Efficacy of aerosolized chlorine dioxide in reducing pathogenic bacteria on washed carrots

Jong-Lak Cho¹ · Chong-Kyung Kim¹ · Jiyong Park² · Jeongmok Kim¹

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Abstract This study aimed to evaluate the effectiveness of aerosolized chlorine dioxide (ClO_2) in reducing *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on washed carrots at various time durations and conditions. Populations of the bacteria on carrots were reduced by 1.5, 1.5, and 1.3 log CFU/g, respectively, in each inoculum after exposure to 300 ppm of aerosolized ClO_2 for 30 min. Populations were further reduced by 2.4, 2.3, and 2.1 log CFU/g, respectively, at 400 ppm, showing a positive correlation between the concentrations of ClO_2 and microbial control. The D-value was 13, 14, and 15 min for *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes*, respectively. ClO_2 residues were 1 ppm or less in all treated carrots, showing no appearance or discoloration defects. As a result, effectiveness of aerosolized ClO_2 in reducing bacterial pathogens and maintaining the quality of fresh carrots is signifying the prospects of aqueous ClO_2 as a non-thermal disinfectant.

Keywords Washed carrot · Chlorine dioxide · Antimicrobial effect · Aerosolization

Introduction

Demand for fresh produce is rising among today's health-conscious, busy consumers as they look for fresh and ready-to-eat nutritious meals. Minimally processed fruits and vegetables are peeled, trimmed, sliced, and washed

before packaging in various ready-to-eat formats. However, fresh produce represents a critical issue given that microbial contamination of fruits and vegetables may not be completely removed through the non-thermal process. That is, microbial contamination of fresh produce can pose a threat to the health of the people consuming fresh product. The most significant pathogens in minimally processed fresh produce include *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* [1–3]. Carrot is one of the most common raw vegetables and prone to microbial contamination. Carrots are consumed directly as juice or in salads, thus threatening food safety. However, pathogenic microbes on the surface of carrots after washing and bacterial biofilm formation on the surface may pose a food safety risk [4]. Non-thermal treatments including gamma ray exposure [5], UV-C exposure and immersion in ozone water [6], electrolyzed water [7], and aqueous chlorine [8] have been applied to freshly cut carrots. Among those treatments, chlorine is the most commonly used sanitizer for reducing pathogens on fresh produce. Use of chlorine solutions lead to bacterial counts of 1–2 log CFU/g or less on the surface of fruits and vegetables [9] and lose their effectiveness by reacting with organic compounds in foods, resulting in halogenated compounds [10]. Chlorine dioxide (ClO_2) has emerged as an alternative to chlorine as it has better antimicrobial effectiveness with a higher solubility, shorter response time, and wider pH range [11]. It does not react with ammonia to form chloramines. Many studies are underway to explore the applications of ClO_2 in gaseous and aqueous formations in the produce industry [12, 13]. However, regulatory permission of gaseous ClO_2 for application to food is not established. Gaseous ClO_2 cannot be stored or transported in its radical forms owing to its explosive nature, and sophisticated apparatus is required for ClO_2 gas generation.

✉ Jeongmok Kim
jmkim@mokpo.ac.kr

¹ Department of Food Engineering, Mokpo National University, Muan, Jeonnam 58554, Korea

² Department of Biotechnology, Yonsei University, Seoul 03722, Korea

Sanitizers are applied to the food industry by the spraying or dipping methods. However, these methods decrease the antimicrobial activity in fresh produce by affecting the surface conformation and substances that obstruct the sanitizer to contact the pathogens. Aerosol technology is designed to generate aerosol mist by turning liquid into finedroplets. Aerosolized sanitizers overcome the drawbacks of aqueous solutions of sanitizers [14, 15]. Aerosol technologies have been applied to medical treatment for respiratory diseases [16] and disinfection purposes in farms and hospitals [17, 18]. However, its applications to the food industry have been insignificant. This study was therefore aimed to investigate the antimicrobial effectiveness of aerosolized ClO_2 for fresh carrots.

Materials and methods

Bacterial strains and cell suspension

A streptomycin-resistant (Str^R) mutants of *E. coli* O157:H7 (ATCC 43895), *S. Typhimurium* KCTC 2515, and *L. monocytogenes* 4244 were used as experimental bacteria. Each strain was sub-cultured more than three times in tryptic soy broth (TSB, Difco, USA) on a tryptic soy agar plate containing 0.1% streptomycin (TSA-STR, Sigma, St. Louis, MO, USA) and incubated at 37 °C for 24 h. The strains showed a time-related growth pattern similar to that of the parent strain in TSB. A single colony from each Str^R strain was transferred to 15 mL TSB and incubated for 24 h at 37 °C and 100 rpm in a shaking incubator (VS-8480SF, Vision Scientific Co., Ltd., Osan, Korea). Following incubation, the culture suspension was centrifuged (VS-5000 N, Vision Scientific Co.) for 10 min at 4000 rpm. Isolated pellets were diluted in 0.1% peptone water. The culture suspension with a microbial population of approximately 10^8 – 10^9 log CFU/mL was used to inoculate the prepared carrots.

Sample preparation and inoculation

Pre-washed fresh carrots (*Daucus carota*, L) were purchased at a supermarket (Mokpo, Korea) and washed for 2 min with running water. Washed carrots were cut into pieces of the same size ($3 \times 3 \times 1 \text{ cm}^3$) with a sterile knife on a cutting board. A spot inoculation method was used to inoculate the pathogenic bacteria on carrots [19]. A 100- μL aliquot of each culture was spotted (10 droplets) with a micropipette on the surface of carrots in a biosafety hood (VS-1400, Vision Scientific Co.). Next, the carrots were placed on a sterile perforated tray and air-dried in a biosafety hood at room temperature for 30 min prior to aerosol ClO_2 treatments.

Preparation of aerosol ClO_2

A ClO_2 stock solution was prepared from sodium chlorite and phosphoric acid using a ClO_2 generator (K-CIDE, Envio Corp., Korea). This stock solution was used to prepare various working solutions (100, 200, 300, and 400 ppm) based on the content of the total available ClO_2 (TACD expressed as mg/mL ClO_2), as determined by iodometric and *N,N*-diethyl-*p*-phenylene-diamine (DPD) ferrous titration methods [20]. Both the stock and working ClO_2 solutions were freshly prepared on each day of experimentation. The ultrasonic nebulizer (MH5518, Woori-Tec Co., Korea) used in this study had an aerosol output of 80 mL/h, and an ultrasonic vibrator used in the humidifier (MH-150B, M-Tec Co., Gimhae, Korea) had a frequency of 1.6 MHz.

Treatment of inoculated carrots with aerosol ClO_2

All experiments were conducted using the plexiglass glove-box chamber ($100 \times 60 \times 80 \text{ cm}^3$). The flow of aerosol ClO_2 was controlled by a regulation valve to maintain the desired ClO_2 concentration in the treatment chamber. The aerosol ClO_2 content in the treatment chamber was constantly mixed using a fan (UF12A series, Fulltech Electric Co. Ltd., Taiwan) to maintain constant relative humidity and ClO_2 concentration during treatment. While samples were being treated in the test chamber, ClO_2 aerosol density was measured at 5 min intervals using a continuous monitoring system (InterscanTM, Interscan Corp., Chatsworth, CA, USA).

Measurement of the concentration and pH of aqueous ClO_2

After aerosol delivery by an ultrasonic nebulizer at 100, 200, 300, and 400 ppm of ClO_2 working solutions, 10-mL aerosol liquid was collected into a 50-mL tube to measure the pH (420A + , Thermo Scientific Orion) and concentration of ClO_2 . The concentration of aqueous ClO_2 was measured using the iodometric and DPD ferrous titration methods [20]. Antimicrobial effects were measured at 5, 10, and 30 min after an hour of aerosol stream flow of ClO_2 into the plexiglass glove-box chamber using an ultrasonic nebulizer at a velocity of $80 \pm 10 \text{ mL/h}$. Inoculated carrots were placed in sterile plastic containers inside the treatment chamber. Carrot samples were removed from the treatment chamber at 5, 10, and 30 min for microbial enumeration to determine the surviving cell populations.

Bacterial enumeration and D-value determination

Inoculated and treated carrots were transferred to sterile 400-mL stomacher bags (Fisher Scientific, Pittsburgh, NJ)

and stomached for 2 min with a neutralizing buffer (1:5 w/v, Difco Laboratories, Sparks, MD) in a bag mixer (Interscience Lab, Inc., Boston, USA). Serial 10-fold dilutions were prepared in 0.1% peptone water (Difco Laboratories, Sparks, MD), and then the samples were spread on TSA-STR and incubated for 24 h at 37 °C. Colonies were counted, and results were expressed as log CFU/g of carrots. A first-order kinetic model (linear model) was used to analyze each of the data value for obtaining the log of the surviving microorganisms per treatment time. The efficacy of four different concentrations, 100, 200, 300, and 400 ppm, of ClO₂ treated for 0, 5, 10, and 30 min was determined.

Aqueous ClO₂ residues and changes in chromaticity

Each ClO₂-treated carrot sample was blended with 16-mL distilled water in a 50-mL cap test tube for 2 min and filtered through a paper filter (No. 1, Advantec, Toyo Roshi Kaisha, Japan). The ClO₂ residue in the solution was measured using a pocket colorimeter (Pocket Colorimeter II Analysis System, Hach Co., USA). The chromaticity of the ClO₂-treated carrots was measured three times at different spots on the surface of the samples before and after each treatment using CR-300 (Konica Minolta Holdings Inc., Tokyo, Japan) and expressed as lightness (L), red (a), and yellow (b) values. The Hunter scale L, a, and b values based on standards of whiteness were 97.06, +0.04, and +1.84, respectively. The values of the changed colors after treatment were expressed as chromaticity difference ($\Delta E = \Delta L^2 + \Delta a^2 + \Delta b^2$). The whiteness index (WI) was indicated as $WI = 100 - [(100 - L)^2 + a^2 + b^2]^{0.5}$ by adjusting the L, a, and b values according to the Bolin and Huxsoll method [21].

Statistical analysis

The results were statistically analyzed using SPSS 19 (IBM, New York, USA). Significance levels of the difference between the experimental bacteria was evaluated by Duncan's multiple range test following the analysis of variance ($p \leq 0.05$).

Results and discussion

Preparation and purity of aqueous ClO₂

The concentration of aqueous ClO₂, comprising 8% sodium chlorite and 8% phosphoric acid, was 700 ± 20 ppm. The purity ranged between 96 and 98%, which met the FDA requirement of over 90% purity.

Concentration and pH of aerosolized ClO₂

Aqueous ClO₂ was introduced into the test chamber at 100, 200, 300, and 400 ppm using an ultrasonic nebulizer. Next, 10-mL aerosol liquid was collected into a 50-mL tube to measure the pH and concentration of ClO₂ using the iodometric and DPD methods (Table 1). The concentration of aerosolized ClO₂ was 23, 34, 45, and 61 ppm, respectively, at 100, 200, 300 and 400 ppm of ClO₂ working solution concentration, respectively, showing significant differences from baseline measurements. The significant low concentration in aerosolized ClO₂ likely resulted from the evaporation of ClO₂ during the process of particles formation in the nebulizer. The pH level was negatively correlated with the concentration of aqueous ClO₂. The pH level decreased after aerosolization, but the difference was not significant. The changes in the concentration of ClO₂ with time were measured after ClO₂ was introduced into the test chamber at a rate of 150 mL/min.

Effects of aerosolized ClO₂ on pathogenic microbes

To facilitate the differentiation of natural flora on carrots from inoculated bacteria, the Str^R mutant of pathogen was used to spike carrot samples. Fresh carrots artificially contaminated with *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* were exposed to aerosolized ClO₂ in a plexiglass glove-box chamber in which aerosolized ClO₂ was introduced at 100, 200, 300, and 400 ppm, and the air was circulated by a fan. The changes in bacterial numbers in carrots inoculated with *E. coli* O157:H7 were measured at different times after exposure to aerosolized ClO₂ (Fig. 1A). The baseline concentration was 7.8 log units before aerosolization. After exposure to aerosolized ClO₂ at 100 ppm, the total bacterial count was 7.7, 7.6, and 7.1 log, respectively, after 5, 10, and 30 min, exhibiting a negative correlation with treatment time. The negative correlation became more significant as the concentrations of aerosolized ClO₂ and time increased, showing 7.6, 7.3, and 6.8 log at the same intervals at 200 ppm, 7.5, 7.2, and 6.3 log at 300 ppm, and 7.2, 6.7, and 5.4 log at 400 ppm, respectively. The total bacterial count was reduced by 0.7, 1.0, 1.5, and 2.4 log units, respectively, at each concentration level of aerosolized ClO₂ after 30 min of treatment. When samples were treated with 400 ppm of aerosolized ClO₂ for 5 and 10 min, the total bacterial count was reduced by 0.6 and 1.1 log, respectively, showing antimicrobial effects similar to those obtained after exposure to 100 and 200 ppm aerosolized ClO₂ for 30 min. The D-value was 43, 31, 20, and 13 min, respectively, at 100, 200, 300, and 400 ppm (Table 2). The results of fresh carrots inoculated with *S. Typhimurium* are presented in Fig. 1(B). The baseline concentration was 7.8 log, which is

Table 1 Changes in the concentration and pH of chlorine dioxide before and after aerosolization

Before aerosolization		After aerosolization	
Chlorine dioxide concentration (ppm)	pH	Chlorine dioxide concentration (ppm)	pH
100	4.39 ± 0.04 ^{Aa}	23.73 ± 3.90 ^a	4.36 ± 0.01 ^{Aa}
200	4.07 ± 0.05 ^{Ab}	33.73 ± 6.75 ^b	3.87 ± 0.03 ^{Ab}
300	3.95 ± 0.03 ^{Abc}	44.97 ± 4.12 ^c	3.71 ± 0.02 ^{Abc}
400	3.71 ± 0.03 ^{Ac}	60.71 ± 7.13 ^d	3.69 ± 0.02 ^{Ac}

Mean values with different lowercase letters in the same column for pH are significantly different ($p < 0.05$)

Mean values with different uppercase letters in the same row are significantly different ($p < 0.05$)

similar to that of *E. coli* O157:H7. The total bacterial count after treating it with aerosolized ClO_2 for 5, 10, and 30 min each was 7.6, 7.6, 7.1 log, 7.6, 7.3, 6.7 log, 7.4, 7.2, 6.3 log, 7.1, 6.4, 5.5 log, respectively, at each concentration. The total bacterial count was reduced by 0.7 log at 400 ppm for 5 min, showing a microbial effect similar to that obtained at 100 ppm for 30 min. These results were identical with those of *E. coli* O157:H7. After treatment for 10 min, the bacterial population was reduced by 1.4 log. A similar reduction was also observed at 300 ppm after 30 min. However, the reduction rates were different from those of *E. coli* O157:H7 at each concentration and time. After treatment with 100, 200, 300, and ppm of aerosolized ClO_2 for 30 min, the total bacterial count was reduced by 0.7, 1.1, 1.5, and 2.3 log, respectively. This result is similar to that of *E. coli* O157:H7. The D-value was 47, 28, 21, and 13 min, respectively. The results of fresh carrots inoculated with *L. monocytogenes* are presented in Fig. 1(C). The baseline concentration was 7.5 log, which was lower than that of *E. coli* O157:H7 and *S. Typhimurium*. The total bacterial count after treating it with aerosolized ClO_2 for 5, 10, and 30 min each was 7.4, 7.3, 6.8 log, 7.3, 7.1, 6.6 log, 7.1, 6.8, 6.2 log, 7.0, 6.3, 5.4 log, respectively, at each concentration. After treatment at 100 ppm for 30 min, the bacterial population was reduced by 0.7 log, which is similar to that of *E. coli* O157:H7 and *S. Typhimurium*. After treatment at 400 ppm for 5 min, the bacterial population was reduced by 0.5 log, showing a lower reduction compared to that of *E. coli* O157:H7 and *S. Typhimurium*. After treatment for 10 min, the bacterial population was reduced by 1.2 log, showing an antimicrobial effect similar to that of *S. Typhimurium* but different from that of *E. coli* O157:H7. After treatment at 100, 200, 300, and 400 pm for 30 min, the total bacterial count was reduced by 0.7, 0.9, 1.3, and 2.3 log, respectively, exhibiting somewhat lower reductions at 200, 300, and 400 ppm than *E. coli* O157:H7 and *S. Typhimurium*. The D-value was 42, 34, 25, and 15 min, respectively. After treatment at 400 ppm for 30 min, *E. coli* O157:H7, *S. Typhimurium*, and *L.*

monocytogenes were reduced by 2.4, 2.3, and 2.1 log, respectively. This result is similar to the reduction levels of 2.47, 2.55, and 2.17 log found in lettuce inoculated with the same bacterial population and treated with 2% malic acid for 30 min [14]. However, Oh et al. [15] reported the bacterial reductions of 2.2, 3.3, and 2.7 log after treatment with peroxyacetic acid for 30 min. Thus, the microbial control for *S. Typhimurium* varies among studies. Kim et al. [22] claimed higher antimicrobial activity of smaller particle sizes after treating iceberg lettuce inoculated with the same bacteria used in this study. They aerosolized 200 ppm of sodium hypochlorite solution using vibrators with frequencies of 1.6 and 2.4 MHz. They have also reported reduction of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* of 0.2, 0.3, and 0.3 log at vibration frequency of 1.6 MHz and 1.6, 0.6, and 0.5 log at 2.4 MHz for 30 min treatment. However, Lee et al. [23] asserted a higher antimicrobial activity at 1.6 MHz against *E. coli* O157:H7 after aerosolizing five different disinfectants at vibration frequencies of 1.6 and 2.4 MHz. They suggested a higher microbial activity at 2.4 MHz against *L. monocytogenes*, concluding that no correlation was established between the sizes of aerosol particles and antimicrobial effects. Their study also revealed that hydrogen-peroxide-based disinfectants proved better performance in controlling *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* among disinfectants for commercial use. Chlorine-based disinfectants demonstrated a reduction of *L. monocytogenes* by 1.56 log and a reduction of *E. coli* O157:H7 and *S. Typhimurium* by 0.33 log or lower. ClO_2 is considered as an alternative to chlorine and an effective sanitizer for fresh produce in gaseous and aqueous formations [14]. This study also confirmed high microbial activity of aerosolized ClO_2 . Izumi [24] reported that the total microbial count of carrot slices treated with electrolyzed water (pH 6.8) containing 50 ppm chlorine for 3 min was reduced by 1.1 log₁₀CFU/g on the surface. Choi et al. [25] reported a reduction of over 3 log in a stainless steel coupon inoculated with *E. coli* O157:H7, *S. Typhimurium*,

Fig. 1 Viable cell numbers of *E. coli* O157:H7 (A), *S. Typhimurium* (B), and *L. monocytogenes* (C) on inoculated carrot surface after aerosolized chlorine dioxide treatment by an ultrasonic nebulizer for various times and concentrations

Table 2 D-values of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* on freshly cut carrot by aerosolized chlorine dioxide treatment

Chlorine dioxide concentration (ppm)	D-value (min)		
	<i>E. coli</i> O157:H7	<i>S. Typhimurium</i>	<i>L. monocytogenes</i>
100	42.6	47.2	42.4
200	30.8	28.5	34.2
300	19.6	21.3	24.7
400	13.0	14.4	14.9

and *L. monocytogenes* and treated with disinfectants containing 0.25 and 0.5% hydrogen peroxide-based disinfectants. Park et al. [26] reported bacterial reduction in range of 0.5–3.6 and 2.8–5.8 log, respectively, in their study in which stainless steel and PVC coupon with biofilm cells of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* were treated with aerosolized sodium hypochlorite and peracetic acid at 100 ppm for 50 min. Ha et al. [27] reported that bacterial population was reduced in the range of 0.3–3.8 log when stainless steel coupons inoculated with *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* were treated with aerosolized grapefruit extract and acetic acid, citric acid, and lactic acid for 5 min. They confirmed the effectiveness of the combined use of natural and chemical sanitizers in aerosol formation in the food industry. ClO_2 gas treatment needs humidity for its antimicrobial activity and therefore requires a humidifier. However, the advantage of aerosol ClO_2 is that it does not need humidifier. Relative humidity in the chamber can be generated and controlled using an ultrasonic humidifier.

Residual concentration and changes in chromaticity after exposure to aerosolized ClO_2

ClO_2 residues in fresh carrots after exposure are presented in Fig. 2. Aerosolized ClO_2 residues were not detected when samples were treated with 100 and 200 ppm aerosolized ClO_2 for 5 min. However, residues of 0.16 and 0.38 ppm, respectively, were detected at 100 ppm of aerosolized ClO_2 for 10 and 30 min and 0.22 and 0.42 ppm at 200 ppm aerosolized ClO_2 for the same treatment times. Residues of 0.12, 0.31, and 0.62 ppm were detected at 300 ppm and 0.18, 0.56, and 0.98 ppm were detected at 400 ppm. That is, ClO_2 residues were correlated with concentration and time. However, residue concentrations were below 1 ppm in all experimental bacteria. Gómez-López et al. [28] described that no residue was detected after 6 min when carrots were treated with gaseous ClO_2 of 1.33 mg/L for 30 s. Lee [29] suggested that ClO_2 residues are affected by temperature and pH and decrease with time. Thus, ClO_2 is known to be decomposed by heat and light. Changes in color are presented in Table 3. Color changes in fresh produce are an important factor affecting the quality of produce. Bleaching is defined as the color

Fig. 2 Change of residual chlorine dioxide contents in washed carrot treated with aerosolized chlorine dioxide

change induced when lignin and pigment such as β -carotene are decomposed [28, 30]. Bleaching is caused by the contamination of microbes during storage and distribution or by textural damage or dryness during the process. Moreover, a study claimed that bleaching is accelerated when the concentration of ClO_2 increases [31]. Minimally processed carrots lose their bright orange color quickly during storage, developing a white blush on its surface [21]. Cisneros-Zevallos et al. [32] reported a biopolymer-based edible coating that can control microbial growth and white blush on the surface of minimally processed carrots. However, it was found that WI decreased when carrots were treated with aerosolized ClO_2 . Low bleaching is explained by the aerosol technology in which particles stick to the surface without affecting the surface. Moreover, lightness is improved by aerosol particles just after treatment.

This study provides information for aerosolized ClO_2 applications with traditional sanitizing procedures. Application of aerosolized ClO_2 can be incorporated into the existing process during post-harvest treatment. In conclusion, the aerosolized ClO_2 treatment on carrots can be useful in controlling foodborne pathogens. In addition, different components in various types of fresh produce may influence the interaction with aerosolized ClO_2 , and other environmental factors affecting antimicrobial effectiveness need to be studied.

Table 3 Changes in the color of carrot treated with aerosolized chlorine dioxide for various times and concentrations

Concentration and time (min)	Before treatment			After treatment			ΔE	Whiteness index
	L	a	b	L	a	b		
Control								
5	53.1 ± 0.18 ^A	15.3 ± 0.23 ^A	27.8 ± 0.19 ^A	54.0 ± 0.17 ^B	15.2 ± 0.24 ^A	28.2 ± 0.15 ^A	1.07 ± 0.35 ^{bcd}	43.35 ± 0.02 ^e
10	52.9 ± 0.11 ^A	15.3 ± 0.13 ^A	27.7 ± 0.14 ^A	53.9 ± 0.12 ^B	15.1 ± 0.21 ^A	27.8 ± 0.22 ^A	1.05 ± 0.28 ^{bcd}	43.16 ± 0.19 ^e
30	52.8 ± 0.14 ^A	15.3 ± 0.23 ^A	27.7 ± 0.13 ^A	53.6 ± 0.16 ^A	15.6 ± 0.16 ^B	28.3 ± 0.23 ^B	1.07 ± 0.22 ^{cde}	43.09 ± 0.12 ^e
100								
5	53.7 ± 0.24 ^A	16.7 ± 0.10 ^A	29.7 ± 0.16 ^A	53.7 ± 0.11 ^A	16.2 ± 0.14 ^B	29.3 ± 0.13 ^A	0.64 ± 0.06 ^{abc}	42.52 ± 0.20 ^d
10	53.9 ± 0.13 ^A	16.8 ± 0.21 ^A	29.8 ± 0.17 ^A	53.7 ± 0.17 ^A	16.3 ± 0.19 ^A	29.4 ± 0.20 ^A	0.62 ± 0.35 ^{abc}	42.62 ± 0.12 ^d
30	53.8 ± 0.15 ^A	16.8 ± 0.15 ^A	29.9 ± 0.12 ^A	53.7 ± 0.16 ^A	16.3 ± 0.26 ^A	29.3 ± 0.14 ^B	0.78 ± 0.30 ^{abcd}	42.49 ± 0.19 ^d
200								
5	53.8 ± 0.13 ^A	16.8 ± 0.19 ^A	29.7 ± 0.16 ^A	53.9 ± 0.19 ^A	16.4 ± 0.20 ^A	29.4 ± 0.22 ^A	0.51 ± 0.29 ^{abc}	42.57 ± 0.13 ^d
10	53.9 ± 0.19 ^A	16.8 ± 0.21 ^A	29.9 ± 0.10 ^A	54.0 ± 0.21 ^A	16.9 ± 0.11 ^A	29.5 ± 0.26 ^A	0.51 ± 0.18 ^{abc}	42.48 ± 0.21 ^d
30	53.9 ± 0.24 ^A	16.9 ± 0.11 ^A	29.9 ± 0.23 ^A	54.0 ± 0.18 ^A	16.8 ± 0.13 ^A	29.8 ± 0.14 ^A	0.21 ± 0.23 ^a	42.45 ± 0.17 ^d
300								
5	52.8 ± 0.17 ^A	17.3 ± 0.21 ^A	29.4 ± 0.10 ^A	53.1 ± 0.29 ^A	16.9 ± 0.12 ^A	29.8 ± 0.17 ^B	0.66 ± 0.08 ^{abcd}	41.81 ± 0.12 ^e
10	52.4 ± 0.21 ^A	16.9 ± 0.19 ^A	29.1 ± 0.16 ^A	51.6 ± 0.11 ^B	16.4 ± 0.18 ^B	29.3 ± 0.23 ^A	1.31 ± 0.19 ^e	41.73 ± 0.17 ^{bc}
30	52.1 ± 0.19 ^A	16.8 ± 0.15 ^A	29.9 ± 0.13 ^A	53.1 ± 0.15 ^B	16.5 ± 0.19 ^A	29.3 ± 0.16 ^B	1.26 ± 0.21 ^{de}	41.06 ± 0.05 ^a
400								
5	51.8 ± 0.21 ^A	16.7 ± 0.20 ^B	28.9 ± 0.11 ^A	51.9 ± 0.13 ^B	16.7 ± 0.25 ^A	28.2 ± 0.17 ^B	0.64 ± 0.27 ^{abcd}	41.39 ± 0.19 ^{ab}
10	51.8 ± 0.11 ^A	16.7 ± 0.14 ^B	28.8 ± 0.23 ^B	49.9 ± 0.18 ^B	16.6 ± 0.15 ^A	28.4 ± 0.10 ^B	1.97 ± 0.28 ^f	41.44 ± 0.20 ^b
30	51.9 ± 0.13 ^A	16.7 ± 0.21 ^B	28.8 ± 0.19 ^B	52.1 ± 0.15 ^B	16.7 ± 0.25 ^A	28.9 ± 0.15 ^A	0.23 ± 0.13 ^{ab}	41.53 ± 0.20 ^{bc}

Mean values with different lowercase letters in the same column are significantly different ($p < 0.05$)Mean values with different uppercase letters in the same row are significantly different ($p < 0.05$)

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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