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Antimicrobial photodynamic activity by water-soluble curcumin against foodborne pathogens

Atividade fotodinâmica antimicrobiana de uma curcumina solúvel em água contra patógenos de origem alimentar

Actividad fotodinámica antimicrobiana de la curcumina soluble en agua contra patógenos transmitidos por los alimentos

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Abstract
Foodborne diseases and microbiological control represent the major challenge for the food industry. New technologies using natural agents have attracted increasing interest. Therefore, this study aimed to evaluate the *in vitro* susceptibility of *Staphylococcus aureus* and *Escherichia coli* to water-soluble curcumin (WSC) combined with acidic pH and blue LED light. The minimum inhibitory concentration (MIC) and bacterial photoinactivation were conducted using different photosensitizer concentrations. For *S. aureus*, the combination of WSC with lactic acid and 2.5 min illumination time reduced MIC from 500 µg/mL to 15.62 µg/mL, and WSC with citric acid reduced MIC from 125 µg/mL to 7.81 µg/mL after 5 min of light exposure. WSC without illumination did not inhibit *E. coli* growth (MIC >1,000 µg/mL), however, when applied with photodynamic therapy (5 min blue LED illumination), WSC at 62.5 µg/mL with lactic acid and WSC at 7.81 µg/mL with citric acid eliminated *E. coli* cells. The results obtained suggest that water-soluble curcumin with organic acids when combined with a blue LED light was effective against foodborne pathogens.
Keywords: Curcumin; Foodborne pathogens; Photodynamic therapy.

Resumo

As doenças transmitidas por alimentos e o controle microbiológico representam um grande desafio para a indústria alimentícia. Novas tecnologias que empregam agentes naturais têm despertado um grande interesse. Portanto, este estudo tem como objetivo avaliar a suscetibilidade in vitro de *Staphylococcus aureus* e *Escherichia coli* à curcumina solúvel em água (CSA) associada a pH ácido e luz LED azul. A concentração inibitória mínima e a fotoinativação bacteriana foram realizadas utilizando diferentes concentrações de fotossensibilizador. Para *S. aureus*, a combinação de CSA com ácido lático e 2,5 min de iluminação reduziu a CIM de 500 µg/mL para 15,62 µg/mL, e CSA com ácido cítrico reduziu a CIM de 125 µg/mL para 7,81 µg/mL após 5 min de exposição à luz. CSA sem iluminação não inibiu o crescimento de *E. coli* (CIM > 1000 µg/mL), no entanto, quando aplicado na terapia fotodinâmica (5 min de iluminação com LED azul), CSA a 62,5 µg/mL com ácido lático e CSA 7,81 µg/mL com ácido cítrico, nenhuma célula viável foi recuperada. Os resultados obtidos sugerem que a curcumina solúvel em água com ácidos orgânicos quando combinada com uma luz LED azul foi eficaz contra patógenos de origem alimentar.

Palavras-chave: Curcumina; Patógenos transmitidos por alimentos; Terapia Fotodonâmica.

Resumen

Las enfermedades transmitidas por los alimentos y el control microbiológico representan el mayor desafio para la indústria alimentaria. Las nuevas tecnologias que emplean agentes naturales han despertado um interés cresciente. Por lo tanto, este estudio tuvo como objetivo evaluar la susceptibilidad *in vitro* de *Staphylococcus aureus* y *Escherichia coli* a la curcumina soluble em água (CSA) associada com pH ácido y luz LED azul. La concentración inhibitoria mínima y la fotoinactivación bacteriana se realizaron utilizando diferentes concentraciones de fotosensibilizados. Para *S. aureus*, la combinación de CSA con ácido láctico e 2.5 min de tempo de iluminacción redujo la CIM de 500 µg/mL a 15.62 µg/mL, y el CSA con ácido cítrico y redujo la CIM de 125 µg/mL a 7.81 µg/mL Después de 5 min de luz de exposición. CSA sin iluminación no inhinió el crecimiento de *E. coli* (CIM > 1000 µg/mL), cuando se aplico em terapia fotodinámica (LED azul com 5 min de tempo de iluminación) CSA a 62.5 µg/mL com ácido láctico y CSA a 7.81 µg/mL com el ácido cítrico erradicó las células de *E. coli*. Los resultados obtenidos sugieren que la curcumina soluble em agua con ácidos orgânicos cuando se combina con una luz LED azul fue efectiva contra los patógenos transmitidos por los alimentos.

Palabras clave: Curcumina; Patógenos transmitidos por los alimentos; Terapia fotodnámica.

1. Introduction

Safe and nutritious foods are essential to ensure the quality of life, however, foodborne diseases represent a serious health problem, caused by pathogenic microorganisms or toxic substances in water and food (Fung et al., 2018). Foodborne diseases are capable of causing gastrointestinal symptoms, such as vomiting, diarrhea, and other more serious illnesses, which can lead to even death (World Health Organization, 2020). According to the World Health Organization (2015), it is assumed that 600 million people fall ill after eating contaminated food, and of these, 420,000 die each year.

In addition to having health consequences, food contamination affects social, environmental, and economic development; therefore, food sectors seek to adopt the culture of food safety and implement microbial control alternative methods to ensure the quality of the product offered (Fung et al., 2018; Scharff, 2015; World Health Organization, 2015; Powell et al., 2011). In fact, methodologies currently used are not always effective for microbial control and are often limited to the type of food, resulting in changes in sensory properties and in the emergence of microorganisms resistant to antibiotics or sanitizing agents, generating a health and food safety risk (Faille et al., 2018; Gutiérrez-del-Río et al., 2018; Yang et al., 2017). Thus, there is a need to explore alternative methods that guarantee a better antimicrobial activity.

For all these reasons, the number of studies seeking to find natural alternatives for food preservation is increasing (Bouarab Chibane et al., 2019; Gutiérrez-del-Río et al., 2018). Among them, curcumin, a yellow compound derived from the *Curcuma longa* rhizome, is widely used by the food industry as a food additive with coloring, flavoring, and preservative properties. Also, it is applied in the pharmaceutical industry for its anti-inflammatory and antioxidant properties (Delgado et al., 2021; Hewlings & Kalman, 2017). Curcumin and its derivatives have shown antimicrobial activity against both Gram-positive and Gram-negative bacteria, and when combined with visible light at a specific wavelength, it is photoexcited and its antimicrobial activity is enhanced by the production of reactive oxygens species (ROS), resulting in cells damage and microorganism death (Polat & Kang, 2021; Praditya et al., 2019; Adamczak et al., 2020; de Oliveira et al., 2018).

The use of curcumin is restricted because it is extremely hydrophobic and unstable (Silva et al., 2018). As result, new alternatives are sought to increase its solubilization and stability, such as formulations encapsulated in nanoparticles and water-soluble formulations, to obtain better uptake, release and antimicrobial activity compared to free curcumin (Dias et al., 2021; Gao & Matthews, 2020; Mirzahosseinipour et al., 2020; dos Santos et al., 2019; Mangolim et al., 2014). Studies also indicate the highest action of curcumin at low pH, increasing antimicrobial activity (Wang et al., 2021; de Oliveira et al., 2018). Similarly, our research group has demonstrated the greatest photoinhibition effect of curcumin in nanoparticles in acidic environments (Dias et al., 2021).

So, the present study evaluated the combination of WSC with citric and lactic acids, as they are natural food additives generally recognized as safe (GRAS) by U.S Food and Drug Administration (Food and Drug Administration, 2021). We evaluated the *in vitro* susceptibility of *Staphylococcus aureus* and *Escherichia coli* to non-irradiated and photoactivated water-soluble curcumin as photosensitizer (PS) combined with organic acids.

2. Methodology

The research was characterized as *in vitro* experiments and was developed at the Food Microbiology Laboratory of the Clinical Analysis and Biomedicine of the State University of Maringá.

2.1 Compound

Water-soluble curcumin (WSC, Natural Powder Curcumin Water Soluble) was kindly provided by IFC Solutions (Liden, NJ, USA) and obtained in partnership with Federal Technological University of Paraná (Campo Mourão *campus*).

The blue LED light system used in the assays was constructed to illuminate 96-well plates and was composed of 20 LEDs (3 W) with an irradiance of 16 mW/cm², and a wavelength of 450 nm. The absolute irradiance of LEDs was evaluated in a Spectroradiometer USB2000 + RAD (Ocean Optics, Winter Park, FL, USA) and the spectral emission was obtained using a spectrofluorometer (Varian Gary Eclipse, San Diego, United States). The maximum light dose (fluency) was 15.0 J/cm², calculated by multiplying irradiance by illumination time.

2.2 Microorganism and culture conditions

The bacterial strains used were *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 provided by the Laboratory of Food Microbiology, State University of Maringá, state of Paraná, Brazil. These strains were stored at -20ºC in Brain Heart Infusion (BHI, Difco, Le Pont-de-Claix, France) supplemented with 20% glycerol (v/v).

2.3 Determination of the minimum inhibitory concentration and the minimum bactericidal concentration

The minimum inhibitory concentration and the minimum bactericidal concentration of WSC were determined according to the Clinical and Laboratory Standards Institute (CLSI, 2020), using the broth microdilution method in 96-well microplates. WSC was serially diluted in 100 µL Mueller Hinton Broth (MHB, Difco, Le Pont-de-Claix, France) with final pH adjusted to 7.0 and 5.0 (with 1 M lactic or citric acids) at concentrations of 1.95 – 1,000 µg/mL. Standardized bacterial suspensions were prepared in 0.85% saline using a McFarland scale at a 0.5-interval and diluted 1:20, and 10 µL was inoculated in each microplate well. Microplates were incubated at 35 ºC for 24 h, and the MIC was visually determined as the lowest concentration of WSC at which bacterial growth was not observed. The positive control consisting of MHB and bacterium inoculum, and controls with lactic acid and citric acid in MHB were included. After MIC determination, 20 µL were taken from wells in which bacterial growth was not observed and inoculated onto Trypticase Soy Agar (TSA, Difco, Le Pont-

de-Claix, France), incubated at 35 ºC for 24 h. MBC was determined as the lowest concentration at which no bacterial growth was observed. The experiment was performed in triplicate with three repetitions.

2.4 Antimicrobial photodynamic therapy

Conditions for antimicrobial photodynamic therapy (aPDT) were established based on the results obtained in MIC assays and previous studies from our research group. Before assays, each bacterial strain was grown in BHI at 35 ºC overnight, centrifuged at 4,500g for 5 min, washed three times and resuspended in 0.85% saline solution. The bacterial inoculum was standardized at 10^7 CFU/mL and the aPDT was performed according to Dias et al. (2021), with some modifications. For that, 7.9 µL inoculum was mixed with 150 µL WSC solution (WSC in acidic saline pH 5.0, adjusted with lactic or citric acid) in 96-well microplates, with concentrations ranging from 3.90 to 125 µg/mL and kept in the dark for 10 min. Samples were subjected to treatments with different illumination times (2.5, 5 and 10 min). After treatment, samples were serially diluted in 0.85% saline solution, plated on TSA, and incubated at 35 ºC for 24 h. Results were expressed as log CFU/mL. Each assay was performed in duplicate with at least three repetitions.

Seven control groups were used: bacterium inoculum, no curcumin or irradiation – $WSC_{(-)}L_{(-)}$ (positive control), bacterium inoculum and WSC, no irradiation – $WSC_{(+)}L_{(-)}$ (dark control), bacterium inoculum exposed to LED – $WSC_{(-)}L_{(+)}$ (light control), bacterium inoculum in acidic saline with lactic acid, no curcumin or irradiation – $LA_{(+)}WSC_{(-)}L_{(-)}$ (lactic acid control), bacterium inoculum in acidic saline with lactic acid, no curcumin, exposed to LED – $LA_{(+)}WSC_{(-)}L_{(+)}$ (lactic acid light control), bacterium inoculum in acidic saline with citric acid, no curcumin or irradiation – $CA_{(+)}WSC_{(-)}L_{(-)}$ (citric acid control) and bacterium inoculum in acidic saline with citric acid, no curcumin, exposed to LED – $CA_{(+)}WSC_{(-)}L_{(+)}$ (citric acid light control).

2.5 Statistical analysis

Results were expressed as mean and standard deviation and data were tested by to analyses of variance (ANOVA) with 5% level of significance and compared using Tukey's test. Statistical analysis was performed using the GrandPad Prism 7.04 Software.

3. Results and Discussion

3.1 Determination of the minimum inhibitory concentration and the minimum bactericidal concentration

Values of MIC and the MBC of WSC are listed in Table 1. For *S. aureus*, the most effective activity was observed using WSC in acidic pH with citric acid (MIC 125 µg/mL). For *E. coli*, there was no growth inhibition at the highest concentration evaluated regardless of pH.

To the best of our knowledge, the antimicrobial activity of curcumin has been reported only at neutral pH. Bhawana et al. (2011) investigated the antimicrobial effect of curcumin dissolved in DMSO compared to nanocurcumin soluble in water against *S. aureus* and *E. coli*. Curcumin in DMSO showed a MIC of 150 µg/mL for *S. aureus* and 300 µg/mL for *E. coli*, while for nanocurcumin soluble in water, the MIC was 100 µg/mL for *S. aureus* and 250 µg/mL for *E. coli*. On the other hand, Adamczak et al. (2020) observed an inhibitory concentration of 250 µg/mL and 2,000 µg/mL for *S. aureus* ATCC 29213 and *E. coli* ATCC 25922, respectively. Alippilakkote and Sreejith (2018) tested a curcumin loaded poly (lactic acid) nanocapsule and reported a MIC of 468 µg/mL for *S. aureus* and 937 µg/mL for *E. coli*. When investigating the antimicrobial activity of curcumin using inhibition zone measurements, Belma et al. (2021) reported the strongest activity of curcumin solutions at a concentration of 500 µg/mL, resulting in an inhibition zone of 14.7 mm against *S. aureus* and 13.7 mm against *E. coli*.

Table 1 – Minimum inhibitory concentration and minimum bactericidal concentration for water-soluble curcumin against *Staphylococcus aureus* and *Escherichia coli.*

Species	WSC (neutral pH)		WSC and lactic acid		WSC and citric acid	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
Staphylococcus aureus	1,000	> 1,000	500	500	125	250
Escherichia coli	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000

Source: Prepared by the authors (2022).

According to Holetz (2002), the antimicrobial activity of natural compounds was classified into the following groups regarding to the MIC values: i) high antimicrobial activity: MIC < 100 µg/mL; ii) moderate activity: MIC: 100 – 500 µg/mL; iii) low activity: MIC: 500 – 1,000 µg/Ml and iv) inactive: MIC > 1,000 µg/mL. Despite the variation in biological activity found in the studies above, according to Holetz (2002), all presented a moderate antimicrobial activity of curcumin against *S. aureus*, while for *E. coli*, it demonstrated a low active or inactive activity. These findings agree with the literature available that indicated the more effective antimicrobial activity of curcumin on Gram-positive than on Gram-negative bacteria, which could be explained by the difference in the structure of bacterial cell walls (Belma et al., 2021; Bhawana et al., 2011).

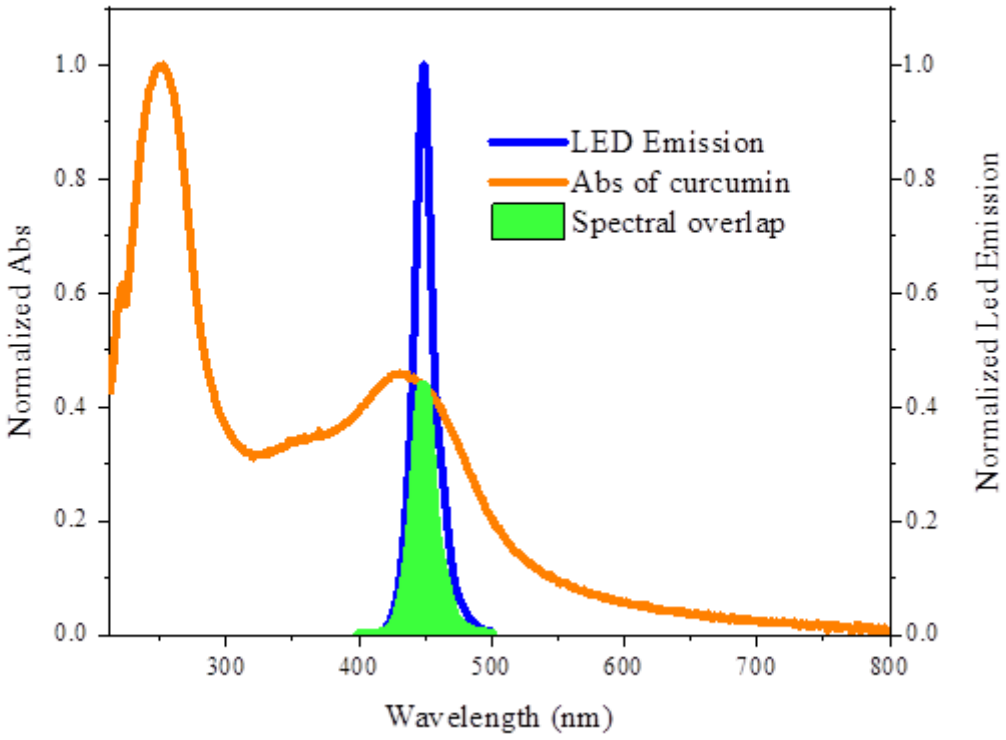
Another point that should be considered is the result obtained against *Staphylococcus aureus* when combining WSC and lactic and citric acids. Hilmi et al (2019) tested citric acid as a potential antimicrobial agent, and found that *S. aureus* was more sensitive to the citric acid treatment than *E. coli*. Although the mechanism of action is not fully elucidated, it is believed that undissociated forms of organic acids can easily penetrate the bacterial cell. And once internalized into the cytoplasm, they dissociate into protons and anions, decreasing intracellular pH and causing changes in cytoplasm and cellular metabolism (Burns et al., 2021; Kim et al., 2020; Lund et al., 2014). However, according to Burel et al. (2020), *E. coli* showed greater sensitivity to citric acid when exposed to neutral and high pH values, where acids are in their tribasic form and able to chelate a large number of divalent cations present in the membrane, causing a rupture of the cell membrane.

3.2 Antimicrobial photodynamic therapy

From the results obtained in MIC and MBC assays and considering curcumin as a potential PS, we sought to evaluate the potentiation of antimicrobial activity, combining the compound with visible blue light.

Fig 1. shows the visible absorption spectrum of WSC and the light-emitting diode power (P_{LED} emitted). The absorption maxima were consistent with literature (Priyadarsini, 2009), which shows that in polar solvents curcumin has absorption maximum at ~420 nm, and in hydrogen bond donor and acceptor solvents, around 430 – 434 nm. The power density was 16 mW/cm², as a result, light dose of 2.5, 5 and 10 J/cm² corresponded to 2.5. 5 and 10 minutes of light exposure.

Figure 1. Light-emitting diode emitted power (P_{LED} emitted) and PS electronic absorption spectra.



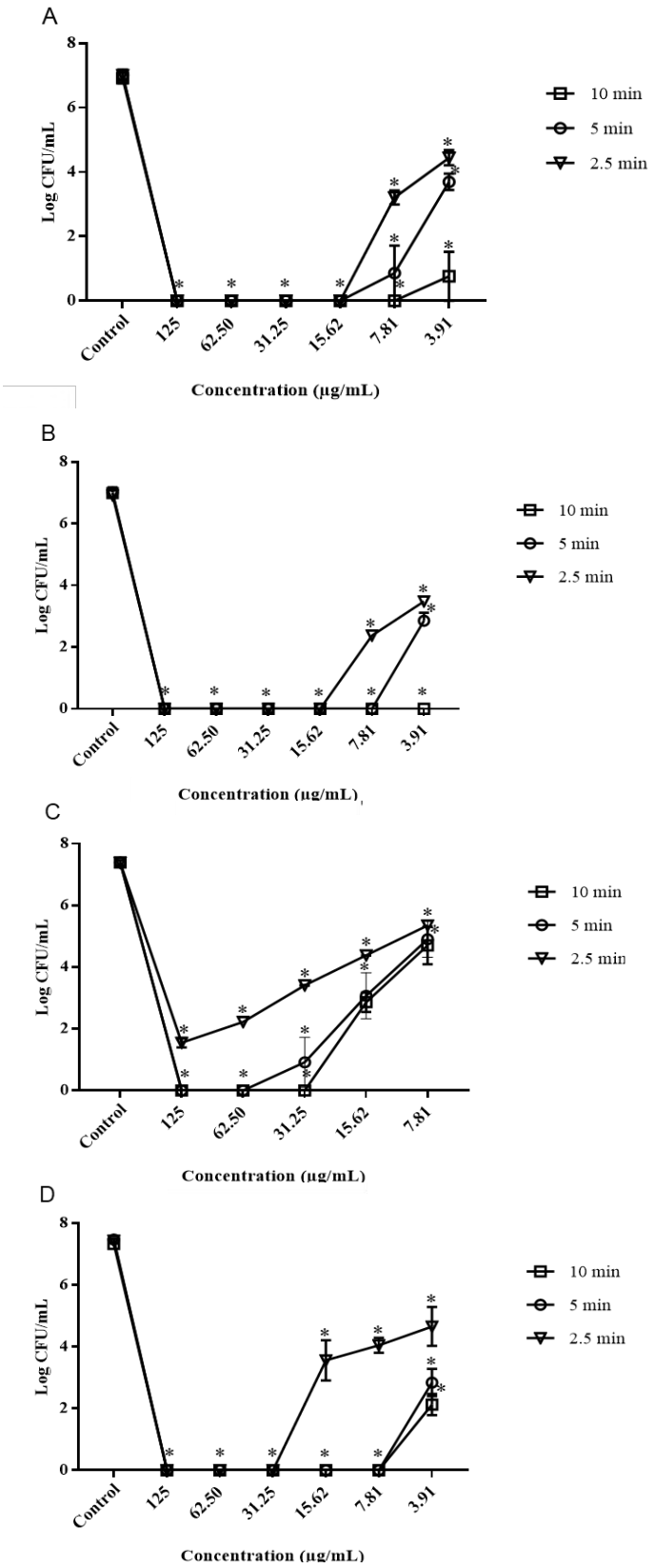
Source: Prepared by the authors (2022).

Although the efficiency of curcumin as a PS to control foodborne pathogens (Gao & Matthews, 2020; dos Santos et al., 2019; Lin et al., 2019), few studies have evaluated the effect of curcumin at different pH (Dias et al., 2021; de Oliveira et al., 2018). Furthermore, previous studies in our laboratory demonstrated a better activity of curcumin as a PS at acidic pH (Dias et al., 2021). So, in this study, we sought to investigate the potential activity of curcumin combined with organic acids (lactic and citric acids) in different photosensitizer concentrations (ranging from 3.90 to 125 µg/mL) and illumination times (2.5 - 10 min) (Figure 2).

Antimicrobial photodynamic therapy mediated by WSC was efficient in inactivating *S. aureus* and *E. coli*. The evaluated controls had counts of approximately 7 log CFU/mL, and no reduction in bacterial viability was found when compared to the positive control, indicating that the acids, light, and WSC alone do not have an antimicrobial effect.

Total inactivation of *S. aureus* was found after 10, 5 and 2.5 min of LED light exposure when concentrations of 125 – 15.62 µg/mL WSC with lactic acid were used (Figure 2A). Cell recovery occurred after 5 and 2.5 min of illumination at a WSC concentration of 7.81 µg/mL, with the growth of approximately 1.0 and 3.5 log CFU/mL, respectively. The treatment with citric acid and WSC at lower concentration (3.90 µg/mL) after 10 min of light exposure was able to lead to complete inactivation of microorganisms. Bacterial eradication was also achieved with WSC at 7.81 µg/mL at an illumination time of 5 min (Figure 2B).

Figure 2. Effect of different illumination times and WSC concentrations on the photodynamic inactivation of *Staphylococcus aureus:* (A) WSC with lactic acid and (B) WSC with citric acid; *Escherichia coli:* (C) WSC with lactic acid and (D) WSC with citric acid. The control group represents cells in saline solution. Data are presented as mean values and error bars indicate standard deviations (* $p < 0.05$).



Source: Prepared by the authors (2022).

Among the studies evaluating aPDT mediated by WSC at neutral pH as an alternative to microbial control, dos Santos et al. (2019) found a greater reduction in *S. aureus* with 750 µg/mL and 5 min LED illumination (450 nm; 32.1 mW/cm²; 10 J/cm²). In contrast, Mirzahosseinipour et al. (2020) observed no significant reduction in *S. aureus* population using nanocurcumin-silica (50 µg/mL) illuminated with a LED source for 10 min. Regarding the influence of pH, few studies demonstrated the activity of aPDT in acidic pH. Dias et al. (2021) analyzed curcumin in Pluronic® P123 nanoparticle against *S. aureus* and compared the influence of pH on antimicrobial activity. At pH 7.2, bacterial counts were reduced by approximately 1.5 – 2.0 log CFU/mL after exposure to LED for 15 and 30 min and curcumin nanoparticle at 31.25, 15.62, and 7.80 µmol/L. At pH 5.0 with hydrochloric acid, a greater antimicrobial reduction was demonstrated. Under the same conditions of illumination time and curcumin concentrations, a total inactivation of *S. aureus* was found.

Gram-negative bacteria demonstrated greater resistance to treatments. So higher concentrations of WSC (125 and 62.5 µg/mL) and exposure time to illumination (10 and 5 min) were necessary to completely inactivate *E. coli* when WSC was combined with lactic acid (Figure. 2C). Under the same conditions, WSC with citric acid at 31.25 and 15.62 µg/mL and 10 and 5 min of light exposure resulted in the complete elimination of *E. coli* (Figure 2D). De Oliveira et al (2018) compared the effect of pH on curcumin-mediated photoinactivation against *E. coli* O157:H7, and found that, at pH 3.0, the combination of UV-A light with curcumin (5 µg/mL) caused a reduction of more than 5 log CFU/mL after 2 min of illumination, while at pH 6.0, no reduction was observed.

Importantly, results demonstrated that the use of WSC as a PS was significantly more effective for inhibiting microbial growth, requiring lower concentrations when compared to MIC. For *S. aureus*, the combination of WSC with lactic acid and 2.5 min illumination time reduced the MIC from 500 µg/mL to 15.62 µg/mL, and WSC with citric acid and 5 min light exposure reduced the MIC from 125 µg/mL to 7.81 µg/mL. In turn, for *E. coli*, curcumin that previously did not inhibit bacterial growth, when exposed to 5 and 2.5 min illumination time, inhibited growth with 62.5 µg/mL WSC with lactic acid and 31.25 µg/mL with citric acid, respectively.

Photosensitive compounds, in the presence of oxygen, are excited when exposed to light, producing energy that results in the production of reactive oxygen species, such as singlet oxygen and/or hydroxyl radicals, superoxide, and hydrogen peroxide, causing a series of cytotoxic oxidative reactions and leading to an apoptotic response (do Prado-Silva et al., 2022; Wang et al., 2021; Cieplik et al., 2018).

The combination of WSC with organics acids showed good results in aPDT; citric acid promoted a greater reduction in bacterial counts compared to lactic acid. The results were consistent with Wang et al. (2021), who observed that pH lower than 5.2 with phosphoric acid-citric acid buffer could lead to an enhanced inhibitory effect of curcumin, reducing cell viability. According to de Oliveira et al. (2018), citric acid can increase the susceptibility of cells to PS and/or improve curcumin photoactivity. Furthermore, acidic pH contributes to increasing the solubility and stability of curcumin and improve cell permeability, making the cell more susceptible to aPDT (Lund et al., 2014).

The mechanism of action that improved aPDT in acidic pH has not been elucidated in the present study, but according to Lund et al. (2014), although organic acids are considered weak, causing stress to bacteria, as they are less dissociated, they freely cross the membrane causing a collapse of the pH gradient across bacteria cells. Thus, lactic acid and citric acids can disrupt the integrity of the cell wall, promoting the entry of PS into the cell (Zhu et al., 2021). Lactic acid can act by releasing lipopolysaccharides from the cell wall, compromising the integrity and exposing membrane lipids, while citric acid, being a chelating agent, removes Ca^{2+} and Mg^{2+} ions from the cell wall, increasing membrane permeability and releasing phospholipids and lipoproteins. Since lipids and proteins are the main targets of ROS during aPDT, citric acid may have potentiated aPDT, exposing these compounds (Ghate et al., 2015).

4. Conclusion

The results demonstrated that WSC combined with a blue LED light source is effective against *S. aureus* and *E. coli*, requiring lower concentrations compared to MIC. Short illumination time and low WSC concentration combined with organic acids are enough for elimination of bacterial growth, and *S. aureus* is more susceptible to aPDT. WSC mediated aPDT was most effective when combined with citric acid. Therefore, when WSC was applied in aPDT, it enables the development of a promising alternative to be applied in foodborne pathogen control.

The aPDT mediated by WSC associated with organic acids with LED light allows future research to evaluate its application in naturally acidic foods, mainly fruits.

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