Antimicrobial efficiency of non-thermal atmospheric pressure plasma processed water (PPW) against agricultural relevant bacteria suspensions

Uta Schnabel[1*], Rijana Niquet[2], Christian Schmidt[3], Jörg Stachowiak[4], Oliver Schlüter[5], Mathias Andrasch[6], Jörg Ehlbeck[7]

[1,2,3,4,6,7]Leibniz Institute for Plasma Science and Technology, Felix-Hausdorff-Str. 2, 17489 Greifswald, Germany
[5]Leibniz Institute for Agricultural Engineering Potsdam-Bornim, Max-Eyth-Allee 100, 14469 Potsdam, Germany

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, phosphate buffered saline (PBS) or nutrient broth to generate plasma processed water (PPW), plasma processed PBS (PPP) or plasma processed broth (PPB) which can be applied for the decontamination of packaging material, fresh produce and processing equipment. This is a new and innovative method for the generation of antimicrobial active plasma processed liquids (PPL). In our experiments, bacterial suspensions 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 10^6 cfu · ml^{-1} and subsequently treated with PPW, PPP, PPB and HNO_3 were investigated. For PPL production, the plasma was ignited for 5, 15 or 50 s. After a post-plasma treatment with PPL of maximum 5 minutes, a decrease of bacterial load up to 6 log steps were detected for examined bacteria. Furthermore, an exclusive inactivation by acidification of PPL was excluded. 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.*

Keywords— fresh food, microbial inactivation, non-thermal atmospheric pressure plasma, plasma processed water.

Highlights:

- microwave plasma processed liquids used successfully for bacterial inactivation
- 3 different plasma processed liquids, distilled water, PBS and nutrient broth were investigated and their antimicrobial efficacy compared among each other and to HNO_3 solution
- decontamination by plasma up to 6.0 log steps for potential human and phytopathogens were achieved.

I. INTRODUCTION

The consumption of about 400 g up to 800 g per day of fresh fruits and vegetables is recommended by many organizations like the World Health organization (WHO) [1], the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) [2] as well as the German Nutrition Society e. V. (DGE).

Fruits and vegetables are the supplier of vitamins and minerals, of dietary fiber and phytochemicals with a low energy density [3].

Investigations lead to the conclusion that the more fruits and vegetables eaten, the lower the risk is not only for certain types of cancer, but also for obesity, hypertension and coronary heart disease [4-10].

However, 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. The U.S. Food and Drug Administration (FDA) listed under the ten riskiest foods in their Center for Science in the Public Interest (CSPI) Report 2009 5 times fruits and vegetables. Whereby leafy greens are on the top [11]. 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 fruits and vegetables, too [12, 13].

The European Food Safety Authority (EFSA) described in their zoonoses report of 2011 [14] 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.

Initial microbiological load of fresh vegetables ranges between 10^2 and 10^7 cfu · g^{-1}, whereas most germs are harmless Gram-negative bacteria like *Pseudomonas* spp. and *Pectobacterium* spp. For food safety relevant foodborne pathogens are in particular bacteria like *Enterobacteriaceae* (*Escherichia* spp., *Samonella* spp.*)* and *Listeria* spp. [15, 16]. Due to the low infection dose of *E. coli*, the guidance level for it can be reduced to 100 cfu · g^{-1} [17, 18].

In case of fresh fruits and vegetables, preservation methods such as heat treatment and freezing are not applicable because of the resulting loss of freshness properties. 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 [19, 20]. 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 [21, 22].

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 [23].

Alternative methods for both effective and safe disinfection of fresh food, especially fruits and vegetables, are needed to guarantee safe consumption of high-quality products.

One possible alternative method could be the application of non-thermal atmospheric pressure plasma.

Plasma is generated by supply of energy to a gas leading to an excitation as well as ionization of gas atoms or molecules, giving the opportunity to a direct absorption of electrical power. A wide range of different plasma types are known. One type is the non-thermal atmospheric pressure plasma [24, 25]. Plasma is always a cocktail of a variety of species including excited and reactive atoms, molecules, ions and radicals, but also radiation (VUV, UV) [26-28]. Plasma is currently used in various industrial fields such as electrical engineering, textile and packaging industry, optics, automotive industry, printing as well as environmental technology, and much more [25, 29, 30].

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 [31]. 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.

Recent reports include special applications like modification of seed germination or active packaging of fruits [32, 33], but also plasma applications for decontamination of different food products, in most cases with the objective of further shelf-life or storage-time extension [34-39].

However, independent of the application a humid or wet environment is given by microbial suspensions, biofilms, and cell tissue, fresh or liquid food. Therefore, the presence of a gas-liquid environment and a gas-liquid interaction is always given.

The aim of the presented work was to investigate the antibacterial efficacy of plasma processed water (PPW) against food-related microorganisms in suspension. Investigations of buffering effects by phosphate buffer and nutrient broth should give an insight for the capability to use PPW in food washing plants. To exclude the pH value as single responsible antimicrobial component, HNO_3 solution was examined separately.

II. METHODS

2.1 Generation of plasma processed liquids by microwave discharge

The generation of all plasma processed liquids was realized by microwave driven discharge processed gas in contact with sterile, distilled water; phosphate buffered saline (PBS, after Sörensen, pH 7.2) or nutrient broth. 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 plasma processed air (PPA) was introduced into distilled water, PBS or nutrient broth and the resulting plasma processed water (PPW), plasma processed PBS (PPP) or plasma processed broth (PPB) were then used to inactivate the bacterial suspensions. The discharge was ignited for 5, 15 or 50 s. The suspensions were treated for 1, 3 and 5 minutes with the PPW, PPP or PPB (post-treatment time). The observed inactivation of microorganisms depended on the storage with long-lived reactive chemical species in the liquids and acidification during post-plasma treatment time.

For comparability and to investigate if only the acidification is responsible for the inactivation of bacterial suspensions, HNO_3 solutions with 3 different pH values were used the same way like the other liquids, but without PPA treatment. The chosen pH values for HNO_3 solutions correspond to pH values received for PPW after PPA treatment.

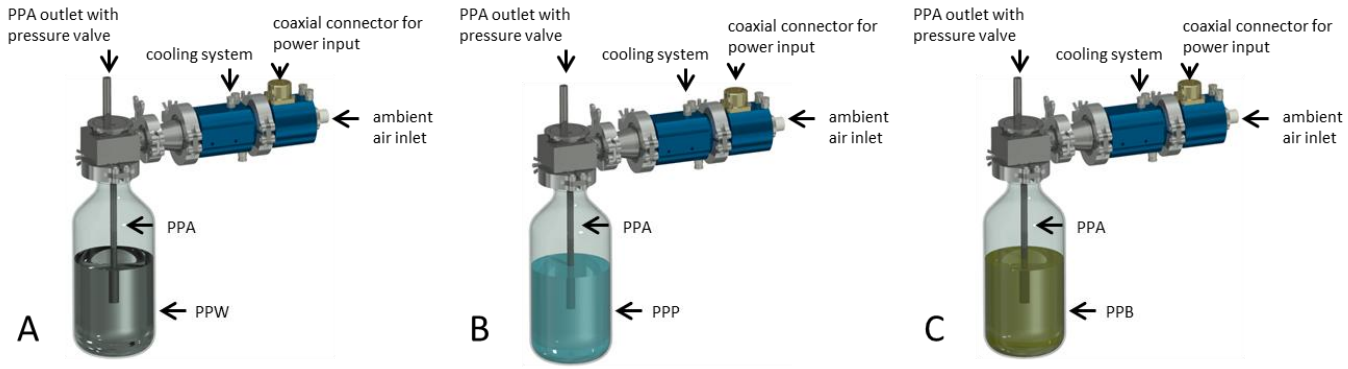

FIGURE 1. SCHEME OF THE MICROWAVE-SETUP FOR THE GENERATION OF A: PPW (PLASMA PROCESSED WATER), B: PPP (PLASMA PROCESSED PBS) AND OF C: PPB (PLASMA PROCESSED BROTH) [40].

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, PPP or PPB generation. The observed pH values in dependency of the plasma-on time are shown in **Table 2**. The pH value for the used HNO_3 solutions is also shown in **Table 1**.

TABLE 1
PH VALUES OF PPW (PLASMA PROCESSED WATER), PPP (PLASMA PROCESSED PBS), PPB (PLASMA PROCESSED BROTH) AND HNO_3

pre-treatment time (s)	PPW	PPP	PPB	HNO_3
0	6.1	7.2	7.1	-
5	1.7	7.0	6.6	3,5
15	1.5	6.6	5.3	2,5
50	1.1	2.8	3.0	1,5

2.2 Investigated microorganism suspensions

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 10^6 cfu · ml^{-1} suspended in sterile, distilled water, see also **Table 2**. *E. coli* K12 (DSM 11250) and *L. innocua* (DSM 20649) were chosen due to their relationship to enterohemorrhagic *E. coli* (EHEC) O157:H7 and *L. monocytogenes*, both human pathogens which could occur on food. However, the chosen strains are classified as risk level 1 and therefore easy to handle.

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

TABLE 2
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	

2.3 Treatment of bacterial suspensions and recovery of bacterial load

The treatment of bacterial suspensions was realized by transferring 2.5 ml PPW, PPP, PPB or HNO_3 to 2.5 ml bacterial suspension for a specific treatment time. These treatment times were 1, 3 and 5 minutes. Afterwards the antibacterial reaction was stopped with 5 ml nutrient broth (tryptic soy broth from Merck KGaA, Darmstadt, Germany or standard nutrient broth I from Carl Roth GmbH+Co.KG, Karlsruhe, Germany) for plasma processed liquids with 5 and 15 seconds plasma on time as well as HNO_3 solution with pH 3.5 and 2.5. In the case of 50 seconds plasma on time and pH 1.5 of HNO_3 20 ml nutrient broth was used to stop the antibacterial reaction. 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 realized and 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 $\cdot$ ml^{-1}. If the number 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 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.

III. RESULTS

The investigation of antibacterial effects of PPW, PPP, PPB and HNO_3 on different bacterial suspensions was based on a previous work with the use of PPA and PPW [41-43].

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

3.1 Inactivation of bacterial suspensions of phytopathogen *P. fluorescens*

The investigations with bacterial suspensions of *P. fluorescens*, DSMZ and RIPAC strain, in combination with PPW, PPP, PPB and HNO_3 were done under the aspect to compare a non-buffered plasma processed liquid which is commonly used in food industry and elsewhere with weak and strong buffered plasma processed liquids as well as a non-plasma processed liquid with comparable pH values and containing RNS (reactive nitrogen species). These possible RNS are also produced within the used microwave generated PPA and could dissolve in distilled water.

By pipetting, the plasma processed liquids (PPL) and HNO_3 were added to the bacterial suspensions of *P. fluorescens* in concentrations of 10^6 cfu · ml^{-1}.

The PPA, used for the generation of PPW, PPP and PPB, 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 plasma processed liquids were 1, 3 and 5 minutes. The timescales reflected the time of contact between bacterial suspensions and PPL, before nutrient broth was used to stop the possible reactions.

The analysis with plasma processed PBS was done due to the fact that this solution would have a mild buffering effect. The dissolved salts of sodium chloride, potassium chloride and phosphates should react with chemical reactive compounds of PPA and therefore less antibacterial species may be available to inactivate the bacteria in the suspension. To investigate the buffering effect of non-organic and organic components to the antimicrobial efficacy of PPW is important due to its possible applications in food industry on organic matter.

Commonly, microorganisms which occur in food industry and processing are in contact with complex media, like the product itself or washing and rinsing liquids. Within this environment biofilm forming can occur easily. Therefore, the agents used for cleaning should be compatible to the buffering capacity of these complex media. This means the antibacterial components should not be decomposed or a sufficient amount of antibacterial species should remain.

All bacterial suspensions were treated with PPB generated by microwave PPA treatment of nutrient broth. The used nutrient broth was dependent on the used bacterial strain. 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 PPB were 1, 3, and 5 minutes. This was the time of contact between the bacterial suspension and the PPB.

The experiments with PPL made from distilled water, PBS and nutrient broth showed a dependency of bacterial inactivation between pre-treatment time, post-treatment time and the pH-value. However, a complex chemistry also happens when PPA gets into contact with the PPLs. To exclude that the pH-value change is not the only cause for the observed inactivation kinetics, a HNO_3 solution in different antimicrobial effective pH-values was investigated. Therefore, pH-values comparable to the lowest ones of each PPL, pH 3.5 of HNO_3 compared to pH 3.0 of PPB after 50 s pre-treatment time, pH 2.5 of HNO_3 compared to pH 2.8 of PPP after 50 s pre-treatment time and pH 1.5 of HNO_3 compared to pH 1.1 of PPW after 50 s pre-treatment time, were examined.

Experimental results (**Fig. 2**) showed an antibacterial reduction of 6.6 log-steps for *P. fluorescens* (DSMZ strain) and 7.0 log-steps for *P. fluorescens* (RIPAC strain) maximum. Inactivation kinetics observed for 5 seconds/pH 3.5 and 50 seconds/pH 1.5 pre-treatment are comparable for both *P. fluorescencs* strains. Five seconds plamsa treated PBS and nutrient broth as well as HNO_3 solution with a pH value of 3.5 resulted in 0.6 log-step reduction for DSMZ strain and in 2.4 log-steps reduction for RIPAC strain maximum. Only the PPW treatment led to higher reduction rates. For *P. fluorescens* DSMZ strain 4.4 log-steps and for RIPAC strain 5.0 log-steps inactivation was received. In nearly all cases for this treatment parameters a tailing after 1-minute post-treatment time was gained. In the case of 50 seconds plasma treated liquids or a HNO_3 solution with a pH value of 1.5 the lowest decrease for *P. fluorescens* was 0.9 and 1.3 log-steps, respectively. The worst inactivation kinetics with a maximal decrease of 4.5 and 3.3 log-steps was seen for HNO_3 solution. The detection limit was reached for both strains with PPW after 1-minute post-treatment time, with PPP after 3 minutes' post-treatment and with PPB within 1 up to 3 minutes. In between these results the ones for 15 seconds pre-treatment of PPL and HNO_3 with pH value of 2.5 are located. Here the strains showed different behavior. However, PPW had the strongest (5.4 and 7.0 log-steps) inactivation capacity and PPP the lowest (0.6 log-steps). The combination of *P. fluorescens* DSMZ strain and PPB led to no inactivation, with HNO_3 this strain had up to 3.1 log-steps reduction. In the case of *P. fluorescens* RIPAC strain the behavior was slightly different. Here the treatment with 15 seconds pre-treated PPB led to 2.4 log-steps decrease and HNO_3 with pH 2.5 to 2.8 log-steps inactivation maximum.

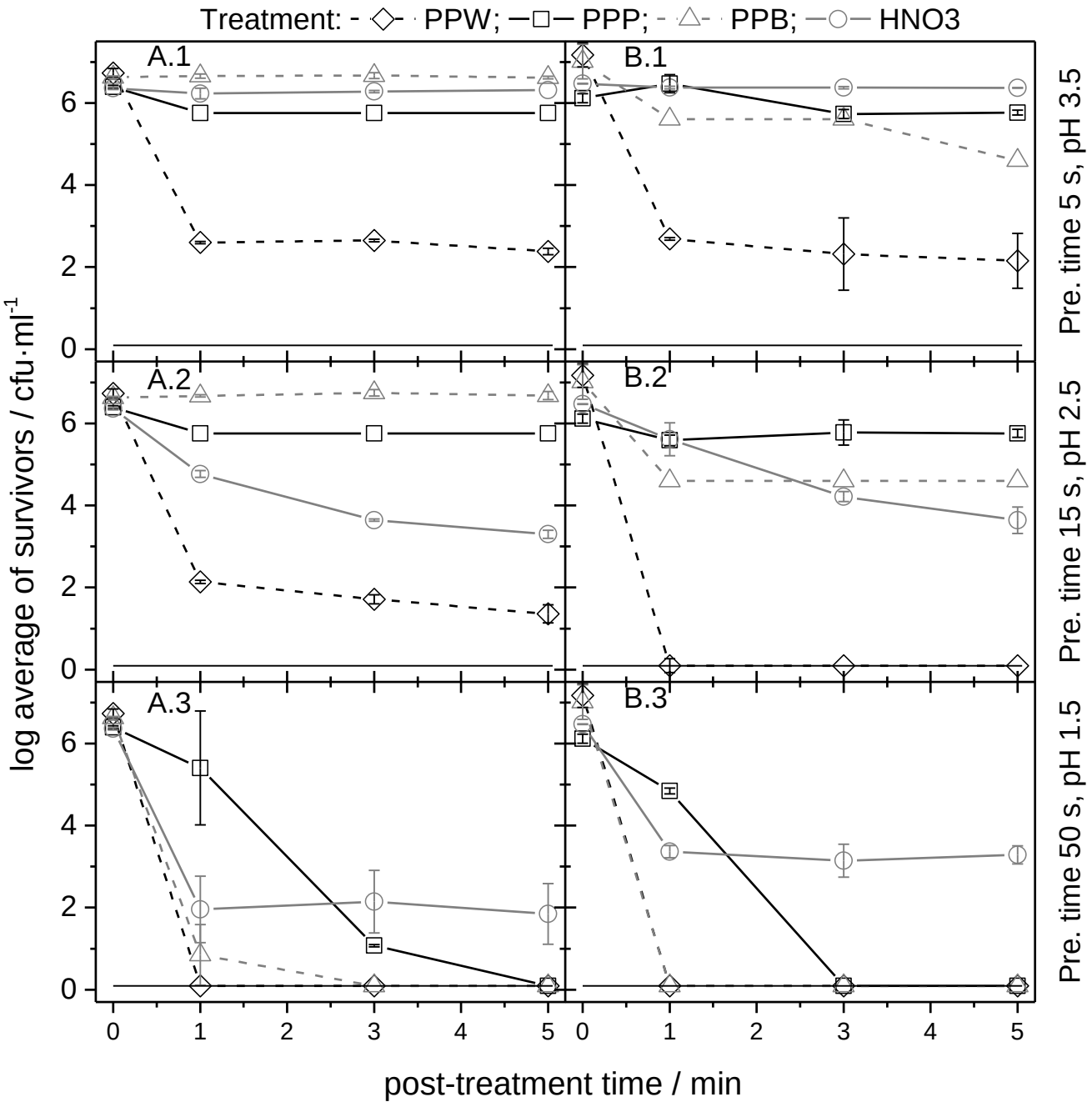

FIGURE 2. RESULTS OF THE PPL AND HNO_3 TREATMENT OF BACTERIAL SUSPENSIONS (2.5 ml). P. FLUORESCENS DSM 50090 (A) AND P. FLUORESCENS RIPAC (B) IN CONCENTRATIONS OF 10^6 cfu · ml^{-1} WERE INVESTIGATED. AFTER A PLASMA IGNITION FOR 5 SECONDS (PRE-TREATMENT TIME) OR PH 3.5, RESPECTIVELY (A1/B1), FOR 15 SECONDS OR PH 2.5, RESPECTIVELY (A2/B2) AND FOR 50 SECONDS OR PH 1.5, RESPECTIVELY (A3/B3), THE SUSPENSIONS WERE INCUBATED WITH PLASMA PROCESSED LIQUIDS (PPL) OR HNO_3 IN DURATIONS OF 1, 3 AND 5 MINUTES (POST-TREATMENT TIME). THE AVERAGE OF THREE EXPERIMENTS IS SHOWN. EXPERIMENTS WERE DONE WITH N=3.

3.2 Inactivation of bacterial suspensions of phytopathogens *P. marginalis* and *P. carotovorum*

Experimental results (**Fig. 3**) showed an antibacterial reduction of 6.2 log-steps for *P. marginalis* and 6.3 log-steps for *P. carotovorum* maximum. Inactivation kinetics observed for 5 seconds/pH 3.5 and 50 seconds/pH 1.5 pre-treatment are comparable for both investigated strains, furthermore the gained kinetics for *P. marginalis* are very close to the ones received for *P. fluorecens* strains (**Fig. 2 A** and **B**). Five seconds plasma treated PBS and nutrient broth as well as HNO_3 solution with

a pH value of 3.5 resulted in 0.1 log-step reduction for *P. marginalis* and in 0.3 log-steps reduction for *Pectobacterium* strain maximum. Only the PPW treatment led to higher reduction rates. For *P. marginalis* 6.2 log-steps and for *P. carotovorum* 6.3 log-steps inactivation was received. In all cases of PPP, PPB and HNO_3 for this treatment parameters a tailing after 1-minute post-treatment time was gained. In the case of 50 seconds plasma treated liquids or a HNO_3 solution with a pH value of 1.5 the lowest decrease for *P. marginalis* was 1.7 and for *P. carotovorum* 4.3 log-steps, respectively. The worst inactivation kinetics was seen for PPB and HNO_3 solution within *P. marginalis* treatment and for PPP in *P. carotovorum* treatment. The detection limit was reached for both strains with PPW after 1-minute post-treatment time, with PPP after 1-minute and 5 minutes' post-treatment, with PPB and HNO_3 within 5 minutes' post-treatment time (*P. marginalis*) and 1-minute post-treatment for *P. carotovorum*. In between these results the ones for 15 seconds pre-treatment of PPL and HNO_3 with pH value of 2.5 are located (**C2/D2**). Here the strains showed different behavior. However, PPW had the strongest (6.2 and 6.3 log-steps) inactivation capacity and PPP as well as PPB the lowest (0.0 to 0.4 log-steps). The combination of *P. marginalis* and HNO_3 led to 2.2 log-steps inactivation. In the case of *P. carotovorum* the behavior was different. Here the treatment with HNO_3 with pH 2.5 resulted in 0.8 log-steps inactivation maximum.

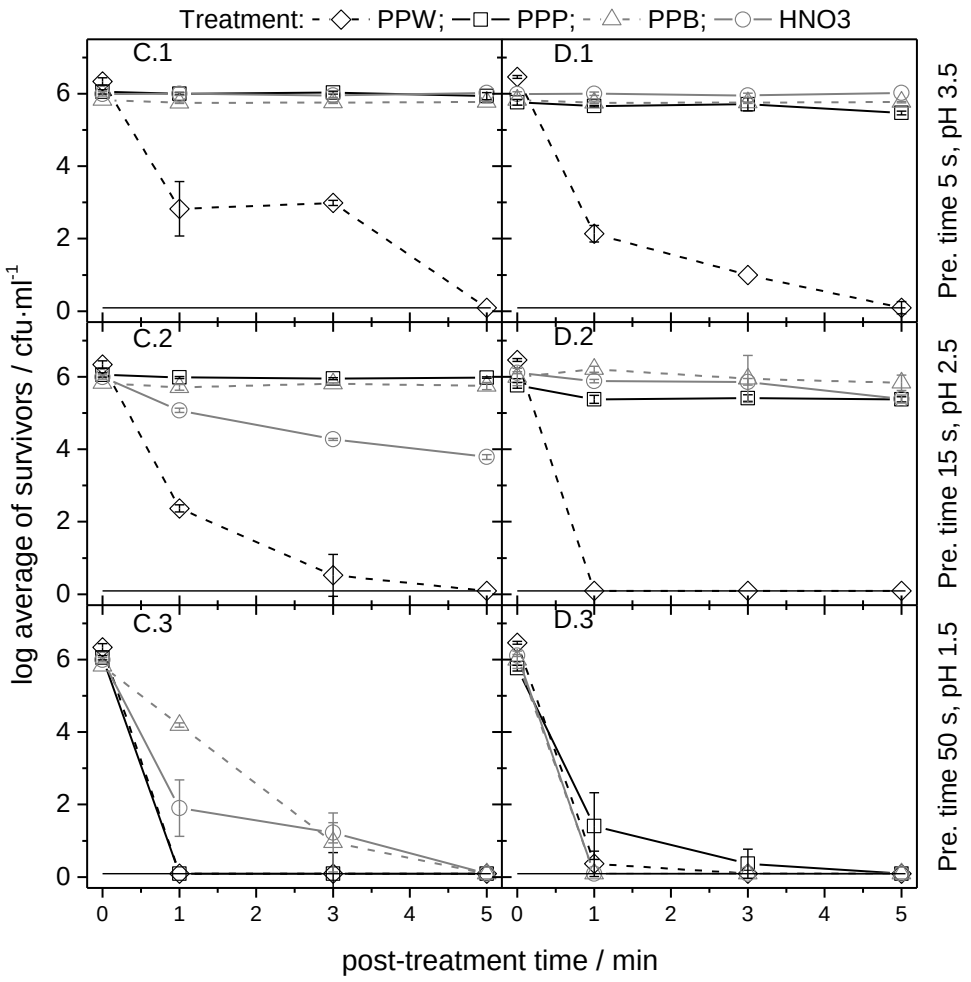

FIGURE 3. RESULTS OF THE PPL AND HNO_3 TREATMENT OF BACTERIAL SUSPENSIONS (2.5 ml). P. MARGINALIS (C) AND P. CAROTOVORUM (D) IN CONCENTRATIONS OF 10^6 cfu · ml^{-1} WERE INVESTIGATED. AFTER A PLASMA IGNITION FOR 5 SECONDS (PRE-TREATMENT TIME) OR PH 3.5, RESPECTIVELY (C1/D1), FOR 15 SECONDS OR PH 2.5, RESPECTIVELY (C2/D2) AND FOR 50 SECONDS OR PH 1.5, RESPECTIVELY (C3/D3), THE SUSPENSIONS WERE INCUBATED WITH PLASMA PROCESSED LIQUIDS (PPL) OR HNO_3 IN DURATIONS OF 1, 3 AND 5 MINUTES (POST-TREATMENT TIME). THE AVERAGE OF THREE EXPERIMENTS IS SHOWN. EXPERIMENTS WERE DONE WITH N=3.

3.3 Inactivation of bacteria suspensions of human pathogens *E. coli* and *L. innocua*

Experimental results (**Fig. 4**) showed an antibacterial reduction of 6.5 log-steps for *E. coli* and 6.2 log-steps for *L. innocua* maximum. Inactivation kinetics observed for 5 seconds/pH 3.5 and 15 seconds/pH 2.5 pre-treatment are comparable for both investigated strains. The inactivation kinetics in all used parameter combinations are different from the ones gained for the phytopathogens (**Fig. 2** and **Fig. 3**). Five seconds plasma treated PBS and nutrient broth as well as HNO_3 solution with a pH value of 3.5 resulted in 0.5 log-step reduction for *E. coli* and in 0.0 log-steps reduction for *L. innocua* maximum. Only the PPW treatment led to higher reduction rates. For *E. coli* 2.0 log-steps and for *L. innocua* 0.8 log-steps inactivation was received. In the case of 15 seconds plasma treated liquids or a HNO_3 solution with a pH value of 2.5 the lowest decrease for *E. coli* was 0.0 and for *L. innocua* 0.0 log-steps, respectively. The worst inactivation kinetics was seen for PPP, PPB and HNO_3 solution within both strains. The detection limit was not reached for both strains with PPW after 5-minutes' post-treatment time. However, a maximum reduction of 3.0 log-steps for *E. coli* and 2.4 log-steps of *L. innocua* was seen. The results for 50 seconds pre-treatment of PPL and HNO_3 with pH value of 1.5 are shown in **Fig. E3/F3**. Here the strains showed a little different behavior. However, PPW and PPP had the strongest (6.5/ 5,9 and 6.2/ 5,8 log-steps) inactivation capacity and HNO_3 as well as PPB the lowest (1.6 to 0.6 log-steps).

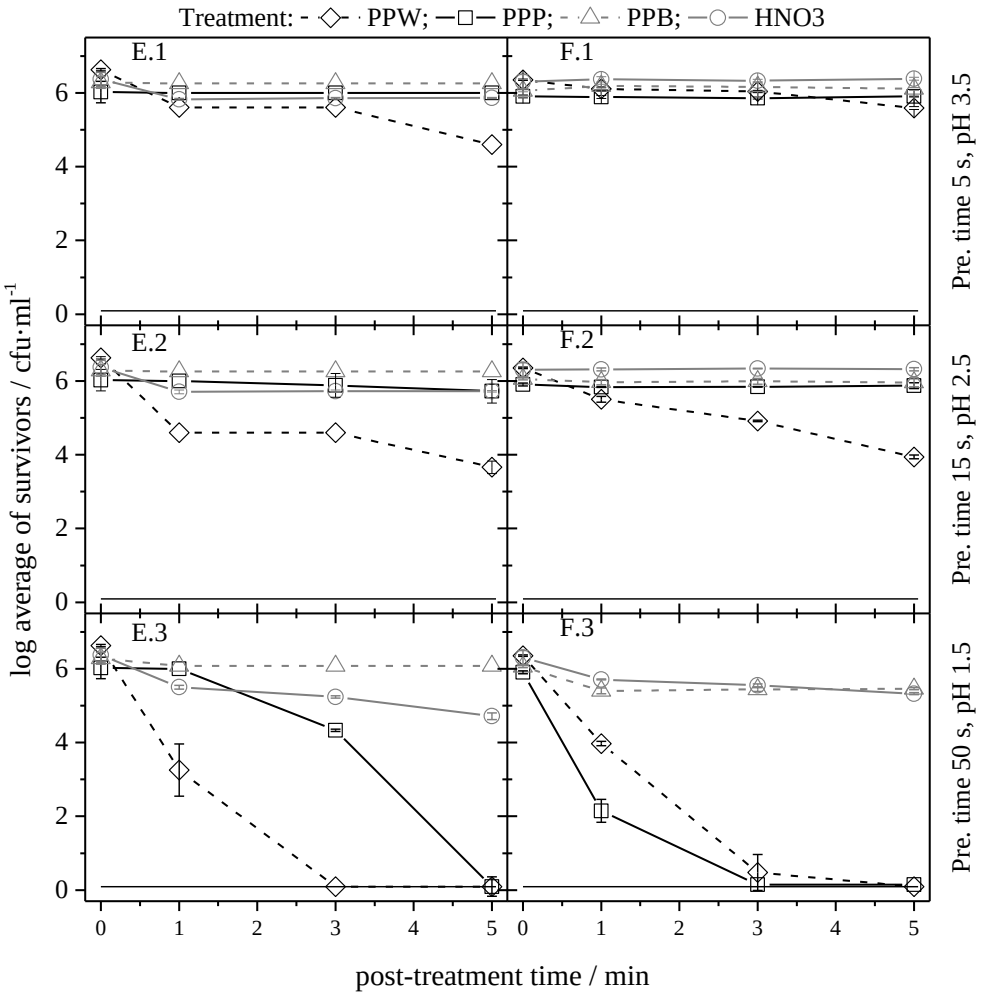

FIGURE 4. RESULTS OF THE PPL AND HNO_3 TREATMENT OF BACTERIAL SUSPENSIONS (2.5 ML). E. COLI (E) AND *L. INNOCUA* (F) IN CONCENTRATIONS OF 10^6 CFU · ML^{-1} WERE INVESTIGATED. AFTER A PLASMA IGNITION FOR 5 SECONDS (PRE-TREATMENT TIME) OR PH 3.5, RESPECTIVELY (E1/F1), FOR 15 SECONDS OR PH 2.5, RESPECTIVELY (E2/F2) AND FOR 50 SECONDS OR PH 1.5, RESPECTIVELY (E3/F3), THE SUSPENSIONS WERE INCUBATED WITH PLASMA PROCESSED LIQUIDS (PPL) OR HNO_3 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 gained results within these experiments clearly showed that not only the pH-value is responsible for the observed inactivation of bacteria by PPW. A deeper insight into the gas composition of PPA and more chemical analysis of PPW may offer new aspects for the responsible inactivation mechanisms of PPW. This will be investigated in future studies.

3.4 Statistical Analysis

The results of statistical analysis of the experimental data presented in **Fig. 2** to **4** are shown in the **Tables 3** to **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 PPW (PLASMA PROCESSED WATER). THE SHOWN VALUES ARE THE P-VALUES MULTIPLIED BY 1000 FOR BETTER READABILITY OF THE TABLE.

	PPW (plasma processed water)								
	p-values x 1000								
pre-reatment time (s)	5			15			50		
post-treatment time (min)	1	3	5	1	3	5	1	3	5
E. coli	2	2	2	2	2	2	2	2	2
P. fluorescens DSM	25	25	25	25	25	25	25	25	25
P. fluorescens RIPAC	78	78	78	78	78	78	78	78	78
P. marginalis	15	15	15	15	15	15	15	15	15
P. carotovorum	3	3	3	3	3	3	3	3	3
L. innocua	1	0	0	0	1	1	1	1	1

TABLE 4
RESULTS OF STATISTICAL ANALYSIS DONE WITH THE T-TEST FOR PPP (PLASMA PROCESSED PBS). THE SHOWN VALUES ARE THE P-VALUES MULTIPLIED BY 1000 FOR BETTER READABILITY OF THE TABLE.

	PPP (plasma processed PBS)								
	p-values x 1000								
pre-reatment time (s)	5			15			50		
post-treatment time (min)	1	3	5	1	3	5	1	3	5
E. coli	896	896	896	896	522	272	896	89	86
P. fluorescens DSM	5	5	5	5	5	5	0	3	3
P. fluorescens RIPAC	212	46	62	33	67	44	25	22	22
P. marginalis	422	623	239	123	40	70	0	0	0
P. carotovorum	187	679	36	17	21	22	12	12	12
L. innocua	641	211	836	114	124	584	3	3	3

TABLE 5
RESULTS OF STATISTICAL ANALYSIS DONE WITH THE T-TEST FOR PPB (PLASMA PROCESSED BROTH). THE SHOWN VALUES ARE THE P-VALUES MULTIPLIED BY 1000 FOR BETTER READABILITY OF THE TABLE.

	PPB (plasma processed broth)								
	p-values x 1000								
pre-reatment time (s)	5			15			50		
post-treatment time (min)	1	3	5	1	3	5	1	3	5
E. coli	750	750	750	750	750	750	182	182	182
P. fluorescens DSM	469	417	676	117	170	499	1	1	1
P. fluorescens RIPAC	237	237	225	225	225	225	224	224	224
P. marginalis	27	9	44	94	425	235	0	0	0
P. carotovorum	359	342	410	302	846	556	226	226	226
L. innocua	50	299	582	136	244	271	0	1	2

TABLE 6
RESULTS OF STATISTICAL ANALYSIS DONE WITH THE T-TEST FOR HNO_3. THE SHOWN VALUES ARE THE P-VALUES MULTIPLIED BY 1000 FOR BETTER READABILITY OF THE TABLE.

	HNO_3								
	p-values x 1000								
pH value	3.5			2.5			1.5		
post-treatment time (min)	1	3	5	1	3	5	1	3	5
E. coli	112	0	0	0	0	0	0	0	0
P. fluorescens DSM	139	0	0	0	0	0	0	0	0
P. fluorescens RIPAC	8	0	0	0	0	0	0	0	0
P. marginalis	921	460	478	7	6	6	6	6	6
P. carotovorum	150	303	528	2	6	0	2	2	2
L. innocua	637	846	542	982	806	938	101	85	74

IV. 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 phytopathogens 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 phytopathogens 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, PPP and PPB described within, only physical stresses by chemical, acidic and biocidal 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 with PPW, PPP, PPB and HNO_3 are very different. The buffering effect of different solutions was clearly demonstrated. If there was an inactivation by the plasma processed liquid its best result was gained for a 50 seconds pre-treatment time.

Here, the reason could be the accumulation of antibacterial agens 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, food borne bacteria cannot grow at pH-values lower than about 4.0. During the treatment of bacterial suspension in this study, an acidification on the PPW, PPP and PPB 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 ml distilled water or other liquids, 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 process´s.

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 also be observed in our results. However, no significant difference in the inactivation kinetics for gram-negative *E. coli* as well as the gram-positive *L. innocua* is observed. Maybe *E. coli* has specific defense mechanisms against RNS and/or acidification. 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.

Due to the fact that nitric acid could be generated in the plasma processed liquids, especially in the PPW it was investigated separately with low pH-values. The results showed that only a very strong acidified HNO_3 solution led to comparable inactivation rates. An antimicrobial effect of PPW exclusively based on HNO_3 formation and increased acidification can be excluded. However, the acidification strongly supports the inactivation process, which was proved by using the buffering solutions PBS and nutrient broth.

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

V. 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, with microwave plasma processed PBS (PPP) and with microwave plasma processed broth (PPB). A significant dependency of inactivation efficiency due to used microorganism, their resistance to plasma-chemical components, acidification and the treatment times was detected. Buffering solutions and environments can affect the antibacterial efficacy of PPW. With regard to the final pH-value in the sanitizing solution this effect is not excluding this plasma process for decontamination processes. 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 should be further investigated.

VI. CONCLUSION

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REFERENCES

[1] WHO: Diet, nutrition, and the prevention of chronic diseases. Technical report series 797, Genf (1990)

[2] World Cancer Research Fund/American Institute for Cancer Research: Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Washington, DC (2007)

[3] Thompson HJ, Heimendinger J, Diker A et al.: Dietary botanical diversity affects the reduction of oxidative biomarkers in women due to high vegetable and fruit intake. J Nutr 136 (2006) 2207–2212

[4] Deutsche Gesellschaft für Ernährung: Stellungnahme: Obst und Gemüse in der Prävention chronischer Krankheiten. www.dge.de/fileadmin/public/doc/ws/stellungnahme/Stellungnahme-OuG-Praevention-chronischer-Krankheiten-2007-09-29.pdf

[5] Deutsche Gesellschaft für Ernährung (Hrsg.): Ernährungsbericht 2008. Bonn (2008)

[6] Takachi R, Inoue M, Ishihara J: Fruit and vegetable intake and risk of total cancer and cardiovascular disease. Japan Public Health Center-based Prospective Study. Am J Epidemiol 167 (2008) 59–70

[7] Buijsse B, Feskens EJM, Schulze MB et al.: Fruit and vegetable intakes and subsequent changes in body weight in European populations: results from the project on Diet, Obesity, and Genes (DiOGenes). Am J Clin Nutr 90 (2009) 202–209

[8] Wright ME, Park Y, Subar AF et al.: Intakes of fruit, vegetables, and specific botanical groups in relation to lung cancer risk in the NIH-AARP Diet and Health Study. Am J Epidemiol 168 (2008) 1024–1034

[9] Slattery ML, Berry TD, Potter J, Caan B: Diet diversity, diet composition, and risk of colon cancer (United States). Cancer Causes and Control 8 (1997) 872–882

[10] Franceschi S, Favero A, la Vecchia C et al.: Influence of food groups and food diversity on breast cancer risk in Italy. Int J Cancer 63 (1995) 785–789

[11] THE TEN RISKIEST FOODS REGULATED BY THE U.S. FOOD AND DRUG ADMINISTRATION. http://cspinet.org/new/pdf/cspi_top_10_fda.pdf (accessed on 17 11 2014)

[12] opinion no. 017/2011 (9.05.2011) of the Federal Institute for Risk Assessment (BfR)

[13] Stiftung Warentest" volume 06/2013 „Da haben wir den Salat…" (available in German)

[14] EFSA and ECDC: The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011, EFSA Journal 2013; 11(4): 3129

[15] Francis, G.A., Thomas, C., O'beirne, D. 1999. The microbial safety of minimally processed vegetables. International Journal of Food Science & Technology 34: 1-22.

[16] Oliveira, M., Usall, J., Vinas, I., Solsona, C., Abadias, M. 2011. Transfer of Listeria innocua from contaminated compost and irrigation water to lettuce leaves. Food Microbiology 28, 590-596.

[17] DGHM. 2010 Veröffentlichte mikrobiologische Richt- und Warnwerte zur Beurteilung von Lebensmitteln. Deutsche Gesellschaft für Hygiene und Mikrobiologie. Hannover. (available in German)

[18] EC. 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria on foodstuffs.

[19] White, G. C. Handbook of Chlorination and Alternative Disinfectants, 4th ed. Wiley-Interscience: New York, United States of America, 1999.

[20] Rico, D.; Martín-Diana, A.B.; Barat, J.M.; Barry-Ryan, C. Extending and measuring the quality of fresh-cut fruit and vegetables: a review. Trends Food Sci. Technol. 2007, 18, 373–386.

[21] Itoh, Y.; Sugita-Konishi, Y.; Kasuga, F.; Iwaki, M.; Hara-Kudo, Y.; Saito, N.; Noguchi, Y.; Konuma, H.; Kumagai, S. Enterohemorrhagic Escherichia coli O57:H7 present in radish sprouts. Appl. Environ. Microbiol. 1998, 64, 1632-1535.

[22] Lang, M.M.; Ingham, B.H.; Ingham, S.C. Efficacy of novel organic acid and hypochlorite treatments for eliminating Escherichia coli O157:H7 from alfalfa seeds prior to sprouting. Int. J. Food Microbiol. 2000, 58, 73-82.

[23] Matthews, K.R. (Editor) Emerging Issues in Food Safety. Microbiology of Fresh Produce. American Society for Microbiology Press (ASM Press) 2006.

[24] Conrads, H., Schmidt, M. 2000. Plasma generation and plasma sources. Plasma Sources Science and Technology 9: 441-454.

[25] Tendero, C., Tixier, C., Tristant, P., Desmaison, J., Leprince, P. 2006. Atmospheric pressure plasmas: a review. Spectrochimica Acta B 61: 2-30.

[26] Ehlbeck, J., Schnabel, U., Polak, M., Winter, J., von Woedtke, Th., Brandenburg, R., von dem Hagen, T., Weltmann, K.-D. 2011. Low temperature atmospheric pressure plasma sources for microbial decontamination. Journal of Physics D: Applied Physics 44: 013002.

[27] Fridman A. 2008. Plasma chemistry. Cambridge University Press. New York, NY.

[28] Moisan, M., Barbeau, J., Moreau, S., Pelletier, J., Tabrizian, M., Yahia, L'H. 2001. Low-temperature sterilization using gas plasmas: a review of the experiments and an analysis of the inactivation mechanisms. International Journal of Pharmaceutics 226: 1-21.

[29] Kogelschatz, U. 2004. Atmospheric-pressure plasma technology. Plasma Physics and Controlled Fusion 46: B63-B75.

[30] Suchentrunk, R., Staudigl, G., Jonke, D., Fuesser, H.J. 1997. Industrial applications for plasma processes – examples and trends. Surface and Coatings Technology 97: 1-9.

[31] Knorr, D., Fröhling, A., Jäger, H., Reineke, K., Schlüter, O., Schössler, K. 2011. Emerging technologies in food processing. Annual Review of Food Science and Technology 2: 203–235.

[32] Volin, J.C., Denes, F.S., Young, R.A., Parker, S.M.T. 2000. Modification of seed germination performance through cold plasma chemistry technology. Crop Science 40: 1706-1718.

[33] Fernández-Gutierrez, S., Pedrow, P.D., Pitts, M.J. 2010. Cold atmospheric-pressure plasmas applied to active packaging of apples. IEEE Transactions on Plasma Science 38: 957-965.

[34] Niemira, B.A., Sites, J. 2008. Cold plasma inactivates Samonella Stanley and Escherichia coli O157:H7 inoculated on golden delicious apples. Journal of Food Protection 71: 1357-1365.

[35] Perni, S., Shama, G., Kong, M.G. 2008. Cold atmospheric plasma disinfection of cut fruit surfaces contaminated with migrating microorganisms. Journal of Food Protection 71: 1619-1625.

[36] Perni, S., Liu, D.W., Shama, G., Kong, M.G. 2008. Cold atmospheric plasma decontamination of the pericarps of fruit. Journal of Food Protection 71: 302-308.

[37] Ragni, L., Berardinelli, A., Vannini, L., Montanari, C., Sirri, F., Guerzoni, M.E., Guarnieri, A. 2010. Non-thermal atmospheric gas plasma device for surface decontamination of shell eggs. Journal of Food Engineering 100: 125-132.

[38] Selcuk, M., Oksuz, L., Basaran, P. 2008. Decontamination of grains and legumes infected with Aspergillus spp. and Penicillium spp. by cold plasma treatment. Bioresource Technology 99: 5104-5109.

[39] Shama, G., Kong, M.G. 2012. Prospects for treating foods with cold atmospheric gas plasmas. In: Machala, Z., Hensel, K., Akishev, Y. (Eds.), Plasma for bio-decontamination, medicine and food security. NATO Science for Peace and Security Series, Series A: Chemistry and Biology. Springer. Berlin. Pp.: 433-443.

[40] DE, 102005043278, 2005. INP Greifswald e.V., U. Krohmann, T. Neumann, J. Ehlbeck

[41] Schnabel, U.; Niquet, R.; Krohmann, U.; Winter, J.; Schlüter, O.; Weltmann, K.D.; Ehlbeck, J. Decontamination of microbiologically contaminated specimen by direct and indirect plasma treatment. Plasma Process. Polym. 2012, 9, 569-575.

[42] Schnabel, U.; Niquet, R.; Krohmann, U.; Polak, M.; Schlüter, O.; Weltmann, K.D.; Ehlbeck, J. Decontamination of microbiologically contaminated seeds by microwave driven discharge processed gas. J. Agricultural Sci. Applications 2012, 1, 100-106.

[43] Schnabel, U.; Sydow, D.; Schlüter, O.; Andrasch, M.; Ehlbeck, J. Decontamination of fresh-cut iceberg lettuce and fresh mung bean sprouts by non-thermal atmospheric pressure plasma processed water (PPW). Modern Agricultural Sci. Technol. 2015, 1, 23-39.