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Cold plasma processing of powdered *Spirulina* algae for spore inactivation and preservation of bioactive compounds

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ABSTRACT

Technologies for controlling microbial risks in a heat and humidity sensitive food powder are still limited. To preserve bioactive compounds while inactivating Bacillus subtilis spores in powdered Spirulina microalgae (Arthrospira platensis) with a non-thermal atmospheric plasma is the challenge presented in this paper. Artificially contaminated powder was treated with a custom-made surface micro-discharge cold atmospheric pressure plasma (SMD-CAPP) at the effective, specific surface energy of the plasma (E_s) of 7–15 mW/cm^2. The inactivation of spores in air plasma was faster than in nitrogen plasma. The final effect after 5 min exposure time of close to 2 $\log_{10}$ reduction could be achieved with both plasma types but at different E_s. Matrix effects resulted in bi-phasic inactivation kinetics, while single-phasic kinetics were observed for exposure without powder matrix. Chlorophyll-a, carotenoid, and phycobilin concentrations were more reduced by an exposure of the powder to an air plasma, compared to nitrogen plasma. Unexpectedly, the total phenolic content (TPC) increased by a factor of up to 2 at a nitrogen plasma treatment, while a decreasing TPC was observed with increasing plasma (or discharge) energy in an air plasma. Similar effects were identified for the Trolox equivalent antioxidant capacity (TEAC). The liberation of phenolic compounds from biopolymers and the decrease of scavenging compounds by the plasma treatment are supposed being responsible for simultaneous but opposite reactions influencing TPC and TEAC values. Furthermore, the scavenging capacity might reduce the inactivation of spores as observed with the nitrogen versus the air plasma. The failure of a higher spore inactivation relates to structure effects of the powder and improvements are supposed to be reached by a powder fluidization. The application of nitrogen plasma is preferred to that of air plasma for the decontamination of *Spirulina* powders when the preservation of bioactive compounds is the paramount objective.

1. Introduction

Spirulina, with the scientific name *Arthospira*, is a genus of gram-positive, non-toxic cyanobacteria. Dietary food supplements are mainly produced from *A. platensis* and *A. maxima* (Caporgno & Mathys, 2018), because of the high contents of proteins, lipids, vitamins, minerals, and antioxidants. Main antioxidants are carotenoids, like β-carotene, and a hydrophilic, blue-colored biliprotein, the C-phycocyanin (Kilimtzidi et al., 2019; Wu et al., 2016). *Spirulina* is considered as a sustainable source of valuable bioactive compounds, with gut microbiota stimulating, anti-inflammatory, anti-cancer, antioxidant, and immunomodulatory effects (Fernando, Kim, Son, Jeong, & Jeon, 2016; Raposo & de Morais, 2015).

Photo- or heterotrophic cultivation of algae is performed in open ponds or closed bioreactors. One of the most important constraints in algae mass cultivation are bacterial contaminations depending on the type of cultivation and the low growth rate of algae. Notable contaminants are *Alteromonas* spp., *Flavobacterium* spp., *Cytophaga* spp., *Myxobacter* spp., *Pseudomonas* spp., and *Bacillus* spp. (Liu et al., 2013; Wang, Zhang, Chen, Wang, & Liu, 2013). Microbial contamination control in *Chlorella vulgaris* cultures was successful with nanosecond pulsed electric field processing (Buchmann, Bloch, & Mathys, 2018).

Down-stream from the fermentation, a separation based on fluid-mechanical principles was demonstrated to be efficient for reducing the bacterial contamination of a green algae culture (Godino, Jorde, Lawlor, Jaeger, & Duschl, 2015). Microorganisms surviving down-stream processing or would be introduced in this context in the final product. Rifna, Singh, Chakraborty, and Dwivedi (2019) reviewed thermal and non-thermal techniques for powder decontamination and concluded shortcomings on fundamental knowledge on inactivation

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Abbreviations

CAPP	Cold atmospheric pressure plasma
DBD	Dielectric barrier discharge
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SMD	Surface micro-discharge
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content

mechanisms with non-thermal technologies. Some of the non-thermal technologies including high hydrostatic pressure, pulsed light, ozone, and cold plasma processing have been scaled for batch-wise manufacturing of food, but not for steady-state, high throughput processing and/or inactivation of spores. Cold plasma processing has a potential to inactivate microbial spores (Hertwig, Steins, et al., 2015; Jeon, Klämpfl, Zimmermann, Morfill, & Shimizu, 2014; Pina-Perez, Martinet, Palacios-Gorba, Ellert, & Beyrer, 2019). It can be driven at atmospheric pressure. Large area plasma can be created with dielectric barrier discharge (DBD) or surface micro-discharge (SMD) planar electrodes. For industrial application, the product might be continuously conveyed by a belt or vibrating chute to control the residence time in the plasma. An SMD differentiates from a DBD plasma chamber as the sample is exposed to an afterglow plasma containing non-charged species only, while charged species will be retained between the sandwiched electrodes of the SMD setup. Many non-charged species including ozone and nitrogen oxides interplay in non-thermal air plasmas. A temporal evolution from ozone to nitrogen dioxide was observed. At room temperature, the ozone concentration increases in a processing time of 100 s, while the nitrogen dioxide generation starts subsequently and relates to a reduction of the ozone concentration (Park, Choe, & Jo, 2018). Ozone as such does not play a major role in inactivation of bacteria. Furthermore, a metabolism of ROS is supposed for prokaryotic cells, with the polysaccharide membrane as a primary target (Dobrynin, Fridman, Friedman, & Fridman, 2009).

The matrix-free inactivation of spores with an SMD plasma achieves a level of 3–7 $\log_{10}$ reduction (Klämpfl et al., 2012; Pina-Perez et al., 2019) and is thus suited for a sterilization process, while the inactivation in powders is less effective (Mosovská et al., 2018; Pina-Perez et al., 2019). The plasma dose and the size to volume ratio of the exposed particles enables selective effects, make a tuning between the damage of bacteria and the damage of the matrix possible (Dobrynin et al., 2009), especially when contaminants are deposited on the surface of the matrix particles. An approximately 3 log reduction of *Cronobacter sakazakii* after 120 s plasma treatment do not modify the amino acid composition of not-fat dry milk powder or the total phenolic content but modifies slightly noticeable the color of particles surface (Chen et al., 2019). Color, piperine and volatile oil content of black peppercorns are not or only slightly modified by a 30 min exposure for inactivation of *Salmonella* spp. and *Bacillus* spores in an atmospheric remote plasma (Hertwig, Reineke, Ehlbeck, Knorr, & Schluter, 2015). Surface diagnostics of black peppercorns exposed to a CAP ambient air plasma for inactivation of bacterial endospores showed no significant changes in the characteristic bonds, e.g. piperine (Mosovská et al., 2018). In contrast, DBD CAP treatment decreases the diameter of zein micelles, reduces the denaturation temperature and increases free sulfhydryl group concentration in addition to other techno-functional properties of a zein powder at a relatively high humidity of 45% (Dong, Gao, Xu, & Chen, 2017). Also, amylose and amylopectin can be cross-linked, starch depolymerized, or starch granules etched for a modification of starch properties by a CAP treatment (Thirumdas, Kadam, & Annapure, 2017). The different examples demonstrate applications on target and random modifications of biopolymers. Research on the toxicologic and allergenic potential of plasma induces modifications of biomolecules was rarely published (Boehm, Heslin, Cullen, & Bourke, 2016).

Spirulina is an important source of antioxidants which contributes also to the TPC (Wu et al., 2016). The behavior of polyphenols and the antioxidant capacity of plasma-treated plant materials seems to depend strongly on the experimental conditions (Rodriguez, Gomes, Rodrigues, & Fernandes, 2017). Most authors observed an increase of polyphenols and other antioxidants after a short plasma treatment and a decrease after longer or more powerful treatment. The increase was explained by the liberation of covalently bound antioxidant polyphenols, by disintegration or breakdown of the cell membrane and by depolymerization of polymeric polyphenols (Herceg et al., 2016; Kovacevic et al., 2016).

The study aims to gain information about the physical intensity of the plasma treatment and the composition of the plasma on (i) the inactivation kinetics of bacterial spores on a *Spirulina* powder and (ii) on the preservation of bioactive compounds. Such knowledge is central for the optimization of non-thermal plasma devices for proper control of microbial risks.

2. Material and methods

2.1. Spirulina powder and reagents

Commercial *Spirulina* powder was acquired from the Phytopharma, S.A. (Grandvillard, Switzerland) with > 80% purity (*Spirulina plus*).

Ethanol absolute was purchased from Cochimy (Martigny, Switzerland). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Acros Organics (Geel, Belgium). Folin & Ciocalteu's phenol reagent, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and gallic acid were acquired from Sigma-Aldrich (Buchs, Switzerland). All other reagents used were of analytical grade. Deionized sterile water was obtained using a Milli-Q purification system (Millipore AG, Zug, Switzerland).

2.2. Contamination of Spirulina powder with Bacillus subtilis spores

Bacillus subtilis spores were used as a surrogate for *B. cereus* to test the SMD-CAPP bactericidal effectiveness. The *B. subtilis* strain was obtained from the culture collection of the HES-SO Valais-Wallis, Institute of Life Technologies (Sion, Switzerland). *B. subtilis* endospores were transferred from stock cultures on tryptic soy agar (TSA, Biolife, Italy) and incubated for 24 h at 37 °C. Bacteria from incubated plates were harvested and inoculated in tryptic soy broth (TSB, Biolife, Italy) for 24 h under agitation at 150 rpm at 37 °C. The vegetative cells were separated by centrifugation at 7000 × *g* and 4 °C for 10 min. Subsequently, the pellet was washed twice with sterile peptone water (0.85% NaCl and 0.1% neutralized bacteriological peptone). The pellet was re-suspended in 0.1% buffered peptone water (BPW, Biolife, Italy). A volume of 600 µL of each spore suspension was layered onto TSA in 12 × 12 cm square Petri dishes and incubated 10 days at 37 °C for sporulation. After the incubation period, sporulation was examined by contrast phase microscopy. Spores were harvested from the surface of the culture plates by rubbing the upper layer and using a sterile glass spatula. The spore suspension was transferred to a 50 mL glass tube and heated in a water bath at 80 °C for 20 min to kill any vegetative cells. Heat-treated suspensions were then centrifuged at 500 × *g* and 4 °C for 20 min. The pellet was re-suspended in water to constitute the stock suspension with 10^9 spores/mL. This preparation was maintained under refrigeration at 4 °C for 1 month. Routinely, spore dispersions were checked by phase-contrast microscopy for purity.

Spirulina powder was inoculated with *B. subtilis* by using 1 mL of the spore suspension applied on 10 g of algae powder: 50 µL of spores' stock suspension was spotted on a thin powder layer and homogenized by mixing according to Kim, Oh, Won, Lee, and Min (2017). Samples of contaminated powder (0.1 g) were homogeneously spread in thin layers on sterile standard flat glass slides as carriers (0.1 g/carrier; powder

load = 5 mg/cm^2; ≈10^5 spores per g) immediately before SMD-CAPP treatments.

Methods on powder-free inactivation of *B. subtilis* spores were presented before and results will be cited as a baseline of inactivation (Pina-Perez et al., 2019).

2.3. Surface micro-discharge cold atmospheric pressure plasma (SMD-CAPP) treatment

A tailor-made SMD equipment was designed by the Institute of Systems Engineering (HES-SO Valais, Wallis, Sion, Switzerland) and described in detail before (Pina-Perez et al., 2019). Briefly, filamentary discharges with a length of up to a few millimeters were formed in a powered stainless steel mesh electrode (total surface area = 149.76 cm^2; mesh size = 9.8 × 9.4 mm^2), mounted together with no gap with a dielectric barrier made from Teflon, and a grounded planar water-cooled aluminum electrode (Fig. 1). The frequency was fixed at 10 kHz. The discharge power was calculated from total current and voltages curves measured at the output of the transformer (Lissajous-method), using a Testec HVP 15 HF (Testec Elektronik GmbH, Germany) voltage probe and a Pearson model 4100 current probe.

Experiments were carried out in the range of 10–20 W total power, corresponding to discharge power densities of 7 mW/cm^2 to 15 mW/cm^2. The device was operated at ambient pressure, using air or nitrogen in individual trials as process gas. A K-type thermocouple (Thermocoax, France) was used to measure the temperature inside the cooled aluminum electrode. This temperature remained below 35 °C for all treatments (data not shown). Plasma diagnostics for this setup and air as process gas are presented in Pina-Perez et al. (2019). A gap of 6 mm between the sample and the mesh electrode was fixed. *Spirulina* powder (0.1 g) was spread on glass carriers (powder load 5 mg/cm^2) and placed in the SMD-CAPP chamber for treatments in the range of 0–5 min. An exposure time of 0 min represents the control samples for each working session. Experiments were conducted at room temperature and each test was performed in triplicates.

2.4. Microbiological analysis

Plasma treated samples and controls were transferred aseptically into 114 mm × 230 mm transparent sterile stomacher plastic bags (Carl Roth, Germany) immediately after the treatment. Ten mL of sterile 0.1% (w/v) BPW (4 °C) was added and the bags were sealed. The samples were agitated in a stomacher blender (Stomacher Lab Blender Model 400, Seward Medical, London UK) for 1 min at 150 rpm. Then the glass slides were removed using sterile tweezers. Spore dispersions were serially diluted in BPW 0.1% (w/v) and plated in TSA. Colonies were counted after 24 h at 37 °C incubation period. This procedure was performed in triplicates and microbiological counting of each replicate was done in duplicate.

2.5. Measurement of color

The color of SMD-CAPP treated, and control algae powder was analyzed using a CM5 spectrophotometer in reflectance mode (Konica-Minolta Sensing Inc., Japan) as described by Mokrzycki & Tatol (2011). A D_{65} illuminant was used as a light source. Standard black and transparent quartz plates were used to calibrate the spectrophotometer. A 30 mm target mask was used. The 10° standard observer adjustment (10° dihedral angle) was chosen. The L^*, a^*, and b^* values were determined. Each sample was analyzed in triplicates.

The CIELAB color difference parameter (ΔE_{Lab^*}) was calculated from values for the control (c) and the sample (s):

$$\Delta E_{Lab^*} = \sqrt{(L_c^* - L_s^*)^2 + (a_c^* - a_s^*)^2 + (b_c^* - b_s^*)^2} \quad \text{(Eq. 1)}$$

2.6. Carotenoid and chlorophyll content

Extracts from SMD-CAPP treated *Spirulina* powder and control (untreated powder) were prepared according to Guedes et al. (2013). In brief, 0.1 g of SMD-CAPP treated/not treated *Spirulina* powder samples were suspended in ethanol-water (95/5 v/v) and mixed with an Ultra Turrax T 18 homogenizer (IKA, Staufen, Germany) at 20000 × *g* for 30 s. Then, samples were preserved overnight at 4 °C under agitation (150 rpm). The suspension was centrifuged at 8000 × *g*, for 10 min at 4 °C. The obtained supernatant for treated samples and controls was collected.

The quantitative determination of the chlorophyll pigments chlorophyll-a and -*b* (blue-green algae contain exclusively chlorophyll-a) (Bennet & Bogorad, 1973) and carotenoids (xanthophylls and carotenes) in the algae extracts was carried out using UV/VIS spectroscopy (Lichtenthaler & Buschmann, 2001). For calibration and calculation of concentrations, the Lambert-Beer Law was applied:

$$c_a\ (\mu g/ml) = 13.36\ A_{664} - 5.19\ A_{648} \quad \text{(Eq. 2)}$$

$$c_{(x+c)}\ (\mu g/ml) = (1000\ A_{470} - 2.13\ c_a - 97.64\ c_b)/209 \quad \text{(Eq. 3)}$$

where A is the absorbance at a certain wavelength, c_a is the concentration of chlorophyll-a, and $c_{(x+c)}$ is the concentration of carotenoids (xanthophylls and carotenes), and c_b is the concentration of chlorophyll-b, which equals to zero in *Spirulina* powder (compound not present).

2.7. Phycobiliprotein pigments

Phycobiliproteins occur in red algae and blue-green algae (cyanobacteria) and absorb energy in the visible spectrum (390–750 nm). Specifically, Spirulina is known to produce high amounts of C-phycocyanin (C-PC), a blue pigment with supposed bioactive properties, like antioxidant, antimicrobial, anti-carcinogen (Dejsungkranont, Chen, & Sirisansaneeyakul, 2017). These water-soluble pigments were extracted from SMD-CAPP algae powder according to the protocol published by Shanab, Mostafa, Shalaby, & Mahmoud (2012). Suspensions of 0.1 g algae powder and 10 mL demineralized water were mixed with an Ultra Turrax T 18 homogenizer (IKA, Staufen, Germany), at 8000 × *g* for 30 s, and then centrifuged at 5000 × *g* for 10 min at 4 °C. Afterward, the supernatant was collected. The remaining pellet was extracted again with 1 mL demineralized water, centrifuged as described before, and the supernatant was combined with the first fraction, adjusting the volume to 10 mL by water adding. The absorbance of the solution was recorded at 650 nm, 620 nm, 565 nm wavelengths with a spectrophotometer Biochrom Libra S12 (Cambridge, England).

2.8. Total phenolic content (TPC) and Trolox equivalent antioxidant capacity (TEAC)

Extracts from control and SMD-CAPP treated *Spirulina* powder samples were prepared according to Machu et al. (2015) protocol.

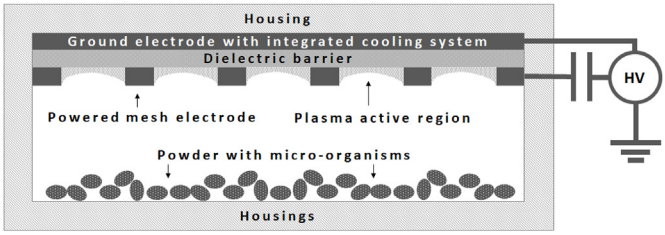

Fig. 1. Schematic illustration of the surface micro-discharge cold atmospheric pressure plasma (SMD-CAPP) chamber. Connections for flushing the chamber with air or nitrogen before plasma igniting, the water-cooling system and details of power electronics are not shown.

Water (10 mL) was used to extract 100 mg of algae powder. The extraction was carried out at 30 °C for 15 min under continuous agitation. After extraction, the suspension was cooled to room temperature and centrifuged at 5000 × *g*, at 4 °C, and for 15 min. The supernatant was immediately used for TPC and TEAC measurements.

The TPC was determined using the Folin-Ciocalteu reagent according to a method of Singleton, Orthofer, and Lamuela-Raventos (1999) with modifications previously published (Horszwald & Andlauer, 2011; Horszwald, Carlen, Héritier, & Andlauer, 2017). For the analysis, 25 µL of standard solutions and methanolic extracts were pipetted into a microwell plate (Nunc, ThermoScientific, Waltham, US) in triplicates. Methanol was used as a blank. The plate was placed in an Infinite M200 Pro microplate reader (Tecan, Switzerland) Folin-Ciocalteu reagent (1:15 v/v, in water) was added by the first injector (TECAN Infinite M200 Pro). After 10 min of incubation, 25 µL of 5% sodium carbonate solution was added by the second injector. The absorbance was measured at λ = 755 nm after 20 min. A calibration curve was prepared using solutions of gallic acid. The results were expressed in mg of gallic acid equivalents (GAE) per g of *Spirulina* powder.

TEAC was assessed as the scavenging activity against $ABTS^{\bullet+}$ according to Re et al. (1999) with the modifications of Horszwald & Andlauer (2011). The antioxidants present in the sample scavenge ABTS radical resulting in a decrease of blue color. Briefly, $ABTS^{\bullet+}$ solution was prepared by reacting ABTS diammonium salt at a concentration of 7 mmol/L with 2.45 mmol/L potassium persulfate at room temperature for 16 h. The obtained stock solution was diluted with ethanol at the ratio 1:50. A volume of 290 µL of the resulting solution was injected into the 96-well plate containing 20 µL of algae extract, blank or Trolox solutions. The absorbance was measured after 6 min at the wavelength λ = 734 nm. The calibration curve was plotted as a function of the percentage of ABTS radical scavenging activity versus the concentration of Trolox. The results were expressed in µmol Trolox eq. (TE) per g *Spirulina* powder.

2.9. Statistical analysis

All data were statistically analyzed (ANOVA) with Statgraphics Centurion XVII (Statpoint Technologies, Inc., USA) to determine the significance (p-value ≤ 0.05) of the influence of studied processing factors (discharge power density and treatment time) on both microbial inactivation and quality properties in treated algae powder.

3. Results and discussion

3.1. Effectiveness of SMD-CAPP plasma on spore reduction in Spirulina powder

The first concern of the cold plasma treatment of *Spirulina* powder was to inactivate a certain number of microorganisms for delivering safe products while preserving to a maximum nutritional and sensory values. The powder was artificially contaminated with *B. subtilis* spores. Increasing the discharge power or increasing the treatment time in the SMD-CAPP was correlated to increases in the efficacy of the inactivation of *B. subtilis* spores (Table 1). An inactivation of about 2 $\log_{10}$ reductions can be achieved with air plasma in 1 min with a very low discharge power density of 15 mW/cm^2. The same magnitude of inactivation was achieved with nitrogen plasma at the same discharge power density, but by extending the exposure time to 5 min. Oxygen and water vapor, which can be decomposed easily by the plasma to hydrogen and oxygen species, are the initial components in ambient air, transformable to oxygen radicals, while nitrogen radicals are involved in chemical reactions with the treated matter in a nitrogen plasma. The composition of air plasma generated with the SMD setup was analyzed before (Pina-Perez et al., 2019).

The efficacy of the inactivation of *B. subtilis* spores is lower in the *Spirulina* powder compared to a matrix-free exposure. Close to 4 $\log_{10}$ reductions have been reported at an exposure on glass carriers at a discharge power density of only 7 mW/cm^2 (Pina-Perez et al., 2019). Amini & Ghoranneviss (2016) reported 4 $\log_{10}$ reductions for *Escherichia coli* in black tea and when treating the sample with a punctiform applied argon plasma over 3 min. Unfortunately, a description of their discharge power density is not available. Kim, Lee, & Min (2014) detected a reduction of *B. cereus* spores of 3.4 ± 0.7 $\log_{10}$ with a combination of a cold plasma (microwave powered plasma, afterglow plasma used, He:O_2 mixture, 900 W, 20 min) or heat treatment (90 °C, 30 min). Compared to such a result, the presented SMD-CAPP is much more efficient.

The inactivation efficacy correlates with the product of process time t and discharge power Pdischarge, or with the surface energy respectively Es (equation (4)). The inactivation of *B. subtilis* spores exposed to the plasma on a smooth surface or glass carrier can be fitted to first-order kinetics in the range of observation (Pina-Perez et al., 2019). Matrix effects in *Spirulina* powder result in non-linear inactivation curves for the function "Surface energy – $\log_{10}$ reduction of spores". The inactivation is bi-phasic on *Spirulina* powder but is single-phasic on glass carriers. Hence, the following fitting function (equation (5)) is proposed for the inactivation in a complex matrix:

Table 1
Common logarithm of the concentration of *Bacillus subtilis* spores (Log_{10} N_f; N_f was determined in CFU/g) in *Spirulina* powder after treatment with varying discharge power densities, treatment times and air (a) or nitrogen plasma (b).

Discharge power (W)	Discharge power density (mW/cm^2)	Treatment time (min)			
(a) Air plasma					
		0	1	3	5
1.1	7	4.56 ± 0.11^a	3.32 ± 0.26^b	2.77 ± 0.18^c	2.84 ± 0.05^c
1.3	9	4.51 ± 0.08^a	3.88 ± 0.29^b	3.22 ± 0.45^b	2.41 ± 0.12^d
1.5	10	4.42 ± 0.07^a	3.47 ± 0.34^b	3.36 ± 0.15^b	2.69 ± 0.11^c
1.8	12	4.77 ± 0.09^a	3.96 ± 0.11^b	3.08 ± 0.12^b	2.46 ± 0.32^d
2.2	15	4.36 ± 0.14^a	2.80 ± 0.40^c	3.03 ± 0.05^c	2.73 ± 0.24^c
(b) Nitrogen plasma					
		0	1	3	5
1.1	7	5.08 ± 0.12^a	5.35 ± 0.15^a	4.90 ± 0.15^a	4.02 ± 0.28^b
1.3	9	5.08 ± 0.12^a	4.06 ± 0.11^b	4.27 ± 0.10^b	3.50 ± 0.27^c
1.5	10	4.97 ± 0.03^a	5.14 ± 0.17^a	4.04 ± 0.04^b	3.21 ± 0.12^c
1.8	12	4.97 ± 0.03^a	5.12 ± 0.23^a	3.44 ± 0.28^c	3.22 ± 0.30^c
2.2	15	5.27 ± 0.13^a	4.20 ± 0.11^b	3.67 ± 0.13^c	3.15 ± 0.37^c

a-d: Mean values followed by different superscript letters per line differ significantly at p-value < 0.05. A 2-way ANOVA was applied to values in Table 1a or b.

$$E_s = \frac{P_{discharge} \times t}{A_s} \quad \text{(eq 4)}$$

$$N_{E_s} = N_{0_1} \times 10^{-k_1 \times (E_s - E_{sc1})} + N_{0_2} \times 10^{-k_2 \times (E_s - E_{sc2})} + N_f \quad \text{(eq. 5)}$$

Where Es is the applied surface energy (J/cm^2), t is the exposure time (s), Pdischarge is the discharge power (W), As is the surface of the powered mesh electrode, $N_{0,1}$ and $N_{0,2}$ are the initial cell counts of microorganisms for each of the two phases (CFU/g), Nf is the final cell count (microorganisms that cannot be inactivated at all with the given conditions) and $N_{E,s}$ is the number of surviving microorganisms after the application to a certain surface energy. The critical surface energy of initial inactivation is described by $E_{s,c1}$ for the first phase of inactivation and $E_{s,c2}$ for the second phase. Appropriate fitting parameters for this second-order, bi-phasic inactivation kinetics are calculated with Origin (Table 2) and fitting curves are plotted for inactivation of *B. subtilits* spores on glass carriers and in *Spirulina* powder, treated whether with an air or nitrogen plasma (Fig. 2).

The critical surface energy $E_{s,c1}$, and $E_{s,c2}$ for inactivating spores increases when the spores were exposed to the nitrogen plasma in the powder compared to air plasma combined with powder or on glass carriers. The discharge power density must be increased to overcome the protective effects of the *Spirulina* powder matrix. The structure of the powder, the particle size, the porosity, and the layer thickness and scavenger effect of algae antioxidants contribute to the protective effects. Scavenger effects will be discussed in chapter 3.4. Inversely, the formal reaction rate in the first phase decreases to approximately 20% when spore's inactivation was done the powder matrix and even more when the air plasma was replaced with a nitrogen plasma. However, the rate of inactivation is slightly higher in the second phase with nitrogen plasma and the final count number similar for both, the air and the nitrogen plasma and exposure of the spores in *Spirulina* powder. Based on the results obtained without algae, it can be concluded that limitations concerning the efficacy of the spores' inactivation are more related to the protective potential of the matrix and less to the chemical character of the plasma.

It was supposed that the interaction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) with food components such as polyphenols, carbohydrates, proteins, or vitamin C influence the efficacy of a plasma in the inactivation of microorganisms (Helgadottir et al., 2017). The antioxidant capacity of green tea is approximately twice of that of black tea (436 compared to 239 mg vitamin C equivalents per g of tea) (Bruno, Bromser, & Ferruzzi, 2014; Lee, Lee, & Lee, 2002) and consequently the inactivation of coliform bacteria, yeasts and molds is less efficient in green compared to black tea (Amini, & Ghoranneviss, 2016).

Besides, the structure of the powder influences the rate and the final effect of inactivation. On corn starch, a non-scavenging powder with a smooth surface, the inactivation effect for *B. subtilis* spores reduces to half compared to the inactivation on glass carriers (1.5 compared to 3.7 $\log_{10}$ reduction with 7 mW/cm^2 in 5 min) (Pina-Perez et al., 2019). The corn starch powder load in this experiment was 25 mg/cm^2. Compared to the attenuation of inactivation effects on *Spirulina* powder this is little, considering as well that the discharge power density was increased to 15 mW/cm^2 to achieve 2 $\log_{10}$ reduction in the *Spirulina* powder. For further differentiation of the character of protective factors and the influence on the inactivation mechanism, the antioxidant potential was determined (chapter 3.4).

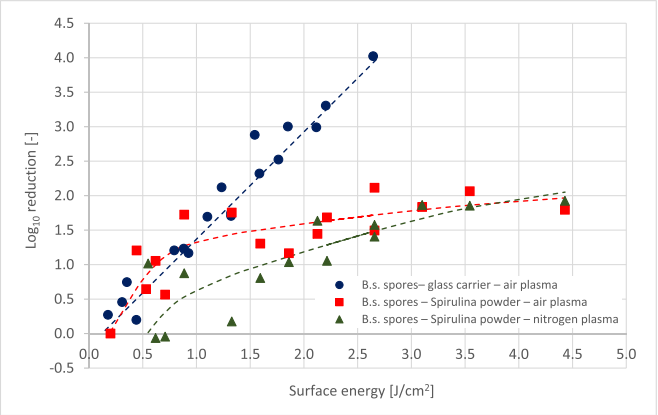

Fig. 2. Inactivation of *B. subtilis* spores as a function of the surface energy, the surface character of the carrier of spores (flat glass or *Spirulina* powder) and the composition of the process gas (air or nitrogen). The surface energy was calculated with equation (4) and fit curves with equation (5). Values of the fitting are represented in Table 2. Values on the inactivation of *B. subtilis* spores deposited on flat glass and treated with an air-plasma were adapted from Pina-Perez et al. (2019).

3.2. Effect of SMD-CAPP on the color of Spirulina powder

The treatment of *Spirulina* powder with an air plasma leads to color modifications, specifically of greenness – a^* and blueness – b^* (Table 3). The difference of color between the non-treated and any treated sample (ΔE_{Lab^*}) equals or excel 2.3, which is the recognized limit of perceptible changes (Mokrzycki & Tatol, 2011). The b^* value decreases stronger than the a^* value and the L^* value even increases at a discharge power up to 1.3 W. In nitrogen plasma the b^* value shifts remarkable, while a^* and L^* values are rather stable compared to treatments with air plasma. However, overall color changes, characterized by ΔE_{Lab^*}, are of the same magnitude for air and nitrogen plasmas at the higher discharge power. Verbalized in tonalities, yellow pigments degrade in both plasma types (or blue pigments increase), while green pigments trend to a higher presence specifically with an air plasma exposition.

Color changes due to plasma treatments have been observed by several authors, specifically when the working gas contained oxygen. Amini, Ghoranneviss, and Abdijadid (2017) found that adding 20% of oxygen to argon or helium leads to a reduction of lightness of plasma-treated saffron. Whether oxygen or air ignited by induction in a packaging discolored fresh spinach leaves (Klockow & Keener, 2009). ROS are supposed to modify pigments rather than RNS.

Color measurements relate to the perception of pigments in a mixture and represent a summary parameter only. Therefore, characteristic pigments such as chlorophyll-a, carotenoids, and phycobilins have been extracted from the *Spirulina* powder and quantified by absorption spectrometry.

3.3. Effect of SMD-CAPP treatment on pigments: chlorophylls, carotenoids, and phycobilins in Spirulina algae powder

The UV/VIS spectrum for *Spirulina* extracts shows two major peaks, corresponding with the blue absorption maximum of chlorophyll-a (from 428 to 432 nm) and the red absorption maximum of carotenoids

Table 2
Values of inactivation kinetics of *B. subtilis* spores exposed to an SMD-CAPP: Plasma ignited in air or nitrogen.

Matrix	Process gas	$N_{0,1}$ [cfu/g]	$E_{s,c1}$ [J/cm^2]	k_1 [cm^2/J]	$N_{0,2}$ [cfu/g]	$E_{s,c2}$ [J/cm^2]	k_2 [cm^2/J]	N_f [cfu/g]	R^2
Glass carrier	**Air**	2.5×10^9	0.12	1.56	0	0	0	0	0.95
***Spirulina* powder**	**Air**	2.5×10^9	0.18	3.00	9.8×10^7	0.87	0.29	1.8×10^7	0.68
***Spirulina* powder**	**Nitrogen**	2.5×10^9	0.48	3.02	7.5×10^7	2.57	0.52	1.3×10^7	0.76

Table 3
Effect of SMD-CAPP on the color of *Spirulina* powder at an exposure time of 5 min.

Discharge power (W)	Discharge power density (mW/cm^2)	L*		a*		b*		ΔE_{Lab}*	
		Air	N_2	Air	N_2	Air	N_2	Air	N_2
0.0	**0**	33.2 ± 2.6	33.2 ± 2.6	−6.3 ± 0.1	−6.3 ± 0.1	8.1 ± 1.6	8.1 ± 1.6	0	0
1.1	**7**	37.3 ± 1.3	31.5 ± 0.1	−8.2 ± 0.1	−6.9 ± 0.1	7.3 ± 0.1	5.2 ± 0.0	4.5	3.4
1.3	**9**	38.5 ± 0.5	32.5 ± 0.8	−8.3 ± 0.1	−6.6 ± 0.3	6.9 ± 0.0	5.6 ± 0.7	5.7	2.6
1.5	**10**	34.3 ± 0.2	32.8 ± 1.4	−7.6 ± 0.1	−6.5 ± 0.1	5.7 ± 0.3	5.3 ± 0.9	2.9	2.7
1.8	**12**	34.5 ± 0.3	32.7 ± 0.0	−7.4 ± 0.4	−7.12 ± 0.1	5.9 ± 0.0	5.0 ± 0.0	2.8	3.1
2.2	**15**	34.8 ± 0.6	34.7 ± 0.1	−7.4 ± 0.1	−6.18 ± 0.0	5.8 ± 0.1	5.9 ± 0.1	2.8	2.6

$\Delta E_{Lab^*} \approx 2.3$ correspond to JND (just noticeable difference).

and xanthophylls (from 660 to 665 nm). The nitrogen plasma applied with a specific power of 7 or 15 mW/cm^2 does not significantly influence the concentration of extractable chlorophyll-a or carotenoids and xanthophylls (Table 4). In contrast, the air plasma reduces the concentration of chlorophyll-a peak, while the carotenes/xanthophylls concentration was significantly, but relatively less modified. Both groups of chromophore substances, chlorophyll-a, and carotenoids have been significantly degraded by an air DBD plasma applied on kiwifruit slices (Ramazzina et al., 2015). The air plasma contains ROS and RNS, while the nitrogen plasma is mainly composed of RNS. The presence of ROS or a combination of RNS and ROS is supposed to be linked to observed changes in the absorption spectrum.

The dominant phycobilin in the commercial *Spirulina* powder is phycocyanin with a concentration of about 7.5 mg/g of powder. Allophycocyanin and phycoerythrin concentrations are lower (1.3–1.5 mg/g of powder). For extraction, water has been used as a solvent. With organic solvents, the concentration of soluble phycocyanin was reported to be higher when extracted from dried *Spirulina* and reaches 96 mg/g of powder (Kilimtzidi et al., 2019). However, Park & Dinh (2019) found a phycocyanin concentration of 3.2 mg/g and allophycocyanin or phycoerythrin at lower concentrations than phycocyanin (0.19 and 0.79 mg/g, respectively).

The spectrophotometric signatures of phycobilins from *Spirulina* extracts (spectrum 500–730 nm) differ strongly between the control and SMD-CAPP treated samples. Calculated total concentrations of phycobilin's, including phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE), are presented in Table 5. For example, the PC signal at λ_{max} = 620 nm decreased for a powder exposed to an air plasma of only 7 mW/cm^2 by 90% and by 83% with an exposition to a nitrogen plasma of the same discharge power density. The residual PC content is inversely correlated to the plasma powder density applied (7–15 mW/cm^2) (p-value < 0.05). In contrast, this is not the case when the powder was exposed to a nitrogen plasma. The initial drop of the phycocyanin concentration is followed by a fluctuation at higher discharge power densities.

For allophycocyanin (absorption peaks at 615 and 650 nm) and phycoerythrin (absorption peaks at 495 and 565 nm), the degradation pattern repeats that observed for phycocyanin. The allophycocyanin and phycoerythrin degradation are likewise a function of the plasma composition and the discharge power density.

In similarity to the supposed impact of ROS and RNS on the chromophore molecule chlorophyll-a, a slight conversion might be affected by RNS in nitrogen plasma and a tremendous degradation is observed in the air plasma, presumably caused by ROS. UV light might initiate an oxidative reaction chain via the generation of ozone and other oxygen radicals (Sinha, Richter, Faddoul, Braun, & Hader, 2002). These observations deliver an explanation of chemical reactions involving non-specifically different sensitive molecules of *Spirulina* cells. The global effect on antioxidants will be discussed subsequently.

3.4. Effect of SMD-CAPP on total phenolic content (TPC) and Trolox Equivalent Antioxidant Capacity (TEAC) of Spirulina algae powder

To get information about the changes in the summary parameters TPC and the TEAC of the *Spirulina* powder, established and well described photometric methods have been used. The TPC of the non-treated control was measured in an aqueous extract and reached 16.40 ± 3.93 mg GAE/g of powder. A comparable level (17–24 mg GAE/g of *Spirulina* powder) was reported by Machu et al. (2015).

The effect of an SMD-CAPP on TPC and TEAC was evaluated after exposure of *Spirulina* powder to a discharge power density of 9 mW/cm^2 to 15 mW/cm^2 over 5 min, conditions required to inactivate *B. subtilis* spores by approximately 2 $\log_{10}$ or higher. Interestingly, TPC of the *Spirulina* powder changed slightly, but not significantly (p-value < 0.05) due to treatment with air plasma (Fig. 2). TEAC values, however, slightly decreased indicating a degradation of antioxidant compounds. Unexpectedly, nitrogen plasma treatment increased significantly (p-value < 0.05) and the increase of TPC and the TEAC values correlated linearly to the discharge power. The increase of TEAC of nitrogen plasma treated samples is approximately 20% at a discharge power density of 15 mW/cm^2, while the decrease of TEAC of air plasma treated samples is approximately 30% at the same discharge power density (Fig. 3).

At least two simultaneous processes are running: on one hand, there is a decrease in TPC and TEAC due to degradation of antioxidants and polyphenols; on the other hand, plasma treatment seems to liberate compounds from the matrix, contributing to the two parameters TPC and TEAC. In the case of air plasma and TPC, degrading processes equal the liberating processes. For nitrogen plasma, liberating effects dominate the degrading effects of the plasma, which leads to an increase of

Table 4
Concentration of chlorophyll-a and carotenoids (carotenes and xanthophylls) (μg/g powder sample) pigments in *Spirulina* algae powder previous and after the exposure to SMD-CAPP treatment at varying discharge power density (exposure time = 5 min).

Pigments concentration (μg/g)	Discharge power density (mW/cm^2)				
	0	7		15	
	Air	Air	Nitrogen	Air	Nitrogen
Chlorophyll-a	3126 ± 155^a	1142 ± 344^b	2926 ± 83^a	1381 ± 126^b	3069 ± 13^a
Carotenoids	840 ± 32^a	549 ± 95^b	708 ± 21^b	645 ± 135^b	713 ± 2^b

a-c: superscript letters are indicating significant differences (per line) between pigment values obtained at different SMD-CAPP treatment conditions.

Table 5
Concentration of phycobilins (mg/g) (phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE)) in extracts from control and SMD-CAPP treated *Spirulina* powder samples at 5 min.

Discharge power (W)	0.0	1.1	1.2	1.8	2.2
Discharge power density (mW/cm^2)	0	7	9	10	15
	Air plasma				
PC (mg/g)	7.46 ± 0.49	0.78 ± 0.01	0.49 ± 0.03	0.50 ± 0.13	0.33 ± 0.05
APC (mg/g)	1.45 ± 0.07	0.84 ± 0.02	0.60 ± 0.01	0.65 ± 0,05	0.51 ± 0.01
PE (mg/g)	1.29 ± 0.02	0.47 ± 0.14	0.35 ± 0.04	0.37 ± 0.02	0.29 ± 0.03
	Nitrogen plasma				
PC (mg/g)	7.46 ± 0.49	1.27 ± 0.11	1.89 ± 0.40	1.81 ± 0.03	1.62 ± 0.22
APC (mg/g)	1.45 ± 0.07	0.67 ± 0.04	1.02 ± 0.01	0.70 ± 0.09	0.91 ± 0.09
PE (mg/g)	1.29 ± 0.02	0.37 ± 0.10	0.46 ± 0.07	0.53 ± 0.15	0.51 ± 0.06

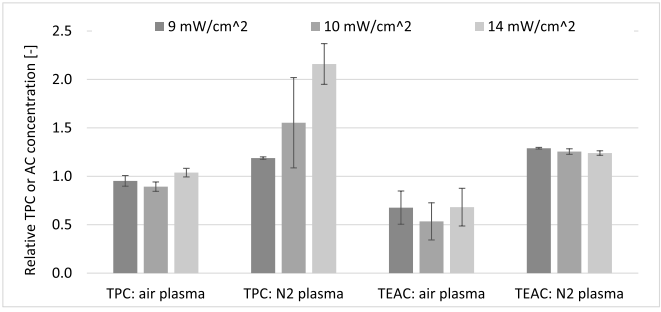

Fig. 3. Relative change (based on untreated samples) of total Phenolic Content (TPC) and Trolox Equivalent Antioxidant Capacity (TEAC) of *Spirulina* powder treated at varying discharge power density for 5 min and with air or nitrogen plasma.

TPC and TEAC after the plasma treatment. Some authors did not find any effects on TPC and TEAC values (Ramazzina et al., 2015). Others describe a decrease of total anthocyanins and phenolic acids, mainly under longer treatment times (Garofulic et al., 2015).

The behavior of TPC and TEAC seems to depend strongly on the experimental conditions. Most authors observed an increase of polyphenols and other antioxidants during plasma treatment, contributing to the TPC and TEAC values. Nitrogen plasma increased the antioxidant activity rapidly, but longer plasma treatment time led to lower antioxidant activities (Rodriguez et al., 2017). The increase was explained by the liberation of cell membrane-bound flavonoids and other phenolic compounds. Free-radical scavenging activity, transition metal-chelating activity, and singlet-oxygen quenching capacity are properties associated with polyphenols and explain their role as antioxidant compounds in vegetables and fruits. For pomegranate juice, a higher anthocyanin (flavonoid) content has been observed and was explained by an improved extractability and disintegration of the cell membrane of cloudy particles (Kovacevic et al., 2016). From another study on pomegranate juice from the same research group, an increase of several phenolic compounds in plasma-treated juice has been reported (Herceg et al., 2016). A cell membrane breakdown, a liberation of covalently bound compounds and depolymerization of polymeric phenols have been hypothesized to explain the increase of the phenolic compounds' concentration. Sarangapani, O'Toole, Cullen, & Bourke (2017), who treated entire blueberries, confirmed a significant increase in TPC and total flavonoids. Also, in this study, longer treatment times led to a slight decrease in both values.

4. Conclusions

Reactive oxygen and reactive nitrogen species play a dominant role in inactivating bacterial spores and interfere with bioactive and nutritionally pertinent ingredients of the treated food matrix. Higher discharge power densities or extension of the treatment time are applied to increase the inactivation efficacy of bacterial spores. A negative consequence is the destruction of bioactive compounds such as phycobilins or carotenoids. However, a simultaneous degradation of biopolymers leading to a liberation or a new formation of scavenger molecules might stabilize or even increase the scavenging capacity of microalgae. Some of the studied chromophore molecules are excellent markers for detrimental plasma effects. The advantage of applying a low energy nitrogen plasma, with low concentrations of ROS for inactivating spores while preserving bioactive compounds from *Spirulina* was clearly shown in the present experiments.

Interestingly, single-phase reaction kinetics of bacterial spores at exposure on flat glass carriers change into bi-phase reaction kinetics at exposure on algae powder particles. This is supposed to be related to a more complex reaction pathway, involving diffusion phenomena of short half-live plasma species in a porous matrix and the scavenging potential of bioactive molecules of the matrix material.

CRediT authorship contribution statement

M. Beyrer: Conceptualization, Methodology, Formal analysis, Data curation, Writing - review & editing. **M.C. Pina-Perez:** Methodology, Investigation, Formal analysis, Writing - original draft. **D. Martinet:** Methodology, Formal analysis. **W. Andlauer:** Writing - original draft.

Declaration of competing interest

Authors have no conflict of interest to declare.

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