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Decontamination of Fresh-Cut Iceberg Lettuce and Fresh Mung Bean Sprouts by Non-Thermal Atmospheric Pressure Plasma Processed Water (PPW)

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Abstract: Currently used methods for decontamination and sanitation are antimicrobial ineffective, generate high costs with a high consumption of water and chemicals additionally. As an alternative, non-thermal plasma at atmospheric pressure could be a versatile tool. Therefore, an experimental set-up based on a microwave-plasma source which generates plasma processed air (PPA) containing manifold RNS-based chemical and antimicrobial compounds was used. The PPA was introduced into distilled water or tap water to generate plasma processed water (PPW) which can be applied for the decontamination of packaging material and fresh produce. This is a new and innovative method for the generation of antimicrobial active water. In our experiments, PET stripes, fresh-cut lettuce, and fresh sprouts were contaminated with six different bacteria; *Escherichia coli* K12 (DSM 11250), *Pseudomonas fluorescens* (DSM 50090), *Pseudomonas fluorescens* (RIPAC), *Pseudomonas marginalis* (DSM 13124), *Pectobacterium carotovorum* (DSM 30168) and *Listeria innocua* (DSM 20649); in a concentration of 10^8 cfu ml^{-1} and subsequently treated with PPW. For PPW production, the plasma was ignited for 5, 15 or 50 s. After a post-plasma treatment with PPW of maximum 5 minutes, a decrease of bacterial load up to 6 log were detected for *P. fluorescens* (DSM-strain) on PET, *P. marginalis* and *P. carotovorum* on salad. For all other bacteria and specimen the inactivation rate was lower. Furthermore, visual examinations after 8 days of storage showed only little influences on the texture and the appearance of the tested specimens. The characteristics of plasma and its generated cocktail of long living chemical compounds in air and in water leading to a high bacterial inactivation and offering a wide range of possible applications.

Key words: biological decontamination, fresh food, microwave plasma, microorganism, plasma processed water

1. Introduction

Fresh and fresh-cut produce have a limited shelf life of several days, which allows only a regional distribution of that produce. The limited shelf life and the associated losses of fresh produce have various causes, but especially depend on microbial contamination at all stages in the value chain. The microbial contamination may also cause foodborne illnesses, which occur annually and worldwide. Especially, produce like fresh-cut salad and fresh sprouts are frequently affected. The U.S. Food and Drug Administration (FDA) listed both, leafy greens and sprouts, under the ten riskiest foods in their Center for Science in the Public Interest (CSPI) Report 2009. Whereby leafy greens are on the top [1]. European institutions and customer organizations like the German Institute for Risk Assessment (BfR) are aware of the risk of food borne illnesses caused by fresh-cut salad and sprouts, too [2, 3].

Both products are pointed out to be loaded with high amounts of bacteria, even with human pathogens like *Listeria monocytogenes*, under non-cooled and cooled conditions. The European Food Safety

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Authority (EFSA) described in their zoonoses report of 2011 [4] 5648 reported food-borne outbreaks for 2011 with more than 200,000 confirmed human cases. The outbreaks were caused by *Bacillus* toxins, *Campylobacter*, *Clostridium*, *E. coli* (mainly Verotoxin-producing *Escherichia coli* (VTEC), *Listeria*, *Yersinia* and some others. Ready-to-eat salad and sprouts were also contaminated with these microorganisms.

In the *E. coli* outbreaks, 50% of the involved products were vegetables and juices and other products thereof. One reason for this was a large outbreak of haemolytic-uraemic syndrome (HUS) and bloody diarrhea associated with shiga-like toxin-producing *E. coli* (STEC) O104:H4 infections occurred primarily in northern Germany from May to July 2011.

This outbreak is the largest recorded to date in Germany and, based on the number of cases of HUS, is the largest outbreak of this sort worldwide.

Fenugreek sprouts were identified as the most likely vehicle of infection. In total, 3,793 STEC cases, including 827 HUS cases with death for 35 patients and 2,966 cases of acute gastroenteritis with death for 18 patients, were reported. Internationally, 137 cases, including 54 HUS cases, in 15 European and non-European countries related to the outbreak were reported.

This is just one example of the remarkable impact of foodborne disease on the health of consumers and resulting direct and indirect consequences, costs and losses.

In general, a great demand regarding gentle sanitation in the production and processing of fresh produce exists because of the significant economic importance.

Conventional methods of decontamination and cleaning of fresh food are based on rinsing with water which may contain high amounts of chemicals, e.g., chlorine (50-200 ppm), chlorine dioxide or ozone. Although the poor stability of chlorine and the association of chlorine with a possible formation of carcinogenic chlorinated compounds in water have called the use of chlorine in food processing applications into question [7, 8]. Water containing disinfectant eliminates 3 to 4 log of microorganisms in solution and prevents them from attaching to the product surface. However, once bacteria are attached or internalized, no effective method exists to remove or destroy the contamination [5, 6].

Therefore the development of environmentally friendly alternative disinfection and cleaning methods is important, but also the product compatibility, costs, environmental impact, impact on product quality and regulatory provisions have to be taken into account [9]. One possible alternative method could be the application of non-thermal atmospheric pressure plasma.

Plasmas are ionized gases with a high proportion of free charged particles such as ions and electrons. The application of non-thermal atmospheric pressure plasma is a discipline with increasing attention in the field of food processing and an emerging non-thermal technology for reducing microbial load on the surface of fresh and processed foods. Thus the potential applications of non-thermal atmospheric pressure plasma for the food industry are manifold and it has specific potential for the treatment of foods [10-12]. For example dry decontamination of food surfaces, granular and particulate foods, and sprouted seeds could be carried out with that method. Furthermore, the surface of packaging material could be sterilized [13-15].

Non-thermal plasma is implemented in the food industry for the decontamination of raw agricultural products such as apples, lettuce, almonds, mangoes, melons, egg surfaces, cooked meat, and cheese [10, 16]. Non-thermal plasma is also suitable for processes, in which high temperatures are not recommended [14, 17].

The results of investigations on inactivation kinetics due to plasma processed water of two microbiological

contaminated fresh produce and one contaminated plastic are presented in this work. As fresh produce fresh-cut iceberg lettuce (*Lactuca sativa*) and fresh mung bean (*Vigna radiata*) sprouts were examined. The investigated plastic was PET (Polyethylene terephthalate), which is a typical packaging material in food industry. The microorganisms used in this study were the gram-negatives *Escherichia coli*, *Pseudomonas marginalis, Pseudomonas fluorescens* and *Pectobacterium carotovorum* as well as the gram-positive *Listeria innocua*.

2. Materials and Methods

2.1 Investigated Microorganisms and Specimens

For microbiological experiments *Escherichia coli* K12 (DSM 11250), *Pseudomonas fluorescens* (DSM 50090), *Pseudomonas fluorescens* (RIPAC), *Pseudomonas marginalis* (DSM 13124), *Pectobacterium carotovorum* (DSM 30168) and *Listeria innocua* (DSM 20649) were used in concentrations of 10^8 cfu ml^{-1} suspended in sterile, distilled water, see also Table 1. As specimens PET-stripes with dimensions of 32×8×2 mm, fresh-cut iceberg lettuce (*Lactuca sativa*) with pieces of 2×2 cm^2 and fresh mung bean sprouts (*Vigna radiata*) were investigated. For the two last, the amount of 10 g was contaminated. The iceberg lettuce and the mung bean sprouts were bought at a local supermarket one day before usage.

Table 1 Bacteria Strains Used in This Work

microorganism	DSM number	ATCC/ NCTC number
Escherichia coli	DSM 11250	NCTC 10538
Pseudomonas fluorescens	DSM 50090	ATCC 13525
Pseudomonas marginalis	DSM 13124	ATCC 10844
Pectobacterium carotovorum	DSM 30168	ATCC 15713
Listeria innocua	DSM 20649	ATCC 33090
***Pseudomonas fluorescens* (RIPAC)**	directly isolated from cantaloupe, RIPAC number: D13-0092-1-1-13	

Escherichia coli K12 (DSM 11250) and *Listeria innocua* (DSM 20649) were chosen due to their relationship to enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 and *Listeria monocytogenes*, both human pathogens which could occur on food. However, the chosen strains are classified as risk level 1 and therefore easy to handle.

Pseudomonas fluorescens (DSM 50090), *Pseudomonas fluorescens* (RIPAC), *Pseudomonas marginalis* (DSM 13124) and *Pectobacterium carotovorum* (DSM 30168) occur in soil or on plants and can cause spoilage of food or storage losses, e.g., by soft rot.

2.2 Contamination of Specimens

The PET-stripes, the fresh-cut lettuce and the fresh sprouts were contaminated with the bacterial suspension by pipetting 100 µl suspension on the stripes or immerse the fresh produce in 200 ml suspension with a concentration of 10^8 cfu ml^{-1} for 5 minutes. The lettuce and the sprouts were dried with a salad spinner or drained on a stainless steel sieve. Afterwards, the fresh products were kept under sterile and cool conditions (7°C) for 30 minutes and the suspension on the PET-stripes could dry under laminar flow for 2 hours.

2.3 Recovery of Microbial Contamination of Each Specimen

The recovery of microbial contamination or its residues on the specimens after decontamination was realized by transferring them from the PPW beakers to ones with nutrient broth (tryptic soy broth from Merck KGaA, Darmstadt, Germany or standard nutrient broth I from Carl Roth GmbH+Co.KG, Karlsruhe, Germany). By shaking both the PET-stripes and the fresh products in 10 ml and 30 ml broth for 10 minutes, respectively and by using the surface-spread-plate count method with tryptic soy agar (Merck KGaA, Darmstadt, Germany) or standard nutrient agar I (Carl Roth GmbH+Co.KG, Karlsruhe,

Germany) plates, the recovery was completed with an overnight cultivation in an incubator. The surface-spread-plate count method is a surface counting method employed for aerobic bacteria. 100 µl of all serial dilutions of the broth were plated out on the whole surface-area of the petri dish. Serial dilutions were performed as a 1 in 10 dilution.

The detection limit of this procedure was 1 cfu ml^{-1}. If the numbers of microorganisms fell below the detection limit, i.e., no viable microorganisms have been found, the values were set at detection limit in the graphical representations.

2.4 Decontamination by Microwave Plasma Processed Water (PPW) and Tap Water (PPtW)

Non-thermal plasma treatment of the contaminated specimens was done with microwave driven discharge processed gas in contact with water. The used microwave driven discharge set-up is shown in Fig. 1. The microwaves had a frequency of 2.45 GHz and the supply power was in the range of 1.1 kW. Accordingly, the gas temperature was about 4000 K at a gas flux of 18 slm air. The so called generated PPA was introduced into distilled water or tap water and the resulting PPW was then used to decontaminate the PET-stripes as well as the fresh produce. The contaminated vegetable specimens were placed in a 250 ml glass beaker and immersed in the PPW and PPtW or placed on a stainless steel sieve and sprinkled with PPW. The contaminated PET-stripes were placed in a lower shell of a sterile petri dish and covered with the PPW by pipetting 200 µl on the specimens. The discharge was ignited for 5, 15 or 50 s. The specimens were treated for 1, 3 and 5 minutes with the PPW or PPtW (post-treatment time). The observed inactivation of microorganisms depended on the storage with long-lived reactive chemical species in the water and acidification during post-plasma treatment time.

Measurements of the pH value were analyzed with a pH-meter (Multi 3420 — WTW Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) and the pH electrode SenTix® Mic (pH 0-14/0-100°C — WTW Wissenschaftlich- Technische Werkstätten GmbH, Weilheim, Germany) directly after PPW or PPtW generation. The observed pH values in dependency of the plasma-on time are shown in Table 2.

2.5 Statistical Analysis

Data presented were mean of the logarithmic values of replicated experiments. Significant differences among non-treated references and countable plasma-treated samples were determined by the independent two-sample t-test for unequal variances also known as Welch's t-test. For calculation the T.Test function implemented in Microsoft® Excel was used.

2.6 Visual Verification of Product Quality after PPW Treatment

For the investigation of food quality for fresh-cut iceberg lettuce and fresh mung bean sprouts after PPW treatment, a visual verification was carried out. Both, the fresh-cut lettuce and the fresh sprouts were treated with the PPW under the same conditions used for decontamination measurements. For each specimen,

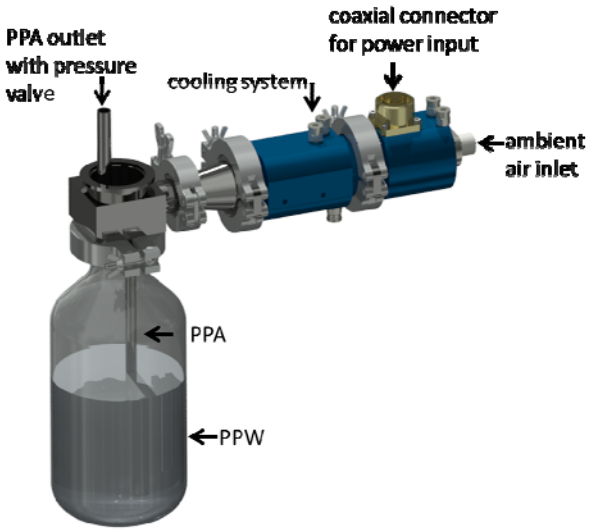

Fig. 1 Scheme of the Microwave-Setup for the Generation of PPW (Plasma Processed Water) and of PPtW (Plasma Processed Tap Water) [48]

Table 2 pH Values of PPW and PPtW

pre-treatment time (s)	PPW	PPtW
0	6.06	6.5
5	1.94	2.45
15	1.56	1.85
50	1.28	1.4

10 g lettuce or sprouts were used. The PPW was generated by PPA of 5, 15 and 50 seconds plasma on time (pre-treatment time). The fresh produce was immersed in the PPW for 5 minutes post-treatment time. After the treatment the specimens were spinned or drained and finally stored at 7°C in a refrigerator for 8 days. At day 0, 1, 6, 7 and 8 after storage all specimens were viewed and photos were taken (Figs. 6 and 7).

3. Results

The investigation of antimicrobial effects of PPW and PPtW (plasma processed tap water) on fresh biological surfaces e.g. fresh produce were based on a previous work with the use of PPA [18, 19].

The optimized plasma parameters for PPA production and the subsequent preparation of PPW and PPtW were taken from the latter publications.

3.1 Inactivation of Different Bacteria on PET-stripes by PPW

The investigations with PET-stripes were done under the aspect to start with an artificial but rough surface which is easy to handle and available.

By pipetting, the complete area of 256 mm^2 was covered with all mentioned microbial suspensions in concentrations of 10^8 cfu ml^{-1}

The surface drying was controlled by visual inspection. All PET-stripes were treated with PPW generated by microwave PPA treatment of distilled water. The PPA was generated in three different concentrations achieved by a 5, 15 and 50 second microwave plasma ignition (pre-treatment time). The post-treatment times of the PPW were 1, 3 and 5 minutes. The timescales reflected the time of contact between PET and PPW.

Experimental results (Fig. 2) showed an antimicrobial reduction of 2.5 log steps for *P. carotovorum* up to 6.0 log-steps for *P. fluorescens* (DSM-strain) maximum. Apart from *P. fluorescens* (DSM-strain), all investigated bacteria showed similar inactivation kinetics as well as a tailing after a 5 second plasma-on time. This indicates, that a prolonged post-treatment time has no further significant influence. However, regarding the increasing pre-treatment time, the inactivation capacity of PPW increased (Fig. 2B and E). For the combination of *E. coli* and PET (Fig. 2A), a tailing also for the pre-treatment time was gained and the need for a certain pre-treatment time which must be exceeded to achieve higher inactivation rates was seen for Fig. 2C, D and F. All gained results showed very small error bars.

3.2 Inactivation of Different Bacteria on Fresh-Cut Iceberg Lettuce by PPW

The analysis with fresh-cut iceberg lettuce was done due to the fact that this produce is commercially important, especially in the field/section of RTE food. The commercial need of such a product is foiled by high production losses and a risk of foodborne illnesses due to microbial contaminations.

Fresh-cut iceberg lettuce is easy to handle, almost always available throughout the year, and offers an uneven surface.

The complete head of lettuce was cut into pieces of 400 mm^2 and for each specimen, 10 g were used. The fresh-cut lettuce was completely immersed in all mentioned bacterial suspensions in concentrations of 10^8 cfu ml^{-1}. After spinning, the specimen was stored in the refrigerator at 7°C for 30 minutes.

All lettuce specimens were treated with PPW generated by microwave PPA treatment of distilled water. The PPA was generated in three different concentrations by a 5, 15 and 50 second microwave

plasma ignition (pre-treatment time) and the post-treatment times of the PPW were 1, 3 and 5 minutes. The timescales reflected the time of contact between fresh produce and PPW.

The experimental results (Fig. 3) showed an antimicrobial reduction of 1.8 log steps for *P. fluorescens* (DSMZ) up to 6.1 log-steps for *P. marginalis* and *P. carotovorum* maximum. The investigated bacteria from A to C (*E. coli*, *P. fluorescens* (DSMZ) and *P. fluorescens* (RIPAC)) showed similar inactivation kinetics as well as a tailing after a 5 second plasma-on time. The bacteria from D to F (*P. marginalis*, *P. carotovorum* and *L. innocua*) had also similar kinetics and showed tailing characteristics. However, with the 50 seconds pre-treatment time the inactivating effects were very strong, in these three cases the detection limit was reached after 1 to 3 minutes post-treatment time with very high inactivation rates.

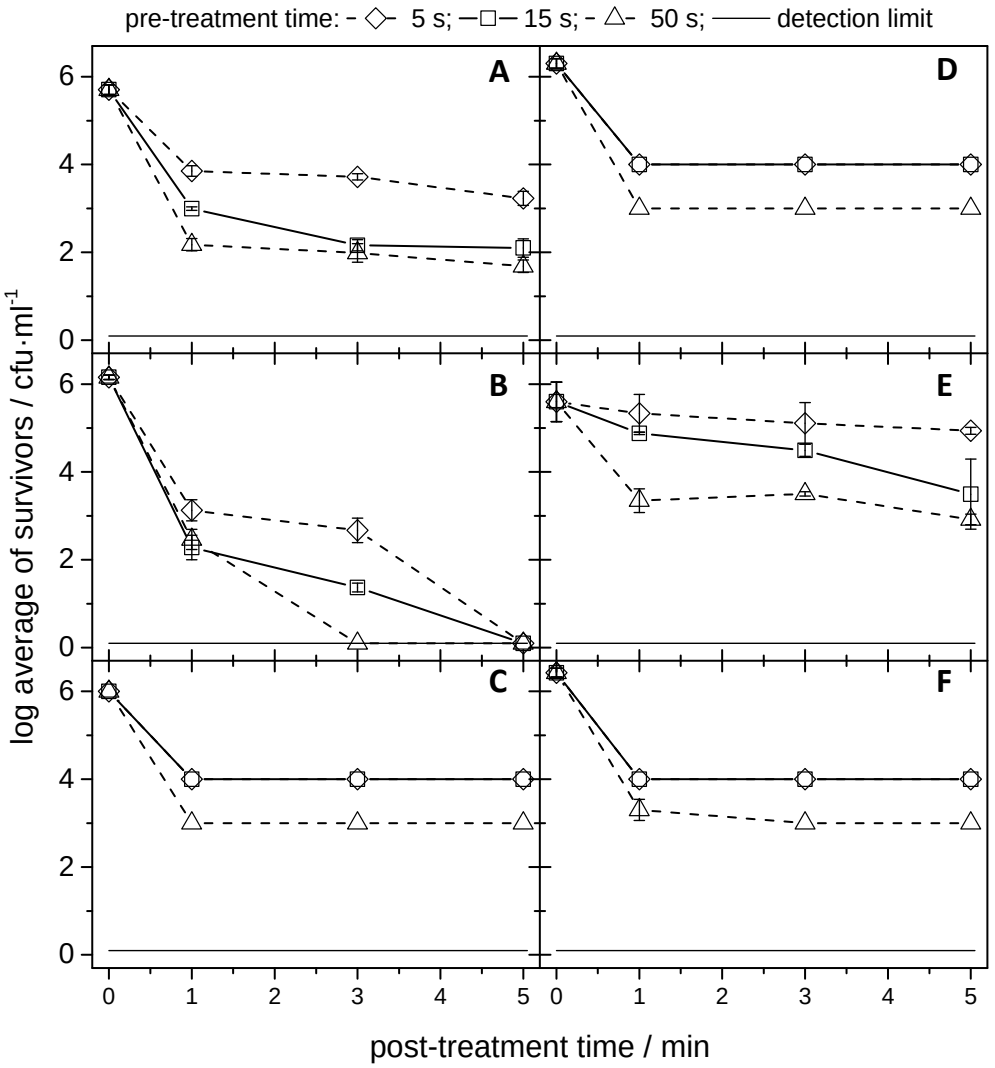

Fig. 2 Results of the PPW Treatment of PET-stripes (32×8×2 mm). PET-stripes Were Contaminated with Six Different Microorganisms – *E. coli* (A), *P. fluorescens* DSM 50090 (B), *P. fluorescens* RIPAC (C), *P. marginalis* (D), *P. carotovorum* (E) and *L. innocua* (F) in Concentrations of 10^6 cfu ml^{-1}. After a Plasma Ignition for 5, 15 and 50 Seconds (Pre-treatment Time) the PET-stripes Were Completely Covered with Plasma Processed Water (PPW) in Durations of 1, 3 and 5 Minutes (Post-treatment Time). The Average of Three Experiments Is Shown. Experiments Were Done with n = 3.

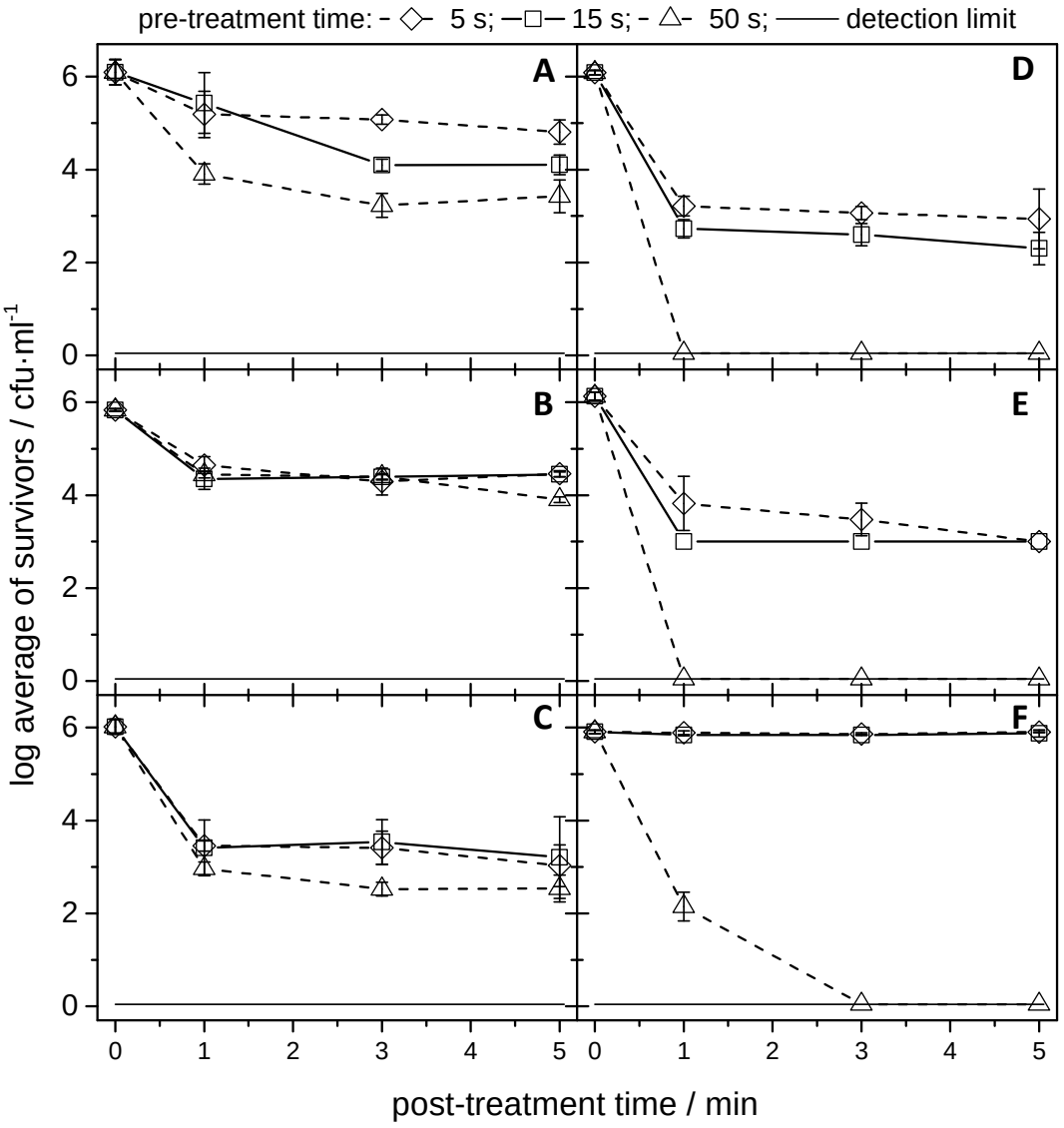

Fig. 3 Results of the PPW Treatment of Fresh-cut Iceberg Lettuce (10 g of 2×2 cm pieces). Lettuce Was Contaminated with Six Different Microorganisms – *E. coli* (A), *P. fluorescens* DSM 50090 (B), *P. fluorescens* RIPAC (C), *P. marginalis* (D), *P. carotovorum* (E) and *L. innocua* (F) in Concentrations of 10^6 cfu ml^{-1}. After a Plasma Ignition for 5, 15 and 50 Seconds (Pre-Treatment Time) the Fresh-Cut Lettuce Was Completely Covered with Plasma Processed Water (PPW) in Durations of 1, 3 and 5 Minutes (Post-Treatment Time). The Average of Three Experiments is Shown. Experiments Were Done with n = 3.

The observed results indicate, that a pre-treatment time for PPW-generation led to increasing inactivation rates for *E. coli*. For the combination of *P. fluorescens* (DSM-strain) and salad (Fig. 3B), a tailing also for the pre-treatment time was gained and the need for a certain pre-treatment time which must be exceeded to achieve higher inactivation rates was seen for Fig. 3C to F. All gained results showed very small error bars.

Due to microbial recoveries from specimen suspended in PPW, microorganisms might strongly adhere to the fresh produce surfaces; the recovery difference from initial concentration to the reference was between 1.9 and 2.2 log steps. Also, the maximal 10% increase in weight due to produce immersion in bacterial suspension at the contamination step may be a reason.

3.3 Inactivation of Different Bacteria on Fresh Mung Bean Sprouts by PPW

Especially in the field of RTE food mung bean sprouts were also of commercial importance, but with a high risk of foodborne illnesses dependent on microbial contamination beginning at the step of packaging. Hence, fresh mung bean sprouts were considered in an own series of tests, too. Fresh mung bean sprouts are easy to handle and almost always available throughout the year. They offer a large surface and thus a high release of cell liquor by breaking inside the packaging and show many shadowing areas by overlapping 10 g of commercially distributed sprouts were used for each sample and completely immersed in all mentioned bacterial suspensions in concentrations of 10^8 cfu ml^{-1}. The contaminated sprouts drained on a stainless steel sieve in the refrigerator at 7°C for 30 minutes.

All sprouts specimens were treated with PPW generated by microwave PPA treatment of distilled water. The PPA was generated in three different concentrations by a 5, 15 and 50 second microwave plasma ignition (pre-treatment time) whereas the post-treatment times of the PPW were 1, 3, and 5 minutes. This was the time of contact between fresh produce and PPW.

The experimental results (Fig. 4) showed an antimicrobial reduction of 2.5 log steps for *P. marginalis and P. carotovorum* up to 3.5 log-steps for all other bacteria maximum. All inactivation kinetics showed a tailing or only a slight decrease after a 5 second plasma-on time. None of the investigated bacteria seemed to be specifically adapted to mung bean sprouts.

The observed results indicate, that a pre-treatment time for PPW-generation led to increasing inactivation rates for three bacterial strains (Fig. 4A, C and E). For *P. fluorescens* (DSM-strain) as well as for *P. marginalis* (Fig. 4B and D), a tailing also for the pre-treatment time was gained and the need for a certain pre-treatment time which must be exceeded to achieve higher inactivation rates was only seen for *P. carotovorum*. All gained results showed very small error bars.

The observed results indicate, that a pre-treatment time for PPW-generation led to increasing inactivation rates for three bacterial strains (Fig. 4A, C and E). For *P. fluorescens* (DSM-strain) as well as for *P. marginalis* (Fig. 4B and D), a tailing also for the pre-treatment time was gained and the need for a certain pre-treatment time which must be exceeded to achieve higher inactivation rates was only seen for *P. carotovorum*. All gained results showed very small error bars.

Due to microbial recoveries from specimen suspended in PPW, microorganisms might strongly adhere to the fresh produce surfaces; the recovery difference from initial concentration to the reference was between 0.8 and 1.6 log steps. Also, the maximal 10% increase in weight due to produce immersion in bacterial suspension at the contamination step may be a reason.

3.4 Inactivation of L. innocua on Fresh-Cut Iceberg Lettuce by Sprinkled PPW or by PPtW

The experiments with PPW made from distilled water and the immersion of the specimens in it were for scientific aspects such as the exclusion of undefined parameters of the used water or to ensure a complete PPW coverage of the produce surface, respectively.

However, due to the amounts of the used water, the laboratorial approach cannot be translated into an industrial practice. Therefore, two different treatment options of PPW for the decontamination of fresh produce were investigated.

The first one was to replace the PPW immersion by a sprinkling technique which covers the lettuce with PPW and secondly, a replacement of the distilled water by tap water. These investigations were carried out for iceberg lettuce contaminated with *L. innocua*.

For sprinkling the lettuce with PPW, it was placed on a stainless steel sieve with minimal contact area.

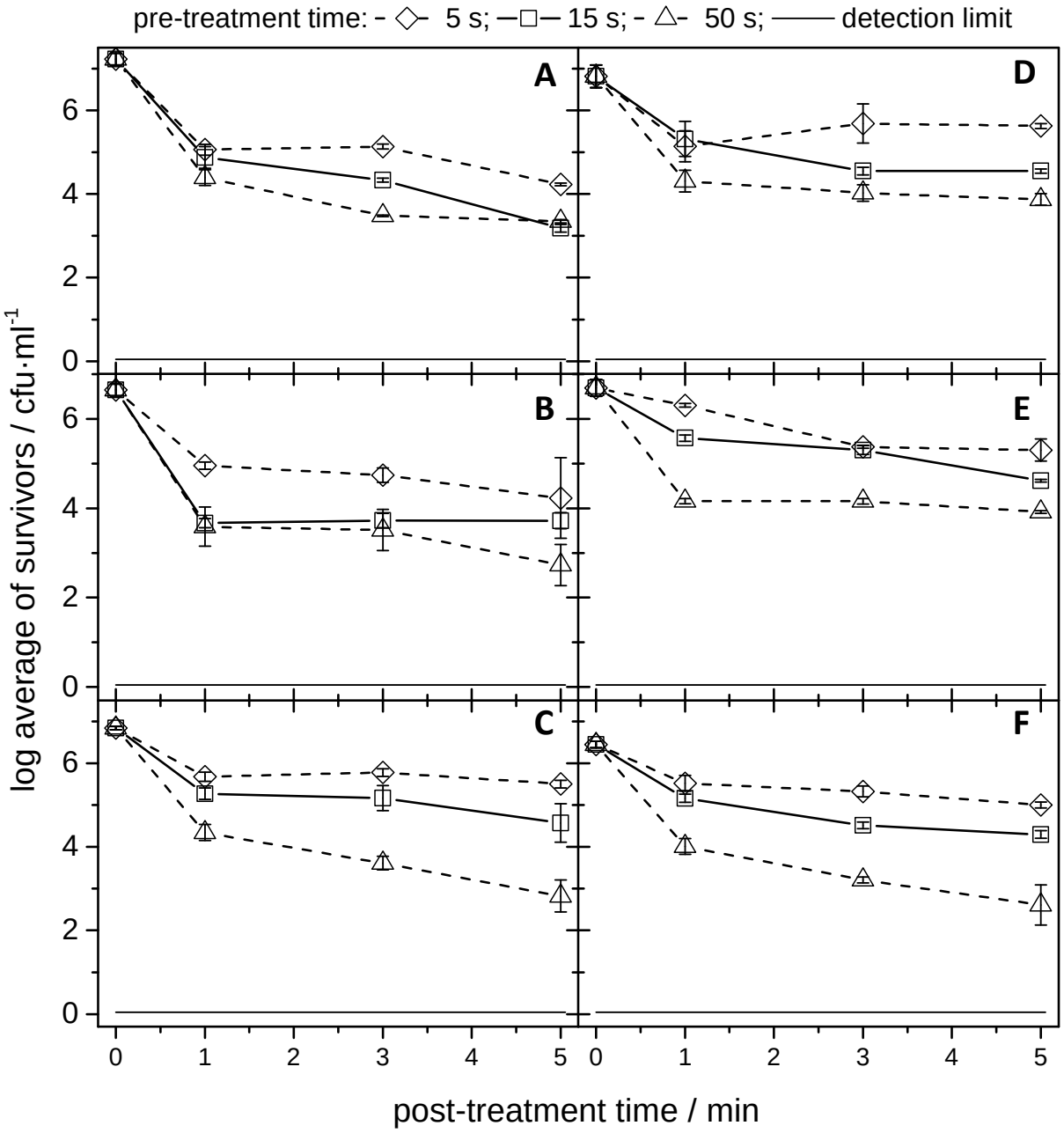

Fig. 4 Results of the PPW Treatment of Fresh Mung Bean Sprouts (10 g). Sprouts Were Contaminated with Six Different Microorganisms – *E. coli* (A), *P. fluorescens* DSM 50090 (B), *P. fluorescens* RIPAC (C), *P. marginalis* (D), *P. carotovorum* (E) and *L. innocua* (F) in Concentrations of 10^6 cfu ml^{-1}. After a Plasma Ignition for 5, 15 and 50 Seconds (Pre-treatment Time) the Sprouts Were Completely Covered with Plasma Processed Water (PPW) in Durations of 1, 3 and 5 Minutes (Post-treatment Time). The Average of Three Experiments Is Shown. Experiments Were Done with n = 3.

The experimental results (Fig. 5) showed a maximum antimicrobial reduction of 2.8 log steps for both options. Compared to the standard PPW technique (Fig. 3F and 5A), the observed inactivation effects for sprinkled PPW and PPtW was half of the values obtained by laboratory-based methods. However, these results were only obtained for specimens treated under 50 seconds' pre-treatment time conditions. For shorter pre-treatment times, both alternatives showed a higher inactivation capability.

Again, the inactivation kinetics showed a tailing after a 5 second plasma-on time.

In Fig. 5 the increasing pre-treatment time for PPW-generation resulted in the need to exceed an inhibition threshold for the standard PPW technique (Fig. 3F and 5A). Usage of sprinkled PPW led to an increased inactivation (B) and of PPtW to a tailing also for the pre-treatment time. All gained results showed very small error bars.

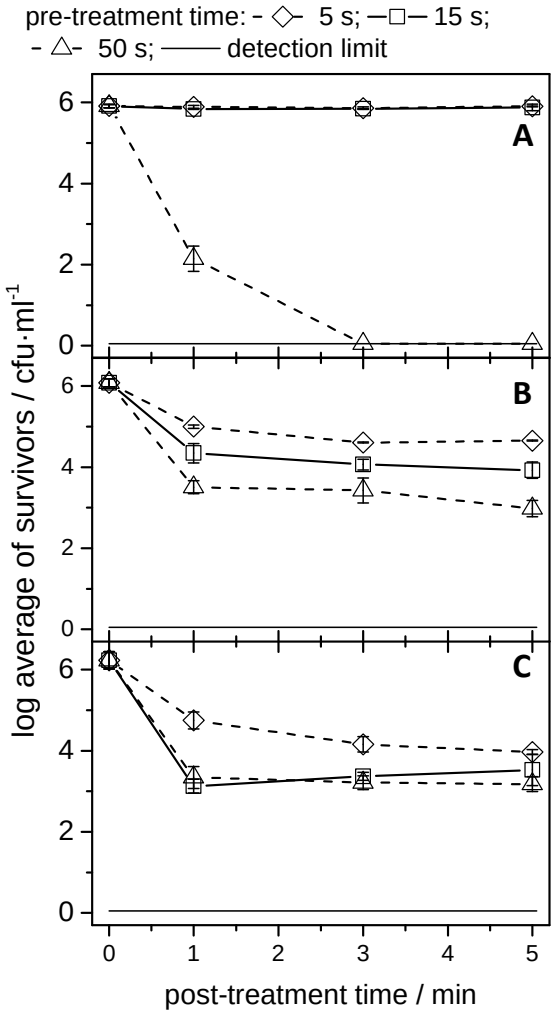

Fig. 5 Results of the PPW Treatment of Fresh-Cut Iceberg Lettuce (10 g of 2×2 cm Pieces). Lettuce Was Contaminated with *L. innocua* in Concentrations of 10^6 cfu ml^{-1}. After a Plasma Ignition for 5, 15 and 50 Seconds (Pre-treatment Time) Fresh-Cut Lettuce Was (A) Completely Covered with PPW, (B) Covered with PPW by a Sprinkling Technique or (C) Completely Covered with PPtW in Durations of 1, 3 and 5 Minutes (Post-Treatment Time). The Average of Three Experiments Is Shown. Experiments Were Done with n = 3.

3.5 Statistical Analysis

The results of statistical analysis of the experimental data presented in Figs. 2-5 are shown in the Tables 3-6. It is obvious that for all reduction above one order of magnitude, the experimental data are significant different to the control.

Table 3 Results of the Statistical Analysis Done with the T-Test For PET-Stripes. The Shown Values Are the P-Values Multiplied by 1000 for Better Readability of the Table.

	p-values × 1000								
	PET-stripes								
pre-reatment time (s)	5			15			50		
post-treatment time (min)	1	3	5	1	3	5	1	3	5
E. coli	23	23	22	22	22	22	22	22	22
P. fluorescens DSM	4	4	4	4	4	4	4	4	4
P. fluorescens RIPAC	76	76	76	76	76	76	74	74	74
P. marginalis	75	75	75	75	75	75	74	74	74
P. carotovorum	518	227	187	178	145	129	129	129	128
L. innocua	15	15	15	15	15	15	15	15	15

Table 4 Results of Statistical Analysis Done with the t-test for Fresh-Cut Iceberg Lettuce. The Shown Values Are the p-values Multiplied by 1000 for Better Readability of the Table.

	fresh-cut iceberg lettuce								
	p-values × 1000								
pre-reatment time (s)	5			15			50		
post-treatment time (min)	1	3	5	1	3	5	1	3	5
E. coli	124	124	114	143	107	107	106	105	105
P. fluorescens DSM	1	1	2	1	2	2	2	2	2
P. fluorescens RIPAC	33	33	33	33	33	33	33	33	33
P. marginalis	4	4	4	4	4	4	4	4	4
P. carotovorum	16	16	16	16	16	16	16	16	16
L. innocua	641	211	836	114	124	584	3	3	3

Table 5 Results of Statistical Analysis Done with the t-test for Fresh Mung Bean Sprouts. The Shown Values Are the p-values Multiplied by 1000 for Better Readability of the Table.

	fresh mung bean sprouts								
	p-values × 1000								
pre-reatment time (s)	5			15			50		
post-treatment time (min)	1	3	5	1	3	5	1	3	5
E. coli	31	33	28	30	28	28	29	28	28
P. fluorescens DSM	33	32	32	32	32	32	32	32	32
P. fluorescens RIPAC	3	3	3	3	3	3	3	3	3
P. marginalis	79	85	85	80	77	77	76	76	76
P. carotovorum	130	58	57	61	57	54	53	53	53
L. innocua	11	14	13	13	13	12	12	12	12

Table 6 Results of Statistical Analysis Done with the t-test for Fresh-Iceberg Lettuce Contaminated with *L. innocua*. The Shown Values Are the p-values Multiplied by 1000 for Better Readability of the Table.

	L. innocua								
	p-values x 1000								
pre-reatment time (s)	5			15			50		
post-treatment time (min)	1	3	5	1	3	5	1	3	5
immereged in PPW	641	211	836	114	124	584	3	3	3
sprinkled with PPW	114	109	109	107	106	106	105	105	105
immereged in PPtW	54	51	51	51	51	51	51	51	51

3.6 Visual Verification of Product Quality after PPW Treatment

The investigations described above were focused on the decontamination of fresh-cut iceberg lettuce and fresh mung bean sprouts.

In the context of food quality after a PPW-plasma treatment, a subsequent visual verification of the lettuce and the sprouts was carried out.

Both, the fresh-cut lettuce and the fresh sprouts were treated with the PPW under the same conditions used for decontamination measurements. However, the investigations were done for the longest post-treatment time of 5 minutes. For each specimen, 10 g of lettuce or sprouts were used. After the treatment the specimens were spinned or drained and finally stored at 7°C in a refrigerator up to 8 days. At day 0, 1, 6, 7, and 8 after storage all specimens were viewed and photos were taken (Figs. 6 and 7).

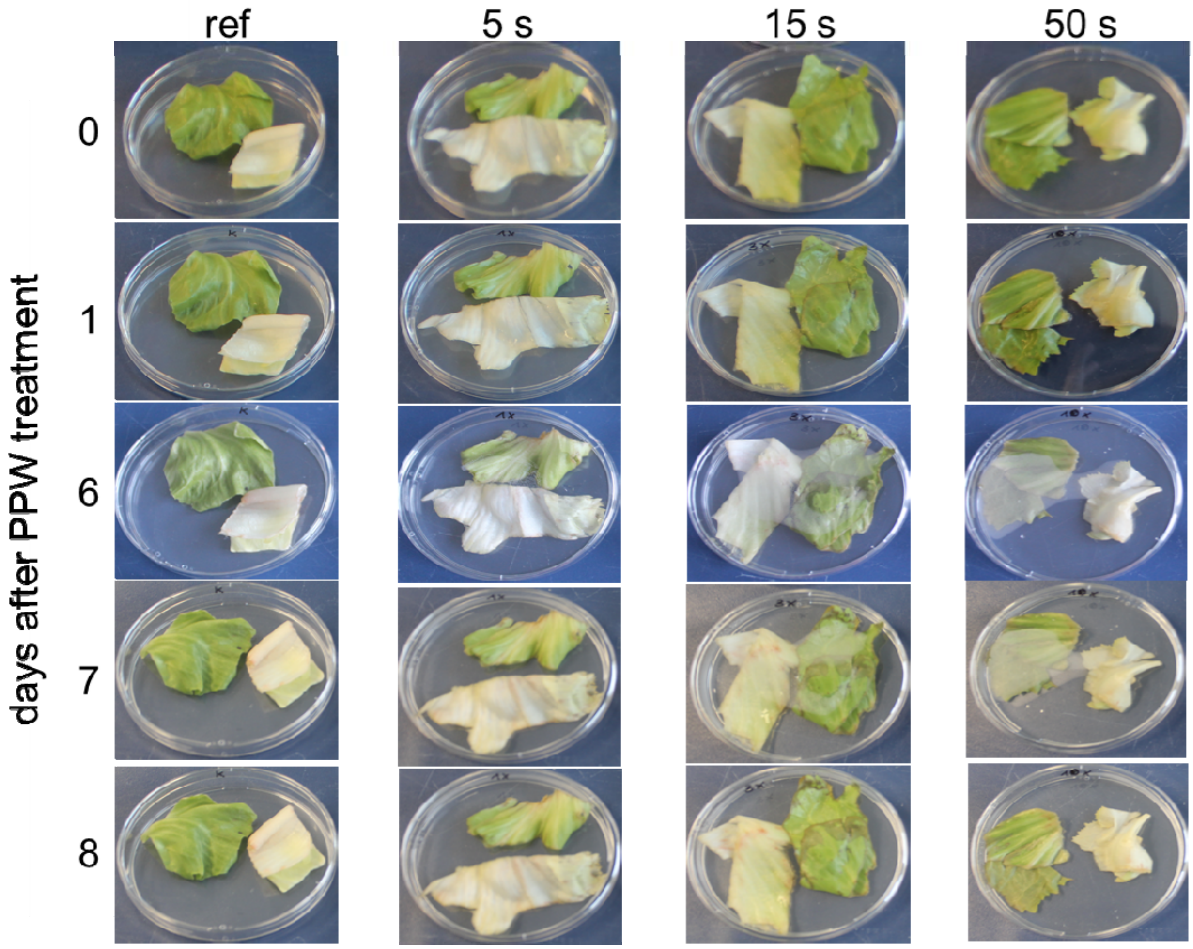

Fig. 6 Results of the Visual Verifications of Fresh-Cut Iceberg Lettuce after PPW Treatment in Duration of 5 Minutes (Post-Treatment Time). PPW Production Was Done by Plasma Ignitions for 5, 15 and 50 Seconds (Pre-Treatment Time). The Specimens Were Completely Covered with PPW.

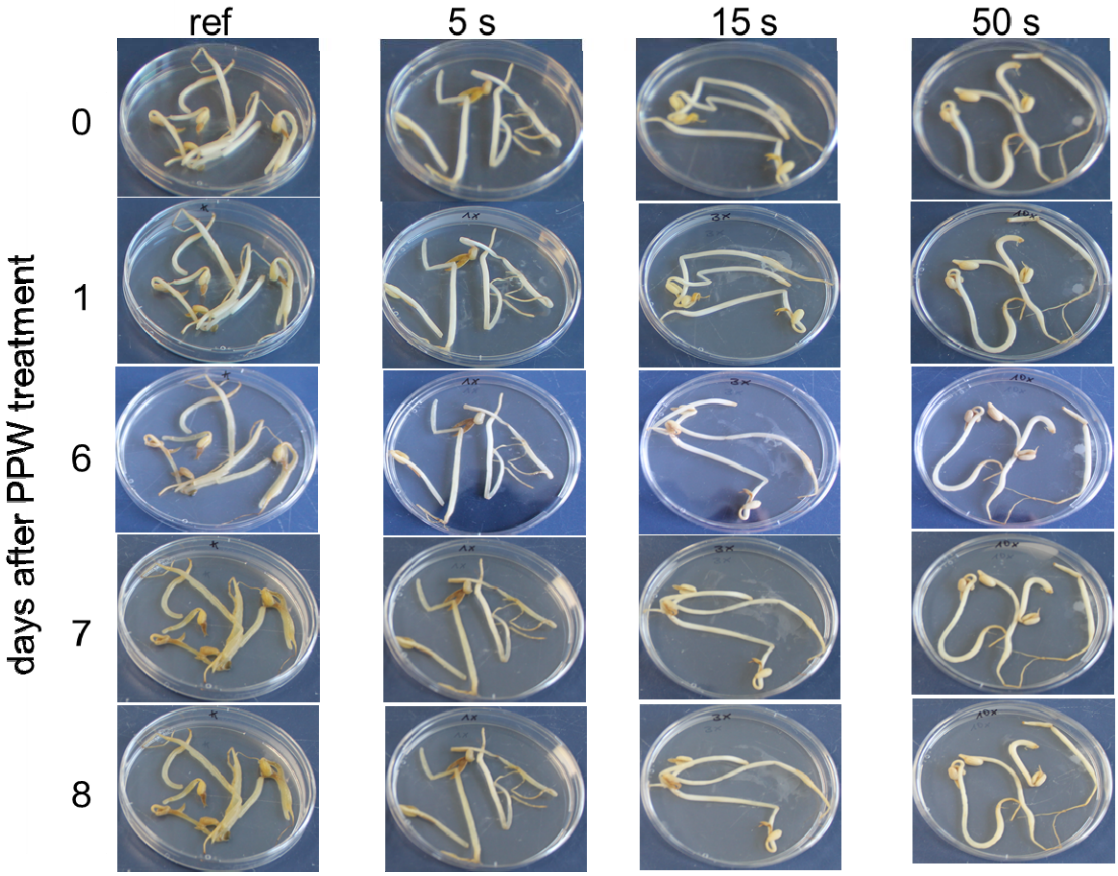

Fig. 7 Results of the Visual Verifications of Fresh Mung Beans after PPW Treatment in Duration of 5 Minutes (Post-Treatment Time). PPW Production Was Done by Plasma Ignitions for 5, 15 and 50 Seconds (Pre-Treatment Time). The Specimens Were Completely Covered with PPW.

Fresh-cut iceberg lettuce had no significant difference to the untreated references. In all cases the sectional areas become slightly glassy and brownish after storage of 8 days. For the fresh mung bean sprouts a significant difference between the untreated and treated specimens was observed. The untreated samples became strongly glassy and cell liquor was released. Compared to the references, the treated samples showed a slight glassiness only for the 5 second plasma-generated PPW, for the 15 and 50 second ones no influences were observed.

4. Discussion

In the manufacture of food, good hygiene is a key part of the quality assurance, i.e., ensuring that the product is within the microbial specifications appropriate to its use. Poor hygienic conditions and inadequate sanitation will result in healthcare-associated infections and foodborne diseases as well as high production losses in food industry. Therefore, the inactivation of human- and phyto-pathogens is of great interest in many social and economic fields.

Some typical human pathogens which can be found in food are *E. coli*, *L. monocytogenes*, *Salmonella*, *Yersinia*, *S. aureus* — even MRSA (methicillin-resistant *S. aureus*) and ORSA (oxacillin-resistant *S. aureus*), *Clostridium* and *Aspergillus*. In a selection of phyto-pathogens many molds (e.g., *Fusarium*), oomycetes, *Xanthomonas*, *Erwinia* including the new group of *Pectobacteria* and *Pseudomonas* can be found [4, 20].

The investigated bacteria represent possible food contaminations, gram-positive and gram-negative,

which are often responsible for human or plant diseases.

Non-thermal plasma treatment of foods is a promising technology in that it acts rapidly, does not leave toxic residuals on processed parts or in the exhaust gas and the temperature rise can be kept at an acceptable level [15].

The combination of plasma species with a non-thermal treatment mode makes non-thermal plasmas particularly suited for decontamination in food processing settings [21-25]. This process is practical, inexpensive, and suitable for decontamination of products where heat is not desirable [26].

For the inactivation of *E. coli*, *Pseudomonas* and other microorganisms by microwave generated PPW and PPtW described within, only physical stresses by chemical, acidic and biocidic agents are important. Other stresses such as temperature, pressure, or radiation can be excluded due to the experimental set-up.

The observed kinetics of antimicrobial inactivation of the investigated microorganisms on PET, lettuce, and sprouts are very different and in most cases a tailing was observed after 1 minute's post-treatment time**.** One reason for this tailing may be an impact of the PPW chemistry to the bacteria in that way that they may attach better to the rough surfaces which would influence the recovery process and the detection in the used proliferation assay.

Apart from a single case (*P. fluorescens* DSM 50090), every PPW- and PPtW-treatment shows its best result for 50 seconds pre-treatment time. Here, the reason could be the strong acidification which was gained by increasing the pre-treatment time from 5 up to 50 seconds.

For sanitizing products, chlorinated water is also used which includes several disadvantages. Other agents are hydrogen peroxide or lactic acid [27, 28]. The inactivation mode of lactic acid can be attributed to an acidification process causing depression of the inner pH of microbial cells by ionization of the undissociated acid molecules or disruption of the substrate transport by alteration of the cell-membrane permeability. Additionally, foodborne bacteria cannot grow at pH-values lower than about 4.0. During the treatment of contaminated specimen in this study, an acidification on the specimen's environment (PPW) was observed and may lead to a similar inactivation mode comparable to the observed lactic acid mode.

Due to the plasma set-up and dry air (below 32% relative humidity) as working gas, chemical reactions and species mainly based on RNS (reactive nitrogen species) are expected. Nitrogen and oxygen in air react to nitrogen monoxide (NO*), which further leads to the generation of nitrogen dioxide (NO_2*) with oxygen (O_2). NO* and NO_2* are two stable radicals with known antimicrobial effectivity. Nitrogen monoxide may also react with ozone (O_3) to nitrogen dioxide and oxygen. Together, both radicals (NO*, NO_2*) can form dinitrogen trioxide (N_2O_3), which may react with ozone to nitrogen trioxide radical (NO_3*) via dinitrogen hexoxide (N_2O_6). Another product might be peroxynitrite ($ONOO^-$) throughout the reaction of NO* with superoxide radical (O_2^{*-}) [29, 30].

All these reactions are possible in dry air after plasma ignition. Taking into account that this processed air was combined with 10 or 30 ml distilled water, other chemical reactions may happen. NO*, O_2 and water (H_2O) react to nitrite (NO_2^-) and hydrogen (H^+). If instead of nitrogen monoxide NO_2* reacts with the other two molecules, H^+ and nitrate (NO_3^-) are the products. N_2O_3 is generated in gas and also gas/water phase and may react with H_2O to nitrite and hydrogen again.

Two radicals, NO_3* and NO_2* form dinitrogen pentoxide (N_2O_5) under the influence of water [29], [30]. The latter can react with water to nitrate and hydrogen. The occurrence of OH radicals was not detected. Reasons may be the absence of oxygen radicals (O*) due to no energy intake. A further

possibility may be water clusters such as $(H_2O)NO$ or $(H_2O)OH$.

The experiments showed a strong acidification which might be a result of nitrous acid (HNO_2) and nitric acid (HNO_3), the final end product of all reactions.

Usually HNO_2 decays to hydrogen (H^+) and nitrite (NO_2^-), but a pH-value beneath 2.75 could lead to a spontaneous forming of OH* and NO* radicals.

Most of the mentioned ions, radicals and molecules are highly toxic for microorganisms and the chemical cocktail as well as the pH shift may result in the gained inactivation. Further investigations on reactive species densities will provide a better insight into the chemical and biochemical processes underlying the antimicrobial effects observed and assumed in the presented work. Apart from that, the exploration of the mechanisms of inactivation of the target microorganisms might reveal relevant details about the plasma inactivation processes'.

Due to their different formation and composition of cell walls and membranes, commonly gram-negative bacteria are less resistant than gram-positives, which are followed by fungus, conidiospores and endospores for the treatment by physical plasmas [31]. This influence could not be observed in our results. No significant difference in the inactivation kinetics for gram-negatives as well as the gram-positive *L. innocua* is observed. Maybe the excluded plasma stresses like temperature, pressure and radiation which occur under direct and semi-direct plasma treatment are more responsible for affecting the bacteria walls. Additionally a higher impact of reactive oxygen species (ROS) like ozone or hydrogen peroxide in air and in water to affect bacteria walls due to lipid oxidation [32, 33] may play a role compared to RNS which are needed to generate the PPW.

It is also very interesting why microorganisms can be inactivated by ROS and RNS and the plant material is not or just slightly affected. In plants a local and systemic defence mechanism against microbicidal pathogens based on ROS and RNS already exist [34], [35]. NO* is an important messenger in the defense signal pathways [36, 37]. It was shown to be a crucial regulator of many physiological processes in plants, including stomatal closure and plant growth as well as development [38-44]. Nitrogen monoxide can regulate these processes directly by governing gene transcription. NO*-regulated genes are included in signal transduction, defence, cell death, transport, basic metabolism, and production as well as degradation of ROS. The radicals react as activators or inhibitors of enzymes, ion channels, transcription factors, and therefore regulate specific processes during stress situations in plants.

As described before, the formation of RNS, especially NO*, occurs in the presented plasma set-up. The achieved microbicidal effects indicate the antimicrobial efficiency of generated RNS.

Then treating consumable food with a novel developed innovative technology like non-thermal atmospheric pressure plasma as well as PPW described within this paper the food quality should be taken under consideration, too.

First publications confirmed an influence, especially when plasma is directly interacting with the product. For instance, references [45] and [46] investigated the influence of a direct treatment of lamb's lettuce by an atmospheric pressure plasma jet to its phenolic profile.

In studies with seeds, the post-plasma storage with PPA did not affect the germination [18].

The food quality of investigated fresh-cut lettuce and fresh mung bean sprouts showed for the first specimen no significant difference to the untreated references. In all cases the sectional areas become slightly glassy and brownish after storage of 8 days. The browning effect is due to enzymatic browning. The processing, e.g., cutting of RTE lettuce leads to injury of the plant tissue by cuts and fractures. Injuries lead to numerous wound stress reactions such as the increased synthesis of phenylalanine-ammonium lyase,

even in uninjured tissue areas. In a first step, this enzyme is responsible for the synthesis of phenolic compounds and catalyzes the formation of cinnamic acid from phenylalanine. Cinnamic acid is the initial compound for the formation of phenolic compounds which are oxidized to brown pigments by plant enzymes such as polyphenol oxidase. Through the influence of oxygen and lack of inactivation of the plant's own enzymes in the production process, enzymatic browning reactions occur within a few days [47].

For the fresh mung bean sprouts a significant difference between the untreated and treated specimens after a post-treatment time of 5 minutes and storage of 8 days at 7°C was observed. The untreated samples became strongly glassy and cell liquor was released. Compared to the references, the treated samples showed a slightly glassiness only for the 5 second plasma-generated PPW, for the 15 and 50 second plasma-generated PPW no influence were seen. The positive results for the food quality of fresh sprouts after an 8-day storage under cooled conditions may be due to the gained inactivation of bacteria on the sprouts surface from very high levels of cfu g^{-1} to medium levels. With the PPW treatment it was possible to prolong the fresh sprouts' shelf-life significantly and to reduce the hazard of foodborne illness for the customer.

5. Conclusions

The new and innovative method for the generation of antimicrobial active water presented within this work showed a possible inactivation of 6 different microorganisms with microwave plasma processed water (PPW) based on distilled water or tap water as by immersion or spraying.

To our knowledge these are the first results of which were published with the direct use of PPW for food decontamination. A significant dependency of inactivation efficiency due to used microorganism, their resistance to plasma-chemical components, and surface was detected. In most cases a tailing in the inactivation kinetics was observed. More structured surfaces of vegetables offered cavities for the bacteria and therefore shade effects, which can inhibit the effects of PPW. Preliminary investigations of quality aspects of PPW-treated fresh produce showed only minimal influences at texture and appearance for lettuce. The detected changes for sprouts showed the possibility to prolong the shelf-life significantly. It can be assumed that these effects be connected to PPW treatment.

The inactivation effects on bacteria by PPW depend on many parameters, such as plasma source, gas mixture, specimen, specimens' surface and bacterial species and concentration. However, the promising results and the advantages of plasma processed water (low-temperature, simple and cheap generation, comparability to tap water rinsing, ozonized water, chlorinated water, electrochemically activated water (ECA)) offer a wide range of possible applications.

The chemical interaction, especially the function of water solved RNS and ROS with respect to microbial inactivation mechanisms and food interaction (food quality) should be further investigated.

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