of intracellular $\cdot O_2^-$. In addition, compared to the cells treated with EGCG, the SOD content of the cells treated with EGCG + 1 mM Ca^{2+} after 4 h was always lower. This indicates that 1 mM Ca^{2+} could enhance the EGCG inhibitory effect on the SOD

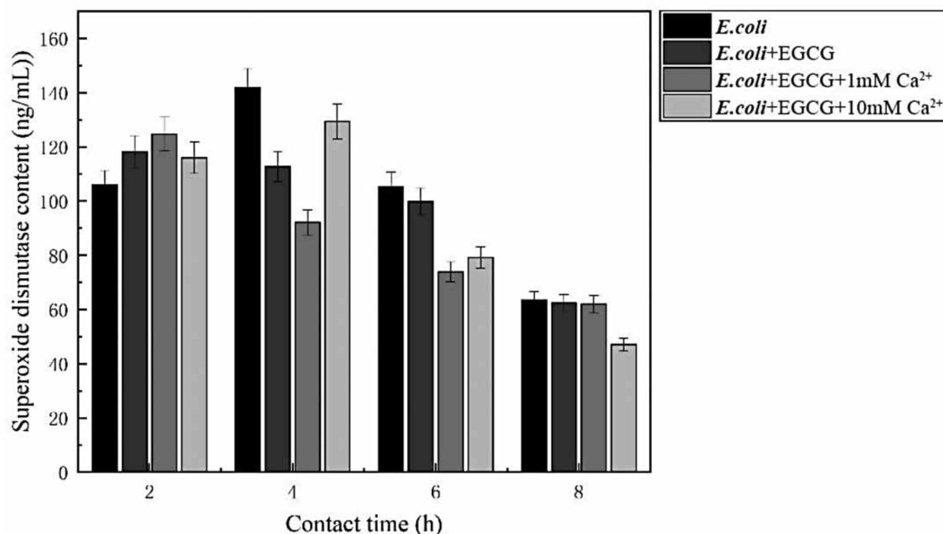

Figure 7 | SOD content.

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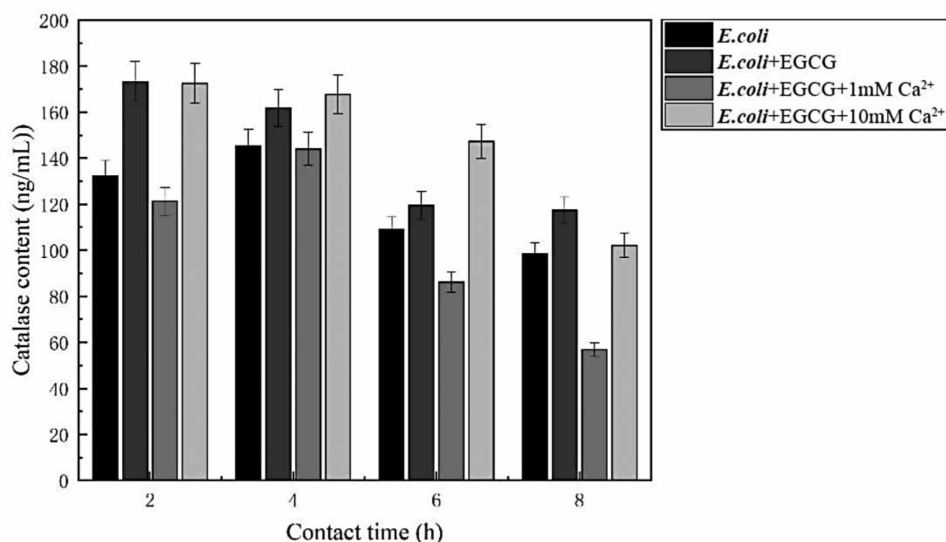

Figure 8 | CAT content.

enzyme, thereby enhancing the oxidative effect of EGCG on bacterial cells. However, the content of SOD enzyme in the cells treated with EGCG + 10 mM Ca^{2+} was significantly higher at 4 h and then lower after that. This indicates that Ca^{2+} has a robust regulatory effect on SOD at high concentrations, which is related to the inhibitory effect of EGCG on SOD, an antagonistic correlation consistent with previously reported results (Chunyan *et al.* 2022). EGCG exerts a better oxidative and bacteriostatic effect by inhibiting SOD, but the concentration of Ca^{2+} significantly affects this process. EGCG's inhibitory effect on SOD can be strengthened by 1 mM Ca^{2+}, but it is weakened by 10 mM Ca^{2+}.

CAT catalyzes the decomposition of hydrogen peroxide to generate oxygen and water in cells. As shown in Figure 8, the content of CAT is higher in *E. coli* cells treated with EGCG compared to the blanks, indicating that EGCG promotes the production of hydrogen peroxide in bacteria via increased CAT content. Compared to the cells treated with EGCG, the CAT content of the cells treated with EGCG + 1 mM Ca^{2+} was significantly lower, and the changing trend was similar to the blank group, indicating that CAT and hydrogen peroxide in the bacteria maintained a relatively balanced state at this time. 1 mM Ca^{2+} could stimulate CAT to decompose excess hydrogen peroxide, leading to a significant reduction in CAT activity, so EGCG is unlikely to cause oxidative damage to *E. coli* when it produces excess hydrogen peroxide in the presence of 1 mM Ca^{2+}. Conversely, the CAT content in the cells treated with EGCG + 10 mM Ca^{2+} was similar to that of those treated with EGCG only, within 4 h of culture, but it was 19% higher after 6 h of culture. This indicates that in the presence of 10 mM Ca^{2+}, EGCG could promote *E. coli* to produce more hydrogen peroxide and CAT with time. EGCG consumption would also increase correspondingly, resulting in a decrease in the oxidative and bacteriostatic effects of EGCG. In conclusion, EGCG can stimulate *E. coli* to produce excessive hydrogen peroxide to achieve the bacteriostatic effect, whereas Ca^{2+} will weaken the persistence of EGCG oxidation and bacteriostasis.

3.2.2. Changes in the content of antioxidant substances

Antioxidants are the second barrier of the intracellular antioxidant system and include GSH, ascorbic acid, vitamin E, etc. Due to the presence of active sulfhydryl groups in the structure of GSH, GSH reacts easily with free radicals and accelerates their depletion, thereby protecting the sulfhydryl-containing proteins from being damaged by excessive amounts of free radicals. Therefore, the reduction of GSH content is considered a potential apoptosis signal (Guangtao *et al.* 2021). Our test detected the changes of GSH in *E. coli*, under different conditions, as shown in Figure 9.

The data reported in Figure 9 show that, compared with the blank group, the overall content of GSH in *E. coli* cells treated with EGCG is lower. However, the GSH content was higher after 8 h of culture. The reason for this is presumably that the sulfhydryl groups of GSH can combine with the hydroxyl group of the EGCG molecule and a small amount of EGCG is consumed when the GSH content declines, preventing EGCG from reacting with the sulfhydryl groups of the cell membrane proteins to maintain membrane integrity (Shunni & Li 2020). Therefore, EGCG reduces *E. coli* GSH content. The increase

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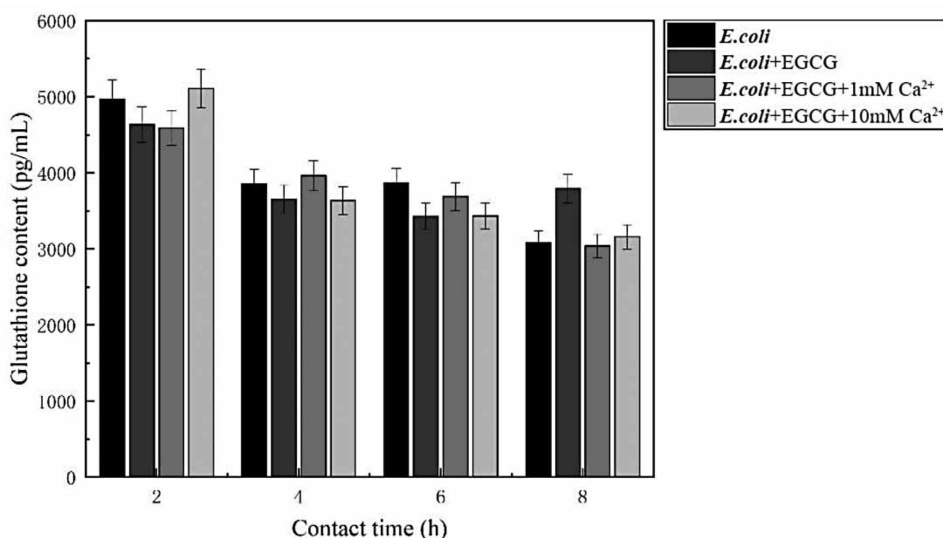

Figure 9 | GSH content.

of GSH amounts in the later stages of the reaction due to the excess of hydrogen peroxide produced by *E. coli* stimulates the antioxidant system to produce a large amount of GSH to ensure the balance of free radicals, while the EGCG content is low in the later stages of the reaction and cannot react with GSH, resulting in an increase of free GSH. Compared with the *cells* treated with ECGC only, the GSH content in the cells treated with EGCG + 1 mM Ca^{2+} decreased over time, although the GSH content gradually approached that of the blank group after 4 h of culture, indicating that 1 mM Ca^{2+} could promote the production of GSH and accelerate the consumption of EGCG, hence the oxidation and antioxidant response in *E. coli* to restore the balance gradually. Although the GSH content change trend in the cells treated with EGCG + 10 mM Ca^{2+} is similar to that of cells treated with EGCG + 1 mM Ca^{2+}, the actual GSH content after 2 h is significantly higher in the former group, indicating that the presence of 10 mM Ca^{2+} stimulates the bacteria to produce excessive H_2O_2. In addition, the antioxidant system in *E. coli* produces a large amount of GSH, indicating that Ca^{2+} could increase the GSH content in bacteria and weaken the oxidative effect of EGCG to a certain extent.

3.3. Oxidative stress response of *E. coli* and its changes

EGCG can stimulate *E. coli* to produce a large amount of ROS, such as $\cdot O_2^-$s and hydrogen peroxide. When the antioxidant system of *E. coli* cannot completely remove ROS excess, it causes cellular oxidative stress, hence inhibition of cell growth and even apoptosis. SoxRS and oxyR regulators are important defense systems against oxidative stress in *E. coli* cells. Once *E. coli* is exposed to oxidative stress, these two regulators are activated and change gene expression and regulate ROS. In this way, they restore the oxidative balance and exert their antioxidant effects in bacteria (Baez & Shiloach 2013). SoxRS regulon is a defense system against excessive $\cdot O_2^-$ stimulation (Kaur & Benov 2020) and its most relevant genes are sodA, soxR, and soxS. The oxyR regulon is a defense system against excess hydrogen peroxide stimulation and its related genes include oxyR, oxyS, ahpC, adpCF, DPS, gor, and katG.

To further explore the effect of EGCG on the oxidative stress response of *E. coli* in the presence of Ca^{2+}, the bacteria were cultured for 6 h in the medium added with EGCG, EGCG + 1 mM Ca^{2+} and EGCG + 10 mM Ca^{2+}. Then, using fluorescence quantitative PCR, the gene expression of the soxRS regulator and the oxyR regulator was detected. The relative expression of the genes was calculated by comparing it to the blank group. The results are shown in Figures 10 and 11.

Figure 10 illustrates that, in contrast to the cells cultured with EGCG only, in the presence of Ca^{2+} the expression levels of sodA, soxR, and soxS defense genes are significantly increased. The expression level of soxS gene in the cells cultured with EGCG + 10 mM Ca^{2+} was nine times higher than that observed in the presence of EGCG only. The protein transcribed from soxS gene activates *E. coli* efflux pumps and promotes the active excretion of drugs from the cytosol, thereby making the bacteria resistant to it (Zhang *et al.* 2017). This indicates that *E. coli* is less sensitive to EGCG and resistant to EGCG in the

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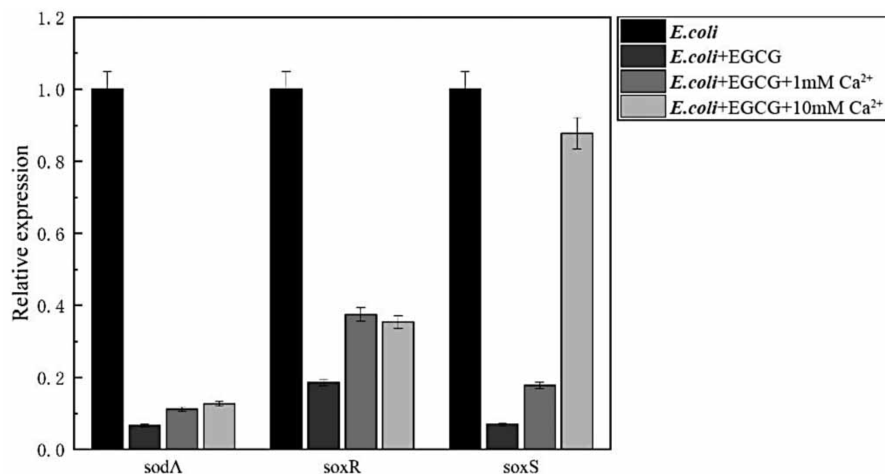

Figure 10 | *E. coli* soxRS regulator gene expression profile.

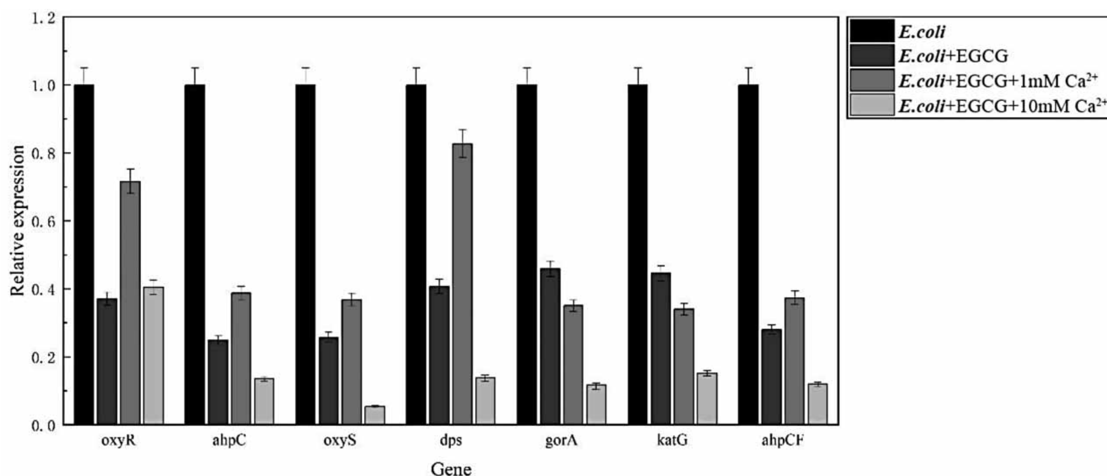

Figure 11 | *E. coli* oxyR regulators gene expression profile.

presence of 10 mM Ca^{2+}, and this experimental condition could cause more bacteria to be injured to a certain extent (Xu *et al.* 2021). The presence of EGCG at 10 mM Ca^{2+} could inhibit bacterial damage.

The oxyR gene is mainly responsible for the recognition and regulation of hydrogen peroxide and plays a vital role in the gene expression of CAT in bacteria. DPS protein binds to DNA forming a tight DPS-DNA complex that protects DNA from oxidative damage (Wen *et al.* 2015). As can be seen from Figure 11, compared with the cells cultured with EGCG only, the addition of 1 mM Ca^{2+} induced a twofold higher expression of most genes related to the oxyR regulator, which indicated that the content of hydrogen peroxide in this group was lower than the control group, and 1 mM Ca^{2+} might attenuate the oxidative damage of EGCG to bacterial DNA.

The expression of oxyR gene in the cells cultured with EGCG + 10 mM Ca^{2+} was slightly higher, indicating that in these experimental conditions, more hydrogen peroxide is produced in *E. coli*, triggering the defense system related to hydrogen peroxide. However, the expression level of other genes was significantly lower. For example, the DPS expression level was only one-third of that in the control group, suggesting that the hydrogen peroxide content observed with EGCG + 10 mM Ca^{2+} exceeds the hydrogen peroxide defense ability of *E. coli*, and the DNA oxidative damage was more evident, which was beneficial to the EGCG-dependent oxidative damage.

Ca^{2+} plays a significant role in the oxidative stress defense system of *E. coli* cells. 1 mM Ca^{2+} improves the hydrogen peroxide defense ability in *E. coli* cells and weakens oxidative stress damage in bacteria. 10 mM Ca^{2+} promotes the production of

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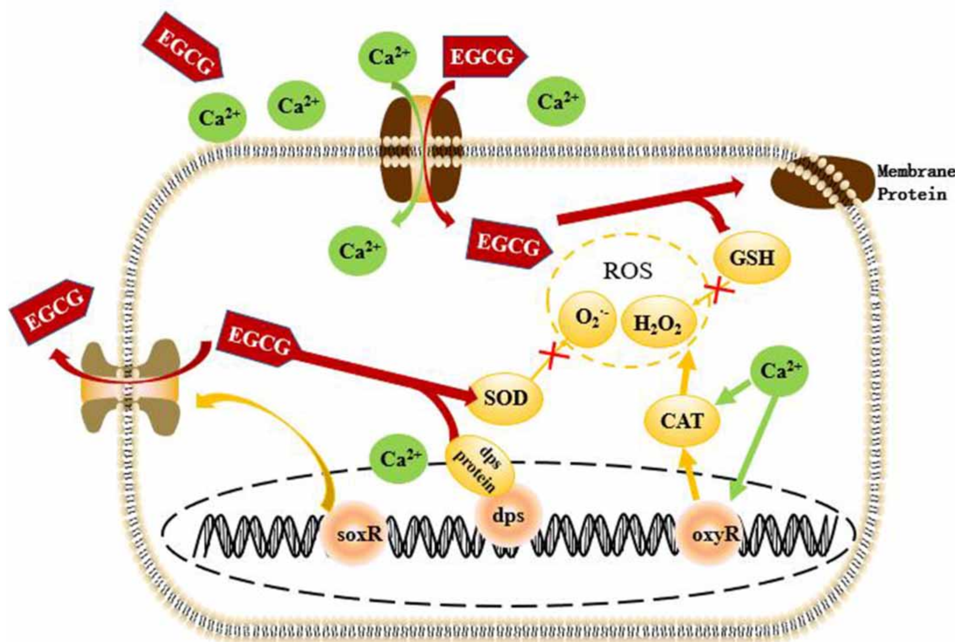

Figure 12 | The oxidative damage process of EGCG to *E. coli* in the presence of Ca^{2+}.

hydrogen peroxide and decreases the expression of defense system-related genes, enhancing the EGCG-dependent oxidative damage to bacterial DNA.

4. CONCLUSION

We showed how the presence of Ca^{2+} is cross-linked to the oxidative damage mechanism of EGCG in *E. coli*. Based on the research herein described, we built a mechanism as reported in Figure 12.

In the presence of Ca^{2+}, the permeability of *E. coli* cell membrane is enhanced, leading to an increased entry of EGCG into the cell. EGCG uses its own oxidation to promote the increase of $\cdot O_2^-$ and hydrogen peroxide content in *E. coli*, augmenting the level of ROS in bacteria. Due to the inhibitory effect of EGCG on the antioxidant enzymes and antioxidant substances in the antioxidant system, the level of ROS in *E. coli* exceeds its own antioxidant capacity, thereby causing oxidative stress in *E. coli* and resulting in oxidative damage. Nevertheless, Ca^{2+} stimulates the expression of oxyR, DPS, and soxS genes, reducing the damage of EGCG to bacterial DNA, and Ca^{2+} may activate efflux pumps, leading to the resistance of *E. coli* to EGCG and leaving the bacteria susceptible to disinfection damage.

EGCG can be complexed with metal ions and the metal complexes formed to have different inhibitory activities and can improve the bacterial inhibitory effect of EGCG. The mechanism of bacterial inhibition is shown in its effect on bacterial cell membranes, proteins, genetic material, and other aspects. EGCG is used for disinfection of drinking water and shows good disinfection characteristics.

Currently, the highest content of metal ions in drinking water is Ca^{2+}. Experimental results show that EGCG has a strong inhibitory effect on the growth of *E. coli* under high concentrations of Ca^{2+}, which can improve the killing ability of EGCG on *E. coli*. In the future, EGCG can also be used in conjunction with new drinking water treatment technologies such as ultrafiltration membranes to ensure the safety of water quality in water supply networks.

ACKNOWLEDGEMENTS

This research is funded by National Natural Science Foundation of China (51678026), Open Research Fund Program of Key Laboratory of Urban Stormwater System and Water Environment (Beijing University of Civil Engineering and Architecture), Ministry of Education (2020) and Beijing University of Civil Engineering and Architecture Postgraduate Innovation Project

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(PG2022061). The authors would like to express their gratitude to EditSprings (https://www.editsprings.cn) for the expert linguistic services provided.

DATA AVAILABILITY STATEMENT

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

CONFLICT OF INTEREST

The authors declare there is no conflict.

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First received 8 October 2022; accepted in revised form 27 February 2023. Available online 10 March 2023

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