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Inactivation of relevant eggshell-related bacterial species on agar plates with atmospheric pressure plasma

Inaktivierung relevanter Eischalen assoziierte Bakterien auf Agarplatten mittels atmosphärischem Plasma

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Summary

Main representatives of the native eggshell flora, namely *Staphylococcus* spp., *Micrococcus* spp. and *Escherichia coli,* as well as the pathogens *Salmonella* Typhimurium, *Salmonella* Agona and *Salmonella* Enteritidis were treated with an atmospheric pressure plasma jet using different process gases (argon; argon:oxygen) and exposure times (15–300 seconds). The microorganism agar test was used to describe the observed effects. On the treated plates, the bacterial species tested showed zones of complete and partial inhibition, which are indicated in cm² as inhibition zone and reduction factor. These zones varied from 0.11 to 16.04 cm². In addition, reduction factors between 0.01 and 2.43 $\log_{10}$ cfu/agar plate were calculated. In 54% of all investigated settings argon treatment resulted in significant larger inhibition zones than argon:oxygen plasma. Contrary to that, argon:oxygen plasma had a higher impact at the partial sterilization at the whole agar plate and therefore reached higher reduction factors in 74% of all investigated settings.

Keywords: atmospheric pressure plasma jet, egg associated microorganisms, microorganism agar test

Zusammenfassung

Die Effektivität von atmosphärischem Plasma gegenüber charakteristischen Bakterien der Eischale wurde mittels Agar-Platten-Test untersucht. Ausgewählt wurden *Staphylococcus* ssp., *Micrococcus* ssp., *Escherichia coli,* aber auch Pathogene wie *Salmonella* Typhimurium, *Salmonella* Agona und *Salmonella* Enteritidis. Die Behandlung mit atmosphärischem Plasma erfolgte mit verschiedenen Gasen (Argon; Argon:Sauerstoff) über 15–300 s. Auf den behandelten Platten zeigten die getesteten Bakterien Zonen kompletter und partieller Hemmung, welche als Hemmzone in cm² und als Reduktionsfaktor angegeben werden. Die Hemmzonen variierten von 0.11 bis 16.04 cm². Zudem wurden Reduktionsfaktoren zwischen 0.01 und 2.43 $\log_{10}$ KbE/Agarplatte berechnet. In 54 % aller Versuche führte eine Behandlung mit Argon-Plasma zu signifikant größeren Zonen kompletter Sterilisation, als mit Argon:Sauerstoff-Plasma. Dafür zeigte die Zumischung von Sauerstoff einen größeren Einfluss auf die partielle Sterilisation auf der gesamten Agar-Platte und erreichte in 74 % aller Versuche höhere Reduktionsfaktoren.

Schlüsselwörter: Plasma-Jet, Ei assoziierte Mikroorganismen, Mikroorganismen-Agar-Test

Introduction

Plasma, the fourth state of matter, is generated by the ionization of gas (Niemira, 2012). The resulting radicals, reactive species, charged particles and UV radiation of the plasma are able to inactivate microorganisms. Two categories of plasma can be distinguished: thermal and non-thermal plasma. Non-thermal plasmas are attained at low or atmospheric pressures and less power (Moreau et al., 2008). In recent years, the use of non-thermal or atmospheric pressure plasma to inactivate microorganisms is gaining in relevance, particularly as a sanitation treatment in the food industry (Niemira, 2012; DFG, 2012). Especially, fresh products of plant or animal origin are in the focus of various studies (Critzer et al., 2007; Dirks et al., 2012; Kim et al., 2014; Schnabel et al., 2014; Ziuzina et al., 2013).

In the present study, atmospheric pressure plasma was applied to egg-associated bacterial species such as isolates from native eggshells and selected *Salmonella* species with regard to a potential application in egg sanitation. Eggs represent an important low-priced protein source, which is reflected by a steady increase of the egg consumption per capita to 230 in Germany in 2017 (Statista, 2019). However, eggs and egg products are also the most frequently reported vehicles (36.8%) for strong-evidence *Salmonella (S.)* foodborne outbreaks (EFSA, 2018). The most commonly reported serovars in human cases were *S.* Enteritidis and *S.* Typhimurium (EFSA, 2018). *S.* Enteritidis is primary isolated from the eggshell (Martelli and Davies, 2012; de Louvois, 1993). In addition to the serovars Enteritidis, *S.* Typhimurium and *S.* Agona (isolated from poultry farming) were included to investigate a higher number of strains. Although *S.* Agona occurs less frequently, it is also confirmed in connection with *Gallus gallus* populations (EFSA, 2015).

The native eggshell flora consists of a wide range of microorganisms (Mayes and Takeballi, 1983; Fehlhaber, 1994). Whereas fungi and viruses play a subordinated role (Fehlhaber, 1994), bacterial species and especially *Micrococcus (M.)* spp. (Board et al., 1964), *Staphylococcus (Staph.)* spp. and Enterobacteriaceae (Bruce and Johnson, 1978; Moats et al., 1980) are frequently represented on the eggshell. To examine the antimicrobial efficacy of a plasma source, the microorganism agar test (MAT) was applied, a common test for such analysis (Laroussi et al., 2006; Matthes et al., 2010 and 2012; Pompl et al., 2009; Shimizu et al., 2008).

Material and methods

Bacterial samples

The eggshell-associated human outbreak strain *S.* Enteritidis (strain 59) was obtained from the Robert Koch-Institute (RKI, Wernigerode, Germany). *S.* Enteritidis strain 21 (isolated from fresh egg yolk), *S.* Typhimurium (isolated from chicken litter) and *S.* Agona (isolated from diseased poultry) were provided from the strain collection of the Institute of Food Hygiene (Leipzig). Representatives of the native egg flora, namely *Staph.* spp., *M.* spp. and *Escherichia coli (E. coli),* were isolated from fresh hen eggs from a local *Salmonella*-free farm, according to Wittmann (2010). The isolates were identified by gram staining, morphology determination and biochemical characterization (oxidase, catalase, anaerobic glucose conversion). The *E. coli* isolate was additional confirmed by indole test and growth onto Escherichia coli Direct (ECD) agar (SIFIN, TN 1235, sifin diagnostics GmbH, Germany). All strains were stored cryogenically (Mast Group, Mast Diagnostica GmbH, Germany) at –74 °C.

The cultivations started with the transfer and incubation of one bead from the cryogenic culture into nutrient broth (SIFIN, sifin diagnostics GmbH, Germany) for 24 hours at 37 °C. The suspensions of the *S.* strains were plated on Xylose Lysine Desoxycholat (XLD-) agar (SIFIN TN1196, sifin diagnostics GmbH, Germany) and incubated for 24 hours at 37 °C. Subsequently, one colony was transferred into nutrient broth and incubated for 24 hours at 37 °C. The suspensions of the native bacterial strains were first plated on Plate-Count (PC) agar (SIFIN TN1189, sifin diagnostics GmbH, Germany) and incubated for 24 hours at 37 °C. Afterwards, one colony was transferred into nutrient broth and incubated for 24 hours at 37 °C.

Two experimental set-ups were performed. The inocula were serially diluted to the desired bacterial cell concentration. For experiments which evaluate the RF (reduction factor), 10^1–10^2 cfu (colony forming unit)/agar plate were plated, as these concentrations assured countable numbers of colonies. 10^6–10^7 cfu/agar plate were plated to characterize the colony-free IZs (inhibition zones), as these concentrations result in plate-covering growth. After plasma treatment, the agar plates were incubated for 24 hours at 37 °C.

Atmospheric pressure plasma treatment

The atmospheric pressure plasma jet (APPJ) kINPen 09® (neoplas tools GmbH, Germany) generating plasma by high-frequency excitation at 1.1 MHz (megahertz) and 2-3 kV (kilovolt) was applied for all experiments. A similar plasma jet has already been described in detail by Weltmann et al. (2009).

The inoculated agar plates were placed at a distance of 8 mm from the plasma nozzle, treated with 5 different time settings (15 s, 60 s, 120 s, 180 s or 300 s) with either argon (purity 99.99%) or argon:oxygen plasma (admixture of 0.5% oxygen) (AIR LIQUIDE Deutschland GmbH, Germany) with a gas flow of 5 slm (standard liter per minute). Each experiment was performed in duplicate, with three plates in each. Inoculated and non-plasma-treated agar plates were maintained as controls for each experiment. To exclude an influence of the gas itself, inoculated agar plates were also treated with argon and argon:oxygen gas and incubated for 24 hours at 37 °C.

Analysis

The reduction factor was calculated with the following formula:

$$\text{Reduction Factor (RF)} = \log_{10}N^{0}_{\text{mean}} - \log_{10}N^{t}_{\text{single}}$$

The IZ was calculated in cm² based on the horizontal and vertical radius of the zone.

GraphPad software version 4.00 for windows (GraphPad Software, US) was applied for data analysis. The Kolmogorov-Smirnov test was used to assess if data sets were normally distributed. Either the t-test (to compare the applied feed gases) or one-Way ANOVA with Bonferroni's post-test (to compare the different treatments times and bacterial species) were performed to compare the treatment conditions. Evaluations were based on a significance level of $p < 0.05$.

Journal of Food Safety and Food Quality 72, Heft 3 (2020), Seiten 59–82

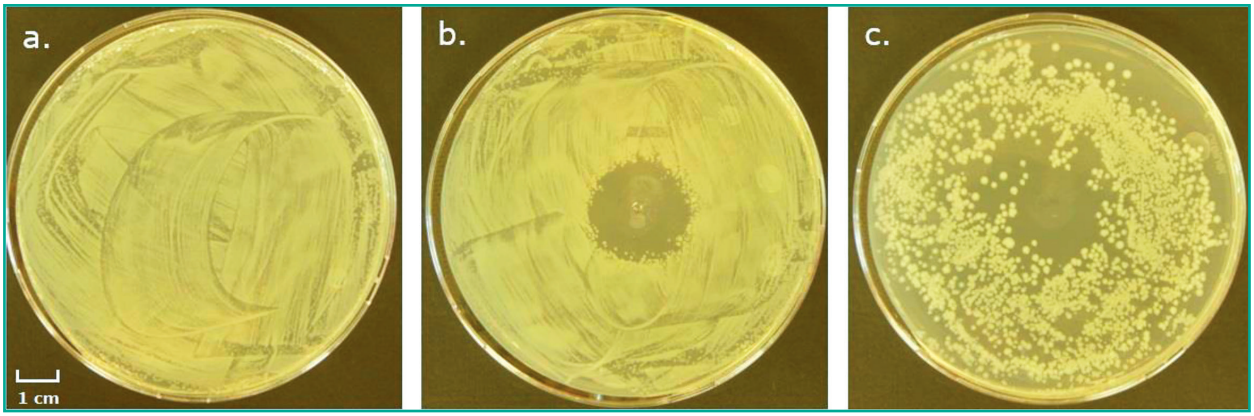

FIGURE 1: *S. Enteritidis 59 on a) untreated and b) 300 s argon or c) 300 s argon:oxygen plasma treated (5 slm gas flow, 8 mm distance, 10^7 cfu/agar plate inoculation) PC-agar plate.*

Results

After plasma treatment using the 10^6–10^7 cfu/agar plate, circular IZs were detected in all experiments, whereas untreated controls and samples treated with pure gas streams exhibited dense growth (Figure 1 a, b). IZs of all tested bacterial species increased steadily with increasing exposure times (Table 1).

Based on the mean values, in 54% of all investigated settings, argon treatment resulted in significantly better reduction than argon:oxygen plasma. There is no general tendency for all species in regard to the effectiveness of the applied feed gas. However, the different bacterial species varied significantly in their sensitivity to oxygen. Whereas *E. coli* reacted in 4 of 5 time settings, and *S.* Enteritidis 21 in 3 of 5, statistically less sensitively to argon than to argon:oxygen plasma, *S.* Typhimurium, *Staph.* spp. and *M.* spp. were significant more inhibited for all settings with argon plasma. The largest IZs were achieved for *Staph.* spp. and *M.* spp. with argon and for *E. coli* with argon:oxygen plasma, respectively.

After treatments with argon:oxygen plasma and with increasing exposure times, not only did the IZs enlarge in size, but bacterial growth also changed across the entire agar plate. Instead of dense growth, single colonies were detectable (Figure 1 c). To verify this phenomenon, a second trail with determination of RFs was examined by using a lower cell concentration.

The calculated RFs are listed in Table 2. RFs increased with rising exposure time for nearly all species and feed gases. Based on the mean values, in 74% of all investigated settings argon:oxygen plasma treatment resulted in significantly better reduction than argon plasma. The individual analysis for each bacterial species showed that argon:oxygen plasma was significantly more effective in 3 of 5 (*M.* spp., *E. coli, S.* Enteritidis 59) and 4 of 5 (*S.* Enteritidis 21, *S.* Agona, *S.* Typhimurium) time settings, but only for longer exposure times (60 s).

Discussion

The examination of colony-free areas of inoculated agar plates after plasma treatment, also known as microorganism agar test, is a common and frequently applied test to describe the antimicrobial efficacy of a plasma source (Daeschlein et al., 2012; Fridman et al., 2007; Laroussi et al., 2006; Matthes et al., 2010 and 2012; Pompl et al., 2009; Shimizu et al., 2008; Weltmann et al., 2009).

A correlation between IZ size and exposure time for non-thermal plasma sources has been shown for different bacterial strains (Matthes et al., 2010 and 2012; Pompl et al., 2009; Shimizu et al., 2008). Laroussi et al. (2006) explained this by the diffusion of reactive species with time, a wider area could therefore be affected. Even with the use of pure noble gas, small amounts of reactive species were generated (Surowsky et al., 2014; Laroussi et al., 2006). In addition to pure argon, an admixture of oxygen was used

TABLE 1: *IZs (cm^2) ± standard deviation of S. Enteritidis 59, S. Enteritidis 21, S. Agona, S. Typhimurium, E. coli, M. spp. and Staph. spp. at different exposure times (15–300 s), a distance of 8 mm, a gas flow rate of 5 slm and different feed gases (argon, argon:oxygen). Different letters indicate significant differences ($p<0.05$), small letters between the different species and capital letters between the tested feed gases of the same species for one treatment time.*

	15 s	60 s	120 s	180 s	300 s
argon					
S. Enteritidis 59	0.63 ±0.15^{a,c}	1.69 ±0.12^{a,A}	1.85 ±0.21^{a,A}	2.43 ±0.84^{a,A}	3.01 ±1.33^{a}
S. Enteritidis 21	0.75 ±0.19^{a,c,A}	1.81 ±0.18^{a}	2.56 ±0.35^{a,c}	3.44 ±0.72^{a,d,A}	5.12 ±0.49^{a,c,A}
S. Agona	0.82 ±0.20^{c,A}	2.02 ±0.31^{a,A}	2.65 ±0.35^{a,c}	3.36 ±0.27^{a,d}	5.20 ±0.81^{a,c}
S. Typhimurium	0.84 ±0.09^{c,A}	1.93 ±0.20^{a,A}	2.70 ±0.30^{a,c,A}	3.88 ±0.61^{a,d,A}	5.89 ±0.81^{c,A}
E. coli	0.71 ±0.08^{a,c,A}	1.98 ±0.34^{a}	3.25 ±0.26^{b,c,A}	4.72 ±0.21^{c,d,A}	5.05 ±0.41^{a,c,A}
M. spp.	0.51 ±0.08^{a,A}	1.62 ±0.12^{a,A}	3.81 ±0.38^{b,A}	7.02 ±0.92^{b,A}	11.45 ±0.71^{b,A}
Staph. spp.	0.18 ±0.24^{b}	1.09 ±0.52^{b}	3.30 ±0.97^{b,c,A}	5.98 ±1.78^{b,c,A}	11.74 ±2.34^{b,A}
argon:oxygen					
S. Enteritidis 59	0.48 ±0.17^{a}	0.79 ±7.30E-007^{a,B}	1.33 ±0.49^{a,B}	1.42 ±0.64^{a,B}	2.45 ±0.75^{a}
S. Enteritidis 21	0.47 ±0.10^{a,B}	1.69 ±0.12^{c,d}	3.32 ±1.03^{b}	5.41 ±0.88^{b,B}	9.59 ±3.29^{b,B}
S. Agona	0.48 ±0.05^{a,B}	1.35 ±0.40^{b,c,B}	2.45 ±0.75^{a,b}	4.07 ±0.79^{c}	4.63 ±0.99^{a}
S. Typhimurium	0.44 ±0.06^{a,B}	1.12 ±0.35^{a,B}	1.29 ±0.44^{a,B}	1.54 ±0.52^{a,B}	3.29 ±1.55^{a,B}
E. coli	0.84 ±0.09^{c,B}	1.82 ±0.28^{d}	6.76 ±0.37^{c,B}	9.10 ±0.89^{d,B}	16.04 ±1.04^{c,B}
M. spp.	0.23 ±0.09^{b,B}	0.9 ±0.18^{a,b,B}	2.42 ±1.07^{a,b,B}	4.36 ±0.60^{b,c,B}	8.08 ±1.14^{b,B}
Staph. spp.	0.11 ±0.03^{b}	0.71 ±0.08^{a}	1.58 ±0.22^{a,B}	2.23 ±0.11^{a,B}	4.73 ±0.59^{a,B}

in these experiments, as a number of studies have shown that this produces a higher amount of reactive species, which leads to optimized decontamination (Fricke et al., 2012; Gweon et al., 2009; Kim et al., 2009; Laroussi et al., 2006; Lim et al., 2007; Surowsky et al., 2014; Uhm et al., 2007).

Regarding the IZs, 54% of the experiments showed significant higher reduction of bacterial cells with pure argon plasma. Maybe these findings are related to a higher emission intensity of hydroxyl radicals ($OH^{\cdot}$) for pure argon than for argon:oxygen plasma. Reineke et al. (2015) assumed that the higher reduction efficiency of *Bacillus (B.) atrophaeus* and *B. subtilis* with pure argon as carrier gas followed by an admixture of argon + 0.135 vol.% oxygen + 0.2 vol.% nitrogen was caused by a higher amount of reactive nitrogen species (RNS) and reactive oxygen species (ROS), especially hydroxyl radicals ($OH^{\cdot}$). Surowsky et al. (2014) also observed a higher relative emission intensity of $OH^{\cdot}$ radicals for pure argon than for argon:oxygen with a kINPen plasma jet.

TABLE 2: *RFs (log_{10} cfu/agar plate) ± standard deviation of all tested microorganisms (S. Enteritidis 59, S. Enteritidis 21, S. Agona, S. Typhimurium, E. coli, M. spp. and Staph. spp.) at different exposure times (15–300 s), a distance of 8 mm, a gas flow rate of 5 slm and different feed gases (argon, argon:oxygen). Different letters indicate significant differences (p<0.05), small letters between the different species and capital letters between the tested feed gases of the same species for one treatment time.*

	15 s	60 s	120 s	180 s	300 s
argon					
S. Enteritidis 59	0.09 ±0.04^{a,A}	0.12 ±0.09^{a}	0.15 ±0.06^{a,c,A}	0.14 ±0.09^{A}	0.25 ±0.08^{a,c,A}
S. Enteritidis 21	0.01 ±0.02^{a}	0.07 ±0.06^{b,A}	0.05 ±0.03^{a,A}	0.07 ±0.03^{A}	0.20 ±0.03^{a,A}
S. Agona	0.09 ±0.060^{a}	0.10 ±0.03^{a,b,A}	0.18 ±0.10^{a,b,A}	0.57 ±0.70^{A}	0.42 ±0.11^{c,A}
S. Typhimurium	0.11 ±0.03^{a}	0.09 ±0.03^{b,A}	0.13 ±0.05^{a,c,A}	0.13 ±0.05^{A}	0.27 ±0.03^{a,c,A}
E. coli	0.25 ±0.11^{b,A}	0.22 ±0.10^{a}	0.22 ±0.12^{b,c,A}	0.23 ±0.09^{A}	0.23 ±0.02^{a,c,A}
M. spp.	0.03 ±0.03^{a,A}	0.22 ±0.06^{a,A}	0.29 ±0.04^{b,A}	0.58 ±0.06^{A}	2.14 ±0.12^{b}
Staph. spp.	0.10 ±0.04^{a}	0.08 ±0.05^{b,A}	0.16 ±0.06^{a,b,A}	0.16 ±0.08^{A}	0.30 ±0.18^{a,c,A}
argon:oxygen					
S. Enteritidis 59	0.04 ±0.03^{a,B}	0.16 ±0.13^{a}	0.64 ±0.22^{a,B}	1.44 ±0.34^{a,B}	1.97 ±0.36^{a,B}
S. Enteritidis 21	0.07 ±0.06^{a}	0.45 ±0.21^{c,d,B}	1.35 ±0.57^{c,B}	1.57 ±0.51^{b,B}	2.28 ±0.28^{a,c,B}
S. Agona	0.05 ±0.04^{a}	0.30 ±0.03^{a,c,B}	0.91 ±0.22^{a,c,B}	1.90 ±0.48^{b,B}	2.43 ±0.06^{c,B}
S. Typhimurium	0.09 ±0.03^{a,b}	0.21 ±0.11^{a,d,B}	0.66 ±0.28^{a,B}	1.67 ±0.47^{b,B}	2.30 ±0.07^{a,c,B}
E. coli	0.04 ±0.03^{a,B}	0.13 ±0.03^{a}	0.35 ±0.07^{a,B}	0.78 ±0.12^{b,B}	2.01 ±0.09^{a,B}
M. spp.	0.19 ±0.10^{b,B}	2.00 ±0.25^{b,B}	2.05 ±0.27^{b,B}	2.15 ±0.30^{b,B}	2.23 ±0.18^{a,c}
Staph. spp.	0.04 ±0.05^{a}	0.48 ±0.10^{c,B}	0.69 ±0.14^{a,B}	1.20 ±0.30^{a,B}	1.36 ±0.13^{b,B}

The global view of the whole agar plates modified the statement of argon efficiency. In 74% of all experiments argon:oxygen plasma showed higher RFs and was therefore significantly more effective than pure argon plasma. Reineke et al. (2015) measured a higher UV-C light emission for argon with an addition of 0.135 vol.% of oxygen compared to pure argon gas. Contrary to those measurements, Surowsky et al. (2014) reported no relevant UV-C emission for argon as carrier gas with admixtures of oxygen. The intensity of the UV irradiance decreased exponentially with higher distance to the plasma nozzle outlet (Reineke et al., 2015; Brandenburg et al., 2009); therefore, likely differences in the emitted UV-C light are not responsible for the differences between the RFs of argon and argon:oxygen plasma. Single colonies instead of a dense growth after a treatment for 300 seconds, as well as the higher RFs at longer exposure times, could be explained by the higher amount of reactive radicals plasma species in the argon:oxygen plasma. This is further confirmed by optical emission spectroscopy measurements of a similar plasma source (kINPen 09®, neoplas tools GmbH) by Surowsky et al. (2014) which prove that higher oxygen concentrations in the feeding gas led to higher amounts of ROS.

With regard to the reaction of gram-positive and gram-negative bacteria, no discernible trend could be observed in the present study. Even Kayes et al. (2007) and Pompl et al. (2009) could not detect differences between representatives of the two gram groups.

Conclusion

The present study examined the suitability of a direct atmospheric pressure plasma treatment by an APPJ to inactivate typical microorganisms of the native eggshell flora and different *S.* serovars. Longer exposure times (300 seconds) led to higher significant bacterial reduction. Evaluating the extent of complete sterilization directly underneath the plasma jet argon plasma was more effective in 54% of the experiments. In contrast, argon:oxygen plasma had a higher impact at a partial sterilization of the whole agar plate. This perception should be taken into particular account for the development of a plasma source for the treatment of the whole egg, as most studies only consider small areas of the treated sample. However, differences in bacterial growth inhibition of the used strains showed that it is not possible to make a general statement about the susceptibility of direct atmospheric pressure plasma for each bacterial species. The present data demonstrated, that large sterilization areas up to 16.04 ±1.04 cm² could be reached, even for the tested S. strains (up to 9.59 ±3.29 cm²). Furthermore, it was possible to achieve a reduction for all tested bacterial strains in the study at hand. Improvements to consumer protection and longer shelf lives as a result of treating eggshells with atmospheric pressure plasma might be expected, but require confirmation through further investigations.

Conflict of interest

The author(s) declare(s) that there is no conflict of interest.

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