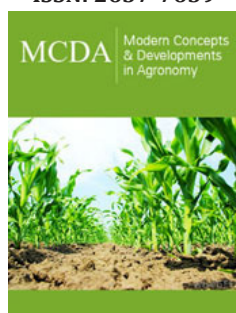


Effect of Curcumin Mediated Photodynamic Technology on *Salmonella typhimurium* Biofilm and Its Bactericidal Mechanism and Application in Milk

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Abstract

The inactivation effect of curcumin-mediated photodynamic technology (PDT), a novel alternative non-thermal technique, on *Salmonella typhimurium* (*S. typhimurium*) biofilm and its preliminary bactericidal mechanism and application in milk were investigated. Biofilm formed from *S. typhimurium* ATCC 14028 was incubated with the photosensitizer curcumin, followed by exposure to blue laser (λ_{\max} 450 nm) for testing antibiofilm effect. Planktonic *S. typhimurium* was taken for exploring the possible bactericidal mechanism. After curcumin-PDT treatment, the damages of bacterial DNA and protein were observed by agarose gel electrophoresis and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) respectively, and the morphology change was visualized by scanning electron microscopy (SEM). Confocal laser scanning microscopy (CLSM) was employed to test bacterial membrane permeability change. Bacterial viability decreased significantly when curcumin concentration and illumination time increased. Curcumin (80 μM) combined with blue light (200 mW/cm^2) illumination for 200 mW inactivated more than 2 lg/(CFU/mL) *S. typhimurium* biofilm. DNA damage, protein degradation, and morphological change of PDT-treated *S. typhimurium* were observed. Curcumin-PDT treatment gained less bactericidal effect on *S. typhimurium* in milk and the inactivation efficacy was related to type of milk, curcumin concentration, illuminated liquid level and liquid transmittance. Therefore, curcumin-PDT is a promising method or assistance to control foodborne *S. typhimurium* in milk

Keywords: Photodynamic technology; Curcumin; *Salmonella typhimurium*; Biofilm; Milk

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Introduction

Rich in nutrients and welcomed by customers of all ages, milk also provides favorable conditions for foodborne pathogens. It has a high risk of contamination by foodborne pathogens such as *Bacillus cereus* [1], *Cronobacter sakazakii* [2] and *Salmonella* spp. [3] *Salmonella* is one of the top-four foodborne pathogens leading to high morbidity and mortality [4,5]. Cases of 93.8 million gastroenteritis are caused by *Salmonella* worldwide each year, resulting in nearly 155,000 deaths [6], and *S. typhimurium* is one of the most common serotypes [7]. Reports on milk in particular infant formula milk powder contaminated by *Salmonella* always caused

widespread social concern and economic loss [8,9]. Besides bacteria existing in raw milk, bacteria contaminating processing equipment can easily form biofilms then contaminate products. Biofilm has been regarded as an intractable problem in dairy industry, since the formation of biofilm will increase the resistance of bacteria to adverse environment and weaken the effect of disinfection [10]. Therefore, the control of *Salmonella* biofilm that may contaminate food contact surfaces and planktonic *Salmonella* in milk is of great importance during dairy product processing.

Traditionally, thermal sterilization is the most commonly employed method to inactivate foodborne pathogens, but it may lead to the loss of heat-sensitive nutrients and the change of organoleptic profiles [11]. Therefore, non-thermal processing technologies such as disinfectant, high-pressure processing and irradiation are considered to be potential for foodborne bacteria disinfection. Recently, however, the shortcomings of these non-thermal techniques were discussed. The problems of toxic residues, high cost, professional requirement for operation and deterioration of nutritional and organoleptic properties of food make it urgent to find a novel alternative non-thermal technique to ensure the quality and safety of milk product [12]. Photodynamic technology (PDT), without above-mentioned disadvantages, is a promising technique to prevent pathogens based on photochemical reactions [13,14].

In this process, the photosensitizer is activated by light at specific wavelength and releases energy to form superoxide, hydroxyl radical or singlet oxygen which can react with adjacent biological molecules to produce bacterial toxicity, leading to damage or death of pathogens [15-17]. In recent years, increasing PDT studies based on natural edible photosensitizers especially curcumin have showed that PDT can inactivate a wide range of foodborne bacteria with marginal damage to quality of various food such as fruits, vegetables, meat and seafood products [18-21]. Comparative research studies pointed out that *S. typhimurium* can be effectively inactivated by PDT although it is more resistant to this treatment as compared to other bacterial species [20,22-24]. To our knowledge, no investigation has been carried out to test curcumin-PDT effect on bacteria in milk and little literature is available on curcumin-PDT to *S. typhimurium* biofilm. The aims of this study, therefore, were to investigate curcumin-PDT inactivation effect on *S. typhimurium* biofilm, explore preliminary inactivation mechanism on planktonic *S. typhimurium* cells, and apply this technique in milk.

Material and Methods

Bacterial culture preparation

Bacterial and biofilm culture and curcumin-PDT preparation were conducted in the Microbiology Laboratory, Department of Food Science and Engineering, Jinan University. *S. typhimurium* ATCC 14028 was gifted by Guangdong Provincial Center for Disease Prevention and Control (CDC), China. To obtain working culture, a single colony was picked and enriched in 5 mL sterile tryptic soy broth (TSB) (Qingdao Hope Bio-Technology Co., Ltd, Qingdao,

China), which was agitated at 37 °C and 120 rpm for 16 h. The planktonic cells were harvested by centrifugation at 3532×g for 10 min, washed three times and re-suspended in phosphate-buffered saline (PBS) (10⁹ CFU/mL).

Photosensitizer and light source

Curcumin powder (>95%, Ci Yuan Biotechnology Co. LTD., Shanxi, China) was dissolved in ethanol (99%) as 20 mM stock solution which was then diluted with sterile water to obtain a series of working concentrations. All the solutions were stored in the dark at 4 °C before use. The ethanol concentration in working solutions was <1% (v/v). It is worth pointing out that curcumin, as a food additive, can be used in a range of 50-500 mg/L or mg/kg in different foods [23].

A blue 450nm laser (LWBL450-10W-F, Beijing Laser wave Optoelectronics Technology Co., LTD, China) was used as the light source and placed at 17cm distance above the sample. Energy density on the surface of sample was (200 mW/cm²) measured with a Laser Power Meter (LI-P20W-A, Beijing Laser wave Optoelectronics Technology Co., LTD, China).

PDT treatment on *S. typhimurium* biofilm

Samples during the PDT processing were illuminated in the Optics Laboratory, Department of Optoelectronic Engineering, Jinan University. Every 200 µL bacterial suspension and 5 mL TSB were transferred to one well of 6-well plate. A microscope cover glass (20 mm×20 mm, 10212020C, Jiangsu, Citotest Scientific Co., Ltd, China) was put in each well and incubated at 30 °C for 48 h to form biofilm. Then the TSB was removed, and the cover glass was washed with sterile PBS for three times. The biofilm on cover glass was incubated with a series of curcumin (0, 10, 20, 40, 80 µM) in the dark at 37 °C for 5 min. Then samples were illuminated at room temperature for 0, 1, 10, 20, 30 min, respectively. Experiments were divided into four groups: L-S- group (no light and curcumin), L-S+ group (curcumin alone), L+S- group (light alone) and L+S+ group (both light and curcumin). After illumination, the cover glass was placed in a centrifuge tube containing 5 mL saline solution and treated by ultrasound for 10 min. One mL treated suspension was taken for 10-fold serial dilution followed by culturing on TSA plate for counting the viable bacteria. Each experiment was in triplicates.

Preliminary mechanism of bactericidal effect on planktonic *S. typhimurium*

Agarose gel electrophoresis analysis of *S. typhimurium* DNA: The planktonic cells were prepared as description in the section of bacterial culture preparation. Each aliquot (1.5 mL) of bacterial suspension with 3.5mL curcumin (80 µM) or PBS was mixed in one well of 6-well plate then illuminated for 30 min. Treated samples were used for extracting bacterial DNA by bacterial total DNA extraction kit (Tian Gen Biochemical Technology Co. LTD., Beijing, China). Harvested DNA products were separated by 1.5% agarose gel electrophoresis, stained by golden view and visualized under UV light.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of *S. typhimurium* protein: Each aliquot (1.5 mL) of bacterial suspension with 3.5 mL curcumin (80 μ M) or PBS was mixed in one well of 6-well plate then illuminated for 30 min. Each sample was collected (4 mL) by centrifugation at 6149 \times g for 1 min. The pellet was re-suspended in 20 μ L sterile water first and 20 μ L 2 \times SDS-gel loading buffer afterwards. After heating for 5 min at 95 $^{\circ}$ C, the samples were centrifuged for another 2 min. Ten μ L supernatant was loaded and separated by 5% stacking gel and 12% separation gel electrophoresis (PowerPac™ Basic, Bio-Rad, America). Then the gel was stained by Coomassie Brilliant Blue.

Scanning electron microscopy (SEM) observation of *S. typhimurium* morphology: Each aliquot (1.5 mL) of bacterial suspension with 3.5 mL curcumin (80 μ M) or PBS was mixed in one well of 6-well plate and then illuminated for 30 min. Each sample was collected (4 mL) by centrifugation at 3532 \times g for 10 min and washed with PBS for three times. The pellet was fixed in 1 mL glutaraldehyde (2.5%) for 12 h at 4 $^{\circ}$ C then dehydrated in ethanol solutions of graded series concentrations (once at 30, 50, 70, 80 and 90%, and twice at 100%) before critical point drying in carbon dioxide. Finally, samples were coated with gold and observed under SEM (Supra55, Zeiss, Germany).

Confocal laser scanning microscope observation of *S. typhimurium* membrane permeability: Change of cell permeability after PDT treatment was measured by LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen, Thermo Fisher Scientific, America). Biofilm was formed on the cover glass and exposed to 80 μ M curcumin combined 30-min light illumination treatment (L+P+) or untreated (L-P-). Subsequently, the glass was picked up and washed for three times by sterile water. Mixture (150 μ L) of SYTO9 and propidium iodide (PI) was added to cover the sample on glass surface, followed by a 15-min dark incubation at 37 $^{\circ}$ C and washing for three times. Then samples were added a drop of anti-fluorescence quenching solution respectively, prior to their observation by confocal laser scanning microscope (CLSM, LSM880, Zeiss, Germany).

PDT treatment on planktonic *S. typhimurium* in milk

Table 1: Instruction of milk solution preparation.

Type of Milk Powder	Milk Power (g)	Sterile Water (mL)
Infant formula	41.25	225
Full cream	35	250
Skim	25	250

Infant formula (Friso, Friesland Campina (Hong Kong) Limited, China), instant milk powder (Devondale, Murray Goulburn Co-Operative Co. Limited, Australia) and instant skim milk powder (Devondale, Murray Goulburn Co-Operative Co. Limited, Australia) were purchased from local supermarket in Guangzhou. Different types of milk solutions were prepared according to instructions given by manufacturers (Table 1). Curcumin stock solution (0.10 mL, 20 mM) compounded with 24.90 mL and 16.57 mL milk solutions respectively to gain 80 μ M and 120 μ M turmeric milk.

To test the effect of curcumin concentration on bactericidal efficacy, 1 mL bacterial aliquot was mixed with the equal volume of PBS, 80 μ M and 120 μ M turmeric milk solutions respectively in a 6-well plate. One mL or 2 mL bacterial aliquot was mixed with the equal volume of PBS and 120 μ M turmeric milk solutions respectively to test the effect of liquid level (0.2 cm or 0.4 cm) on bactericidal efficacy. Then the turmeric milk solutions were diluted to gain double dilution milk, and 1 mL bacterial aliquot was mixed with the equal volume of PBS, 120 μ M raw turmeric milk and 120 μ M double diluted turmeric milk solutions respectively to test the effect of milk dilution on bactericidal efficacy. All plates after incubation in the dark at 37 $^{\circ}$ C for 5 min, were placed under the blue light to illuminate for 30 min. One mL treated sample was then taken for 10-fold serial dilution, and 100 μ L of each dilution was spread on TSA plate and incubated at 37 $^{\circ}$ C for 16 h for counting viable cells.

The infant formula milk solution was diluted to gain twofold and tenfold dilution milk for testing light transmittance. The light transmittance of milk samples was measured by UV spectrophotometer. Each experiment was in triplicates.

Statistical analysis

All data are expressed as mean \pm standard deviation (SD) from three separate experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) using Origin 9.0 software.

Result and Discussion

PDT inactivation of *S. typhimurium* biofilm

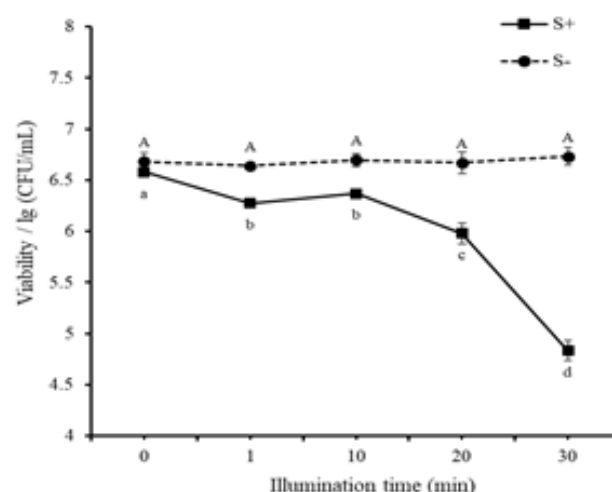


Figure 1: Effect of illumination time on PDT inactivation of *S. typhimurium* biofilm.

Different upper letters and lowercase letters represent significant difference in S- group (without curcumin) and S+ (with curcumin, 20 μ M) group respectively.

Effect of exposure time: The effect of illumination time on PDT inactivation of *S. typhimurium* biofilm was shown in Figure 1. Illumination alone did not affect the *S. typhimurium* biofilm viability

obviously, even the illumination time was prolonged to 30 min. Curcumin (20 μM) combined with blue light had the inactivation effect on *S. typhimurium* biofilm, and 1.9 lg/(CFU/mL) viability decrease was obtained after 30 min PDT treatment.

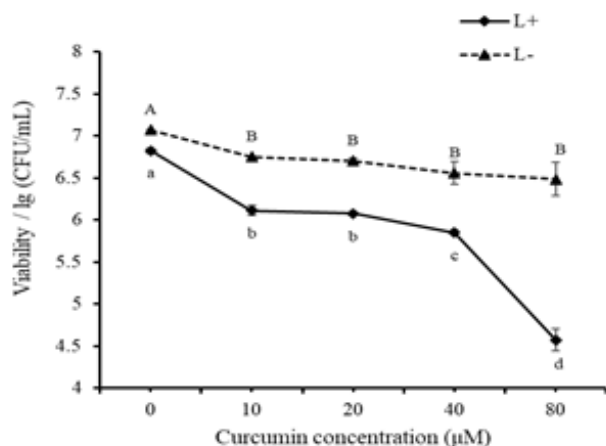


Figure 2: Effect of curcumin concentration on PDT inactivation of *S. typhimurium* biofilm. Different upper letters and lowercase letters represent significant difference in L- (without light) group and L+ (with light, 20 min) group respectively.

Effect of curcumin concentration: Data in Figure 2 demonstrated that curcumin had a mild bactericidal effect by itself. Eighty μM curcumin could reduce approximate 0.6 lg/(CFU/mL) biofilm viability. When combined with blue light, curcumin showed more significant inactivation effect on *S. typhimurium* biofilm. The inactivation effect was strengthened when curcumin concentration increased, and 80 μM curcumin reduced the most biofilm viability (more than 2 lg/(CFU/mL)) after 20-min illumination. A previous study reported that curcumin treatment alone caused cell membrane damage and cell death because the insertion of curcumin into the liposome bilayer enhanced cell membrane permeability [25,26], but it took longer time to achieve the same bactericidal effect as PDT. Previous studies pointed out that gram-positive bacteria were more susceptible than gram-negative bacteria [23,24] and bacterial biofilm inactivation by PDT was more difficult than planktonic bacteria [27,28]. Present viability results suggested that curcumin-PDT could penetrate extracellular polymeric substance (EPS) and injured bacteria inside. Penha et al. [23] examined the bactericidal effect of 75 μM curcumin and 30 min blue LED (λ_{max} 470 nm) illumination, showing a 2.82 log CFU/mL reduction of *S. typhimurium*. This treatment conditions and results were quite similar to that in present study, indicating that curcumin-PDT can also be a potential strategy for controlling both planktonic *S. typhimurium* and its biofilm. Bonin et al. [24] exposed *S. typhimurium* to the combined treatment of 10 μM eosin Y and 15-min green LED (λ_{max} 490-570 nm) illumination, causing a 1.7 log CFU/mL reduction. Santos et al. [22] compared PDT effects mediated by eosin and rose bengal respectively on *S. typhimurium*, and 100 μM eosin combined 15-min illumination

(530 \pm 40 nm) could only lead to about 2 log reduction. In contrast, 50 μM rose bengal with 15-min illumination (530 \pm 40 nm) led to the total inactivation. Although, some photosensitizers in PDT treatment could result in very desirable inactivation effects, they are synthesized dyes and not suitable for food application.

Preliminary mechanism of PDT inactivation of *S. typhimurium* biofilm

Damage of bacterial DNA: In Figure 3, the intensity of DNA band in L-S+ group showed a marginal decrease compared with bands in L+P+ and L+P- groups, indicating that light alone could not damage bacterial DNA but 80 μM curcumin treatment alone might lead to a slight DNA damage. The DNA band in L+S+ group was the weakest, showing the most significant damage compared with other groups, but still not disappeared completely. It is consistent with the DNA damage of *Vibrio parahaemolyticus* by MB-PDT reported by Deng et al. [29]. Other studies have shown that PDT enhanced the control of pathogens by attenuating quorum sensing (QS) dependent factors, such as production of exopolysaccharide and alginate, swimming ability and virulence factors [30-32]. However, this study only showed the PDT effect on total bacterial DNA by SDS-PAGE for a preliminary conclusion. The quantification of gene damage and analyzing specific gene damage caused by PDT can be further carried out by real-time quantitative polymerase chain reaction.

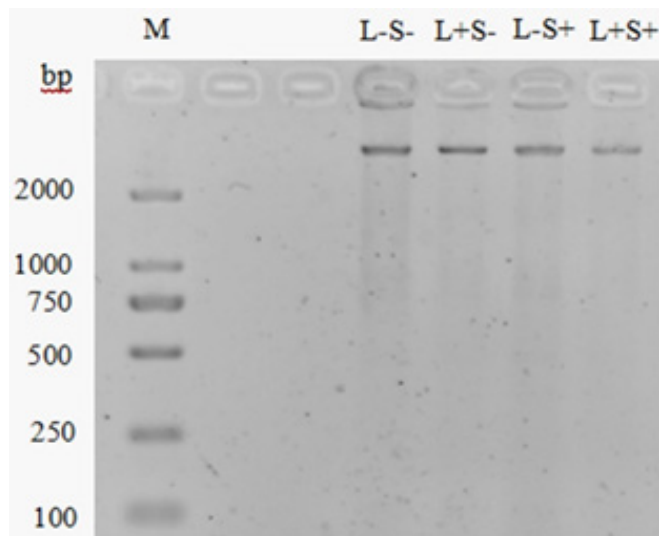


Figure 3: Agarose gel electrophoresis analysis of *S. typhimurium* DNA.

M: Marker, DL 2000. L-S-: no light and curcumin. L+S-: light alone (30 min). L-S+: curcumin alone (80 μM). L+S+: both light (30 min) and curcumin (80 μM).

Damage of bacterial protein: In Figure 4, protein degradation of *S. typhimurium* was analyzed by SDS-PAGE. Intensity of protein bands in L-P- (control), L-P+ (only curcumin), L+P- (only light) groups did not change obviously, showing that compared with control group, neither individual curcumin nor only light treatment could affect *S. typhimurium* protein. In contrast, slight decreasing intensity of protein band was observed in L+P+ group, suggesting

that bacterial protein degradation might be caused by 80 μ M curcumin in combination with 30 min illumination treatment. Results obtained in this study proved curcumin-PDT caused damage of *S. typhimurium* total protein. Similarly, Li et al. [21] observed a slight degradation of total *Salmonella spp.* protein by riboflavin-mediated PDT. Wu et al. [33] showed that PDT damaged bacterial

outer membrane protein. But hardly any report has further studied other PDT-targeted proteins of bacteria. Actually, the oxidative burst of reactive oxygen species (ROS) produced by PDT will break the balance of microorganism homeostasis and attack those substances playing important physiological roles in cells such as RNA and lipid, besides DNA and protein [34].

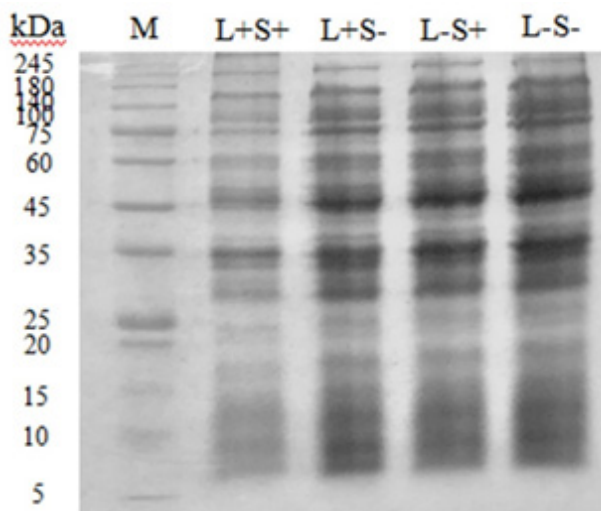


Figure 4: SDS-PAGE analysis of *S. typhimurium* protein.

M: Marker (5-245 kDa). L-S-: no light and curcumin. L+S-: light alone (30 min). L-S+: curcumin alone (80 μ M). L+S+: both light (30 min) and curcumin (80 μ M).

Change of bacterial morphology: Morphological change of *S. typhimurium* after PDT treatment was shown in Figure 5. The bacteria of L-S- group were oval and plump, while bacterial cells in L+S+ group were wrinkled and irregular, even some cracks occurred on the surface (see arrows in Figure 5B). This result visually showed that curcumin-PDT distorted morphology of *S.*

typhimurium but did not cause collapse of cells. Chai et al. [35] observed no significant change of *L. monocytogenes* morphology after curcumin-PDT by SEM, but further transmission electron microscopy (TEM) observation found partial cytoplasm cavitation. Therefore, the main target of PDT might be the disruption of intracellular biological damage.

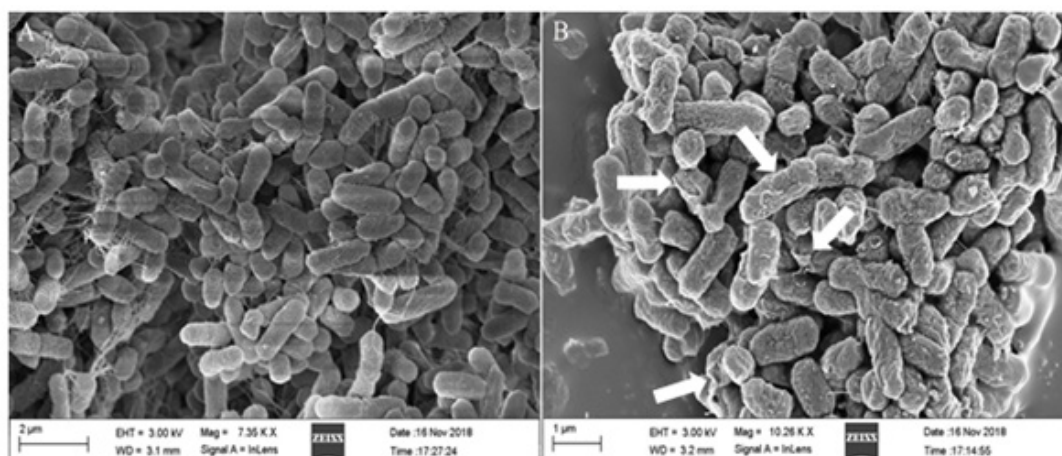


Figure 5: SEM images of *S. typhimurium*.

A: L-S-, no light and curcumin. B: L+S+, both light (30 min) and curcumin (80 μ M).

Change of membrane permeability: Since membrane integrity is indispensable for bacterial survival, based on the different transmembrane ability of SYTO 9 and PI, live and dead cells can be

distinguished by double stains SYTO 9 and PI. SYTO 9 can cross cell membrane and label all bacteria with green fluorescence when used alone while PI can only penetrate cells when membrane injured or

disrupted and present red fluorescence [36]. CLSM was used to observe the permeability of bacteria in biofilms after PDT treatment of 80 μM curcumin in combination with 30-min illumination. As shown in Figure 6, cells in L-S- group were green, showing bacterial membrane remaining intact and blocked PI stain outside the cells. In contrast, most cells in L+S+ group presented red, indicating PDT treatment caused membrane permeability damage and PI can went

inside. Therefore, the result suggested that curcumin-PDT could penetrate or disrupted bacterial EPS and induced *S. typhimurium* permeability damage, leading to lethal injuries of inside bacteria. However, how PDT interacted and affected protective bacterial EPS could not be explained in present study. Further study on PDT effect on EPS in detail needs to be carried out.

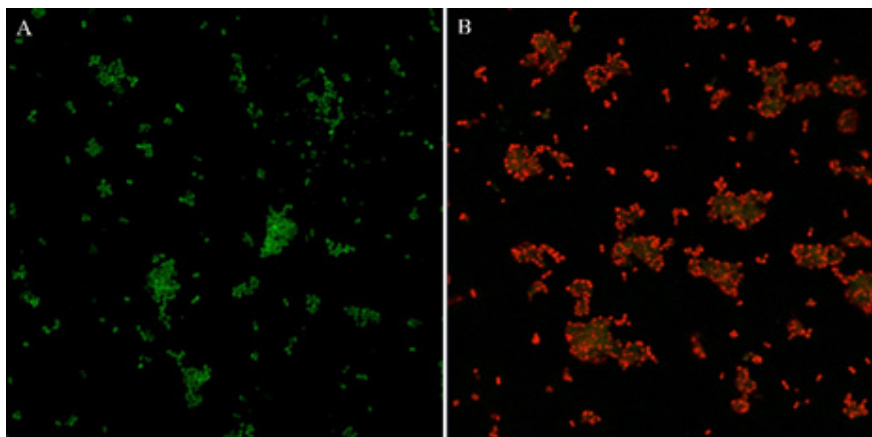


Figure 6: Live/dead fluorescent staining images of *S. typhimurium*. A: L-S-, no light and curcumin. B: L+S+, both light (30 min) and curcumin (80 μM).

PDT inactivation of *S. typhimurium* in milk

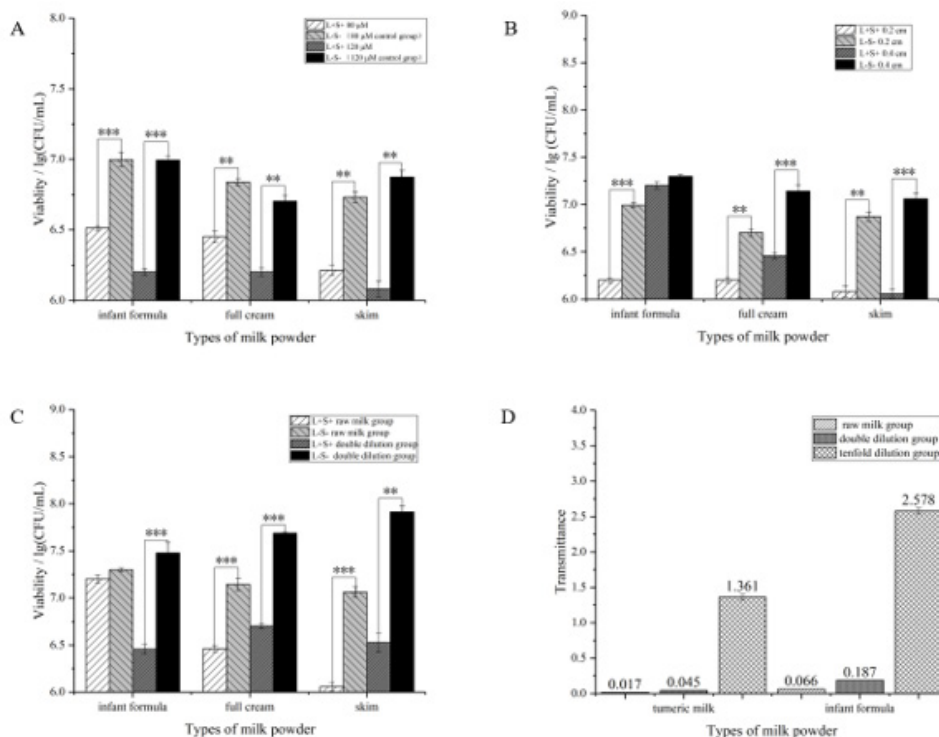


Figure 7: Factors involved in PDT effect on *S. typhimurium* in milk. A. Curcumin concentration (80 μM +0.2 cm; 120 μM +0.2 cm). B. Liquid level (120 μM +0.2 cm; 120 μM +0.4 cm). C. Milk dilution ratio (raw milk; double diluted milk). D. Light transmittance of milk with different dilution ratio.

Results in Figure 7 showed that when PDT was applied in milk, type of milk, curcumin concentration, illuminated liquid level and liquid transmittance all affected inactivation efficacy. In Figure 7A, PDT inactivation effect on *S. typhimurium* in full cream milk was the weakest in terms of three types of milk. Increasing curcumin concentration to 120 μM could decrease milk-borne *S. typhimurium* in full cream milk by around 0.5 lg/(CFU/mL) while about 0.8 lg/(CFU/mL) decline was observed in other two kinds of milk. The difference might be attributed to the lipid content in milk powder. Since the external structural integrity of gram-negative bacteria is closely related to lipopolysaccharide (LPS), the higher level of lipid content in milk may be more conducive to maintaining the lipid soluble structure of bacteria. Undesirable bactericidal effect in milk was also observed by Wang et al. [37] *Staphylococcus aureus* in milk was just reduced by 0.42 log after Na-chlorophyllin-PDT treatment, while 4.5 log bacteria could be reduced in saline solution. Such a significant difference was presumed to be caused by large solid particles in milk which sheltered and returned light to block PDT reaction. Galstyan et al. [38] considered that reduction of bactericidal effect in milk was due to aggregation effect of casein and whey protein on photosensitizer, which changed the maximum absorption spectrum of photosensitizer. The existence of cysteine could also quench the singlet oxygen produced during the photodynamic process. In addition, calcium and magnesium ions in the emulsion could stabilize the negative charge in oligosaccharide chains and might seriously affect the binding of photosensitizer to gram-negative bacteria.

Figure 7B showed that the PDT effect did not change rapidly when the liquid level of full cream and skim milk changed, while the bactericidal effect on infant formula milk was totally inhibited when the illuminated liquid level changed from 0.2 cm to 0.4 cm. It might be that the infant formula milk contained more trace elements and minerals (iron, zinc, vitamins), so blue light could not penetrate the emulsion effectively, and the light intensity reaching the surface and bottom of the sample was unequal, which greatly reduced the bactericidal effect.

Besides, the bactericidal effect was significantly enhanced with the increase of milk dilution ratio (Figure 7C). Especially for skim milk, *S. typhimurium* reduction in double diluted milk was the most, reaching almost 1.4 lg/(CFU/mL). Figure 7D revealed that transmittance of milk increased with the increase of dilution ratio, indicating the positive correlation between PDT efficacy and solution transmittance. Wang et al. [37] also proved it by comparing the PDT inactivation effect on *S. aureus* in clear and cloudy litchi juice. Therefore, application of PDT in cloudy liquid food will be more difficult.

Conclusion

This study showed that curcumin in combination with blue light could inactivate *S. typhimurium* biofilm by more than 2 lg. For planktonic *S. typhimurium*, curcumin-PDT could cause genomic DNA damage and protein degradation, and distorted *S. typhimurium* morphology, induced membrane damage and permeability change,

leading to *S. typhimurium* cell death. When curcumin-PDT was adopted to control *S. typhimurium* in milk, type of milk, curcumin concentration, illuminated liquid level and liquid transmittance all affected PDT efficacy. Curcumin (120 μM) in combination with illumination (30 min) obtained the best bactericidal effect in double diluted skim milk with a liquid level of 0.2 cm.

Even though inactivation effects of curcumin-PDT were observed on both *S. typhimurium* biofilm in vitro and planktic *S. typhimurium* cells in milk, further investigations are necessary to be implemented to illustrate how milk compositions (such as protein and fat) interact with curcumin and light, and if the milk attributes will be changed after treatment.

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