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Impact of a Pilot-Scale Plasma-Assisted Washing Process on the Culturable Microbial Community Dynamics Related to Fresh-Cut Endive Lettuce

Antje Fröhling [1], Jörg Ehlbeck [2] and Oliver Schlüter [1,*]

[1] Leibniz Institute for Agricultural Engineering and Bioeconomy, Quality and Safety of Food and Feed, Max-Eyth-Allee 100, 14469 Potsdam, Germany; afroehling@atb-potsdam.de
[2] Leibniz Institute for Plasma Science and Technology, Felix-Hausdorff-Str. 2, 17489 Greifswald, Germany; ehlbeck@inp-greifswald.de
* Correspondence: oschlueter@atb-potsdam.de

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Abstract: Cold plasma is described as a promising technique for the treatment of fresh food. In particular, the application of plasma-treated water gained interest in fresh-cut produce processing. This study aimed to evaluate the effectiveness of plasma-treated water (PTW) to decontaminate lettuce during washing on a pilot-scale level with special interest in the dynamics of the culturable microbial community in a first approach. PTW was used in pilot-scale washing at different processing steps, and the total viable count (TVC) of endive lettuce was determined after treatment and after storage (seven days, 2 °C). Microflora representatives were identified using MALDI-ToF MS. The highest reduction of TVC (1.8 log units) was achieved using PTW for washing whole lettuce before cutting. The microbial community structure showed high variations in the composition along the processing chain and during storage with a decrease in diversity after washing with PTW. PTW reduced the microbial load of endive lettuce; however, this was not clearly detectable at the end of storage, similar to other sanitizers used in comparable studies. To assure the safety of fresh products, detailed knowledge about the microbial load and the composition of the microbial community close to the end of shelf life is of high interest for optimized process design.

Keywords: plasma-treated water; total viable count; MALDI-ToF MS; pilot-scale lettuce washing

1. Introduction

Processing of ready-to-eat salads includes a washing step to remove dirt, debris, and cellular juice from the product. Additionally, the microbial load can be reduced using a washing step to some extent. However, in addition to the positive impact of washing, attention must be paid to the fact that the wash water can transfer microorganisms [1], and this can lead to a cross-contamination of the products. The addition of disinfectants may improve the reduction of the microbial load; however, during storage of the products, the positive effect seems to disappear, and faster growth of the microorganisms can be observed [2,3]. Furthermore, due to the removal of competitive microorganisms, the growth of potentially pathogenic bacteria might be enhanced [2] and has to be considered during the evaluation of inactivation efficiencies.

Chlorine is widely used as a sanitizing agent in the fresh-cut produce industry due to its low cost and high antimicrobial activity [2]. However, because of potential hazardous by-products formed during chlorine application, the search for chlorine substitutes is still in demand [3]. In addition to chlorine, hydrogen peroxide and acid solutions [4], as well as ozone, organic acids, chlorine dioxide, and electrolyzed oxidizing water came into focus in recent years [5]. The main focus of the research in

this area surrounds the inactivation efficiency directly after the treatment and after defined storage times [2,6–8], whereas only limited information is available about the microbial community changes on the products due to the application of sanitizers. Gu et al. [9] found a shift in the microbial community on spinach after washing with chlorine and subsequent storage, whereas the microbial community after storage was again similar to pre-washing conditions. Dependent on the applied sanitizers, the composition of the microbial community on lamb's lettuce varied irrespective of the storage time [10].

Another alternative technique for the disinfection of fresh produce is cold plasma. The ability of cold plasma to inactivate microorganisms on fresh produce was demonstrated in various studies, and the findings were summarized by Ziuzina and Misra [11]. In addition to the application of plasma gas, cold plasma can be admixed in water, where the generated plasma species interact with the water and changes in the chemical composition occur, resulting in plasma-activated water (PAW). These modifications in the chemical conditions include changes in the conductivity and redox potential, as well as the creation of an acidic environment and the formation of reactive oxygen species and reactive nitrogen, resulting in good conditions for microbial inactivation [12]. PAW can be generated using two different approaches, i.e., plasma discharge above the water surface or plasma discharge in the water [13]. Depending on the generation method, different chemistry and reaction products of the PAW occur [12]. In the biomedical field, plasma-activated liquids are used for the treatment of cancer and the inactivation of microorganisms, while an agricultural application involved observing the impact of PAW on seed germination promotion and seedling stem elongation [14].

Information about the effectiveness of PAW on the decontamination of fresh produce is still rare. Ma et al. [13] investigated the impact of PAW (discharge above the water surface) on strawberries. They inoculated *Staphyloccoccus aureus* on the strawberries and achieved inactivation up to 2.3 log units directly after treatment and after four days of storage (20 °C) using PAW treatment times up to 15 min. *S. aureus* was reduced up to 3.4 log units depending on PAW generation time and PAW treatment time. Additionally, they evaluated the impact of PAW on strawberry quality and found no significant changes in color, firmness, and pH of strawberries. Xu et al. [15] tested PAW (discharge above the water surface) as a postharvest preservation method for button mushrooms. The button mushrooms were soaked in PAW for up to 15 min and stored for up to seven days at 20 °C. The PAW treatment for 10 min reduced the microbial load by 1.5 log units, and fungi were reduced by 0.5 log units during storage. They also observed that the treatment with PAW could lead to a delay in the softening of the mushrooms, and they found no significant changes in color, pH, and antioxidant properties of the button mushrooms, indicating that PAW can be a promising tool for the postharvest preservation of button mushrooms. Other terms for plasma-activated water in the literature include plasma-processed water, ozonated water, and plasma-treated water (PTW) [16].

However, most of these studies were conducted on a laboratory scale using long treatment times which are mostly not suitable for implementation in the industrial processing of fresh produce. Especially for new technologies, it is important to evaluate the effectiveness of the applied decontamination technologies after up-scaling research to pilot-scale or industrial-scale levels [17]. Hence, in this study, the effectiveness of plasma-treated water (PTW) in decontaminating lettuce during washing was evaluated on a pilot-scale level. PTW was used in the washing line at different process steps. The total aerobic mesophilic viable count (TVC), as well as the composition and dynamics of the culturable microbial community, on endive lettuce were determined at different processing steps. The TVC and the composition of the culturable microbial community on endive lettuce was additionally evaluated after a storage time of seven days at 2 °C to obtain knowledge regarding the maintenance of microbial decontamination during storage.

2. Material and Methods

2.1. Experimental Set-Up

Washing of endive lettuce was conducted using a pilot-scale washing process constructed by Jürgen Löhrke GmbH, Lübeck, Germany. Plasma-processed air was generated using a two-stage microwave-driven discharge set-up (PLexc$^{2®}$, INP, Greifswald, Germany). The plasma-processed air was then inserted into tap water to produce plasma-treated water (PTW). The experimental set-up of the pilot scale washing process, as well as the microwave-driven discharge set-up, was previously described in detail by Andrasch et al. [18].

2.2. Experimental Trials

Endive lettuce (*Cichorium endivia*) was purchased from a local producer on the day of usage. The unpackaged lettuce was obtained as harvested without any pre-processing steps. Washing of endive lettuce was performed with or without the addition of PTW at different steps of the washing chain. Thereby, six different washing procedures were tested. Briefly, the washing chain included, if not otherwise stated, 180 s of whole-lettuce dipping (pre-bath), cutting into pieces, 10 s of pre-rinsing, 120 s for the first wash, 180 s for the second wash, and 10 s of final rinsing. Trial A was performed without the addition of PTW (washing with tap water only). The next experiment (trial B) was conducted without washing of whole lettuce before cutting with plasma-treated water added for 10 s during pre-rinsing. The following experiment (trial C) was an analog of trial B, but the time of pre-rinsing was extended to 30 s. The next experiment (trial D) included the washing of whole lettuce in PTW for 180 s, and trial E was conducted again without a pre-bath of whole lettuce with PTW added during the second wash for 180 s. The last experiment (trial F) included washing of whole lettuce in PTW for 180 s, and pre-rinsing with PTW for 30 s, along with the addition of PTW in the second wash for 180 s. Further details regarding the experimental procedure are described by Schnabel et al. [19]. Briefly, 5 kg of endive lettuce was used for each washing process. The lettuce was cut into pieces of approximately 5 × 5 cm. Samples were taken at six different sampling points: (1) raw material; (2) endive lettuce after pre-bath; (3) endive lettuce after pre-rinsing; (4) endive lettuce after first wash; (5) endive lettuce after second wash; (6) endive lettuce after final rinsing. At each sampling point, 150 g of lettuce was taken in triplicate, stored at temperatures below 5 °C in sterile bags (Whirl-Pak®, Carl Roth GmbH, Karlsruhe, Germany), and analyzed either within 24 h or after storage for seven days at 2 °C.

2.3. Total Aerobic Mesophilic Viable Count on Endive Lettuce along the Washing Process Chain

The total aerobic mesophilic viable count (TVC) along the washing process chain was conducted according to the European reference standard (EN ISO 4833-2: 2013) [20]. Briefly, 25 g of endive lettuce was mixed with 225 mL of buffered peptone salt solution and homogenized in a bag mixer (BagMixer® 400 CC®, Interscience, St Nom la Bretèche, France) for 2 min at speed 4. Subsequently, a dilution series with peptone salt solution was conducted, and 100 µL of each dilution step was spread on plate count agar. The TVC was determined after incubation of the agar plates for 72 h at 30 °C with a detection limit of 2 log colony-forming units (cfu)/g. To compare the decontamination efficiency of the different washing procedures, log N/N_0 was calculated, where N_0 represents the TVC of the raw material and N represents the TVC of the endive lettuce after final rinsing. The statistical significance of the variations of the TVC values for different samples was evaluated using Welch's unequal variances *t*-test with a significance level of 0.05.

2.4. Culturable Microbial Diversity on Endive Lettuce along the Washing Process Chain

To evaluate the culturable microbial diversity on endive lettuce along the washing process chain and its changes due to different washing procedures, the grown microorganisms on the plate count agar were identified using MALDI-ToF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry). Colonies showing different color and morphology, as well as randomly selected

colonies, were sampled to obtain the best possible summary of the microbial diversity. Thereby, microorganisms from the highest dilutions were chosen for identification to evaluate the microbial diversity of the most abundant microorganisms. For the analysis, cell material of the microorganisms was overlaid with an α-cyano-4-hydroxy cinnamic acid (CHCA) matrix (RIPAC-LABOR GmbH, Potsdam, Germany) on a target and analyzed using MALDI-ToF MS (Axima Confidence, Shimadzu Deutschland GmbH, Duisburg, Germany) after drying. Recording of the spectra was conducted in a mass range between 3000 and 20,000 m/z using the linear mode with a laser repetition rate of 50 Hz. *Escherichia coli* ribosomal proteins were used for calibration. The obtained mass spectra were compared with the reference mass spectra of the AnagnosTec SARAMIS^{TM} database (Spectral Archive and Microbial Identification System, bioMérieux Deutschland GmbH, Nürtingen, Germany) for identification. A cluster analysis (unweighted pair group method with arithmetic mean (UPGMA) clustering) was conducted using the peak-based similarity coefficient "Dice" within the BioNumerics software (version 7.6; Applied Maths NV, Sint-Martens-Latem, Belgium). Parameters for clustering were set as follows: linear tolerance = 500 ppm; constant tolerance = 1 m/z. The cophenetic correlation was calculated to detect reliable and unreliable clusters. Clusters were classified as identified if a spectrum matched with reference spectra of the AnagnosTec SARAMIS^{TM} database with a confidence level >90%. A confidence level between 75 and 89.9% led only to cluster identification to the family level, whereas a confidence level below 75% resulted in "not identified" clusters.

3. Results and Discussion

3.1. Total Aerobic Mesophilic Viable Count of Endive Lettuce along the Washing Process Chain

Typical processing of fresh-cut salads includes the removal of outer leaves, cutting, washing/disinfection, centrifugal drying, and packaging. The washing step is mainly conducted after cutting of the produce, resulting in leakage of produce ingredients into the wash water which may increase the consumption of sanitizers. It is also proposed that cutting may enable a better attachment of microorganisms onto the surface of products, as well as the internalization of microorganisms [21]. Therefore, a washing-before-cutting approach was proposed by different authors [7,21]. Baur et al. [7] found a 2 log unit reduction of the initial microbial load of iceberg lettuce after pre-washing of whole-lettuce head using chlorine. Palma-Salagdo et al. [21] found a 0.8 log cfu/g higher reduction of *E. coli* O157:H7 on iceberg lettuce when applying the washing-before-cutting process in comparison to the commonly applied cutting-before-washing process.

In this study, the total aerobic mesophilic viable count (TVC) of the endive raw material ranged between 7.5 log cfu/g and 7.7 log cfu/g (Table 1). After storage, the untreated material showed TVC values between 7.4 log cfu/g and 8.3 log cfu/g (Table 2).

For the application of plasma-treated water during washing, the washing-before-cutting process, as well as the cutting-before-washing process, was applied. Washing with water was applied using the washing-before-cutting method (trial A). The TVC was significantly reduced along the processing chain from 7.7 log cfu/g to 6.5 log cfu/g (Table 1), revealing a reduction of 1.2 log units (Table 3). After storage at 2 °C for seven days, the TVC of endive lettuce at the end of the washing was 7.5 log cfu/g and significantly lower than the stored endive raw material with 8.3 log cfu/g (Table 2), which still means a reduction of 0.8 log units (Table 3). The application of plasma-treated water at different processing steps reduced the TVC of the final endive lettuce product to 6.9 log cfu/g, 6.5 log cfu/g, 6.0 log cfu/g, 6.6 log cfu/g, and 6.6 log cfu/g for trials B, C, D, E, and F, respectively (Table 1). The highest reduction on the final endive lettuce product was achieved for trial D (PTW in pre-bath, 180 s) after processing (1.6 log reduction), followed by treatment F (PTW in pre-bath (180 s), pre-rinsing (30 s), and second wash (180 s)) with a reduction of 1.1 log unit (Table 3). However, there was no significant difference in the TVC reduction between washing with water and the addition of plasma-treated water at different processing steps with the exception of trial B (PTW in pre-rinsing, 10 s), which showed a significantly lower reduction of the TVC after processing (Table 3).

Table 1. Total viable count on endive lettuce after washing with or without the addition of plasma-treated water (PTW) at different processing steps. Letters and numbers in square brackets refer to the six trials and six sampling points, respectively, described in Section 2.2.

	[A] Washing with Water and Pre-Bath (180 s) (cfu/g)	[B] Washing with PTW in Pre-Rinsing (10 s) (cfu/g)	[C] Washing with PTW in Pre-Rinsing (30 s) (cfu/g)	[D] Washing with PTW in Pre-Bath (180 s) (cfu/g)	[E] Washing with PTW in Second Wash (180 s) (cfu/g)	[F] Washing with PTW in Pre-Bath (180 s) in Pre-Rinsing (30 s) and in Second Wash (180 s) (cfu/g)
[1] Endive raw material	$7.70^{a} \pm 0.12$	$7.54^{a} \pm 0.18$	$7.53^{a} \pm 0.21$	$7.60^{a} \pm 0.15$	$7.58^{a} \pm 0.09$	$7.74^{a} \pm 0.13$
[2] Endive after pre-bath	$6.77^{b} \pm 0.09$	n.d.	n.d.	$5.85^{b,d,e} \pm 0.19$	n.d.	$6.85^{b,e} \pm 0.05$
[3] Endive after pre-rinsing	$7.22^{c} \pm 0.10$	$6.19^{b} \pm 0.28$	$5.16^{b} \pm 0.23$	$5.99^{b,c} \pm 0.13$	$7.07^{b} \pm 0.16$	$7.18^{b,e} \pm 0.04$
[4] Endive after first wash	$7.40^{d} \pm 0.12$	n.d.	n.d.	$6.34^{c} \pm 0.35$	$7.06^{b} \pm 0.08$	$7.18^{c} \pm 0.04$
[5] Endive after second wash	$6.82^{b} \pm 0.10$	n.d.	n.d.	$5.54^{d} \pm 0.29$	$5.43^{c} \pm 0.12$	5.43^{d} 0.03
[6] Endive after final rinsing	$6.52^{e} \pm 0.16$	$6.90^{c} \pm 0.12$	$6.49^{c} \pm 0.36$	$6.00^{c,e} \pm 0.31$	$6.57^{d} \pm 0.09$	$6.64^{e} \pm 0.50$

n.d.: not determined; cfu: colony-forming units. Columns with different letters indicate statistically significant differences ($p < 0.05$).

Table 2. Total viable count on stored (2 °C, seven days) endive lettuce after washing with or without the addition of plasma-treated water at different processing steps. Letters and numbers in square brackets refer to the six trials and six sampling points, respectively, described in Section 2.2.

	[A] Washing with Water and Pre-Bath (180 s) (cfu/g)	[B] Washing with PTW in Pre-Rinsing (10 s) (cfu/g)	[C] Washing with PTW in Pre-Rinsing (30 s) (cfu/g)	[D] Washing with PTW in Pre-Bath (180 s) (cfu/g)	[E] Washing with PTW in Second Wash (180 s) (cfu/g)	[F] Washing with PTW in Pre-Bath (180 s) in Pre-Rinsing (30 s) and in Second Wash (180 s) (cfu/g)
[1] Endive raw material	$8.26^{a} \pm 0.16$	$7.39^{a} \pm 0.36$	$7.83^{a} \pm 0.16$	$7.76^{a} \pm 0.15$	$7.76^{a} \pm 0.19$	$8.11^{a} \pm 0.16$
[2] Endive after pre-bath	$7.91^{b} \pm 0.26$	n.d.	n.d.	$6.95^{b,c} \pm 0.41$	n.d.	$7.48^{b} \pm 0.23$
[3] Endive after pre-rinsing	$7.63^{b,c} \pm 0.23$	$6.94^{b} \pm 0.16$	$5.97^{b} \pm 0.62$	$6.99^{b} \pm 0.42$	$7.59^{a} \pm 0.15$	$7.13^{c} \pm 0.24$
[4] Endive after first wash	$7.57^{c} \pm 0.22$	n.d.	n.d.	$7.27^{b,d} \pm 0.23$	$7.66^{b} \pm 0.36$	$7.19^{c,d} \pm 0.07$
[5] Endive after second wash	$7.51^{c} \pm 0.07$	n.d.	n.d.	$6.98^{c} \pm 0.14$	n.d.	6.16^{e} 0.39
[6] Endive after final rinsing	$7.47^{c} \pm 0.14$	$6.94^{b} \pm 0.18$	$7.49^{a} \pm 0.34$	$7.48^{d} \pm 0.14$	$7.48^{a} \pm 0.27$	$7.16^{b,c} \pm 0.37$

n.d.: not determined; cfu: colony-forming units. Columns with different letters indicate statistically significant differences ($p < 0.05$).

Table 3. Reduction of total viable count on endive lettuce after washing process with or without addition of plasma-treated water at different processing steps and subsequent storage at 2 °C for seven days. Letters in square brackets refer to the six trials described in Section 2.2.

	After Processing log N/N_0	After Storage log N/N_0
[A] Washing with water and pre-bath (180 s)	$-1.18^{a,c,d} \pm 0.21$	$-0.80^{a,c} \pm 0.20$
[B] Washing with PTW in pre-rinsing (10 s)	$-0.64^{b} \pm 0.16$	$-0.44^{a,b,c} \pm 0.54$
[C] Washing with PTW in pre-rinsing (30 s)	$-1.04^{b,c,d} \pm 0.52$	$-0.34^{b} \pm 0.27$
[D] Washing with PTW in pre wash bath (180 s)	$-1.60^{c} \pm 0.45$	$-0.29^{b} \pm 0.25$
[E] Washing with PTW in second wash (180 s)	$-1.01^{d} \pm 0.09$	$-0.27^{b} \pm 0.41$
[F] Washing with PTW in pre-bath (180 s) in pre-rinsing (30 s) and in second wash (180 s))	$-1.10^{a,c,d} \pm 0.46$	$-0.95^{c} \pm 0.51$

Columns with different letters indicate statistically significant differences ($p < 0.05$).

After storage at 2 °C, the TVC of all samples increased again, but the TVC of the washed endive lettuce was significantly lower than the TVC of the stored endive raw material with the exception of trials C and E, where no significant differences could be detected (Table 2). In contrast to the analyses after processing, the highest reduction was achieved for trial F. However, this reduction was not significantly different from the reduction obtained in trial A (water only). All other treatments with PTW showed a significantly lower reduction in comparison to the washing with water only (trial A; Table 3).

Taking a closer look at the endive lettuce sampled at the point of PTW addition, a varying reduction was achieved for the different experimental trials (Table 1). Dipping of whole endive lettuce in the PTW pre-bath for 180 s led to a reduction of 1.8 log units in trial D, whereas the dipping of whole lettuce into PTW pre-bath for 180 s in trial F only led to a reduction of 0.9 log units. Highest reduction of the TVC was achieved for the endive lettuce directly after 30 s of pre-rinsing with PTW (trial C; 2.4 log units), but a reduction of only 0.6 log units was obtained at the same sampling point at trial F. Sampling of endive lettuce after the second wash with PTW led to similar reductions in trial E and trial F with 2.2 log units and 2.3 log units, respectively. Trial B (10 s of pre-rinsing with PTW) led to a reduction of 1.4 log units on endive lettuce sampled at the point of PTW addition. The corresponding stored endive lettuce showed significantly lower TVC values than the stored endive raw material (Table 2).

The effectiveness of PTW for the reduction of TVC on endive lettuce was different depending on the processing step of PTW application, and was associated with the PTW application time. The dependence of the microbial inactivation on the application time of plasma-activated water was also found for strawberries by Ma et al. [13] and button mushrooms by Xu et al. [15]. In contrast, the microbial reductions on strawberries [13] and button mushrooms [15] were higher after storage than for the endive lettuce after storage observed in this study. However, it has to be taken into account that the strawberries were artificially contaminated with *S. aureus*, which is not comparable with naturally occurring contamination and its reduction. The achieved reduction of the TVC by PTW on endive lettuce at the end of the washing process was within the range of other sanitizers tested for application in the fresh-cut produce industry. Chlorine also reduced the microbial load of fresh-cut produce by only 1–2 log units, and a similar reduction was achieved for ozone, electrolyzed water, and H_2O_2 [22]. The limited reduction of microorganisms on fresh produce using sanitizers is attributed to the presence of biofilms, internalization of microorganisms, and the attachment of the microorganisms to the surface or near stomata [23]. Other studies showed that, despite the reduction achieved after treatment with sanitizers, the microbial count increased again during storage to a level similar to that observed for the unwashed sample, resulting in minor differences in the microbial count between samples treated with sanitizers and samples treated without sanitizers at the end of storage [2,22]. This was also observed for the PTW-treated lettuce in this study, revealing the importance of the evaluation of the microbial load of the products at the end of storage since the reduction of the microbial load must be maintained during storage [2]. However, the sampling of

the endive lettuce at the point of PTW addition showed the ability of PTW to reduce the microbial load of the endive lettuce significantly up to 2.4 log units. However, the reduction did not remain stable; as such, at the end of the washing process, the analysis of the final product revealed a lower reduction. This can be attributed to the inhomogeneity of the biological material and corresponding high variability of the microbial load. Another explanation for the lower reduction of the microbial load on the endive lettuce could be re-contamination of the product during the washing process.

3.2. Microbial Diversity on Endive Lettuce along the Washing Process Chain

Even though some research concluded that the microbial load of a product affects its product quality, other studies demonstrated that the TVC or the specific count of spoilage microorganisms cannot predict the quality of the product. An exception is the count of lactic acid bacteria and yeasts, where a good correlation was found with the product quality [2]. Furthermore, faster growth during storage was observed for epiphytic microbiota of fresh produce after application of sanitizers [3], and Enterobacteriaceae count on iceberg lettuce was higher during storage on samples treated with sanitizers than on untreated samples [24]. This implies that it is not only important to monitor the TVC after certain decontamination treatments and subsequent storage, but it is also important to get insight into the microbial community structure on the products in relation to the treatments and storage. In this study, the culturable microbial community structure on endive lettuce was evaluated using MALDI-ToF MS. Dependent on the grown microorganisms on the plate count agar, an uneven number of microorganisms was selected for the identification via MALDI-ToF MS, and, due to the high amount of samples, it was not possible to evaluate each sample in triplicate. The number of analyzed samples was divided into identified and not identified microorganisms per sample (Supplementary Materials Table S1). However, since the experiments were performed at a pilot scale, the results of this study can help gain knowledge about the dynamic changes on endive lettuces due to the application of PTW during washing.

The culturable microbial community of endive salad before washing with water (trial A) consisted of 37% identified microorganisms, and 63% of the measured microorganisms could not be identified with the applied database. The identified microorganisms were *Flavobacterium* sp., *Herbaspirillum huttiense*, Pseudomonadaceae, *Pseudomonas fluorescens*, *Pseudomonas* spp., *Rahnella aquatilis*, Rhizobiaceae, Staphylococcaceae, and *Pseudoxanthomonas spadix*. After washing with water, the measurement of the culturable microbial community revealed 77% not identified microorganisms and 23% identified microorganisms and, in comparison to the unwashed sample, only *Flavobacterium* sp., *Herbaspirillum huttiense,* and *Pseudomonas* spp. were not detected (Figure 1A). After storage, the culturable microbial community of the endive raw material consisted of 60% not identified microorganisms and 40% identified microorganisms, which were Pseudomonadaceae, *Pseudomonas fluorescens*, Rhizobiaceae, and Staphylococcaceae (Figure 1B). The culturable microbial community structure of the washed and stored endive lettuce showed higher diversity in comparison to the unwashed and stored samples. Specifically, 56% of the measured colonies could not be identified and 44% could be identified, revealing the additional occurrence of *Pseudomonas* spp. and *Pseudomonas viridiflava* in comparison to the unwashed and stored endive lettuce.

After washing with water followed by storage for seven days at 2 °C, the predominant identified bacteria on endive lettuce was *Pseudomonas fluorescens*. Bacteria belonging to the family Pseudomonadaceae act as food spoilage microorganisms, and their presence can lead to textural changes and changes in the sensory quality [25]. A closer look at the not identified microorganisms showed that this cluster can be split into 30 different clusters for endive raw material before storage and into 18 different clusters after storage. The not identified cluster of the washed endive lettuce consisted of 25 sub-clusters before storage and 16 sub-clusters after storage (data not shown).

Regarding the TVC, washing with PTW in the pre-bath for 180 s (trial D) showed the best reduction after washing (1.6 log cfu/g reduction). Since the TVC after storage returned to almost the same

level as the unwashed and stored endive raw material, it is of special interest which microorganisms predominate the microbial community.

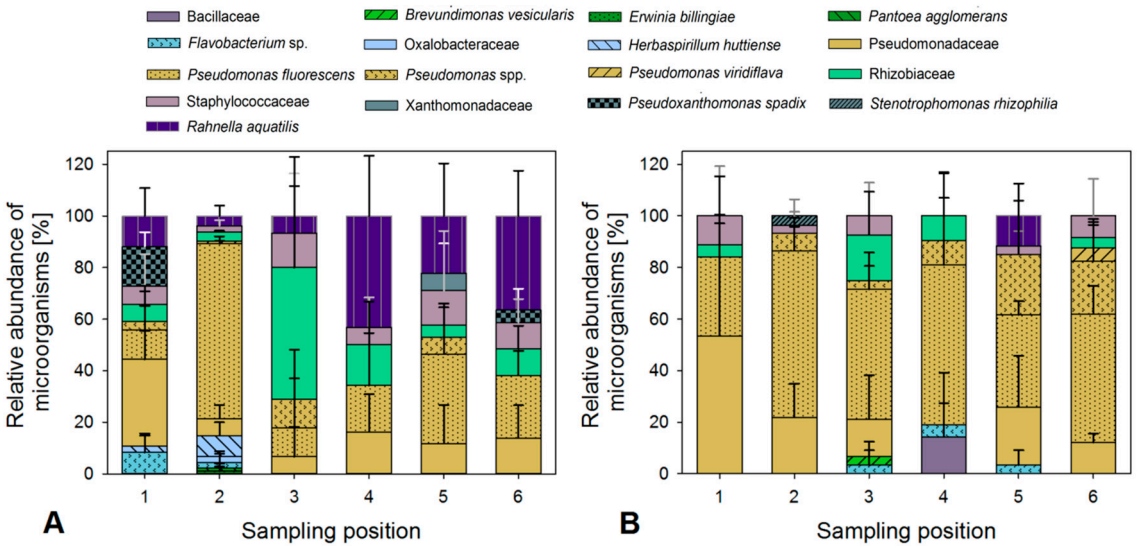

Figure 1. Culturable microbial community structure of endive lettuce (identified colonies only) along the washing process chain. The washing was conducted without the addition of sanitizers (trial A) and the samples were analyzed after washing (**A**) and after storage for seven days at 2 °C (**B**). Sampling positions: (1) endive raw material; (2) endive after pre-bath; (3) endive after pre-rinsing; (4) endive after first wash; (5) endive after second wash; (6) endive after final rinsing.

The analysis of the culturable microbial community of endive raw material before storage revealed 77% not identified microorganisms (43 sub-clusters), and the identified fraction was composed of *Herbaspirillum huttiense*, Moraxellaceae, Pseudomonadaceae, *Pseudomonas fluorescens*, Rhizobiaceae, Sporidiobolaceae, *Stenotrophomonas* sp., and Yersiniaceae (Figure 2A). At the end of the washing process, the microbial community of endive lettuce shifted. The amount of not identified microorganisms increased to 93% (36 sub-clusters) and the identified microorganisms belonged to Arthrodermataceae, Porphyromonadaceae, and Staphylococcaceae. After seven days of storage, none of the microorganisms obtained from the endive raw material could be identified (nine sub-clusters) by MALDI ToF MS using the SARAMIS^{TM} database (Figure 2B). After washing with PTW for 180 s in the pre-bath and subsequent storage of the end product, all identified microorganisms belonged to the family Staphylococcaceae and 89% (14 sub-clusters) could not be identified. Staphylococcaceae were also found in all other samples tested in different abundances. Members of the family Staphylococcaceae can be human pathogens, e.g., *Staphylococcus aureus*, which is able to produce enterotoxins which can cause staphylococcal food poisoning [13]. However, the microorganisms could only be classified to the family level; thus, no statement can be made about the possible pathogenicity of the grown microorganisms. Nevertheless, since members of the family Staphylococcaceae can even grow at refrigerator temperatures [26] and they seem to be less sensitive to the treatment with PTW, their occurrence should be monitored with care.

Directly after cutting, cell exudates have to be removed because they can act as a good reservoir for bacterial growth; hence, an application of sanitizers directly after cutting might enhance microbial inactivation.

The application of PTW directly after cutting in the pre-rinsing step for 10 s (trial B) or 30 s (trial C) showed reductions of TVC of 0.6 log units and 1.0 log units, respectively. After storage, the microbial reduction was lower than that for the samples after washing with water only. The application of PTW for 10 s in the pre-rinsing step led only to minor changes of the culturable microbial community after treatment, which might also be the result of the colony sampling. Furthermore, 87% of the analyzed microorganisms obtained from the endive raw material could not be identified

(17 sub-clusters) and, after the washing process, the amount of not identified microorganisms increased to 92% (10 sub-clusters). Members of the family Moraxellaceae were found before and after washing, whereas members of the family Staphylococcaceae were not found after washing (Figure 3A). However, after storage at 2 °C, members of the family Staphylococcaceae were again detected in the unwashed and washed endive lettuce. Additionally, members of the family Pseudomonadaceae were detected in higher amounts in all samples tested (Figure 3B). The amount of not identified microorganisms after storage was 50% for the endive raw material (nine sub-clusters) and 71% for the washed endive lettuce (nine sub-clusters).

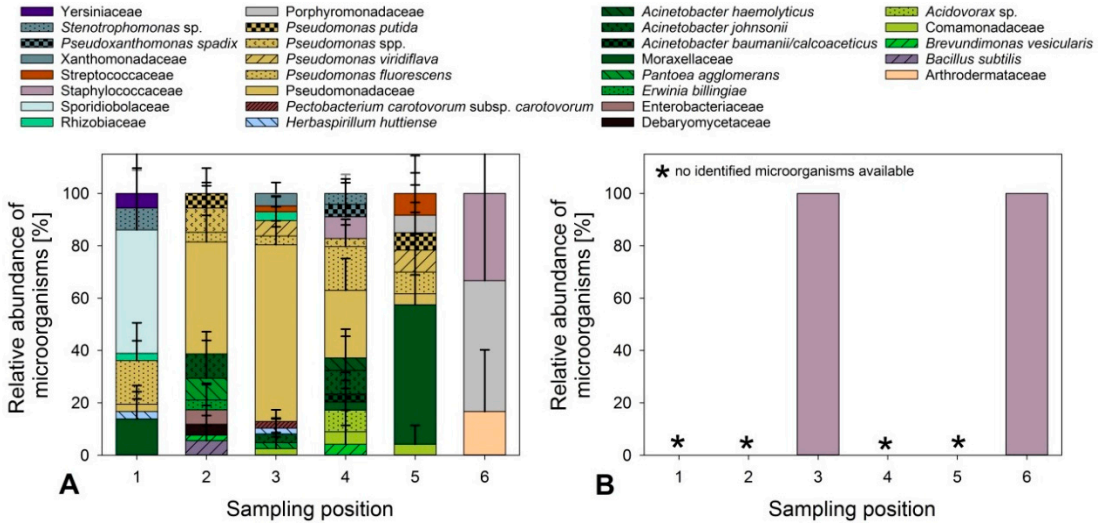

Figure 2. Culturable microbial community structure of endive lettuce (identified colonies only) along the washing process chain. The washing was conducted with the addition of plasma-treated water (PTW) in the pre-bath (trial D) and the samples were analyzed after washing (**A**) and after storage for seven days at 2 °C (**B**). Sampling positions: (1) endive raw material; (2) endive after pre-bath; (3) endive after pre-rinsing; (4) endive after first wash; (5) endive after second wash; (6) endive after final rinsing.

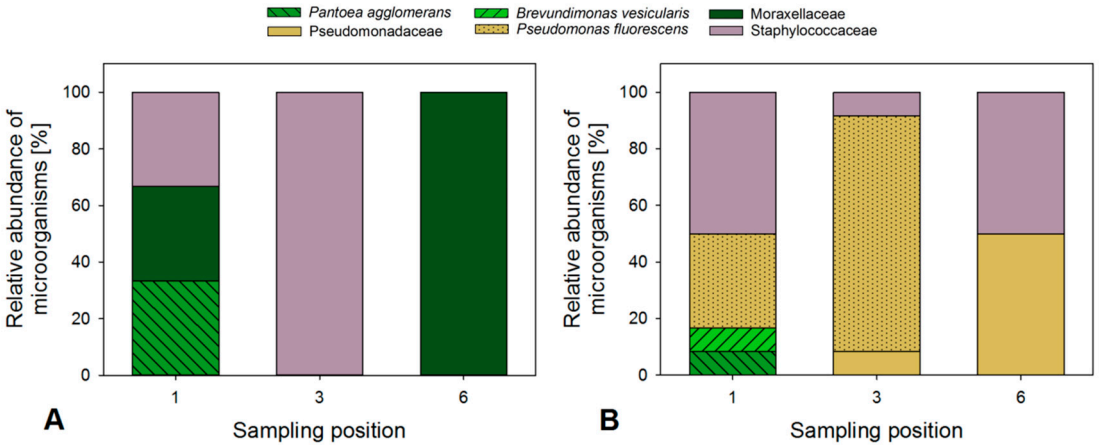

Figure 3. Culturable microbial community structure of endive lettuce (identified colonies only) along the washing process chain. The washing was conducted with the addition of PTW in the pre-rinsing step (10 s) (trial B) and the samples were analyzed after washing (**A**) and after storage for seven days at 2 °C (**B**). Sampling positions: (1) endive raw material; (3) endive after pre-rinsing; (6) endive after final rinsing.

Increasing the application of PTW in the pre-rinsing step to 30 s revealed similar results in comparison to the application of PTW in the pre-rinsing for 10 s. After washing, 60% of the microorganisms obtained from endive raw material could not be identified (13 sub-clusters) and,

at the end of the washing process, the amount of not identified microorganisms increased to 94% (nine sub-clusters). The identified microorganisms belonged mainly to the family Staphylococcaceae and members of the family Pseudomonadaceae were only detected on endive raw material (Figure 4A).

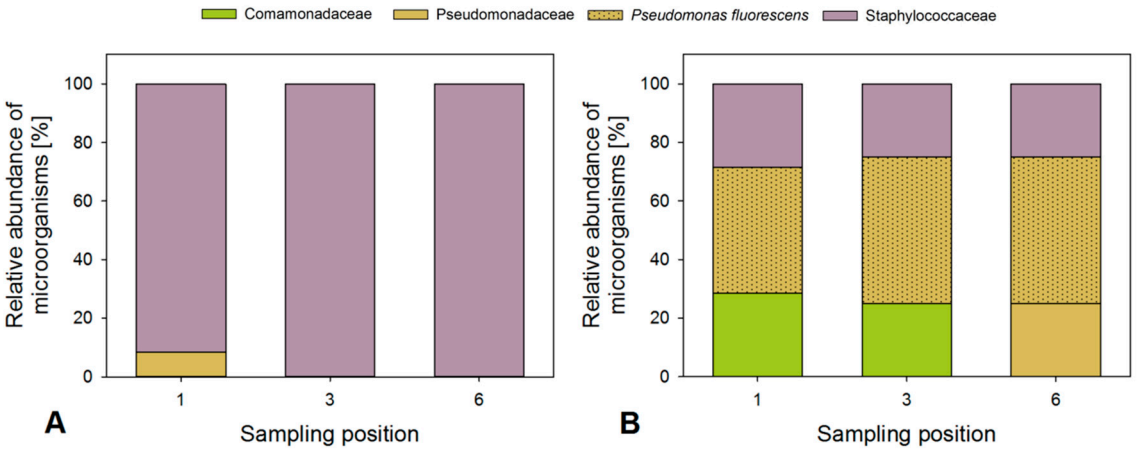

Figure 4. Culturable microbial community structure of endive lettuce (identified colonies only) along the washing process chain. The washing was conducted with the addition of PTW in the pre-rinsing step (30 s) (trial C) and the samples were analyzed after washing (**A**) and after storage for seven days at 2 °C (**B**). Sampling positions: (1) endive raw material; (3) endive after pre-rinsing; (6) endive after final rinsing.

After storage for seven days, 53% of the microorganisms analyzed from the endive raw material were not identified (seven sub-clusters) and the washed endive lettuce showed 81% not identified microorganisms (11 sub-clusters) after storage. Members of the families Comamonadaceae and Staphylococcaceae, as well as *Pseudomonas fluorescens*, were detected in the endive raw material after storage and, on the stored washed endive lettuce, members of the family Comamonadaceae were no longer detected (Figure 4B).

The process step for the addition of sanitizers is primarily the second wash, which is followed by a rinsing step to remove residual sanitizers from the product [21]. PTW was, therefore, added to the second wash with a lettuce retention time of 180 s to evaluate the impact on the culturable microbial community on the endive lettuce. The amount of not identified microorganisms increased from 56% (14 sub-clusters) to 94% (13 sub-clusters) after washing. Staphylococcaceae, Moraxellaceae, and *Pseudomonas fluorescens* were detected on the endive raw material and, after washing, only members of the family Phaffomycetaceae were identified (Figure 5A). After storage, the mainly identified microorganism was *Pseudoxanthomonas spadix* on the endive raw material, as well as on the washed endive sample (Figure 5B). The amount of not identified microorganisms remained almost the same after storage in comparison to the fresh endive samples with 56% (12 sub-clusters) for the stored endive raw material and 96% (14 sub-clusters) for the washed and stored endive lettuce.

Since the application of PTW for 180 s in the pre-bath, for 30 s in the pre-rinsing step, and for 180 s in the second wash led to an inactivation of 1.6 log units, 1 log unit, and 1 log unit, respectively, it was tested whether an addition of PTW in the pre-bath, in the pre-rinsing step, and in the second wash in one washing process could enhance the microbial inactivation. Unfortunately, the application of PTW at three washing steps along the process chain did not enhance the microbial inactivation. Regarding the culturable microbial community, 78% of the analyzed microorganisms obtained from endive raw material could not be identified (14 sub-clusters) and, after washing, the amount increased to 94% (11 sub-clusters). Whereas in the endive raw material members of the family Staphylococcaceae, as well as members of the family Moraxellaceae, were detected, only members of the family Staphylococcaceae were identified after washing (Figure 6A). After storage, the endive raw material showed 52% not identified microorganisms (10 sub-clusters) and the amount increased to 74% (nine sub-clusters) after

washing and storage of the endive lettuce. In addition to Staphylococcaceae and Moraxellaceae, Pseudomonadaceae and *Pseudomonas fluorescens* were also identified on the endive raw material after storage. After washing and storage of the endive lettuce, not only were Staphylococcaceae and Pseudomonadaceae found, but Phaffomycetaceae was also detected (Figure 6B).

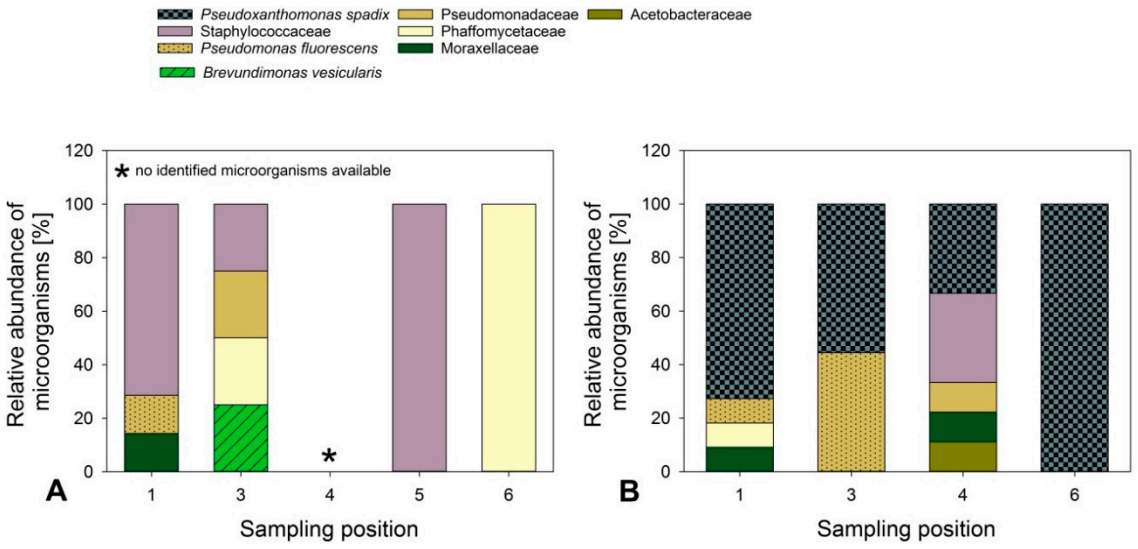

Figure 5. Culturable microbial community structure of endive lettuce (identified colonies only) along the washing process chain. The washing was conducted with the addition of PTW in the second wash (180 s) (trial E) and the samples were analyzed after washing (**A**) and after storage for seven days at 2 °C (**B**). Sampling positions: (1) endive raw material; (3) endive after pre-rinsing; (4) endive after first wash; (5) endive after second wash; (6) endive after final rinsing.

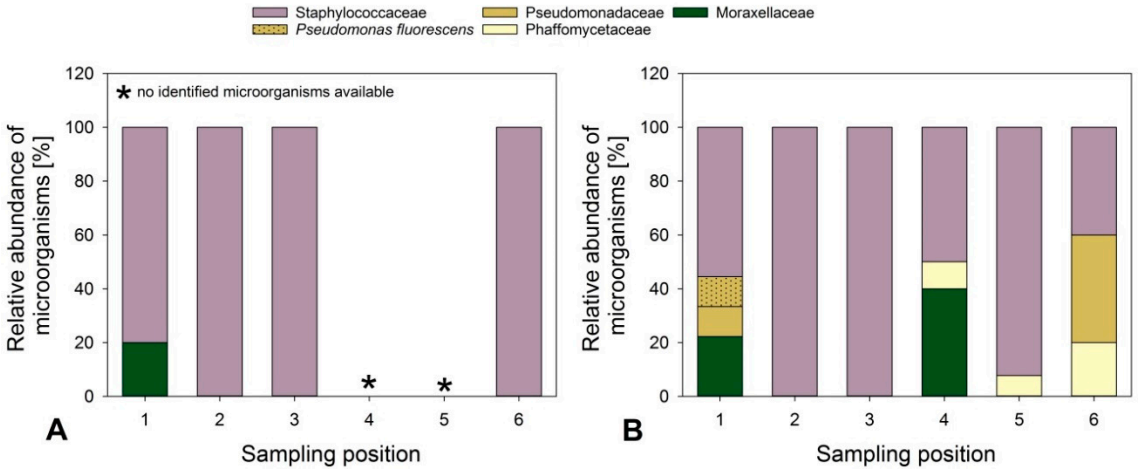

Figure 6. Culturable microbial community structure of endive lettuce (identified colonies only) along the washing process chain. The washing was conducted with pre-bath of the whole lettuce and the addition of PTW in the pre-rinsing step (30 s) and the second wash (180 s) (trial F) and the samples were analyzed after washing (**A**) and after storage for seven days at 2 °C (**B**). Sampling positions: (1) endive raw material; (2) endive after pre-bath; (3) endive after pre-rinsing; (4) endive after first wash; (5) endive after second wash; (6) endive after final rinsing.

Unfortunately, most of the microorganisms analyzed could not be identified using the SARAMIS^{TM} database independently of the applied washing procedure. This hampers a reliable statement about the dynamics of the culturable microbial community structure due to different washing procedures. The lack of identification can either be caused by spectra with poor quality obtained from the grown microorganisms or by the occurrence of unknown microorganisms. However, there is

a lack of reference spectra in the MALDI-ToF MS databases regarding environmental samples [27], since commercially available databases mostly focus on clinically relevant microorganisms [28]; thus, the not identified microorganisms might not be unknown microorganisms, but rather environmental microorganisms that are not yet included in the databases. Low identification percentages for environmental samples were also described by Rahi et al. [28], and most of the grown microorganisms on plate count agar obtained from a spinach processing line could not be identified by MALDI-ToF MS [29]. However, it seems that, after application of PTW in different processing steps, the amount of not identified microorganisms increased. After storage, the amount of not identified microorganisms decreased again. This raises the question whether the application of PTW alters the microorganisms of the endive lettuce, resulting in different mass spectra during analysis and, therefore, to a lack of identification. The MALDI-ToF MS technique is based on the analyses of primarily ribosomal proteins. These ribosomal proteins are synthesized under all growth conditions [28]. Since the proteome changes with changing growth conditions, it is recommended that growth conditions be as stable as possible to achieve reliable results. Additionally, stress conditions may lead to changes in the ribosomal proteins [30]. The application of PTW during washing of endive lettuce led to a pH value below 2 in the wash water as a consequence of the presence of nitrite and nitrate and their reaction with water to form nitric acid and nitrous acid [19]. Hence, it is conceivable that changes in the ribosomal proteins occur in response to the stressful conditions during washing with PTW, and these changes may hamper the identification of the mass spectra. During storage, the microorganisms might regenerate, leading to a decreased amount of not identified spectra. However, this is only a hypothesis, and changes in MALDI-ToF mass spectra of bacteria after the application of certain decontamination processes need to be evaluated and verified in further studies.

Nevertheless, it is obvious that the culturable microbial community structure is diverse and varies greatly between the batches. This can be attributed to the natural microbial diversity on lettuce, but it may also be influenced by the different environmental conditions during the experiments, since there was a temperature increase from 19 °C to 31 °C and an increase in relative humidity from 69% to 95% in the working environment during the experimental trials [19]. Therefore, it has to be taken into account that it is not possible to determine the microbial community structure of endive lettuce during an experiment and transfer the results to other experiments and that it is, therefore, necessary to evaluate the dynamics in the microbial community structure of products during different processing steps singularly. Nevertheless, the results indicate that the culturable microbial community is influenced by the application of PTW during washing, indicating that some microorganisms are more sensitive to PTW than others. Changes in the microbial diversity after certain processing steps were also found in other studies. The microbial diversity of soybean sprouts changed due to washing with bacteriocins [31], and the microbial diversity of lamb's lettuce changed after washing with essential oils and subsequent storage [10]. In our study, members of the family Staphylococcaceae were predominately found after washing with PTW. In comparison to the washing without sanitizers, a decreased culturable microbal diversity was found in all samples after washing with addition of PTW at different processing steps. During storage, changes in the culturable microbial diversity were also observed, which might be attributed, on the one hand, to the application of sanitizers and, on the other hand, to the refrigeration temperatures. The storage temperature of minimally processed produce should be between 1 °C and 4 °C to extend the shelf life of the products [32]; however, psychrotrophic bacteria such as Pseudomonadaceae are able to proliferate at refrigerator temperatures, where even mesophilic bacteria can grow albeit at lower growth rates [33]. Changes of the composition of the microbial community during storage were also found for ready-to-eat baby spinach and mixed salad with Pseudomonadaceae as predominant microorganisms [34].

Even though the TVC of the final product was reduced after application of PTW to some extent, at the end of the storage (seven days, 2 °C), no significant difference of TVC on the final product could be observed between the different treatments applied. This implies that the shelf life of endive lettuce could not be enhanced in comparison to the endive lettuce washed without sanitizers, which

is in accordance with other studies using, e.g., chlorine as a sanitizer. However, the TVC was only tested at the end of storage period of seven days and, in future studies, the TVC should be obtained following different lengths of storage to evaluate the development of TVC during storage, and if any differences between the application of PTW during washing and washing with water only occur. The determination of the culturable microbial community structure of endive lettuce along the processing chain and during storage revealed high variations in the composition of the microbial community. Since the direct sampling at the point of PTW addition revealed higher reduction of the microbial load than the sampling of the final product, the washing process has to be improved to maintain the obtained microbial inactivation. Additionally, the maintenance of the microbial reduction during storage is an important factor and may be enhanced by using suitable packaging and/or storage procedures, e.g., modified atmosphere packaging or controlled atmosphere storage.

The results emphasize the necessity to monitor not only the TVC after treatment with sanitizers, but also at the end of the storage to make sure that the obtained inactivation is still detectable close to the end of shelf life, as well as to monitor the composition of the microbial community to avoid an enhanced growth of potentially pathogenic bacteria as a result of the application of sanitizers. Additionally, re-contamination of the product during the washing process has to be avoided to ensure that the microbial reduction achieved by PTW is also maintained at the end of the washing process and close to the end of storage. A PTW-based clean-in-place procedure might also help derive improved processing strategies. If this information is available, it will possibly create gentle tailor-made processes to increase the microbiological safety of perishable food products.

Supplementary Materials: The following is available online at http://www.mdpi.com/2076-3417/8/11/2225/s1, Table S1: Number of analyzed microorganisms via MALDI-ToF MS. In cases of triplicates, the average of analyzed microorganisms, as well as the standard deviation, is given.

Author Contributions: A.F. conceived and designed the experiments, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. J.E. conceived and designed the experiments, and proof-read the manuscript. O.S. conceived and designed the experiments, contributed reagents, materials, analysis tools, and data, and proof-read the manuscript.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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