Chapter 4
Antibiofilm Application of Cold Plasma in Food Safety

Qisen Xiang, Liyuan Niu, and Yanhong Bai

Abstract Biofilms are defined as aggregates of microorganisms encased in a matrix of extracellular polymeric substances that are attached to a surface. Biofilms can form quickly in food industry environments, which results in serious hygienic problems and significant economic losses. Over the past decade, cold plasma, as a novel nonthermal technology, has shown great potential for safe and sustainable food production, including food decontamination, shelf-life extension, removal of toxins, and degradation of pesticides. This chapter presents an overview of the application of cold plasma for inactivation of microbial biofilms in vitro or on food products in detail. The factors affected the antibiofilm efficacy of cold plasma are well reviewed, including the characteristics of plasma generation, processing parameters of cold plasma, properties of microbial biofilms, and characteristics of the tested samples. The synergistic effect of reactive species, charged particles, UV emission, and electromagnetic fields are responsible for the antibiofilm efficacy of cold plasma. In addition, the synergistic antibiofilm effects of cold plasma combined with other hurdle strategies (such as plant essential oils, disinfecting agents, and chelating agents) are also well reviewed. The perspectives, research needs, and challenges in applying cold plasma for biofilms control in food industry are also discussed in this chapter.

Keywords Biofilms · Cold plasma · Control · Mechanisms

4.1 Biofilms in the Food Industry

Microbes such as bacteria, molds, yeasts, and viruses represent major threats for public health and food safety. In most natural environments, microorganisms predominantly exist as communities of sessile cells that develop as biofilms, which is

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generally recognized as a major threat causing infectious diseases and economic losses (Alvarez-Ordonez et al. 2019; Srey et al. 2013; Yuan et al. 2020).

4.1.1 Overview of Biofilms

As defined by the International Union of Pure and Applied Chemistry (IUPAC), biofilms are referred to as "aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface" (Vert et al. 2012). Microorganisms generally can exist in biofilms on a wide variety of biotic and abiotic surfaces, such as living tissues, medical devices, industrial settings, water system piping, or natural aquatic systems (Donlan 2002). Biofilms may be composed of a single species or a mixture of many microbial species. Single-species biofilms in vitro have been extensively studied. However, most biofilms in nature are generally formed by a mixture of microbial species, including bacteria, fungi, yeasts, viruses (phages), algae, and protozoa Davey and O'toole 2000; Miquel et al. 2016). As compared with planktonic counterparts, microbial cells embedded in biofilms show enhanced resistance to stressful environmental conditions, e.g., antimicrobial agents, oxidative stress, desiccation, nutrient starvation, UV radiation, extreme temperature and pH, high salinity, and high pressure (Bridier et al. 2015; Flemming et al. 2016; Yin et al. 2019).

4.1.2 Biofilms in the Food Industry

Microbial biofilms are considered as potential sources of contamination and represent one of the main problems in a wide range of food industries, e.g., fresh produce, brewing, seafood processing, dairy processing, and meat processing (Srey et al. 2013; Yuan et al. 2020). Generally, foods provide a favorable environment for the growth and replication of most microorganisms. Various pathogenic and spoilage microorganisms can attach to food products and form biofilms on the surfaces (Srey et al. 2013). *Enterobacter* spp., *Listeria* spp., *Salmonella* spp., *Bacillus* spp., *Vibrio* spp., *Aeromonas* spp., *Campylobacter* spp., and psychrotrophic *Pseudomonas* spp. are the most common foodborne biofilm producers in the food industry (Galié et al. 2018; Srey et al. 2013).

Food contact surfaces are recognized as a major source of microbial contamination of food products during production, processing, transportation, packaging, and storage (Srey et al. 2013). Food contact surfaces refer to all surfaces that may contact with food products during the processing and packaging, such as cutting tools (e.g., knives and cutting boards), utensils, stockpots, prep tables, conveyor belts, and packaging material (Galié et al. 2018; Saad et al. 2019). These surfaces are typically made of a range of materials, such as stainless steel, plastic, wood, rubber, glass, and

ceramics (Aviat et al. 2016). During food production and processing, microorganisms may adhere to the food contact surfaces and form biofilms, subsequently leading to cross-contamination in the food industry.

Biofilm-associated microorganisms may adversely affect the safety and quality of food products and result in the outbreaks of foodborne diseases (Srey et al. 2013; Yuan et al. 2020). In addition, biofilms are responsible for many problems in food production, such as equipment corrosion, reduced heat transfer efficiency, increased energy consumption, and blocked membrane pores (Alvarez-Ordonez et al. 2019; Poulsen 1999; Yuan et al. 2020). Thus, appropriate methods should be applied for the prevention of biofilm formation or biofilm removal in the food industry. However, biofilms are more resistant to antimicrobials compared to planktonic cells; it is a great challenge to make their elimination from food processing facilities.

4.1.3 Control Strategies for Biofilms

Some conventional strategies have been widely used for the control of biofilms in the food industry, e.g., chemical cleaning, enzymatic disruption, and steel coatings (Galié et al. 2018). For example, chemical disinfectants such as sodium hypochlorite, sodium hydroxide solutions, hydrogen peroxide, and peracetic acid are widely used for eradicating different kinds of microorganisms in food processing environments including wash water and food contact surfaces. However, the potential toxic effects of these chemical sanitizers have received great attention from the public in the last decades. Some toxic disinfection by-products (DBPs) such as trihalomethanes (THMs) and haloacetic acids (HAAs) are formed from reactions of natural organic matter with chlorine-based compounds and other disinfectants, which pose potential health risks to humans (de Castro et al. 2019; Hung et al. 2017). In the past few years, some novel technologies have been developed for the control of microbial biofilms in food processing, such as cold plasma, high hydrostatic pressure, ultrasound, high-pressure carbon dioxide, and bacteriophage (Erriu et al. 2014; Furukawa 2015; Galié et al. 2018).

4.2 Antibiofilm Activity of Cold Plasma

Cold plasma is a novel nonthermal food processing technology, which has shown great application in the food industry, e.g., inactivation of microorganisms, enzyme inactivation, removal of toxins, degradation of pesticides, shelf-life extension, and packaging modifications (Pan et al. 2019; Pankaj et al. 2018). The engineering principles of cold plasma is well reviewed in Chap. 1. In the past few years, the potential application of cold plasma in the control of microbial biofilms has attracted increasing attention (Fig. 4.1) (Gilmore et al. 2018; Gupta and Ayan 2019; Rao et al. 2020; Zhu et al. 2020).

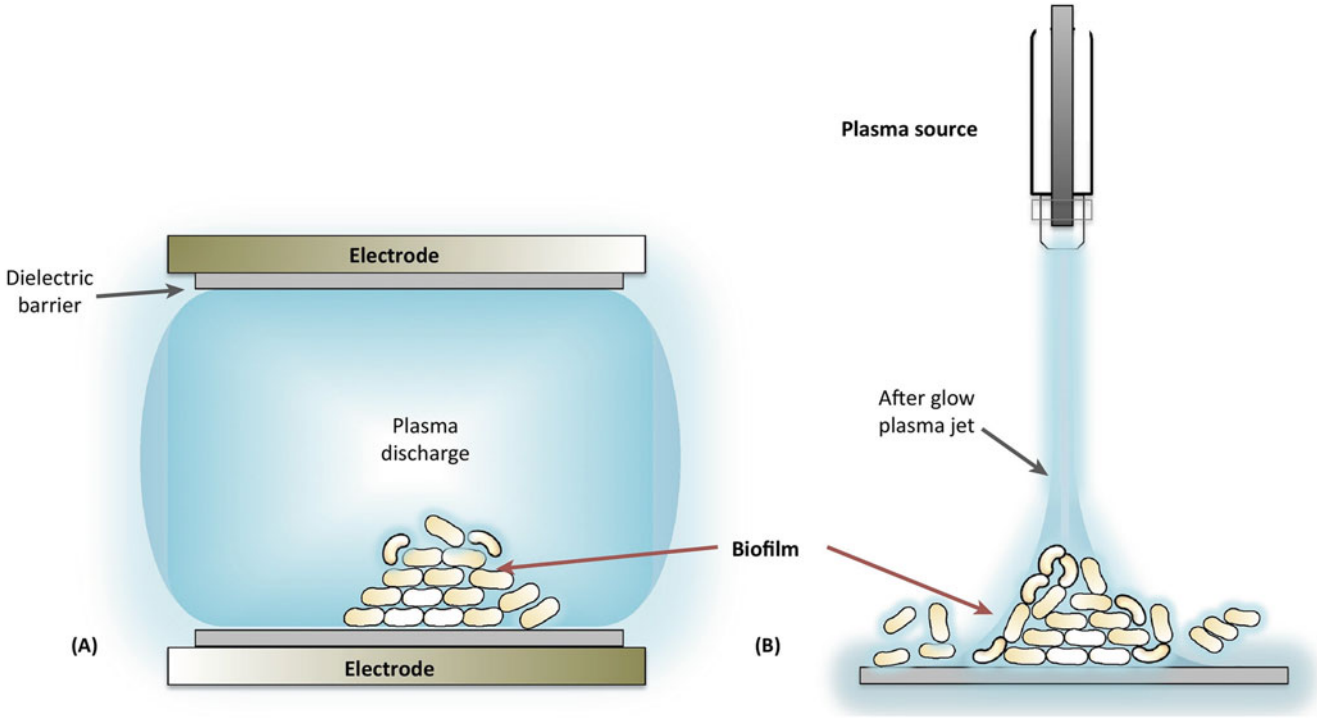

Fig. 4.1 Schematics of (**a**) DBD plasma and (**b**) atmospheric pressure plasma jet for biofilm eradication and control. (Adapted from Gilmore BF, Flynn PB, O'Brien S, et al. (2018) Cold plasmas for biofilm control: Opportunities and challenges. Trends Biotechnol 36(6): 627–638. Copyright (2020) with permission from Elsevier)

4.2.1 *In Vitro Antibiofilm Activity of Cold Plasma*

Several different types of cold plasma have been developed for food and biomedical application. Today, electrical discharge techniques have been commonly utilized to produce nonthermal plasma at atmospheric pressure, including dielectric barrier discharge (DBD), corona discharge (CD), atmospheric pressure plasma jet (APPJ), gliding arc discharge (GAD), resistive-barrier discharge (RBD), surface micro-discharge (SMD), floating electrode-dielectric barrier discharge (FE-DBD), radio-frequency discharge (RFD), plasma needle, and plasma pencil (Hoffmann et al. 2013; Sakudo et al. 2019). The application of cold plasma for in vitro biofilm inactivation is summarized in Table 4.1.

4.2.2 *Cold Plasma for Biofilm Eradication on Food Products*

The applications of cold plasma in the inactivation of bacterial biofilms are reviewed in Table 4.2. It should be noted that the antibiofilm effects of cold plasma on food products are generally lower than that of in vitro tests (Cui et al. 2016b). Thus, an extended time or power was required to reduce the mixed culture biofilm of bacteria on food product as compared with in vitro bacterial biofilms. The inactivation efficacy of cold plasma against biofilms formed on food products is affected by many factors, such as the types of microorganisms, biofilm formation conditions (such as temperature and time), and the nature of the produce's surface (Cui et al. 2016b; Jahid et al. 2015; Patange et al. 2019; Srey et al. 2014).

The influences of cold plasma on the nutritional and sensory qualities of treated foods were well assessed in previous studies. As demonstrated by Srey et al. (2014), the cold plasma treatment at 750 mJ/cm^2 did not cause any statistically significant changes in the color (L^*, a^*, and b^*) and texture of cabbage and lettuce. In the study of Jahid et al. (2015), the 5-min cold plasma treatment did not cause significant changes in the color (L^*, a^*, and b^*), texture, and sensory characteristics of lettuces. However, the obvious damages of lettuce surface were observed after being treated by cold plasma at 400 W for 5 min (Cui et al. 2016b). Therefore, the processing parameters of cold plasma should be well optimized to avoid resulting adverse effects on the nutritional and sensory qualities of treated food products.

4.2.3 *Cold Plasma for Biofilm Eradication on Food Contact Surfaces*

Several studies have investigated the effect of plasma on the biofilm inactivation of food contact surfaces, such as glass slides (Niemira et al. 2014; Vandervoort and Brelles-Marino 2014), stainless steel (Cui et al. 2016a, b), and plastic materials

Table 4.1 Application of cold plasma for in vitro biofilm inactivation

Bacterial strain	Biofilm formation	Cold plasma parameters	Results	Reference
E. coli, *L. monocytogenes*, *S. enterica*, and *P. fluorescens*	96-well plate (30 °C for 24 or 48 h)	Type: DBD; voltage: 120 kV; frequency: 50 kHz; time: 2 min	*E. coli* in 48-h old biofilm formed at 15 °C reduced to undetectable levels, and the populations of *L. monocytogenes*, *S. enterica*, and *P. fluorescens in* biofilm reduced by 3.76, 4.14, and 2.6 log CFU/mL, respectively	Patange et al. (2019)
L. monocytogenes and *P. fluorescens*	96-well plate (30 °C for 24 or 48 h)	Type: DBD; voltage: 120 kV; frequency: 50 kHz; time: 1 min.	*L. monocytogenes* and *P. fluorescens* in mixed biofilm decreased by around 4.2 log CFU/mL	Patange et al. (2019)
E. coli O157:H7	Sterile glass slides (37 °C for 24, 48, or 72 h)	Type: GAD; power: Of 522 to 549 W; gas: Dried air; frequency: 23 to 48 kHz; time: 5, 10, or 15 s. distance from emitter to biofilms: 5 or 7.5 cm	Cold plasma at 5 cm reduced *E. coli* O157:H7 biofilms by 1.41, 2.57, and 3.29 log CFU/mL for 5, 10, and 15 s, respectively, while the efficacy at 5, 10, and 10 s were 0.96, 2.13, and 3.29 log CFU/mL at 5 cm, respectively	Niemira et al. (2018)
E. coli, *L. monocytogenes*, and *S. aureus*	96-well plate (37 °C for 48 h)	Type: DBD; voltage: 80 kV_{RMS}; frequency: 50 Hz; distance: 22 mm; gas: Air; time: 1, 2, or 5 min	The populations of *E. coli* biofilm decreased to undetectable levels after cold plasma treatment for 60 s, whereas 300 s was needed to inactivate *L. monocytogenes* and *S. aureus* biofilms	Ziuzina et al. (2015a)

E. coli, *Bacillus* spp., and *Lactobacillus* spp.	96-well plate (37 °C for 72 h)	Type: DBD; voltage: 80 kV; frequency: 50 Hz; electrode gap: 22 mm; gas: Air; time: 5 min	After cold plasma treatment for 5 min, *E. coli* spp., *B. subtilis* and *Lactobacillus* spp. biofilms reduced by >3 logs. *B. atrophaeus* biofilms showed highest resistance cold plasma	Los et al. (2017)
S. aureus	96-well plate and stainless steel (37 °C for 48 h)	Gas: N_2; flow rate: 1000 SCCM/min; power: 200, 300, 400, 500, and 600 W; time: 0.5, 1, 1.5, 2, and 2.5 min	*S. aureus* biofilms on 96-well plate and stainless steel decreased by 1.90 and 3.02 log CFU/cm^2 after cold plasma treatment at 400 W for 2.5 min, respectively	Cui et al. (2016a)
E. coli O157:H7	Stainless steel (25 °C for 5 days)	—	*E. coli* O157:H7 biofilms on stainless steel decreased by 2.23 log CFU/cm^2 after cold plasma treatment at 400 W for 3 min	Cui et al. (2016b)
L. monocytogenes and *S.* typhimurium	Polystyrene petri dishes (50 mm × 9 mm) 1 or 7 day, at 30 °C for *L.* monocytogenes, 25 °C for *S.* typhimurium	Type: DBD; input voltage: 21.88 V; output voltage: 6.5 kV; power: 7.0 W; gas: He; flow rate: 4 L/min; time: 10 min	After DBD plasma treatment for 10 min, the 1 and 7 days old *L. monocytogenes* biofilms decreased by 2.32 and 0.77 log CFU/cm^2, respectively. Similar findings were also observed in *S.* typhimurium	Govaert et al. (2019b)
C. albicans	24-well microtiter plates (37 °C for 48 h)	Type: Plasma jet; voltage: 15 kV; frequency: 1 kHz; gas: 99.5% He+0.5% O_2 (2 L/min); distances: 1 mm; time: 0, 2, 4, 6, and 8 min	Cold plasma induced significantly inactivation of *C. albicans* biofilms in vitro	He et al. (2020)

(continued)

Table 4.1 (continued)

Bacterial strain	Biofilm formation	Cold plasma parameters	Results	Reference
E. faecalis	Polystyrene petri dishes (35 mm × 10 mm, 37 °C for 24, 48, or 72 h)	Type: SMD; voltage: 3.5 kV; frequency: 4.0 kHz; power: 0.5 to 1 W; gas: Ambient air; time: 1, 3, 5, or 10 min; time: 1–10 min	After cold plasma treatment for 5 and 10 min, the 24–h-old *E. faecalis* biofilms reduced by ≥3 logs and ≥ 5 logs, respectively. The both 48-h-old and 72-h-old *E. faecalis* biofilms were reduced by ≥5 logs after cold plasma treatment for 10 min	Theinkom et al. (2019)
S. aureus, *P. aeruginosa*, and *C. albicans*	96-well plate (37 °C for 24 h)	Type: Plasma jet; voltage: 5 kV; frequency: 61 kHz; power: 2.5–3.5 W; gas: Ambient air; distances: 8, 9, 10, and 11 mm; time: 1, 2, or 3 min	The survival rate of *S. aureus*, *P. aeruginosa*, and *C. albicans* biofilms were 27%, 35%, and 9%, respectively, after 3 min plasma exposure at 8 mm	Taghizadeh et al. (2015)
W. confusa	Cellulose ester membrane (17 cm^2, 0.45 μm, 30 °C for 48 h)	Type: Plasma jet; frequency: 20 kHz; gas: Ambient air	After 20 min exposure to cold plasma, the *W. confusa* biofilms on MRS media with or without sucrose decreased by 2.16- and 2.63-log reduction, respectively	Marchal et al. (2012)
S. aureus	15-mm circular glass coverslips (in 12-well plates, 37 °C for 24 h)	Type: FE-DBD; voltage: 120 V; power: 0.13 W/cm^2; gas: Ambient air; distances: 8, 9, 10, and 11 mm; time: 1, 2, or 3 min	All biofilms of the tested pathogens were inactivated in less than 150 s (19.5 J/cm^2)	Joshi et al. (2010)
C. albicans	Polystyrene 24-well plates (37 °C for 72 h)	Distances: 12 mm; time: 5–60 s	*C. albicans* biofilms decreased by 1.25-log reduction after exposure to cold plasma for 20 s. after 55 s of treatment, *C. albicans* biofilm reduced to undetectable levels	Pu et al. (2019)

Methicillin-resistant *S. aureus*	Silica films (10 mm × 10 mm × 0.5 mm) and 24-well plates (37 °C for 3 d)	Type: Surface discharge; gas: He +1% air (4 L/min), Ar + 1% air (4 L/min), synthetic air (79% N_2 + 21% O_2, 4 L/min), and natural air; time: 1, 2, 3, 4, or 5 min	*S. aureus* in biofilms decreased by 5.0-, 4.8-, 3.7-, and 2.3-log reduction after exposure to plasma with Ar + 1% air, natural air, synthetic air, and He+1% air for 5 min, respectively	Guo et al. (2019)
P. aeruginosa	Borosilicate coupons and the CDC biofilm reactor	Type: Plasma jet; frequency: 13.56 MHz; input power of 35 W; gas: He (20.4 L/min) + N_2 (0.135 L/min); time: 1, 2, 3, 5, 15, or 30 min	Cold plasma effectively inactivated *P. aeruginosa* biofilms in a time-dependent manner (0–5 min)	Vandervoort and Brelles-Marino (2014)
P. aeruginosa	Polycarbonate discs (13 mm × 3 mm) and 24-well microplates (37 °C for the first 48 h, and 23 °C for the following 24 h)	Type: Surface barrier-discharged plasma; voltage: 4 kV; power: 118 mW/cm^2; frequency: 30 kHz; gas: Ar and Ar + 1% O_2, and Ar + H_2O; time: 30, 60, 150, or 300 s	*P. aeruginosa* biofilms decreased from 0.97 log CFU/cm^2 (Ar + H_2O plasma) to 4.88 log CFU/cm^2 (Ar plasma)	Matthes et al. (2014)
P. aeruginosa	Calgary biofilm device (37 °C for 48 h)	Type: Plasma jet; voltage: 6 kV; frequency: 20 to 40 kHz, gas: 0.5% O_2 and 99.5% He (2 SLM); time: 0–240 s	*P. aeruginosa* biofilms decreased in a time- and frequency-depended way after exposure to plasma jet	Alkawareek et al. (2012)
Salmonella spp.	Sterile glass slides (7.62 cm × 2.54 cm, 37 °C for 24, 48, or 72 h)	Type: Plasma jet; power: 522 to 549 W; frequency: 23 to 48 kHz, gas: Air (2 SLM); distance: 5 or 7.5 cm; time: 5, 10, or 15 s	*Salmonella* biofilms reduced maximally by 2.13 logs (15 s, 5 cm)	Niemira et al. (2014)
S. aureus and *E. coli*	96-well plates (37 °C for 24 h)	Type: DBD; voltage: 5 kV; power: 8 W; frequency: 37 kHz; power density: 70 mW/cm^2; gas: Air; distance: 4 mm; exposure area: 120 cm^2; time: 10 to 300 s	*S. aureus* and *E. coli* biofilms reduced maximally by 2.77- and 0.47-log reduction, respectively	Czapka et al. (2018)

(continued)

Table 4.1 (continued)

Bacterial strain	Biofilm formation	Cold plasma parameters	Results	Reference
P. aeruginosa (DBM 3081, DBM 3777, ATCC 10145, and ATCC 15442)	Polypropylene cultivation vessel (37 °C for 24 h, 100 rpm)	Type: DC cometary discharge; voltage: 5 kV; current: 50–70 μA; time: 0.25, 0.5, and 1 h	After 1 h exposure to cold plasma, the metabolic activity of biofilm cells was reduced to 40% for DBM 3081, 20% for DBM 3777, 19% for ATCC 10145, and 13% for ATCC 15442	Paldrychová et al. (2019)
L. monocytogenes and *S.* Typhimurium	Polystyrene petri dish (50 mm × 9 mm, at 25 or 30 °C for 1 or 7 d)	Type: SBD and DBD; input voltage: 21.88 V; frequency: 15 kHz; output voltage: 3.5 kV for SBD, 6.4 kV for DBD; power: 3.2 W for SBD, 7.0 W for DBD; time: 0–30 min	Biofilms reduced by 1.5 to 2.5 logs CFU/cm^2	Govaert et al. (2020a)
B. subtilis, *S. epidermidis*, *K. carniphila*, *P. aeruginosa*, *P. libanensis*, and *E. cloacae*	Steel coupons (1.27 cm × 0.3 cm, 24 h)	Type: kINPen med; frequency: 1.82 kHz; distance: 4 mm; gas: Ar (4.2 SLM); time: 1, 3, and 10 min	After being treated by cold plasma for 10 min, the biofilms of Gram-positive *B. subtilis*, *S. epidermidis*, and *K. carniphila* decreased by 0.6-, 1-, and 2-log reduction, respectively, while the biofilms of gram-negative species (*P. aeruginosa*, *P. libanensis*, and *E. cloacae*) showed high reduction ranging from 3.3- to 3.6-log reduction	Mai-Prochnow et al. (2016)

Note: *SCCM* standard cubic centimeters per minute, *SLM* standard liter per minute

Table 4.2 Applications of cold plasma for biofilm eradication on food products

Bacterial strain	Food products	Biofilm formation	Cold plasma parameters	Results	Reference
L. monocytogenes	Iceberg lettuce and white cabbage (5×3 cm^2)	4 °C for 24 h	Type: Cold oxygen plasma; intensity: 750 mJ/cm^2; time: 5 min for each side	Cold plasma achieved a reduction of 3.85 log CFU/cm^2 on lettuce and 4.09 log CFU/cm^2 on cabbage, respectively, without significant changes in the color and texture quality	Srey et al. (2014)
S. Typhimurium and cultivable indigenous microorganisms (CIM)	Lettuce (3×5 cm^2)	15 °C for 24 h	—	The populations of bacteria in *S.* Typhimurium monoculture, CIM monoculture, *S.* Typhimurium mixed culture, and CIM mixed culture biofilms on lettuce were decreased by 3.74, 4.11, 1.74, and 1.63 log CFU/cm^2, respectively, after 5 min of cold treatment	Jahid et al. (2015)
E. coli O157:H7	Lettuce (3×5 cm^2)	25 °C for 48 h	Power: 400 W; time: 1, 3, or 5 min	*E. coli* O157:H7 biofilms on lettuce were reduced by 0.81 log CFU/cm^2 after cold plasma treatment at 400 W for 3 min	Cui et al. (2016b)
L. monocytogenes and *P. fluorescens*	Lettuce (Irish iceberg) (5×5 cm^2)	4 °C or 15 °C for 48 h	Type: DBD; voltage: 80 kV; frequency: 50 kHz; time: 2 or 5 min	After 5 min of treatment, *L. monocytogenes* and *P. fluorescens* in 48 h biofilm formed at 15 °C were reduced to undetectable levels, while the cells in 48 h biofilm formed at 4 °C reduced by 4.0 and 2.1 logs, respectively	Patange et al. (2019)

Note: *SCCM* standard cubic centimeters per minute, *SLM* standard liters per minute

(Govaert et al. 2020a; Matthes et al. 2014; Pu et al. 2019; Theinkom et al. 2019). Niemira et al. (2014) studied the cold plasma-induced inactivation of *Salmonella* biofilms by using glass slides as a model food contact surface. The *Salmonella* biofilm was significantly inactivated by up to 2.1-log reduction within 15 s of cold plasma treatment. Cold plasma has also been used to control microbial biofilm contamination of 3D-printed food tools. In the research of Muro-Fraguas et al. (2020), 3D-printed poly-lactic acid (PLA) Petri dishes were coated by APPJ using acrylic acid (AcAc) and tetraethyl orthosilicate (TEOS). The results of crystal violet staining revealed that the total biofilm biomass of *L. monocytogenes*, *P. aeruginosa*, and *E. coli* formed on AcAc-coated Petri dishes by APPJ was decreased by 52.3%, 49.6%, and 35.9%, respectively, as compared with the untreated samples without plasma treatment. These findings suggested that plasma coatings can be used to reduce the bacterial attachment and biofilm formation on food contact surfaces.

4.3 Combination of Cold Plasma with Other Technologies

Although the individual treatments of cold plasma demonstrate the ability to inactivate mature biofilms, the limited diffusion of the reactive species inside the biofilms and the short lifetimes of chemical species cause adverse effects on the inactivation efficiency of cold plasma against biofilms. Consequently, the combination of cold plasma with other antimicrobial technologies is still required to improve the safety and quality characteristics of food products. Some new hurdle strategies have been developed, such as the combination treatments of cold plasma with plant essential oils (Cui et al. 2016a, b), disinfecting agents (Govaert et al. 2019b; Koban et al. 2013), vitamin C (Helgadóttir et al. 2017; Pandit et al. 2017a, b), chelating agents (Koban et al. 2013; Tschang and Thoma 2019), and bacteriophages (Cui et al. 2018). The mentioned descriptions are as follows.

4.3.1 Cold Plasma and Plant Essential Oils

Essential oils, a complex mixture of volatile organic compounds extracted from parts of plant materials (such as roots, stems, leaves, flowers, buds, fruits, and seeds), are well known for their excellent antibacterial and antibiofilm properties (Oh et al. 2017; Tariq et al. 2019). The combined treatment of cold plasma and plant essential oils has been well investigated for inactivation of biofilms (Cui et al. 2016a, b). According to Cui et al. (2016a), both *Helichrysum italicum* essential oil (0.5, 1, 2, and 4 mg/mL) and cold nitrogen plasma (400, 500, and 600 W) displayed significant eradication effect on *E. coli* O157:H7 biofilms in vitro. Following the combined treatment of *Helichrysum italicum* essential oil (0.5 mg/mL, 40 min) and cold plasma (400 W, 1 min), the population of *S. aureus* biofilm on 96-well plate and stainless steel were reduced by 6.26 and 5.50 log, respectively, which were

significantly higher than that of the independent treatment (Cui et al. 2016a). The significant population reduction in *S. aureus* biofilms on different food contact surfaces (such as stainless steel, glass sheet, and plastic) was also observed after the synergetic treatment of *Helichrysum italicum* essential oil and cold plasma. The in vitro synergistic antibiofilm efficacy of cold nitrogen plasma and clove oil was also proved using the *E. coli* O157:H7 biofilm models grown on sterile stainless steel (Cui et al. 2016b).

Cui et al. (2016b) also evaluated the synergistic antibiofilm efficacy of cold nitrogen plasma and clove oil on fresh lettuce. The numbers of *E. coli* O157:H7 in biofilms on lettuce decreased by 0.81 and 2.26 $\log_{10}$ CFU/cm^2 after the individual treatment of cold plasma (400 W, 3 min) or clove oil (4 mg/mL, 30 min), respectively. It was demonstrated that the *E. coli* O157:H7 biofilms on lettuce were decreased by 5.48 $\log_{10}$ CFU/cm^2 following the synergetic treatment of cold nitrogen plasma (400 W, 3 min) and clove oil (1 mg/mL, 30 min), which was remarkably higher than that by using plasma or clove oil alone (Cui et al. 2016b). Moreover, the combined treatment of cold nitrogen plasma and clove oil has mild negative effect on the appearance, color, taste, and overall acceptability of lettuce.

4.3.2 Cold Plasma and Disinfectants

Disinfectants are extensively used to eliminate or inactivate microorganisms in the food industry. However, biofilms are more resistant to disinfectants than their planktonic counterparts (Bridier et al. 2011; Sanchez-Vizuete et al. 2015). Thus, the combining strategies should be applied to optimize biofilm control when using disinfectants. The synergistic effect of cold plasma and different disinfecting agents has been well evaluated, namely hydrogen peroxide, sodium hypochlorite, chlorhexidine, octenidine, ethylenediaminetetraacetic acid (EDTA), and polyhexanide (Govaert et al. 2019b; Koban et al. 2013). Govaert et al. (2019b) investigated the synergetic effect of DBD plasma and H_2O_2 on the inactivation of *L. monocytogenes* and *S.* Typhimurium biofilms in vitro. After DBD plasma treatment for 10 min, the 1- and 7-day-old *L. monocytogenes* biofilms decreased by 2.32 and 0.77 $\log_{10}$ CFU/cm^2, respectively. Similar findings were also observed in *S.* Typhimurium. After the cold plasma or H_2O_2 treatment, the 1-day-old *S.* Typhimurium biofilms reduced between 1.96 and 3.70 $\log_{10}$ CFU/cm^2. For the 1-day-old *S.* Typhimurium biofilms, the simultaneous H_2O_2 (0.05%, v/v) and DBD plasma resulted in significant differences between the combined log reduction value and the sum of corresponding individual treatments ($p < 0.05$). So the combined treatment of cold plasma and H_2O_2 may be used as a promising method for the inactivation of biofilms grown on food contact surfaces.

Koban et al. (2013) evaluated the combined antimicrobial effect of plasma with different agents (H_2O_2, sodium hypochlorite, chlorhexidine, octenidine, polyhexanide, and EDTA) against the monospecies (*S. mutans*) and multispecies dental biofilm models grown on Titanium discs in vitro. The combination of cold Ar

plasma and all the tested compounds displayed additive antimicrobial effect against the used multispecies biofilms models.

4.3.3 Cold Plasma and Chelating Agents

Chelating agents, also known as chelators, can react with metal ions to form a stable complex, including ethylenediaminetetraacetic acid (EDTA), trisodium citrate (TSC), and ethylene glycol tetraacetic acid (EGTA). Previous studies have reported that chelating agents, such as EDTA, TSC, EGTA, and alizarin, can effectively destroy bacterial biofilms (Abraham et al. 2012; Banin et al. 2006; Bosma et al. 2010; Lee et al. 2016). Some studies have been conducted to investigate the bacterial biofilm reduction by the combined treatment of cold plasma and chelating agents (Tschang and Thoma 2019; Koban et al. 2013). Koban et al. (2013) found that the antibiofilm effect of Ar plasma against *S. mutans* biofilms was significantly increased by adding EDTA. This may be related to the EDTA-induced disruption of biofilm structure, which subsequently enhances the penetration of chemical species generated in the plasma into the biofilm matrix (Soares et al. 2010).

Consistent findings were also observed by Tschang and Thoma (2019), who studied the inactivation of *E. coli*, *E. faecalis*, and *S. capitis* biofilms induced by the combination of cold plasma and chelating agents (TSC, EDTA, EGTA, and alizarin). The results of colony count assay showed that the combined treatment of EDTA and TSC (10%, w/v) with cold plasma displayed synergistic effect on the inactivation of all the three bacterial biofilms, while the sequential treatment of alizarin and cold plasma had no significant effects on all three bacteria compared with cold plasma treatment alone (Tschang and Thoma 2019).

4.3.4 Cold Plasma and Vitamin C

Vitamin C, also known as ascorbic acid or L-ascorbic acid, is a water-soluble vitamin that is naturally present in fruits and vegetables. Vitamin C shows excellent antimicrobial and antibiofilm activities when used alone or in combination with other treatments (Majtan et al. 2020; Tajkarimi and Ibrahim 2011). Recent studies have proved that vitamin C can be used to enhance the inactivation capacity of cold plasma against biofilms (Helgadóttir et al. 2017; Pandit et al. 2017a, b). Helgadóttir et al. (2017) investigated the influences of vitamin C pretreatment on cold plasma-induced the inactivation of biofilms. The 48-h-old biofilms of *B. subtilis*, *S. epidermidis*, *E. coli*, and *P. aeruginosa* on glass coverslips were reduced by 2.0, 0.1, 1.0, and 0.4 $\log_{10}$ CFU/coverslips, respectively, after being exposed to cold plasma for 5 min. After the individual treatment of vitamin C for 15 min, the populations of *B. subtilis*, *S. epidermidis*, *E. coli*, and *P. aeruginosa* biofilms decreased by 1.0-, 0.3-, 0.8-, and 0.4-log reductions, respectively. The pretreatment

with vitamin C could effectively strengthen the inactivation ability of cold plasma against the 48-h-old biofilms of *S. epidermidis*, *E. coli*, and *P. aeruginosa*. After the pretreatment of vitamin C for 15 min and cold plasma for 5 min, the biofilms of *S. epidermidis*, *E. coli*, and *P. aeruginosa* reduced by 0.8-, 1.7-, and 1.5-log reductions, which were higher than that of the individual treatment of vitamin C and cold plasma (Helgadóttir et al. 2017).

The pretreatment of vitamin C can inhibit the production of the matrix EPS and reduce the structural complexity of biofilms, which enhances the susceptibility of bacterial cells inside the biofilms to various chemical species generated in the plasma (Helgadóttir et al. 2017; Pandit et al. 2017a, b). Besides, vitamin C can exert predominantly pro-oxidant activity in the presence of iron, which leads to the generation of toxic free radicals (Timoshnikov et al. 2020; Vilchèze et al. 2013). Thus, the pro-oxidant activity of vitamin C may be one of the underlying mechanisms for the synergistic antibiofilm efficacy of cold nitrogen plasma and vitamin C.

4.3.5 Cold Plasma and Bacteriophages

Bacteriophages, also known as phages, are the viruses of bacteria. In recent years, the use of phages as an interesting alternative antibacterial approach has attracted great attention (Ferriol-González and Domingo-Calap 2020; Polaska and Sokolowska 2019). Cui et al. (2018) investigated the synthetic antibiofilm effects of the sequential treatment of cold plasma and phage techniques. The results showed that *E. coli* O157:H7 phages could effectively enhance the antibiofilm effects of cold plasma against *E. coli* O157:H7. Following the sequential treatment of cold plasma (400 W for 2 or 3 min) and phage techniques (5% or 10% for 30 min), the *E. coli* O157:H7 adhered on steel coupons were eliminated completely. Similar results were obtained in lettuces, cucumbers, and carrots. The initial concentration of *E. coli* O157:H7 was over 10^6 CFU/g for lettuces and cucumbers, while over 10^5 CFU/g for carrots. After the storage at 4 °C for 14 d, the population of *E. coli* O157:H7 in the control lettuce reduced to 3.79 log CFU/g. During the storage at 4 °C, the population of *E. coli* O157:H7 in lettuce treated by cold plasma and phages reduced to 1.21 log CFU/g on the third day, and no viable bacteria were detected on the ninth day. Similar results were also obtained in cucumbers and carrots. In addition, the sequential treatment of cold plasma and phages also effectively sustains the sensory quality of the types of vegetables.

4.3.6 Cold Plasma and Antibiotic

The combination of nonthermal plasma and antibiotic treatment was also used for the control of biofilms (Guo et al. 2018; Julák et al. 2020). Guo et al. (2018) studied the effect of cold plasma on the antibiotic sensitivity of methicillin-resistant

Staphylococcus aureus (MRSA). The results indicated that the antibiotic sensitivity of MRSA increased after the sublethal treatment of both plasma and plasma-activated saline. The metabolic activity of *S. epidermidis* biofilms was decreased by 75% following the combination of cold plasma (15 min) and erythromycin (10 mg/L for 24 h), which was significantly higher than that of the NTP or erythromycin treatment alone (decreased by 46% and 47%, respectively). The improved decrease in the metabolic activity of *E. coli* biofilms was also observed after the subsequent combination of cold plasma and polymyxin B (Julák et al. 2020).

Taking together, the antimicrobial and antibiofilm properties of cold plasma can be effectively improved by combining with other mild physical or chemical intervention methods (Liao et al. 2020). However, the application of these synergetic methods in the food industry is not well investigated. Thus, further investigations are still required to assess the influences of these cold plasma-based hurdle interventions on microbiological safety and quality characteristics of food products. In subsequent work, the mechanisms underlying the synergistic antibiofilm efficacy of the cold plasma and other technologies also should be elucidated in more detail.

4.4 Factors Affecting the Antibiofilm Activity of Cold Plasma

In order to design effective methods for cold plasma-based biofilm removal, it is important to understand the factors affecting the antibiofilm activity of cold plasma. In general, the antibiofilm activity of cold plasma is affected by the characteristics of plasma generation, processing parameters of cold plasma, and properties of microbial biofilms (Alkawareek et al. 2012; Govaert et al. 2018; Gupta and Ayan 2019; Los et al. 2017; Puligundla and Mok 2017; Šimoncicová et al. 2019). These influencing factors should be taken into account for the practical application of cold plasma technologies in the food industry. The factors that affect the antibiofilm activity of cold plasma are reviewed in the present section (Fig. 4.2).

4.4.1 Characteristics of Plasma Generation

Generally, cold plasmas are generated by certain types of gas discharges at atmospheric pressure. So the discharge parameters affect the antibacterial and antibiofilm efficacy of cold plasma, including types of electrical discharge, device geometries, electrode configurations, voltage, frequency, input power, gas type, and flow rates (Alkawareek et al. 2012; Deng et al. 2007; Guo et al. 2019; Govaert et al. 2019a; Gupta and Ayan 2019; Han et al. 2016a, b; Hähnel et al. 2010; Matthes et al. 2014; Šimoncicová et al. 2019).

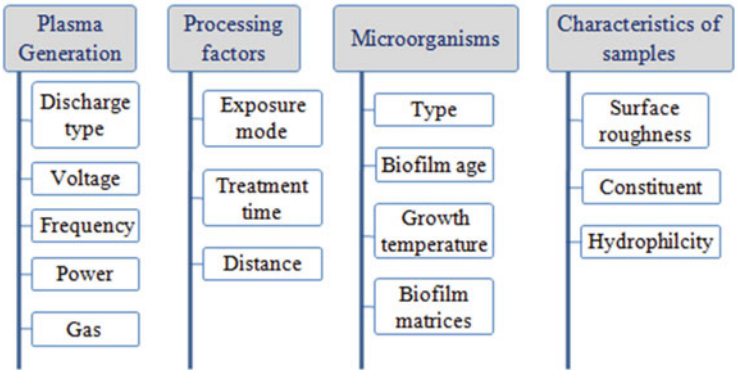

Fig. 4.2 The factors that influence antibiofilm activity of cold plasma

4.4.1.1 Types of Electrical Discharge

The most widely used plasma devices for biological samples are dielectric barrier discharge (DBD), atmospheric pressure plasma jets (APPJs), and corona discharge, which may affect the effectiveness of cold plasma. Govaert et al. (2019a) compared the antibiofilm efficacy of DBD plasma and surface barrier discharge (SBD) plasma. The obtained data suggested that the DBD configuration had the highest biofilm inactivation efficacy. Each discharge mode has its electrical characteristics, which affects the production of reactive chemical species and the antibacterial property. Therefore, the types of discharges should be appropriately chosen for the practical application of cold plasma technologies.

4.4.1.2 Voltage

The energy of electrical discharge is decided by applied voltage and frequency, thus generating different amounts of reactive species in cold plasma. Numerous studies have proved the effect of voltage levels on antimicrobial efficacy of cold plasma against bacteria (Deng et al. 2007; Govaert et al. 2019a; Han et al. 2016a, b; Patange et al. 2019). As reported by Patange et al. (2019), the inactivation abilities of cold plasma toward *S. enterica*, *L. monocytogenes*, and *P. fluorescens* in PBS were significantly enhanced when the applied voltage increased (60 to 80 kV). Similar inactivation patterns were also found in bacterial biofilms. Han et al. (2016a, b) investigated the effect of voltage levels (60, 70, and 80 kV_{RMS}) on DBD plasma-mediated inactivation of *E. coli*, *L. monocytogenes*, and *S. aureus* biofilms in a meat model medium. The results demonstrated that the increase in the voltage levels led to further reductions in the number of bacteria inside the biofilms. This may be due to the more generation of extracellular reactive species in bacterial cells after being treated using DBD plasma with increased voltage levels (Han et al. 2016a, b).

4.4.1.3 Frequency

The plasma operating frequency also significantly affects the antibacterial activity of cold plasma (Alkawareek et al. 2012; Deng et al. 2007). As demonstrated by Deng et al. (2007), the inactivation of *E. coli* increased with the frequency. The populations of *E. coli* cells were reduced by 1.85-, 2.02-, 4.15-, and 5.60-log reduction after being treated by cold plasma for 30 s at 1, 1.5, 2.0, and 2.5 kHz, respectively. According to the studies of Alkawareek et al. (2012), the inactivation rate of *P. aeruginosa* biofilms after being exposed to 40 kHz plasma was higher than that of 20 kHz plasma. The D-values (the time taken to reduce the bacterial population by 90%) for *P. aeruginosa* biofilms with 40 kHz plasma jet were 15.97 s and 69.79 s for the first and the second inactivation phases, respectively, which were both higher than the 23.57 s and 128.20 s for 20 kHz plasma jet (Alkawareek et al. 2012). However, the gas temperature of the plasma jet discharged at 20 kHz was 39 °C, and the temperature increased to about 57 °C for 40 kHz plasma. The higher temperature of the plasma jet may limit its application for heat-sensitive fresh foods and food contact materials.

4.4.1.4 Input Power

The inactivation efficacy of cold plasma against microorganisms in planktonic and biofilm states is increased with an increase in input power (Cui et al. 2016a, b; Pina-Perez et al. 2020). Cui et al. (2016a) investigated the effect of different power on cold nitrogen plasma-mediated inactivation of biofilms. The results indicated that cold nitrogen plasma could inactivate *S. aureus* biofilm in an input power-dependent manner. The numbers of *S. aureus* biofilm on 96-well plates and stainless steel were reduced by 1.69- and 2.02-log reduction when treated with cold nitrogen plasma at 400 W for 1 min, respectively, which were significantly higher than that of samples treated with cold nitrogen plasma at 300 W for 1 min (Cui et al. 2016a). These results were consistent with those observed in cold nitrogen plasma-treated *E. coli* O157:H7 biofilms formed on stainless steel surface (Cui et al. 2016b).

4.4.1.5 Working Gas

The composition, humidity, and flow of working gases are the significant factors affecting the biofilm inactivation induced by cold plasma (Guo et al. 2019; Govaert et al. 2019a; Matthes et al. 2014). Matthes et al. (2014) investigated the influences of carrier gases on the antibiofilm properties of surface barrier-discharged (SBD) plasma. Three working gases were used in their work, which were Ar, Ar + 1% O_2 (v/v), and Ar with 80% relative gas humidity (Ar + H_2O), respectively. After being treated by SBD plasma for 300 s, *P. aeruginosa* biofilms were decreased by 4.88 log CFU/cm^2 (Ar plasma), 2.97 log CFU/cm^2 (Ar + O_2 plasma), and 2.65 log

CFU/cm^2 (Ar + H_2O plasma), respectively. Similar results were also observed by Guo et al. (2019), who investigated the influences of four working gases on the inactivation of methicillin-resistant *S. aureus* biofilms induced by surface discharge plasmas. Four working gases were used in this work, namely He+1% air, Ar + 1% air, synthetic air (a gas mixture of ~79% N_2 and ~ 21% O_2), and natural air (~78% N_2, ~21% O_2, and ~ 1% of rare gas). The experimental results demonstrated that the surface plasmas generated with Ar + 1% air and natural air had higher inactivation effect against *S. aureus* biofilms than that of plasmas generated with He+1% air and synthetic air. The *S. aureus* in biofilms were inactivated by 5.0-, 4.8-, 3.7-, and 2.3-log reduction by plasmas generated with Ar + 1% air, natural air, synthetic air, and He+1% air for 5 min, respectively (Guo et al. 2019). The composition of working gases may affect the kinds and concentrations of reactive chemicals produced by plasma discharge. As demonstrated by Guo et al. (2019), the levels of reactive species in the gas phase (such as $^1O_2$, $O_2\cdot^-$, $\cdot NO_2$, and $ONOO^-$) and liquid phase (such as H_2O_2, NO_2^-, and NO_3^-) produced by surface plasma with Ar + 1% air were remarkably higher than those generated with He+1% air. In addition, the working gases also affected the depths penetration of the reactive species in biofilms. The ROS penetration depths of plasma with Ar + 1% air, natural air, synthetic air, and He +1% air were 78 μm, 72 μm, 48 μm, and 16 μm, respectively, after 3-min plasma treatment; the changing trend was well in accordance with that of the antibiofilm properties of plasma with the four kinds of gases (Guo et al. 2019).

The antibiofilm activity of cold plasma is also influenced by the humidity of working gases. Hähnel et al. (2010) observed that the antimicrobial efficacy of dielectric barrier surface discharge plasma toward *Bacillus* spores was enhanced by increasing the relative air humidity up to 70%. However, as reported by Matthes et al. (2014), the antimicrobial efficacy of cold plasma generated with Ar against *P. aeruginosa* biofilms was decreased when increasing the relative humidity. After being exposed to Ar plasma for 300 s, *P. aeruginosa* biofilms were decreased by 4.88 $\log_{10}$ CFU/cm^2, which was significantly higher than 2.65 $\log_{10}$ CFU/cm^2 of Ar + H_2O plasma. This may be due to the too high relative humidity of Ar used in the research of Matthes et al. (2014).

4.4.2 Processing Factors

The antibacterial and antibiofilm efficacy of cold plasma is also affected by the processing factors of cold plasma treatment, including the mode of plasma exposure, treatment time as well as the distance between the plasma source and the samples (Fridman et al. 2007; Gupta and Ayan 2019; Taghizadeh et al. 2015; Theinkom et al. 2019; Ziuzina et al. 2014).

4.4.2.1 Mode of Plasma Exposure

The applications of cold plasma for biofilm destruction can be classified into three different treatment modes: the direct plasma treatment, indirect plasma treatment, and hybrid plasma treatment (Czapka et al. 2018; Gupta and Ayan 2019; Keidar et al. 2013). Direct cold plasma treatment is defined as exposure of the samples placed directly under the plasma plume discharge (Morris et al. 2009; Ziuzina et al. 2014) or where the sample itself serves as an electrode that participates in the plasma discharge (Fridman et al. 2007). By contrast, the indirect or "remote" cold plasma treatment refers to the samples placed away from the plasma discharge area (Fridman et al. 2007; Morris et al. 2009; Ziuzina et al. 2014, 2015a). The hybrid plasma treatment represents a combination of direct and indirect plasma treatment (Keidar et al. 2013).

As described by Fridman et al. (2007), the bacteria inactivation induced by the direct treatment of DBD plasma was much faster than that of the indirect treatment. This may be because the cells are subjected to all plasma agents including reactive species, charged particles, photons, and electric field during the direct exposure (Laroussi 2020). However, contradictory findings have been reported by Ziuzina et al. (2015a), who evaluated the effect of direct or indirect DBD plasma treatment on the elimination of *E. coli*, *L. monocytogenes*, and *S. aureus* biofilms. According to the results of XTT assay, there was no significant difference between the antibiofilm efficacy of direct and indirect plasma exposure. So the plasma treatment mode should be fully considered in the practical application of cold plasma technologies.

4.4.2.2 Treatment Time

The antibiofilm performance of cold plasma is significantly affected by the treatment time. Generally, the inactivation efficacy of cold plasma against biofilm is increased with an increase in treatment time. As reported by Theinkom et al. (2019), the 48-h-old biofilms of *E. faecalis* were reduced by 1.8-, 1.8-, 2.6-, and 5.7-log reduction by surface micro-discharge plasma for 1, 3, 5, or 10 min, respectively. For 72-h-old biofilms of *E. faecalis*, the bacterial populations decreased by 1.7-, 2.0-, 2.4-, and 4.9-log reduction after being exposed to surface micro-discharge plasma for 1, 3, 5, or 10 min, respectively. Similar findings have also been observed in other studies (Cui et al. 2016a, 2016b; Han et al. 2016a, b; Niemira et al. 2018; Taghizadeh et al. 2015; Ziuzina et al. 2015a). According to Cui et al. (2016b), the *E. coli* O157:H7 biofilms on stainless steel decreased by 1.51 and 2.23 log CFU/cm^2 after cold plasma treatment at 400 W for 1 or 3 min, respectively. Similar trends were found in *E. coli* O157:H7 biofilms formed on fresh lettuce. However, some obvious damage of lettuce surface was observed after exposure to cold nitrogen plasma at 400 W for 5 min (Cui et al. 2016b). Thus, the treatment time of cold plasma should be optimized when used for the food products.

4.4.2.3 Distance Between the Plasma Source and the SAMPLES

The distance between the plasma source and the sample also significantly affects the inactivation efficacy of cold plasma against biofilms. Taghizadeh et al. (2015) investigated the influences of the distance between the plasma nozzle and the sample on the inactivation of *S. aureus* biofilm. The survival rate of *S. aureus* biofilm on 96-well plate increased when upon increasing the distance between the plasma source and the sample. The *S. aureus* biofilm was effectively killed at 8 mm distance in comparison to the other distances (9, 10, and 11 mm). Almost no reduction in *S. aureus* biofilm was observed at a distance of 11 mm (Taghizadeh et al. 2015). Similar findings are also observed by Niemira et al. (2014). The distance between the plasma source and the sample may affect the amounts of reactive species that can reach the sample (Anzai et al. 2019). As reported by Anzai et al. (2019), the levels of reactive species (such as •OH, H_2O_2, and nitroxide radicals) are gradually decreased when the distance between the solution surface and the plasma jet nozzle increased. Thus, the inactivation efficiency of cold plasma against biofilm inactivation can be directed by varying the distance between the plasma source and the treated samples.

4.4.3 Properties of Microbial Biofilms

In general, the efficacy of cold plasma treatment is also determined by the biological properties of microbial biofilms, such as the microbial type, biofilm age, growth temperatures, the thickness of biofilm, the spatial structures and matrix components of biofilm, and initial inoculum (Govaert et al. 2018; Los et al. 2017; Puligundla and Mok 2017; Ziuzina et al. 2015a).

4.4.3.1 Type of Microorganism

The efficiency of cold plasma treatment against biofilms is strongly affected by phenotype and strain of bacteria studied. The tolerance of microbial biofilms to cold plasma is variable between species, strains of the same species. It has been reported that the biofilms of Gram-positive bacteria are more resistant to cold plasma treatments than that of Gram-negative bacteria (Los et al. 2017; Mai-Prochnow et al. 2016; Ziuzina et al. 2015a). As reported by Ziuzina et al. (2015a), the populations of Gram-negative *E. coli* in biofilms reduced to undetectable levels from an initial 5.4 log CFU/mL after the DBD plasma treatment for 60 s, whereas an extended treatment for 300 s was needed to inactivate the Gram-positive *L. monocytogenes* and *S. aureus* biofilms. After being treated by cold plasma for 10 min, the biofilms of Gram-positive *B. subtilis*, *S. epidermidis*, and *K. carniphila* decreased by 0.6-, 1-, and 2-log reduction, respectively, while the biofilms of Gram-negative species (*P. aeruginosa*, *P. libanensis*, and *E. cloacae*) showed high reduction ranging

from 3.3 to 3.6 log reduction (Mai-Prochnow et al. 2016). These differences may due to the differences in the bacterial cell wall structure and the EPS (such as thickness, composition, and quantity) (Vu et al. 2009; Ziuzina et al. 2015a). For example, Gram-positive bacteria possess a thick (20–80 nm) cell wall than Gram-negative bacteria (<10 nm) (Mai-Prochnow et al. 2016). As analyzed by Mai-Prochnow et al. (2016), there is a good correlation of CFU log reduction of biofilms with cell wall thickness after 1, 3, and 10 min of cold plasma treatment. Czapka et al. (2018) also found that the biofilm of *E. coli* ATCC 10231 shown higher resistance to DBD plasma than biofilm of *S. aureus* ATCC 25922.

Generally, the single-species biofilms have been used extensively to assess the in vitro activity of antibiofilm approaches. However, most biofilms in nature are actually formed by multiple microbial species. Mixed-species biofilms are ubiquitous in the food industry, which are generally associated with higher resistance to disinfectants and antimicrobials (Burmolle et al. 2006; Jahid et al. 2015; Jahid and Ha 2014; Yuan et al. 2020). As founded by Jahid et al. (2015), the mixed cultures of *S.* Typhimurium and cultivable indigenous microorganisms (CIM) showed significantly greater resistance to cold plasma as compared to monoculture biofilms on lettuce. So more attention should be paid to the effect of cold plasma treatments on mixed-species biofilms on the surface of either real foods or packaging materials.

4.4.3.2 Biofilm Age

Biofilm age is also a decisive factor in determining the susceptibility of the resident microorganisms to disinfectants and antimicrobials treatment (Wong et al. 2010). The susceptibility of biofilms of different ages to cold plasma was well evaluated (Govaert et al. 2018, 2019b; Vleugels et al. 2005; Ziuzina et al. 2015b). Govaert et al. (2018) investigated the inactivation efficiency of DBD plasma against *L. monocytogenes* and *S.* Typhimurium biofilms with five different ages (1, 2, 3, 7, and 10 days). It was found out that the 7 days old *L. monocytogenes* and *S.* Typhimurium biofilms were more resistant toward DBD plasma treatment (Govaert et al. 2018). Similar findings were also reported by Ziuzina et al. (2015a) that the 48-h-old *Salmonella*, *L. monocytogenes*, and *E. coli* biofilms developed on lettuce exhibited higher resistance to cold plasma than 24-h-biofilms. The increased tolerance of the 7 days old biofilms to cold plasma may be due to the higher amount EPS matrix, which probably reduces the diffusion of the reactive species generated during plasma discharge into the biofilms, and, consequently, protects the biofilm-associated cells from DBD plasma-mediated cell death (Govaert et al. 2018; Jiang et al. 2017). Vleugels et al. (2005) also reported similar findings that 24-h-old *P. agglomerans* biofilms had stronger resistance to glow discharge plasma than that of 12-h-old samples. However, inconsistent findings were reported by Niemira et al. (2018) indicating that the biofilm age (24, 48, or 72 h) was not a significant factor influencing the inactivation efficiency of gliding arc plasma against *E. coli* O157:H7 biofilms.

4.4.3.3 Growth Temperatures of Biofilm

The temperature for biofilm formation also significantly affects the inactivation efficiency of cold plasma against bacterial biofilms. As reported by Patange et al. (2019), the mixed biofilms of *L. monocytogenes* and *P. fluorescens* formed on lettuce at 4 °C were more resistant to DBD plasma than that of biofilms formed at 15 °C. The results demonstrated that the DBD plasma treatment for 5 min significantly reduced *L. monocytogenes* and *P. fluorescens* in 48 h biofilm formed at 15 °C reduced to undetectable levels, while the cells in 48 h biofilm formed at 4 °C reduced by 4.0- and 2.1-log, respectively (Patange et al. 2019). In contrast, Jahid et al. (2014) reported that the biofilm populations of *A. hydrophila* formed on lettuce at higher temperatures (≥15 °C) displayed enhanced resistance to cold oxygen plasma. After 5 min of cold oxygen plasma treatment, a 5-log reduction was observed in biofilm populations of *A. hydrophila* formed on lettuce at 4 °C, 10 °C, and 15 °C. However, the populations of *A. hydrophila* in biofilms formed at 20 °C, 25 °C, and 30 °C were reduced maximally by 3.9-, 3.2-, and 3.0-log, respectively. These results may be related to the modulating effect of temperature on quorum sensing and biofilm formation of *A. hydrophila* on lettuce surfaces and stomata (Jahid et al. 2014).

4.4.3.4 Biofilm Matrices

The bacterial biofilm matrix is comprised of self-produced extracellular polymeric substances (EPS) like exopolysaccharide, proteins, extracellular DNA (eDNA), and extracellular RNA (eRNA) (Flemming and Wingender 2010; Karygianni et al. 2020). The EPS matrix offers effective protection microbial cells from a variety of unfavorable environmental stresses (Guillonneau et al. 2018; Yin et al. 2019). The polymeric matrix in biofilms may prevent the penetration of cold plasma into its deep layer, weakening its bactericidal capacity against bacterial cells at the bottom of the biofilms. The penetrating abilities of plasma reactive species are affected by plasma generation parameters (e.g., voltage, gas type), microbial biofilm properties, etc. As reported by Alkawareek et al. (2012), the cold plasma generated with a gas mixture (0.5% O_2 + 99.5% He) can penetrate deeply and eradicate the *P. aeruginosa* biofilms of 40 to 80 μm thickness. In the work of Xiong et al. (2011), the cold oxygen plasma can penetrate the 15-μm-thick biofilms of *P. gingivalis* and effectively deactivate the biofilm cells. Thus, the matrices and thickness of bacterial biofilms are the significant factors affecting the inactivation efficiency of cold plasma (Gupta and Ayan 2019; Zhu et al. 2020).

4.4.4 Characteristics of the Treated Samples

In addition to the above-mentioned factors, the inactivation efficacy of cold plasma against biofilms formed on food products is also affected by the nature and characteristics of the tested samples (Cui et al. 2016b; Patange et al. 2019; Srey et al. 2014). The interactions between plasma and food products can be influenced by various factors, such as surface roughness, adsorption of diffusing plasma, components, and moisture, which may affect the resistance of microorganisms to cold plasma (Bhide et al. 2017; Yong et al. 2015). Bhide et al. (2017) showed that the microbial inactivation efficacy of cold plasma against *E. aerogenes* on sandpapers and the fruit peel surfaces significantly decreased as the surface roughness increased. For instance, the roughness values of the apple, orange, and cantaloupe peel surfaces were 6.12 μm, 13.07 μm, and 16.98 μm, respectively. After treatment with cold plasma for 492 s, the populations of *E. aerogenes* cells on the apple, orange, and cantaloupe peel surfaces decreased by 1.86-, 0.77-, and 0.61-log reduction, respectively. So the nature and characteristics of the food samples should be taken into consideration when applying cold plasma technologies in the preservation of foods.

4.5 Inactivation Agents of Cold Plasma

Plasma is an ionized gas composed of heat, UV emission, charged particles (electrons and ions), and reactive species (ROS, RNS, and free radicals), and electromagnetic fields (Ji et al. 2019; Weltmann and von Woedtke 2017). Many of these physical or chemical factors are well known to have antimicrobial efficacy (Niedźwiedź et al. 2019). It is widely recognized that the synergistic effect of these active agents is responsible for the antimicrobial and efficacy of cold plasma (Fig. 4.3) (Gilmore et al. 2018; Gupta and Ayan 2019; Laroussi 2005; Weltmann and von Woedtke 2017; Zhu et al. 2020).

4.5.1 Reactive Species

During plasma discharges, considerable amounts of reactive chemical species are generated through various collisional pathways such as hydroxyl radical (•OH), ozone (O_3), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), nitric oxide (NO), and nitrogen dioxide (NO_2), which are well known to have antimicrobial efficacy (Laroussi 2005; Vatansever et al. 2013). Thus, it has always been recognized that the reactive species in cold plasma play an important role in its antimicrobial properties. These reactive species can directly or indirectly damage the structure (such as cell wall and cell membrane) and cellular components

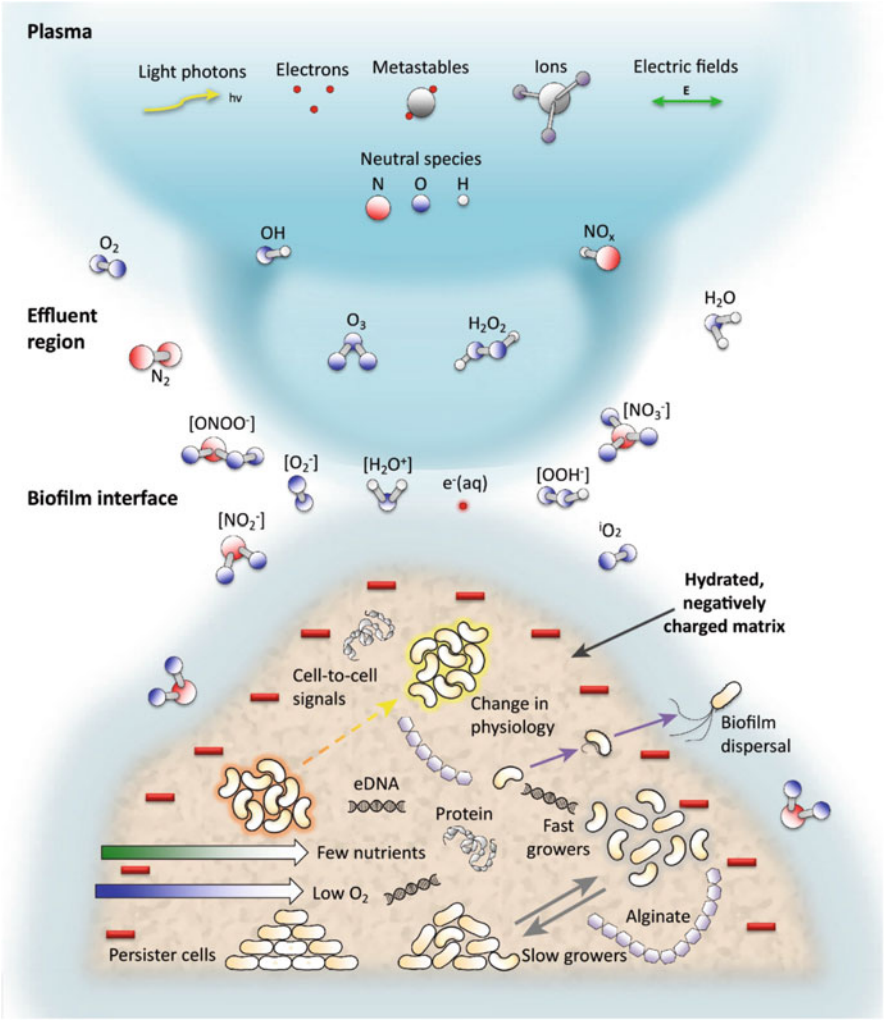

Fig. 4.3 The interaction between cold plasma and biofilms. (Adapted from Gilmore BF, Flynn PB, O'Brien S, et al. (2018) Cold plasmas for biofilm control: Opportunities and challenges. Trends Biotechnol 36(6): 627–638. Copyright (2020) with permission from Elsevier)

(e.g., DNA, proteins, and lipids) of microbial cells, which subsequently lead to the death of microorganisms (Han et al. 2016a, b; Liao et al. 2017a).

In the work of Schneider et al. (2011), they developed an experimental setup to effectively separate UV photons and reactive particles (such as accelerated ions and radicals) in a plasma jet with He gas flow. The complete-jet treatment showed the strongest antibacterial efficacy against *E. coli*, followed by the reactive particles-jet, while the UV photons-jet alone had little effect on the inactivation of *E. coli* cells at the same condition. These data indicated that the reactive species are mainly

responsible for the microbial inactivation induced by cold plasma. Similar findings were also observed by Govaert et al. (2020b), who investigated the role of reactive species in the cold plasma-induced inactivation of biofilms by covering the biofilms with a quartz disc. The quartz disc can prevent all reactive species plasma, apart from UV photons, to interact with the biofilm samples (Herrmann et al. 1999). The results showed that the log reductions of *L. monocytogenes* and *S.* Typhimurium model biofilms obtained without quartz disc were quite higher than the corresponding values with quartz disc. In summary, the reactive species play important roles in the cold plasma-induced inactivation of microbial cells in both planktonic and biofilm states.

4.5.2 UV Radiation

The antibacterial and antibiofilm efficacy of UV radiation has been well identified. The ultraviolet photons are produced by radiative de-excitation of atoms or molecules during plasma discharge. Generally, the UV light produced by atmospheric pressure air cold plasma is less than that by low-pressure plasma (Lackmann et al. 2013; Laroussi 2009; Laroussi and Leipold 2004). The UV photons produced in the plasma may also contribute to its bactericidal and antibiofilm effect. In the work of Govaert et al. (2020b), the generation of UV light was detected by means of UV strips (wavelength ranging between 250 and 420 nm, intensities ranging between 5 and 60 mJ/cm^2). After the DBD plasma treatment using He/O_2 gas mixture at 21.88 V for 10 min, obvious color change in UV strips was observed, suggesting the generation of UV light during DBD discharge. The authors further investigated the role of UV light in the cold plasma-induced inactivation of biofilms. The remarkable inactivation of *L. monocytogenes* and *S.* Typhimurium biofilms was observed when the biofilms were covered with a quartz disc, which allowed UV photons and heat to pass through but prevented all chemical species in plasma (Govaert et al. 2020b). Therefore, ultraviolet photons may be involved in the biofilms inactivation induced by cold plasma, but the impact may be relatively weak (Lackmann et al. 2013; Schneider et al. 2011).

4.5.3 Charged Particles

Generally, the roles of reactive species and UV radiation in cold plasma-induced microbial inactivation have been well discussed, while much less attention is given to charged species (e.g., electrons and ions). Though the exact role of charged particles is not yet fully understood, their importance is indicated by several studies (Fridman et al. 2007; Laroussi 2002; Stoffels et al. 2008). It is suggested that charged particles may play a significant role in the electrostatic disruption of the outer membrane of *E. coli* cells (Laroussi 2002). They suggested the generated

electrostatic force by charged particles can overcome the tensile strength of the membrane when sufficient charged particles are accumulated on the outer surface of bacterial cells, eventually leading to the disruption of the cell membrane structures (Laroussi 2002). According to Fridman et al. (2007), the direct treatment of cold plasma yielded better sterilization efficiency than the indirect treatment. This may be related to the charged particles, because the charged particles can only contact the microbial cells directly during the direct treatment of cold plasma (Laroussi 2009). Due to the complex structure of microbial biofilms, the charged particles may only contact the cells located on the surface layer of the biofilms. So much more work is needed to reveal the exact role of charged particles in cold plasma-induced eradication of biofilms.

4.5.4 Heat

Though the final temperature of cold plasma-treated samples increases in a time-depended way, heat is not an important factor in the inactivation process of bacterial cells (Laroussi 2009). As reported by Lacombe et al. (2017), the average surface temperature of berries increased to 46.6 °C from initial 23.2 °C after plasma jet treatment for 120 s at a working distance of 7.5 cm. The effect of plasma temperature on biofilm inactivation was also investigated (Fridman et al. 2007; Niemira et al. 2018; Vandervoort and Brelles-Marino 2014). Niemira et al. (2018) monitored the temperature of glass slides during the treatment of plasma jet, which was obtained with an infrared camera. Following cold plasma treatment at a 5 cm and 7 cm spacing, the maximum temperatures of the slides were 35.0–45.7 °C and 38.1–53.4 °C, respectively. Similar findings were also reported by Vandervoort and Brelles-Marino (2014) that the temperature of the coupon surface during cold plasma treatment (once a minute for 10 min) reached to 35 °C within a few minutes and remained constant over time. These obtained temperatures are too low to inactivate bacterial cells within the biofilms (Chmielewski and Frank 2004; Stringer et al. 2000). These data indicate that the thermal effects do not play an important role in the biofilm inactivation induced by cold plasma. However, to maintain the sensory and nutritional characteristics of food products, the untoward thermal effects of cold plasma on the samples should be avoided with appropriate measures such as forced air cooling (Lacombe et al. 2017).

4.5.5 Gas Flow

The effect of gas flow on the inactivation of the biofilm has also been assessed in previous studies (Joaquin et al. 2009; Vandervoort and Brelles-Marino 2014). Vandervoort and Brelles-Marino (2014) investigated the roles of gas flow (He flow of 20.4 L/min + N_2 of 0.135 L/min) in cold plasma-induced inactivation

of biofilms. The reduction level of *P. aeruginosa* biofilms was 5 to 10% after being exposed to gas in the absence of plasma (plasma source turned off), which was much lower than the inactivation level by cold plasma. The findings of this study were consistent with previous studies by Joaquin et al. (2009). The above data indicate that the inactivation of biofilms is due to plasma treatment and not due to excessive cell drying from the gas flow.

4.6 Antibiofilm Mechanisms of Cold Plasma

How cold plasma induces the inactivation of microbial biofilm is an interesting and complicated question. Several proposals have been suggested for the cold plasma-induced biofilm eradication (Gilmore et al. 2018; Gupta and Ayan 2019; Zhu et al. 2020).

4.6.1 The Roles of Reactive Species

The reactive species present in the plasma possess strong oxidizing potentials and are believed to be mainly responsible for the antimicrobial and antibiofilm efficacy of cold plasma. These reactive species can cause severe oxidative damages to cellular structures and important cellular components (such as DNA, proteins, and lipids), which subsequently led to the death of microorganisms (Han et al. 2016a, b; Liao et al. 2017a; Montie et al. 2000). For instance, oxidative DNA damage has been revealed in bacterial cells after exposure to cold plasma. Joshi et al. (2011) showed that the formation of 8-OHdG in planktonic *E. coli* cells, a ubiquitous marker of oxidative DNA damage, was significantly increased after being exposed to plasma treatment. The PCR results also revealed multisite genomic DNA strand breakage in *E. coli* and *L. monocytogenes* cells after DBD plasma treatment (Lu et al. 2014). DNA damage was also observed in *L. monocytogenes* and *S.* Typhimurium biofilms for most of the cold plasma treatment conditions (Govaert et al. 2020b). Joshi et al. (2011) assessed the malondialdehyde (MDA) levels in *E. coli* cells during plasma treatment. The results demonstrated that DBD plasma induced lipid peroxidation in intact *E. coli* cells in a time-dependent manner.

Besides the direct damage effect of reactive species, cold plasma also induces the generation of intracellular ROS or RNS in bacteria cells (Brun et al. 2018). In the work of Delben et al. (2016), the intracellular ROS levels in plasma-treated biofilms were measured with fluorescent probe dihydrorhodamine 123 (DHR 123). The results revealed that more cells in the biofilms of *C. albicans* and *S. aureus* exhibited green fluorescence compared with the untreated control, suggesting the presence of ROS in all plasma-treated biofilms. The consistent findings were also revealed by Govaert et al. (2020b).

4.6.2 Dispersion of Extracellular Matrix

In most biofilms, the matrix can contribute to more than 90% of the total dry mass, while the microorganisms account for less than 10% (Flemming and Wingender 2010). The biofilm matrix is mainly composed of water and EPS. EPS are the natural organic polymers of high molecular weight secreted by microbes, which are primarily comprised of polysaccharides, extracellular proteins, extracellular DNA (eDNA), and lipids (Flemming and Wingender 2010; Di Martino 2018). EPS can also act as a scaffold to protect the biofilm cells from a wide range of external environmental stresses, e.g., UV radiation, oxidizing agents, desiccation, antibiotics, and biocides (Vandervoort and Brelles-Marino 2014; Yin et al. 2019). In addition to their protective role, EPS also play important roles in the biofilms formation and development, such as mediating biofilm adhesion to surfaces, providing mechanical and structural stability, mediating, controlling gene regulation, facilitating quorum sensing, and acting as nutrient sources (Flemming and Wingender 2010; Hobley et al. 2015).

Though many studies have been conducted on the biofilm eradication induced by cold plasma, the damages inflicted to the biofilm matrix are not still well investigated. Vandervoort and Brelles-Marino (2014) studied the effect of DBD plasma on the *P. aeruginosa* biofilm matrix. By using the fluorescent dyes Calcofluor White and SYTO9, DBD plasma caused a reduction to almost complete removal of the biofilm matrix in a time-dependent manner. Besides, the noticeable lipid oxidation products and damaged DNA were also observed in *P. aeruginosa* biofilm matrix after plasma exposure. The above data suggest that the reactive agents in DBD plasma cause damages to polysaccharides, proteins, eDNA, lipids, and other components of the biofilm matrix. In addition, the disrupted heterogeneous biofilm structures were also observed in the work of Ziuzina et al. (2014) and Ziuzina et al. (2015a). These chemical and structural changes on the biofilm matrix may contribute to the plasma-mediated biofilm inactivation (Vandervoort and Brelles-Marino 2014; Ziuzina et al. 2014, 2015a).

4.6.3 Inhibition of Quorum Sensing

Quorum sensing (QS) is a type of population density-dependent cell to cell signaling system, which is based on the effect of low-molecular-weight extracellular signal molecules called autoinducers (AIs) (Rutherford and Bassler 2012). Autoinducers can be classified into three main types based on their structure and specific function: *N*-acyl-homoserine lactones (AHLs), autoinducing peptides (AIPs), and autoinducer-2 (AI-2). AHLs primarily exist in Gram-negative proteobacteria as well as some cyanobacteria and bacteroidetes, AIPs are mainly synthesized in Gram-positive bacteria, and the AI-2 QS system is found in both Gram-negative and -positive bacteria (Hense and Schuster 2015; Rutherford and Bassler 2012). QS

signaling plays important roles in biofilm formation (such as attachment, maturation, aggregation, and dissolution), antimicrobial resistance development, and virulence factors secretion (Parsek and Greenberg 2005; Rutherford and Bassler 2012). Thus, the disruption of QS seems to be an attractive strategy for biofilm control (Muhammad et al. 2020; Sadekuzzaman et al. 2015).

Flynn et al. (2016) confirmed that the cold plasma caused chemical modification and degradation of AHL molecules in a time-dependent manner, which subsequently inhibited the production of QS-regulated virulence factors (such as pyoverdine and pyocyanin). Significant reductions of QS-regulated virulence factors, such as pyocyanin and elastase were also observed in *P. aeruginosa* following the exposure to DBD plasma (Ziuzina et al. 2015a). For the biofilms of non-hospital strains of *P. aeruginosa* (DBM 3777, ATCC 10145, and ATCC 15442), the production of AHLs was decreased after the cold plasma exposure, and the eradication of the biofilm was also achieved. These data suggested that cold plasma may reduce the formation of biofilm by inhibiting the AHL-dependent QS systems in non-hospital strains of *P. aeruginosa* (Paldrychová et al. 2019). The production of AHLs in hospital strains of *P. aeruginosa* was also significantly decreased after exposure to cold plasma (ATCC 27853 and DBM 3181), but this decrease did not result in significant changes in the total biofilm biomass and metabolic activity of the two stains. The biofilm formation of the two clinical isolates is probably controlled by another QS system, which is not affected by cold plasma (Paldrychová et al. 2019). Therefore, it is summarized that QS may be involved in the biofilm inactivation induced by cold plasma, while the underlying exact mechanisms are still not widely recognized.

4.6.4 Damage of Cytoplasmic Membranes

As reported in the literature, membrane damage is one of the primary mechanisms of microbial inactivation induced by cold plasma, including changes in membrane permeabilization, membrane potential, membrane depolarization, and membrane lipid peroxidation (Brun et al. 2018; Joshi et al. 2011; Kvam et al. 2012; Lu et al. 2014). Joshi et al. (2011) observed that the membrane potential in *E. coli* cells decreased in a dose-dependent way after exposure to DBD plasma. Govaert et al. (2020b) investigated the effect of cold plasma on the membrane integrity of the biofilm-associated bacterial cells by measuring the leakage of intracellular DNA, an indicator of membrane damage. The authors found that cold plasma caused leakage of intracellular DNA in *L. monocytogenes* and *S.* Typhimurium cells encased within biofilms, suggesting the severe destruction of membrane integrity (Govaert et al. 2020b).

4.7 Conclusion

This chapter reviews the unique opportunities of cold plasma for the control and eradication of biofilms on food products. Numerous studies have shown that cold plasma is a promising and multi-targeting approach to combat biofilms formed in food products and food processing environments. The antibiofilm efficacy of cold plasma is affected by plasma equipment, processing parameters, properties of microbial biofilms, etc. The synergistic effects of reactive species, charged particles, UV emission, and electromagnetic fields mainly contribute to the antibiofilm activity of cold plasma. In order to achieve a higher rate of biofilm inactivation, the cold plasma should be combined with other hurdle strategies, such as plant essential oils, disinfecting agents, and chelating agents. The present studies are mostly focused on the in vitro effect of cold plasma on microbial inactivation. In future studies, special attention should be paid to the application of cold plasma for biofilm eradication on food products and food processing environments. Besides, the influences of cold plasma on the nutritional and sensory qualities of food products should also be investigated in detail. In addition, microbial cells may enter into the sublethal injury or viable-but-nonculturable (VBNC) state after cold plasma exposure, which pose a potential threat to food safety (Liao et al. 2017b; Rowan et al. 2007; Ziuzina et al. 2015a). Therefore, further studies are still needed to clarify the stress responses elicited by food-associated microorganisms to cold plasma.

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