Innovative Food Science and Emerging Technologies 72 (2021) 102745

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Optimizing the application of plasma functionalised water (PFW) for microbial safety in fresh-cut endive processing

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ARTICLE INFO

Keywords:
Food safety
Leafy greens
Microwave-driven discharge
Non-thermal processing
Ready-to-eat produce

ABSTRACT

The microbiological profiles and responses of native microflora of endive were investigated using a model process line, to establish where a defined PFW should be optimally applied to retain or improve produce microbiological quality. The PFW processes were compared with tap water and ClO_2. The antimicrobial efficacy of PFW was quantified by determining the reduction in microbial load, the microbial viability and vitality. Depending on the stage of application of PFW, up to 5 log_{10}-cycles reduction was achieved, accompanied by a reduction of metabolic activity, but not necessarily with a decrease in metabolic vitality. Multiple application (3-step-PFW-application) was more effective than single application (1-step-PFW-application) and PFW showed stronger antimicrobial effect in pre-cleaned endive. High concentrations of nitrite (315 mg l^{-1}) and nitrate (472 mg l^{-1}) in PFW were the main factors for the antimicrobial efficacy of PFW against bacteria. Furthermore, H_2O_2 and an acidic pH supported the mechanism of action against the endive microflora. These results identify the pathway to scale up successful industrial application of PFW targeting microbiological quality and safety of fresh leafy products.

Industrial relevance: The safety, quality and shelf life of freshly cut vegetables, e.g. lettuce, are strongly influenced by the microbial load. In addition, the hygienic design of production line, and a good handling/ production practice are indispensable. This study shows that the application of PFW, as a promising non-thermal sanitation technology, enables the inactivation of native microbial contamination on fresh-cut endive depending on the process stage of application. It further describes the impact of PFW on the metabolic activity and metabolic vitality of the lettuce-associated microflora. For higher acceptance, the mechanism of action of PFW was assumed based on previous chemical analyses and compared to the industrial standard of ClO_2. The results contribute to the understanding and product-specificity of PFW-induced effects on safety, quality and shelf life of fresh cut lettuce and could be a basis for a possible industrial implementation and complement of common technologies.

1. Introduction

As with many other vegetables, fresh-cut lettuce is a minimally processed product that is harvested, cut, washed, centrifuged and packaged (Baur, Klaiber, Wei, Hammes, & Carle, 2005; Odumeru et al., 1997). These activities may be associated with mechanical damage to plant tissue, which causes biochemical and physiological reactions (e.g. enzymatic browning, increased respiration and sensory and structural decay) (Saltveit, 1997). Cutting the lettuce leaves releases cell fluid that supports the growth of microorganisms. The natural microflora of fresh-cut lettuce and other vegetables is mostly characterized by Gram-negative bacteria, in particular representatives of the Pseudomonadaceae and Enterobacteriaceae (King, Magnuson, Török, & Goodman, 1991), but human pathogens such as *Listeria monocytogenes*, *Salmonella*

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https://doi.org/10.1016/j.ifset.2021.102745
Received 18 January 2021; Received in revised form 17 June 2021; Accepted 24 June 2021
Available online 26 June 2021

sp. or *Escherichia coli* O157:H7 are occasionally detected. Foodborne outbreaks and diseases may then be caused by these pathogens and may be associated with the consumption of raw fresh vegetables such as lettuce (Heaton & Jones, 2008; Lynch, Tauxe, & Hedberg, 2009). Recently, the United States Department of Agriculture (USDA) and the Centers for Diseases Control and Prevention (CDC) recalled lettuce products due to possible *Escherichia coli* O158:H7 contamination (CDC, 2019; USDA, 2019). Between November 2017 and January 2018, a large outbreak due to *E. coli* contaminated leafy greens in the USA and in Canada was recognized, including hospitalization and death (FoodSafety magazine, 2018). The European Food Safety Authority (EFSA) reported 5146 foodborne outbreaks (including waterborne ones) for 2018 with nearly 350,000 confirmed human cases (EFSA & ECDC, 2019). Among others, the outbreaks were caused by *Campylobacter* (almost 70% of all reported zoonoses), *Salmonella*, Shiga toxin-producing *E. coli* (STEC infections), *Yersinia*, and *Listeria* (highest number of hospitalization and case fatality). Thermal processes to reduce or eliminate microbial risks are not applicable to fresh-cut vegetables, so non-thermal strategies are needed to reduce microbial load and ensure safety (Chen, Zhu, Zhang, Niu, & Du, 2010). Washing the fresh-cut lettuce is used to remove field heat, dirt, microorganisms, possibly pesticide residues and cell exudates (Gil, Selma, Lopez-Galvez, & Allende, 2009; Zagory, 1999). Washing stages are therefore particularly important for the microbial safety of fresh-cut lettuce. However, the process water can also be a source of microorganisms and lead to cross contamination. Therefore, where the use is legally permitted, water additives, mostly chemical, are used to reduce the microbial load in the washing water. Chlorine-based compounds are the most common and widely used disinfectant (Ölmez & Kretzschmar, 2009; Tomas-Callejas et al., 2012). However, the use of chemicals is not permitted in the production of organic food and in conventional food processing the use of chemical disinfectants is not without concern, as they can lead e.g. in the case of ClO_2 to the formation of potentially harmful haloform by-products (chloramines, trihalomethanes) (Gopal, Coventry, Wan, Roginski, & Ajlouni, 2010). Other wash water additives or treatments already in industrial use include chemical sanitizers like ozone, hydrogen peroxide, electrolyzed water and peracetic acid, or physical treatments such as high hydrostatic pressure, pulsed electric field, oscillating magnetic field, ultra violet (UV) or gamma irradiation and high-power ultrasound (Baier et al., 2014; Biazotto Bachelli, Álvares Amaral, & Benedetti, 2013; Hägele et al., 2016; Issa-Zacharia, Kamitani, Muhimbula, & Ndabikunze, 2010; Ramos, Miller, Brandao, Teixeira, & Silva, 2013; Wulfkühler et al., 2013). The development of sustainable disinfection methods is important and challenging, but product quality compatibility, cost, environmental impact, and regulatory provisions must be met additionally (Matthews, 2006).

An innovative strategy demonstrated to reduce the microbial load of process water and on fresh cut produce is the use of plasma functionalised water (PFW) (Patange et al., 2019), The application of non-thermal plasma (NTP) generated at atmospheric pressure is a promising physical approach (Bourke, Ziuzina, Boehm, Cullen, & Keener, 2018; Schlüter et al., 2013). Plasmas are ionized gases containing neutral- and free charged particles such as ions and electrons (Kogelschatz, 2004). In food processing, the application of NTP in direct and remote mode is of interest, as these can be used to treat the food at low temperatures (<70 °C). In addition, processes carried out at atmospheric pressure are preferable in the food sector because they allow continuous process control and do not accelerate undesirable phase transitions, compared to applications at reduced pressure ($p < 1013$ mbar) or low pressure (p < 10 mbar) (Schlüter et al., 2013). PFW could be used as transport medium of reactive species and antimicrobial components for food and surface sanitation (Zhang et al., 2013). PFW is comparable to ozonized or chlorinated water with regard to the mode and stages of application as well as antimicrobial effects. The chemical composition of the PFW used within this work was previously characterized by Schnabel et al., 2021a concerning the acidic pH, and the reactive oxygen and nitrogen species (RONS) (Schnabel et al., 2021a). Both low pH and RONS are known to support and to cause the antimicrobial mechanisms of action, therefore the chemical composition of PFW should be known and controllable if PFW is to be approved as a process wash water. In the present study, PFW is investigated with respect to its antimicrobial efficacy, mechanisms of action, and its suitability as a wash water for fresh-cut lettuce. This works investigates where a PFW should be applied to optimally address the critical parameter of antimicrobial efficacy. Therefore, the PFW efficacy is determined with respect to the stage of application within a fresh cut endive processing line to ascertain where this alternative strategy is best applied for retention of microbiological quality and safety.

2. Material and methods

2.1. Plasma source PLexc^2

The PLexc^2 plasma source is a two-stage microwave-driven device (2.45 GHz), operated at atmospheric pressure. The single stage plasma torch PLexc, which was used as the technical basis, is described in detail elsewhere (Ehlbeck et al., 2011; Krohmann, Neumann, & Ehlbeck, 2005). The supply of additional microwave energy in the second plasma stage leads to a re-ignition of the effluent of the first stage to an active plasma, which processes the second gas supply. In Fig. 1 the two plasma stages are schematically illustrated. The technical parameters for the plasma device used are given in Table 1.

2.2. Generation of PFW and its chemical characterization

As described in our previous work (Schnabel et al., 2021a), the plasma was used to generate plasma-processed air (PPA). Subsequently PPA was used to functionalise distilled water. This leads to the formation of PFW. In brief, the distilled water was treated with PPA in a tumbler (diameter of 700 mm) in order to have the possibility to treat from 1 l up to a maximum of 20 l of water simultaneously. Instead of distilled water, which is preferred for scientific analyses, the use of tap water is also possible (Schnabel et al., 2019).

The consistently used plasma parameters are in Table 1. The production rate of PFW by PPA is 60 l per hour if the water used has a pH of 7–8 and the applied PFW should achieve a pH < 2, which is in corre-

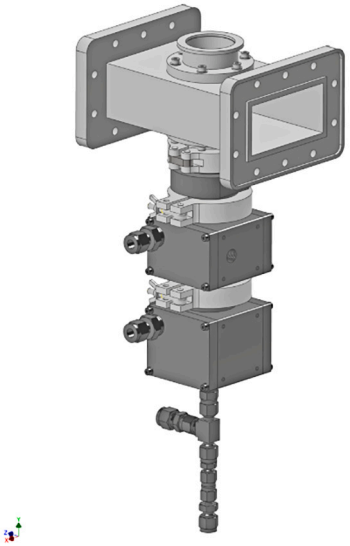

Fig. 1. Schematic diagram of the microwave plasma sources PLexc^2.

Table 1
Technical operating parameters for the experiments.

Technical parameters	
Power (P)	4.3 kW
Frequency (f)	2.45 GHz
Volume flowrate (Γ)	72 slm
gas	air

lation to high RNS concentrations. The contact surface is kept stable and defined by the tumbler diameter and the rotation speed of 12 rpm with 0.44 m s^{-1} at an angle of 30°.

The chemical composition of PPA and PFW was characterized in detail by emission spectroscopy (ES, plasma fingerprint), Fourier transform infrared spectroscopy (FTIR, process gas composition), chronoamperometry (CA, hydrogen peroxide detection) (Pipa, Andrasch, Rackow, Ehlbeck, & Weltmann, 2012; Schnabel et al., 2021a), and ion chromatography (IC, nitrite and nitrate detection). In the ES analysis of the microwave-induced plasma, nitrogen monoxide radical (·NO), nitronium cation (NO_2^+) and hydroxide anion (OH^-) were detected as the main emitting species. In the FTIR analysis, the main detected components of PPA beside nitrogen (N_2) and oxygen (O_2) were nitrogen monoxide radicals (·NO), nitrogen dioxide radicals (·NO_2) and water (H_2O). Chronoamperometry identified H_2O_2 in the PFW. Finally, the IC measurements identified high values for NO_2^- and NO_3^- by anion separation. All measured concentrations of RONS are given in Table 2.

As the storability of PFW may play an important role for industrial application, e.g. high amounts of PFW requested in very short times, the duration of the retention of the antibacterial effect of the PFW generated using this system was investigated. The high antibacterial effects on *Pseudomonas fluorescens* ATCC 13525 were observed for 48 h minimum post generation, where the PFW was kept at 10 °C (Weihe et al., 2021). Longer storage terms may be achievable by cooling or freezing (Tsoukou, Bourke, & Boehm, 2020) but this is also likely dependent on specific chemistry and should be confirmed for this PTW formulation.

2.3. Processing of fresh-cut endive

The endive (*Cichorium endivia* L.) was bought at a local organic market in Greifswald, Germany. Subsequently, the whole endive heads were stored for a maximum of 24 h at 7 ± 0.1 °C, and cut directly before experimental use. The outermost leaves were removed, but the stalk was retained. Both, the softer leaf parts and the harder stalk parts of the lettuce were mixed to provide representative samples. The processing of the fresh-cut endive was performed on a washing line mimicking a common industrial production process. To test different process variants, the washing line ultimately consisted of up to five main sections – pre-bathing, pre-rinsing, pre-washing, main washing and post-rinsing. The investigated process variants are given in Table 3 and as an example, the reference with tap water is illustrated in Fig. 2. After the last washing step, the samples were briefly stored over a sieve for draining, but were not spun.

Table 2
Detected RONS concentration in PPA and PFW by FTIR, chronoamperometry and IC analysis.

RONS concentration in PPA and PFW	
PPA	
NO	2900 ppm (7.79 10^{22} m^{-3})
NO_2	76 ppm (2.04 10^{21} m^{-3})
H_2O	9200 ppm (2.47 10^{23} m^{-3})
PFW	
NO_2^-	315.83 mg l^{-1}
NO_3^-	472.8 mg l^{-1}
H_2O_2	5.61 mg l^{-1} (29.39 mM)

2.4. Characterizing the antimicrobial efficacy and mechanisms of PFW

2.4.1. Antimicrobial efficacy (CFU-assay)

To determine the natural contamination on the fresh-cut endives before and after treatment, the samples were homogenized in bags with 40 ml homogenizer solution (0.85% NaCl, 10% Tween80 and 10 mg l^{-1} sodium thiosulfate, all chemicals from Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for 30 s at a speed of 400 rpm. This suspension was used to prepare a decimal dilution series in maximum recovery diluent (MRD). Volumes of 100 µl aliquot were used for surface-spread-plate counts using Plate Count Agar (Roth GmbH, Karlsruhe, Germany), and plates were incubated for 24 h at 30 °C under aerobic conditions. The detection limit of this method was 10 cfu·ml^{-1}.

2.4.1.1. Calculation of reduction factor and standard deviation of reduction factor. For fresh-cut lettuce specimens the reduction (R) and reduction factor (RF) were calculated as follows:

$$R = \frac{n_{MO}^{Ref}}{n_{MO}^{Sam}} \tag{1}$$

n_{MO}^{Ref} : *concentration of microorganismn of the reference*

n_{MO}^{Sam} : *concentration of microorganismn of the treated sample*

$$RF = log_{10}(R) \tag{2}$$

The standard deviation of the reduction (ΔR) was calculated as follows:

$$\Delta R = \sqrt{\left(\frac{\partial R}{\partial n_{MO}^{Ref}} \cdot \Delta n_{MO}^{Ref}\right)^2 + \left(\frac{\partial R}{\partial n_{MO}^{Sam}} \cdot \Delta n_{MO}^{Sam}(i)\right)^2} \tag{3}$$

$$\frac{\partial R}{\partial n_{MO}^{Ref}} = \frac{1}{n_{MO}^{Sam}(i)} \tag{4}$$

$$\frac{\partial R}{\partial n_{MO}^{Sam}} = -\frac{n_{MO}^{Ref}}{n_{MO}^{Sam}(i)^2} \tag{5}$$

$$\Delta R = \sqrt{\left(\frac{1}{n_{MO}^{Sam}(i)} \cdot \Delta n_{MO}^{Ref}\right)^2 + \left(\frac{n_{MO}^{Ref}}{n_{MO}^{Sam}(i)^2} \cdot \Delta n_{MO}^{Sam}(i)\right)^2} \tag{6}$$

Δn_{MO}^{Ref} : *error of reference*

$\Delta n_{MO}^{Sam}(i)$: *error of sample i*

ΔR : *error of reduction*

2.4.2. Metabolic activity (XTT-assay)

The XTT assay was used to determine cell viability before and after treatment (AppliChem, St. Louis, USA) according to manufacturer instructions. This assay demonstrates cell viability as a function of the redox potential resulting from electron transport through the transplasmic membrane (Scudiero et al., 1988). The sample was the same suspension that was used for the CFU- and the fluorescence-assay. The 96-well plate was incubated at 37 °C with continuous horizontal shaking (80 rpm) overnight (16–20 h) in the dark. The 96-well plate was then scanned at a wavelength of 470 nm using the Varioskan Flash® device (Fisher Scientific GmbH, Schwerte, Germany). The values obtained were blank corrected with XTT and activation solution mixture without sample, scanned at a wavelength of 670 nm. The experiments were repeated three times with $n = 3$, resulting in $n = 9$.

2.4.3. Vitality (Fluorescence-assay)

The LIVE/DEAD BacLight™ Bacterial Viability Kit (Fisher Scientific

Table 3
The investigated process variants and the stage of application of PFW in a model process line. NA is used for "not analysed". Colours are used for better visualization.

Process variant	*Unwashed (0)*	*Pre-bathing (1)*	*Pre-rinsing (2)*	*Pre-washing (3)*	*Main washing (4)*	*Post-rinsing (5)*
Duration of washing [s]	0	180	30	180	180	30
tap-water (A)	NA	water/ NA	water	water	water	water
PFW (B)	NA	PFW	water	water	water	water
PFW (C)	NA	NA	PFW	water	water	water
PFW (D)	NA	NA	water	water	PFW	water
PFW (E)	NA	PFW	PFW	water	PFW	water
ClO_2 (F)	NA	NA	water	water	ClO_2	water

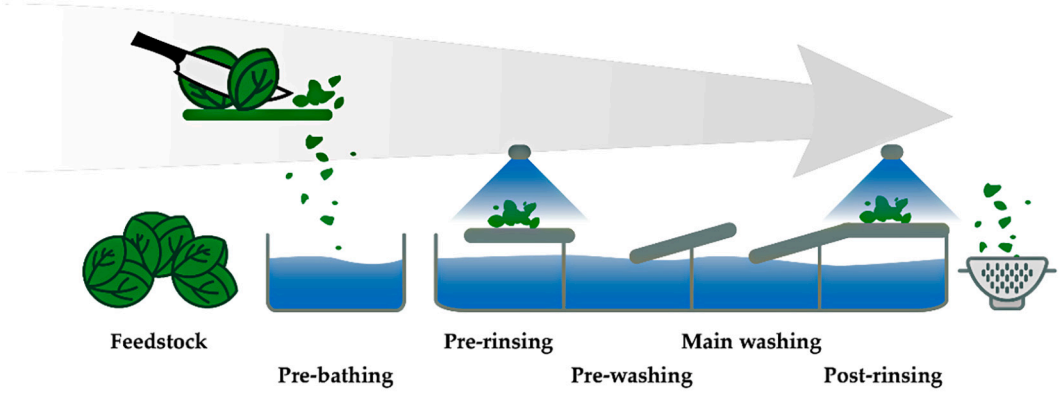

Fig. 2. The model process line employed for tap water process variant (adapted from Schnabel et al., 2021).

GmbH, Schwerte, Germany) was used according to the manufacturer instructions. Briefly, 0.9 µl of the dye mixture was added to 300 µl of the suspension, and then placed on a rotary shaker in the dark at room temperature for 20 min. Using a fluorescence microplate reader (Varioskan-Flash®, Fisher Scientific GmbH, Schwerte, Germany) the fluorescence of each well of a 96-well plate was determined using an excitation wavelength of 470 nm and an emission wavelength of 530 nm and 630 nm for green (G) and red (R) fluorescence respectively. A G/R ratio was then calculated by dividing the intensity value of the green fluorescence by the value of the red fluorescence. The experiments were repeated three times with $n = 3$, resulting in $n = 9$.

2.5. Nitrite and nitrate concentration in the washing water analysed by IC measurements

At each step, where PFW was applied, it was freshly prepared, used and not reused. To determine the nitrite and nitrate concentration in the washing water (tap water and PFW) before and after fresh-cut lettuce application, ion chromatography measurements were done.

Measurements were carried out using a Dionex ICS 6000 system (Thermo Scientific, Dreieich, Germany) with a conductivity detector. The system was controlled by Dionex Chromeleon Version 7.1.3.1541. Separation of the ions was performed via an anion-exchange column (Dionex IonPac AS 18, Thermo Scientific, Dreieich, Germany) with a guard column (Dionex IonPac AG 18, Thermo Scientific, Dreieich, Germany). Ultrapure water (GenPure Pro Barnstead, Thermo Fisher Scientific, Dreieich, Germany) in combination with a 23 mM KOH cartridge (Thermo Scientific, Dreieich, Germany) was used as the eluent.

Injection of samples was realized by a partial loop injection method with a volume of 5 µl. Each sample was diluted 1:25, except the tap water reference which was diluted 1:10. The runtime for each measurement was 20 min with an eluent flow of 0.25 ml min^{-1}. Samples were stored in the autosampler at 10 °C (± 5 °C). The experiments were repeated three times with n = 3, resulting in n = 9.

The identification and quantification was based on the Dionex 7-ion standard solution (Thermo Scientific, Dreieich, Germany), diluted with ultrapure water in samples of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128. The concentrations of nitrite and nitrate were calculated by the usage of the linear regression function of the measured standards (7).

$$c = \frac{(AUC \pm y_{intercept})}{x_{slope}} \quad (7)$$

Where *AUC* is the integrated area under the conductivity peak over the retention time of the detected anion, x_{slope} is the slope of the regression function and $y_{intercept}$ is the interception of the y-axis of the regression function.

In these experiments only the process variants A and E (Table 3) were examined, to provide insight of the possible depletion of antimicrobial RNS – nitrite and nitrate – by interaction with the fresh-cut lettuce at the specific washing steps.

3. Results

3.1. Antimicrobial efficacy

In Fig. 3 the reduction factor (RF) by which the population density is reduced, has been quantified. The RF is the number of $\log_{10}$-cycles that the microbial population declines. Washing fresh-cut endive with tap water provided a reduction of nearly 2 $\log_{10}$-cycles (Fig. 3a to e, black triangles; Table 3, variant A). When fresh-cut endive was washed with PFW, the dynamic of the inactivation curves changed. In variant B (Fig. 3a, red dots), when PFW was used at the first extraction point, the RF was greater than 2 and remained above this value until the last extraction point, even though the lettuce was washed with tap water from then onwards. The survival of the native microflora of the lettuce was negatively affected by the PFW.

Fig. 3b shows the results for variant C. Here, PFW was used at the second extraction point (pre-rinsing), and again, the RF was above 2. However, the RF declined faster than in variant B (Fig. 3a) to a value close to 1 in the following washing stages, which may be due to the shorter treatment time of 30 s with PFW and to the pre-rinsing stage of application instead of pre-bathing. There was a significantly different kinetic in response to PFW application at the fourth washing stage (main washing) presented as process variant D (Fig. 3c), where the RF increased to more than 3 and decreased only slightly below 3 at the post-rinsing washing stage. This significantly higher RF achieved for variant D (Fig. 3c) compared to variants B (Fig. 3a) and C (Fig. 3b) could be explained by the use of the washing stages 2 and 3 prior to the application of the antimicrobial PFW. Washing stages 2 and 3 may clean and loosen dirt particles, which then no longer interfere with the mechanism of the antimicrobial substances present in the PFW. In variant E (Fig. 3d), PFW was used across washing stages 1, 2 and 4, which led to very high reductions between 4 and 5 $\log_{10}$ cycles. The antimicrobial effect of ClO_2 with a concentration of 15 ppm and when applied at washing stage 4 as per industrial practice, (Fig. 3e, variant F) showed a reduction comparable with tap water.

3.2. Metabolic activity

The results of the XTT assay, which assesses the cell viability as a function of the redox potential, are presented in Fig. 4.

The absorption of formazan dye at 470 nm is given as a percentage of the reference (extraction point 0 (unwashed) in Table 3) over the extraction points for the washing process variants. Metabolically active cells can reduce the tetrazolium salt XTT to the orange colored compound formazan. The higher the percentage, the higher the metabolic activity retained in the membranes of the microbial cells obtained from

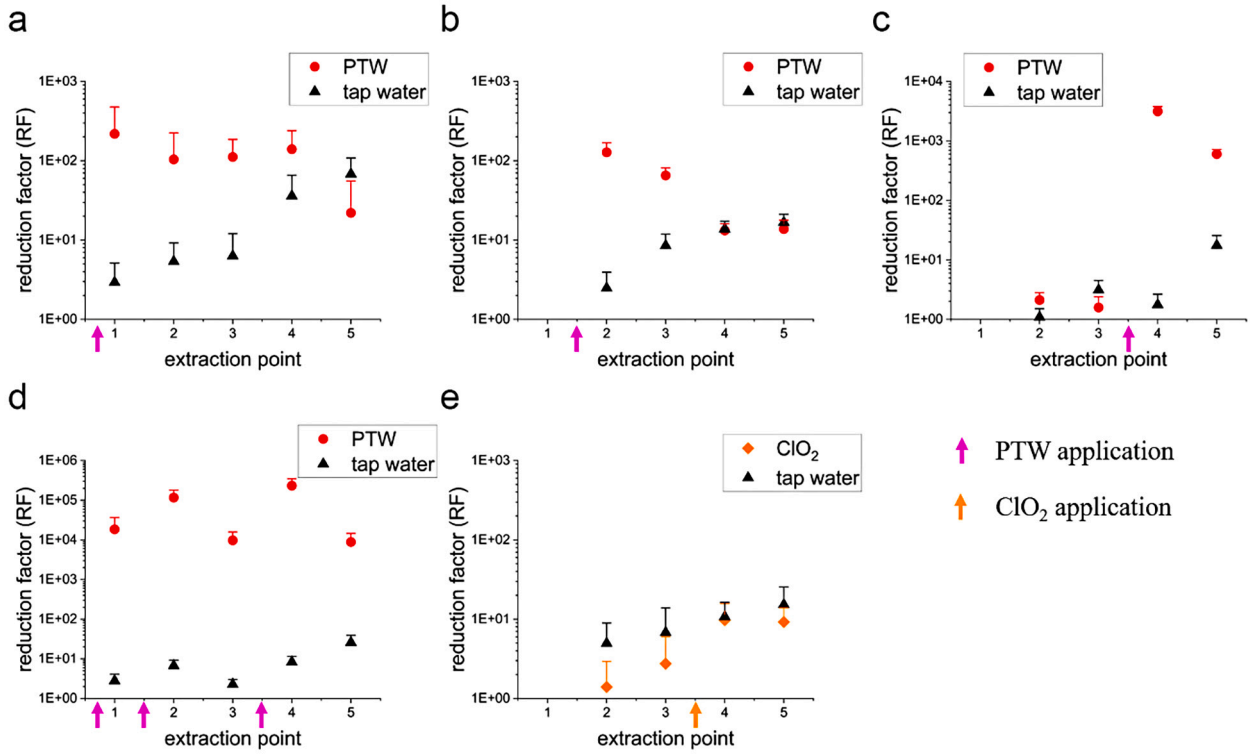

Fig. 3. Antimicrobial efficacy – reduction factor - of the process variants applied. All RFs are related to the unwashed lettuce as the reference point. a) In red – variant B: PFW at extraction point 1 (180 s pre-bathing), extraction points 2 to 5 with tap water; in black – tap water used at extraction points 1 to 5. b) In red – variant C: PFW at extraction point 2 (30 s pre-rinsing), extraction points 3 to 5 with tap water; in black – tap water used at extraction points 2 to 5. c) In red – variant D: PFW at extraction point 4 (180 s main washing), extraction points 2, 3 and 5 with tap water; in black – tap water used at extraction points 2 to 5. d) In red – variant E: PFW at extraction point 1, 2 and 4 (180 s pre-bathing, 30 s pre-rinsing, 180 s main washing), extraction points 3 and 5 with tap water; in black – tap water used at extraction points 1 to 5. e) In orange – variant F: ClO_2 at concentration of 15 ppm at extraction point 4 (180 s main washing), extraction points 2, 3 and 5 with tap water; in black – tap water used at extraction points 2 to 5. The initial concentration was 10^6 to 10^7cfu g^{-1}. All experiments were repeated threefold with $n = 3$ resulting in $n = 9$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

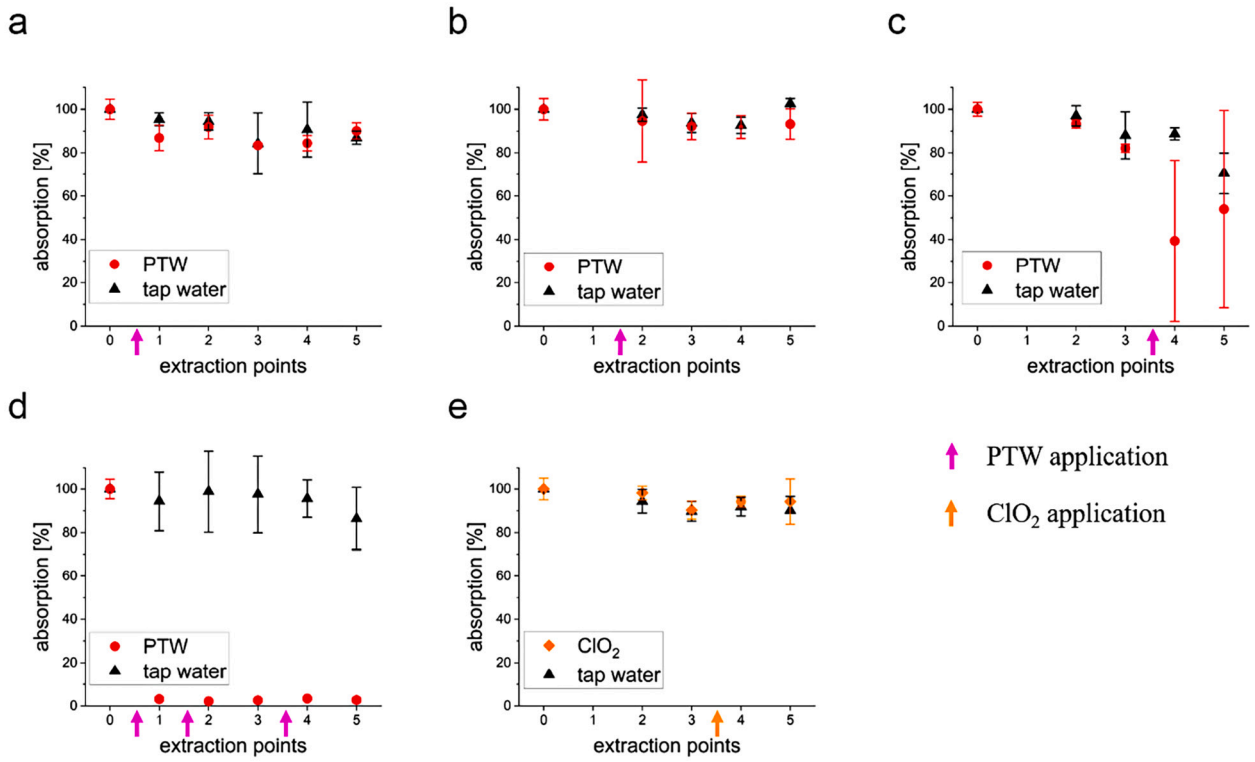

Fig. 4. The effect of process variant on the metabolic activity using the XTT assay. All data points are related to the unwashed lettuce (reference, extraction point 0). a) In red – variant B: PFW at extraction point 1 (180 s pre-bathing), extraction points 2 to 5 with tap water; in black – tap water (variant A) used at extraction points 1 to 5. b) In red – variant C: PFW at extraction point 2 (30 s pre-rinsing), extraction points 3 to 5 with tap water; in black – tap water used at extraction points 2 to 5. c) In red – variant D: PFW at extraction point 4 (180 s main washing), extraction points 2, 3 and 5 with tap water; in black – tap water used at extraction points 2 to 5. d) In red – variant E: PFW at extraction point 1, 2 and 4 (180 s pre-bathing, 30 s pre-rinsing, 180 s main washing), extraction points 3 and 5 with tap water; in black – tap water used at extraction points 1 to 5. e) In orange – variant: ClO_2 at concentration of 15 ppm at extraction point 4 (180 s main washing), extraction points 2, 3 and 5 with tap water; in black – tap water used at extraction points 2 to 5. All experiments were repeated threefold with $n = 3$ resulting in $n = 9$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the lettuce. The data for the extraction point 0 showed the metabolic activity for the unwashed lettuce as 100%. Introducing PFW at the different stages within process variants B, C and F (Fig. 4a, b and e) had no difference in the effect on the metabolic activity by comparison with that recorded for tap water, PFW or ClO_2 treatments of washed fresh-cut lettuce at all extraction points. However, using PFW at the main washing step - variant D (Fig. 4c) affected the metabolic activity of the microorganisms, demonstrated by the decrease to 40% metabolic activity at extraction points 4 and 5 in variant D. Variant E (Fig. 4d) where PFW was used at three process stages, showed an extremely steep decrease in the metabolic cell activity to approx. 3% for all extraction points.

3.3. Vitality assay

With regard to the effect of the stage of application of PFW on microbial vitality, in most of the process variants, there was no difference observed between the cell envelope of microorganisms washed with tap water, PFW or ClO_2 (Fig. 5).

The results obtained showed less differences than those observed for the antimicrobial activity (CFU, Fig. 3) and metabolic activity (XTT, Fig. 4). For variants B, C and F (Fig. 5a, b and e), the data points lead to the conclusion that the cell membrane was severely damaged. However, for each of these variants, a normal metabolic activity was detected in the XTT assay and in the proliferation assay (CFU); only a reduction of 1 to 2 $\log_{10}$-cycles was detected for the same data points.

In contrast, there was a very high reduction of 3 to 5 $\log_{10}$-cycles for the variants D and E (Fig. 3c and d) in the proliferation assay and a strong reduction of metabolic activity (Fig. 4c and d) was shown. However, the vitality of remaining populations showed small differences (Fig. 5c and d) to the variants B, C and F (Fig. 5a, b and e), as well as for tap water.

3.4. Nitrite and nitrate concentration in the washing water (tap water or PFW) before and after application

Table 4 depicts the average nitrite and nitrate concentrations at each extraction point for process variant A and E (Table 3). All extractions from process variant A contained detectable nitrate, but nitrite was undetectable. This was expected as only tap water was used for washing and the tap water reference also showed only nitrate anions. However, an increase in the nitrate concentrations in for all extraction points from process variant A was seen compared with the tap water reference. This may be caused by the leaching of nitrate of the fresh-cut washed lettuce.

Regarding process variant E, the nitrate concentrations for the pre-bathing, pre-rinsing and main washing are within the standard deviation of the PFW reference; no significant influence of the lettuce itself is noted. For the pre-washing and post-rinsing of process variant E an increase of the nitrate and nitrite concentration in contrast to the tap water reference was recorded. These increases are most likely caused by carry-over effects of the prior PFW-washing-step, since these steps contained PFW with higher nitrite and nitrate concentrations.

4. Discussion

This study sought to compare PFW with standard washing treatments for fresh cut produce, and also to determine at which stage or stages in the washing process PFW can be optimally used to maximise effects prior to packaging. A comparison of the application of PFW, i.e. whether

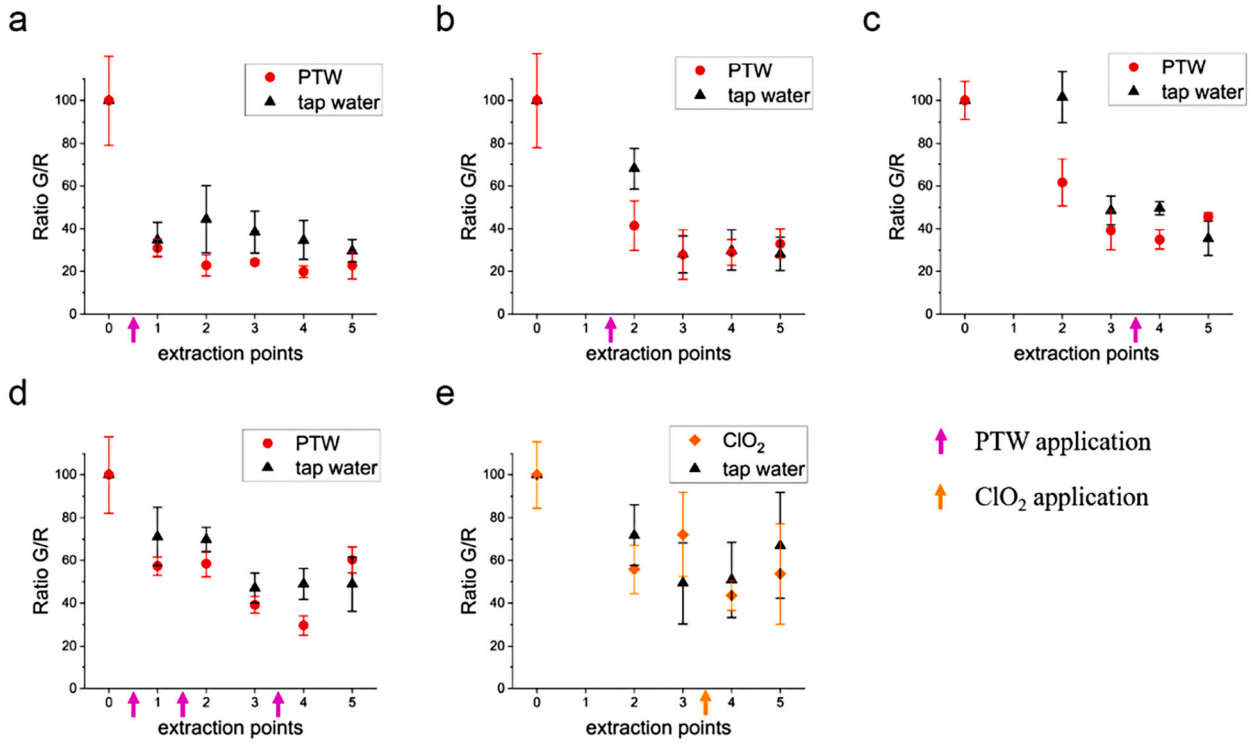

Fig. 5. The effect of PFW unit processes on the microbial vitality of the native microflora of endive. All data points are related to the unwashed lettuce (reference, extraction point 0). a) In red – variant B: PFW at extraction point 1 (180 s pre-bathing), extraction points 2 to 5 with tap water; in black – tap water (variant A) used at extraction points 1 to 5. b) In red – variant C: PFW at extraction point 2 (30 s pre-rinsing), extraction points 3 to 5 with tap water; in black – tap water used at extraction points 2 to 5. c) In red – variant D: PFW at extraction point 4 (180 s main washing), extraction points 2, 3 and 5 with tap water; in black – tap water used at extraction points 2 to 5. d) In red – variant E: PFW at extraction point 1, 2 and 4 (180 s pre-bathing, 30 s pre-rinsing, 180 s main washing), extraction points 3 and 5 with tap water; in black – tap water used at extraction points 1 to 5. e) In orange – variant: ClO_2 at concentration of 15 ppm at extraction point 4 (180 s main washing), extraction points 2, 3 and 5 with tap water; in black – tap water used at extraction points 2 to 5. All experiments were repeated threefold with n = 3 resulting in n = 9. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
The average concentrations of nitrite and nitrate in mg l^{-1} of each extraction point detected via IC. ND is used for "not detected".

Process variant	Extraction point	c $_{NO2}$ [mg l^{-1}]	c $_{NO3}$ [mg l^{-1}]
A	Pre-bathing	ND	71.1996 (±1.9342)
	Pre-rinsing	ND	69.7848 (± 2.8053)
	Pre-washing	ND	70.4478 (± 0.5705)
	Main washing	ND	72.9161 (± 5.2735)
	Post-rinsing	ND	68.5368 (± 2.9195)
E	Pre-bathing	241.1622 (± 72.9661)	466.3155 (± 27.5796)
	Pre-rinsing	160.3129 (± 50.0431)	482.6958 (± 24.3364)
	Pre-washing	15.8799 (± 4.6234)	123.9002 (± 3.7866)
	Main washing	218.2333 (± 57.6505)	420.7843 (± 19.0138)
	Post-rinsing	43.4312 (± 7.4343)	161.8436 (± 24.2576)
Reference (100% tap water)		ND	58.9111 (± 3.1769)
Reference (100% PFW)		315.8344 (± 95.0439)	472.8019 (± 34.6399)

it is nebulized in the pre-rinsing stage (variant C) or as a bath in the pre- and main washing stages (variants B and E), shows that the higher wetting of the lettuce surface during bathing is advantageous compared to the lower wetting during rinsing. However, the exposure time during rinsing was also significantly lower due to the experimental setup. In a comparison of variants B and D (washing with PFW, same exposure time), it can be concluded that a pre-cleaned lettuce washed with tap water (variant D) allows a better antimicrobial effect of PFW than if the fresh-cut lettuce is still contaminated (variant B). If PFW is used multiple times in the washing process (variant E), the antimicrobial effect is increased compared to a single application (variants B, C, D) and the reduction achieved is retained at this very high level up to the final product. In addition, the multiple or late use of PFW in the washing process (variants D and E) not only had an effect on the reduction of the total bacterial count, but also on the metabolism and the vitality of the microorganisms remaining on the lettuce. This can have a positive effect on the shelf life of the fresh-cut lettuce. Our analyses show how and where PFW can be optimally applied in the washing process of fresh-cut lettuce, depending on industrial conditions. However, challenging the optimized protocols for reduction of representative pathogens and their subsequent behaviour over extended storage life is required. The composition of PPA influences the chemical characteristics of PFW and thus its antimicrobial properties. Knowing the chemical composition of PPA and PFW allows conclusions to be drawn about the antimicrobial effect of PFW as a process water against produce contaminants and where PFW may be optimal. The chemical analyses of PFW (Schnabel et al., 2021a) showed a strong acidification, which can support important antimicrobial chemical reactions in bacterial cells. The main compounds detected in PFW were NO_2^- and NO_3^- (Schnabel et al., 2021a),

and the antimicrobial properties of high concentrations of NO_2^- and NO_3^- in an acidic environment are well described in literature (Naïtali, Kamgang-Youbi, Herry, Bellon-Fontaine, & Brisset, 2010; Oehmigen et al., 2011). The inhibition of the antioxidant pathways, the damage of membrane protein repair chaperones and DNA repair cascades by reactive nitrogen and oxygen species (RONS) may lead to microbial inactivation (Pomposiello & Demple, 2002; Vatansever et al., 2013). High concentrations of NO_2^- in combination with H_2O_2 play a key role in the microbial inactivation by PFW (Shaw et al., 2018; Shen et al., 2016). Both chemicals can form peroxynitric acid (O_2NOOH), which subsequently leads to the formation of superoxide (O_2^-) (Ikawa, Tani, Nakashima, & Kitano, 2016; Arts, Gennaris, & Collet, 2015). O_2^- is among other ROS significant for bacterial inactivation ()[27,41]. $ONOO^-$, which can be produced by H_2O_2 and nitrous acid (HNO_2), is also responsible for the inactivation of microorganisms (Lukes, Dolezalova, Sisrova, & Clupek, 2014). However, this molecule was not detected in the PFW. Chemical reactions of RONS are very complex in PFW (Traylor et al., 2011). Microorganisms, especially under aerobic conditions, can survive oxidative and nitrosative stresses. The cell wall and membranes of bacteria are the first protection barrier from the extracellular environment; therefore, they have many repair mechanisms to maintain their cell integrity. Bacteria produce RONS as natural byproducts during their metabolic activity (Vatansever et al., 2013), therefore, microorganisms have defense mechanisms like superoxide dismutase (SOD) and catalase, which convert O_2^- to H_2O_2 and H_2O_2 to H_2O and O_2. Furthermore, the presence of nitrate reductases, allows bacteria to utilize NO_3^- as a nitrogen source or use NO_3^- as an alternative electron acceptor during ATP synthesis (Moreno-Vivián, Cabello, Martínez-Luque, Blasco, & Castillo, 1999).

H_2O_2, was also detected in this PFW, but at a very low concentration of 5.61 mg l^{-1} (29.39 mM) (Schnabel et al., 2021a). Commonly, a 3% (882 mM) to 6% (1764 mM) solution is used (Linley, Denyer, McDonnell, Simons, & Maillard, 2012). H_2O_2 has a strong antimicrobial effect at high concentrations, but needs long exposure times at lower concentrations. While the structure of the bacterial cell wall makes Gram-positive bacteria more susceptible than Gram-negative ones, Gram-positive bacteria have catalase and peroxidase proteins to increase their tolerance against H_2O_2 at lower concentrations (McDonnell & Russell, 1999). Anaerobic microorganisms are more sensitive than aerobic ones and yeast and molds are in most cases more resistant to H_2O_2 than bacteria (Block, 2001). To realize the antimicrobial effect of H_2O_2, the treatment environment should be at low pH as an alkaline environment would decompose H_2O_2 rapidly (Wallhäußer, 1995). H_2O_2 is known to produce hydroxyl radicals (·OH). The strong oxidizing potential of H_2O_2 can lead to the destruction of cell components like oxidation-sensitive metabolic enzymes. Subsequently, this may be followed by oxidation of structural elements with sulfhydryl groups or disulfide bridges. As a result, these structures lose their functionality and the cell metabolism collapses.

The cell envelope is exposed to the oxidizing molecules, based on the plasma reactive species, which target the microorganisms. Elevated levels of reactive species can lead to intracellular damage to DNA, proteins as well as membrane lipids leading finally to cell death. Therefore, the presence of oxidative stress defense mechanisms is crucial for cell survival. Thus, bacteria, like nearly all other living organisms, contain enzymatic systems to deal with oxidative stress. These defense mechanisms occur in bacteria in the cytoplasm and the cell membranes. The cell envelope of bacteria consists of membranes, a cell wall made of peptidoglycan and the periplasmic space. Gram-negative bacteria have an outer and an inner membrane with a thin layer of peptidoglycan whereas Gram-positive bacteria have only one inner membrane but a thick peptidoglycan cell wall. Active $O_2\cdot^-$ cannot easily penetrate cell membranes (Arts et al., 2015). Therefore, SODs are active in the cytoplasm and periplasm to detoxify $O_2\cdot^-$ such as released by the respiratory systems. Commonly, the SODs are synthesized at stationary phase. In contrast, H_2O_2 diffuses through biological membranes and is detoxified in the bacterial cytoplasm by catalases and peroxidases. Up to now, only one enzyme in the periplasm for peroxide reduction has been reported in the literature (Cho et al., 2012). The defense mechanism of bacteria against oxidative stress deals with two options: 1) direct inactivation of RONS and 2) protein repair after oxidative damage. Thus, minimal membrane defects do not lead to cell death.

The proposed mechanisms of action of PFW against Gram-negative and Gram-positive bacteria are based on the results for the chemistry of PFW and the CFU, XTT and LIVE/DEAD assays for possible and identified ROS in Fig. 6 and in Fig. 7 for possible and identified RNS.

Reactions are dependent on pH and the availability of oxygen. Arranged after (Biology of the Nitrogen Cycle, 2007; Cammack et al., 1999; Chan, Xing, Magliozzo, & Bloom, 1992; Shank, Silliker, & Harper, 1962; Shiva, 2013; Torres et al., 2016; Vázquez-Torres & Bäumler, 2016).

As the total native load of the fresh-cut lettuce was investigated in this study, a distinction between Gram-positive and Gram-negative bacteria was not possible. Based on the proposed mechanism of action by (Han et al., 2016) and the observed higher tolerance of Gram-positive bacteria compared to Gram-negative ones (Pericone, Overweg, Hermans, & Weiser, 2000) additional investigations are needed concerning the microbial diversity of the native load (e.g. selective agar) and the possible inactivation outcomes (e.g. cell leakage, DNA damage). The prevalent microbial populations are modified on fresh produce as a function of preparation, processing treatments, storage conditions, temperature and time. Gram-negative psychrophiles and psychrotolerant populations are predominant spoilers of produce in refrigerated storage, although some Gram-positive populations such as lactic acid bacteria can also persist. Gram-negative bacteria seem to be more tolerant against $O_2\cdot^-$ due to more periplasmic space with SODs, and less tolerant against H_2O_2 due to less peptidoglycan compared to Gram-positive bacteria. Gram-positive bacteria seem to be more tolerant against H_2O_2 and more sensitive to $O_2\cdot^-$.

Since Gram-negative bacteria have a different cell wall structure than Gram-positive bacteria, the RNS present in the PFW may influence the respective mechanism of inactivation. For example, the NO_3^- in PFW can penetrate directly through the porins in the cell wall of Gram-negative bacteria, whereas in Gram-positive bacteria it diffuses through the thick peptidoglycan layer (Fig. 7). Thus, the NO_3^- concentration in the periplasmic space could be increased in different ways, thereby delaying the activation of bacterial defense mechanisms. Since Gram-negative bacteria have a larger periplasm in which many degradation processes of NO_3^- can take place by different reductases (nitrate reductase, nitrite reductase, nitric oxide reductase, nitrous oxide reductase and nitrogenase), it is conceivable that Gram-negative bacteria defenses react more effectively to the antimicrobial effect of this PFW.

There was also an overlap with the ROS mechanisms (Fig. 6), since SODs not only degrade $O_2\cdot^-$ to H_2O_2 but can also inhibit the formation of NO_2^- in the periplasm (Fig. 7) and thus prevent further degradation by nitrite reductase to NO or NH_4^+. This allows more NO_3^- to enter the cytoplasm, where it can then react via NO_2^- to NO. In combination with $O_2\cdot^-$ and H_2O_2 the latter can lead to DNA strand breaks and thus to cell death. All antimicrobial RONS (NO_3^-, NO_2^- and H_2O_2) detected in the PFW interact with each other and can support each other in their effect, just as the low pH $<$ 2 promotes the antimicrobial effect. Since $O_2\cdot^-$ also plays an important theoretical role, the persistence and concentration in this PFW should be established.

Considering the collated results, an inactivation of the natural load on fresh-cut lettuce is possible. The success of PFW in fresh produce processing is clearly a function of the stage of application, and also the PFW volume to product ratio, the means of the application (rinsing or washing), and treatment time duration (contact time). However, PFW alone is likely to cause only minor cell membrane damage, whereas metabolic activity could be significantly affected. Chlorine dioxide at a concentration of 15 ppm (15 mg l^{-1}) used for 180 s caused comparable effects to tap water. Here 15 ppm represents a higher

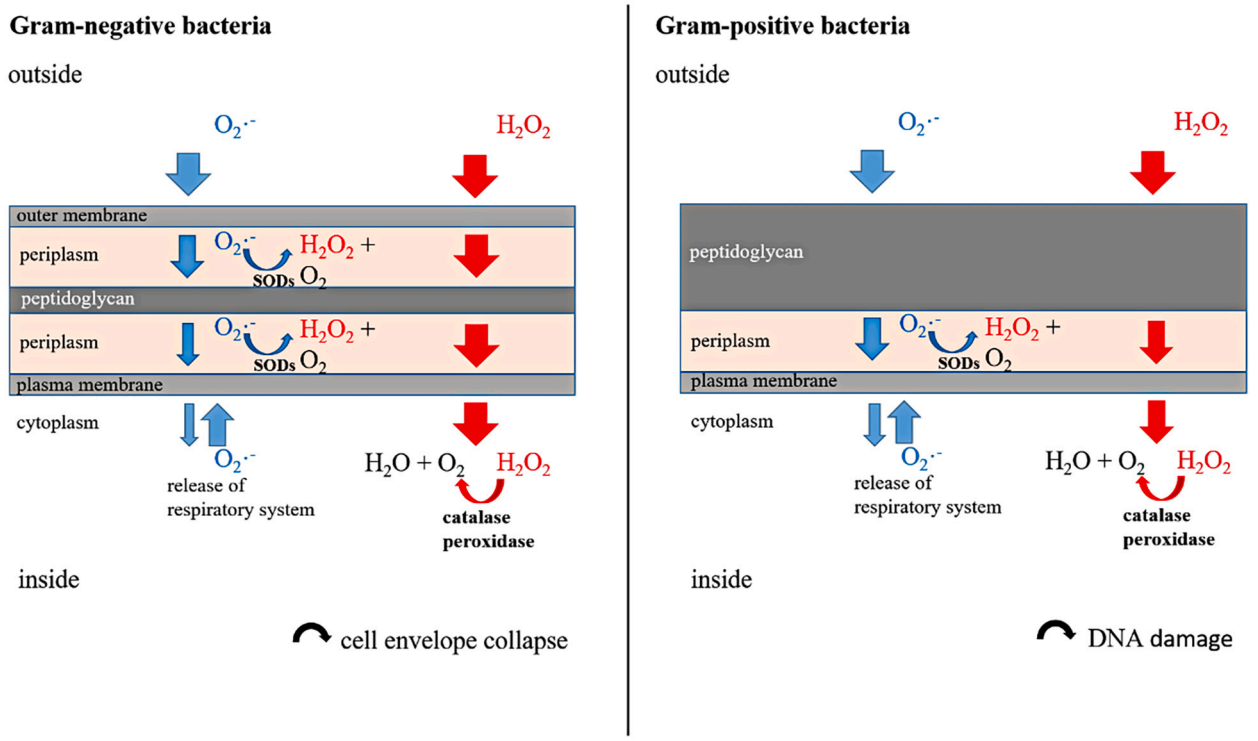

Fig. 6. Proposed mechanism of action of PFW based on reactive oxygen species (ROS). Adapted from (Han et al., 2016).

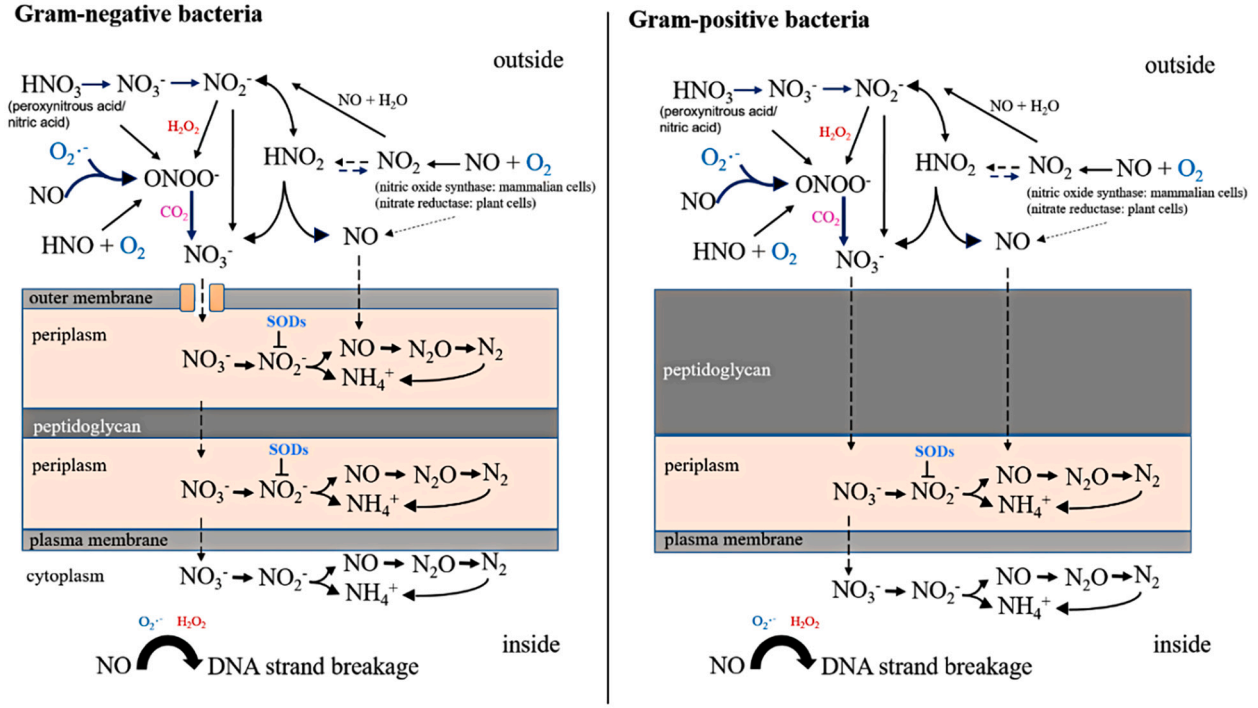

Fig. 7. Proposed mechanism of action of PFW based on reactive nitrogen species (RNS).

concentration than is usually used for industrial washing of lettuce with 3 mg l^{-1} for 30–300 s (FAO and WHO, 2009).

For industry application, up-scaling of the device and/or its mode of operation would be necessary to align with industry scale processor needs. However, the scalability of bench or pilot scale devices in the sense of pure size adjustment is mostly insufficient for industrial application.

Bench scale trials are set up to explore key parameters and provide a "proof of principle" under laboratory conditions. Therefore, questions of scalability and efficiency can play a subordinate role, but these questions should be the focus of implementation on a pilot and industrial scale.

In addition to expanding the capacity of the used system, the efficiency of the individual process steps, their reliable technical

implementation and an operating concept that allows the circle of operators to be trained to include non-scientific personnel must also be carried out. Furthermore, safety, occupational health and safety and life cycle environmental aspects must be integrated into the overall concept to a completely different extent when operating complex systems on a pilot or industrial scale and with the associated increased substance turnover.

Based on pilot scale experiments described by Schnabel et al. (2019) the applied technique has already demonstrated scalability. The capacity of the current pilot set up generates 60 $l\ h^{-1}$ PFW (with $pH < 2.0$) per module with costs of about 1.5 $ct\ l^{-1}$ (related to actual German energy costs). Therefore, application to an industrial scale of 400 kg lettuce per hour is a realistic prospect.

An identified problem of the pilot scale setup was the process efficacy. Therefore, in the present study, the focus was placed on realising process effectiveness, in order to use the PFW generated as efficiently as possible. For this purpose, the laboratory system consisted of several process treatment stations, which were built on the basis of the pilot scale system in Schnabel et al. (2019), but offered the flexibility to change conditions for comparing different treatment scenarios. Future steps include the adaptation of these processing parameters to an industrial scale set-up under real industrial processing conditions.

5. Conclusions

The present study demonstrated that PFW, when introduced at the correct process stage(s) represents an effective process water to improve conventional washing methods for fresh-cut produce. PFW exhibited strong antimicrobial activity against endive-associated microorganisms from 1 to 5 log_{10}-cycles. The antimicrobial active agents quantified in PFW included H_2O_2, NO_2^- and NO_3^- the acidification to low pH values. The promising results and the advantages of this PFW including low-temperature application, simple and cheap generation based on air and water and enhanced efficacy by comparison with current procedures such as tap water rinsing and chlorinated water, offers a timely and sustainable strategy for fresh produce microbiological quality retention.

Funding

This research was partly funded by the Federal Ministry for Food and Agriculture (BMEL) of Germany, grant number 2816IP005 and by the Federal Office for Agriculture and Food (ptBLE) within the program “Deutsche Innovationspartnerschaft Agrar” (DIP), through SFI grant number SFI/16/BBSRC/3391 and through the FIRM program (17/F/275), administered by Department of Agriculture, Food and Marine, Ireland.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

The authors like to thank Carsten Desjardin for assistance with visualization of figures, graphs and tables.

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