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The Combination of Plasma-Processed Air (PPA) and Plasma-Treated Water (PTW) Causes Synergistic Inactivation of *Candida albicans* SC5314

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Abstract: Microwave-induced plasma was used for the generation of plasma-processed air (PPA) and plasma-treated water (PTW). By this way, the plasma was able to functionalize the compressed air and the used water to antimicrobial effective agents. Their fungicidal effects by single and combined application were investigated on *Candida albicans* strain SC5314. The monoculture of *C. albicans* was cultivated on specimens with polymeric surface structures (PE-stripes). The additive as well as the synergistic fungicidal potential of PPA and PTW was investigated by different process windows of plasma exposure time (5–50 s) and sample treatment time with PPA/PTW (1–5 min). For a single PTW or PPA treatment, an increase in the reduction factor with the indicated treatment time was observed (maximum reduction factor of 1.1 and 1.6, respectively). In comparison, the combined application of PTW and then PPA resulted in antagonistic, additive and synergistic effects, depending on the combination. An application of the synergistically acting processes of PTW for cleaning and PPA for drying can be an innovative alternative to the sanitary processes currently used in production plants.

Keywords: antimicrobial; atmospheric pressure; decontamination; microwave discharge; cold plasma

1. Introduction

The sources of contamination along the entire value chain of fresh and fresh-cut products are numerous and diverse (raw materials, processing tools, surfaces in contact with food, lack of hygiene among employees). A contaminated product will most likely lead to a negative impact along the entire production chain. Therefore, the internal process hygiene is especially monitored, and not only traditional, commercial measures are taken into account; new, innovative techniques are also gaining more and more attention. Non-thermal plasmas (non-equilibrium plasmas) are plasmas in which the cooling of ions and uncharged particles is more effective than the transfer of energy from energetically excited electrons to the latter particles. This has the effect that the gaseous environment does not heat up, which is usually the case in thermal plasmas [1]. Non-thermal plasmas are now used in some areas of medicine and other industries. Their high antimicrobial potential in combination with a non-thermal application is very advantageous [2].

Biofouling, a problem caused by microbial biofilms, leads to high resource and economic losses, especially in the food industry. Causes for the formation of biofilms can be the microbiological contamination of processed products such as meat, fish, vegetables or fruit [3], but also abiotic materials such as processing surfaces due to increased corrosion, increased fluid friction resistance and heat flow [4].

Normally, the typical microbial life cycle begins with adhesion to solid surfaces, and through subsequent multiplication this leads to the formation of three-dimensional, multicellular aggregates

called biofilms, which finally and protectively embed themselves in a self-generated extracellular matrix (ECM; [5,6]). In particular, microorganisms such as *Candida albicans*, which can be responsible for product spoilage and food-related illnesses, preferentially form biofilms on abiotic materials such as stainless steel, aluminum, glass, polytetrafluoroethylene (PTFE) seals and polyamide (PA). These materials are typically found in food processing plants [7–9]. For example, *Candida* spp. has often been isolated from conveyor belts in the food and beverage industry [10,11].

Economic losses in the food and health sector and a more sensitive public awareness are bringing food safety to the fore. For this reason, uniform guidelines for good manufacturing practice (GMP) and the Hazard Analysis Critical Control Point (HACCP) for the food industry have been introduced within the European countries. Nevertheless, the European Food Safety Authority has identified 49,950 food-borne outbreaks in 2016, resulting in illness, 3869 hospitalizations and 20 deaths [12]. There are also various hygienic standards in food production worldwide, but 841 food-borne disease outbreaks have been reported in the United States (USA), for example, resulting in 14,481 illnesses, 827 hospitalizations and 20 deaths in 2017 [13].

Conventional methods (high-pressure cleaners, brushing, wiping), which are supposed to help reduce biofilms, do lead to a loss of biomass, but not efficiently and sustainably enough. If a reduced biofilm remains after mechanical cleaning, it will most likely grow back with increased resistance and resilience [14,15]. Currently, the majority of sterilization and disinfection methods are based on wet chemical processes (e.g., peracetic acid, hydrogen peroxide, chlorine dioxide, chlorine, active oxygen, ammonium compounds, isopropanol, phosphoric acid, formic acid or nitric acid), which are not only highly effective but can also promote the development of new resistances [16]. Despite the integration of cleaning and disinfection cycles in numerous process chains for the removal of organic (proteins, lipids, carbohydrates) and inorganic (salts) contaminations, it remains a major challenge to develop new and uniform safety standards. Alternative, innovative techniques and concepts can help to overcome the existing economic, political and social barriers in the future.

Furthermore, the complex geometries of food processing plants made of different materials (stainless steel, glass, glass fiber, polymers, rubber and polytetrafluoroethylene (PTFE)) pose an aggravating challenge for effective cleaning. For example, even routine processes can lead to incomplete microbial decontamination, so that human-pathogenic microorganisms survive on surfaces and contaminate the rest of the process chain [17–19]. Under these circumstances, the risk of microbial adhesion and biofilm formation is particularly high. Cross-contamination, bacterial adhesion and biofilm formation can be reduced or avoided completely by an optimized choice of materials and the use of efficient, sustainable antimicrobial agents [20,21]. Food producers are therefore confronted with a broad spectrum of resource and cost-intensive challenges (environmental, energy and water consumption, residues as well as storage and disposal, etc.), which has greatly increased the demand for innovative process concepts that complement existing systems in recent decades. The need for novel strategies to control and prevent microorganisms, especially biofilm-forming ones, in the food industry is unbroken.

The use of non-thermal plasmas for the decontamination of microorganisms is an interesting, innovative field of research, which is constantly increasing and leads to new knowledge about the antimicrobial effect and mode of action [22–27].

For a plasma treatment based on a microwave-driven discharge for the generation of antimicrobial functionalized plasma-processed air (PPA) or functionalized plasma-treated water (PTW), first antimicrobial effects on abiotic (glass, plastic) and biological (fruit, vegetables, meat) surfaces have already been investigated [28–33]. Furthermore, Handorf et al. (2019) demonstrated the successful inactivation of *C. albicans* biofilms by PPA [34].

So far, the fungicidal effects of PTW itself, based on the microwave plasma technology investigated here, and the additive/synergistic effects of a combined treatment with PTW/PPA for *C. albicans* have not been investigated and are therefore the subject of this study. Our results suggest a serial application of the two antimycotics PTW and PPA. If the hygienic measures in the respective food

production process allow an application of PTW and PPA, it is recommended to apply a PPA drying step to a PTW rinsing/washing step. Most conventional processes in the food industry meet these requirements, which allows the PTW/PPA process under investigation here a broad spectrum for industrial applications against short-term adapted yeast cells and already grown biofilms.

2. Materials and Methods

2.1. Contamination of PE-Stripes with C. albicans SC5314

C. albicans SC5314, a strain frequently used in laboratory experiments, was grown on Sabouraud agar with 4% glucose (Roth, Karlsruhe, Germany) at 37 °C. The yeast was then pipetted onto biocompatible polyethylene (PE) polymer strips measuring $32 \times 8 \times 2$ mm^3 for inoculation of the test samples. PE is often used in the food industry for conveyor belts, transport or collection containers, and packaging material, and is therefore well suited to investigate our research question. The preparation of the yeast suspension used for inoculating the PE strips was achieved by a 24-h cultivation from an overnight culture. For this purpose, 1 mL overnight culture was pipetted to 30 mL fresh nutrient broth (tryptic soy broth; Roth, Karlsruhe, Germany). The yeast suspension (1.3 mL) thus obtained was then added to a screw cap tube in which the PE strip was already contained. The PE strips prepared in this way were incubated again for 24 h at 37 °C so that the sample was then completely covered with yeast cells in the stationary growth phase. Finally, the inoculated PE strips were washed three times with phosphate-buffered solution (PBS, pH 7.0) to discard loose cells. The finished test specimens were transferred to a new 2 mL screw cap tube and stored sealed for a short time before PTW/PPA treatment. The final concentration of yeast cells per PE strip was 10^4 cfu mL^{-1}, based on three biological and experimental replications.

2.2. Plasma Source and Specimens' Treatment

2.2.1. Plasma-Processed Air (PPA) Generation and Plasma-Treated Water (PTW) Functionalization

The functionalization of compressed air for PPA production for the PPA treatment of the *C. albicans* inoculated PE strips was carried out with a microwave-driven discharge device [35,36]. The setup used is shown in Figure 1A. The microwaves used had a frequency of 2.45 GHz with an input power of 1.1 kW, which results in a gas temperature of about 3500–4000 K at a gas flow of 18 slm air. By cooling the PPA to room temperature (20–25 °C), the functionalized gas could be used directly for the decontamination of the PE strips or for further functionalization of the water to PTW (Figure 1B). The given gas temperature was measured in pure nitrogen by optical emission spectroscopy (OES) using the first negative system of $N_2{}^+$. This method is a common method for temperature measurements at high temperatures such as those found in arc jets or microwave torches and has been described in detail by Laux et al. (2001) [37]. A temperature of about 4000 K was then calculated. Unfortunately, this method is not applicable in air plasmas due to the presence of oxygen. Therefore, an alternative method described in Ehlbeck et al. (2008) was used [38]. Based on the OH spectrum between 300 nm and 320 nm, a temperature of 3000 K ± 500 K is assumed. If the Fourier-transform infrared spectroscopy (FTIR) spectrum measured in the plasma outflow is taken into account [39] and compared with the chemical composition of the air plasma of the entire temperature range in Drost (1978) [40], it becomes clear that the temperature of the plasma torch must be in the range of 3500 K–4000 K.

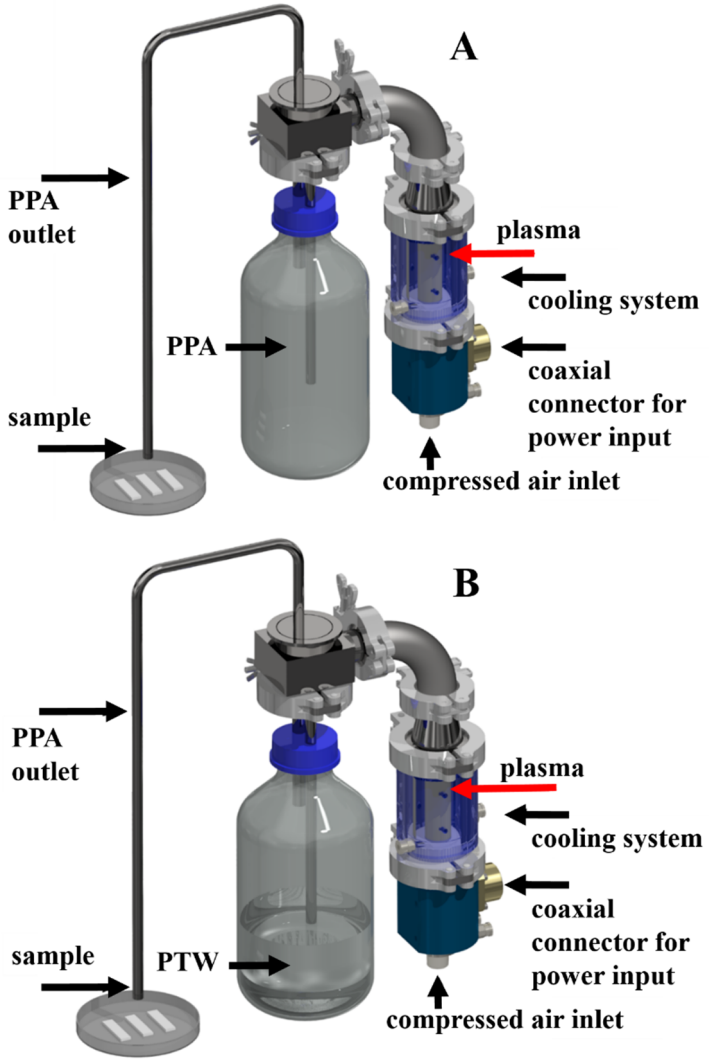

Figure 1. Scheme of the microwave-setup for (**A**) the generation of PPA (plasma-processed air) and (**B**) the functionalization of PTW (plasma-treated water).

The main chemical components of PPA are reactive nitrogen species (RNS) such as nitric oxide (·NO), nitrogen dioxide (·NO_2), nitrous acid (HNO_2) and nitric acid (HNO_3) and water (H_2O) [31,39]. However, previous mass spectrometric investigations (OmniStar™ GSD3001O1, Pfeiffer Vacuum (Asslar, Germany)) [31] showed that 97% of the air used as working gas remained compressed air after microwave discharge treatment and cooling at the point of application, so that only 3% was functionalized to PPA [31]. As described in [31], a mass spectrometric analysis were done under atmospheric pressure. The plasma gas was fed through a heatable stainless steel capillary (inner diameter of 1/16" and length of 1 m) into the chamber for analysis. A gas flow of 1 to 2 sccm was generated. The detection range of the mass spectrometer was 1 to 100 amu. The measurements and evaluations were performed with the software QuadStar™ (Pfeiffer Vacuum). Nitrogen dioxide (NO_2) was measured by two different methods. The first is described in [33] in detail and is based on a self-constructed ·NO_2 sensor, which was calibrated by usage of test gas (Linde AG, Pullach, Germany: test gas ·NO_2 (3.19%) and synthetic air). The sensor is based on absorbance measurements of ·NO_2-bands at 400 nm [41]. The second method is described in [39] in detail and is based on FTIR measurements. The FTIR (Vertex 70v, Bruker Cooperation, Billerica, MA, USA) was equipped with a variable long path cell, 0.25–1 m (Bruker Cooperation), which was calibrated for a path length of 0.25 m. In order to get species concentrations, the experimental data were processed by a MATLAB (The MathWorks Inc., Paderborn, Germany) code developed at our institute based on the

spectroscopic data of the HITRAN database (High-Resolution Transmission molecular absorption database, Harvard-Smithsonian Center for Astrophysics, Cambridge, MA, USA). For the measurements, the microwave plasma device was connected directly to the long path cell via a 9-fold gas distributor. Furthermore, small amounts of hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), were detected [39]. Both RNA and ROS are known as antimicrobial agents.

PPA was subsequently used to functionalize distilled, sterile water to produce PTW (Figure 1B). The volume of water used was 10 mL in a 1-L glass bottle. A volume of 1.3 mL was used for PTW treatment of the inoculated PE strips. The most important antimicrobial components of PTW are nitrite (NO_2^-), nitrate (NO_3^-) and H_2O_2 [39].

2.2.2. Single and Combined Treatment with PTW and PPA

PPA Treatment

A plasma-on time of 5, 15 or 50 s was selected for the single treatment of the inoculated PE strips with antimicrobial functionalized PPA (pre-treatment time). In these time windows, the compressed air was functionalized to PPA. Subsequently, the samples were treated with the functionalized PPA for 1, 3 or 5 min (post-treatment time). The post-treatment time was stopped by means of pure compressed air introduced into the sample-loaded reaction chamber. Thus, the PPA was displaced. This step was repeated twice in a row. The treatment chamber had a volume of 4.5 l ($270 \times 185 \times 150$ mm^3), and the samples were fixed freestanding in the chamber to keep the contact areas as small as possible.

The inactivation of the yeast cells thus depended on the amount of short- and long-lived reactive chemical species (RONS) produced during the pre-treatment time and which could act as a fungicide during the post-treatment time with PPA. For reference, the post-treatment time was given as 0 min, which means that these samples were exposed to an airflow instead of a PPA flow.

PTW Treatment

With the help of PPA, distilled, sterile water was functionalized to PTW. For this purpose, the produced PPA was introduced into a 1-L glass bottle with 10 mL water. Here too, the plasma-on time was 5, 15 or 50 s (pre-treatment time), and the subsequent post-treatment time was again limited to 1, 3 or 5 min. During this post-treatment time, the antimicrobial functionalized PTW could act on the inoculated PE strips. Therefore, a volume of 1.3 mL PTW was added to the prepared PE strips in the screw cap tubes to start the inactivation process. Stopping the post-treatment time was done by decanting the PTW. Finally, the PTW-treated PE strips were transferred to 10 mL nutrient broth (tryptic soy broth). The nutrient broth has a very high buffer capacity, which prevented any chemical reaction. The surviving/proliferating yeast cells were then detected as described in Chapter 2.3. The observed inactivation of yeast cells depended on incubation with long-lived reactive species (RONS) in PTW with low pH during the post-treatment time.

Combined Treatment

To demonstrate possible additive and synergistic effects of a combined PTW/PPA treatment, the individual steps described above for the PTW and PPA single treatments were coordinated.

In a first step, PTW was added to the inoculated PE strips to be decontaminated, and in a second step, these treated samples were dried and decontaminated with PPA. All investigated different pre- and post-treatment times of the single treatments with PPA and PTW were also tested for the combination of PTW and PPA.

2.3. Recovery and Counting of Surviving C. albicans SC5314 on PE Stripes

Subsequently to a single or combined PTW and PPA treatment, the surviving colony forming units (CFUs) were recovered. Based on Equation (1), the CFU mL^{-1} was calculated [42,43]:

$$\frac{CFU}{ml} = \frac{10^x}{v} \cdot \frac{\sum c_y + \sum c_{y+1}}{n_y + 0,1 n_{y+1}} \quad (1)$$

10^x = the dilution factor for the lowest dilution
v = the volume of diluted cell suspension per plate in mL
$\sum c_y$ = the total number of colonies on all (n_y) plates of the lowest evaluated dilution level 10^{-x}
$\sum c_{y+1}$ = the total number of colonies on all (n_{y+1}) plates of the next highest dilution level evaluated $0^{-(x+1)}$.

After the calculation of Equation (1) for the CFU mL^{-1}, the reduction factor (RF) was determined as follows via Equation (2):

$$RF = MV_c log_{10} - MV_s log_{10} \quad (2)$$

$MV_c log_{10}$ = mean value of the CFU mL^{-1} of the references (control)
$MV_s log_{10}$ = mean value of the CFU mL^{-1} of the treated specimens

For the detection of CFU mL^{-1}, the treated PE strips were transferred into 10 mL nutrient broth as described above and shaken for 15 min at 300 rpm. The resulting *C. albicans* suspensions were used to prepare the dilution series. Using the surface-spread-plate count method, the CFUs were countable after incubation at 37 °C for 12–18 h. The volume of 100 µL of all serial dilutions was plated out. Serial dilutions were performed as 1 to 10 dilutions. The detection limit was 10 CFU mL^{-1}. CFUs were counted manually.

2.4. Calculations of the Reduction Factor ($\widetilde{RF}$) of the Single and Combined PTW/PPA Treatment

The combination of the PTW- and PPA-treatment may result in different effects. They can be additive, synergistic or simply reveal a decreased antimicrobial efficacy (antagonistic).

The experiments were evaluated by determining the non-logarithmic reduction factor (RF). This was calculated as the ratio between the concentration of yeast cells in the reference and the concentration of yeast cells in a treated sample (Equation (3)):

$$RF = \frac{n_{MO}^{Ref}}{n_{MO}^{Sam}} \quad (3)$$

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

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

$$\widetilde{RF} = \log_{10}(RF) \quad (4)$$

The standard deviation of the reduction factor (ΔRF) was calculated via Equations (5)–(8):

$$\Delta RF = \sqrt{\left(\frac{\partial RF}{\partial n_{MO}^{Ref}} \cdot \Delta n_{MO}^{Ref}\right)^2 + \left(\frac{\partial RF}{\partial n_{MO}^{Sam}} \cdot \Delta n_{MO}^{Sam}(i)\right)^2} \quad (5)$$

$$\frac{\partial R}{\partial n_{MO}^{Ref}} = \frac{1}{n_{MO}^{Sam}(i)} \quad (6)$$

$$\frac{\partial R}{\partial n_{MO}^{Sam}} = -\frac{n_{MO}^{Ref}}{n_{MO}^{Sam}(i)^2} \quad (7)$$

$$\Delta RF = \sqrt{\left(\frac{1}{n_{MO}^{Sam}(i)} \cdot \Delta n_{MO}^{Ref}\right)^2 + \left(\frac{n_{MO}^{Ref}}{n_{MO}^{Sam}(i)^2} \cdot \Delta n_{MO}^{Sam}(i)\right)^2} \quad (8)$$

$$\Delta n_{MO}^{Ref} \ : error\ of\ reference$$

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

$$\Delta RF \ : error\ of\ reduction\ factor$$

3. Results and Discussion

Comparing two inactivation processes, an important factor is whether these processes were added in the linear or logarithmic scale. This depends on the procedures themselves (in our case, the PTW and PPA treatments) and the corresponding treatment conditions. In the best case, for hygiene aspects, both treatments act completely independently of each other.

In this case, the reduction factor ($\widetilde{RF}$) of both treatments could be added in the logarithmic scale ($\widetilde{RF}_{comb} = \widetilde{RF}_{add}$). In order to identify the kind of interaction, the logarithmic scale of the combined treatments ($\widetilde{RF}_{comb}$) was compared with the sum in the logarithmic scale of the individual treatments ($\widetilde{RF}_{add}$). Therefore, if the measured reduction factor for the combined procedure ($\widetilde{RF}_{comb}$) was smaller than the sum of the individual procedure steps ($\widetilde{RF}_{add} = \widetilde{RF}_{PTW} + \widetilde{RF}_{PPA}$), then the individual inactivation steps did not work wholly independently from each other. This case is called antagonistic and is marked in Table 1 in red for better allocation ($\widetilde{RF}_{comb} < \widetilde{RF}_{add}$). If the result was a combined reduction factor equal to the added value ($\widetilde{RF}_{comb} = \widetilde{RF}_{add}$) in the experiments, the two inactivation processes operated completely independently, and this was called additive and was indicated by yellow in Table 1. If additional inactivation mechanisms were gained, which means $\widetilde{RF}_{comb} > \widetilde{RF}_{add}$, this was called synergistic and was labeled in green in Table 1.

Table 1. Experimental ($\widetilde{RF}_{comb}$) and calculated $\widetilde{RF}_{add}$ data of the single and combined applications of PPA (plasma-processed air) and PTW (plasma-treated water) under different pre- and post-treatment times for antimicrobial effects on *Candida albicans* SC5314. Color code for effects of combined PTW/PPA treatments: red = antagonistic, yellow = additive, green = synergistic. w/o = without. Exp. = experimental, Calc. = calculated.

PPA		**PTW**									
		w/o	**5 s + 1 min**	**5 s + 3 min**	**5 s + 5 min**	**15 s + 1 min**	**15 s + 3 min**	**15 s + 5 min**	**50 s + 1 min**	**50 s + 3 min**	**50 s + 5 min**
w/o	Exp.		0.13 ±0.80	0.69 ±0.29	0.80 ±0.12	0.66 ±0.3	0.66 ±0.12	0.92 ±0.51	0.74 ±0.61	1.04 ±0.43	1.17 ±0.39
	Calc.										
5 s + 1 min	Exp.	0.91 ±0.35	1.27 ±0.35	1.51 ±0.18		1.78 ±0.58	1.42 ±0.05		1.02 ±0.19	1.06 ±0.04	
	Calc.		1.04 ±0.57	1.60 ±0.32		1.57 ±0.32	1.57 ±0.23		1.65 ±0.48	1.95 ±0.39	
5 s + 3 min	Exp.	1.22 ±0.45	1.37 ±0.18	1.7 ±0.08		1.73 ±0.05	1.53 ±0.24		1.36 ±0.61	1.17 ±0.30	
	Calc.		1.35 ±0.62	1.91 ±0.37		1.88 ±0.37	1.88 ±0.28		1.96 ±0.53	2.26 ±0.44	
5 s + 5 min	Exp.	1.28 ±0.10	1.46 ±0.12	2.00 ±0.29		1.97 ±0.11	1.89 ±0.18		1.11 ±0.34	1.50 ±0.16	
	Calc.		1.41 ±0.45	1.97 ±0.19		1.94 ±0.20	1.94 ±0.11		2.02 ±0.35	2.32 ±0.26	
15 s + 1 min	Exp.	1.52 ±0.17	1.60 ±0.46	1.73 ±0.15		1.52 ±0.37	1.47 ±0.37		1.67 ±0.49	2.21 ±0.15	
	Calc.		1.65 ±0.48	2.21 ±0.23		2.18 ±0.23	2.18 ±0.14		2.26 ±0.39	2.56 ±0.30	

Table 1. *Cont.*

PPA		PTW									
		w/o	5 s + 1 min	5 s + 3 min	5 s + 5 min	15 s + 1 min	15 s + 3 min	15 s + 5 min	50 s + 1 min	50 s + 3 min	50 s + 5 min
15 s + 3 min	Exp.	1.65 ±0.00	1.72 ±0.21	1.92 ±0.19		1.85 ±0.17	1.66 ±0.33		1.94 ±0.58	3.47 ±0.00	
	Calc.		1.78 ±0.40	2.34 ±0.14		2.31 ±0.15	2.31 ±0.06		2.39 ±0.30	2.69 ±0.21	
15 s + 5 min	Exp.	1.65 ±0.00	1.77 ±0.19	1.94 ±0.22		2.16 ±0.23	1.72 ±0.41		2.24 ±0.82	3.47 ±0.00	
	Calc.		1.78 ±0.40	2.34 ±0.14		2.31 ±0.15	2.31 ±0.06		2.39 ±0.30	2.69 ±0.21	
50 s + 1 min	Exp.	1.65 ±0.00	1.59 ±0.19	1.54 ±0.21		1.66 ±0.27	1.62 ±0.17		3.49 ±0.00	3.47 ±0.00	
	Calc.		1.78 ±0.40	2.34 ±0.14		2.31 ±0.15	2.31 ±0.06		2.39 ±0.30	2.69 ±0.21	
50 s + 3 min	Exp.	1.65 ±0.00	1.71 ±0.13	1.93 ±0.17		1.73 ±0.22	1.73 ±0.43		3.49 ±0.00	5.57 ±0.00	
	Calc.		1.78 ±0.40	2.34 ±0.14		2.31 ±0.15	2.31 ±0.06		2.39 ±0.30	2.69 ±0.21	
50 s + 5 min	Exp.	1.65 ±0.00	2.03 ±0.10	2.05 ±0.03		1.75 ±0.05	1.94 ±0.21		3.49 ±0.00	3.47 ±0.00	
	Calc.		1.78 ±0.40	2.34 ±0.14		2.31 ±0.15	2.31 ±0.06		2.39 ±0.30	2.69 ±0.21	

Table 1 displays the reduction factors for *C. albicans* obtained in single and combined PTW and PPA treatments. In general, the single treatment with PTW and PPA resulted in an $\widetilde{RF}$ of 1.1 and 1.6 maximum, respectively. However, the $\widetilde{RF}$ for the single PPA treatment was higher than for the single PTW treatment. The reason for this is perhaps the increased ability of PPA, a gas, to penetrate the entire exposed surface. If the combined treatment of PTW/PPA was applied to the *C. albicans* inoculated PE stripes, surprisingly, the results showed an antagonistic result in most cases (~51.8%), followed by additive (~31.4%) and synergistic (~16.6%) effects. Best results were gained for combinations of the PPA treatment of 50 s + 5 min with the PTW treatment of 5 s + 1 min, of the PPA treatment of 50 s + 1 to 5 min with the PTW treatment of 50 s + 1 min, and of the PPA treatment of 15 s + 3 and 5 min as well as 50 s + 1 to 5 min with the PTW treatment of 50 s+ 3 min. Maybe the PTW treatment should be elongated to a 5 min post-treatment-time (e.g., 5 s + 5 min, 15 s + 5 min and 50 s + 5 min) to achieve an increase in the reduction.

These results are in line with the ones published by [44], where different Gram-positive (*Listeria monocytogenes*) and Gram-negative bacteria (*Escherichia coli*, *Pectobacterium carotovorum*), as well as *Bacillus atrophaeus* endospores, were investigated under comparable conditions. The inactivation effects observed after the single and especially after the combined PTW/PPA treatment strongly depended on the investigated microorganism and used treatment times. However, in all cases synergistic process windows were found and could be used for further investigations and applications.

There are many possible mechanisms induced by PTW/PPA treatment, which may underlie the observed results. However, the effective inactivation mechanisms and any involved signal pathways induced by indirect plasma treatment based on PPA and PTW are still unidentified.

A first aspect of inactivation could be an acidification, as is known for lactic acid [45]. Previous investigations showed pH values of 1.7 (5 s pre-treatment time), 1.5 (15 s pre-treatment time) and 1.1 (50 s pre-treatment time) for PTW [46]. Causing a pH decrease inside a bacterial cell by ionization of the undissociated acid molecules or a disturbance of the substrate transport by changing the permeability of the cell envelope could be a mechanism of action that causes cell inactivation. In addition, pH values below 4.0 have a negative effect on the growth of most food-borne microorganisms. Due to the chemical behavior of PTW and PPA, whether used separately or in combination, acidification is expected on the sample surface and thus in the direct microbial environment [31,46–48].

Another reason for the inactivation of microorganisms by PTW and PPA can be moisture in the PPA and PTW processes, which is provided by the environment/compressed air or by the water itself.

Maeda et al. (2003) have reported on the influence of humidity in the air on bacterial inactivation [49].

They observed an obvious dependence of microbiological inactivation of *E. coli* on air humidity.

The microorganism under investigation showed a maximum inactivation rate at 43% relative humidity, which is frequently found in laboratory/room air. The significance of plasma gas humidity for the inactivation of *Bacillus subtilis* endospores and *Aspergillus niger* conidiospores was investigated by Muranyi et al. (2008) [50]. They showed a reduction of up to 5 $\log_{10}$ cycles within 7 s at 0% relative humidity and of up to 4 $\log_{10}$ cycles at 80% relative humidity for the spores.

The third and perhaps most important explanation could result from the chemical composition of PTW and PPA. Against this background, there are antimicrobial active reactive nitrogen species and a few reactive oxygen species that come into the fore and have been detected in our PTW and PPA. Therefore, experimental studies of the chemical composition of PPA and PTW are crucial for understanding the biochemical reactions responsible for microbial inactivation. Chemical analysis showed that reactive species (RONS) were detectable in both PTW and PPA.

The chemical species detected in PPA were nitric oxide ($\cdot NO$), nitrogen dioxide ($\cdot NO_2$), nitrous acid (HNO_2), nitric acid (HNO_3), hydrogen peroxide (H_2O_2) and water (H_2O). In the PTW, nitrite (NO_2^-), nitrate (NO_3^-) and very small amounts of hydrogen peroxide (H_2O_2) were measured.

Nitrogen monoxide ($\cdot NO$) is produced by nitrogen (N_2) and oxygen (O_2), both of which are contained in the compressed air used as working gas.

In the presence of O_2, $\cdot NO$ reacts further to form nitrogen dioxide ($\cdot NO_2$). The long-lived radicals $\cdot NO$ and $\cdot NO_2$ are known for their antimicrobial effectiveness.

In addition, peroxynitrite ($ONOO^-$), which is believed to have a strong antimicrobial activity, could be a product of the reaction of NO with a superoxide radical ($O_2\cdot^-$) [45,51]. The analysis of $O_2\cdot^-$ in PPA will be part of future investigations. With regard to PTW, other reactions responsible for the mechanism of action may occur, as H_2O may be an additional reaction component. $\cdot NO$, O_2 and H_2O react to form NO_2^- and hydrogen (H^+). If $\cdot NO_2$, instead of NO, reacts with O_2 and H_2O, H^+ and NO_3^- are the reaction products.

The PTW used in our experiments showed a strong acidification, which could be a consequence of the radicals $\cdot NO$ and $\cdot NO_2$, which react with H_2O to nitrous acid (HNO_2) and nitric acid (HNO_3). Normally, HNO_2 decomposes to H+ and NO_2^-, but a pH below 2.75 could lead to the spontaneous formation of $\cdot OH$ and $\cdot NO$ [45,51]. Many of the detected and postulated ions, radicals and molecules are known for their strong antimicrobial activity. In combination with the antimicrobial potential of a pH shift into an acidic environment, the occurrence of such compounds, which are also influenced by pH, may lead to the observed antimicrobial mechanism of action of PPA and PTW. Further investigations are necessary and will provide a better insight into the chemical and biochemical processes underlying the antimicrobial effects observed and assumed in the present work.

The high quality and safety of food is achieved by the high hygienic standard in its production. The importance of production hygiene and quality assurance increases with the level of microbiological requirements to be met. In the food industry in particular, inadequate hygiene leads to health risks for consumers due to food-borne diseases and leads to economic risks due to high production losses.

The high nutrient content and relatively high pH value (pH 5.5 to 7) of some fruit and vegetable varieties can promote the growth of pathogens [52]. Bacterial and fungal contamination of food itself or of the production environment, as well as interrupted freezing and cooling chains, can be a cause of food-borne diseases, as well as production and quality losses. *L. monocytogenes*, *Salmonella* sp., *Clostridium* sp., *E. coli*, *Staphylococcus aureus* and *Aspergillus* sp. are human pathogens typically found on and in food. Phyto-pathogens include many molds (e.g., *Fusarium* sp.), Oomycetes, *Xanthomonas* sp., *Erwinia* sp. and *Pseudomonas* sp. These can have a negative effect on product quality.

Regular cleaning and disinfection cycles of production facilities and rooms to reduce the microbiological load can mean high costs, lower productivity, possibly heavily contaminated waste water and material damage for the food producer. Therefore, there is a great need for innovative cleaning concepts in the food industry, which supplement or replace existing plants with resource-saving, versatile and efficient technologies.

Against this background, the use of the plasma-based PTW/PPA decontamination process investigated in this study seems promising. The functionalization of air and water to fungicidal and antimicrobial agents by non-thermal plasma leads to a fast acting decontamination agent without leaving toxic residues on food or production surfaces and can be applied in adjustable, product-specific process windows at room temperature.

These advantages, coupled with an on-demand application and easy integration into existing cleaning processes, make cold plasmas particularly attractive for decontamination in food processing [53,54]. As mentioned above, microorganisms such as *C. albicans* can contribute to biofilm formation on surfaces. The essential step of biofilm formation is the transition from a reversible attachment of the cells on surfaces to irreversible adhesion [55,56]. Therefore, fungicidal and antimicrobial cleaning and inactivation can make a positive contribution to biofilm prevention.

In addition to the observed antagonistic, additive and synergistic inactivation effects, another important point for the combined treatment of *C. albicans* and the previously published bacteria and endospores was observed [44]. In disinfection and sterilization processes, deviations from the exponential inactivation kinetics are often found. In the semi-logarithmic representation, these deviations can show concave curves up to the so-called tailing instead of the expected linear relationship. In the case of tailing, the inactivation strives towards a lower limit value, which thus represents a process limit that cannot be overcome even by a massive extension of the process times. The reasons for the non-exponential inactivation kinetics can be manifold and have been discussed in the literature for a long time with no agreement having been reached up to now. Cerf (1977) provides a good review of this topic [57]. Under certain conditions, tailing can also occur in the two plasma processes described here. Therefore, it is of crucial importance for an economic use of the process to take measures to reduce or eliminate tailing completely. In the combination of the two processes, it was shown in our work that this is possible under certain parameters. Further scientific investigation of the mechanisms behind this effect is necessary for a process-technical implementation.

4. Conclusions

In our investigations, a microwave-based plasma source was used to generate two antimicrobial active agents (PPA and PTW), which can be used individually or in combination as an innovative cleaning and decontamination method. The microwave-based plasma source setup allows for the variation of process parameters and can functionalize air and/or water to two different fungicidal agents. These are subsequently flexible in application and combination. Our microbiological investigations showed an antimicrobial effect of PTW and PPA against *C. albicans* in single and combined application, and regarding the combined application new information about the interaction of PTW and PPA (antagonistic, additive and synergistic) against *C. albicans* could be obtained. The previous knowledge about the chemical composition of PPA and PTW in combination with the observed microbiological results also allowed theoretical considerations about possible mechanisms of action of PPA and PTW, which can serve as a basis for discussion and research for further investigations. The technical implementation and upscaling of PPA/PTW technology into industrial processes, with self-sufficient supply, on-demand operation and simultaneous resource conservation, will be one of our future challenges.

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