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Influence of plasma-treated air on surface microbial communities on freshly harvested lettuce

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ABSTRACT

Plant-based foods like lettuce are an important part of the human diet and worldwide industry. On a global scale, the number of food-associated illnesses increased in the last decades. Conventional lettuce sanitation methods include cleaning either with tap or chloritized water. Beside these water-consuming strategies, physical plasma is an innovative and effective possibility for food sanitation. Recent studies with plasma-treated water showed an effective reduction of the microbial load. Plasma-processed air (PPA) is another great opportunity to reduce the microbial load and save water. To test the efficiency of PPA, the surface microbiome of treated lettuce was analyzed via proliferation assays with special agars, live/dead assays and tests for respiratory activity of the microorganisms. PPA showed a reduction of the colony forming units (CFU/mL) on all tested microbial groups (Gram-negative and Gram-positive bacteria, yeasts and molds). These results were supported by the live/dead assay. For further insights, the PPA-ingredients were detected with Fourier Transformation Infrared Spectroscopy (FTIR), which revealed NO_2, NO and N_2O_5 as the main reactive species in the PPA. In the future, PPA could be an outstanding, on-demand sanitation step for higher food safety standards, especially in situations where humidity and high temperature should be avoided.

1. Introduction

Plant-based foods are one of the pillars on which human diets are based. Among others, lettuce is a popular part of the human diet. The Statistics Division of the Food and Agriculture Organization of the United Nations lists lettuce with a yearly turnover of 24.6 m t (Berg et al., 2014). Endive (*Cichorium endivia* L.) and other leafy greens could act as a deliverer of vitamins and nutrients (Cardinale et al., 2015; Erlacher et al., 2015). Lettuce is mostly consumed raw. Accordingly, a high level of food safety is obligate. Furthermore, this safety must be achieved without visually damaging the plant tissue that is purchased by consumers (Cardinale et al., 2015; Schnabel et al., 2021).

The phyllosphere could host up to 10^5 – 10^8 colony forming units (CFU) per gram lettuce fresh weight (Itohan et al., 2011; Paillart et al., 2017; Bencardino et al., 2018). Of these colonizers, bacteria are the most abundant (Williams et al., 2013; Erlacher et al., 2015). Every year, there are 600 million foodborne illnesses (420,000 deaths) worldwide (Lee and Yoon 2021). Pathogens causing these infections are, among others, *Listeria monocytogenes,* Campylobacter spp. and *Escherichia coli*, which could lead to illnesses resulting in hospitalization and death (Abadias et al., 2008; Williams et al., 2013; McManamon et al., 2017; Lee and Yoon 2021). Most of the time, animal-based food are the primary vectors for illness, but vegetables could also be potential carriers of pathogens (Heredia and García 2018; Lee and Yoon 2021).

Some microorganisms within the native microbial load could also cause spoiling. In Germany alone, annual harvest and market losses of vegetables are listed in the range of 1.1 million tons, with losses of 30% considered common (Schnabel et al., 2012). Typical spoilage-associated bacteria like *Pseudomonadaceae* and *Enterobacteriaceae*, which could decrease possible storage times of packed lettuce (Gram et al., 2002; Lopez-Velasco et al., 2011), may be found within the microbial loads (Gram et al., 2002; Lopez-Velasco et al., 2011). However, it seems that not all members of the *Pseudomonas* phylum are harmful. For example, strains of *Pseudomonas fluorescence* could build antifungal compounds and thus promote plant growth (Duan et al., 2013; Hol et al., 2013; Hernández-León et al., 2015; Dorjey et al., 2017). Further, *Pseudomonas*

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syringae could be harmless on leaves, or it could be a major plant pathogen (Hirano and Upper 2000). These two *Pseudomonas* examples show that more than the sheer presence of potentially pathogenic organisms seems to have an impact on storability. Therefore, the current industrial methods for extending food shelf life is the reduction of the total microbial count in general (Pirovani et al., 2004; Wang et al., 2004; Rose et al., 2012).

The food industry is aware of the high variability in performance of sanitation methods to achieve a hygienic and consumer-safe lettuce. Some conventional methods are the use of pure water, or adding both chlorine dioxide and ozone to prepare the wash water for reuse and generate an option for water recycling (Delaquis et al., 2004; Klaiber et al., 2005; Najafi and Khodaparast 2009).

Air drying tunnels like LDT-50/5D (Sormac, Venlo; Netherlands) are used to dry the surface of the lettuce after washing. This drying method often uses pure air. This step of lettuce preparation also has the potential to reduce the bacterial load. Different studies show that non-thermal physical plasma could reduce the microbial load on different abiotic and biotic surfaces due to its ingredients (RNOS) and UV, which damage microbial cells on different levels (Ehlbeck et al., 2010; Handorf et al., 2020; Chautrand et al., 2022). Some foods cannot be treated with water-based techniques (such as spices, tea and herbs) because it negatively affects their shelf life and quality. Thus, a dry-cleaning method has a wide potential range of applications.

In general, physical plasma describes a gas particle mixture of ions and free electrons; however, it can also contain neutral atoms or molecules, and therefore contain free charge carriers (Kogelschatz 2004, Handorf et al., 2020). If physical plasma hits a medium, it generates RNOS (reactive nitrogen/oxygen species) (Oehmigen et al., 2011).

The MidiPLexc is a microwave-driven plasma torch that uses compressed air as working gas (Handorf et al., 2020). The MidiPLexc generates a complex competition of reactive molecules in the plasma-processed air (PPA) (Schmidt-Bleker et al., 2015). Compounds in the PPA are nitric oxide (NO*), nitrogen dioxide (NO_2*) and hydrogen peroxide (H_2O_2), as well as other metastable RNOS (Schnabel et al., 2012).

Our hypothesis (H_1) is that PPA has a negative impact on the native microbial community and reduces it. Our null hypothesis (H_0) is that PPA has no influence on the microbial load.

To test our hypotheses, we investigated the development of the CFU/mL after different treatment times on plate-count agar (PCA) under controlled temperatures. After the identification of optimal treatment times, these were tested in combination with different special agars. In addition, the live/dead ratio and metabolic activity of the native microbial load on lettuce were examined. In order to attribute potential effects to the components of the PPA, the composition of the PPA was analyzed by means of FTIR. Both untreated lettuce samples and air-treated lettuce samples were used as controls in our experiments.

2. Materials and methods

2.1. Plasma source

In our experiments, the in-house developed MidiPLexc (Fig. 1) was used. Previously, Handorf et al. (2021) described its exact structure in detail, as well as the protocol for PPA generation (Handorf et al., 2021). Briefly, the MidiPLexc is a microwave-driven plasma device that uses compressed or ambient air. The device generates plasma-treated air (PPA), which contains nitrogen oxides as well as other RNOS. Parameters used for the running the MidiPLexc are given in Table 1.

2.2. Sample preparation and PPA-treatment

In this study, the lettuce used was endive (*Cichorium endivia* L.). The endive was planted and harvested on an open field (region of Heilbronn, Germany) under conventional conditions. Freshly-harvested lettuce heads were cooled initially to 4 °C. The cooled lettuce heads were sent to our laboratory in Greifswald (Germany) overnight under the same conditions. After arriving in Greifswald, the outer lettuce leaves were discarded and the heads were stored at 4 °C. Before each experiment, one lettuce head was cut down to bite-sized pieces, which were then mixed. The mixed pieces were weighed into 5 g samples. The 5 g samples were put into 1 L sterile glass bottles and closed until they were treated with PPA (Fig. 2).

Before PPA treatment, the glass bottles were stored at room temperature for a maximum of 5 minutes. The 5 g samples were processed in three parallel treatments. The treatment times were 0s (untreated), 60s PPA treatment (PPA_{60s}), 120s PPA treatment (PPA_{120s}) and 240s PPA treatment (PPA_{240s}). A 240s compressed air treatment (AIR_{240s}) was also tested as a negative control. After the treatments, the gases were vacuumed from the bottle within 1-3 s.

The longest applicable treatment time was 240s, due to subsequent temperature increases above 38 °C. Thus, possible reductions beyond this time would no longer be solely attributable to the PPA components.

The treated lettuce pieces were transferred into blending bags (400

Table 1
Parameters of PPA generation with MidiPLexc.

Frequency [GHz]	2.45
Forward power [W]	50
Reverse power [%]	1-5
Volumina flow [SLM]	5

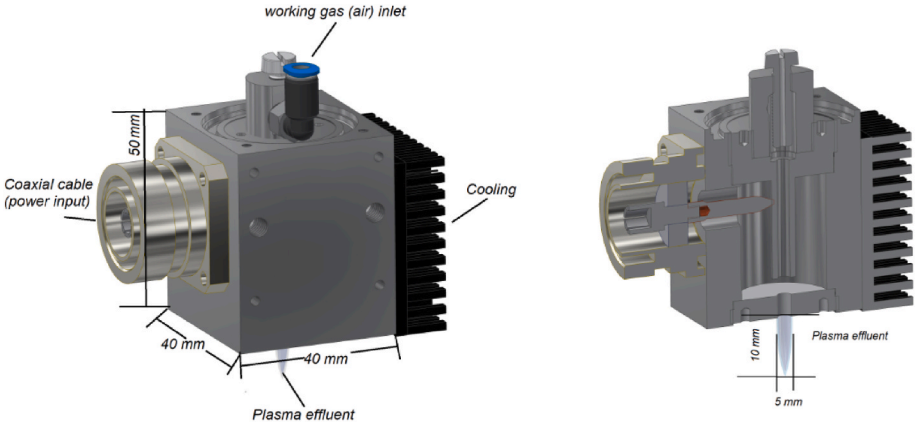


Fig. 1. Construction of the MidiPLexc. On the left side, the MidiPLexc with dimensions. On the right side, the cross section of the MidiPlexc with the dimensions of the plasma effluent.

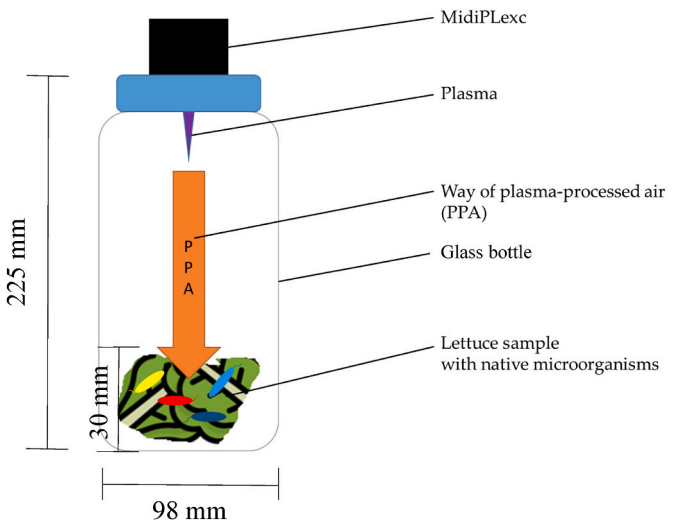


Fig. 2. Overview. Setup of the experiment with the plasma source and the native lettuce sample.

mL, VWR, Germany) and immediately blended with 20 mL PBS (Phosphate buffer with pH 7.2 after Sörensen). Blending was done with the blender ECN 710-0873 (VWR, Germany) for 30 seconds at 160 rpm and finally, 1 mL of the lettuce-microorganism-PBS suspension was removed and used for further tests.

2.3. Detection of lettuce drying while storage

It was necessary to determine the endive water content before measurements with the Fourier-transform infrared spectroscopy (FTIR) could take place. As humidity could reduce the chemical species concentration of the plasma, the possible humidity of the lettuce sample could influence the chemical composition detection during FTIR analysis.

In addition, lettuce water content losses during 4 °C storage were detected to analyze the impact of this factor on quality loses or microbial reduction, as most microorganisms need water activity values (a_w values) above 0.9.

After delivery, freshly-harvested lettuce heads were stored in a 4 °C fridge. Lettuce samples were measured on the day of delivery, as well as on the first, second and third day. For each measurement, 100 g of lettuce were cut into small pieces. Approximately 2 g of lettuce pieces were measured by Halogen moisture analyzer HE73 (230V) (Mettler-Toledo, USA) in five parallels.

2.4. Characterization of PPA composition by fourier transformation infrared sepctroscopy (FTIR)

To detect and analyze the chemical compounds of the PPA, the Fourier Transform Infrared spectroscopy (FTIR, Vertex V70; Bruker, Billerica, Massachusetts) was used. The resolution of the FTIR was 1 cm^{-1} and the absorption path length was 0.15 m. The main reactive components of the PPA were identified downstream of the plasma effluent. Therefore, different operation conditions were used. Measurements were made 1.5 m from the plasma effluent of the MidiPLexc. The plasma source was operated with 50 W power and an airflow of 1.5 SLM. Thus, compressed air was converted to PPA by means of the ignited plasma source.

2.5. Detection of culturable microbial cell counts (CFU/mL)

PCA was used for the identification of the optimal treatment times. After establishing the treatment times, different agars were used to test various groups of the microbial community (Table 2).

One mL suspension samples of bite-sized lettuce were taken after the PPA and air treatments. The suspensions were diluted by adding between 100 and 900 μL of recovery dilutant (8.5 $g*L^{-1}$ NaCl and 1.0 $g*L^{-1}$ tryptone). Next, the samples were diluted stepwise up to 10^{-5} of the original concentration. The dilutions (10^0 – 10^{-5}) were plated out on the different agars via the micro-dilution-method (Miles et al., 1938). The diluted samples were incubated at room temperature (except EA, which was incubated at 37 °C) for 5 days. Colonies were counted on days 2, 3, 4 and 5. The detection limit was 10^2 CFU/mL.

2.6. Detection of live-dead-ratio of treated samples

The LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific, Germany) was used for the detection of damaged cell envelopes by the ratio of Green/Red (G/R) after treatment. The kit contains two dyes: the green fluorescent SYTO9 (alive) and the red fluorescent propidium iodide (PI, dead). The kit was used according to instructions: nine μL of PI and nine μL of SYTO9 were mixed into three mL of ultrapure water. After that, 100 μL of the mixture was added together with 100 μL of the samples into a 96-well plate. Then the 96-well plate was incubated for 15 minutes (80 rpm, under light termination, at room temperature) on an orbital shaker (PSU-20i, Biosan, Latvia). After 15 minutes, the fluorescence signal of each well was detected by a plate reader (Varioskan-Flash®, Thermo Scientific, Germany). Measurements were made at an excitation wavelength of 485 nm and an emission wavelength of 530 nm for SYTO9. For PI, the excitation wavelength of 485 and emission wavelength of 630 nm for fluorescence were used. For the calculation of the G/R-ratio, the green absorbance value was divided by the red absorbance value. Finally, the G/R ratio of control samples was set to 100% to calculate the potential relative reduction after different treatments.

2.7. Detection of metabolic activity via XTT-kit

The TACS XTT CELL Proliferation assay (R&D Systems, USA) was used to determine the expected reduction in the agar-based experiments. The XTT assay was based on the solute yellow tetrazolium salt (XTT),

Table 2
Compositions of the different agars.

Agar type	Cultivable microbial groups	Agar composition
Plate count agar (PCA) [a]	Full medium agar	23.5 g/L
Standard count agar (MM) [a]	Minimal medium agar	25 g/L
MAcConkey agar (MCA) [a]	Special agar for Gram-negative	50 g/L
PCA with collistinesulfate and nalidixinacid (CNA)	Special agar for Gram-positive	PCA [a] After autoclaving: 10 mg/L collistinsulfat [a] 15 mg/L nalidixineacid [a]
Sabouraud agar with 4% dextrose (S4) [b]	Special agar for yeasts and molds	65 g/L
Glycerol yeast extract agar (GYEA)	Special agar composition for sporulating bacteria	15 g/L agar-agar [a] 5 g/L glycerol [c] 2 g/L yeast extract [a] 1 g/L di-potassium phosphate) [a]
Crystal violet bile lactose agar (CVBLA) [a]	Special agar for coliforms (room temperature)	41.5 g/L
Endo agar (ENDO)	Special agar for coliforms (37 °C)	36 g/L Endo agar base agar [a] 5 mL/L ethanolic fuchsin solution [a]

[a] Carl Roth (Germany).
[b] Merck KGaA (Germany).
[c] TH Geyer (Germany).

which is degraded by the dehydrogenase activity of living cells. This degradation results in a color change from yellow to orange, by which the available metabolic activity is detectable.

According to the protocol of the manufacturer, 50 μL of XTT solution was pipetted into single wells of 96-well plates. Then, the 100 μL samples from different PPA treatment times were pipetted in three parallel wells. The XXT sample solution was then shaken for 24 hours (37 °C, under light closure) by Linear shaker SU1000 (Sunlab Instruments, Germany).

After this 24 h reaction time, the samples in the 96-well plates were measured by a Varioskan-Flash® ELISA plate reader (Thermo Scientific, USA). The plate reader measured the fluorescence at the wavelengths of 470 nm and 670 nm (blanks).

2.8. Statistical analysis

To test the significance of the hypotheses, the differences in average total viable count (TVC) was tested with the non-parametric Kruskal-Wallis test (for n = 9 and α = 5%) in Origin Pro 2022b. To specify the difference in average TVC for each group (0s, 60s, 120s, 240s and 240s only air), a post-hoc analysis with Dunn's test with an adjusted α (due to multiple testing) was carried out. The α was adjusted using the Bonferroni method (equations (1)–(3)).

$$\alpha_{adjusted} = \frac{\alpha}{k} \tag{1}$$

$$\text{with } k = \frac{\#groups \cdot (\#groups - 1)}{2} \tag{2}$$

$$\text{such that } \alpha_{adjusted} = \frac{0,05}{\frac{5 \cdot 4}{2}} = 0,005 \tag{3}$$

3. Results

3.1. Lettuce water content measurements during storage

As expected, the lettuce consistently lost water while in storage (Table 3). Before storage, the outer leaves were discarded and the whole lettuce heads were placed on a plastic plate. They were then stored in a fridge (4 °C) for three days. The water content of the lettuce decreased slightly after longer storage times in the fridge. After three days of storage, 5 g of lettuce lost 0.07 g free water compared to the freshly delivered lettuce (day 0). These small differences in the free water content should not have a high influence on the following FTIR analysis and can be considered negligible. However, water loss may cause a loss of quality, especially in texture, which could be pertinent to other research questions.

3.2. Components of the PPA

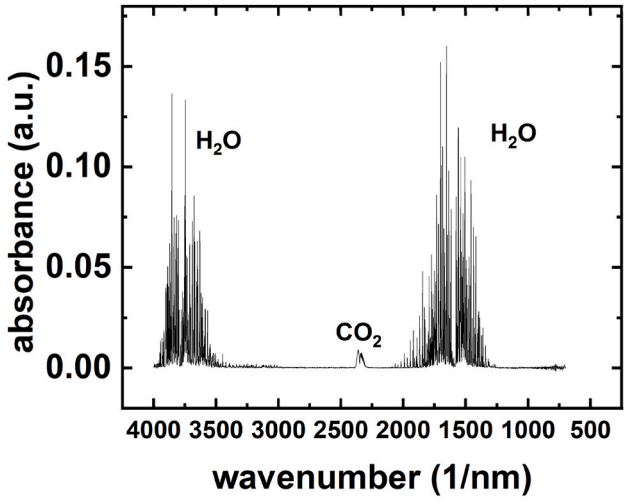
Among others, nitrogen oxide (NO), nitrogen dioxide (NO_2) and dinitrogen pentoxide (N_2O_5) were detected as long-living components of the PPA. NO_2 accounts for more than 70% of all reactive species in the PPA. The qualitative and quantitative analysis of PPA components by FTIR were carried out on a 1 cm^{-1} resolution and an absorption path length of 0.25 m. Concentration values were obtained from the calibration curve of respective reference gases. Therefore, NO_2 was used as an indicator for determining the optimal process parameters.

Table 3
Overview of the water loss while storage, in percent of fresh weight and extrapolation to 5 g lettuce.

Day	Water content [% of MC]	Water content [g] of 5 g lettuce	Loose of water [g] compared to day 0
0	94.18 ± 0.53	4.71 ± 0.03	0
1	93.66 ± 0.73	4.68 ± 0.04	- 0.03
2	93.00 ± 0.41	4.65 ± 0.02	- 0.06
3	92.98 ± 0.47	4.64 ± 0.01	-0.07

The FTIR facilitated several insights into the PPA composition (Fig. 3a and b). Firstly, the untreated lettuce was measured in a glass bottle (Fig. 2). The peak clusters in the ranges of 1260 – 2092 cm^{-1} and 3393 - 4047 cm^{-1} characterize water from non-treated, freshly-cut lettuce (Fig. 3a), which is expected considering that lettuce has a free water content of more than 94% (see 3.1). In addition, a peak at 2358 cm^{-1} characterizes CO_2 in the glass bottle, which was most likely introduced into the system while opening the glass bottle to place samples.

Fig. 3b depicts the FTIR measurement of PPA during the lettuce treatment. NO_2, NO and N_2O_5 are expected in the PPA as main components (Weihe et al., 2023). The exhaust of the glass bottle (Fig. 2) was connect with the inlet of the FTIR in transmission mode for measuring gas samples. However, due to the existence of water introduced by the lettuce into the system, parts of the peaks of NO_2, NO and N_2O_5 are overlapping with the peaks of water in the range of 1260 – 2092 cm^{-1}. From the comparison of the two spectra of PPA chemical composition with and without lettuce, and excluding H_2O and CO_2, no additional chemical compounds were found in the system due to the presence of lettuce. Conclusively, the introduction of the lettuce food matrix seemed to have no influence on the chemical composition of main reactive



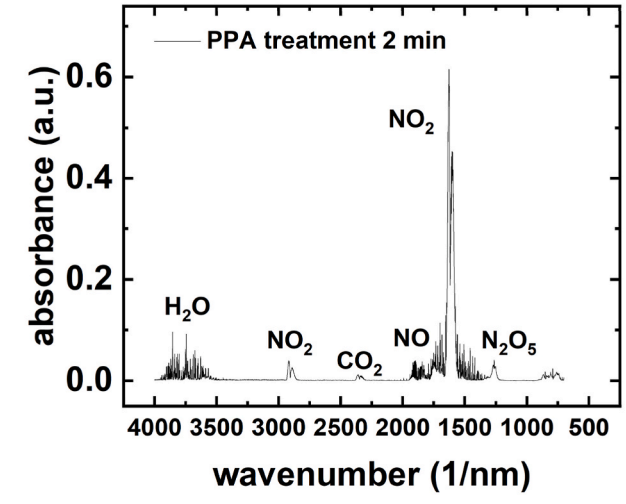


Fig. 3. FTIR spectra of lettuce in the glass bottle without (a) and with (b) PPA.

species in the PPA.

3.3. Influences of the PPA on the culturable native microbiome

3.3.1. Proliferation assay based on TVC count

The cultivable microbial community was analyzed with special agars for the groups of Gram-negative bacteria (particularly the group of coliform bacteria), Gram-positive bacteria (particularly the group of sporulating bacteria) and the group of yeasts and molds. All tested groups had concentrations in the range of 10^5 to 10^6 CFU/mL.

The PPA treatment showed a stronger reduction of the CFU/mL following longer treatment times (Fig. 4). Four minutes of treatment with compressed air flowing through the MidiPLexc without plasma ignition showed non-reductive effects on the CFU/mL. The CFU/mL of the untreated controls and the compressed air-treated samples were equivalent.

In contrast, the PPA treatment showed a reduction in the CFU/mL across all tested groups. The reductions on the full medium (PCA) were nearly equivalent to the reductions on the minimal medium (MM) agar (Fig. 4). The lowest CFU/mL after PPA treatment was detected for the group of Gram-negative bacteria (MCA) and coliform bacteria on the ENDO agar (10^3 CFU/mL). The CFU/mL of the coliform bacteria on the KVGL (cultivated at room temperature) and ENDO (cultivated at 37 °C) agars showed nearly the same reduction dynamics. Gram-positive bacteria (CNA) were detected in higher concentrations than Gram-negative bacteria and fungi (S4) after the PPA treatment. The subgroup of

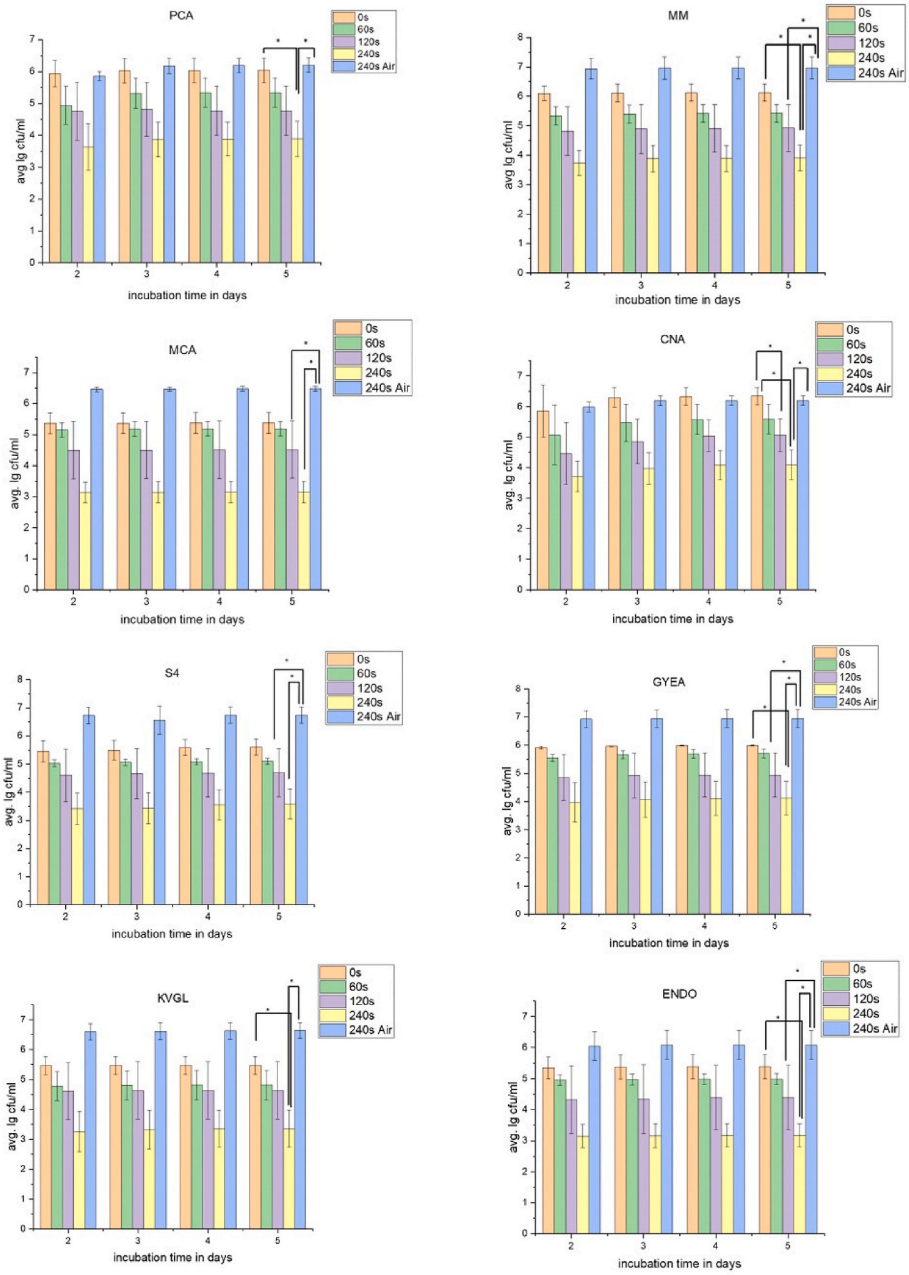


Fig. 4. TVC of the microbial communities on different special agars following different treatment times and 5 days of incubation. The agars were plate-count agar (PCA), minimal medium agar (MM), MacConkey agar (MCA), PCA with added collistinsulfate and nalidixin (CNA), Sabouraud agar with 4 & dextrin (S4), glycerine yeast extract agar (GYEA), crystal violet bile lactose agar (KVGL) and ENDO-agar. The PPA treatment times were 0s, 60s, 120s, 240s as well as 240s treatment with only compressed air as a control. The stars indicate significant differences after alpha adjustment as described in the statistical methods. The detection limit was at 10^2 CFU/mL.

sporulating Gram-positive bacteria were the least negatively affected by the PPA treatments. After the PPA treatment, yeasts and molds (S4) still had lower concentrations than the Gram-positive bacteria (CNA) and slightly higher concentrations than the Gram-negative bacteria (MCA). However, it seems the fungi group (S4) survived the PPA treatment better than the bacterial groups (MCA, CNA, GYEA, KVGL and ENDO) (Fig. 4).

3.3.2. *Live/dead assay*

The fluorescent dye-based assay shows differences between the live/dead ratio after the different PPA treatment times (Fig. 5). After 60 seconds of PPA treatment, the ratio was reduced by ~20%. The PPA treatment time of 120 seconds reduced the ratio by about 25%. After the longest possible PPA treatment time of 240 seconds, the ratio was lowered by nearly 27%.

3.3.3. *XTT assay*

The XTT assay can provide insights into the activity of the respiratory chain after PPA treatment (reproductive metabolism). The results of the yellow-orange dye-based XTT assay (Fig. 6) were not as clear as the results of the live/dead assay. After 1 min of treatment, a small reduction was detectable. This reduction was negligible after longer PPA treatment times. These results may be due to wavelength detection limits. With the blending of the lettuce pieces after the PPA treatments, the PBS also became slightly yellow. This yellow coloring of the medium by the native lettuce ingredients may overlap with the dyes in the kit. On the other hand, it could indicate that the metabolic activity of the surviving and still proliferating microorganisms are not affected, and that the gained reduction in the proliferation (CFU-assay, 3.3.1) as well as in the cell envelope integrity (live/dead assay, 3.3.2) are due completely to the killing mechanisms of PPA. In addition, it could mean that PPA did not induce the viable but non-culturable (VBNC) state. A finally conclusion based on the XTT-assay is not possible under the chosen experimental design and setup.

4. Discussion

Our main goal was to analyze the antimicrobial activity of PPA on the native microbiome of fresh-cut lettuce and to have an insight into the resulting PPA composition. Therefore, we used cultivation-based proliferation assays as well as cultivation-independent live/dead assays to detect influences of PPA on both the whole cultivable microbiome and specific microbial groups. As assumed in our hypothesis that PPA would have a negative impact on the native microbial community and reduce

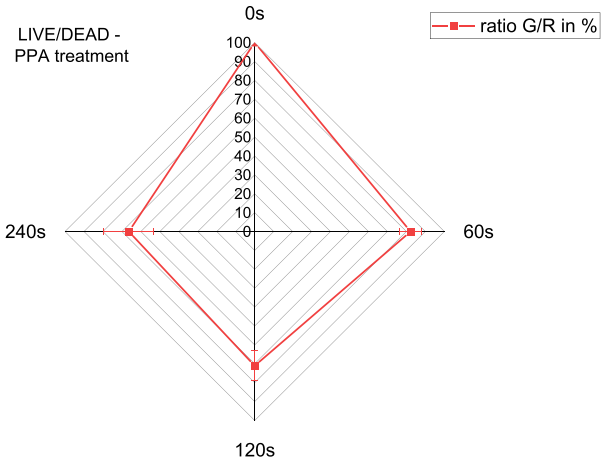


Fig. 5. Live/dead assay results of the native lettuce microbiome after plasma treatment.

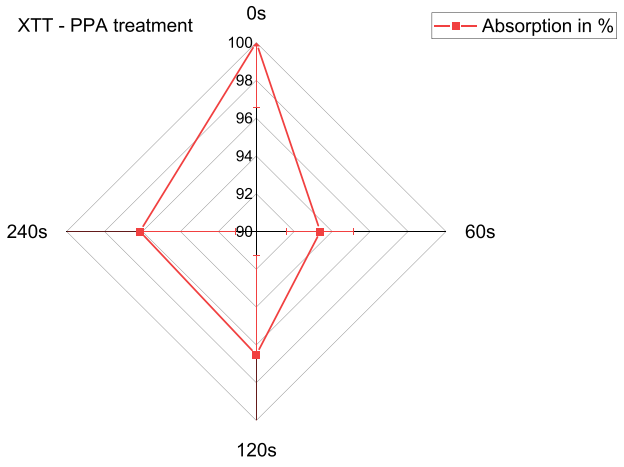


Fig. 6. Results of the XTT assay of the lettuce microbiome after plasma treatment.

it, the PPA treatment reduced the CFU/mL of all tested groups. Thus, it can be inferred that PPA can cause a general reduction in the native microbiome of the lettuce. Further, it appears that Gram-negative bacteria are reduced more strongly than the other groups tested.

The FTIR results showed a high concentration of NO_2, NO and N_2O_5. These molecules are expected to be main components of PPA. A further study from Weihe et al. showed comparable results (Weihe et al., 2023). Because of the high energy transfer to the MidiPLexc, a high gas temperature within the discharge dissociated the air molecules (Schnabel et al., 2012).

The thermal formation of NO starts with an initial attack of an oxygen atom on the triple bond of N_2. This step requires high energy consumption and is rate-limiting. The resulting free nitrogen atoms were oxidized rapidly with OH or O_2 (Abian et al., 2015):

$$O + N_2 \rightleftharpoons NO + N$$

$$N + OH \rightleftharpoons NO + H$$

$$N + O_2 \rightleftharpoons NO + O$$

Following the NO generation, NO_2 could possibly emerge by reaction of NO and O_2 (Huie 1994):

$$2\ NO + O_2 \rightleftharpoons 2\ NO_2$$

The reactive N_2O_5 could possibly emerge after NO_2 reacts with O_2 to NO_3, then in a second reaction NO_3 and NO_2 react together in the physical plasma (Fitzsimmons et al., 1999):

$$2\ NO_2 + O_2 \rightleftharpoons 2\ NO_3$$

$$NO_3 + NO_2 \rightleftharpoons N_2O_5$$

Also possible is the generation of HNO_3, which was not detectable in this study (Van Doren et al., 1990).

$$N_2O_5 + H_2O \rightleftharpoons 2\ HNO_3$$

Nitric oxide (NO*) and nitrogen dioxide (NO_2*) are highly reactive molecules that can react quickly with other molecules. NO* can modify the redox-active cysteine residue of proteins, and subsequently can deactivate them or could react with enzymatic iron-sulfur-complexes ([4Fe-4S]), and hereafter inhibit these enzymes (Liew and Cox 1991; Vázquez-Torres and Bäumler 2016). NO_2, NO and N_2O_5 are reactive nitrogen species (RNS) that could be generated by non-thermal plasmas (Chae 2003, Pankaj et al., 2018; Wang et al., 2023). NO_2 negatively affected different groups of surface bacteria at different levels (Janvier

et al., 2020).

We were also able to compare the degree of reduction across different microbial groups following PPA treatment. Most of the samples had a natural bacterial load of 10^5-10^6 CFU/mL, whiles studies show a natural bacterial load of 10^5-10^8 CFU/mL (Itohan et al., 2011; Paillart et al., 2017; Bencardino et al., 2018). These differences could be due to the natural heterogeneity of native samples, removal of the outer lettuce leaves or storage conditions, among other possible causes. However, the investigated initial load fit within the commonly detected range.

Although the microbiome within all tested groups was reduced, the Gram-negative bacteria appeared to be most susceptible to the PPA treatment. NO_2 and NO have the capacity to damage bacterial cells on different levels (DNA, proteins and lipids), and it seems that the hydrophobic part of the membranes could favor the reactive activity of RNS with other molecules (Chautrand et al., 2022). Lipids are a major target for reactive species (Cabiscol Català, Tamarit Sumalla et al., 2000). An intact barrier to the environment is essential for microbial survival, and when phospholipids are oxidized, membrane integrity cannot be maintained and cell death follows (Meyer et al., 2015; Banthia et al., 2019; Chautrand et al., 2022). The temperature of the medium could also affect the concentration of RNOS (such as NO_2) in water (Pang et al., 2022). Considering that lettuce is largely composed of water, it is possible that adjusting the temperature of the lettuce prior to treatment could further enhance the reduction effects.

The Gram-positive bacteria (particularly sporulating bacteria) seem to tolerate the PPA agents better than the other two groups. This is probably due to endospore formation. Bacterial endospores can survive harsh conditions (Held 2004, Koopman et al., 2022). This survival strategy can ensure the survival of Gram-positive bacteria regardless of the type of stress (Held 2004, Munk et al., 2008; Koopman et al., 2022). Endospores can survive for a long time (up to years) (Vreeland et al., 2000; Munk et al., 2008). Under improved conditions, the spores can once again develop into the original bacterial cells.

In addition to endospore formation, Gram-positive bacteria have a much thicker and more stable cell wall than Gram-negative bacteria. This thick cell wall also appears to be another advantage over the Gram-negative bacteria concerning PPA treatment. In Gram-negative bacteria, the much thinner murein layer is surrounded on both sides by an inner and an outer membrane that includes lipopolysaccharides (Costerton et al., 1974; Lüderitz et al., 1982; Beveridge 1999). Between the murein layer and the respective membranes, periplasmic layers can be found in Gram-negative bacteria, in which specific structures exist that are likely weak points of the Gram-negative bacteria concerning some antimicrobial PPA components like NO or NO_2 (Miller 1969; De Groote and Fang 1995; Beveridge 1999; Jones et al., 2009; Hibbard and Reynolds 2019).

The efficient reduction of Gram-negative bacteria is beneficial for food-safety, because the group of coliform bacteria includes many potential human pathogens like *Escherichia*, *Enterobacter* and *Citrobacter* (Feng et al., 2002). Thus, a general reduction of this group is beneficial to obtain safe food.

Interestingly, the TVC on the CNA and GYEA differs slightly. While on the GYEA the TVC is slightly higher, the TVC is slightly lower on the special agar for all Gram-positive bacteria (CNA). This indicates that non-sporulating Gram-positive bacteria (like *Listeria*) were reduced more strongly than the sporulating Gram-positive bacteria (like *Bacillus*). Some members of *Listeria* could act as human pathogens (Gandhi and Chikindas 2007). A stronger reduction of this microbial group would be beneficial for industry and human health.

Yeasts and molds also have thick envelopes. Therefore, it is possible that the better survival of the fungal members of the microbiome is based on protection from this thick envelope composed of cross-linked β-1,6-glucan with β-1,3-glucan, mannoproteins and high amounts of chitin (up to 20% of the dry mass) (Aguilar-Uscanga and Francois 2003). Some fungi develop a special response for pH switches in the environment, such as the Rim/Pal pathway (Selvig and Alspaugh 2011; Vylkova 2017). Additionally, fungi can modulate the pH of the host tissue in the process of molding (Vylkova 2017).

The results of the live/dead assay support the results of the proliferation assays. Longer PPA treatment times led to a stronger reduction in the ratio of living cells. However, the live/dead assays showed a reduction of >30% in the living cells, while the proliferation assays showed a constant reduction of >90% (more than 1 lg) of the CFU/mL. These differences could be an indication for the VBNC (viable but non-cultivable) status. Bacterial and fungal cells in the VBNC are still alive but cannot grow on agars (Oliver 2005, Salma et al., 2013; Zhang et al., 2015). However, this does not quite fit with the XTT results. This is possibly due to the impact of a time-delay in the CFU assay, since the L/D assay was run directly after treatment and the proliferation assay after 5 days. This inability to grow could be due to cellular reparation, which requires so much of the cellular energy capacity that the cells cannot simultaneously divide. This maintenance metabolism takes precedence over reproductive metabolism. However, after the repairing a re-growth of cells is possible (Oliver 2005, Ramamurthy et al., 2014).

As hypothesized, the treatment without ignited plasma (only air flow) for 4 min showed no reduction of the microbial load. These results confirmed that the observed reductive effect is due to the PPA ingredients.

In summary, our results demonstrate a clear tendency in the reduction of living, cultivable cells by PPA treatment. Thus, we can infer that PPA reduces the microbiome in total number. Because of this effective reduction, simple handling, cheap source gas (compressed air) and reduction of water usage, PPA can be a worthwhile alternative to or extension of industrial cleaning methods.

5. Conclusion

The PPA treatment reduced the microbiome on lettuce due to its ingredients. Cultivable microbial groups of bacteria and fungi were reduced in the range from 10^4 to 10^3. The antimicrobial efficiency of PPA is based on the RNOS such as NO*, NO_2* and N_2O_5. These substances could act on different modes of action and potentially damage the bacterial lipids, proteins and DNA. With this wide spectrum of possible targets, microorganisms could hardly build efficient defense strategies against the PPA.

In the future, PPA could be a great opportunity to support higher food security standards. This method has the capacity to improve food supplies in the future. However, this work focused primarily on the antimicrobial properties of PPA. Possible effects of PPA on lettuce in terms of stability and possible flavor or color changes are relevant aspects of the treatment that could be investigated in following studies. In addition, PPA treatment of other sensitive vegetable foods like tomatoes or spinach is possible.

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CRediT authorship contribution statement

Hauke Winter: Investigation, Data curation, Formal analysis, Writing – original draft. **Robert Wagner:** Formal analysis, Writing – original draft. **Yijiao Yao:** Investigation, Data curation, Writing – original draft. **Jörg Ehlbeck:** Funding acquisition. **Uta Schnabel:** Conceptualization, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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