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Chlorine dioxide for the reduction of human pathogens in lettuce washing process

During recent years, the consumption of prepackaged ready-to-eat salads has strongly increased. To facilitate the production of microbiological safe fresh-cut products, the potential of chlorine dioxide for decontamination of iceberg lettuce during washing and its effects on external and internal quality parameters of the produce were investigated. ClO_2 application results in 5 to 6 log reduction of microorganisms in the washing water, depending on its chemical oxygen demand (COD). Colour parameters and vitamin C content of leaves were not affected by chlorine dioxide washing. Therefore, chlorine dioxide is an appropriate sanitizer to minimize human pathogens in lettuce washing water.

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Abstract
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During recent years, the popularity of and the demand for fresh minimally processed cut salads has rapidly increased. Growth rates of consumption of such products have been estimated as 10 to 20 % per year [1, 2]. Interestingly, consumers are both private households and key accounts such as canteens, hospitals and restaurants. When purchasing packed cut convenience salads, consumers' decision is normally guided by sensory criteria such as fresh and crisp appearance of the salad; even more important, however, is the microbial safety of the fresh and highly perishable minimally processed products.

Worldwide, numbers of reports on outbreaks of food-borne diseases increases. Many of them are caused by human pathogenic microorganisms on fresh vegetables and salads [3]. Most dramatic was the epidemic in Germany in 2011, caused by a Shigatoxin producing *Escherichia coli* (STEC) strain. In this case, a total of 3785 seriously ill and 45 dead people had to be registered [4].

Production processes of fresh ready-to-eat (RTE) cut salads are very simply designed. The vegetables are sorted, cut, washed, dried and packed in plastic bags. To guarantee a high product quality till the end of minimum shelf-life, the Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM, German Society of Hygiene and Microbiology) released guidance and warning limits for relevant microbial contamination of packed cut salads (**Table 1**).

Producers of RTE salads must warrant these limits until the end of minimum guaranteed shelf life; on the other hand, they cannot any longer control the produce or its storage conditions once it has left the production site. Interruptions of cool chains for these highly perishable products easily result in substantial rates of spoilage and trashing. It is assumed that up to 26 % of all avoidable and partially avoidable food waste in German households consists of vegetables [5].

The risk of microbial contamination of fresh-cut salads demands the introduction of new sustainable but also gentle sanitation techniques. During recent years, various sanitizers have been tested for their suitability as additives in the vegetable washing process. Besides the application of chemical substances such as ozonated water, chlorine, hydrogen peroxide or organic acids (e.g. citric acid, lactic acid or acetic acid) the potential of physical methods such as UV-C irradiation, pulsed light or hydrostatic high pressure has been investigated [4, 6, 7]. Nevertheless, these investigated methods were only very limited in their effectiveness [e. g. 5, 8] and/or caused product damages such as texture changes or tissue browning [1, 9, 10, 11]. In addition, the technical implementation of such techniques appears to be problematic in some cases. This may be due to particular chemical properties of sanitizers (e. g. corrosivity of ozone and acetic acid) and also other influencing factors such as temperature, water turbulences and, not at least, pollution of washing water with oxidisable substances [12, 13, 14]. These substances may largely reduce the oxidative efficiency of the sanitizers. This

Table 1

Guidance and warning levels for prepackaged mixed lettuce from the DGHM (2007)

	Richtwert/*Guidance level*	**Warnwert/*Warning level***
Aerobe mesophile Koloniezahl/*Aerobic mesophilic colony counts*	5 x 10^7 KbE g^{-1}/*CFU* g^{-1} [1)]	—
Salmonella spp.	—	Nicht nachweisbar in 25 g *not detectable in 25 g*
E. coli	1 x 10^2 KbE g^{-1}/*CFU* g^{-1}	1 x 10^3 KbE g^{-1}/*CFU* g^{-1}
L. monocytogenes	—	1 x 10^2 KbE g^{-1}/*CFU* g^{-1}
Hefen/*Yeasts*	1 x10^5 KbE g^{-1}/*CFU* g^{-1}	—
Schimmelpilze/*Moulds*	1 x 10^3 KbE g^{-1}/*CFU* g^{-1}	1 x 10^4 KbE g^{-1}/*CFU* g^{-1}

[1)] kbE: koloniebildende Einheit/*CFU: colony-forming unit.*

effect can be characterized by the so called chemical oxygen demand (COD). The influence of COD on sanitation success during washing of cut salads has not been systematically investigated and described up to now.

Hence, the aim of the present study was to test the suitability of chlorine dioxide (ClO_2) for the elimination of undesired microorganisms in the washing process of cut salads. The tests of the sanitation efficiency of ClO_2 were performed in a model washing water with predefined COD of 350 and 1 000. A COD of 350 represents an average value in the washing in a practical processing line for cut salads. On the other hand, a COD of 1 000 may reflect an incident, potentially caused by a malfunction of the fresh water supply.

At the same time, produce quality should not be impaired by the treatment. Therefore, experiments were performed to evaluate the potential of chlorine dioxide to effectively sanitize the salad washing water. To assess potential negative effects on product quality, colour measurements were conducted after ClO_2 treatment and during simulated shelf life and vitamin C content was determined in lettuce leaves.

Material and Methods

The experiments on the inactivation of relevant microorganisms were conducted in synthetic salad washing water. To produce it, the core of an iceberg salad was cut out and discarded; then, the remaining head was cut into pieces of approx. 2 cm x 2 cm and these finely puréed with a hand-held blender. The purée was pressed through a tea strainer and the obtained juice separated from the remaining solid matter by filtration (PE filter, pore size 330 µm). The COD of the filtrate was measured photometrically (spectral photometer CADAS 200, Dr. Lange, Berlin, Germany; thermostat LT 200 (at 148 °C for 2 h), cuvette test LCK 014, both Hach Lange, Berlin, Germany) and the requested COD adjusted by adding exact volumes of tab water.

Chlorine dioxide was produced from the Dr. Küke two component product (Dr. Küke GmbH, Hannover, Germany). According to instructions, 250 ml of DK-DOX aktiv (component 1) was activated by mixing it with 3.85 g DK-DOX component 2. The final product is fully activated after a reaction time of 24 h at 30 °C. ClO_2 concentrations were measured photometrically (spectral photometer DR 2800; cuvette test LCK 310, both Hach Lange, Berlin, Germany) and the requested concentration adjusted by dilution of the stock solution with tap water.

Microorganisms necessary for the study, i. e. *Escherichia coli* (DSMZ 19206), *Listeria monocytogenes* (DSMZ 20600) and *Salmonella enterica* (DSMZ 17058), were stored in cryogenic culture (Carl Roth GmbH, Karlsruhe, Germany) at -80 °C. For each test, one cryo bead was incubated in 5 ml Nutrient Broth (NB; Carl Roth GmbH, Karlsruhe, Deutschland; *E. coli* and *S. enterica*) and Brain Heart Infusion Broth (BHIB; Carl Roth GmbH, Karlsruhe, Germany; *L. monocytogenes*) at 37 °C for 24 h. Subsequently, the optical density of bacterial suspensions was measured at a wave length of 600 nm (BioPhotometer plus, Eppendorf, Hamburg, Germany). According to the optical density, the main culture was inoculated in NB or BHIB, respectively, and shaken at 170 rpm and 38 °C for 18 h to obtain bacterial suspensions of approx. 10^9 cfu ml^{-1}. Cell numbers were determined with a Multisizer™ 3 Coulter Counter® (Beckman Coulter, Krefeld, Germany) and suspensions were diluted according to the required test parameters to yield initial bacterial counts of approx. 10^6 cfu ml^{-1}. A control was always run in parallel to each treatment.

Inactivation tests were always performed in 50 ml polypropylene tubes (Falcons, Sarstedt, Nümbrecht, Germany). In the falcons, an aliquot of lettuce sap was mixed with bacterial suspension, the requested volume of ClO_2 solution ($c(ClO_2$ = 0, 10, 20 and 30 mg l^{-1}) added and the tube content thoroughly blended. After 1 and 2 min, respectively, inactivation was stopped by adding an equivalent amount of sodium thiosulfate pentahydrate (AppliChem, Darmstadt, Germany). The effect of the treatments was evaluated by determination of bacterial counts with the spread plate method. For this, serial dilutions in Ringer's solution (Merck, Darmstadt, Germany) were created of controls and treated samples, plated on nutrient agar (Carl Roth GmbH, Karlsruhe, Germany) and incubated at 37 °C for 24 h. The detection limit was 100 cfu ml^{-1}.

Surface colour was measured photometrically (CM-2600d portable spectrophotometer, Konica Minolta Inc., Tokyo, Ja-

pan) on single iceberg lettuce leaves, which were washed with aqueous ClO_2 solutions of various concentrations (10, 20 and 30 mg l^{-1}) for 1 or 2 min, respectively, rinsed with tap water and blotted dry. Subsequently, leaves were sealed in plastic bags and stored at 4 °C for up to 5 d. Colour measurement was repeated every day with 5 repetitions.

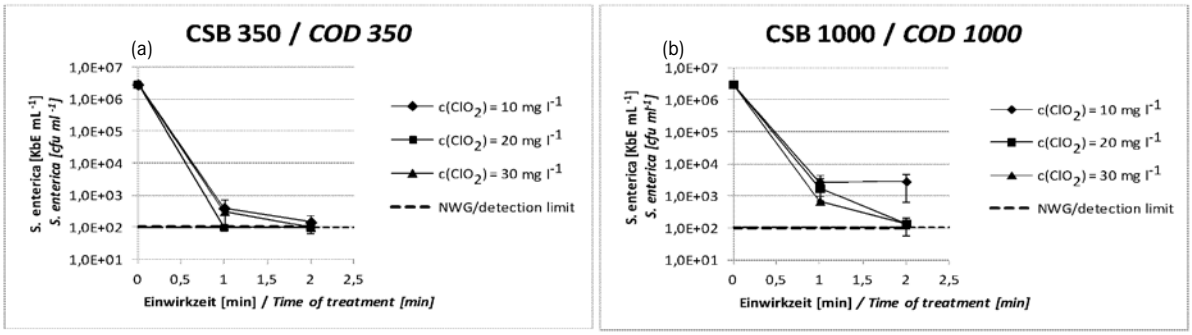
Vitamin C content of romaine lettuce leaves was measured after washing them in aqueous ClO_2 solutions ($c(ClO_2)$ = 0, 10, 20 and 30 mg l^{-1}) for 1 or 2 min, respectively, with a Reflectoquant test set (Merck KGaA, Darmstadt, Germany) according to the instructions of the manufacturer. For this, 3 to 4 leaves were combined to a mixed sample. Each leaf was cut into two halves along the midrib. The midrib was detached and discarded. One half of the leaf was used as a control while the other was treated. Samples were further cut into bite-size pieces (approx. 2 cm x 2 cm), washed with tap water or the respective ClO_2 solution. Subsequently, all samples were washed with tap water and carefully blotted dry with paper towels. Controls and treated samples were always analysed in parallel for their vitamin C content. Leaves pieces were rapidly frozen in liquid nitrogen, finely ground, the pulp filtrated with a paper filter and the resulting filtrate collected in a 2 ml reaction vial (Roth, Karlsruhe, Germany). For determination of the vitamin C contents, samples were diluted (v/v = 1 : 5) with distilled water. For each variant, three samples were analysed and measured three times each.

Results

Inactivation of microorganisms in salad washing water

The germ-killing activity of ClO_2 solution strongly depends of its COD, i.e. the contamination of the washing water with organic matters, as exemplarily shown for *S. enterica* (**Figure 1**). At a COD of 350 and a reaction time of 2 min, *S. enterica* loads of the synthetic washing water can be reduced below detection limit. In contrast, this effect is not obtainable at a COD of 1 000. Nevertheless, compared to controls, *S. enterica* loads were significantly reduced under all investigated treatment conditions. From the results it is additionally obvious that the germ-killing activity of ClO_2 solutions increases with the concentration.

Fig. 1



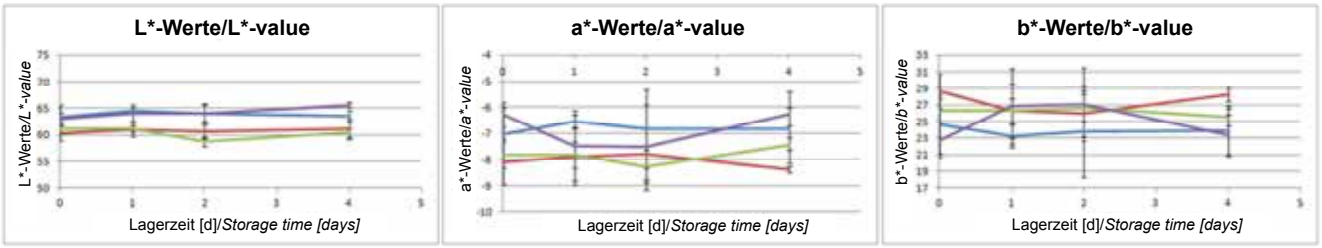
Effects of ClO_2 concentrations on S. enterica in solutions of COD 350 (a) and 1 000 (b), respectively, n = 3, means ± SD, detection limit: 10^2 CFU ml^{-1}

Table 2

Effect of ClO_2 concentrations on E. coli and L. monocytogenes in solutions of COD 350 and 1 000, respectively, n = 3, means ± SD, detection limit: 10^2 CFU ml^{-1}

$c(ClO_2)$ [mg l^{-1}]	CSB 350/*COD 350*			CSB 1 000/*COD 1 000*		
	t [min]					
	0	1	2	0	1	2
	E. coli [KbE ml^{-1}] / *E. coli [CFU ml^{-1}]*					
0	$2\ 10^6 \pm 5{,}3\ 10^5$			$2\ 10^6 \pm 4{,}5\ 10^5$		
10		$1\ 10^2 \pm 1{,}7\ 10^2$	$< 10^2$		$4{,}1\ 10^2 \pm 3{,}4\ 10^2$	$4{,}9{,}10^2 \pm 1{,}1\ 10^2$
20		$< 10^2$	$< 10^2$		$< 10^2$	$5{,}7\ 10^2 \pm 8{,}9\ 10^2$
30		$< 10^2$	$2{,}5\ 10^2 \pm 4{,}1\ 10^2$		$< 10^2$	$< 10^2$
	L. monocytogenes [KbE ml^{-1}] / *L. monocytogenes [CFU ml^{-1}]*					
0	$1{,}5\ 10^6 \pm 7{,}4{,}10^5$			$6{,}9{,}10^5 \pm 7{,}9{,}10^5$		
10		$< 10^2$	$1{,}7\ 10^2 \pm 2{,}8\ 10^2$		$< 10^2$	$1\ 10^3 \pm 1{,}7,\ 10^3$
20		$< 10^2$	$< 10^2$		$< 10^2$	$< 10^2$
30		$< 10^2$	$< 10^2$		$< 10^2$	$< 10^2$

Fig. 2



L-, a*-, b*-values of iceberg lettuce during storage after ClO_2 treatment (blue: control, red: 10 mg l^{-1} ClO_2, green: 20 mg l^{-1} ClO_2, purple: 30 mg l^{-1} ClO_2)*

Results determined for *E. coli* and *L. monocytogenes* were always very close to those obtained for *S. enterica* (**Table 2**). At a COD of 350, *E. coli* bacterial loads in the synthetic washing water could be reduced below detection limit with ClO_2 concentrations of 30 mg l^{-1} and its application for 1 min and with 10 mg l^{-1} for 2 min. One exceptional result was, however, recorded for $c(ClO_2)$ = 30 mg l^{-1} and t = 2 min. At COD of 1 000, a complete reduction below detection limit could only be achieved at ClO_2 concentrations of 30 mg l^{-1}. Irrespective of the COD investigated, ClO_2 concentrations of 20 mg l^{-1} and an application time of 1 min are enough to inactivate *L. monocytogenes* below detection limit.

Influence of ClO_2 treatments on product quality

Irrespective of the ClO_2 concentrations and the application time, treatment with ClO_2 containing washing water did not significantly affect the colour of iceberg leaves. This was also valid after 5 d of storage. Even after this time, no significant differences in lightness (L*) and in the colour parameters a* (red to green) and b* (yellow to blue) between treated leaves and controls could be monitored (**Figure 2**).

For the analysis of potential effects of ClO_2 treatments on the vitamin C content of leaves, romaine salad was used because of its higher ascorbic acid content. This could facilitate determination of ClO_2 washing-related changes in this value adding component. In leaves of iceberg lettuce, vitamin C concentration was generally low, in particular when harvested late in the season. Furthermore, pronounce natural leaf-to-leaf variability in ascorbic acid content of each salad heads must be taken into consideration for the meaningful evaluation of ClO_2 potential effects. In the presented study, even the differences between the two halves of a leaf were 8 % on average. Keeping this in mind, ClO_2 treatments in the investigated concentration range of up to 30 mg l^{-1} did not significantly affect the vitamin C content.

Conclusions

Experiments on the sanitizing efficiency of ClO_2 treatments on human pathogenic bacteria on lettuce were performed in synthetic washing water. This was essential, because sanitizing agents added to salad washing water also rapidly react with organic decontaminations [13]. This easily wastes the sanitizer, which in turn, is no longer available for hygienisation of lettuce leaves. This, in consequence, largely increases the demand of the sanitizing agents. Contaminations in washing water urgently need to be taken into account for successful produce sanitation during salad washing. The presented results indicated that ClO_2 treatments may reduce loads of all investigated bacteria below detection limit at the practically relevant COD of 350. In case of *E. coli* and *L. monocytogenes,* ClO_2 concentrations of 20 mg l^{-1} at application times of 1 min are necessary, while for *S. enterica*, 20 mg l^{-1} ClO_2 at 2 min is sufficient.

If COD is as high as 1000, the reaction time required to inactivate *E. coli* and *L. monocytogenes* below detection limit increased to 2 min. In addition, the ClO_2 concentration must be enhanced to 30 mg l^{-1} in case of *E. coli*. At this COD, loads of *S. enterica* could not be reduced below detection limit. The highest reduction (4 log units) of this bacterial load was obtained at a ClO_2 concentration of 20 mg l^{-1} and an application time of 2 min.

According to current knowledge, the addition of ClO_2 ($c(ClO_2)_{max}$ = 30 mg l^{-1}, t_{max} = 2 min) to the washing water of cut lettuce is suitable to effectively inactivate relevant human pathogenic bacteria in the water. Investigations on relevant parameters of produce quality indicated that this washing water decontamination can be achieved without negatively affecting leaf colour or vitamin C content. Nevertheless, potential treatments effects on important sensory attribute of cut salads need to be included in future investigations.

In addition, it must also be verified that ClO_2 successfully inactivates microorganisms closely attached to the produce surfaces. Furthermore, it still needs to be investigated whether ClO_2 decontamination is sustainable till the end of shelf life and whether there are any residues of the sanitizer or of its degradation products detectable on the produce. Occasionally, an additional washing step might be necessary in that case.

For future technical implementations of this treatment, the observance of worker protection requirements is of highest priority. An adequate washing line should be constructed as a closed system, being equipped with efficient warning and exhaust systems for any case of average.

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