MICROBIAL DECONTAMINATION BY NOVEL TECHNOLOGIES – MECHANISMS AND APPLICATION CONCEPTS

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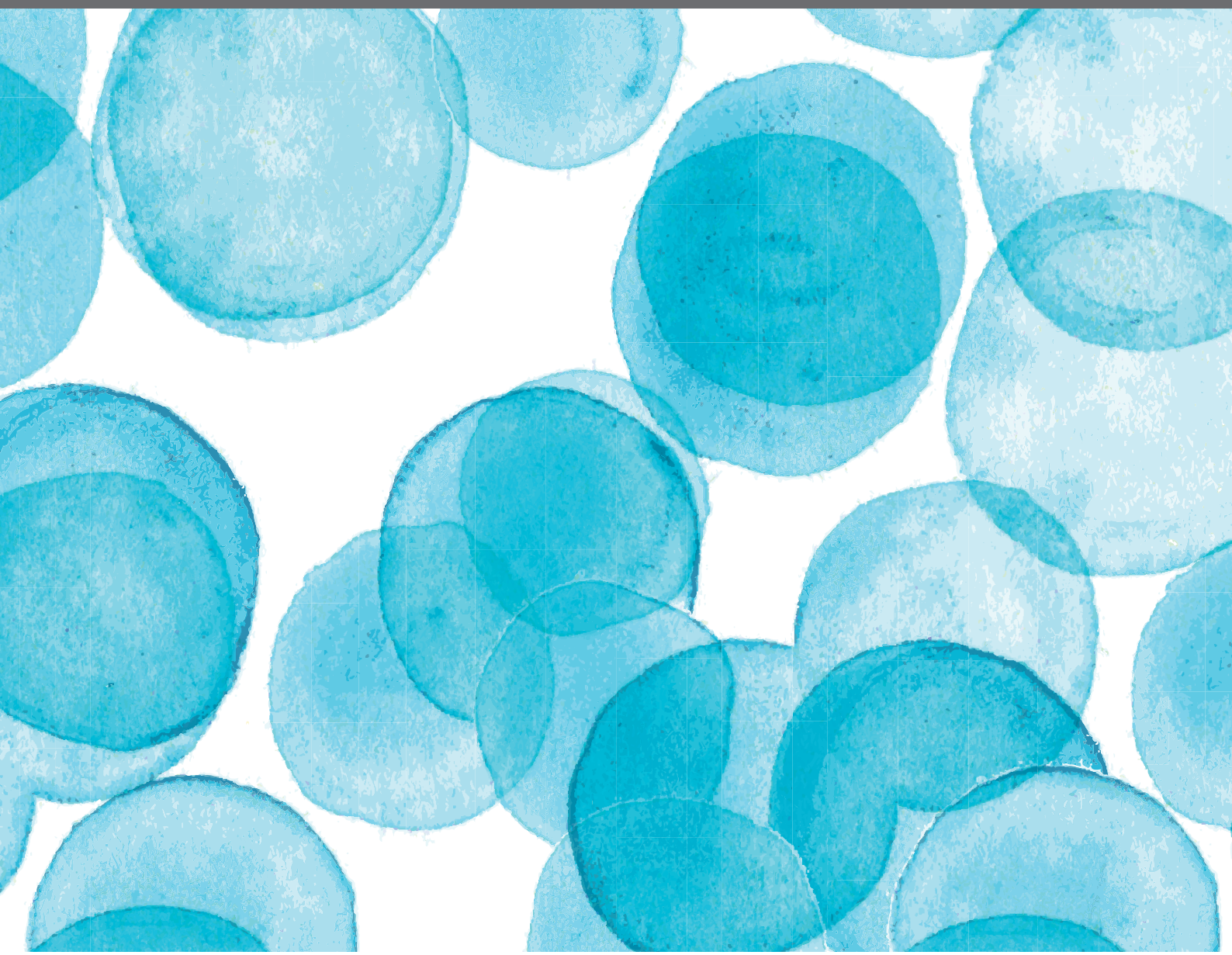

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MICROBIAL DECONTAMINATION BY NOVEL TECHNOLOGIES – MECHANISMS AND APPLICATION CONCEPTS

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EDITORIAL
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Editorial: Microbial Decontamination by Novel Technologies – Mechanisms and Application Concepts

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Keywords: inactivation mechanisms, non-thermal technologies, food safety, minimal processing, process validation

Editorial on the Research Topic

Microbial Decontamination by Novel Technologies – Mechanisms and Application Concepts

Microbial food safety and effective preservation constitutes the fundamental aspects of global food production systems. Despite of being well-developed in certain domains such as thermal processing; the quality and organoleptic properties of the treated products are limited. Since many years, significant efforts have been made to improve food quality, and in parallel, enabling similar microbial safety levels that of commercial products.

Emerging preservation technology concepts form the core focus of these developments. The main aim of this Research Topic for "Frontiers in Microbiology" is to provide an overview of the recent studies focusing on the inactivation mechanisms during novel non-thermal decontamination treatments and their implication on the development of application and validation concepts.

This Research Topic comprises of 9 original articles (including 2 reviews), contributed by 45 authors.

The fundamental concept and backbone of these research articles is the multi hurdle technology (MHT) concept, suggested by Leistner and Gorris (1995). It includes a broad variety of physical (thermal, electromagnetic, mechanical), physico-chemical, or biological hurdles, where different multi-hurdle combinations lead to targeted additive or even better synergistic effects against microorganisms. The overall goal is the tailored stabilization of individual food products while retaining the beneficial qualities and organoleptic properties.

However, the MHT concept is based on a sensitive balance of specific hurdles for targeted food products or categories. Removing one or more effective hurdles and replacing them by non-thermal technologies is still not well investigated and understood. On this perspective, Schottroff et al. reviewed different non-thermal technologies with a focus on pulsed electric fields, pulsed light, ultraviolet radiation, cold atmospheric pressure plasma, and high isostatic pressure and their cellular as well as molecular mechanisms of action. The authors had focused on sub-lethal injury and the viable but non-culturable (VBNC) states of microorganisms after the novel decontamination treatments. An understanding of these mechanisms based on the advanced analytical measures is essential to ensure effective microbial food safety during storage as well. This becomes more relevant if the inhibition-based hurdles (such as low pH) are replaced with inactivation-focused hurdles, such as high isostatic pressure.

Another review, by Zhang and Mathys, discusses the control of the most resistant form of microorganisms, the bacterial spores. Here, population heterogeneities could occur by different germination and inactivation mechanisms. The so-called super dormant spores could

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germinate extremely slowly or totally fail to germinate. Although germination is the target of some non-thermal technologies such as high isostatic pressure, understanding the detailed mechanisms of their effects on germination deficiency by proper isolation techniques and analytical characterization is essential.

Three research articles focused on low and high energy electron beam. Depending on the kinetic energy of the electrons, an electron beam could be distinguished either as a high (HEEB; >300 keV) or a low energy electron beam (LEEB; ≤300 keV).

Hieke and Pillai and Bhatia and Pillai used HEEB with a 10 MeV, 15 kW eBeam linear accelerator. The first group of authors applied a lethal target dose of 7.0 kGy and investigated the associated sub-lethal injury with the potential VBNC states in *Escherichia coli*. After investigating the overall cellular functionality via analyzing bacteriophage infection, ATP level, metabolic activity, membrane integrity and DNA double-strand breaks; the authors concluded that the irradiated *E. coli* cells resembled viable, non-treated cells more closely than the thermal inactivated cells. After irradiation, the cells were still metabolically active up to 9 days.

Bhatia and Pillai investigated a similar research focus with inactivated pathogens *E. coli* 026:H11 and *Salmonella Typhimurium* after HEEB-inactivation and lethal doses of 2–3 kGy. The cells were noted as metabolically active even after the β-alanine, alanine, aspartate, and glutamate metabolic pathway analyses. Hence, they suggested the term "Metabolically Active yet Non-Culturable" for HEEB-inactivated bacterial cells.

Zhang et al. investigated the LEEB-based spore inactivation by using different energy levels of 80–200 keV and the energy inputs up to 9.8 kGy. LEEB, as an emerging non-thermal technology, can perform surface decontamination with less quality losses than the alternative thermal or chemical based treatments. The authors revealed that the appearance of LEEB-based inactivation efficiency is comparable to the other ionizing radiation techniques, like HEEB. However, the employed indicator for irradiation-based sterilization, *Bacillus pumilus* (DSM 492), was more sensitive than *Geobacillus stearothermophilus* (ATCC 7953) spores.

A technology with physical and chemical mode of action under investigation was cold atmospheric pressure plasma (CAPP). Waskow et al. applied a diffuse coplanar surface barrier discharge to inactivate the pathogens and fungal spores from the seeds. The seeds could germinate even after the CAPP treatment, upon which the seed surface was speculated to be sufficiently decontaminated. In terms of mechanisms, the authors could separate the effect of ultraviolet light from other plasma components and suggested physical damage to the cell envelope after advanced analysis of the treated *E. coli*.

Durek et al. focused on *Aspergillus niger* and *Penicillium verrucosum* (inoculated on barley) inactivation and the production of one of the most abundant food-contaminating mycotoxins, ochratoxin A (OTA). Under certain conditions, OTA levels increased even with reduced mold concentrations, possibly due to stress reactions that demonstrated the importance of mechanistic understandings and adapted process settings.

Besides, physical hurdles being the main focus of the Research Topic; innovative chemical hurdles such as natural antimicrobials, especially citral, carvacrol, (E)-2-hexenal, and thyme essential oils (EOs) were investigated by Braschi et al. The authors analyzed the morpho-physiological changes of *Listeria monocytogenes* Scott A and *E. coli* MG 1655 after different EO treatments by flow cytometry. They also developed a protocol to screen EO efficiency with different microorganisms.

Finally, biological hurdles were considered in the study of Li et al. who investigated the microbial community in primary dark tea during the pile-fermentation process.

Hence, to conclude the whole Research Topic, innovative MHT concepts are still not well-understood. So far, very limited combinations from a diverse hurdle portfolio have been applied. Mechanistic understanding of the novel MHT concepts is essential to leverage all the potential benefits and to produce higher quality, safe food products.

AUTHOR CONTRIBUTIONS

AM wrote the manuscript. All authors critically reviewed the manuscript for the intellectual content and approved the final version.

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REVIEW
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Sublethal Injury and Viable but Non-culturable (VBNC) State in Microorganisms During Preservation of Food and Biological Materials by Non-thermal Processes

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The viable but non-culturable (VBNC) state, as well as sublethal injury of microorganisms pose a distinct threat to food safety, as the use of traditional, culture-based microbiological analyses might lead to an underestimation or a misinterpretation of the product's microbial status and recovery phenomena of microorganisms may occur. For thermal treatments, a large amount of data and experience is available and processes are designed accordingly. In case of innovative inactivation treatments, however, there are still several open points with relevance for the investigation of inactivation mechanisms as well as for the application and validation of the preservation processes. Thus, this paper presents a comprehensive compilation of non-thermal preservation technologies, i.e., high hydrostatic pressure (HHP), pulsed electric fields (PEFs), pulsed light (PL), and ultraviolet (UV) radiation, as well as cold plasma (CP) treatments. The basic technological principles and the cellular and molecular mechanisms of action are described. Based on this, appropriate analytical methods are outlined, i.e., direct viable count, staining, and molecular biological methods, in order to enable the differentiation between viable and dead cells, as well as the possible occurrence of an intermediate state. Finally, further research needs are outlined.

Keywords: viable but non-culturable (VBNC), sublethal injury, high hydrostatic pressure (HHP), pulsed electric fields (PEFs), pulsed light (PL), ultraviolet (UV) radiation, cold plasma (CP), flow cytometry

INTRODUCTION

Depending environmental conditions and stresses, microorganisms on exist in different metabolic states and growth phases whereas active replication of cells is not included in all the states (Davis, 2014). In this context, viability is commonly referred to as the existence of replication in culture media (Espina et al., 2016) and the cultivation based viability assay is still considered the "gold standard" for the determination of bacterial viability, even though the absence of growth is not a clear indicator for the absence of microbial life (Emerson et al., 2017). The definition of life or

dead is difficult for microorganisms because the route from life to death as well as the reverse way of recovery is still uncertain and includes many different states. The growth or the lack of growth on or in culture medium allows different interpretation of the results. The formation of colonies on growth medium means that at least one viable cell was able to replicate. Additionally, it is possible that more than one viable cell concur at the same place and form only one colony which might lead to an underestimation of viable cells. The lack of colony growth on culture medium implies that no viable cell is in the sample. However, another interpretation is the use of incorrect growth medium and conditions, i.e., temperature and time, or damaged or stressed cells that are not able to form colonies. Nevertheless, these cells can be viable (Davey, 2011). This again might lead to an underestimation of viable cells. The loss of culturability of microorganisms can be the consequence of damages to essential cellular components or the lack of essential cellular components. This damage can be of a temporary, i.e., sublethal damage, or permanent nature, i.e., lethal injury (Kell et al., 1998). Since there is often an improper classification of injured cells as VBNC cells (Pinto et al., 2015), a description of the different metabolic states of microorganisms is given here. The formation of bacterial endospores is a well-known long-term survival mechanism of Gram-positive bacteria (Colwell, 2009). Dormant bacteria are in a physiological state characterized by a shutdown of the metabolism. These cells show neglectable metabolic activity that cannot be detected by vital assays and they might not be culturable. Upon specific stimuli these cells regain activity and can thus be cultivated again (Kell et al., 1998). Persistent cells are defined as an antibiotic-resistant subpopulation, whereas the other cells in the population remain sensitive to the antibiotics (Li et al., 2014). Low numbers of these cells are formed during growth (Pinto et al., 2015). The viable but non-culturable (VBNC) state of cells can be defined as inactive form of life that is induced by stressful conditions (Colwell, 2009) and undergoes recovery under suitable conditions (Ramamurthy et al., 2014). VBNC cells show low but detectable metabolic activity, they maintain membrane integrity, and express genes at low levels but the formation of colony forming units (CFUs) on culture media is inhibited (Ayrapetyan and Oliver, 2016). The process of recovery of VBNC cells is called resuscitation and describes the transition from the non-cultural to the cultural state (Kell et al., 1998) without any change in cell number due to regrowth (Bogosian and Bourneuf, 2001). Resuscitation can be triggered by the increase of nutrient concentration, decrease or increase of the temperature, addition of chemical stimuli, and co-cultivation with host cells. However, the greatest challenge is to distinguish real resuscitation from regrowth of residual culturable cells (Zhao et al., 2017). The formation of VBNC cells occurs upon longer periods of incubation under stressful conditions (Pinto et al., 2015). In contrast to VBNC cells, sublethally injured species still possess the ability to grow, however, solely and predominantly on non-selective growth media (Li et al., 2014). Sublethal injury in bacteria is induced by the exposure to chemical or physical processes. Reversible damage of cell structures and loss of cell functions can be the result of a sublethal treatment (Wesche et al., 2009). These injured cells usually possess the ability to repair their damages under suitable conditions and consequently they are able to grow again (Espina et al., 2016). Severely injured cells that cannot be resuscitated under laboratory conditions may enter the VBNC state and maintain their pathogenicity (Wesche et al., 2009).

It is well known that less severe preservation intensities result in non-injured, injured, and inactivated bacterial populations (Silva et al., 2012; Wu, 2014; Schottroff et al., 2017), and the VBNC state can also be induced by preservation treatments (Oliver, 2010; Ayrapetyan and Oliver, 2016). Thus, the detection of injured and VBNC bacteria is of distinct relevance for industrial food production, to avoid false-positive or false-negative results (Wu, 2014). Especially an under-estimation of the product's safety status might lead to severe consequences, as the presence and potential re-growth of microorganisms in a physiologically infringed state might lead to a reduction of the shelf life or to an outbreak of foodborne illnesses, and thus may represent a potential risk for food safety (Wesche et al., 2009; Silva et al., 2012). Therefore, reliable detection methods for sublethally damaged cells and VBNC cells are necessary to study inactivation mechanisms, to assess the effectiveness of preservation treatments, to provide suitable validation concepts and to ensure food safety.

Thus, the present paper gives an overview of non-thermal decontamination technologies and their principles of action. The occurrence of sublethal injury and the VBNC state as induced by the treatments are discussed. Consequently, a set of methodologies to differentiate the individual physiological states is presented.

OVERVIEW OF NON-THERMAL INACTIVATION TECHNOLOGIES

Food preservation techniques should fulfill special requirements, including prolonged shelf life of perishable products and maintenance of the individual safety status, by inactivation of pathogenic bacteria and spoilage microorganisms. Additionally, organoleptic and nutritional properties of the products should not be changed and the formation of process induced contaminants should be avoided (Birlouez-Aragon et al., 2010). Last but not least, the preservation technique should be cheap and convenient to apply and there should be no concerns from legislation and consumers (Raso and Barbosa-Cánovas, 2003). An increasing focus on minimal processing with the aim to maintain or improve food safety while lowering the negative impact of processing on product quality leads to the growing implementation of non-thermal preservation technologies. Thus, in the following paragraph promising non-thermal preservation technologies will be summarized and their principles of action will be explained. More detailed insights into application concepts for different food and biological products are given by Knorr et al. (2011).

High Hydrostatic Pressure

High hydrostatic pressure treatment (HHP) is the most established non-thermal technology in industrial food

production and capable of the successful reduction of vegetative pathogenic and spoilage bacteria, with a minimal degradation of valuable food constituents (Gayan et al., 2017).

Principle of the Technology

Current applications of HHP are solely operated in batch mode, with the treatment of the final product being carried out within a flexible packaging. For this purpose, the packaged goods are placed in a pressure vessel, which is linked to a high pressure pump and a corresponding pressure intensifier (Elamin et al., 2015). The vessel is filled with a pressure transmitting liquid, e.g., water, and additional water is pumped into the system in order to increase the pressure. During the pressure built-up, the so-called adiabatic heating occurs, i.e., a product-dependent temperature increase caused by compression (Ragstogi, 2013). Thus, HHP units are further equipped with temperature sensors, in order to be able to monitor this adiabatic heating. HHP treatment is a so-called isocratic process, i.e., the pressure transmission is immediate and its distribution is multidirectionally homogeneous. Consequently the same pressure treatment applies to all goods within the treatment chamber, regardless of product size and shape (Elamin et al., 2015). However, temperature non-uniformity may occur during the treatment due to heat losses toward the wall of the pressure vessel. After the dwell time, the pressure is released and the product can be further handled. Industrial processes are typically carried out in a pressure range of 200–600 MPa, with holding times of up to 10 min with typical temperature increase rates of around 3 to 9°C per 100 MPa, thus allowing pasteurization at distinctly lower temperatures compared to thermal processes (Ting, 2010; Knorr et al., 2011; Ragstogi, 2013).

Inactivation and Mechanisms

In general, the effect of HHP on microorganisms is based on the principle of Le Châtelier and Braun, i.e., the external force applied on a product in the form of an increased pressure causes a shift of the thermodynamic equilibrium in such a way that (biochemical) molecules are reduced in volume (Gayan et al., 2017). Furthermore, the principle of microscopic ordering states that under HHP conditions, the degree of ordering of molecules is increased, further changing the thermodynamic behavior of molecules, such as melting temperature (Balny and Masson, 1993).

Vegetative forms of microorganisms typically present in food, especially pathogenic and spoilage organisms, are the target of the HHP treatment, as they can effectively be inactivated at ambient temperature (Mota et al., 2013; Georget et al., 2015; Wang et al., 2016). The inactivation effectiveness of the HHP process on vegetative microorganisms is based on a multitude of different factors affecting cell components (see **Figure 1**). Thus, pressure acts on morphology and internal structures of cells but also on the metabolism (Bartlett, 2002; Mota et al., 2013). Primarily, membranes, ribosomes, as well as proteins and enzymes are affected.

The effect of HHP on membranes is based on pressure-induced phase transitions and alterations in the membrane fluidity, causing membrane disintegration as well as denaturation of membrane-associated proteins and the associated decrease of the membrane's barrier function (Winter and Jeworrek, 2009). Furthermore, membrane disruption also seems to be associated with the emergence of reactive oxygen species (ROS) and an accompanying oxidative stress, leading to a further inactivation in *Escherichia coli* (Aertsen et al., 2005; Kimura et al., 2017).

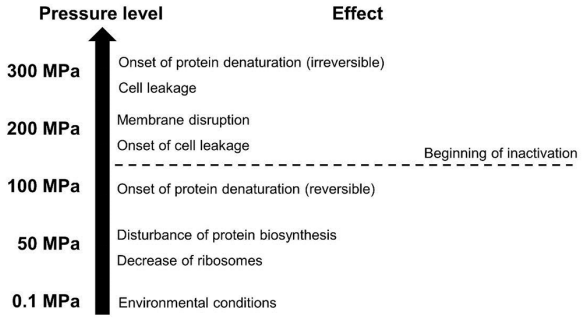

FIGURE 1 | General effects of different pressure levels on microbial cells (adapted from Lado and Yousef, 2002).

Moreover, ribosomes are disrupted by pressure, thus disturbing protein biosynthesis and the associated intracellular metabolism (Niven et al., 1999). The main target of the pressure, however, is the denaturation of proteins and enzymes. This is caused by weakening of non-covalent chemical bonds, such as electrostatic or hydrophobic interactions. Thus, the three-dimensional tertiary and quaternary structures of proteins are altered under pressure, in order to reduce the total volume (Silva, 1993; Winter and Dzwolak, 2005). Above a pressure level of 400 MPa protein monomers are denatured, resulting in the so-called molten globule configuration (Roche et al., 2012; Gayan et al., 2017).

Another distinct influence factor on the inactivation of microorganisms during HHP treatment is the change of intracellular pH due to the shift of the dissociation equilibrium under pressure, which is also related to the pressure-induced denaturation of pH-buffering enzymes and membrane disruption (Molina-Gutierrez et al., 2002). As a stable pH within the cells is inevitable for the maintenance of an intact metabolism and viability, this pH shift also contributes to microbial death under high pressure (Knorr et al., 2011).

Due to genetic as well as phenotypical variations among different forms of microbial life, variations in pressure resistance are present. Thus, eukaryotic species are usually less resistant than prokaryotes, with Gram-negative bacteria being less resistant than Gram-positive ones (Considine et al., 2008; Dumay et al., 2010; Georget et al., 2015).

The effectiveness of the treatment can be increased by the use of slightly elevated process temperatures (Knorr et al., 2011), and especially for sterilization applications the use of higher temperatures (90–100°C) is necessary to achieve a sufficient level of inactivation (Wimalaratne and Farid, 2008; Sevenich et al., 2014; Sevenich and Mathys, 2018).

Inactivation kinetics of high pressure treated microorganisms typically exhibit a so-called tailing behavior, i.e., a decrease in inactivation levels compared to a linear progression, toward the end of the treatment, with increasing process intensities. Reasons for this tailing might be due to the occurrence of microbial subpopulations with different individual pressure resistances due to genetic diversity, as well as adaption to external stresses and selection (Mota et al., 2013).

Role of Sublethal Injury and the VBNC State

Pressurized microorganisms react to the external force by the execution of different stress responses, leading to potential changes in physiology, and associated adaption as well as an increased resistance against the treatment (Mota et al., 2013). Depending on the pressure level and dwell time, as well as intrinsic microbial factors, repair mechanisms might lead to recovery subsequent to the treatment, i.e., pressure-induced injuries may be reversed, with the consequence of regrowth (Bozoglu et al., 2004).

Several studies demonstrated the recovery of microorganisms subsequent to a HHP inactivation treatment, indicating sublethal damage caused by the process and the associated repair of the damage.

Bozoglu et al. (2004) evaluated the influence of temperature on the recovery of *Listeria*, *Staphylococcus*, *Escherichia*, and *Salmonella* species after HHP treatment (maximum 550 MPa, 10 min) in milk and found that at 4°C only *Listeria monocytogenes* was able to regain viability, whereas at 22 and 30°C also colonies of the other cultures could be detected. Similar results were obtained by Bull et al. (2005) for *Listeria monocytogenes*, emphasizing the distinct temperature dependence of recovery after HHP treatment and the associated implications for food safety. Ritz et al. (2006) treated *L. monocytogenes* and *Salmonella* Typhimurium in buffers at pH 7 and 5.6, with pressures of 350–600 MPa for 10 min. They evaluated the behavior of the seemingly inactivated cultures during storage at 4 and 20°C and found resuscitation for all trials below 600 MPa, with a higher amount of viable populations present at increased storage temperature.

Ananta et al. (2004) processed *Lactobacillus rhamnosus* in PBS buffer in the range of 100–600 MPa. Using flow cytometry and propidium iodide as well as cFDA as dyes (see the Section "Detection of Physiological and Structural Changes"), it could be shown that although cultivation on agar plates was not possible, the cell's metabolism (esterase activity) was not in all cases completely shut down. Also in the process window of a lethal treatment, cases were presented where the bacterial membrane was still (partially) intact. Kilimann et al. (2005) showed that upon a HHP treatment of *E. coli* in the range of 200–400 MPa pH homoeostasis was permanently disturbed. On the other hand, the recovery of the cells subsequent to the treatment was distinctly increased when glutamate, which is known to stabilize the intracellular pH, was added to the medium at acidic conditions (pH 4). Kimura et al. (2017) investigated the high pressure inactivation of *E. coli* cells in the range of 400–600 MPa and analyzed viability using flow cytometry as well as culturability of the cells subsequent to the treatment. They found that sublethal injury and recovery was present for the treatments, especially in the lower range of the investigated pressure levels, as distinctly higher CFUs were present after incubation when oxygen-scavenging pyruvate was added to the agar plates. Further, growth temperature after the HHP treatment influenced the recovery of the cells, as higher counts were detected at 25°C incubated cultures, in comparison to 37°C. Also, growth of pressure injured cells was associated to a distinct lag phase at 25°C, indicating an adaption to the growth conditions and the onset of repair mechanisms. In general, specific product properties can exert pronounced effects on the recovery of microorganisms, especially in terms of osmoregulation. Thus, high sugar or salt concentrations in the matrix can decrease the susceptibility of the cells toward high pressure, associated with the accumulation of compatible solutes inside the cell (Molina-Hoppner et al., 2004; Gayan et al., 2017).

Besides, pressure resistance acquisition in bacteria is possible, when subpopulations that survived HHP treatment are re-grown and treated again. This resistance formation was described for a variety of microorganisms with implications for food safety and product quality, involving enterobacteriaceae, *L. monocytogenes, S. cerevisiae,* and *Lactobacillus* spp. (Mota et al., 2013).

Furthermore, Bozoglu et al. (2004) differentiated between two states of injury, one less severe type being associated to the cell envelope, and a more pronounced damage, linked to a negative interference of metabolic processes, similar to the observations made for sublethally injured cells by PEF treatment (Jaeger et al., 2009), with the latter state being associated with the VBNC state (no growth on non-selective agars).

As inactivation kinetics of HHP treated microorganisms often exert a so-called tailing, indicating the presence of resistant subpopulations, as well as the possibility of resuscitation and the associated regaining of viability, it has to be ensured that preservation processes involving high pressure are designed in such a way that they are severe enough to completely inactivate the bacterial target populations or to design processing concepts for the specific control and avoidance of recovery, based on the hurdle concept. Thus, traditional culturability tests might not be sufficient to assess the status of the microorganisms after the treatment. Consequently, adequate analytical methods (see the Section "Detection of Physiological and Structural Changes") have to be carried out, in order to obtain reliable data considering the microbial status of a decontaminated product.

Pulsed Electric Fields

Pulsed electric fields (PEFs) are used in a variety of different food processing applications, such as stress induction in microorganisms, mass transfer enhancement for food products or shelf life extension by microbial inactivation. Especially for the latter purpose, this technology bears a great potential, as it enables the efficient inactivation of vegetative microorganisms at distinctly lower process temperatures, in contrast to conventional heat treatments (Schottroff et al., 2017).

Principle of the Technology

In general, PEFs are generated by charging of a capacitor bank and controlled discharging, e.g., via spark gaps or semiconductor

switches. Depending on the type of switch, the pulse shape can either be exponentially declining or rectangular. The applied voltage is usually in the kilovolt range, with electric field strengths (ratio of voltage and electrode distance) between 10 and 50 kV cm^{-1} for microbial inactivation purposes. Furthermore, monopolar or bipolar modes of operation can be realized, with the latter being beneficial for the reduction of electrode corrosion (Barsotti et al., 1999). The electric field exposure of the product is realized within a treatment chamber, consisting of at least two electrodes, usually from stainless steel, which are separated by a non-conductive insulator. The most widespread treatment chamber configurations are parallel plate for batch applications and the co-linear configuration for continuous processes (Huang and Wang, 2009; Toepfl, 2011).

The duration of one electric pulse is usually in the range of several microseconds (μs) to a few milliseconds (ms) (Heinz et al., 2002).In order to achieve an effective inactivation and the targeted log reduction in microbial counts, a number of pulses are applied, and the total specific energy input (W_{spec}) is usually used as the criterion for the required process intensity. This cumulative parameter condenses the values of electrical and process parameters, and comprises voltage (U), current (I), pulse duration (τ), frequency (f) as well as the product mass (m) or mass flow (ṁ). Typical values of W_{spec} are in the range of 40–200 kJ kg^{-1}, with a corresponding ΔT of up to 45 K (Toepfl, 2006; Amiali and Ngadi, 2012). As the specific energy input is directly linked to temperature increase, this value is often used to control and monitor the process (Witt et al., 2017). An overview of relevant process variables to be considered during a PEF treatment are given by Raso et al. (2016).

Inactivation and Mechanisms

The inactivation mechanism during PEF treatment is based on electroporation and can be considered non-thermal (Knorr et al., 2011). However, an energy-dependent increase in process temperature is present during PEF treatment, due to electric current flow and the individual resistance of the product and may contribute to additional inactivation effects. The technology can be used for the shelf life extension of heat sensitive products, predominantly liquid foods (Lasekan et al., 2017) as an alternative to the conventional thermal pasteurization. An overview of different physiological states of microbial cells after PEF treatment and selected measures for their evaluation are given in **Figure 2**.

In order for non-thermal inactivation effects to occur, the electric field strength has to exceed a certain, microorganism-dependent value, usually in the range of 10–15 kV cm^{-1} for bacteria and around 5 kV cm^{-1} for yeasts (Grahl and Märkl, 1996). Above this critical value, the occurring electro-compressive forces across the membrane, which are emerging due to electric field-induced accumulation and attraction of oppositely charged ions on the inside and outside of the microbial cell wall – are strong enough to cause a perforation of the membrane (Neumann and Rosenheck, 1972). Depending on the intensity of the electric field, the electroporation effect can either be reversible, i.e., the pores can be sealed again, or irreversible, with an associated permanent loss of the membrane's barrier function leading to death of the microbial cell (Zimmermann et al., 1974; Kinosita and Tsong, 1977).

The onset of non-thermal effects during PEF treatment strongly depends on the size of the microorganism to be treated, with larger cells being distinctly easier to inactivate than smaller cells, due to a higher surface area exposed to the electric field (Schwan, 1957; Heinz et al., 2002). Furthermore, also the Gram behavior of a bacterium influences its resistance against the PEF treatment. In general, Gram-negative organisms are more sensitive to the treatment than Gram-positive species – due to the alteration of the membrane's electrical properties by the peptidoglycan layer – and can therefore be inactivated more easily (Hulsheger et al., 1983). However, more precise studies have shown a pH dependence of this resistance and pH can also be considered as one of the major matrix variables to affect the sensitivity of microbial cells toward PEF inactivation.

García et al. (2005b) compared the inactivation behavior five different Gram-positive and five different Gram-negative strains and found that resistance against PEF treatment was greatest at pH 7 for Gram-positive bacteria, whereas Gram-negatives were more resistant at pH 4.

Moreover, not only microbiological factors influence the inactivation but also further properties of the medium the

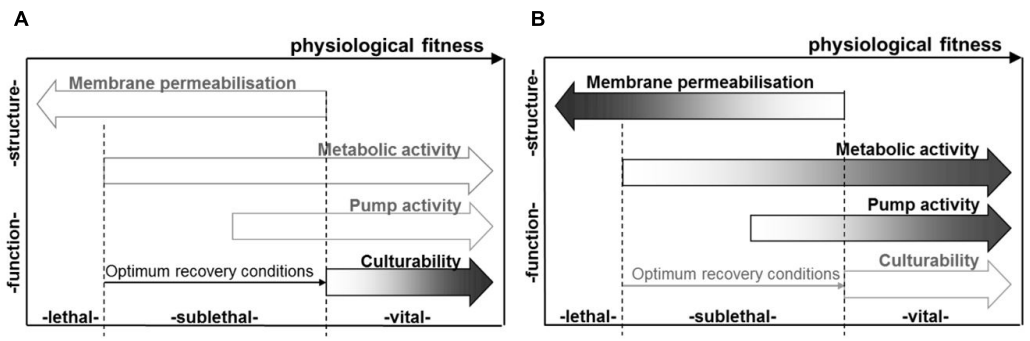

FIGURE 2 | Factors relating to the physiological state of microbial populations subsequent to PEF treatment, as determinable based on cultural methods, e.g., using selective media **(A)**, as well as membrane integrity, metabolic and pump activity, as indicated, e.g., by staining procedures **(B)**. Reprinted by permission from Miklavčič (2017).

microorganisms are suspended in. As the electrical conductivity influences the current flow through a medium, this strongly influences the energy to be delivered by each electrical pulse (Grahl and Märkl, 1996). Thus, at a constant voltage and pulse width, the energy per pulse decreases with a decreasing conductivity, due to a decreasing current. In order to obtain a constant total specific energy input, the frequency would have to be increased or the mass flow would have to be decreased (Schottroff et al., 2017). Moreover, the pH of the treated product may exert strong effects on the inactivation efficiency by PEF. During PEF treatment of microorganisms in a low pH medium, the molecules of the acid might diffuse through the electrically induced pores into the cytoplasm, with the consequence of a lowered intracellular pH and the associated disturbance of the cell's metabolism. Similar effects can be observed when antimicrobials are present within the treated matrix (Barbosa-Canovas et al., 2000; Garcia et al., 2007).

Considering bacterial endospores, however, the electric field alone is not effective for inactivation purposes, due to the lack of a distinct cytoplasmic membrane in spores. On the other hand, it could be shown that in combination with elevated temperatures PEF may lead to a more pronounced inactivation than heat alone (Siemer et al., 2014; Reineke et al., 2015). However, further research is necessary in this field in order to obtain mechanistic insights and a broader database on the effects of PEF assisted thermal sterilization on different spore species in various matrices.

Role of Sublethal Injury and the VBNC State

As the electric field mainly affects the cell wall and membrane of microorganisms (Pillet et al., 2016), especially in case of reversible electroporation it is possible that the damage of the membrane is not severe enough to cause an inactivation of a microbial cell. Thus, under certain circumstances, e.g., presence of nutrients, suitable water activity, ideal growth temperature and pH, the microorganism might be able to reseal the membrane pores and consequently regain viability (Garcia et al., 2007). Thus, subsequent to PEF treatment, three different states of a microbial population might occur, i.e., alive, dead, and sublethally injured subpopulations, with sublethal injury referring to a physiological state in-between alive and dead (Wang et al., 2018). Depending on microbial and media factors as well as treatment intensity, only one of these subpopulations might be present, but also several subpopulations can co-exist. During storage, the status of some of the individual populations can also merge into each other (Schottroff et al., 2017). From a mechanistic point of view, a loss of the cell's metabolic activity as well as the membrane's barrier properties refers to the inactivated, e.g., dead, subpopulation, whereas an active state of these two microbial constituents refers to the living state. If the membrane is perforated but the metabolic activity is still active, the sublethal condition is present. In this case, the injured population can subsequently develop into a dead or alive subpopulation (Ulmer et al., 2002; Jaeger et al., 2009; Schottroff et al., 2017). The occurrence of sublethal injury depends on a variety of different factory, including product properties, microbial species, and treatment severity (García et al., 2003, 2005a; Zhao et al., 2011). García et al. (2005b) showed a pH-dependent emergence of sublethal injuries, with the greatest amount of sublethally injured cells being associated to the pH level at which the greatest resistance to the treatment was determined. On the other hand, no sublethal subpopulations were present at pH levels with a low resistance against the treatment. Subpopulations being able to overcome the sublethal state are usually not fully harmed and are thus able to recover and repair the damage caused by the PEF treatment, i.e., repair of the membrane but also of metabolic disturbances (Garcia et al., 2007). Due to individual resistances within a certain microbiological population, resealing of PEF-induced membrane damage can also appear above the critical field strength, and at pH levels which would usually lead to an inactivation of the cell (Sagarzazu et al., 2013). As shocks from an electric field are a phenomenon which is not present in nature, it triggers a variety of different stress responses within the microbial cell. Thus, also the recovery process involves a distinct range of individually regulated genes. An overview of stress responses and repair mechanisms subsequent to PEF treatment is given by Schottroff et al. (2017).

Furthermore, some microorganisms are able to distinctly enter the VBNC state subsequent to PEF treatment. Rowan (2004) describes the occurrence of this physiological state for PEF-treated *Bacillus cereus* and *Listeria monocytogenes*, whereas the same research group later showed that the VBNC state occurred after heat, but not after PEF treatment of *E. coli*, *Bacillus cereus,* and *Listeria monocytogenes* (Yaqub et al., 2004). Thus, further research on this issue is needed, also with regard to the significance of this physiological state for an improved food safety.

Pulsed Light and Ultraviolet Radiation

Pulsed light (PL) is also known as pulsed UV-light (PUV), intense pulsed light (IPL), high-intensity pulsed UV light (HIPL), high-intensity broad-spectrum UV light (BSPL), intense light pulsed (ILP), and pulsed white light (PWL) (Heinrich et al., 2016b). The application of this decontamination technology has been well demonstrated in the packaging industry. Also, the application of ultra violet (UV) could successfully be implemented for food and packaging surface decontamination, as it is effective against a great variety of pathogens (including bacterial endospores) and spoilage microorganisms (Van Impe et al., 2018).

Principle of the Technology

After several adaptions and patents of the PL technology, the FDA approved the application "in the production, processing and handling of food" (Food and Drug Administration, 1996). PL can be applied in several processing steps in the food chain (Heinrich et al., 2016b). In principle, during PL treatment, short-duration, high-power electromagnetic pulses are emitted from a specific flash lamp filled with inert gas. Different lamp types are available in various shapes and materials almost exclusively filled with xenon gas and partly with other noble gases (Dunn et al., 1989; Gómez-López et al., 2007). Considering the application of xenon-flash lamps the emitted broad-spectrum radiation ranges from 180 to 1,100 nm, which encompasses infrared

(700–1,100 nm), visible light (400–700 nm) and a fraction of ultraviolet light (180–400 nm) (Dunn, 1996). The lamps are positioned above, below or surrounding the target object in a tightly closed treatment chamber or tunnel. The components of the basic equipment comprise (i) a power unit for generation of high-power pulses (ii) treatment chamber where pulses are transformed into high-power light pulses (Heinrich et al., 2016b).

Pulsed light is described as cost-effective, non-thermal decontamination technology without unwanted residuals on foods. It has been further developed from the conventional continuous-wave (CW) UV light of defined wavelength. It has been demonstrated that PL is more effective than CW-UV due to its high peak power (Kramer et al., 2016). Nevertheless, a specific UV range is proven to be more effective and varies with the target organism (Kramer et al., 2016). The mode of inactivation between the two technologies is still under discussion.

The principle parameters to describe the effect of PL is the fluence rate (F) [W m^{-2}], the fluence (F) [J m^{-2}], the number of pulses (n), pulse width (t) [s], exposure time ($t_{tot} = n^*t$) [s], frequency [Hz], and the peak power [W] (Heinrich et al., 2016b). Fluence is photochemically seen the most appropriate parameter to describe PL efficacy as it allows to measure the amount of energy impinging the target object (Rowan et al., 2015). Hence, the microbial inactivation dynamics only depend on the fluence applied (Rowan et al., 2015).

Considering several publications on the applicability of PL on different food types and surfaces, basic information on the parameters use is lacking which makes research outcomes incomparable (e.g., lamp manufacturer, geometry of target matrix, inoculation trials, etc.). In food processing PL is mainly used for surface decontamination of food and non-food products. Decontamination efficacy increases when reflection is low, and absorption and transmission coefficients are increased (Gómez-López et al., 2007; Heinrich et al., 2016b). Hence, a surface without huge irregularities and light absorbing matter might protect the target microorganism from the light source. The application of colored agar or concentration dependent protein solutions hamper light penetration mainly at the UV-C range (Kramer et al., 2016).

Inactivation and Mechanisms

Pulsed light is able to inactivate a range of microorganisms in short processing times (seconds) on different matrices. Depending on the type of microorganism up to 6 log units of inactivation are reported whereas on rough surfaces, such as meat products, lower colony count reduction up to 3 log units were found (Heinrich et al., 2016b; Zunabovic et al., 2017; Van Impe et al., 2018).

The characterization of the inactivation kinetics has been properly demonstrated by several authors (Rowan et al., 2015; Heinrich et al., 2016a; Van Impe et al., 2018).

A "typical" PL inactivation curve is mostly postulated to be non-linear with sigmoid shape in three phases. Firstly, (i) cell injury at initial plateau or shoulder effect (ii) fast increase of the inactivation and finally (iii) the tailing phase due to several factors of survival and strain-specific effects (McDonald et al., 2000; Gómez-López et al., 2007; Farrell et al., 2010). Heinrich et al. (2016a) studied the inactivation kinetics on different *Listeria monocytogenes* strains in single application instead of strain cocktails. The substantial decline of cells with less pronounced shoulder was shown already after 0.46 J cm^{-2}. The shoulder effect is absent in case of high initial fluence (Farrell et al., 2010). It must be emphasized here that the application of kinetic models requires particular combinations of conditions (e.g., amount of data points, minimum log reduction, etc.).

Several constituents of microorganisms are known to contribute to the susceptibility toward PL due to the current physiological state and density of the population, and further the growth rate and lag time (Dunn et al., 1989). Pigmentation of microorganisms (e.g., *Aspergillus* conidiospores, melanin, alginate slime, pyocyanin) exhibit higher resistance to PL (Kramer et al., 2016). In addition, a Gram-behavior dependent susceptibility of microorganisms to PL was shown in several studies, with a generally greater tolerance of Gram-positive bacteria, compared to Gram-negative species (Heinrich et al., 2016b; Kramer et al., 2016).

The inactivation mechanisms according to Van Impe et al. (2018) for PL/UV-based technologies can be summarized in a range from strong to lower relevance: (i) damage incl. oxidative damage to cell membrane and damage to DNA (ii) damage to spore coat and (iii) inactivation of key enzymes and chemical modification of in spore core incl. cortex.

The bactericidal effect of PL is contributed by the UV fraction (mainly UV-C), causing photochemical alterations of the genome by emergence of cyclobutane thymine dimers (CPD) and other DNA lesions (Goosen and Moolenaar, 2008; Rowan et al., 2015). At the level of RNA single stranded breaks and formation of dimers are observed (Pollock et al., 2017). Photophysical effects comprise cell death through irreversible structural damage of cells and photothermal mechanisms achieve cell death due to disruption and explosion (Dunn et al., 1989; Wekhof, 2000; Cheigh et al., 2012; Rowan et al., 2015). The type of effect/damage strongly depends on the microorganism and the experimental setup. These inter-related mechanisms act in parallel or in sequence. *Escherichia coli* for instance is inactivated at 270 nm due to high absorption of the DNA at this wavelength (Wang et al., 2005). In addition, the evolutionary adaption of some bacteria frequently exposed to sunlight might contribute to higher PL resistance. DNA repair repertoires (photolyase, glycosylase, endonuclease or nucleotide excision repair) reducing UV-induced lesions reduce susceptibility of certain bacterial strains. These repair pathways are thoroughly discussed in the review of Goosen and Moolenaar (2008). Mucoid and pigment forming bacteria at high cell densities showed increased PL resistance (e.g., *Pseudomonas aeruginosa* strains) (Farrell et al., 2010). Fungal and bacterial spores show different susceptibility after PL treatment due to absorbing spore colors (Dunn et al., 1991; Levy et al., 2012). The size of bacteria is another crucial factor for PL resistance, as larger cells are generally more susceptible to the treatment than smaller organisms (Wekhof, 2000).

Studies on the viral inactivation on food-related surfaces are scarce. The application of the technology in the drinking water sector is more prominent in scientific literature. Here,

mainly poliovirus and rotavirus 4 to 10 log reduction in relation to water turbidity is studied (Vimont et al., 2015). Even though PL is described as non-thermal, product heating cannot be excluded after long operation times. This may additionally lead to microbial inactivation with regard to the target matrix.

Role of Sublethal Injury and the VBNC State

Kinetic data attributed to PL treatments reveal concrete differences due to processing conditions, initial contamination level, matrix effects, etc. Nevertheless, the underestimation of microbial survivors is evident as these kinetic models rely on growth-dependent methodologies (culturability). Limited molecular and cellular based studies are available on the examination of VBNC fractions after PL treatments. As expected, the differences between culture and non-culture based techniques are huge (Rowan et al., 2015). Immediate loss of bacterial vitality after PL application could not be shown even if a cultural 6 log destruction was examined (Rowan et al., 2015). Cell populations with elevated metabolic activity are more vulnerable against PL treatment than older cultures that obviously exhibit better repair mechanisms (Kramer et al., 2016). This has been shown for *E. coli* and *Candida* strains. Gómez-López et al. (2005) observed so-called photoreactivation after PL treatments. This recovery form of PL-treated microorganisms arises after exposure to visible-light. The so-called photolyase enzyme reverses the joinings of two adjacent pyrimidines under light in the near UV/blue light region. Photoreactivation was also shown for *L. innocua*, especially after immediate illumination *post* PL (Kramer et al., 2016). Photoreactivation has been demonstrated a time dependent mechanism, most effective within the first 30 min for some bacterial cultures (Kramer et al., 2016). Further details on the UV repair mechanisms are reviewed by Goosen and Moolenaar (2008). Cellular responses of *Candida* strains to PL treatments depend on the UV-dose applied. An increase of the cell membrane permeability correlated with specific patterns of ROS during treatments (Rowan et al., 2015). Sublethal PL exposure of yeast cells proved cellular repair. Flow cytometric examinations showed early loss of culturability of *S. cerevisiae* strains rather shutdown of vitality indicators (Ferrario et al., 2014). This could also be observed for Gram-positive and negative representative bacteria, such as *Listeria innocua* and *E. coli*. Despite the reduction of colony counts below the detection limit at fluences of 0.76 J cm^{-2}, cellular functions remained at different levels (Berney et al., 2006).

The capability to synthesize ATP after PL treatment (0.1–1.0 J cm^{-2}) was examined with *E. coli* DSM 498, *L. innocua* DSM 20649, *Staphylococcus aureus* DSM 346 and *Salmonella enterica* ATCC BAA-1045. These bacteria still could generate ATP at different levels in a dose dependent manner. However, *L. innocua* and *E. coli* proved to be more resistant to PL (Kramer et al., 2017). Residual cellular activity was also measured through membrane potential, esterase activity, glucose uptake and pump activity (Kramer et al., 2016). The VBNC state was shown for *S. typhi* after PL treatment (Ben-Said et al., 2012).

Hilton et al. (2017) tested a potential synergistic effect between environmental temperatures (5–40°C) and PL. The results indicate that among tested bacterial strains, only *L. innocua* was slightly more inactivated at process temperatures of 40°C.

Tolerance and resistance development after PL application resulted in different outcomes. Heinrich et al. (2016a) described tolerance behavior of *L. monocytogenes* strains after homologous (multiple PL circles) and heterologous (combination with heat) stress application. *Ps. aeruginosa* and *E. faecalis* also seem to adapt to PL stress at low energy doses (Massier et al., 2012, 2013). Sublethal stress factors simulating technological hurdles (e.g., heat, salt, acid) may result in variable susceptibility. These aspects need more detailed evaluation. Also, a combination with other mild treatments, such as PEF and thermosonication was shown to improve PL efficiency (Kramer et al., 2016).

Cold Plasma

Cold plasma (CP) treatment is a promising tool for the decontamination of food surfaces. However, the assessment of the plasma process is difficult because there is a lack of standardization of the process (use of different plasma sources, working gases, process parameters, etc.) and for each product a new assessment has to be conducted (Schlüter et al., 2013). Nevertheless, the following chapter gives an overview on CP treatment of foods, the underlying mechanisms, as well as the role of the VBNC state.

Principle of the Technology

In general, options for taking advantage of the so called fourth state of matter are manifold and widely used in various industries, e.g., illumination, material design, medicine, etc. However, when compared to the other non-thermal processes discussed in this chapter, cold atmospheric pressure plasma (CAPP) is a technique not industrially used for direct food treatment, yet.

Plasma is an ionized gas and can be generated in different ways. Basically, a process gas is forced to pass through an electric field. At a certain energy input [e.g., electron mean energy > 5 eV for air (Whitehead, 2016)] an ionizing process occurs at atmospheric pressure resulting in free electrons accelerated in the electric field. These free electrons can colloid with gas atoms or molecules resulting in an energy transfer and the generation of highly reactive species. These reactive species can then interact with food surfaces (Schlüter et al., 2013). Sources for the generation of CAPP are commonly plasma jets, corona discharges, dielectric barrier discharges (DBDs), and microwave discharges (Ehlbeck et al., 2011; Surowsky et al., 2015). The application of plasmas to food can be direct, semi-direct and indirect. In case the temperature does not exceed a value of 70°C the term CP was defined according to the blanching temperature as mild heat treatment (Schlüter et al., 2013). However, to treat heat-sensitive foods the maximum treatment temperature is often kept below 40°C.

In principle the treatment can be generated and applied batch wise (e.g., in a vacuum system), semi batch wise (e.g., in package when the electrodes are attached to the packaging material) or continuously for example in a bath of plasma processed water (PPW).

Inactivation and Mechanisms

The plasma-based inactivation of microorganisms is dependent on the mode of plasma application and matrix related effects. The effects of plasma on different matrices is described elsewhere (Surowsky et al., 2016). However, the plasma source and the mode of application strongly define the category of main species [e.g., ions, radicals, photons, ROS, reactive nitrogen species (RNS), etc.] and related effects (e.g., perforation, disruption, etching, photo desorption, diffusion, oxidation) (**Figure 3**). This is also of legal relevance since UV irradiation for example might result in a specific labeling of the treated food product.

There are various names and definitions for plasma applications available, but often the impact of the selected system on the main inactivation effect remains unclear. In **Figure 3**, the main aspects are categorized with respect to different set-ups of plasma application in food processing: direct, semi-direct, and indirect. Contact of the target organism with all sorts of species, including short-living species is just possible in the direct mode of action at shortest distances to the exited plasma. Semi-direct mode of operation means contact with selected reactive species. Depending on the system, contact with long living species and/or UV photons is intended. In a former

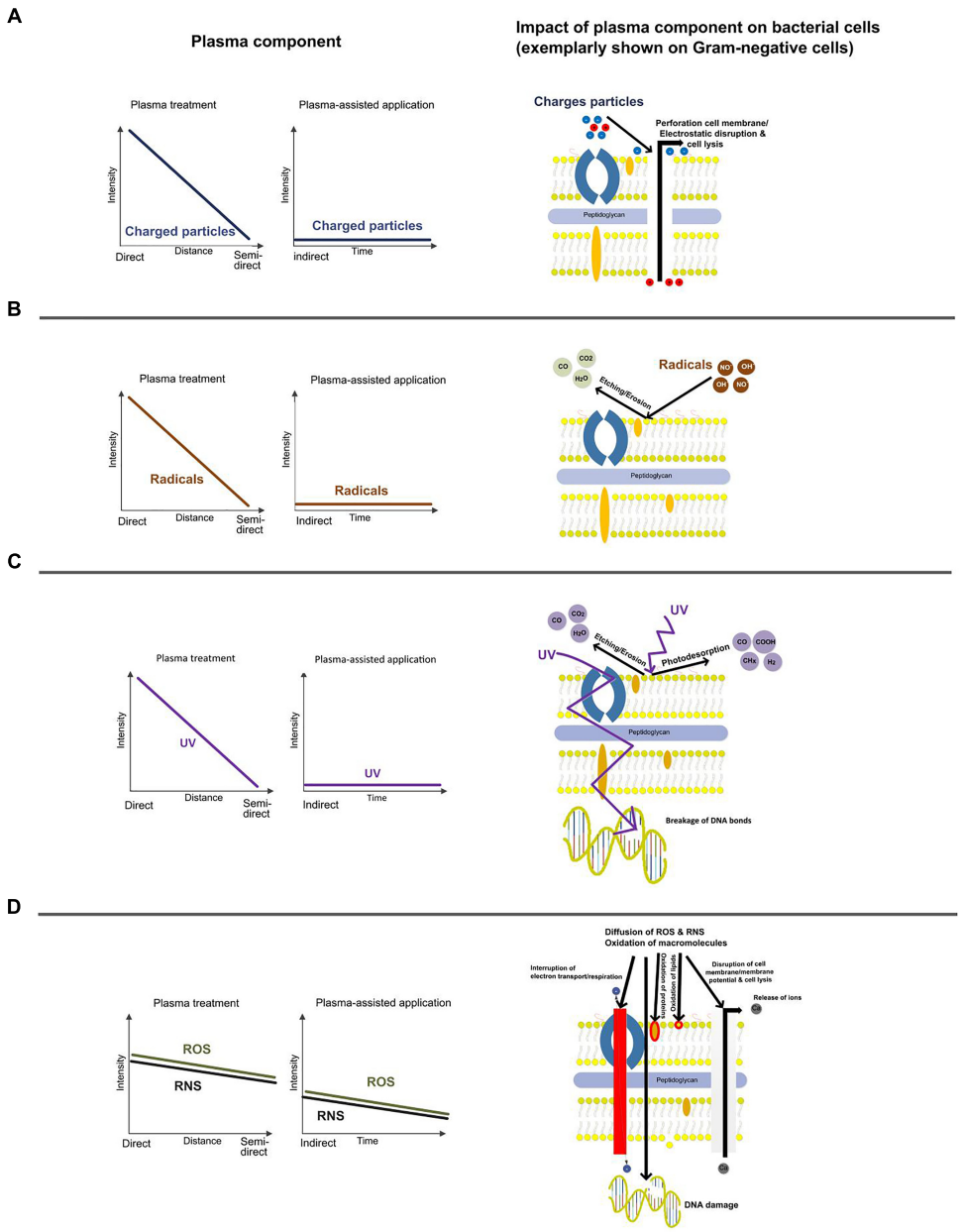

FIGURE 3 | Schematic drawing of the main plasma effects on bacteria and their intensities with respect to the mode of operation. Impact of ions **(A)**, radicals **(B)**, UV-light **(C)**, and ROS/RNS **(D)** on Gram-negative bacteria.

description indirect treatment was defined as treatment with UV and/or VUV light and for plasma treatment of gases and liquids (Schlüter et al., 2013). Since PPW is a new option for fresh produce treatment on pilot scale (Andrasch et al., 2017), the term "indirect" must be re-defined here. More consistent "indirect" means that direct UV light is excluded and plasma species are suspended or dissolved in a transmitting media (e.g., PPW) and the PPW is subsequently used for a treatment. Since UV photons are part of the reactive species also UV lamps belong to the term "semi-direct."

Since plasma interacts with the cell surface in the first instance, the properties of the cell envelope are a key aspect. Gram-negative bacteria contain a cell wall composed of two membranes: an outer membrane and an inner, cytoplasmic membrane. Compared to Gram-positive bacteria, only one layer of murein is present, located between the two membranes. Furthermore, lipopolysaccharides are attached to the outer membrane, acting as endotoxins after the destruction of the cell. Lipoproteins connect the outer membrane and the murein layer with each other, and although the outer membrane shows a low permeability, it contains pores (porins) which regulate the influx into the cell.

According to Schlüter and Fröhling (2014), perforation of microbial cell membranes, similar to the effect of PEF, can be the result of a plasma treatment. If the total tensile force of the membrane is exceeded by the total electric force an electrostatic disruption of the membrane occurs. The electric force is the result of a concentration of surface charge and in case of surface irregularities the electric force is even raised (**Figure 3A**).

Plasma treatment can result in erosion and etching induced by radicals attack ($OH^{\cdot}$ or $NO^{\cdot}$) of the cell membrane. The formation of volatile component (etching) is caused by absorption of radicals into the bacterial surface. Oxygen atoms or radicals emitted from the plasma are slowly combusted leading to an erosion of the microorganisms, atom by atom, through etching (**Figure 3B**).

UV light can induce intrinsic photodesorption which causes a breakage of chemical bonds in microorganisms and then the development of volatile by-products, such as CO, COOH, and CHx, from intrinsic atoms of the microorganisms. Dependent on the source and distance, UV-irradiation might lead to a destruction of genetic material (**Figure 3C**).

It must be considered that ROS react with cellular macromolecules, too. The cell envelope of Gram-negative bacteria consists of a thin layer of peptidoglycan and lipopolysaccharide, which is the major target for ROS. The inactivation is therefore mainly caused by cell leakage, accompanied by some DNA damage (**Figure 3D**). According to Han et al. (2016), the thick, rigid layer of peptidoglycan in the cell wall of Gram-positive bacteria remains intact upon ROS attack, leading mainly to inactivation due to intracellular damage such as DNA breakage.

However, the intensity of the described inactivation effects can be influenced by the mode of operation. The intensity generally decreases from direct to semi-direct treatment, but the effect of long-living ROS/RNS is then more pronounced and assumingly dominating the bacteria inactivation during plasma-assisted applications.

Role of Sublethal Injury and the VBNC State

Physical stresses like low or high temperatures, drying, irradiation, oxidative stress, starvation, PEFs, PL, and high pressure carbon dioxide or chemical disinfectants are known to induce bacteria to enter the VBNC state (Zhao et al., 2017) or bacteria are sublethally damaged (Silva et al., 2012). It is assumed that CP treatment also induces the VBNC state in bacteria which has to be taken into account during evaluation of inactivation efficiencies. Most studies dealing with the inactivation of bacteria by CP treatment do not consider uncultured bacteria (Brelles-Mariño, 2012). Food samples cannot generally be considered as free from pathogens after preservation treatments if plate count methods showed no colony growth because of the possible presence of VBNC or sublethally damaged bacteria. Besides the culturability of bacteria, detection of sublethally injured populations as well as the induction of the VBNC state after plasma application has to be evaluated to obtain a reliable assessment of the treatment. In case of laboratory experiments using pure cultures and non-selective media at least injured bacteria might also be detected. However, the inactivation of bacteria on food samples requires a specific detection of target microorganisms. Therefore the application of selective medium to suppress the background flora is necessary, which might lead to an underestimation of injured bacteria (Blackburn and McCarthy, 2000).

The treatment of *E. coli* in liquids by an atmospheric pressure plasma jet led to 7 log reduction according to plate count methods. In contrast, using the LIVE/DEAD Baclight Viability Kit only a 1 log reduction of *E. coli* was observed. Dolezalova and Lukes (2015) assumed that *E. coli* entered the VBNC state after plasma treatment but they were not able to resuscitate *E. coli* in nutrient broth even though they detected metabolic activity by mRNA analysis. Additionally, they observed plasma-induced DNA damages which hindered the replication and therefore the resuscitation was not successful. Thus, they proposed that the cells were in an active-but-non-culturable state and still able to be virulent because the cells still remained intact 3 months after plasma treatment. However, the measurement of membrane integrity is only one viability criterion and further analysis of metabolic activity would have been necessary to prove that the cells were really in an active-but-non-culturable state. The induction of the VBNC state by plasma treatment was also observed for *Bacillus stratosphericus* (Cooper et al., 2009). Cells of *B. stratosphericus* were treated with a DBD plasma either in suspension or surface-dried. Depending on the plasma dose applied, different viability states were obtained. With increasing plasma doses the viability status of *B. stratosphericus* shifted from viable and culturable to VBNC or disintegrated bacteria. Directly after plasma treatment the respiratory level of the bacteria cells was very low but 24 h later the respiratory activity increased eightfold. This implies the importance to evaluate the directly obtained inactivation but also the long-term inactivation efficacy. A dose-depending inactivation was also observed for *Chromobacterium violaceum* biofilms treated with

an atmospheric pressure plasma jet (Joaquin et al., 2009). After 5 min exposure time almost all culturable biofilm cells were inactivated while a higher respiration rate was determined and even after 60 min of exposure residual metabolic activity was measured. It was proposed that the cells enter the VBNC state after 5 min of plasma treatment. However, no further analyses of the presumably VBNC cells were conducted to verify this assumption and to exclude the occurrence of sublethal damage instead of the VBNC state. Another study using an atmospheric pressure plasma jet to inactivate *Pseudomonas aeruginosa* biofilms also revealed the loss of culturability of the biofilm cells while showing intact cell membranes using the LIVE/DEAD viability kit (Mai-Prochnow et al., 2015). A presumably VBNC state after plasma treatment was also shown for *E. coli* biofilms (Ziuzina et al., 2015). Metabolic activity of *E. coli* biofilm cells was still measured after 300 s of DBD plasma treatment whereas the culturability was already lost after 60 s. Moreover, a rapid inactivation of planktonic methicillin-resistant *Staphylococcus aureus* (MRSA), *P. aeruginosa*, and *C. albicans* cells due to surface damage occurred after DBD plasma treatment (Kvam et al., 2012). With increasing treatment time membrane integrity was decreased, followed by leakage of intracellular components and finally a complete dissolution of the cell. The authors concluded that longer exposure times can avoid the induction of the VBNC state. However, all of the studies have in common that no further analyses of the presumably VBNC cells were conducted. Sublethal injury of bacterial cells as a result of plasma treatment is described in various studies (Rowan et al., 2008; Fröhling et al., 2012; Surowsky et al., 2014; Fröhling and Schlüter, 2015; Smet et al., 2016, 2017; Liao et al., 2017; Vaze et al., 2017; Dasan et al., 2018).

DETECTION OF PHYSIOLOGICAL AND STRUCTURAL CHANGES

In order to detect the individual physiological state of microbial populations and subpopulations after a novel decontamination treatment, a variety of different methodological tools is available. The selection of the appropriate method of analysis depends upon several factors, including the cellular target of the individual treatment, ease of use, and costs of investment for particular equipment, amongst others.

As injured cells are difficult to detect with traditional microbiological methods, culturing on conventional growth media is usually not possible (Kimura et al., 2017). Sublethally injured bacteria are typically analyzed by differential enumeration using non-selective and selective growth media (Espina et al., 2016). Alternative methods for the detection of bacterial viability are based on the measurement of cellular integrity, e.g., membrane potential and integrity, metabolic activity, e.g., DVC, detection of respiration, and detection of mRNA synthesis, or presence of nucleic acids, e.g., polymerase chain reaction (PCR), hybridization, cytochemical staining, reverse transcriptase PCR, nucleic acid sequenced-based amplification, strand displacement amplification; propidium monoazide (PMA)-PCR (viability PCR), loop-mediated isothermal amplification (LAMB) (Keer and Birch, 2003; Li et al., 2017). More specifically, applied methods for the detection of VBNC cells are acridine orange direct count (AODC), DVC, direct fluorescent antibody methods, p-iodonitrotetrazolium violet (INT) viability staining, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining, LIVE/DEAD^{TM} BacLight^{TM} Bacterial Viability Kit, microautoradiography, laser scanning cytometer-scanRDI, and flow cytometry (Babu et al., 2014), as well as Alexa Fluor^{TM} hydrazide (AFH) assay (Emerson et al., 2017), among others. The most relevant of these methods are explained in more detail in the following. Beforehand, some details on resuscitation from the VBNC state will be given, as basis for the afterward described analytical techniques.

Resuscitation From the VBNC State

When kept at favorable conditions, microbial survivors, which underwent physiological stresses, e.g., during a decontamination treatment, and therefore developed sublethal injury or the VBNC state, can regain the ability to form colonies on growth media and therefore become fully vital again (Oliver, 2005; Ramamurthy et al., 2014). This process is called resuscitation and involves a variety of different metabolic pathways, gene expression, and repair mechanisms (Ayrapetyan and Oliver, 2016; Kimura et al., 2017). A crucial factor in this process, however, is the so-called resuscitation-promoting factor (Rpf), a cytokine (Mukamolova et al., 2002) present in bacteria able to enter the VBNC state, independent of their Gram behavior (Ramamurthy et al., 2014). Considering colony formation on microbiological growth media, the addition of agents able to convert ROS, e.g., catalase or sodium pyruvate, can help to reduce the content of said compounds, which can evolve during autoclaving of the media (Kong et al., 2004). Moreover, it was shown that if agar is replaced by other media, growth can be promoted again. This is associated with the occurrence of furan derivates, i.e., so-called neoformed contaminants that form during heat treatment (Ramamurthy et al., 2014). In some cases, an inversion of the stress responsible for the occurrence of the VBNC state can induce resuscitation, whereas other cultures require specific matrices and growth conditions (Ayrapetyan and Oliver, 2016). An overview of different pathogenic bacterial species known to enter the VBNC state and the corresponding resuscitation conditions is given by Li et al. (2014).

Advanced Culture-Based Methods

The fact that injured cells cannot form colonies on selective media, whereas non-selective media enable the recovery and regrowth of the organisms, can be used to differentiate individual physiological states (Kell et al., 1998). For an effective recovery and detection of sublethally damaged bacteria the addition of sodium pyruvate, 3,3′-thiodipropionic acid, catalase, superoxide dismutase, Tween 80 or oxyrase is recommended to overcome possible toxic effects of ROS that are present in the growth media (Wesche et al., 2009; Wu, 2014). Liquid repair methods for the recovery of injured cells include the incubation of the sample in non-selective media for 1–5 h at 25–37°C, followed by subsequent enumeration by direct plating or most probable number techniques. Unfortunately, it is possible that non-injured

and non-target cells multiply before the target cells are repaired. Even though the damaged cells can grow on non-selective media, the application for mixed cultures is hampered since the target cells cannot be differentiated (Wu, 2014). A more direct method for the differential detection of injured bacteria are solid-repair methods, e.g., pour-overlay plating, surface overlay plating, thin-agar-layer, agar underlay, as well as membrane or solid support-based methods. These methods combine non-selective agar media for recovery with the specific detection of target cells using selective media (Wu, 2008). However, high temperatures of the overlay methods may further affect injured cells (Wu, 2014). Additionally, the possible occurrence of enduring lag phases and vulnerability toward growth conditions exacerbate the detection of stressed or injured microorganisms (Joux and Lebaron, 2000; Hewitt and Nebe-von-Caron, 2004), and VBNC cells cannot be detected by culture-based methods (Colwell, 2009).

Staining

A common methodology to visualize microbial physiology and viability is based on staining of cells using different functional dyes (see **Figure 4**). In general, these dyes possess certain properties, which allow them to enter cells or bind to certain regions inside the cell or to be metabolized. The occurrence of fluorescence then allows to draw conclusions on the binding or alteration of these dyes and consequently on the physiology of the cells. Common targets include the cell membrane, metabolic and enzyme activity, DNA, and the intracellular pH (Ueckert et al., 1995; Nebe-von-Caron et al., 2000). The prevailing staining methods are compiled hereafter.

The most commonly used method for the determination of bacterial viability is the measurement of membrane integrity using the LIVE/DEAD^{TM} BacLight^{TM} Bacterial Viability Kit. This method is based on the fact that dead cells are considered to have disrupted and/or broken membranes whereas viable cells are intact (Stiefel et al., 2015). The LIVE/DEAD^{TM} viability kit includes the membrane impermeant propidium iodide and the membrane permeant SYTO 9. A double staining with both dyes allows the discrimination of cells with compromised cell membranes from those with intact cell membranes. Evaluation can consequently be carried out microscopically and by flow cytometry (Boulos et al., 1999; Berney et al., 2007).

There are different fluorescent stains available to measure metabolic activity of bacteria. Alexa Fluor^{TM} hydrazide (AFH), a photostable fluorescent molecule, penetrates membrane-compromised cells and binds to carbonyl groups in irreversibly damaged proteins of dead, dying, and aging cells. The AFH assay is used for the detection of respiring bacteria in food and environmental samples. A double-staining with SYBR Green or $DiOC_2(3)$ showed that the majority of the dead cells would not be detected with DNA-binding dyes alone (Emerson et al., 2017).

The respiration activity of bacterial cells is often measured using CTC. In respiring cells the CTC is reduced to a red-fluorescent formazan molecule and accumulated within the cells. The reduced formazan can be detected and quantified in low concentrations. However, the detection by flow cytometry is more accurate than by epifluorescence microscopy (Sieracki et al., 1999). The INT assay also measures the activity of the electron

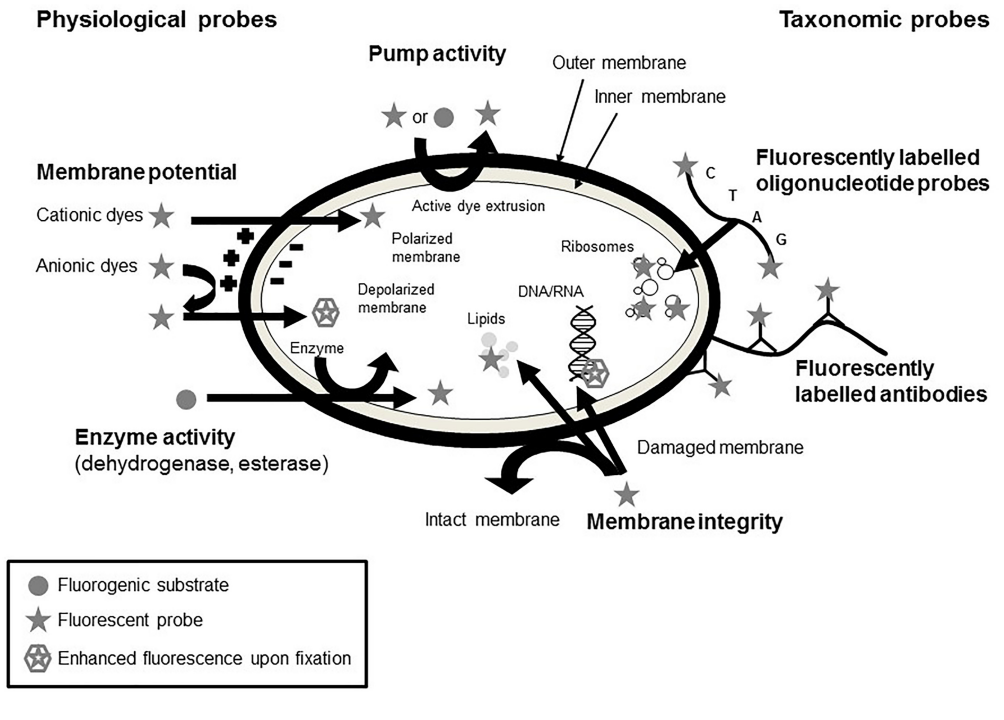

FIGURE 4 | Mechanisms of action of different staining procedures for the evaluation of physiological fitness of microorganisms (adapted from Joux and Lebaron, 2000; Ben Amor, 2004).

transport system. INT is reduced to formazin in metabolically active cells and the formation and accumulation of formazin in the cells is an indicator for an active electron transport system (Li et al., 2014). However, studies on heat stressed cells showed an overestimation of viable *L. monocytogenes* using INT whereas CTC lead to an underestimation of viable cells but after a period of resuscitation it proved to be a good indicator for injured cells (Bovill et al., 1994).

The membrane potential of bacteria is involved in the glucose transport, chemotaxis, the generation of adenosine triphosphate (ATP), bacterial autolysis, and survival at low pH and is therefore an crucial part of bacterial metabolism (Novo et al., 1999). Membrane potential measurements are often conducted using merocyanine dyes, cyanine dyes, and oxonol dyes (Waggoner, 1979). Anionic lipophilic dyes, e.g., oxonol dyes, concentrate within cells and bind to lipid-rich components in case of a decreased membrane potential of the cells. The use of bis-(1,3-dibutylbarbituric acid)trimethine oxonol [$DiBAC_4(3)$] to measure the membrane potential of Gram-negative and Gram-positive bacteria requires a pretreatment with ethylenediaminetetraacetic acid (EDTA). However, it has to be taken into account that the uptake of oxonol dyes is more dependent on membrane integrity than on the depolarization of the membrane (Joux and Lebaron, 2000). In contrast, cationic lipophilic cyanine dyes are able to easily enter into cells if they have a negative interior membrane potential gradient. Upon depolarization of the cells the dyes are released and upon hyperpolarization more dyes can enter the cells (Shapiro, 1994; Novo et al., 2000). Diethyloxacarbocyanine iodide [$DiOC_2(3)$] allows to estimated membrane potential by radiometric techniques precisely. When excited at 488 nm, $DiOC_2(3)$ emits at 530 nm with an additional peak at <600 nm upon use of $DiOC_2(3)$ in elevated concentrations. The green fluorescence signal is not influenced by the membrane potential, whereas the red fluorescence signal is changing with an alteration in the membrane potential. The calculation of the ratio of the red to green fluorescence signal allows the determination of the membrane potential independently of the cell size (Novo et al., 1999). Another possibility to determine the maintenance of membrane potential is the utilization of the dye 2-NBDG ((2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose). As this fluorescent molecule shows distinct analogies to glucose, it can enter the cells via the phosphoenol pyruvate phosphotransferase system (Yoshioka et al., 1996; Natarajan and Srienc, 1999). After uptake of 2-NBDG, a green fluorescence is emitted, which is gradually decreased as the molecule is degraded by the cell's metabolism (Yoshioka et al., 1996). In cells with a reduced viability, this glucose uptake cannot take place anymore and thus indicates the loss of the membrane potential (Berney et al., 2006).

Cellular viability, in terms of enzyme activity, more specifically esterase activity, is another parameter that is measured by flow cytometry. Lipophilic, uncharged and non-fluorescent fluorogenic substrates are generated by esterification of polar, membrane permeable fluorescent dyes with non-fluorescent acetyl or acetoxymethyl esters. The non-fluorescent dye enters cells and is then hydrolyzed by esterases resulting in a fluorescent product which remains in cells with intact cell membranes. Hydrolyses of fluorescein diacetate to fluorescein leads only to low fluorescent signals and retention within the cells is limited. In contrast, carboxyfluorescein diacetate (cFDA), chloromethylfluorescein diacetate (CMFDA), and carboxyfluorescein diacetate acetoxy methyl ester (cFDA-AM) are hydrolyzed into hydrophilic products and therefore, the retention within the cells is more pronounced (Ueckert et al., 1995; Joux and Lebaron, 2000; Veal et al., 2000).

For the microautoradiographic method the sample is exposed to radioactive isotypes and the labeled radioactive isotope can be located within the cells. The combination of radiography with fluorescence *in situ* hybridization (FISH) allows the direct detection of viable cells in complex environments (Babu et al., 2014). The laser scanning cytometer, scanRDI, is a solid-phase cytometry technique where a fluorescent stain is used to evaluate the metabolic activity and membrane integrity of viable cells by scanning the complete membrane. This allows the detection of a low number of fluorescently labeled cells (Babu et al., 2014).

A comprehensive overview on fluorescent dyes for the assessment of microbial physiology as well as detailed mechanisms of action are given by Joux and Lebaron (2000).

Direct Viable Count (DVC)

Using DVC, viable cells can be identified microscopically. The samples are incubated after addition of nutrients and a specific antibiotic (nalidixic acid), which is capable of inhibiting DNA gyrase (Kogure et al., 1979). The cell division is inhibited but available nutrients are metabolized, resulting in the elongation of the cells. These cells are considered as viable, whereas non-elongated cells are considered to be metabolically inactive (Buchrieser and Kaspar, 1993; Besnard et al., 2000a). Unfortunately, Gram-positive species are resistant to nalidixic acid and therefore not inhibited so that this method is not reliable. However, the combination of DVC with FISH can enhance the reliability of the method (Babu et al., 2014). The substitution of nalidixic acid with another antibiotic, ciprofloxacin, allows the application for Gram-negative as well as Gram-positive bacteria (Buchrieser and Kaspar, 1993). AODC is a differential staining method, in which acridine orange (AO) interacts with RNA to red-orange fluorescent and with DNA to green fluorescence complexes. This allows the differentiation of viable (red) from dead (green) cells. However, the concentration of AO, the pH of the growth medium, and the growth medium used as well as the incubation time lead to a high variance in fluorescence color and hampers the clear classification of injured, dead and viable cells (Babu et al., 2014). A further possibility is double staining of the cells using CTC and 4′,6-diamidino-2-phenylindole (DAPI). In this method, total counts emit red and blue fluorescence, whereas the viable cells can be distinguished by their red color. The blue staining is due to DAPI, which colors all cells, the CTC, on the other hand, is reduced by the active cells and consequently shows red fluorescence (Rodriguez et al., 1992; Besnard et al., 2000b).

The technique can be used to analyze microorganisms in a variety of different foods and biological media (Comas-Riu and Rius, 2009), such as dairy (Pettipher et al., 1983;

Rowe and McCann, 1990) and meat (Shaw et al., 1987; Duffy and Sheridan, 1998) products, as well as alcoholic beverages, including wine (Divol and Lonvaud-Funel, 2005) and beer (Siegrist et al., 2015). Thus, DVC is a fast and versatile method for the analysis of the physiological status of microbial cells. However, several sources of interference exist, i.e., stains can unspecifically start reactions with compounds of carbon present in the matrix, and interact with preservatives, such as sorbic acid (Comas-Riu and Rius, 2009).

Molecular Biological Methods

As live/dead staining using microscopy or flow cytometry is unspecific, an identification of the stained bacteria is not possible. Therefore, the combination of PCR with viability dyes allows the monitoring of decontamination efficiencies in environmental samples and of a product's natural microflora (Elizaquível et al., 2014). Viability PCR is commonly performed using ethidium bromide monoazide (EMA) or propidium iodide monoazide (PMA). The samples are stained with EMA or PMA, which can enter perforated cell membranes and bind to DNA. The photooxidation at 464 nm leads to irreversible damage of the nucleic acids. In consequence, these cells cannot be amplified and only cells with intact membranes are amplified (Trevors, 2012). The analysis of samples with and without PMA allows the evaluation of live and dead ratios of a microbial population (Emerson et al., 2017). However, there are some drawbacks of these methods. Cells can remain intact but do not show metabolic activity resulting in false-positive detection of viable cells. Additionally, viable species can have perforated cell membranes during growth, cell wall synthesis or during injury resulting in false-positive detection of dead cells (Stiefel et al., 2015).

Flow cytometry, as a method to continuously analyze a great amount of individual microorganisms within a population, is widely used due to its rapid and versatile detection abilities. By isolating cells and individually passing them through a laser beam, the particle size can be determined by detection of scattering (Comas-Riu and Rius, 2009). Furthermore, the use of different fluorescent dyes for viable staining (see the Section "Staining") allows the evaluation of individual physiological states and cellular targets of antimicrobial treatments, among others (Li et al., 2014; Ayrapetyan and Oliver, 2016). This includes membrane intactness and potential, activity of efflux pumps, as well as activity of several enzymes. By using taxonomic probes, e.g., fluorescently labeled oligonucleotides or antibodies, it is further possible to taxonomically classify the analyzed microbial cultures (Joux and Lebaron, 2000).

Moreover, several assays exist to detect the physiological status of microorganisms. Among them is the deoxyribonuclease (DNase) I protection assay, which indicates the level of membrane integrity, as only perforated membranes admit the intrusion of the enzyme and the associated degradation of genomic DNA. It has already successfully been implemented for the determination of the VBNC state in *Yersinia pestis* (Pawlowski et al., 2011). Using the p-iodonitrotetrazolium violet (INT) assay, metabolic activity can be evaluated (see the Section "Staining"). This assay can thus be used to detect the VBNC state, e.g., as shown by Rahman et al. (1994) for *Shigella dysenteriae* Type 1. Another assay, BacTiter-Glo^{TM}, uses luciferase to detect ATP concentration as an indication of metabolically active organisms. Exemplarily, it could contribute to detect the VBNC state in *L. monocytogenes* (Lindbäck et al., 2010).

FURTHER RESEARCH NEEDS

A successful implementation of novel preservation technologies in the production chain requires a detailed knowledge of inactivation effects and recovery phenomena in order to derive necessary treatment parameters and concepts. To ensure product safety, inactivation data cannot only rely on colony count monitoring immediately after the treatment but have to be verified during and at the end of the shelf life. In addition, a variety of cellular targets needs to be investigated in order to comprehensively describe the physiological and structural state of a microbial cell. For this purpose, online analytical tools are required. Flow cytometry is a promising tool and miniaturized versions are in development to be implemented before and after the inactivation step in order to gain information on cellular damage as well as the recovery potential. An online-flow cytometric measurement firstly requires an optimization of the staining methods as well as an improvement of the analysis and interpretation of flow cytometric data to allow a reliable application of prediction models to assess the impact of decontamination treatments on bacterial physiology. Additionally, the development of new fluorescent dyes that allow the simultaneously detection of physiological and structural properties of bacteria would improve the detection of the viability status of bacteria after inactivation treatments.

In addition, selective inactivation and colony count reduction by different mechanisms and its implication on the development of the microbial population and selective recovery, growth and adaptation of microorganisms needs to be studied. In this context, the microbial diversity changes as result of inactivation treatments should also be considered to avoid potential (re)growth of human pathogenic bacteria due to the lacking presence of naturally occurring phyllospheric and endophytic bacteria after inactivation treatments.

CONCLUSION

In summary, it can be stated that both the VBNC state as well as sublethal injuries can pose significant threats to food safety, as an underestimation of the microbiological status after a decontamination treatment can occur. Under favorable conditions, microbial subpopulations might be able to overcome this inactive state and become active again, thus being able to reproduce within the respective matrix. Hence, recovery phenomena need to be taken into account when evaluating the inactivation effectiveness in particular in the case of novel technologies. In order to avoid the necessity to apply

severe treatment intensities with sufficient safety margins but overprocessing and quality losses at the same time, it is crucial to not only understand inactivation of microbial cells but also their recovery strategies and to develop suitable processing schemes, e.g., based on the hurdle concept. Otherwise, an increased spoilage can occur, leading to an unacceptable product quality loss already prior to reaching the "best before" date. More importantly, the presence of pathogenic species in food may cause a serious outbreak of certain foodborne diseases. Hence, cellular targets of novel preservation technologies such as HHP, PEF, PL, and CP and related inactivation and recovery mechanisms need to be studied in order to design safe processes with minimized alteration of product quality.

This also underlines the importance of specific and accurate analytical tools being able to distinguish between the individual states of microbiological subpopulations, i.e., alive, VBNC/sublethal injury, and dead species. The presented methodologies differ in accuracy and in rapidity of the detection, as well as in the individual level of sophistication, costs of analysis, and ease of use. Depending on the method, a false-positive or false-negative detection of certain species might occur. Exemplarily, this can be the case if intact cells appear to possess no more metabolic activity (false-positive) or due to naturally occurring phenomena, such as the occurrence of pores in the cell membrane during certain growth stages (false-negative). This, however, may lead to an over or underestimation of the food safety status of the analyzed product. Therefore, it is recommended that several viability indicators (e.g., culturability, metabolic activity, membrane integrity) should be measured for a reliable detection of viable cells (Elizaquível et al., 2014).

AUTHOR CONTRIBUTIONS

MZ-P contributed to the pulsed light section. AK and HJ contributed to HPP and PEF section. OS contributed to plasma section. FS and AF equally contributed to the manuscript as co-first authors.

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Superdormant Spores as a Hurdle for Gentle Germination-Inactivation Based Spore Control Strategies

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Bacterial spore control strategies based on the germination-inactivation principle can lower the thermal load needed to inactivate bacterial spores and thus preserve food quality better. However, the success of this strategy highly depends on the germination of spores, and a subpopulation of spores that fail to germinate or germinate extremely slowly hinders the application of this strategy. This subpopulation of spores is termed 'superdormant (SD) spores.' Depending on the source of the germination stimulus, SD spores are categorized as nutrient-SD spores, Ca^{2+}-dipicolinic acid SD spores, dodecylamine-SD spores, and high pressure SD spores. In recent decades, research has been done to isolate these different groups of SD spores and unravel the cause of their germination deficiency as well as their germination capacities. This review summarizes the challenges caused by SD spores, their isolation and characterization, the underlying mechanisms of their germination deficiency, and the future research directions needed to tackle this topic in further depth.

Keywords: bacterial spore, superdormant, germination, inactivation, isolation, characterization, mechanism, gentle spore control

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INTRODUCTION

Bacterial spores are widely distributed and can cause spoilage and food-borne diseases, leading to economic losses and endanger public health (Setlow et al., 2012; Banawas et al., 2013). They are extremely resistant to heat, dehydration, and chemical or physical stresses, making them the main challenge of sterilization processes (Setlow, 2006, 2007; Setlow and Johnson, 2007; Patrignani and Lanciotti, 2016; Zhang et al., 2018). Because of their resistance, intensive wet heat treatment, generally at a temperature higher than 100°C, is usually applied to inactivate spores in food products (Storz and Hengge, 2010; Georget et al., 2013), and such processing procedures often cause an unwanted loss of food quality (Sevenich and Mathys, 2018). Therefore, development of effective gentle non-thermal spore decontamination strategies is currently of high interest (Storz and Hengge, 2010; Zhang et al., 2018).

Research has revealed that spores lose their extreme resistance after germination and become easier to kill, e.g., by milder heat inactivation (Collado et al., 2004; Setlow, 2006; Abee et al., 2011; Lovdal et al., 2011). Moreover, spore germination can be artificially triggered by nutrient germinants (van der Voort et al., 2010; Baier et al., 2011; Sevenich and Mathys, 2018), as well as non-nutrient stimuli, e.g., Ca^{2+}-dipicolinic acid (Ca^{2+}-DPA), and isostatic high pressure (HP) (Gould, 1970, 2006; Baier et al., 2011; Reineke et al., 2013). The overview of germination stimuli

and proposed germination pathways for *Bacillus subtilis* spores is shown in **Figure 1**. Based on this overview, gentle spore control strategies could be developed to achieve spore decontamination without largely compromising the food quality at the same time. For example, so-called "germination-inactivation" methods that first artificially trigger the germination of spores, and then eliminate those spores which lost their extreme resistance during germination with a mild inactivation step (Gould, 2006; Lovdal et al., 2011; Nerandzic and Donskey, 2013).

However, the germination behavior of spores is highly heterogeneous (Chen et al., 2006; Gould, 2006; Indest et al., 2009; Eijlander et al., 2011; Stringer et al., 2011; Setlow et al., 2012). Most spores can germinate rapidly after being exposed to germinant stimuli, but a subpopulation referred to as superdormant (SD) spores remained dormant or germinated extremely slowly (Gould, 2006; Ghosh and Setlow, 2009; Zhang et al., 2010; Rodriguez-Palacios and LeJeune, 2011; Sevenich and Mathys, 2018). These SD spores are the major limitations of the germination-inactivation spore control strategy. With the increased awareness of the importance of this subpopulation, more research has progressively shifted their focus to better understand this subpopulation, either in aggregate or at single cell level (Davey and Kell, 1996; Margosch et al., 2004; Ghosh and Setlow, 2009; Eijlander et al., 2011; Kong et al., 2011; Wang et al., 2011; Zhang et al., 2012; Perez-Valdespino et al., 2013). This review summarizes the challenges that SD spores cause, their isolation and characterization, the mechanisms of their superdormancy, and potential future research directions.

CHALLENGES ASSOCIATED WITH SD SPORES

Owing to their germination deficiency, SD spores are considered to be the main obstacle to the effective application of germination-inactivation spore control methods (Ghosh and Setlow, 2009; Lovdal et al., 2011; Wang et al., 2012; Markland et al., 2013a; Olguin-Araneda et al., 2015). For example, the tyndallization strategy is based on a germination-inactivation concept (Tyndall, 1877), and is considered to not be fully reliable due to the presence of superdormant spores (Gould et al., 1968; Gould, 2006).

Additionally, the presence of SD spores complicates spore quantification and presents potential limitations for the reliability of challenge and sterilization tests. They may stay dormant and remain undetectable during recovery, but germinate later and proliferate, causing spoilage or even foodborne diseases (Deng et al., 2015; Silvestri et al., 2015). For example, spores formed by some *Clostridium* species could recover from superdormancy during long-term storage and become viable afterward, posing a potential risk (Esty and Meyer, 1922; Deng et al., 2015, 2017).

Moreover, the presence of SD spores also complicates decisions regarding the duration of antibiotic treatment for *Bacillus anthracis* infection. A number of antibiotics can destroy germinated *B. anthracis* spores, but SD spores can remain unaffected. Therefore, the ability of SD spores to stay in a dormant state and germinate after antibiotic treatment is stopped makes them capable of causing fatal diseases (Brookmeyer et al., 2003; Heine et al., 2007; Setlow et al., 2012).

Furthermore, SD spores were found to be much more resistant than the overall spore population (Ghosh et al., 2009; Markland, 2011; Rodriguez-Palacios and LeJeune, 2011; Markland et al., 2013b). For example, isolated *Bacillus* nutrient-SD spores had increased heat resistances as compared to the initial spore population (Ghosh et al., 2009). This indicates SD spores might be the main contribution to the $\log_{10}$ non-linear tailing phenomenon of spore inactivation kinetic curves, which complicates the prediction and modeling of spore behavior (Eijlander et al., 2011; Doona et al., 2012, 2016b,c, 2017; Sevenich and Mathys, 2018). Furthermore, their above-average resistance is a clear concern for the food industry, since the treatment intensity that inactivates the majority of the population might not be able to inactivate SD spores, leading to insufficient sterilization.

SD SPORE ISOLATION AND CHARACTERIZATION

Superdormant is a relative term and it describes a subpopulation of spores that is phenotypically different on their germination capacity compared to the rest of the population. Notably, it is not a static subpopulation of spores but rather a subpopulation that depends largely on the germination/isolation conditions and the cut-off point, e.g., germination trigger intensity and maximum treatment time, defined by the researchers. However, the subpopulation that fails to germinate after intensive germination stimuli is generally referred as SD spores (Ghosh and Setlow, 2009). SD spores are grouped into different categories according to their germination stimuli, e.g., nutrient-SD spores, Ca^{2+}-dipicolinic acid SD (Ca^{2+}DPA-SD) spores, dodecylamine-SD spores, and high pressure superdormant (HPSD) spores. Buoyant density centrifugation method was used to isolate nutrient-SD spores. The main principle of this method is that dormant spores have higher wet densities thus would pellet during centrifugation in a density gradient medium such as Nycodenz®. The germinated spores, which have significantly lower densities, would float (Ghosh and Setlow, 2009). This method was shown to effectively isolate Ca^{2+}DPA-SD and dodecylamine-SD spores as well (Ghosh and Setlow, 2010; Perez-Valdespino et al., 2013). Additionally, new tools have been developed to characterize SD spores. These include Raman spectroscopy, differential interference contrast and phase-contrast microscopy (Zhang et al., 2010; Kong et al., 2011), and tracking of single cell germination/outgrowth using microtiter plates containing one spore per well (Webb et al., 2007; Wells-Bennik et al., 2016). Details, additional methods and tools to study spore heterogeneity were summarized by Setlow et al. (2012) and Wells-Bennik et al. (2016).

In addition to the isolation and characterization of SD spores, the mechanisms underlying their germination deficiency have also been investigated (Ghosh and Setlow, 2010). So far, nutrient-SD spores have been studied extensively, while Ca^{2+}DPA-SD spores and dodecylamine-SD spores have been characterized to a

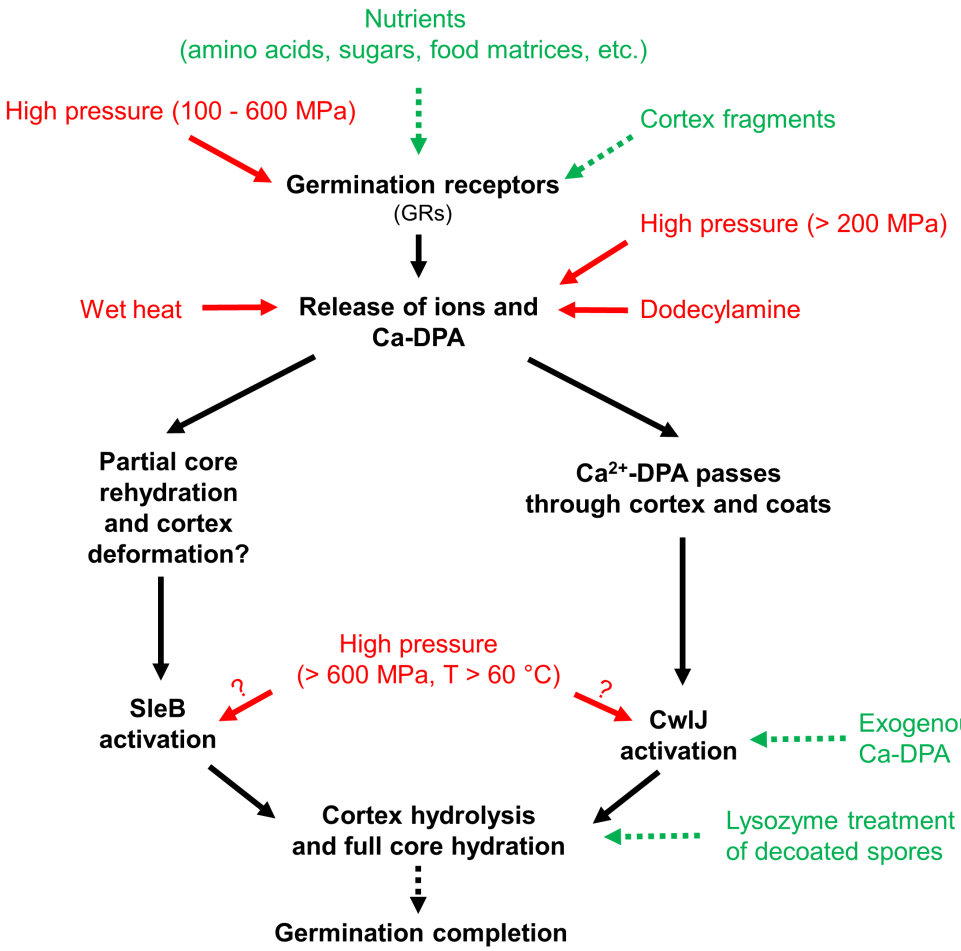

FIGURE 1 | Overview of germination stimuli and proposed germination pathways of *Bacillus subtilis* spores. Stimuli that lead to germination are shown as green with dashed arrows and stimuli that lead to germination and possible inactivation are shown as red with solid arrows. Graph modified from Reineke et al. (2013), with permission from Elsevier.

limited extent, but HPSD spores have not yet been isolated and studied for their properties. More information can be seen in **Table 1**.

Nutrient-SD Spores

The frequency of nutrient-SD spores among the total spore population generally varies between 1 and 12% with rich germinants (Ghosh and Setlow, 2009, 2010; Ghosh et al., 2009, 2012). Better germination conditions and heat activation result in a lower abundance of nutrient-SD spores (Ghosh and Setlow, 2009, 2010). However, the influence of heat activation was less significant when the spores were germinated in a nutrient-rich environment or with a mixture of nutrients that trigger multiple germination receptors (GRs) (Ghosh et al., 2009; Ghosh and Setlow, 2010). Generally, factors that influence the germination capacity of bacterial spores also affect the SD spore amount. These factors include stimulus type and intensity, heat activation, water activity, and so on (Setlow, 2003, 2014; Zhang et al., 2010; Abee et al., 2011; Lovdal et al., 2011; Christie, 2012).

The germination capacities of isolated nutrient-SD spores to different nutrient stimuli were intensively investigated (Ghosh and Setlow, 2009, 2010; Ghosh et al., 2009, 2012; Zhang et al., 2012). It was found that nutrient-SD spores require a heat activation temperature around 8–15°C higher than the initial dormant population (Ghosh et al., 2009). Nutrient-SD spores germinated poorly with the germinants that were originally used in their isolation and are more sensitive to a decrease in germinant concentration (Ghosh and Setlow, 2009; Ghosh et al., 2009; Zhang et al., 2012). A high-concentration mixture of nutrients increased the germination of nutrient-SD spores, but their germination efficiency and speed were still not as good as the initial dormant spores (Ghosh and Setlow, 2009; Ghosh et al., 2009). The germination of nutrient-SD spores with nutrients targeted to other GRs shows various behaviors. Some research has shown that they still germinate poorly (Ghosh and Setlow, 2009, 2010; Ghosh et al., 2009; Wei et al., 2010). Another research has indicated their germination was better as compared with the nutrients that were used to isolate them, but still worse than the initial dormant spores (Ghosh et al., 2012). Other

TABLE 1 | Percentage of superdormant (SD) spores in various isolation conditions and proposed superdormancy mechanisms.

SD spore type	Germination stimulus	Species	ca. % SD spores	Proposed superdormancy mechanisms	Reference
Nutrient-SD	Valine (10 mM)	*B. subtilis*	1.1	Permanent cause: lower GR levels Transient cause: activation status (Ghosh et al., 2009, 2012; Wei et al., 2010; Zhang et al., 2012)	Chen et al., 2014
	Valine (10 mM)	*B. subtilis*	4		Zhang et al., 2012[1]
	Valine (10 mM)	*B. subtilis*	3.8		Ghosh and Setlow, 2009
	Valine (300 μM)	*B. subtilis*	58		Ghosh and Setlow, 2009
	10 × LB medium[2]	*B. subtilis*	0.7		Ghosh and Setlow, 2009
	AGFK[3]	*B. subtilis*	12		Ghosh and Setlow, 2009
	AGFK[4]	*B. subtilis*	6		Zhang et al., 2012[1]
	Glucose (10 mM)	*B. megaterium*	3.5		Ghosh and Setlow, 2009
	Glucose (200 μM)	*B. megaterium*	38		Ghosh and Setlow, 2009
	10 × LB medium	*B. megaterium*	0.5		Ghosh and Setlow, 2009
	Alanine (50 mM)	*B. cereus*	5.3		Ghosh and Setlow, 2010
	Inosine (5 mM)	*B. cereus*	2.3		Ghosh and Setlow, 2010
	Inosine (250 μM)	*B. cereus*	12		Ghosh and Setlow, 2010
	Inosine (5 mM, no heat activation)	*B. cereus*	12		Ghosh and Setlow, 2010
Ca^{2+}DPA-SD	Ca^{2+}-DPA (60 mM)	*B. subtilis*	0.9 (0.5–1.6)	Coat defect, low levels of CLE CwlJ	Perez-Valdespino et al., 2013
Dodecylamine-SD	Dodecylamine (1.2 mM)	*B. subtilis*	0.4 (0.1–1.1)	Not clear	Perez-Valdespino et al., 2013
High pressure SD	No reported isolation			Different to nutrient superdormancy	Wei et al., 2010

Unless stated otherwise, heat activation was applied prior to nutrient germination for different species: B. subtilis: 75°C, 30 min; B. megaterium: 60°C, 15 min; B. cereus: 65°C, 20 min. [1]Spores were heat activated at 70°C for 30 min. SD spore percentages were calculated based on observation of >440 spore for listed cases; [2]LB medium: Luria-Bertani medium; [3]AGFK (12 mM L-asparagine, 13 mM D-glucose, 13 mM D-fructose, 12.5 mM KPO_4 buffer [pH 7.4]); [4]AGFK (10 mM L-asparagine, 10 mM D-glucose, 10 mM D-fructose, 10 mM KCl in 25 mM KPO_4 buffer [pH 7.4]).

authors have stated that nutrient-SD spores exposed to nutrients targeted to other GRs germinated almost as well as the initial dormant population (Zhang et al., 2012), or even more rapidly (Chen et al., 2014). The cause for the differences is unclear. Possibly due to slight differences in sporulation, germination, and isolation conditions, which could lead to differences on SD spore properties.

Although nutrient-SD spores germinate poorly with nutrient germinants, they germinate normally with Ca^{2+}-DPA and dodecylamine (Ghosh and Setlow, 2009, 2010; Zhang et al., 2012). They were also reported to germinate similarly to the initial dormant population with bryostatin and purified peptidoglycan fragments (Wei et al., 2010). Moreover, it seems they can germinate as well as the initial spore population under HP treatment at both 150 MPa (37°C) and 500 MPa (50°C) (Wei et al., 2010). This is surprising, as it is generally considered that *Bacillus* spores germinate at 150 MPa via nutrient germination pathways. The discussed experimental results indicate that the cause of nutrient superdormancy is not the same as HP superdormancy. On the other hand, the isolation steps could have influenced the properties of SD spores. As reported by Chen et al. (2014) previously, some differences in protein levels between dormant and SD spores are similar to the protein changes during germination. This suggest that although SD spores were not committed to germination, small changes already took place in a non-committal way during the exposure to the nutrient germinants. These changes might be the cause that they could germinate normally under HP treatment, but not with the nutrient(s) that was used to isolate them.

Concerning the germination speed, nutrient-SD spores had a much longer individual lag time (T_{lag}, which is the mean time between the spores coming into contact with nutrient germinants and the start of Ca^{2+}-DPA release) (Zhang et al., 2012). Factors that influence the T_{lag} correlate with the factors that influence the SD spore level (Zhang et al., 2010), indicating that the T_{lag} represents the main cause of differences in germination speed between SD spores and the dormant spore population.

Ca^{2+}DPA-SD and Dodecylamine-SD Spores

Most SD spore studies have focused on nutrient SD spores, and only a limited amount of research has targeted populations that are reluctant to germination under the triggers of Ca^{2+}-DPA and dodecylamine. The amounts of the Ca^{2+}DPA-SD spores and dodecylamine-SD spores of *B. subtilis* are much lower than nutrient-SD spores. The amount of Ca^{2+}DPA-SD spores is around 0.9% and dodecylamine-SD spores is around 0.4%, whereas that of nutrient SD spores is around 1–12% (Ghosh and Setlow, 2009; Perez-Valdespino et al., 2013). Research has revealed Ca^{2+}DPA-SD spores germinate well with nutrient germinants and dodecylamine but poorly with Ca^{2+}-DPA, while dodecylamine-SD spores germinate as well as the initial dormant population with nutrients and dodecylamine but germinate more slowly with Ca^{2+}-DPA.

High Pressure Superdormant Spores

High pressure processing inactivates bacterial spores by triggering relevant germination mechanisms. Notably, different HP treatments could induce the germination process, but the germination might be blocked in the intermediate phases and cannot be completed. However, as long as the relevant resistances of the spores are lost or significantly reduced, the following

inactivation step could still inactivate them. Current state of art HP treatments alone cannot induce 100% germination (Mathys, 2008; Knorr et al., 2010; Reineke, 2012; Georget et al., 2014c,d; Dong et al., 2015; Georget, 2015; Sevenich and Mathys, 2018). The percentage of SD spores that remain dormant after HP treatment highly depends on the treatment conditions, including pH, water activity, pressure level, temperature, and dwell time (Mills et al., 1998; Wuytack et al., 1998; Considine et al., 2008; Reineke et al., 2013; Georget et al., 2014b; Bolumar et al., 2015; Rao et al., 2018). For example, decrease in water activities largely inhibits the germination of *B. cereus* spores by HP treatment (Al-Holy et al., 2007; Rao et al., 2018). The remaining SD spores are resistant and can survive HP treatment, thus limiting the application of HP processing as a milder non-thermal spore inactivation strategy. However, to our knowledge, there have been no reports of isolation and characterization of HPSD spores so far.

Superdormant Spores of *Clostridium* Species

Spores of *Clostridium* species exhibit a similar germination heterogeneity like *Bacillus* species and *Clostridium* SD spores also occur (Webb et al., 2007; Rodriguez-Palacios and LeJeune, 2011; Stringer et al., 2011; Wang et al., 2011, 2012; Nerandzic and Donskey, 2013; Deng et al., 2015, 2017; Olguin-Araneda et al., 2015; Doona et al., 2016a). Similar to *Bacillus* species, the amount of *Clostridium* SD spores also depends on the factors that influence the germination efficiency. These influencing factors include heat activation, stimulus type and intensity (Wheeldon et al., 2008; Wang et al., 2011; Doona et al., 2016a). However, although the germination of *Clostridium* species and *Bacillus* species share some similarities, they also have a number of differences (Paredes-Sabja et al., 2011; Xiao et al., 2011; Christie, 2012; Brunt et al., 2014; Setlow, 2014; Setlow et al., 2017). For example, heat activation generally decreases the amount of nutrient-SD spores in *Bacillus*. In comparison, the effect of heat activation is more complex for *Clostridium* species (Ghosh and Setlow, 2009, 2010; Luu et al., 2015). The effect seems to be dependent on the germination/plating media (Montville, 1981), and on species, e.g., heat activation could stimulate the germination of *Clostridium perfringens* but not of several *Clostridium difficile* strains (Wang et al., 2011, 2015; Dembek et al., 2013; Doona et al., 2016a). The differences in germination between *Clostridium* and *Bacillus* species might indicate that their mechanisms of spore superdormancy are different. However, there has been much less work focusing on SD spores in *Clostridium* species than *Bacillus* species and there has been no report of the isolation of *Clostridium* SD spores.

POTENTIAL MECHANISMS OF SPORE SUPERDORMANCY

Superdormancy has been suggested to be an extreme form of germination heterogeneity and a strategy to ensure the survival of the entire population in a fast-changing environment (Veening et al., 2008; Ghosh and Setlow, 2010; Ghosh et al., 2012; Dembek et al., 2013). Obtaining a better understanding of spore superdormancy and its underlying mechanisms is crucial for the development of spore control strategies that are based on the germination-inactivation principle. Therefore, several research groups are currently investigating the genotypic and phenotypic differences between SD spores and their dormant counterparts. Currently, the exact causes of spore superdormancy are unclear and there is no consistent conclusion on whether the superdormancy of isolated SD spores is stable (Keynan et al., 1964; Ghosh and Setlow, 2010; Zhang et al., 2012). Previous research reported that nutrient-SD spores stored at −20°C for several months or even years could germinate similarly well compared to freshly isolated ones (Zhang et al., 2012), indicating the superdormancy could be permanent or at least stable for long time. However, another study reported that the isolated nutrient-SD spores stored at 4°C slowly lost their superdormancy. Even when they were stored at −20°C or −80°C, their germination ability still increased, but the rate of increase was significantly slower. Notably, although the germination capacity of nutrient-SD spores increased during cold storage, it did not reach the level of the initial dormant spore population (Ghosh and Setlow, 2010). Nevertheless, this indicates that the superdormancy of isolated SD spores is not permanent and it decreases over time.

Based on their findings, Ghosh and Setlow (2010) proposed that there are probably at least two causative factors for spore nutrient superdormancy, one permanent and one transient (Ghosh and Setlow, 2010). For the transient cause, Ghosh and Setlow (2010) suggested it might be related to the activation status of the spores, since heat activation, which is reversible, influences the frequency of nutrient-SD spores (Ghosh and Setlow, 2010). For the permanent cause, research has revealed that it is not because of genetic changes, since re-sporulated nutrient-SD spores showed the same germination capacity as the initial dormant population (Ghosh and Setlow, 2009; Chen et al., 2014). It was suggested that the phenotypic heterogeneity in germination may correspond to the presence of lower GR levels in the nutrient-SD spores (Ghosh and Setlow, 2009, 2010; Wei et al., 2010).

Lower GR levels as a cause for spore nutrient superdormancy has been proposed in many studies (Ghosh and Setlow, 2009, 2010; Ghosh et al., 2012). For example, in the study of Ghosh and Setlow (2009), the frequency of SD spores decreased dramatically when the level of GerB receptor increased. In their later research (Ghosh et al., 2012), they found that the level of GRs in SD spores was 6–10 fold lower than that in the initial dormant spores. Moreover, Chen et al. (2014) also found significant lower abundance of GerAC, GerKC, and GerD for *B. subtilis* nutrient-SD spores and proposed that a deficiency of GerD could be a reason for spore nutrient superdormancy. Lower GR levels as a causative factor of spore nutrient superdormancy is also supported by other evidence. First, the average amount of GRs per spore is low, thus, stochastic variation in the number could lead to the situation that a small proportion of spores have very few GRs and would probably germinate more slowly (Paidhungat and Setlow, 2001;

Cabrera-Martinez et al., 2003; Setlow et al., 2012). Second, heat activation, which improved GR-mediated germination, can decrease the frequency of nutrient-SD spores (Ghosh and Setlow, 2009). Third, nutrient-SD spores germinate normally with Ca^{2+}-DPA and dodecylamine, which both trigger spore germination through mechanisms that do not involve GRs (Paidhungat et al., 2001).

However, a lower number of GRs does not seem to explain the existence of other types of SD spores. For example, Ca^{2+}DPA-SD spores were reported to have higher levels of GRs compared to the initial spore population. Their superdormancy could be due to lower levels of CwlJ, which is one of the cortex-lytic enzymes, and coat deficiency (Perez-Valdespino et al., 2013). Moreover, previous research suggested that the cause of HP superdormancy is different from that of nutrient superdormancy, since nutrient-SD spores can germinate normally with HP treatment (Wei et al., 2010).

Furthermore, *Bacillus* nutrient-SD spores showed a lower spore core water content than their dormant counterparts (Ghosh et al., 2009). This finding is consistent with the observation that spores sporulated at a higher temperature, which leads to a lower water content of the spore core (Melly et al., 2002), germinated less well than spores sporulated at a lower temperature (Gounina-Allouane et al., 2008; Markland, 2011; Markland et al., 2013b). This might indicate that a lower spore core water content could also be a cause of spore nutrient superdormancy (Cowan et al., 2003; Sunde et al., 2009; Setlow et al., 2012). One of the factors that leads to a difference in spore core water content is the DPA content of the spore. Although the DPA content of nutrient-SD spores is identical to that of initial dormant spores (Ghosh and Setlow, 2009), the environment of the DPA was found to be different, since the Raman spectral peaks of spore DPA differed between dormant and SD spores (Ghosh et al., 2009).

FUTURE RESEARCH NEEDS

So far, several types of SD spores have been characterized and mechanisms have been proposed for their superdormancy. However, the state of knowledge about some types of SD spores is still rudimentary and the exact mechanisms are not fully clear. Therefore, further research is needed to better understand SD spores, which represent one of the biggest challenges to the application of germination-inactivation as a milder non-thermal spore control strategy.

First, attention should be paid to HPSD spores in future research. To our knowledge, there have been no reports of the isolation and characterization of HPSD spores so far. This is somewhat surprising because from the applied perspective, there are advantages to triggering germination by HP rather than by nutrient/chemical stimuli. For example, HP can be used to evenly treat the final packed products without raising a risk of recontamination, while nutrient/chemical germination triggers need to be added and distributed into the foods. Moreover, HP triggers germination more homogeneously, while added nutrients or chemicals might have an inhomogeneous distribution, especially in solid foods, leading to inconsistent germination within the products. Furthermore, HP treatments can simultaneously trigger germination and inactivate the germinated spores, while spores germinated under nutrient/chemical triggers require further inactivation steps (Gould and Sale, 1970; Knorr et al., 1998, 2010; Georget et al., 2014a,c; Sevenich and Mathys, 2018). Additionally, previous research has suggested that the cause of spore HP superdormancy is different from spore nutrient superdormancy (Wei et al., 2010). Therefore, it would be beneficial to isolate and characterize HPSD spores regarding the mechanisms of their superdormancy. Such research would strongly support the implementation of milder HP-based spore control strategies.

Second, more attention should be paid to SD spores of *Clostridium* species, which have been far less studied than the SD spores of *Bacillus* species (Rodriguez-Palacios and LeJeune, 2011; Wang et al., 2011; Crowther et al., 2014; Deng et al., 2017). Since germination behavior varies among bacterial genera, further research is needed to clarify the properties of *Clostridium* SD spores and the underlying mechanisms of their superdormancy (Rodriguez-Palacios and LeJeune, 2011; Xiao et al., 2011; Deng et al., 2015).

Third, improvement of enumeration and culturing methods would be beneficial. Classic plate count methods based on quantifying colony-forming units are widely used to assess the viability of microbes. However, the number of colony-forming units is a measure of the highest physiological fitness of microbes (Bunthof, 2002), which might not be the best indicator for SD spores, because the possibility that these spores would not germinate on culture plates might lead to a risk of underestimation their numbers (Wells-Bennik et al., 2016). Therefore, tools such as flow cytometry or phase-contrast microscopy should be used to facilitate the enumeration of SD spores in future research. On the other hand, the amounts of SD spores are largely dependent on the germination conditions. Therefore, efforts should be put on improving the culturing methods to increase the recovery/germination of the SD spores. This is important for the accuracy of antimicrobial susceptibility tests, sterilization controls, and challenge tests (Silvestri et al., 2015; Wells-Bennik et al., 2016; Pereira and Sant'Ana, 2018).

Fourth, in order to successfully apply a germination-inactivation technology as a gentle safety control, several other aspects need to be considered besides spore germination. For example, the timing to apply the inactivation step is crucial. On one hand, it should be applied after the majority of spores lost most of their resistance. Spores should have enough time to pass germination stage II or at least to lose most of the Ca^{2+}-DPA and reach a sufficient core hydration before a following inactivation step is considered (Moir et al., 1994; Setlow, 2003; Luu and Setlow, 2014). This time can vary, depending on spore species, germination stimuli and intensities. Notably, not all spores would finalize all their germination steps under a certain trigger (Wuytack et al., 1998; Reineke, 2012), but as long as a relevant

spore resistance is lost, they could be efficiently inactivated by a gentle inactivation step.

On the other hand, the germination-inactivation approach focuses on the elimination of bacterial spores to ensure the microbiological safety of the products, but the absence of spores does not guarantee the absence of toxins. Some pathogenic spore-forming bacteria can produce toxins, which could endanger consumers. Different situations need to be taken into account if the germination-inactivation approach is considered as a food safety control in this case. First, special focus needs to be put on spore species that can produce toxins during the growth phase after their germination. For example, *B. cereus* can produce diarrheagenic or emetic toxins during the exponential or the stationary phase of growth respectively (Roberts and Tompkin, 1996; Brown, 2000; Ceuppens et al., 2012), while *Clostridium botulinum* and *C. difficile* synthesize toxins in the late exponential growth phase and beginning of the stationary phase (Voth and Ballard, 2005; Proft, 2009). It is essential to consider the germination velocity rates and control the time intervals between the germination and inactivation steps to ensure food safety for these cases. Notably, in any case, an inactivation needs to be performed before germinated spores could sporulate again. The time needed to complete sporulation varies, and it takes approximately 8–10 h in *B. subtilis* (Robleto et al., 2012). Proper processing time windows need to be identified using predictive models and experimental validation tests to ensure that the inactivation step is performed in the specific time period where the majority of spores lost most of their resistances but did not start producing toxins or sporulation, yet.

Another situation is where toxins are already present in the product, either produced by vegetative cells in their late growth phases or during sporulation, e.g., *C. perfringens* produces heat sensitive enterotoxin during sporulation and releases the toxin when the mother cell lysis (Duncan et al., 1972; Uemura, 1978). In this case, the following inactivation step needs to be able to degrade the present toxins, e.g., for heat sensitive toxins a mild heat inactivation step could be applied. For heat stable toxins, e.g., *B. cereus* emetic toxin, a mild heat step after germination might remove the sensitized spores but not the toxins. In this case, other approaches to control the toxin levels are needed. Generally, it is important to control the quality of raw material inputs, ingredients and their storage conditions to prevent the toxin formation before germination-inactivation steps.

Finally, knowledge obtained from SD spore research could be used to develop milder spore control strategies. On one hand, germination-inactivation technologies by first triggering spore germination and followed by a gentle inactivation step to inactivate the sensitized spores could be further developed and improved. Spore germination could be maximized when we understand the mechanisms and the influencing factors for spore superdormancy. For example, germination percentages can be increased by combining various germination triggers or controlling the influencing factors. Important influencing factors include heat activation, germination stimulus type and intensity (Wei et al., 2010; Lovdal et al., 2011). Besides that, from the application point of view, it is important to understand the germination behavior of spores that are formed and present in the food products. This is especially relevant as the sporulation conditions, which influence the spore germination properties, are often unknown and not controlled in this case (Wells-Bennik et al., 2016). Moreover, spores germination behaviors might be completely different when spores are germinated in food matrices compared to buffer systems. For example, the germination of *Bacillus* spores by nutrient and HP were inhibited when they are present in foods with low water activity (Al-Holy et al., 2007; Rao et al., 2018). Therefore, future research is needed to investigate the mechanisms of how different factors influence spore germination. On the other hand, since only germinated spores proliferate and cause problems, hurdles can be put in place to inhibit the germination/outgrowth of the remaining SD spores. Examples of these hurdles can be pH, temperature, or bacteriocins such as nisin (Markland et al., 2013a; Nerandzic and Donskey, 2013; Patrignani and Lanciotti, 2016; Wells-Bennik et al., 2016).

CONCLUSION

Research on SD spores will help reveal factors that contribute to their superdormancy and allow for the identification of the underlying mechanisms that lead to their extremely low germination capacity as compared to the whole population. It will also contribute to improved predictive models that take germination heterogeneity into account, which can provide a mechanistic understanding of spore germination processes. Additionally, it will provide a foundation for developing milder non-thermal spore control strategies based on the germination-inactivation principle. This could help to ensure microbial safety and quality retention of food products, contributing significantly to providing fresher and more nutritional foods for consumers. Moreover, aside from the food sector, the medical, pharmaceutical, and (bio)chemical sectors, where spore eradication is needed, will also benefit from research on SD spores, especially for the sterilization of heat-sensitive products.

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YZ and AM contributed to the manuscript at all stages.

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Escherichia coli Cells Exposed to Lethal Doses of Electron Beam Irradiation Retain Their Ability to Propagate Bacteriophages and Are Metabolically Active

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Reports in the literature suggest that bacteria exposed to lethal doses of ionizing radiation, i.e., electron beams, are unable to replicate yet they remain metabolically active. To investigate this phenomenon further, we electron beam irradiated *Escherichia coli* cells to a lethal dose and measured their membrane integrity, metabolic activity, ATP levels and overall cellular functionality via bacteriophage infection. We also visualized the DNA double-strand breaks in the cells. We used non-irradiated (live) and heat-killed cells as positive and negative controls, respectively. Our results show that the membrane integrity of *E. coli* cells is maintained and that the cells remain metabolically active up to 9 days post-irradiation when stored at 4°C. The ATP levels in lethally irradiated cells are similar to non-irradiated control cells. We also visualized extensive DNA damage within the cells and confirmed their cellular functionality based on their ability to propagate bacteriophages for up to 9 days post-irradiation. Overall, our findings indicate that lethally irradiated *E. coli* cells resemble live non-irradiated cells more closely than heat-killed (dead) cells.

Keywords: electron beam, ionizing radiation, bacteria, DNA damage, bacteriophages

INTRODUCTION

Ionizing radiation and its three main sources, electron beam (eBeam), X-rays, and γ (gamma) rays, are cornerstone technologies of the medical device sterilization and food pasteurization industries (Dorpema, 1990; Follett, 2002; Farkas and Mohacsi-Farkas, 2011; Pillai and McElhany, 2011; Pillai and Shayanfar, 2015). The underlying premise is that at appropriate lethal doses of ionizing radiation, the microbial cells are inactivated, in other words, they are unable to multiply. There are a number of reports detailing the response of viruses, bacteria, and protozoa to ionizing radiation (Purdie et al., 1974; Weiss et al., 1974; Daly and Minton, 1995b; Miyahara and Miyahara, 2002; Kimura et al., 2006; Praveen et al., 2013). There are also a number of studies detailing the possible resistance mechanisms of bacterial cells to ionizing radiation (Daly and Minton, 1995a,b, 1996, 1997; Daly et al., 2007; Holloman et al., 2007; Makarova et al., 2007). Studies have also suggested

that ionizing radiation causes structural damage to the DNA in the cells (Krasin and Hutchinson, 1977; Hutchinson, 1985; Daly and Minton, 1995a). However, reports in the literature as well as previous studies in our laboratory suggest that irradiated bacterial cells retain residual metabolic and transcriptional activity. For example, Magnani et al. (2009) demonstrated that lethally gamma irradiated *Brucella melitensis* cells had lost their ability to replicate but still possessed metabolic and transcriptional activity. The cells also persisted in macrophages, generated antigen-specific cytotoxic T cells, and protected mice against virulent bacterial challenge (Magnani et al., 2009). Secanella-Fandos et al. (2014) observed that lethally gamma irradiated *Mycobacterium bovis* cells were metabolically active and exhibited similar tumor growth inhibition and induction of cytokines compared to live cells. In our laboratory, we also observed that when *Salmonella* spp. cells were exposed to lethal doses of eBeam irradiation, the cells were no longer able to multiply. However, the cells had intact membranes and retained their surface antigens (unpublished data). The findings that lethally irradiated cells have DNA double strand breaks, yet are metabolically active and have intact membranes, but are unable to multiply present a scientific conundrum.

The overall objective of this study was to characterize the response of *Escherichia coli* cells (K-12 wild-type strain MG 1655) to a lethal dose of eBeam radiation. Specifically, we investigated the structural damage to the cells' DNA, their membrane integrity, their metabolic activity (electron transport activity and ATP levels) and whether irradiated cells could serve as hosts for bacteriophages λ, T4, and T7. These bacteriophages require the host cell's machinery to varying degrees to produce progeny phage particles. Phage λ relies completely on the host cell to reproduce, T4 requires specific cellular components of the host cell, and T7 requires the host's machinery only at the very beginning of infection (Hendrix and Casjens, 2006; Little, 2006; Molineux, 2006; Mosig and Eiserling, 2006). Non-irradiated, live cells, and heat-killed cells were used as positive and negative controls, respectively. The underlying hypothesis was that eBeam irradiated *E. coli* cells retain enough of their cellular structure and function to serve as host cells for bacteriophage propagation, thereby confirming the metabolic activity and viability of lethally eBeam irradiated bacterial cells.

MATERIALS AND METHODS

Preparation and eBeam Irradiation of Bacterial Cultures

Overnight cultures of the *E. coli* K-12 wild-type strain MG 1655 were grown in Luria-Bertani (LB) broth at 35°C in a shaking water bath. The day of the irradiation, log-phase cultures of *E. coli* were prepared by seeding LB broth with the fresh overnight culture at a ratio of 1:100. The culture was allowed to grow at 35°C to an OD_{600} of ca. 0.5 resulting in approximately 1×10^8 colony forming units (CFU)/ml. The log-phase culture was subsequently chilled on ice for 10 min to arrest cell growth. Aliquots of the log-phase culture in LB broth were packaged for eBeam irradiation. In order to comply with the biosafety regulations of Texas A&M University, aliquots of the cell suspensions were placed in heat-sealed double-bagged Whirl Pak bags (Nasco, New York, NY, United States). These heat-sealed bags were then placed inside 95 kPa specimen transport bags (Therapak, Buford, GA, United States).

Previous studies in our laboratory have shown that irradiating cell suspensions in flat plastic bags produced a dose uniformity ratio (DUR) close to 1.0. A DUR of 1.0 indicates complete dose uniformity throughout the sample. Samples were held at 4°C for less than 2 h prior to irradiation and transported on ice in a Saf-T-Pak transport box (Saf-T-Pak, Hanover, MD, United States). Non-irradiated aliquots of the log-phase culture in LB broth were used as a positive control. The positive control samples were packaged the same way as the experimental samples and were transported to the irradiation facility to eliminate possible differences in survival due to transport and handling. Heat-killed cells (70°C for 60 min) were used as a negative control. The eBeam irradiations were carried out at the National Center for Electron Beam Research (NCEBR) at Texas A&M University in College Station, TX, United States using a 10 MeV, 15 kW eBeam linear accelerator. All eBeam irradiations were carried out at ambient temperature (ca. 25°C). Based on a prior dose-response experiment, it was determined that a dose of 7.0 kilo Gray (kGy) was needed to render 1×10^8/ml *E. coli* cells in LB broth replication incompetent (*data not shown*). Thus, samples were irradiated to a lethal target dose of 7.0 kGy by conveying the samples across the incident eBeam. To confirm that the cells had lost their replication capabilities, cells were plated on LB plates and incubated at 37°C for 4 days.

Membrane Integrity of eBeam Irradiated *E. coli* Cells

We used the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes®, Grand Island, NY, United States), a two-color fluorescent dye system, to characterize the membrane integrity of eBeam irradiated cells. The SYTO® 9 green-fluorescent nucleic acid stain can penetrate cells with either intact or damaged membranes. On the other hand, the red-fluorescent nucleic acid stain, propidium iodide, penetrates only cells with damaged membranes. When used in combination, this dye system stains cells with intact membranes green and cells with damaged membranes red.

Following eBeam irradiation and heat treatment, the *E. coli* samples were stored at 4°C in the LB broth they had been treated in and the membrane integrity was examined at the following time points: 0, 4, 24, and 216 h (9 days). The LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit was used according to the manufacturer's instructions with minor modifications. Briefly, 0.5 ml of the sample were centrifuged for 1 min at RT at maximum speed in a microcentrifuge. The cell pellet was resuspended in 0.5 ml 0.85% sodium chloride (NaCl) solution. 1.5 μl of the dye mixture (equal volume SYTO® 9 and propidium iodide) were added protected from light. The sample was vortexed and incubated for 15 min at RT in the dark. Slides with 10 μl of sample were prepared for fluorescent

microscopy. Images were taken immediately with an Olympus BX50 fluorescent microscope with a FITC/Texas Red filter and a 200× magnification.

Visualization of DNA Double-Strand Breaks in eBeam Irradiated *E. coli* Cells

We used the neutral comet assay, adapted for bacteria, to visualize DSBs under a fluorescent microscope. This assay, also known as single-cell gel electrophoresis, offers direct visualization of DSBs through the appearance of DNA tails or comets. Cells of interest are immobilized in low melting agarose, lysed, and electrophoresed. This allows the DNA to migrate out of the cell in a pattern determined by the extent of DNA damage (Ostling and Johanson, 1984; Lemay and Wood, 1999).

Following eBeam irradiation, the *E. coli* samples were transported to the laboratory on ice and stored at 4°C for 1–2 h until the comet assay could be performed. The neutral comet assay was performed using the Trevigen CometAssay® protocol (Reagent Kit for CometAssay®, Catalog # 4250-050-K) with modifications. Briefly, a 50 μl aliquot (1×10^7 cells/ml) of the appropriate bacterial cell suspension (eBeam irradiated, non-irradiated positive control, and heat-killed negative control) was mixed with lysozyme (final conc. 0.5 mg/ml) and RNase A (final conc. 5 μg.ml) prior to adding 500 μl of molten Comet LMAgarose (0.5% low-melting agarose) (Trevigen Inc., Gaithersburg, MD, United States) kept at 37°C. After mixing the sample, a 50 μl aliquot was pipetted onto the CometSlide (Catalog # 4250-050-03, Trevigen Inc., Gaithersburg, MD, United States), resulting in approximately 50,000 cells per sample area. The slides were incubated at 4°C for 10 min in the dark. Following gelling of the agarose disk, the slides were placed in plastic Coplin Jars containing lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1% sodium lauroyl sarcosinate, 1% Triton X-100 (added fresh)] and incubated for 1 h at RT. Following cell lysis, slides were placed in an enzyme digestion buffer [2.5 M NaCl, 10 mM EDTA, 10 mM Tris pH 7.4, 1 mg/ml Proteinase K] for 2 h at 37°C. After draining the excess buffer, slides were immersed in pre-chilled 1× electrophoresis buffer [100 mM Tris pH = 9, 300 mM sodium acetate] and incubated for at least 30 min at 4°C; slides may also be stored overnight at this point. Slides were placed in a horizontal electrophoresis unit (Owl; Model B-2) containing fresh 1× electrophoresis buffer and electrophoresed at 1 V/cm for 1 h at RT. The slides were then placed in 1 M ammonium acetate in ethanol for 30 min at RT. DNA precipitation was followed by ethanol dehydration of the agarose. Slides were immersed in absolute ethanol for 1 h at RT and air-dried, followed by 70% ethanol for 15 min at RT and then air-dried. Slides were then stained with 50 μl of freshly prepared SYTO 9 solution (1.25 μM in 0.04% DMSO) for 15 min in the dark. The excess SYTO 9 stain was removed by gently tapping the slide on a KimWipe. Slides were then air-dried for 30 min in the dark, followed by 5 min at 40°C in the dark. Observations were made using an Olympus BX50 fluorescent microscope with a FITC filter and a 1000× magnification. CFU counts were obtained by plating the *E. coli* samples on LB agar and incubating them at 37°C for 4 days.

Metabolic Activity in eBeam Inactivated *E. coli* Cells

To investigate the metabolic activity in eBeam inactivated cells over time, we chose an assay that uses cellular reducing conditions to monitor metabolic activity/cell health. Resazurin, the active ingredient, is a non-fluorescent compound. Upon entering the cell, it is converted to resorufin, a highly fluorescent compound, via the cell's reducing environment. Alive and healthy cells have more reducing power than injured/dead cells and will produce a higher fluorescent signal (Squatrito et al., 1995; Nakayama et al., 1997; Rampersad, 2012).

Following eBeam irradiation and heat treatment, the *E. coli* samples were stored at 4°C in the LB broth they had been treated in and the metabolic activity was examined at the following time points: 0, 4, 24, and 216 h (9 days). Metabolic activity was measured with the redox indicator alamarBlue® (Invitrogen, Grand Island, NY, United States) according to the manufacturer's instructions. Briefly, 10 μl of the alamarBlue® reagent were added to 100 μl of cells (in a black 96-well plate), mixed, and incubated in the dark at 37°C for 1 h. Following the 1-h incubation, the fluorescence was measured with a Perkin Elmer Wallac 1420 VICTOR2™ microplate reader. Two independent experiments were performed.

ATP Levels in eBeam Inactivated *E. coli* Cells

Since ATP, an indicator of metabolically active cells, can be detected via a bioluminescence assay (Squatrito et al., 1995), we determined the cellular ATP levels with the BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Madison, WI, United States) according to the manufacturer's instructions with minor modifications. Following eBeam and heat treatment, the *E. coli* samples were stored at 4°C in the LB broth they had been treated in and the ATP levels were examined at the following time points: 0, 4, 24, and 216 h (9 days) by adding 10 μl of the BacTiter-Glo reagent to 10 μl of cells (in a white 384-well plate). Samples were mixed and incubated for 5 min at RT. Following the incubation, the luminescence was measured with a Perkin Elmer Wallac 1420 VICTOR2™ microplate reader. The cellular ATP concentrations were interpolated from a standard curve.

Bacteriophage Multiplication in eBeam Inactivated *E. coli* Cells

To study the overall cellular functionality of eBeam inactivated cells we tested their ability to propagate bacteriophages. Following eBeam and heat treatment, the *E. coli* samples were kept at 4°C in the LB broth they had been treated in and the overall cellular functionality was determined at the following time points: 0, 4, 24, and 216 h (9 days). One milliliter of each sample was centrifuged for 1 min at RT in a microcentrifuge at maximum speed. The cell pellet was resuspended in 50 μl amended LB broth (5 mM $CaCl_2$ and 5 mM $MgSO_4$) and 50 μl of the bacteriophage (lambda vir 101, T4D or T7), also in amended LB broth, were added at a multiplicity of infection (MOI) of 0.01 (10^8 CFU/ml to 10^6 PFU/ml). The mixture was vortexed and incubated in a 37°C shaking water bath for 24 h. Following

the incubation, samples were placed on ice, diluted in amended LB broth and spot plated on LB agar using the top agar overlay method (Adams, 1959). Ten microliters from each dilution (−0 to −8) were spotted to determine the dilutions that would yield countable numbers. LB plates were incubated at 37°C for 16–18 h. Following spot plating, the samples were stored at 4°C overnight and full plate titrations, also using the top agar overlay method, of the appropriate dilutions were performed the next day. LB plates were incubated at 37°C for 16–18 h and then counted for plaque forming units (PFUs). The ability of the *E. coli* cells to replicate (or not) was confirmed by plating survivors on LB agar plates and incubating them at 37°C for 4 days. Two independent experiments were performed.

Statistical Analysis

Statistical significance (P-value <0.05) was determined through pairwise Student's t-tests using the JMP statistical software (version 11).

RESULTS

Membrane Integrity of eBeam Inactivated *E. coli* Cells

The results indicated that, as expected, the live (non-irradiated) *E. coli* cells had intact membranes at all the time points (**Figure 1**). At both the 24 h and day 9 time points, the live control showed a few cells with damaged membranes. In contrast, the heat-killed cells had only damaged membranes for all the time points (**Figure 1**). Overall, the eBeam inactivated cells had intact membranes similar to the live cells (**Figure 1**). At 0 and 4 h post-irradiation, the eBeam inactivated cultures showed a few cells with damaged membranes. As the incubation continued, the number of cells with damaged membranes increased. At day 9 of incubation in LB broth at 4°C, approximately half of the eBeam inactivated cells showed signs of membrane damage (based on qualitative analysis) (**Figure 1**). These microscopic images are presented without enlargement to highlight the finding that the majority of cells in the field of view are viable (green) and as the incubation proceeds to day 9, the number of cells with compromised membranes (red) increase. Though we unfortunately did not perform quantitative image analysis to quantify the % red and green cells, these microscopic images highlight the intactness of the cellular membrane after eBeam irradiation as compared to the membrane damage that occurs during heating.

Visualization of DNA Double-Strand Breaks in eBeam Inactivated *E. coli* Cells

The neutral comet assay was performed to visualize the DNA double-strand breaks (DSBs) in *E. coli* cells exposed to a lethal eBeam irradiation dose (7 kGy), a lethal heat treatment (70°C for 60 min) or no treatment (live control). The measured eBeam dose for *E. coli* cells irradiated in LB was 7.04 kGy. The live cells showed only a few DSBs as seen by a few long DNA tails whereas eBeam inactivated cells showed extensive DSBs as seen by no distinct DNA tails. The extent of DNA damage in heat-killed cells was not as severe as for eBeam inactivated cells, as indicated by the DNA tails protruding from some cells. Nonetheless, the DNA damage in heat-killed cells was more pronounced than in the live cells, since not every cell had distinct DNA tails (**Figure 2**).

Metabolic Activity in eBeam Inactivated *E. coli* Cells

Live *E. coli* cells maintained a high level of metabolic activity over the entire 9 day incubation period (LB broth at 4°C), whereas heat-killed cells exhibited no metabolic activity (**Figure 3A**). In fact, the heat-killed cells were significantly different ($p < 0.0001$) from eBeam inactivated and live cells (**Figure 3A**). Metabolic activity in eBeam inactivated *E. coli* cells was maintained at levels comparable to the live cells over a period of 24 h post-irradiation. By day 9, the metabolic activity in the eBeam inactivated cells had significantly ($p < 0.0001$) decreased compared to the live cells (**Figure 3A**).

ATP Levels in eBeam Inactivated *E. coli* Cells

The ATP levels for the live *E. coli* cells increased over the 9 day incubation period (0 h: 1.06 μM; 4 h: 0.94 μM; 24 h: 1.19 μM; day 9: 1.73 μM) (**Figure 3B**). In contrast, heat-killed cultures maintained constant ATP levels throughout the entire 9 day incubation period (0 h: 0.67 μM; 4 h: 0.63 μM; 24 h: 0.63 μM; day 9: 0.66 μM) (**Figure 3B**). ATP levels for eBeam inactivated *E. coli* cells were much more variable compared to heat-killed and live cells (0 h: 1.4 μM; 4 h: 0.92 μM; 24 h: 1.56 μM; day 9: 0.38 μM) (**Figure 3B**). At 0 h, the eBeam inactivated cells had the highest ATP levels compared to live and heat-killed cells. In addition, the ATP levels were significantly different ($p < 0.0062$) from the heat-killed cells. At 4 h, all three groups had very similar ATP levels. At 24 h, eBeam inactivated cells had the highest ATP levels and heat-killed cells the lowest. The ATP levels in the eBeam inactivated cells were significantly different ($p < 0.0011$) from the heat-killed cells. After 9 days of incubation at 4°C, eBeam inactivated cells had the lowest levels of ATP and the live cells the highest and eBeam inactivated and heat-killed cells had ATP levels that were significantly different ($p < 0.0001$ and $p < 0.0003$, respectively) from the live cells (**Figure 3B**).

Bacteriophage Multiplication in eBeam Inactivated *E. coli* Cells

Phage λ was able to reproduce in healthy *E. coli* host cells (PC) as indicated by the significant difference ($p < 0.0001$) to the no host cell negative control (NC) at every time point. The average log PFU increase was 3.18 ± 0.02 across all the time points (**Figure 4**). Phage λ was able to propagate in eBeam inactivated (EB) host cells that were incubated for 24 h post-irradiation (in LB broth at 4°C) (**Figure 4**). At this time point, a statistically significant difference ($p < 0.05$) based on a log PFU increase of 0.61 was observed between EB and NC. At the other 3 time points (0 h, 4 h, and 9 days), there was no statistically significant difference between the PFU counts for phage λ incubated with EB cells and no host cells (NC). However, a slight increase in log PFU numbers

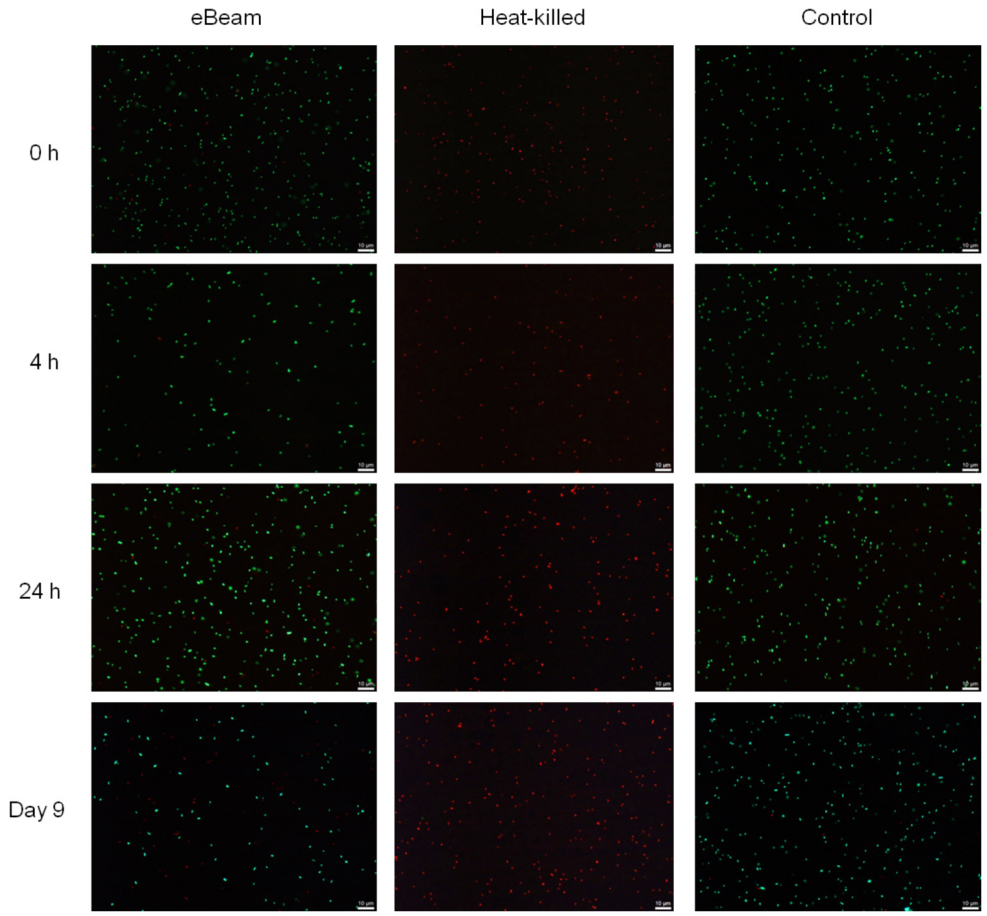

FIGURE 1 | Representative images depicting membrane integrity in eBeam inactivated, heat-killed, and live *Escherichia coli* cells. Cultures were incubated at 4°C in LB broth post-treatment and images were taken at 0, 4, 24 h, and 9 days.

(ca. 0.3) was observed at these three time points (**Figure 4**). Phage λ was not able to reproduce in heat-killed (HK) host cells. In fact, a 0.3 log reduction in PFU counts was observed at all four time points (**Figure 4**). A significant difference ($p < 0.05$) was observed between eBeam inactivated and heat-killed host cells at every time point (**Figure 4**).

Phage T4D was able to reproduce in healthy *E. coli* host cells (PC) as indicated by the significant difference ($p < 0.001$) to the no host cell control (NC) at every time point. The average log PFU increase was 2.04 ± 0.15 across all the time points (**Figure 5**). Phage T4D numbers in eBeam inactivated host cells (EB) remained at the same levels as the NC for all the time points, indicating that T4D was unable to propagate in EB cells (**Figure 5**). Heat-killed host cells (HK) turned out to be a net sink for T4D phages, reducing its numbers by 2.88 logs on average, as indicated by the significant difference ($p < 0.0001$) to the no host cell (NC) control (**Figure 5**).

Phage T7 was able to reproduce in healthy *E. coli* host cells (PC) as indicated by the significant difference ($p < 0.0001$) to the no host cell control (NC) at every time point. The average log PFU increase was 3.57 ± 0.15 across all the time points (**Figure 6**). Phage T7 was able to produce progeny particles in eBeam inactivated host cells (EB) at every time point (0, 4, 24 h, and 9 days post-irradiation) (**Figure 6**). Phage T7 numbers in EB cells were significantly different ($p < 0.0001$) from the no host cell control (NC), increasing by at least 2.6 logs at every time point (**Figure 6**). There was no significant difference between the T7 phage numbers in heat-killed host cells (HK) compared to the NC, indicating that T7 phages were unable to propagate in HK host cells (**Figure 6**). However, HK host cells were not a net sink for T7 phages as they were for T4 phages.

DISCUSSION

Membrane Integrity of eBeam Inactivated *E. coli* Cells

The vast majority of eBeam inactivated *E. coli* cells maintained their membrane integrity up to 24 h post-irradiation when kept in LB broth at 4°C. These results are congruent with a study by Jesudhasan et al. (2015), which found that a large majority of *Salmonella* Enteritidis cells had intact membranes after exposure to a lethal 2.5 kGy eBeam dose. Only after 9 days of incubation did the membrane damage in the eBeam inactivated *E. coli* cells

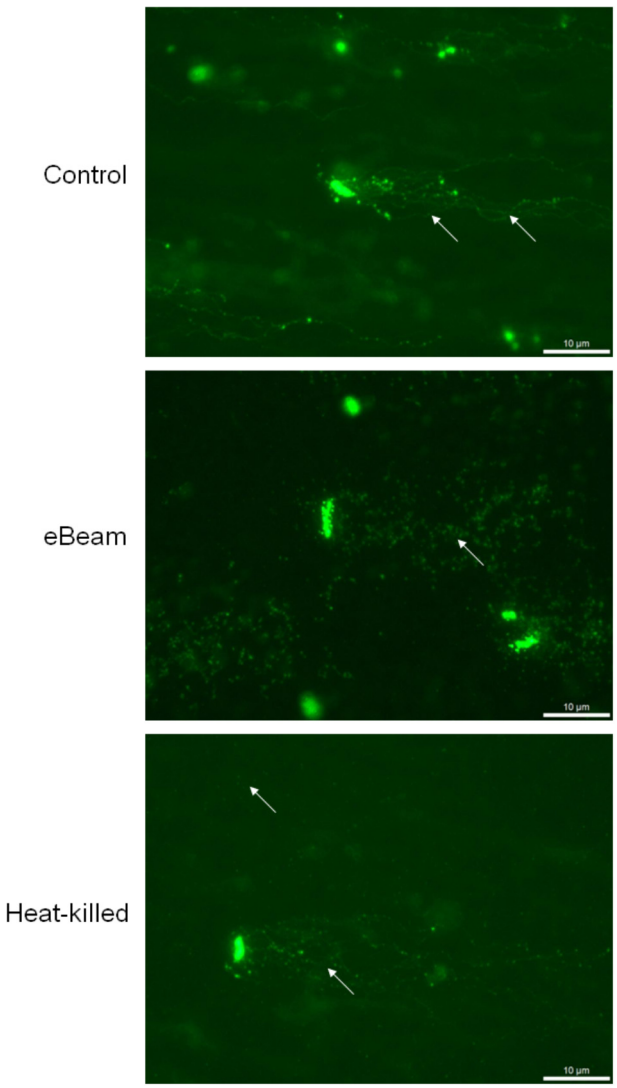

FIGURE 2 | Representative images showing the detection of DNA double-strand breaks in *E. coli* cells using the neutral comet assay. Cells were exposed to either a lethal eBeam irradiation dose (absorbed dose: 7.04 kGy), a lethal heat treatment (70°C for 60 min) or no treatment. Arrows indicate DNA tails (control), putative DNA fragments (eBeam) or both (heat-killed).

become more prevalent (**Figure 1**). This is in stark contrast to heat-killed cells, which showed membrane damage immediately following the heat treatment (**Figure 1**). Our results indicate that eBeam inactivated cells resemble live cells more closely with respect to their membrane integrity than heat-killed cells.

Visualization of DNA Double-Strand Breaks in eBeam Inactivated *E. coli* Cells

Ionizing radiation is known to cause DNA DSBs (Hutchinson, 1985). DSBs are the most lethal form of DNA damage and most organisms can generally tolerate only a few of them (Krasin and Hutchinson, 1977). To confirm that a lethal eBeam dose results in extensive DNA damage, the neutral comet assay was performed on *E. coli* cells irradiated in LB broth. The fluorescent images obtained from the comet assay showed extensive DSBs in the cells after exposure to lethal eBeam irradiation. This is evident by the complete absence of distinct DNA tails/comets (**Figure 2**). The extensive DNA damage in eBeam inactivated cells makes the quantification of DSBs extremely difficult. On the other hand, live healthy *E. coli* cells showed only minor DNA damage as seen by a few long DNA tails, while heat-killed cells exhibited both patterns (**Figure 2**). The lack of distinct DNA tails in the eBeam inactivated cells is a result of the large number of DSBs. It has been estimated that 100 Gy of ionizing radiation cause approximately 1 DSB per one million base pairs (Mbp) (Daly et al., 2010). For the genome of *E. coli* strain K-12 (4.6 Mbp) this translates roughly to 3–5 DSBs per 100 Gy (Blattner et al., 1997; Thomson et al., 2008). Therefore, a dose of 7 kGy would result in 210–350 DSBs per genome. The paper by Singh et al. (1999) is the only other published report that utilized the neutral comet assay to visualize DSBs in irradiated bacteria. They studied x-ray irradiated *E. coli* cells at 0.125–1 Gy and were able to quantify the DNA tails. However, considering the substantial difference in dose, 1 Gy versus 7000 Gy, and the theoretical number of DSBs (0.03 vs. 210 per cell), it is not surprising that distinct or countable DNA tails were not observed in this study. It needs emphasis that DNA comet assays are not particularly useful for prokaryotes because of their low cellular DNA content. Therefore attempting to quantify DNA tails or "spots" would be prone to serious errors. These are key drawbacks of utilizing DNA comet assays in prokaryotes. In preliminary studies using DNA fragmentation analysis, we have observed that DNA fragments in the 10^3 bp predominate after eBeam irradiation as compared to the 10^4 bp fragments that are present in un-irradiated cells (*data not included*).

Metabolic Activity in eBeam Inactivated *E. coli* Cells

eBeam irradiated *E. coli* cells incubated in LB broth at 4°C maintained metabolic activity levels on par with the positive control cells for the first 24 h (**Figure 3A**). This trend was also observed for lethally irradiated *S.* Typhimurium cells incubated in TSB at 4°C. We hypothesize that the lethally irradiated cells are adapting to the cold environment and are adjusting their metabolic needs to focus on DNA repair (Daly and Minton, 1996; Panoff et al., 1998; Liu et al., 2003; Kimura et al., 2006; Dillingham and Kowalczykowski, 2008). By day 9 of incubation the metabolic activity in irradiated cells had decreased significantly compared to the control (**Figure 3A**). This trend could signify the beginning of the cell death phase and is congruent with an observed decrease in membrane integrity (**Figure 1**) (Wanner and Egli, 1990; Finkel, 2006).

ATP Levels in Lethally Irradiated *E. coli* Cells

ATP levels for eBeam irradiated *E. coli* samples were more variable compared to heat-killed and control samples (**Figure 3B**). In general, irradiated samples resembled control samples more closely than heat-killed ones, except on day 9 of

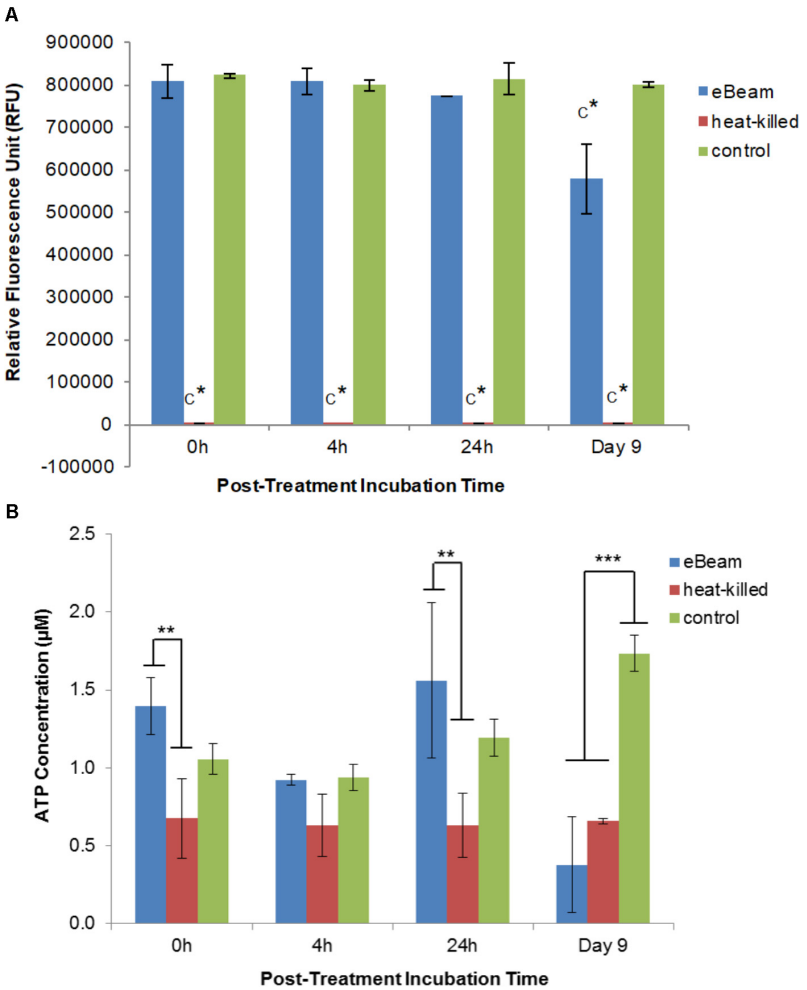

FIGURE 3 | (A) Metabolic activity of eBeam inactivated, heat-killed, and live *E. coli* cells. Heat-killed cells did not have measurable levels. Bacterial cultures were incubated at 4°C in LB broth post-treatment and measurements were taken at 0, 4, 24 h, and 9 days. Two independent experiments were performed, with standard deviations shown. C* denotes statistical significance ($p < 0.0001$). **(B)** ATP levels of eBeam inactivated, heat-killed, and non-treated *E. coli* cells. Samples were incubated at 4°C in LB broth post-treatment and measurements were taken at 0, 4, 24 h, and 9 days. Two independent experiments were performed, with standard deviations shown. **Denotes statistical significance ($p < 0.01$); ***denotes statistical significance ($p < 0.001$).

incubation, at which point the ATP levels in lethally irradiated cells had decreased significantly. The observed trend in ATP levels indicates that irradiated cells were metabolically active (to varying degrees) over the 9 day incubation period. These observations together with the results from the redox indicator (alamarBlue®) support our hypothesis that lethally irradiated cells remain metabolically active for extended periods of time after irradiation. Similar results were obtained by Magnani et al. (2009) and Secanella-Fandos et al. (2014) with lethally gamma irradiated *Brucella melitensis* and *Mycobacterium bovis* cells, respectively.

Bacteriophage Multiplication in Lethally Irradiated *E. coli* Cells

All of the three bacteriophages tested, namely λ, T4, and T7, are tailed, double-stranded DNA phages belonging to the order *Caudovirales* (Ackermann, 2006). All three of them require their host cell's machinery to varying degrees for their propagation. Phage λ relies completely on the host cell to reproduce, T4 requires certain components of the host cell, and T7 only requires the host's machinery at the very beginning of infection (Hendrix and Casjens, 2006; Molineux, 2006; Mosig and Eiserling, 2006).

Phage λ is most dependent on its host cell and it also has one of the best-understood complex regulatory systems. λ is a temperate phage, with an ability to choose between two alternative life styles: the lytic and the lysogenic growth cycles. The decision between the two cycles is made within the first 10–15 min of infection and depends both on the MOI and the physiological state of the host cell (Little, 2006). λ uses the energy of the host cell's metabolism and its biosynthetic machinery to produce ca. 50–100 progeny virions (Little, 2006). Cell lysis occurs after ca. 1 h of infection in healthy host cells (Little, 2006). The results of

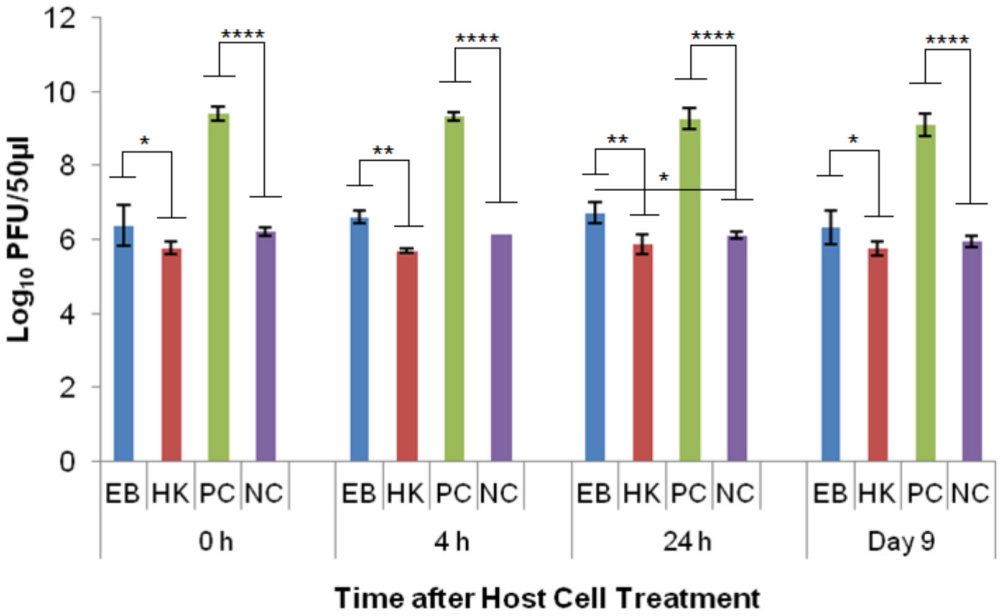

FIGURE 4 | Bacteriophage λ numbers after incubation (at 37°C for 24 h) with eBeam inactivated host cells (EB), heat-killed host cells (HK), non-treated live host cells (PC – positive control), and no host cells (NC – negative control). The 0, 4, 24 h, and day 9 time points represent the time after host cell treatment. Two independent experiments were performed, with standard deviations shown. *Denotes statistical significance ($p < 0.05$); **denotes statistical significance ($p < 0.01$); ****denotes statistical significance ($p < 0.0001$).

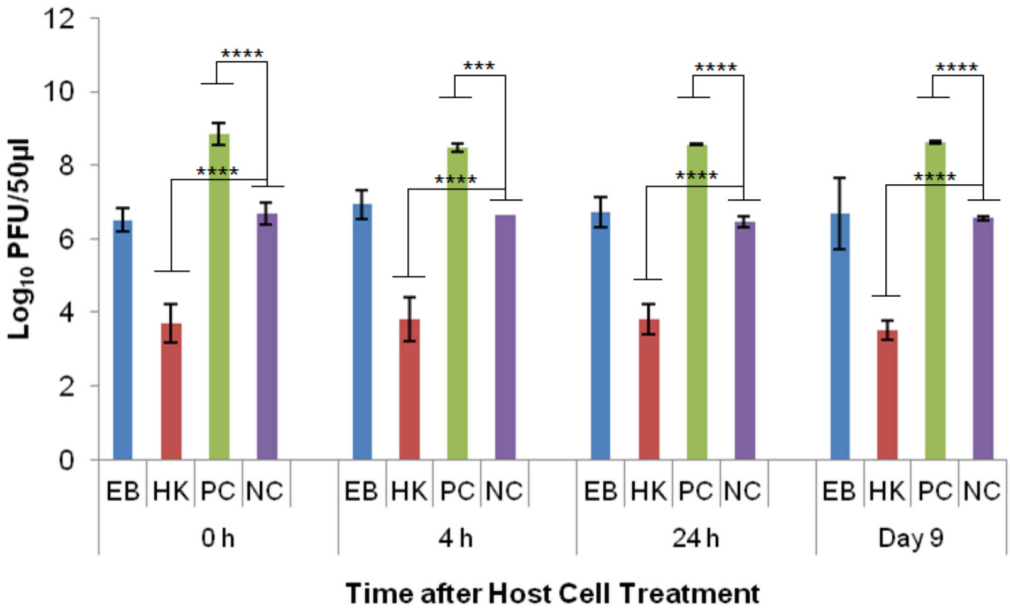

FIGURE 5 | T4 bacteriophage numbers after incubation (at 37°C for 24 h) with eBeam inactivated host cells (EB), heat-killed host cells (HK), non-treated live host cells (PC – positive control), and no host cells (NC – negative control). The 0, 4, 24 h, and day 9 time points represent the time after host cell treatment. Two independent experiments were performed, with standard deviations shown. ***Denotes statistical significance ($p < 0.001$); ****denotes statistical significance ($p < 0.0001$).

this study showed that at the 24 h time point (post-irradiation), there was a statistically significant difference ($p < 0.05$) between eBeam irradiated host cells and the no host cell control (**Figure 4**), indicating that phage λ was able to propagate successfully in eBeam irradiated cells. A similar trend was observed for the remaining three time points (0 h, 4 h, and day 9), but the increase in virus numbers was not statistically significant (**Figure 4**). Since λ phages are able to propagate inside eBeam irradiated

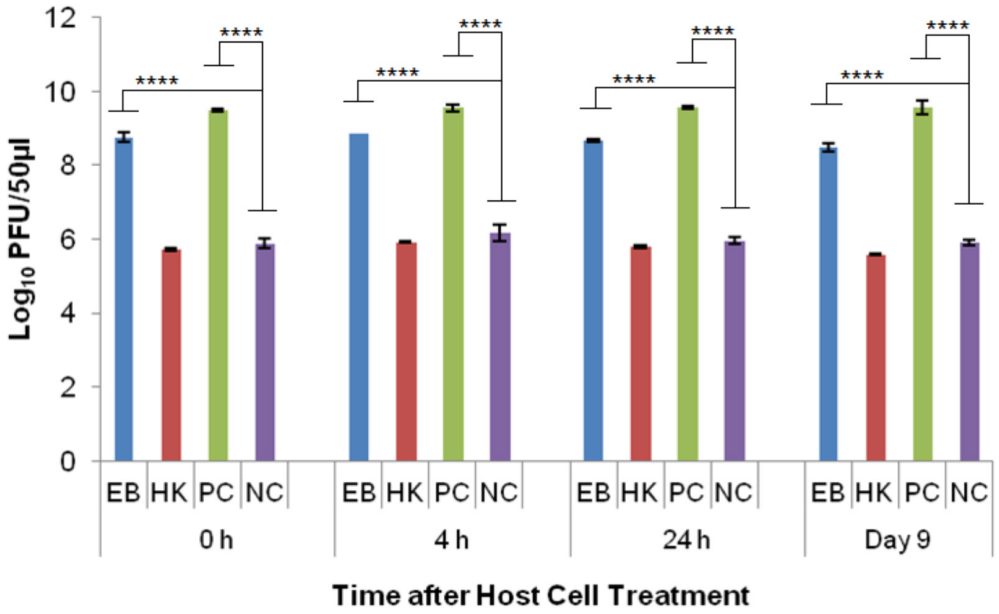

FIGURE 6 | T7 bacteriophage numbers after incubation (at 37°C for 24 h) with eBeam inactivated host cells (EB), heat-killed host cells (HK), non-treated live host cells (PC – positive control), and no host cells (NC – negative control). The 0, 4, 24 h, and day 9 time points represent the time after host cell treatment. Two independent experiments were performed, with standard deviations shown. ****Denotes statistical significance ($p < 0.0001$).

E. coli host cells, we hypothesize that all of the necessary cellular resources/machineries are still functioning within the irradiated cells. Phage λ requires the host's (1) RNA polymerase for all its transcription needs, (2) entire DNA replication apparatus for its phage DNA replication, and (3) translation machinery to make its proteins (Baranska et al., 2001; Hendrix and Casjens, 2006). All of these cellular functions must still be in "good working order" in eBeam irradiated cells; otherwise phage λ would not be able to propagate. It is possible that λ used the host cell's pre-formed RNA polymerase as well as other macromolecules to carry out the transcription and translation of its DNA. Whether or not pre-formed molecules were used, these results prove that the host cell's RNA polymerase is able to transcribe DNA and the ribosomes are able to translate and make proteins after a lethal eBeam irradiation dose.

The results for the T4 phage experiments revealed that they were unable to propagate in eBeam irradiated host cells. Interestingly, heat-killed host cells were a net sink for T4 phages, reducing phage numbers by approximately 3 logs (**Figure 5**). T4 phages also depend on many vital host structures and functions, such as membranes, energy metabolism, transcriptional and translational machines, and some chaperones (Adelman et al., 1997; Bhagwat and Nossal, 2001; Mosig and Eiserling, 2006). T4 phages use and modify the core host RNA polymerase, through phage-induced proteins, to selectively transcribe the hydroxymethylcytosine (HMC) residue containing phage DNA rather than the cytosine residue containing host DNA (Drivdahl and Kutter, 1990; Kashlev et al., 1993; Mosig and Eiserling, 2006). In fact, all host DNA and mRNA present at the time of infection, are rapidly degraded and the breakdown products are used to synthesize phage DNA and RNA. Furthermore, after infection, the translation of host messages ceases and ribosomes are re-programmed to translate T4 messages (Duckworth, 1970). Other than phage λ, T4 phage codes for all the components of its own DNA replication and recombination complexes (Mosig, 1998; Mosig et al., 1998; Kolesky et al., 1999; Mosig and Eiserling, 2006). It is unclear which structural or functional component(s) of the eBeam irradiated host cells were not functioning properly to prevent T4 propagation. It is possible that all of the host cell modifications (i.e., RNA polymerase) initiated by T4 phages increased the overall oxidative stress within the host cells, rendering them ineffective for phage propagation. Krisko and Radman (2010) found that the decline of what they called biosynthetic efficacy (measured by λ propagation) was correlated to radiation-induced oxidative damage. Targeted studies are needed to address this issue as well as the sink phenomenon observed in heat-killed host cells (**Figure 5**).

The results for the T7 phage experiments showed that they were the most successful in utilizing eBeam irradiated host cells for their propagation out of all the phages tested (**Figure 6**). T7 growth is remarkably independent of host enzymes; it only requires the host's translational apparatus and biosynthetic machinery for precursor synthesis (Molineux, 2006). The host cell's RNA polymerase is used to make early RNAs, but most of the transcription is catalyzed by the T7 RNA polymerase (once it has been synthesized by the host cell). T7 DNA replication and recombination are also independent of host proteins, except for thioredoxin (Martin et al., 1988; Imburgio et al., 2000; Molineux, 2006). Just like T4 phages, T7 phages attach to the lipopolysaccharides of the outer membrane and translocate their DNA via a self-made channel into the

host cell's cytoplasm. DNA translocation is highly temperature dependent and requires membrane potential (Molineux, 2001, 2006). Since T7 phages require the least amount of host cell resources and functionalities, this may be the reason why they were able to propagate so efficiently in eBeam irradiated host cells (**Figure 6**). Furthermore, the results indicate that all of the cellular components (i.e., RNA polymerase) needed by the phage to replicate are functioning properly in eBeam irradiated host cells. It would further appear that irradiated cells kept at 4°C post-irradiation are "frozen in time" (in terms of their cellular activities), since T7 phages were able to propagate in cells that had been stored for 9 days just as well as in freshly irradiated cells (**Figure 6**). This is in contrast to post-irradiation incubation at 37°C. Marsden et al. (1972) found that sub-lethally irradiated *E. coli* cells rapidly lost their ability to support T4 phage growth after 2 h of post-irradiation incubation at 37°C. Even though, T7 phages were able to propagate well in irradiated cells, the increase in numbers was still significantly different ($p < 0.0001$) from the non-irradiated (control) cells (**Figure 6**). This leads to the conclusion that some cellular components, apart from the DNA, were rendered less functional due to the irradiation with a lethal eBeam dose. These results are in line with earlier studies that examined phage growth in x-ray irradiated *E. coli* host cells (Labaw et al., 1953; Pollard et al., 1958). The results presented here with eBeam irradiated *E. coli* host cells have raised many more interesting questions (i.e., do phages use pre-formed or newly synthesized RNA polymerase) and warrant further investigation. Using bacteriophages to investigate the functionality of lethally irradiated bacterial cells may prove to be a very elegant model system.

CONCLUSION

The results presented indicate that lethally irradiated *E. coli* cells resemble live (non-irradiated) cells more closely than heat-killed cells. Despite their extensive DNA damage, lethally irradiated cells have intact membranes, are metabolically active, and are able to support the propagation of bacteriophages.

AUTHOR CONTRIBUTIONS

A-SH and SP conceived the experiments and wrote the manuscript. A-SH performed the experiments and data analysis.

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A Comparative Analysis of the Metabolomic Response of Electron Beam Inactivated *E. coli* O26:H11 and *Salmonella* Typhimurium ATCC 13311

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Ionizing radiation such as Electron beam (EB) and gamma irradiation inactivate microbial cells preventing their multiplication. These cells, however, are structurally intact and appear to have residual metabolic activity. We were interested in understanding the metabolic pathways that were still functional in EB-inactivated cells. Therefore, the primary objective of this study was to compare the metabolites accumulating in EB-inactivated pathogens *E. coli* 026:H11 and *S.* Typhimurium immediately after EB inactivation and 24 h post inactivation. Defined aliquots (10^9 CFU/mL) of *E. coli* O26-H11 (TW 1597) and *S.* Typhimurium (ATCC 13311) suspended in phosphate-buffered saline were exposed to lethal EB doses of 3 kGy and 2 kGy, respectively. Complete inactivation (inability of cells to multiply) was confirmed by traditional plating methods. An untargeted analysis of the primary metabolites accumulating in un-irradiated (control) cells, EB-inactivated cells immediately after irradiation, and EB-inactivated cells that were incubated at room temperature for 24 h post EB inactivation was performed using gas chromatography/mass spectrometry. A total of 349 different metabolites were detected in the EB-inactivated *S*. Typhimurium and *E. coli* O26:H11 cells, out of which, only 50% were identifiable. In *S*. Typhimurium, 98 metabolites were expressed at statistically different concentrations ($P < 0.05$) between the three treatment groups. In *E. coli* O26:H11, 63 metabolites were expressed at statistically different concentrations ($P < 0.05$) between the three treatment groups. In both these pathogens, the β-alanine, alanine, aspartate, and glutamate metabolic pathways were significantly impacted ($P < 0.01$). Furthermore, the metabolomic changes in EB-inactivated cells were amplified significantly after 24 h storage at room temperature in phosphate-buffered saline. These results suggest that EB-inactivated cells are very metabolically active and, therefore, the term Metabolically Active yet Non-culturable is an apt term describing EB-inactivated bacterial cells.

Keywords: electron beam, inactivation, metabolomics, *E. coli* O26:H11, *Salmonella* Typhimurium

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INTRODUCTION

Enteric pathogens such as of Shiga-toxin producing *E. coli* and *Salmonella* Typhimurium account for numerous foodborne outbreaks around the world. *Salmonella* is one of the top 5 foodborne pathogens that cause foodborne illness, while pathogenic *E. coli* are one of the top 4 foodborne pathogens that lead to illnesses and hospitalizations in the United States (CDC, 2018). With significant numbers of outbreaks attributed to fresh produce or minimally processed foods, there is an increasing need for effective non-thermal food processing technologies. Electron beam (EB) processing is a form of ionizing radiation used for food pasteurization in the food industry (Miller, 2005; Hallman, 2011; Lung et al., 2015; Prakash, 2016). The technology is currently used for pasteurizing frozen ground beef in the United States. The EB dose applied to a product is optimized to the intended food product, with the U.S. Food and Drug Administration currently approving doses up to 4 kGy and 7 kGy for lettuce/spinach and for frozen poultry meat, respectively (FDA, *Code of Federal Regulations, title 21, sec. 179.26*). When bacterial cells are exposed to ionizing radiation such as EB, multiple double stranded DNA breaks occur preventing DNA replication and therefore, preventing bacterial multiplication (Urbain, 1986; Tahergorabi et al., 2012; Pillai and Shayanfar, 2017). Complete inactivation of defined titers of bacterial cells by lethal EB doses is achievable based on the knowledge of the target organism's D_{10} value. Previous studies in our laboratory have shown that when bacterial cells are EB-inactivated, the cell membranes are structurally intact (Jesudhasan et al., 2015). More recent studies in our laboratory have shown that EB-inactivated cells exhibit very defined gene expression patterns and metabolic activity for prolonged periods post the actual EB irradiation (Hieke, 2015; Hieke and Pillai, 2018). Therefore, determining the specific metabolic pathways that are still operating in EB-inactivated cells was of significant interest to us.

The objective of this study was to understand the metabolomic profile of EB-inactivated bacterial pathogens *E. coli* O26:H11 and *S.* Typhimurium ATCC 13311 immediately after EB inactivation and 24 h post EB irradiation and compare these metabolomic profiles to un-irradiated cells. The underlying hypothesis of this study was that even though EB irradiation inactivates bacterial cells, the bacterial cells are capable of maintaining their metabolic pathways even up to 24 h post EB irradiation.

MATERIALS AND METHODS

Bacterial Cell Preparation

E. coli O26:H11 (TW 1597) obtained from the USDA-ARS culture collection (USDA-ARS-FFSRU, College Station, TX, United States) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) (ATCC 13311) were used for this study. Triplicate overnight cultures of each strain were prepared independently by transferring a single colony to Tryptic Soy Broth (TSB) and incubating overnight at 37°C in a shaking water bath. The overnight cultures were washed three times with phosphate-buffered saline (PBS) by centrifugation (4000 × *g*, 10 min) and suspended in PBS following the last wash. Samples were triple packaged in Whirl-Pak bags (Whirl-Pak, NASCO, Fort Atkinson, WI, United States) (to meet university biosafety protocols) and transported on ice to the EB facility across the campus. Aliquots of the un-irradiated samples were also triple packaged and transported to the EB facility. After processing of irradiated samples, the un-irradiated samples were serially diluted and enumerated on Tryptic Soy Agar (TSA) plates in order to determine the starting titer of the cultures used.

Electron Beam Inactivation

Electron beam inactivation experiments were performed at the EB facility of the National Center for Electron Beam Research at Texas A&M University in College Station, TX, United States. A 10 MeV, 15 kW linear accelerator delivered the EB doses. Alanine (L-α-alanine) dosimeters were used to confirm the delivered dose. Sample packages were previously dose-mapped to ensure a dose uniformity ratio of one. *E. coli* O26:H11 and *Salmonella* Typhimurium cells were irradiated at target doses of 3 kGy and 2 kGy, respectively. Aliquots of the irradiated bacterial cells were plated on TSA plates immediately after irradiation and 24 h after irradiation to confirm complete inactivation.

Metabolite Detection and Identification

The primary metabolites in the experimental treatments (namely, un-irradiated control, freshly EB-irradiated cells, and EB-irradiated stored for 24 h at room temperature post irradiation) were detected and analyzed using an untargeted approach using GC-MS at the University of California-Davis metabolomics laboratory. There were three biological replicates for each experimental treatment, and each biological replicate analyzed three times on the GC-MS as technical replicates. Amino acids, hydroxy acids, carbohydrates, sugar acids, sterols, aromatics, nucleosides, amines as well as other co-purifying miscellaneous compounds were extracted, detected, and analyzed using previously published methods (Fiehn et al., 2008, 2010). An Agilent 6890 gas chromatograph (Agilent, Santa Clara, CA, United States) fitted with a 30 m long, 0.25 mm internal diameter Rtx-5Sil MS column with 0.25 μm 95% dimethyl-5% diphenyl polysiloxane film and additional 10 m integrated guard column (Restek, Bellefonte, PA, United States) was used to separate compounds. A volume of 0.5 μl was injected using a Gerstel MPS2 automatic liner exchange system (Mülheim an der Ruhr, Germany). The temperature was increased to 250°C at a rate of 12°C/s. The mobile phase was pure helium (>99.9% purity) at a flow rate of 1 ml/min. The column was held at 50°C for 1 min, ramped to 330°C at a rate of 20°C/min, and held at 330°C for 5 min. Mass spectrometry was performed by a Leco Pegasus IV time of flight mass spectrometer (St. Joseph, MI, United States) with a −70 eV ionization energy, 1800 V detector voltage, 230°C transfer line temperature, and 250°C ion source temperature. Mass spectra were acquired with unit mass resolution at 17 spectra/s from 80 to 500 Da.

The raw data files were pre-processed directly after data acquisition and all entries were processed by the metabolomics BinBase database. Identified metabolites were reported if present within at least 50% of the total samples. Data was reported

as peak heights for the quantification ion (m/z value) at the specific RI. Peak heights were used because peak heights are more precise than peak areas at quantifying metabolites found in low concentrations. Any metabolite with more than one peak, were summed and reported as a single value. The raw data was transformed and normalized by dividing each raw metabolite value by the sum intensities of all known (excluding unknown) metabolites in that sample. This value was then multiplied by a constant factor to obtain whole numbers. This transformation was done in order to normalize the data to total known metabolite content, disregarding unknowns that could potentially contain artifact peaks or chemical contaminants.

Statistical Analysis

A statistics-based analysis of the primary identified and unknown metabolites was performed using MetaboAnalyst 4.0, a web-based metabolomics processing tool[1]. The data was normalized using log transformed and Pareto scaling feature of MetaboAnalyst. A one-way ANOVA using Tukey's HSD was used to determine significant features. Partial Least Squares - Discriminant Analysis (PLS-DA) was used to examine differences between the treatment groups. For pathway analysis using MetaboAnalyst 4.0, the mean peak value was considered to analogous to mean metabolite concentration. Global test and relative-betweenness centrality algorithms were used for pathway enrichment and pathway topology, respectively. Furthermore, all P values were adjusted for the False Discovery Rate (FDR). The FDR was subsequently used for all significance tests. *S.* Typhimurium and *E. coli* O26:H11 samples were analyzed separately. Given the close taxonomic relatedness between *S.* Typhimurium and *E. coli,* and the lack of publicly available metabolite pathway databases for *S.* Typhimurium, the metabolite library of *E. coli* K-12 MG1655 was used as the pathway library for both *S.* Typhimurium and *E. coli* pathway analysis.

RESULTS

Confirmation of Bacterial Inactivation

The starting titer of *E. coli* and *S.* Typhimurium was 9.36 ± 0.02 log CFU/ml and 9.24 ± 0.20 log CFU/ml, respectively. The *S.* Typhimurium samples received a measured dose of 1.94 kGy and *E. coli* samples received a measured dose of 3.02 kGy. No growth was detected in any of the irradiated samples, confirming that these EB doses achieved complete inactivation of the experimental samples.

Metabolomic Analysis

A total of 349 metabolites were detected, out of which only 175 were identifiable (**Supplementary Data 1, 2**). Overall, only 50% of the detected metabolites in *E. coli* O26:H11 and *S.* Typhimurium were identifiable using publicly available databases such as KEGG and PubChem. In *E. coli* O26:H11, 63 metabolites were expressed at different concentrations ($P < 0.05$) between the three *E. coli* experimental treatment groups (**Supplementary Data 3**). Ninety eight (98) metabolites were found in significantly different concentrations ($P < 0.05$) between the *S.* Typhimurium treatment groups (**Supplementary Data 4**).

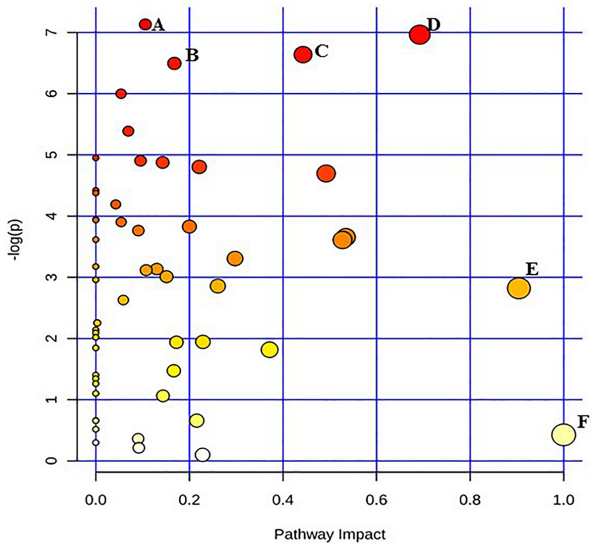

FIGURE 1 | Pathway analysis highlighting important pathways of *E. coli* O26:H11 immediately after irradiation (EB 0 h) compared to un-irradiated cells (0 kGy Control). The *Y* axis represents metabolic pathways containing metabolites that are significantly different between treatment groups. They *X* axis represents the impact these significantly changed metabolites have on the overall pathway based on their position within the pathway. A: Pentose and glucuronate interconversions; B: Pantothenate and CoA biosynthesis; C: Starch and sucrose metabolism; D: Beta alanine; E: Alanine, aspartate, and glutamate metabolism; F: Inositol phosphate metabolism.

Metabolic Pathway Analysis

Given the current technical challenges in analyzing more than two metabolic pathways simultaneously, two separate metabolic pathway analyses were conducted for each of the two target pathogens. One comparison was between the un-irradiated control cells and the freshly irradiated cells. The other comparison was between freshly irradiated cells and irradiated cells that were stored at room temperature for 24 h. **Figure 1** shows the results of the analysis between un-irradiated *E. coli* O26:H11 cells and freshly irradiated cells (control vs. 0 h). **Figure 2** shows the results of the analysis of the freshly irradiated *E. coli* O26:H11 cells and the irradiated cells stored for 24 h (0 h vs. 24 h). Eleven pathways were significantly different ($P < 0.05$) between the *E. coli* O26:H11 control and 0 h groups (**Supplementary Data 5**), while 6 pathways were significantly different ($P < 0.05$) between the 0 h and 24 h *E. coli* O26:H11 (**Supplementary Data 6**). **Figure 3** shows the results of the pathway analysis of un-irradiated *S.* Typhimurium and freshly irradiated *S.* Typhimurium (control vs. 0 h). **Figure 4** shows the results from the comparison of the metabolic pathways from the freshly irradiated

[1]http://www.metaboanalyst.ca/

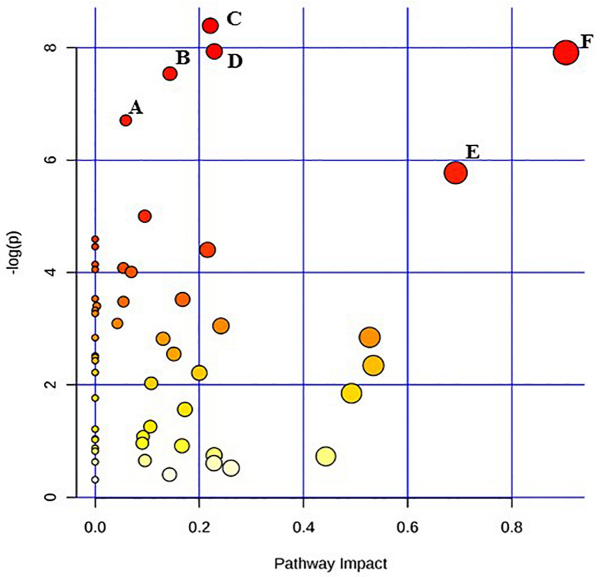

FIGURE 2 | Pathway analysis highlighting important pathways of *E. coli* O26:H11 24 h post EB exposure (EB 24 h) compared to freshly irradiated cell (EB 0 h). The *Y* axis represents metabolic pathways containing metabolites that are significantly different between treatment groups. They *X* axis represents the impact these significantly changed metabolites have on the overall pathway based on their position within the pathway. A: Butanoate metabolism; B: Nicotinate and nicotinamide metabolism; C: Cysteine and methionine metabolism; D: Citrate cycle (TCA cycle); E: beta-Alanine metabolism; F: Alanine, aspartate and glutamate metabolism.

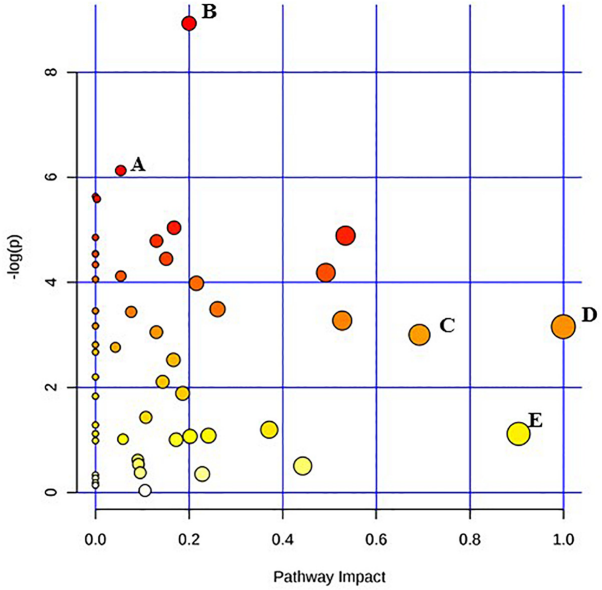

FIGURE 3 | Pathway analysis highlighting important pathways of *S.* Typhimurium immediately after irradiation (EB 0 h) compared to un-irradiated cells (0 kGy Control). The *Y* axis represents metabolic pathways containing metabolites that are significantly different between treatment groups. They *X* axis represents the impact these significantly changed metabolites have on the overall pathway based on their position within the pathway. A: Propanoate metabolism; B: Tryptophan metabolism; C: beta-Alanine metabolism; D: Inositol phosphate metabolism; E: Alanine, aspartate and glutamate metabolism.

S. Typhimurium and EB-irradiated *S.* Typhimurium stored for 24 h (0 h vs. 24 h). Four pathways were significantly different ($P < 0.05$) between the *S.* Typhimurium control and 0 h groups (**Supplementary Data 7**), while 27 pathways were significantly different ($P < 0.05$) between 0 and 24 h *S.* Typhimurium.(**Supplementary Data 8**).

DISCUSSION

Based on PLS-DA, there were clear differences between the *E. coli O26:H11* treatment groups, with 35.2% of the variance explained by component 1 (**Figure 5**). In *S.* Typhimurium, there were also clear differences between treatment groups, with 34.4% of the variance explained by component 1 (**Figure 6**). The PLS-DA scores plots for both target pathogens show tightly clumped biological replicates of the control and 24 h post EB cells. The biological replicates of freshly irradiated cells were more spread out, indicating that there was more variance in the metabolites contributing to the key features of this treatment group. The high variance between the biological replicates of freshly irradiated cells, seen with both pathogens suggests that the immediate response to ionizing radiation is quite variable. Multiple pathways may be expressed in order to protect and repair the bacteria after exposure to lethal EB.

Pathway enrichment analysis is based on quantitative analysis that directly utilizes metabolite concentration values. This type of analysis allows for detecting subtle differences in metabolites involved in the same biological pathway (Xia and Wishart, 2016; Chong et al., 2018). A global test algorithm was used to test for patterns of differentially expressed metabolites between treatment groups (Goeman et al., 2004; Hendrickx et al., 2012). Pathway topology analysis was performed to identify the impact a particular metabolite had on a particular metabolic pathway. Metabolic pathways that were considered the "most significant" were those that had a high impact value above 0.6 and/or statistical significance above –log(p) 6. **Table 1** summarizes the most impacted pathways in *E. coli* O26:H11 and *S.* Typhimurium ATCC 13311 after EB irradiation.

Irrespective of whether it is *E. coli* O26:H11 or *S.* Typhimurium, the primary metabolites in these cells are very distinct depending on whether they are un-irradiated or freshly irradiated or stored for 24 h post EB irradiation (**Figures 1**, **2**). The response of bacterial cells to stressors such as acid, temperature, and ionizing radiation have been extensively studied (Foster, 1991; Arsène et al., 2000; Castanié-Cornet et al., 2010; Daly, 2012; Pin et al., 2012; Swenson et al., 2012; Castillo and Smith, 2017; Villa et al., 2017). Recently, we have shown that *E. coli* O26:H11 when exposed to acid stress (pH 3.6), the key differentially expressed pathways were peptidoglycan biosynthesis, purine metabolism,

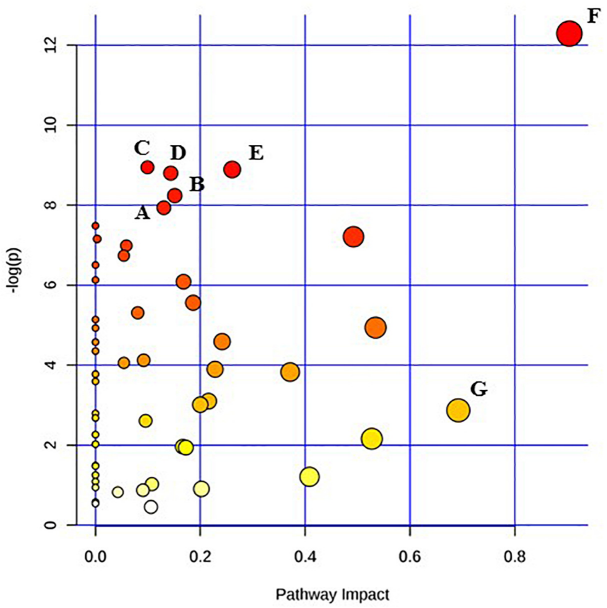

FIGURE 4 | Pathway analysis highlighting important pathways of *S.* Typhimurium 24 h post EB (EB 24 h) exposure compared to freshly irradiated cell (EB 0 h). The *Y* axis represents metabolic pathways containing metabolites that are significantly different between treatment groups. They *X* axis represents the impact these significantly changed metabolites have on the overall pathway based on their position within the pathway. A: Aminoacyl-tRNA biosynthesis; B: Glyoxylate and dicarboxylate metabolism; C: Cysteine and methionine metabolism; D: Nicotinate and nicotinamide metabolism; E: Glycerolipid metabolism; F: Alanine, aspartate and glutamate metabolism; G: beta-Alanine metabolism.

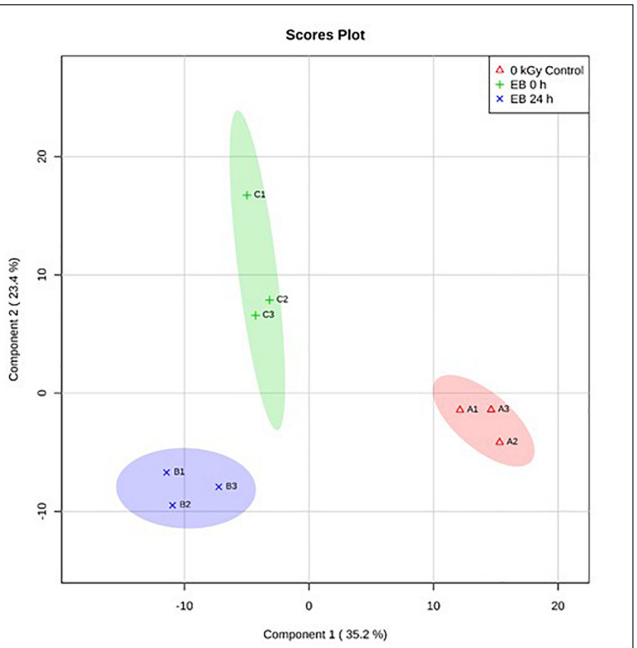

FIGURE 5 | Partial Least Square-Discriminant Analysis (PLS-DA) scores plot showing differences between the un-irradiated (0 kGy Control), irradiated (EB 0 h), and 24 h post irradiation (EB 24 h) *E. coli* O26:H11.

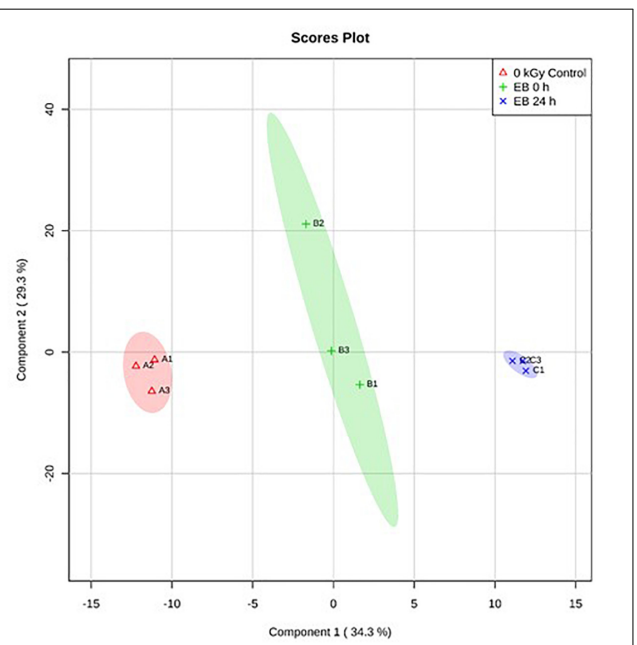

FIGURE 6 | Partial Least Square-Discriminant Analysis (PLS-DA) scores plot showing differences between the un-irradiated (0 kGy Control), irradiated (EB 0 h), and 24 h post irradiation (EB 24 h) *S.* Typhimurium.

D-Glutamine/D-glutamate metabolism, nitrogen metabolism, unsaturated fatty acid biosynthesis, and inositol phosphate metabolism (Shayanfar et al., 2018 *J. Appl. Microbial. submitted manuscript*). The differentially expressed pathways post EB irradiation match these pathways very closely except for peptidoglycan synthesis (**Table 1**). It is noteworthy that the cell wall associated peptidoglycan pathway is not affected during EB irradiation and this is reflected in our findings that the cellular structure is unaffected during EB irradiation (Jesudhasan et al., 2015). Similarity in the perturbations of the other pathways between acid stress exposure and EB irradiation exposure suggests that these pathways are part of the general stress response in bacterial cells. Across all treatments and considering both *E. coli* O26:H11 and *S.* Typhimurium, the β-alanine and alanine, aspartate, and glutamate pathways were significantly impacted (**Table 1**). Increase in amino acid synthesis is a stress response commonly seen in *E. coli.* Stapleton reported that irradiated *E. coli* cells require amino acids such as glutamic and aspartic acid for recovery from radiation injury (Stapleton, 1955). Thus, it is indicative from these studies that the irradiated cells are attempting to synthesize these critically important amino acids. Increased levels of alanine, aspartate, and glutamate have also been observed in the cold stress response (Jozefczuk et al., 2010). Since ionizing radiation at 2 kGy and 3 kGy does not significantly impact proteins, the increase of amino acids could also be a result of intentional protein breakdown (Mandelstam, 1963; Willetts, 1967; Tsakalidou and Papadimitriou, 2011).

TABLE 1 | Most significantly impacted metabolic pathways in *E. coli* O26:H11 cells and *S.* Typhimurium ATCC 13311 when exposed to a lethal EB dose as a function of time post EB exposure.

Comparing 0 kGy and 3 kGy exposed *E. coli* O26:H11 cells (0 kGy Control and EB 0 h)	**Comparing 3 kGy exposed *E. coli* O26:H11 cells immediately after exposure and 24 h post EB exposure (EB 0 h and EB 24 h)**
Pentose and glucuronate interconversions	Butanoate metabolism
Pantothenate and CoA biosynthesis	Nicotinate and nicotinamide metabolism
Starch and sucrose metabolism	Cysteine and methionine metabolism
Beta-alanine metabolism	Beta-Alanine metabolism
Alanine, aspartate, and glutamate metabolism	Alanine, aspartate and glutamate metabolism
Inositol phosphate metabolism	Citrate cycle (TCA cycle)
Comparing 0 kGy and 2 kGy *S.* Typhimurium ATCC 13311 exposed cells (0 kGy Control and EB 0 h)	**Comparing 2 kGy exposed *S.* Typhimurium ATCC 13311 cells immediately after exposure and 24 h post EB exposure (EB 0 h and EB 24 h)**
Propanoate metabolism	Propanoate metabolism
Tryptophan metabolism	Phenylalanine, tyrosine and tryptophan biosynthesis
Beta-Alanine metabolism	Beta-Alanine metabolism
Inositol phosphate metabolism	Cysteine and methionine metabolism
Alanine, aspartate, and glutamate metabolism	Alanine, aspartate, and glutamate metabolism
	Butanoate metabolism
	Nicotinate and nicotinamide metabolism
	Glycerolipid metabolism
	Benzoate degradation via CoA ligation
	Arginine and proline metabolism
	Phenylalanine metabolism
	Tyrosine metabolism
	Pantothenate and CoA biosynthesis
	Aminoacyl–tRNA biosynthesis
	Glyoxalate and dicarboxylate metabolism

Immediately after EB treatment, *E. coli* had increased β-alanine and Pantothenate metabolism. β -alanine is a direct precursor for pantothenate synthesis, explaining why the two are seen together immediately after irradiation in *E. coli* and after 24 h in *Salmonella* (Schneider et al., 2004). Pantothenate has been reported to be involved in cell wall and cell membrane biosynthesis (Toennies et al., 1966). Therefore the enhancement of the pantothenate metabolism suggests that the EB irradiated cells are possibly attempting to repair possible damages to the cellular membrane and walls that are yet undetectable. Both species showed significant increases in inositol phosphate metabolism immediately after irradiation, with the significant increase in the concentration of myo-inositol showing no change after 24 h. This is noteworthy as myo-inositol does not accumulate in bacteria; rather, it is converted to soluble phosphate esters (Roberts, 2006). While there have been very few studies conducted on the role of these compounds in prokaryotes, they are important signaling molecules in yeast cells and other eukaryotes, and exploration into their metabolic function would be of significant biologic importance (Wilson et al., 2013). Significant changes in metabolic activity occurred in both *E. coli* O26:H11 and *S.* Typhimurium even 24 h post exposure to lethal doses of EB. While not immediately triggered, there was a significant increase in the citrate cycle in *E. coli* 24 h after exposure to EB. The TCA cycle is one of the most important metabolic pathways in all oxidative organisms driving not only ATP generation, but also the defense against reactive oxygen species (Shimizu, 2013). Specifically, α-ketoglutarate plays a very important role in the detoxification of H_2O_2 and O_2^- (Mailloux et al., 2007). Interestingly, α-ketoglutarate levels initially increased after irradiation, but after 24 h, they decreased. This suggests that while there is an intial spike in concentration, over the course of 24 h, the cell either cannot or does not need to maintain elevated levels of this metabolite. In both species, succinic acid levels were increased immediately after irradiation, and continued to increase 24 h later. Accumulation of succinic acid has been previously observed and is a biomarker of oxidative stress (Mailloux et al., 2007). Furthermore, the accumulation of succinic acid is also used as a signaling mechanism in oxidatively stresed eukaryotic cells (Tretter et al., 2016). *S.* Typhimurium appears to have a more delayed metabolic reaction to EB, compared to *E. coli* O26:H11. Many of the pathways were impacted only 24 h after exposure to EB (**Table 1**). These include multiple pathways devoted to amino acid metabolism, namely the biosynthesis and metabolism of cysteine, methionine, alanine, aspartate, glutamate, arginine, proline, phenylalanine, tyrosine, and tryptophan. This is similar to the response seen in *E. coli* O26:H11, but on a much larger scope. Previous studies have also demonstrated distinct differences in the rate at which these two pathogens react to sudden changes (Sargo et al., 2015; Burgess et al., 2016). A major drawback of untargeted primary metabolomic analysis is the large presence of unknown metabolites. In this dataset, 50% of the metabolites detected were unknown. Many of the unknown metabolites had significantly different concentrations, and could potentially be key biomarkers of irradiation exposure. However, because their identity was unknown, these metabolites were excluded from the pathway analysis.

Overall, the results of this study clearly show that metabolites in both *E. coli* O26:H11 and *S.* Typhimurium exhibit fluxes in concentration even 24 h post EB irradiation exposure. The origin of these fluxes needs to be clearly understood. Since we did not compare the metabolites of un-irradiated cells stored for 24 h, we did not account for the possible likelihood that auto-oxidation of metabolites occurring. Are changes in metabolite concentration a function of the residual gene expression that we observed in these cells (Hieke, 2015)? Or are these metabolite concentrations changing due to purely abiotic effects within the cells? The continued gene expression coupled with our previous studies showing increased H+ exchanges within the cells (based on alamar blue staining) suggests that even after 24 h post lethal EB irradiation, bacterial cells are metabolically active and still attempting to repair their damage (Praveen, 2014). Detailed

studies are needed to identify whether the presence of certain unique metabolites in bacterial cells can be used as biomarkers of exposure to ionizing radiation exposure.

AUTHOR CONTRIBUTIONS

SB and SP designed the experiments. SB performed the laboratory experiments. SB and SP were involved in preparing the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00694/full#supplementary-material

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Geobacillus and *Bacillus* Spore Inactivation by Low Energy Electron Beam Technology: Resistance and Influencing Factors

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Low energy electron beam (LEEB) treatment is an emerging non-thermal technology that performs surface decontamination with a minimal influence on food quality. Bacterial spore resistance toward LEEB treatment and its influencing factors were investigated in this study. Spores from *Geobacillus* and *Bacillus* species were treated with a lab-scale LEEB at energy levels of 80 and 200 keV. The spore resistances were expressed as *D*-values (the radiation dose required for one $\log_{10}$ reduction at a given energy level) calculated from the linear regression of $\log_{10}$ reduction against absorbed dose of the sample. The results revealed that the spore inactivation efficiency by LEEB is comparable to that of other ionizing radiations and that the inactivation curves are mostly $\log_{10}$-linear at the investigated dose range (3.8 – 8.2 kGy at 80 keV; 6.0 – 9.8 kGy at 200 keV). The *D*-values obtained from the wildtype strains varied from 2.2 – 3.0 kGy at 80 keV, and from 2.2 – 3.1 kGy at 200 keV. *Bacillus subtilis* mutant spores lacking α/β-type small, acid-soluble spore proteins showed decreased *D*-values (1.3 kGy at 80 and 200 keV), indicating that spore DNA is one of the targets for LEEB spore inactivation. The results revealed that bacterial species, sporulation conditions and the treatment dose influence the spore LEEB inactivation. This finding indicates that for the application of this emerging technology, special attention should be paid to the choice of biological indicator, physiological state of the indicator and the processing settings. High spore inactivation efficiency supports the application of LEEB for the purpose of food surface decontamination. With its environmental, logistical, and economic advantages, LEEB can be a relevant technology for surface decontamination to deliver safe, minimally processed and additive-free food products.

Keywords: bacterial spore, low energy electron beam, inactivation, influencing factors, surface decontamination, ionizing radiation, DNA damage

INTRODUCTION

Bacterial spores are the largest hurdle for perishable food preservation due to their extreme resistance to processing steps. Conventionally, food industries apply intensive decontamination processing steps (e.g., thermal preservation) alone or in combination with water activity and pH reduction to control bacterial spores. However, currently, consumers prefer to have fresh-looking and minimally processed food products that are safe and additive-free (Gould, 1996). Therefore, food industries and scientists have been continuously searching for novel non-thermal decontamination processes that can ensure microbiological safety as well as better preserve the freshness and nutritional value of the food products. Among the emerging decontamination technologies, low energy electron beam (LEEB) treatment has proved to be an effective bacterial inactivation with a minimal influence on food quality (Radomyski et al., 1994; Hayashi et al., 1997; De Lara et al., 2002; Arthur et al., 2005; Hertwig et al., 2018). LEEB treatment was introduced into the food industry as a sterilization method for packaging material in 2012 (Comet Group, 2012b) and recently entered into the spice and herb industries for decontamination purposes (International Irradiation Association [IIA], 2017).

Electron beam (EB) is a novel non-thermal sterilization technology, which is noninvasive and chemical-free. EB is a particle-based ionizing radiation, similar to photon-based X-rays and gamma rays, and inactivates bacteria by generating electrons. Generated electrons ionize, leading to breakage of target molecules through direct and indirect effects. Direct effects are damages caused by energy transfer of electrons to the target molecules, while indirect effects are damages induced by free radicals generated in the reaction of electrons with, e.g., water molecules (Tahergorabi et al., 2012; Lung et al., 2015). The exact target of the EB and its inactivation mechanism are still unclear, but it is suggested to be DNA, as seen in other ionizing irradiation technologies (Nicholson et al., 2000; Moeller et al., 2008, 2014).

Depending on the kinetic energy of the electrons, an EB can be distinguished as either a high energy electron beam (HEEB; >300 keV) or LEEB (≤300 keV) (ISO/ASTM 51818, 2009; Tallentire et al., 2010). The kinetic energy of the electrons and the density of the treated material determine the penetration depth. The higher the kinetic energy and the lower the density of the target are, the deeper the electrons can penetrate (Urgiles et al., 2007). The electrons with high kinetic energy can penetrate food products up to several cm, while the penetration depth of electrons with low kinetic energy is limited to a micrometer scale (Jaczynski and Park, 2003; Urgiles et al., 2007).

The emerging EB technology has some advantages over other ionizing irradiation technologies and conventional decontamination technologies, e.g., fumigation with chemicals and dry heat decontamination. Compared to radiation with gamma rays, EB technology does not use radioactive sources (Jaczynski and Park, 2003; Black and Jaczynski, 2006). While it takes gamma radiation minutes to hours to deliver a certain dose, EB can deliver the same dose in few seconds due to a higher dose rate (Silindir and Ozer, 2009; Fan et al., 2017). Moreover, since the electrons are generated electronically, EB can be tuned for the desired intensity and can be switched on or off instantly, which increases the control and flexibility of the application of this technology (Urgiles et al., 2007; ISO/ASTM 51818, 2009). Moreover, on top of these features, LEEB has shown some advantages in comparison to HEEB. LEEB technology deposits electron energy close to the surface where microorganisms are present, resulting in a higher efficiency for surface decontamination (Urgiles et al., 2007). Since the energy deposits are close to the surface, the product-process interactions occur mainly on the surface, resulting in less impact on food quality (Hayashi, 1998; De Lara et al., 2002; Kikuchi et al., 2003). For example, research suggested LEEB can achieve microbial decontamination without inducing much starch degradation (Hayashi et al., 1997) or influencing seed germination (Trinetta et al., 2011; Fan et al., 2017). Furthermore, with its compact size and a minimal need for shielding, LEEB technology is scalable to continuous processes and can be easily implemented in existing processing lines (Bugaev et al., 1994; Hayashi, 1998; Chalise et al., 2007).

Despite all the advantages mentioned above, LEEB is not yet widely applied in the food industry as a decontamination technology. One of the reasons for that might be the lack of consumer acceptance for irradiated foods (Schweiggert et al., 2007; Frewer et al., 2011; Junqueira-Goncalves et al., 2011; Finten et al., 2017). Part of the consumer resistance is due to lack of information and understanding of food irradiation or wrongly associating irradiated food with radioactive food (Maherani et al., 2016). In some cases, the consumers are concerned about the possible side effects of inductive radiation on irradiated food products and the use of radioactive energy (Sahasrabudhe, 1990; De Lara et al., 2002). However, consumer resistance toward this novel decontamination technology appears to be decreasing as consumers and food industries recognize that irradiation can be an effective alternative to chemical additives to preserve foods (Monk et al., 1995; DeRuiter and Dwyer, 2002; Sabharwal, 2013). Moreover, studies showed that consumer acceptance toward irradiated foods can be further improved by consumer education (DeRuiter and Dwyer, 2002; Nayga et al., 2005).

The other reason for its limited application in food industry so far could be that compared to other well-studied irradiation technologies such as gamma irradiation, only a limited amount of studies support the use of LEEB treatment as an efficient decontamination technology (De Lara et al., 2002; Tallentire et al., 2010; Fan et al., 2017). Most of the present LEEB studies are on specific foods, focusing on naturally presented microbial flora and often using different treatment setups (Hayashi, 1998; Rahman et al., 2006). Moreover, the inactivation efficiency was often reported as a reduction of microbial load instead of *D*-values (the radiation dose required for one $\log_{10}$ reduction at a given energy level), and often, the absorbed dose was not measured (Hayashi, 1998; Hayashi et al., 1998; Rahman et al., 2006). These reasons make it difficult to compare the inactivation efficiency of LEEB technology between different LEEB studies (Hayashi et al., 1997; Baba et al., 2004; Imamura et al., 2009), and to that of other conventional spore inactivation technologies, making it more challenging to validate this emerging technology.

Moreover, the efficiency and mechanism of LEEB on bacterial spore inactivation are rarely studied (Fiester et al., 2012). Therefore, more research must be performed for this technology to be utilized as a decontamination step. Bacterial spores are generally more resistant to irradiation treatment than vegetative cells, yeasts, and molds (van Gerwen et al., 1999; De Lara et al., 2002; Setlow, 2006, 2014). For example, Thayer and Boyd (1994) confirmed that *B. cereus* spores have a higher irradiation resistance than that of vegetative cells, and van Gerwen et al. (1999) showed that spores have significantly higher *D*-values than those of most vegetative bacteria, based on a total 539 *D*-values from 38 irradiation studies. A few vegetative bacteria have similar or higher irradiation resistance than that of bacterial spores (e.g., *Deinococcus radiodurans*), but those species are less relevant in the food industry and are not pathogenic.

In this study, we evaluated the spore inactivation efficiency of LEEB by determining the *D*-values for relevant *Geobacillus* and *Bacillus* species, calculated from the linear regression of $\log_{10}$ reduction against absorbed dose of the spore sample. The potential spore LEEB resistance influencing factors, including spore species, sporulation conditions and treatment kinetic energy, were also investigated. Additionally, the possible mechanism of spore inactivation by LEEB treatment was also investigated by using a mutant lacking α/β-type small, acid-soluble spore proteins (SASP), the major protection of spore DNA against damage (Setlow, 1995; Moeller et al., 2008, 2009).This study provided a comparison of LEEB spore inactivation efficiency with other published ionizing radiation decontamination data and gave additional information on the potential target of LEEB technology that induces spore inactivation. This will support the validation and application of the novel LEEB decontamination technology and help in the future delivery of safe, minimally processed and additive-free food products.

MATERIALS AND METHODS

Bacterial Strains, Sporulation and Sample Preparation

In total, three bacterial species and one *B. subtilis* mutant were used in this study. This included *Geobacillus stearothermophilus* ATCC 7953, the biological indicator for the wet-heat sterilization process; *B. pumilus* DSM 492, the biological indicator for the irradiation sterilization process (Prince, 1976); *B. subtilis* wild-type PS 832, a model strain frequently used in spore research and its isogenic mutant *B. subtilis* PS 578 (termed as $\alpha^-\beta^-$) that lacks the genes encoding the two major α/β-type small acid-soluble spore proteins (Nicholson and Setlow, 1990a; Fairhead et al., 1993).

Bacillus subtilis PS 832 and PS 578 were kindly provided by Dr. Peter Setlow and Dr. Barbara Setlow. *B. pumilus* DSM 492 was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). Spores of *G. stearothermophilus* ATCC 7953 were obtained as a commercial spore suspension from MesaLabs (France). Except for *G. stearothermophilus* ATCC 7953, all the others were sporulated at 30°C using modified Difco sporulation media (mDSM) agar plates, with nutrient broth pH 6.9 and without NaCl, from Sigma-Aldrich (Sigma-Aldrich, United States) instead of Difco, and the pH was adjusted to 7.2 (Nicholson and Setlow, 1990b). *B. subtilis* PS 832 was also sporulated at 37°C on mDSM and 2 × SG, a modified Schaeffer's medium described previously (Leighton and Doi, 1971) to investigate the influence of the sporulation conditions on spore resistance toward LEEB treatment. Sporulation cultures were checked with a phase-contrast microscope (Leica, Germany) to estimate the percentages of dormant spores (phase-bright). Spores were harvested when the phase-bright spore percentage was >98%. Harvesting was performed by adding H_2O (4°C) to the surface of the cultivation plates and suspending the overgrown colonies containing spores with sterile spreading sticks. The suspension was then transferred to a 50 ml centrifuge tube and washed with H_2O (4°C) to remove the remaining vegetative cells, cell debris, and germinated spores. Spore stocks were stored in H_2O at 4°C until usage.

A volume of 1 ml of spore suspension (around 10^9 spores/ml; except for *G. stearothermophilus* ATCC 7953 which had an inoculation concentration of around 10^6 spores/ml) was carefully dropped and spread on the upper surface of an autoclaved sterile microscope glass slide (Thermo Fisher Scientific, United States) that laid on a petri dish. The spore suspension stayed on the surface, and all slides were air-dried in a biosafety bench at room temperature. Afterward, the samples were stored and transported for treatment.

Low Energy Electron Beam Treatment and Recovery

Samples were treated in the petri dish without a lid using a LEEB system EBLab-200 (Comet Group, Switzerland) at energy levels of 200 and 80 keV. The schematic of a LEEB lamp can be found elsewhere (Hertwig et al., 2018). Samples were either run through the machine without the electron source being turned on (0 kGy) or at nominal doses of 4, 5, 6, and 7 kGy. Due to the limited stability of the EB lamp at low electric current, treatment at lower doses was not performed. The distance between the emission window to samples was approximately 18 mm. Samples were treated under a N_2 atmosphere (residual O_2 < 210 ppm). All treatments were conducted at room temperature (approximately 23°C). Three independent samples were treated at each dose (results were calculated based on absorbed dose shown in **Table 1**) for all investigated spore strains.

After samples were treated with LEEB, recovery was performed to enumerate cultivable survivors. Treated samples on microscope slides were put into 50 ml falcon tubes filled with 20 ml of 10 mM phosphate buffered saline (PBS, VWR International, United States) containing 0.04% Tween 80 (Sigma-Aldrich, United States). After vigorous shaking for 4 min, microscope slides were removed using flame-sterilized tweezers. The solution, which contained spores that washed off from the glass slides, was plated in triplicates onto tryptic soy agar (TSA, Sigma-Aldrich, United States) plates at appropriate dilutions. Plates were incubated at 37°C for *B. subtilis* and *B. pumilus* and 55°C for *G. stearothermophilus*. After incubation,

TABLE 1 | Absorbed dose of spore samples at 80 and 200 keV.

Nominal dose (kGy)	4	5	6	7
Absorbed dose at 80 keV (kGy)	3.8 ± 0.39	4.7 ± 0.64	6.6 ± 0.94	8.2 ± 0.86
Absorbed dose at 200 keV (kGy)	6.0 ± 0.28	7.0 ± 0.58	8.2 ± 0.62	9.8 ± 0.85

the colony forming units (CFU) were counted. To derive the *D*-values reflecting the inactivation efficiency, spore survival fraction (N/N_0) was plotted against the absorbed dose on a semi-logarithmic scale. Regression analysis was performed using Origin 9.1 (OriginLab Corporation, United States). The *D*-values were calculated from the slope of the linear regression of $\log_{10}$ reduction against absorbed dose according to equation (1). An average *D*-value ($n = 3$) was calculated for each strain. Differences between datasets were analyzed with Excel 2016 (Microsoft, United States), using two-tailed *t*-test with equal variance and a significance level of 0.05.

$$D\ value = -\frac{1}{m} \quad (1)$$

Herein *m* is the slope of linear regression of $\log_{10}$ (N/N_0) against absorbed dose.

Dosimetry

The routine dosimeters used in this study were Risø B3-12 films (Risø High Dose Reference Laboratory, Denmark), which are 18 μm thick. The films were taped on microscope slides, placed in petri dishes and treated under the same conditions as the samples. The surface dose at each nominal dose used in this study was measured with three films placed at the same location as the samples with three replicate treatments. In total, nine films for each setting were irradiated and analyzed. Since electrons with low kinetic energy can be absorbed over a few micrometers, a dose gradient is created across the thickness of the Risø B3-12 dosimeter films that were used for dose measurement (Tallentire et al., 2010). The measured doses using Risø B3-12 dosimeter films were corrected to D_μ, which is the absorbed dose in the first micrometer of the absorbing medium (Helt-Hansen et al., 2010). D_μ-values were evaluated using Risøscan software with a calibration, which was obtained with the help of the Risø High Dose Reference Laboratory (HDRL, Denmark) for each applied energy level (80 and 200 keV) (Helt-Hansen et al., 2005). This calibration ensured that the reported doses from the low energy electron irradiations had measurement traceability to national standards (Helt-Hansen et al., 2010). The overall estimated uncertainty at $k = 2$ (a coverage factor $k = 2$ is close to a 95% confidence interval) of one dose measurement is around 10.6%. The overall uncertainty covers the uncertainty associated with calibration with alanine dosimeters, measurement of alanine dosimeter and D_μ determination.

In our situation, the spore samples were 1–2 μm thick, while the Risø B3-12 films, which is the thinnest standard dosimeter, are 18 μm thick. Therefore, the spore layer sits directly on the glass slide that served as a sample holder on the bottom, while the first micrometer of Risø B3-12 dosimeter are not directly in contact with the glass slide. Since a glass slide gives a stronger backscatter compared to the dosimeter, the spore samples were actually getting higher doses than D_μ that is measured by the dosimeter. Therefore, further simulations were done by the Risø High Dose Reference Laboratory (Denmark) concerning the effect of backscatter from different materials at different energy levels. Correction factors were obtained for the backscatter from the glass slide and from the dosimeter based on the simulation output. The measured D_μ-values were further corrected to the absorbed dose of the spore samples based on the correction factors.

RESULTS

Absorbed Dose of Spore Samples

Accurate dosimetry is essential for acquiring exact results, so the minimum and maximum measured doses were included when reporting EB inactivation experiments (Pillai and Shayanfar, 2017). Acquiring accurate surface doses for the low-energy range (e.g., 80 keV) was challenging due to the dose gradients within the treated dosimeter films. In this study, depth-dose distribution was established, and the surface dose D_μ was obtained using Risøscan software, calculated from measured apparent dose D_{app} (Helt-Hansen et al., 2010). The absorbed doses for our spore samples were corrected based on the surface dose D_μ and simulation output as described in Section "Dosimetry." The absorbed doses of the spore samples at each nominal doses are shown in **Table 1**.

Spore Inactivation

To investigate the spore inactivation efficiency by LEEB treatment and its influencing factors, spores obtained from different species and sporulation conditions were treated with LEEB at different kinetic energy levels, and their *D*-values were calculated and compared.

Kinetics

The regression analysis indicates a linear relationship between $\log_{10}$ reduction and absorbed dose used in this study for all species tested. The inactivation curves of different wildtype strains exhibited $R^2 > 0.95$. The mutant PS 578 showed lower resistance to LEEB and when treated at 9.8 kGy (200 keV), the survivors were below detection limit. Therefore, only four data points were obtained under this condition and the R^2 is higher than 0.95. All inactivation curves are shown in **Figure 1**.

Efficiency and Influencing Factors

The *D*-values obtained from this study for the wildtype strains varied from 2.2 – 3.0 kGy at 80 keV and 2.2 – 3.1 kGy at 200 keV. Different bacterial species showed diverse resistance toward LEEB treatment and the sporulation conditions, and the treatment energy levels showed influences on the spore inactivation efficiency. The spore resistance, expressed as *D*-values, is shown in **Table 2**. *B. subtilis* sporulated at 30°C on mDSM showed the lowest resistance toward LEEB treatment,

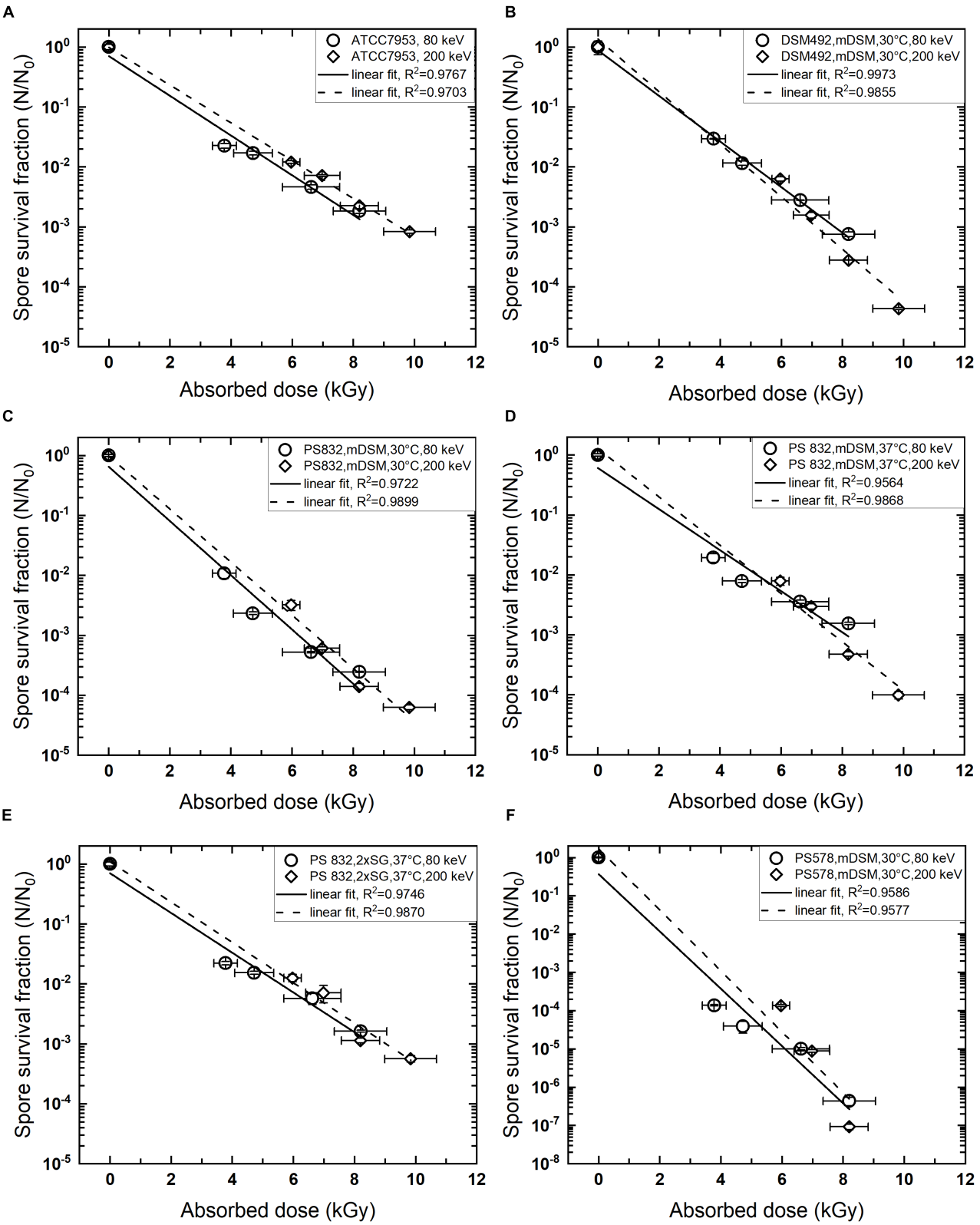

FIGURE 1 | Kinetics of low energy electron beam spore inactivation. **(A)** *Geobacillus stearothermophilus* ATCC 7953, commercial spore suspension; **(B)** *Bacillus pumilus* DSM 492, sporulated on mDSM, 30°C; **(C)**: *B. subtilis* PS 832, sporulated on mDSM, 30°C; **(D)** *B. subtilis* PS 832, sporulated on mDSM, 37°C; **(E)** *B. subtilis* PS 832, sporulated on 2 × SG, 37°C; **(F)** *B. subtilis* PS 578 ($\alpha^-\beta^-$ mutant), sporulated on mDSM, 30°C. Data are average ± standard deviation.

TABLE 2 | *D*-values of spore inactivation under low energy electron beam treatment.

	D_{80keV} (kGy)	D_{200keV} (kGy)
G. stearothermophilus ATCC 7953	$3.0 \pm 0.03^{a*}$	$3.1 \pm 0.05^{e*}$
B. pumilus DSM 492, mDSM, 30°C	$2.6 \pm 0.00^{b*}$	$2.3 \pm 0.02^{f*}$
B. subtilis PS 832, mDSM, 30°C	2.2 ± 0.01^{c}	2.2 ± 0.02^{g}
B. subtilis PS 832, mDSM, 37°C	$3.0 \pm 0.07^{a*}$	$2.5 \pm 0.03^{h*}$
B. subtilis PS 832, 2 × SG, 37°C	2.9 ± 0.08^{a}	2.9 ± 0.06^{i}
B. subtilis PS 578, mDSM, 30°C	1.3 ± 0.07^{d}	1.3 ± 0.02^{j}

*G. stearothermophilus ATCC 7953 was obtained as a commercial spore suspension. Different lower case letters indicate statistically significant differences within the same column ($p < 0.05$). *indicate statistically significant different within the same row ($p < 0.05$).*

with *D*-values of 2.2 kGy at 80 and 200 keV. Interestingly, *B. pumilus* DSM 492, which is suggested to be the biological indicator for irradiation sterilization, did not show higher resistance in most cases: it had lower resistance than that of *G. stearothermophilus* ATCC 7953, especially at the energy level of 200 keV, and it was less resistant than *B. subtilis* sporulated at 37°C on mDSM as well as on 2 × SG.

From **Table 2**, it was observed that *B. subtilis* PS 832 spores sporulated at the higher temperature had higher resistance to LEEB treatment at both energy levels. The *D*-values for spores that were sporulated at 37°C were approximately 0.3 kGy (200 keV) and 0.8 kGy (80 keV) higher than those sporulated at 30°C, even though both sets were sporulated on mDSM agar plates. At the same time, the nutrient richness of the sporulation media also showed influences on spore resistance to LEEB, as the spores sporulated on 2 × SG had higher resistance than that of the ones sporulated on mDSM when treated at 200 keV. The mutant *B. subtilis* PS 578 showed the smallest *D*-value, meaning it was much more sensitive to LEEB treatment than the other strains tested. The results obtained in this study suggest that in some cases there were no significant differences ($p > 0.05$) between LEEB treatment under 80 and 200 keV. Although for some samples, D_{80keV} is significantly different compared to D_{200keV}, there was not a clear trend. For *G. stearothermophilus*, the *D*-values were slightly but statistically significantly ($p = 0.01$) lower at 80 keV compared to at 200 keV, with an average *D*-value of 3.0 kGy at 80 keV compared to 3.1 kGy at 200 keV. For *B. subtilis* PS 832 spores sporulated on mDSM, 37°C, the *D*-value at 200 keV was 0.5 kGy lower than at 80 keV, while for *B. pumilus* DSM 492, the *D*-value at 200 keV was 0.3 kGy lower than at 80 keV (in both cases, $p < 0.01$). These results should be taken with caution, as due to the lack of accurate dose measurement techniques at 1–2 μm layers, it is not possible to determinate accurately what the absorbed doses are. A more accurate dose assessment method might have an impact on the *D*-values.

DISCUSSION

Spore Inactivation Kinetics and Efficiency

Linear spore inactivation kinetics ranging from 3.8 – 8.2 kGy at 80 keV and 6.0 – 9.8 kGy at 200 keV for different species were revealed in this study. Due to the limited stability of the EB lamp at low electric current, spore inactivation at lower doses was not investigated, and therefore, the possibility of a potential nonlinear $\log_{10}$ behavior at the range of lower doses cannot be excluded. Nevertheless, the linear inactivation found in the dose range used is in accordance with previous reports on LEEB inactivation of *B. pumilus* spores (Tallentire et al., 2010). The linear inactivation kinetics were also revealed in spore inactivation research of HEEB. For example, Fiester et al. (2012) reported a linear inactivation curve for *B. atrophaeus* treated at 5 MeV. However, nonlinear spore inactivation curves by HEEB were also reported in previous studies. For example, a nonlinear $\log_{10}$ inactivation curve with a shoulder formation was found for specific strains (Ito and Islam, 1994) and for *B. subtilis* spores that were sporulated in plate count agar (De Lara et al., 2002).

The *D*-values of the investigated wildtype strains in this study were in the range of 2.2 – 3.0 kGy at 80 keV and 2.2 – 3.1 kGy at 200 keV. These *D*-values were slightly higher compared to other LEEB studies (Urgiles et al., 2007; Tallentire et al., 2010). For example, the *D*-value for *B. pumilus* at 80 keV derived from this study was 2.6 kGy, while in the study by Tallentire et al. (2010) the *D*-value was 1.58 kGy, and by Urgiles et al. (2007), was 1.34 kGy. However, those differences in *D*-values could be due to different sample preparation methods, treatment conditions (e.g., at ambient atmosphere or N_2 atmosphere) and recovery methods. For example, in the study of Urgiles et al. (2007), spores were inoculated on Al and Ti coupons, while in our study, spores were inoculated on glass slides. Moreover, the recovery solution and incubation temperature were also different.

The *D*-values obtained in this study are comparable to those reported for HEEB (Ito and Islam, 1994; De Lara et al., 2002). This is consistent to previous research (Tallentire et al., 2010; Gryczka et al., 2018). For example, Gryczka et al. (2018) described that under the treatment conditions applied, HEEB and LEEB treatments have a comparable efficiency in lowering the bacterial load of the food products investigated. Moreover, Tallentire et al. (2010) reported the response of *B. pumilus* spores found to be the same when treated with HEEB and LEEB. On the other hand, another study revealed that the *D*-values for *B. pumilus*, *B. subtilis,* and *B. megaterium* were slightly lower at LEEB treatment compared to HEEB, with *D*-values at 10 MeV were 2.12, 2.05, and 4.11 kGy, respectively, and at 100 keV were 1.34, 1.01, and 3.46 kGy (Urgiles et al., 2007).

In some studies, the inactivation efficiency of EB was similar to that of other ionization radiation types (Ohki et al., 1990; Ito and Islam, 1994; Van Calenberg et al., 1998; De Lara et al., 2002; Tallentire et al., 2010; Fiester et al., 2012). For example, Ohki et al. (1990) reported that the radiation sensitivity was almost equivalent when treated with gamma rays, X-rays, or EB, and the *D*-values obtained were 1.5 – 1.6 kGy for *B. pumilus* and 1.4 – 1.5 kGy for *B. subtilis*. The *D*-values obtained in our study are higher than those found by Ohki et al. (1990). They are also slightly higher than the estimated average *D*-values concluded by van Gerwen et al. (1999) for spores under irradiation treatment. In their study, the estimated *D*-value for spores treated with various irradiation processes was approximately 2.11 kGy, excluding some exceptions and specific conditions. However,

as also stressed in their study, comparison of *D*-values from different projects should be acknowledged with care, since often the irradiation source is not the only influencing factor. In this study, spore species and sporulation conditions were also shown to affect spore inactivation efficiency of LEEB technology.

Influencing Factors on Spore Inactivation Efficiency of LEEB

Spore Species

From our results, it can be seen that spores of *B. subtilis* and *B. pumilus* sporulated on mDSM, 30°C and *G. stearothermophilus* showed significant differences ($p < 0.05$) in their resistance to LEEB treatments (**Table 2**). This observation is consistent with other ionizing radiations, which revealed that spores of different species or even different strains showed different resistances (Anellis et al., 1972; Ito and Islam, 1994; Monk et al., 1995; van Gerwen et al., 1999). For example, previous research revealed that pathogenic *B. cereus* was more resistant than *B. subtilis* (De Lara et al., 2002). Therefore, for specific food applications, process parameters should be adjusted for relevant contamination microbiota to ensure sufficient inactivation.

Our study also revealed that the *D*-values of *G. stearothermophilus* ATCC 7953 were significantly higher ($p < 0.01$) than *B. pumilus* DSM 492, which is suggested to be the biological indicator for irradiation-based sterilization. The great resistance of *G. stearothermophilus* was also reported for other irradiation sources (Anellis and Koch, 1962; Briggs, 1966; Harnulv and Snygg, 1973; Radomyski et al., 1994; van Gerwen et al., 1999). For example, previous research revealed that *G. stearothermophilus* had greater gamma irradiation resistance than that of *Bacillus* species (e.g., *B. subtilis*, *B. megaterium*, and *B. cereus*) (Briggs, 1966; Harnulv and Snygg, 1973). Therefore, *G. stearothermophilus* might be more suitable as a biological indicator for LEEB treatment than *B. pumilus*. If using *G. stearothermophilus* ATCC 7953 spores as an indicator for radiation doses at 10 kGy, which is recommended as the maximum applicable dose to food (FAO/WHO Codex Alimentarius Commission, 2017), more than 3 $\log_{10}$ reduction can be achieved by using LEEB technology with the *D*-value obtained in this study. However, when applying to real food matrices, the spore inactivation efficiency of LEEB might be different, as the matrices might affect it. For example, the location of spores in food matrices, the water content, and the nutrient profile of the food matrices can influence the inactivation efficiency. On the other hand, the use of *B. megaterium* spores as a biological indicator should also be considered, since they displayed an even higher resistance toward LEEB than that of *G. stearothermophilus* in some studies (Ohki et al., 1990; Pillai et al., 2006).

Sporulation Conditions

It was found that the sporulation conditions also influence the inactivation of LEEB, with the higher sporulation temperature leading to increased spore resistance. This result could be because increased sporulation temperature lowers the spore core water content, leading to less indirect damage from hydroxyl radicals formed during irradiation (Beaman and Gerhardt, 1986; Moeller et al., 2008). Moreover, the sporulation media also played a role in spore resistance toward LEEB treatment, as the more nutrients (2 × SG) that were in the sporulation media, the more resistant the spores were toward the treatment. Sporulation media also showed an influence on spore inactivation by HEEB technology. For example, *B. subtilis* spores sporulated on plate count agar had a *D*-value of approximately 3.6 kGy, while when sporulated on nutrient agar, the *D*-value was approximately 1.5 kGy (De Lara et al., 2002). However, the nutrient richness of plate count agar and nutrient agar is comparable, so it might be the salt content in the media that influenced the spore resistance. Moreover, in their study, the inactivation kinetics for the spores that sporulated in these two different media were different. When sporulated on nutrient agar plates, the inactivation curve was linear, while with plate count agar, the inactivation curve appeared biphasic. This might also influenced their *D*-value evaluation. In our case, at the evaluated dose range (3.8 – 8.2 kGy at 80 keV and 6.0 – 9.8 kGy at 200 keV), although the resistance was different, the inactivation curves were all $\log_{10}$ linear. However, the possibility of a potential biphasic behavior out of the evaluated dose range cannot be excluded. Nevertheless, these influences mean that the physiological status of microorganisms should also be considered when evaluating the effectiveness of new decontamination technologies such as LEEB.

Kinetic Energy

Within the current limitations on dosimetry and the impact this might have on *D*-value calculations, our results showed that the kinetic energy level does not influence significantly the spore resistance for half of the investigated samples. No clear trend was found for the other half of samples, as 80 keV lead to a higher inactivation efficiency for *G. stearothermophilus*, while 200 keV lead to higher inactivation efficiencies for *B. pumilus* (mDSM, 30°C) and *B. subtilis* (mDSM, 37°C). Different theories concerning the influence of energy level of electrons on inactivation efficiency were proposed by previous studies (Nikjoo and Goodhead, 1991; Urgiles et al., 2007; Nikjoo and Lindborg, 2010; Krieger, 2012; Bellamy and Eckerman, 2013). A previous research stated that the inactivation is due to DNA bond-breakage, and these bond energies are approximately a few eV, which is considerably less than the energies of the irradiating electrons. Therefore, it should be the absorbed dose, instead of the energy of individual electrons, that determines the level of spore damage (Urgiles et al., 2007). Some other studies proposed that low-energy electrons lead to higher linear energy transfer, which is the amount of energy of an ionizing particle transferred to the biomolecule per unit distance, that in turn increases the relative biological effectiveness (Nikjoo and Goodhead, 1991; Nikjoo and Lindborg, 2010; Krieger, 2012; Bellamy and Eckerman, 2013).

Other Influencing Factors

Food matrices might also influence the spore inactivation efficiency of LEEB technology. In our study, 9 kGy at 80 keV and 200 keV could induce approximately 3 – 4 $\log_{10}$ reduction of different spore species on glass slides, while previous research revealed that approximately 9 kGy only induced

around 2 $\log_{10}$ reduction of microorganisms present on soybeans (Kikuchi et al., 2003). Previous studies also revealed a great difference in spore resistance, depending on the kind of media on which spores were irradiated (Shamsuzzaman and Lucht, 1993). In addition, the presence of O_2 during the treatment was suggested to influence the inactivation efficiency as well (Ito and Islam, 1994; Fiester et al., 2012). For example, the *D*-value of *B. megaterium* spores was increased from 1.8 to 5.1 kGy when they were vacuum-sealed under treatment (Ito and Islam, 1994). This could also be one of the reasons that the *D*-values in this study are slightly lower than some reported *D*-values from literature.

In summary, all these influencing factors should be taken into consideration when evaluating *D*-values. The *D*-values obtained from a model system might give a general information on the resistances of tested microorganisms toward LEEB treatment, but they might change their resistance significantly due to different sporulation and treatment conditions. Moreover, a more accurate dosimetry methodology should be developed for measurement of surface dose as this has a direct impact on the estimation of *D*-values. Therefore, it is very important to validate this novel LEEB technology for specific applications with the right dosimetry.

DNA as One of the Targets for LEEB Spore Inactivation

From the results, we can observe the mutant that lacking SASP, which is the major protection of spore DNA, showed significant lower resistance than that of their isogenic wildtype ($p < 0.01$). This observation indicates that DNA is one of the targets of LEEB spore inactivation, which is similar to the findings using HEEB treatment (Fiester et al., 2012). In Fiester et al. (2012), they also found structural damage of the spore inner membrane and coat, in addition to DNA fragmentation, when *B. atrophaeus* spores were treated with HEEB at 5 MeV, and all of these changes correlated with the applied dose. This finding indicates that DNA is not the only target for HEEB spore inactivation, and whether this is also the case for LEEB requires additional investigation. Moreover, other studies revealed that the mutant lacking SASP also showed increased sensitivity to other ionizing irradiations (e.g., X-ray and high-energy charged iron ions); however, it seems that the lack of SASP does not affect spores' resistance to gamma radiation (Nicholson et al., 2000; Setlow, 2006, 2007; Moeller et al., 2008, 2014).

CONCLUSION AND RECOMMENDATION

This study quantified the spore inactivation efficiency of LEEB treatment by evaluating the *D*-values for relevant species. The inactivation efficiency of LEEB technology is in a comparable range to that of the other ionizing irradiation technologies. However, the comparison between different studies should be taken with care, as disclosed in this study that several factors, including spore species and sporulation media can influence the spore inactivation efficiency of LEEB. This result indicates that for the application of this emerging technology, special attention should be paid to the choice of biological indicator, physiological state of the indicator, dosimetry, and the processing settings. Moreover, the highly efficient surface decontamination of LEEB treatment comes with a low penetration depth, which means the location of the food contaminants should also be carefully considered. The *B. subtilis* mutant experiments also revealed that one of the spore inactivation mechanisms of LEEB technology is to cause DNA damage. Future research on investigation of the nature and level of DNA damages and other damages induced by LEEB, as well as how can spores overcome the damages should be conducted to understand the inactivation mechanism of LEEB.

In general, high spore inactivation efficiency supports the application of LEEB technology for the purpose of food surface decontamination (e.g., for spices or sprouting seeds). Due to the environmental, logistical, and economic advantages of LEEB treatment, it would be a more practical alternative to other irradiation technologies for surface decontamination and could help deliver safe, minimally processed and additive-free food products.

AUTHOR CONTRIBUTIONS

YZ and ST performed the experiments with the support of all authors. GA contributed on the absorbed dose evaluation. All authors discussed the results and implications and commented on the manuscript at all stages.

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Characterization of Efficiency and Mechanisms of Cold Atmospheric Pressure Plasma Decontamination of Seeds for Sprout Production

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The consumption of fresh fruit and vegetable products has strongly increased during the past few decades. However, inherent to all minimally processed products is the short shelf life, and the risk of foodborne diseases, which have been increasingly related to such products in many parts of the world. Because of the favorable conditions for the growth of bacteria during the germination of seeds, sprouts are a frequent source for pathogenic bacteria, thus highlighting the need for seed decontamination to reduce the risk of foodborne illness. Consequently, this study focused on cold atmospheric pressure plasma (CAPP) treatment of artificially inoculated seeds in a diffuse coplanar surface barrier discharge to determine the inactivation efficiency for relevant foodborne pathogens and fungal spores. Plasma treatment of seeds resulted in a highly efficient reduction of microorganisms on the seed surface, while preserving the germination properties of seeds, at least for moderate treatment times. To characterize the mechanisms that contribute to microbial inactivation during plasma treatment, an experimental setup was developed to separate ultraviolet light (UV) and other plasma components. The combination of bacterial viability staining with confocal laser scanning microscopy was used to investigate the impact of ozone and other reactive species on the bacterial cells in comparison to UV. Further characterization of the effect of CAPP on bacterial cells by atomic force microscopy imaging of the same *Escherichia coli* cells before and after treatment revealed an increase in the surface roughness of treated *E. coli* cells and a decrease in the average height of the cells, which suggests physical damage to the cell envelope. In conclusion, CAPP shows potential for use as a decontamination technology in the production process of sprouts, which may contribute to food safety and prolonged shelf life of the product.

Keywords: cold atmospheric pressure plasma, seed decontamination, sprout production, atomic force micorscopy (AFM), dielectric coplanar surface barrier discharge

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INTRODUCTION

Increased consumption, large scale production and more efficient distribution of fresh produce have contributed to an increase in the number of illness outbreaks caused by this commodity over the past two decades (Olaimat and Holley, 2012). In particular, sprouts have gained worldwide popularity due to their nutritional values and health benefits (Kylen and Mccready, 1975;

Kocharunchitt et al., 2009). However, despite the healthy image associated with sprouts, they represent one of the most common vehicles for produce-associated bacterial foodborne illnesses, and have been identified as the primary source of pathogens in many outbreaks (Sikin et al., 2013). A variety of foodborne pathogens such as pathogenic *Escherichia coli*, *Salmonella*, *Listeria monocytogenes*, and *Bacillus cereus* are frequently isolated from sprouted seeds and documented as causative agents of outbreaks of foodborne illness associated with sprouts (NACMCF, 1999). The key aspect that increases the risk of foodborne disease from sprouts compared to other fresh produce is the rapid growth of the bacteria due to the favorable conditions during the sprouting process such as high water activity, warm temperature, neutral pH, and availability of nutrients (Prokopowich and Blank, 1991). Therefore, decontamination of seeds is requested by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 1999). Many sprout manufacturers apply 20,000 ppm of calcium hypochlorite solution to seeds before germination as recommended by the U.S. Food and Drug Administration (FDA). However, chlorine-based treatment achieves on average a reduction of 1–3 log CFU/g only and is associated with negative health effects and environmental issues (Sikin et al., 2013). Furthermore, in Switzerland and many EU countries application of chlorine as wash-based disinfectant for food production is strictly restricted due to the release of excessive amounts of potentially harmful disinfection by-products in the water (Van Haute et al., 2013).

In Japan, heat treatment represents the most common decontamination method for mung bean seeds (Bari et al., 2010). Although treatment for 5 min at 57°C or 60°C reduced *Salmonella* to <1 CFU/g, without substantial loss of germination properties, slightly higher temperatures and prolonged treatment times caused significant declines in seed germination (Jaquette et al., 1996). This narrow temperature range between treatment efficacy and seed injury makes it difficult to rely solely on heat for the elimination of pathogens. Consequently, most of the recent work in this area has involved the evaluation of combination treatments and the most successful strategy for adequate inactivation of microorganisms while maintaining germination properties is coupling heat with chlorine-based or organic sanitizers. However, such combinations turned out to not be sufficient for complete elimination of pathogens from different types of seeds (Ding et al., 2013). Although many methods have been even more effective than the FDA recommended method of 20,000 ppm hypochlorite, so far no treatment has been able to completely remove pathogens from sprout seeds while maintaining the germination capacity of the seeds (Montville and Schaffner, 2004; Bourke et al., 2018).

In conclusion, reliable methods for thorough decontamination of all types of seeds are still lacking and alternative intervention approaches have to be considered (Ding et al., 2013). Decontamination of seeds by cold atmospheric pressure plasma (CAPP) represents a promising alternative to conventional sterilization methods (Hertwig et al., 2018). Plasma is a fully or partly ionized gas, often referred to as the fourth state of matter. Besides plasmas in nature (e.g., sun, aurora borealis, lightning), there is also a large variety of technical applications ranging from fusion reactors with temperatures of more than 10^8°C to non-thermal plasma with gas temperatures below 100°C, which is also called non-equilibrium (non-thermal) or cold plasma. Such cold plasma has the unique feature of a low gas temperature, due to low-energy neutrals and ions, but high reactivity due to energetic electrons, which in turn generate a very reactive cocktail of active particles, such as charged particles, excited species, reactive neutrals and ultraviolet light (UV) photons that can cause detrimental effects on microorganisms (Hertwig et al., 2018). During the last decade, the effectiveness of cold plasma was demonstrated for the inactivation of a wide range of different microorganisms (Kayes et al., 2007; Selcuk et al., 2008; Misra et al., 2011; Tseng et al., 2012; Ziuzina et al., 2014; Butscher et al., 2016a,b). However, the precise inactivation mechanisms of cold plasma treatment and the contribution of the single components to the inactivation of microorganisms is still controversially discussed in literature (Lerouge et al., 2000; Boudam et al., 2006; Hertwig et al., 2018).

Non-thermal plasma processes have already been applied in many applications such as light sources, surface coatings, or ozone generation. In terms of food processing, non-thermal atmospheric plasma is a very recent technology (Knorr et al., 2011). However, as reviewed by Bourke et al. (2018), various studies about the application of CAPP for safe and sustainable food production were published during the last few years. The first studies on cold plasma treatment of plant seeds showed that the microbial load can be reduced while preserving the germination capacity of seeds and growth of sprouts (Selcuk et al., 2008; Bormashenko et al., 2012; Mitra et al., 2014; Butscher et al., 2016a). Moreover, several studies reported that cold plasma treatment of agricultural seeds has the potential to even improve the germination properties of the seeds and the growth parameters of seedlings (Randeniya and de Groot, 2015; Thirumdas et al., 2015; Butscher et al., 2016a).

In conclusion, the use of CAPP for controlling seed-borne microbial contamination seems to be a promising approach due to its benefits regarding efficient energy use, effective antimicrobial activity and seed health. Thus, the objectives in this study were the investigation of CAPP inactivation of relevant foodborne pathogens and fungi on lentil seeds in a diffuse coplanar surface barrier discharge (DCSBD) system, and the characterization of the microbial inactivation mechanisms by confocal laser scanning microscopy and atomic force microscopy (AFM) analysis of plasma treated bacteria.

MATERIALS AND METHODS

Organism Strains and Culture Conditions

Strains of bacteria and fungi used in this study are listed in **Table 1**. All bacterial strains were maintained at −80°C in Brain-Heart-Infusion (BHI) broth (Biolife Italiana, Italy) supplemented with 20% glycerol. Working stocks were maintained on BHI agar (Biolife Italiana, Italy) at 4°C for a maximum of 1 month. Prior to each experiment, fresh overnight cultures were prepared by inoculating an isolated colony into 10 ml BHI broth (Biolife Italiana, Italy). Cultures were incubated for 16 h at 37°C with

TABLE 1 | Organisms used in this study.

Organism	Origin	Description
E. coli ATCC 8739	DSMZ, Germany	Strain Crooks, isolated from feces
E. coli B174 FAM 21843	Agroscope, Switzerland	Heat-tolerant Serovar O178:H12
E. coli B176 NCTC 12900	Agroscope, Switzerland	Serovar O157:H7, EHEC surrogate, non-STEC (stx-, eaeA+)
L. monocytogenes WS1001	Food Microbiology, ETH Zurich, Switzerland	Serovar 4b
Salmonella enterica DT7155	Food Microbiology, ETH Zurich, Switzerland	Serovar Typhimurium
S. aureus ATCC 25923	DSMZ, Germany	FDA, strain Seattle 1945, clinical isolate
Geobacillus stearothermophilus	Merck KGaA, Germany	Suspension of endospores
Penicillium decumbens	Food Microbiology, ETH Zurich, Switzerland	Isolated from food
Aspergillus niger	Food Microbiology, ETH Zurich, Switzerland	Isolated from food

gentle shaking at 170 rpm. To verify growth of the bacteria, the optical density was measured using a Libra S22 (Biochrom; Cambridge, United Kingdom) spectrometer at 600 nm.

Aspergillus niger and *Penicillium decumbens* were grown on YGC agar plates (Biolife Italiana, Italy) incubated at room temperature. After 18 days of growth in the dark, spores were harvested by pouring 10 ml of phosphate buffered saline (PBS) over the agar plate. The spores were agitated using a spatula to dissolve them in PBS and fungal hyphae and conidia were removed from the PBS solution by filtration through four layers of Mediset® Faltkompressen (IVF Hartmann AG; Germany). To verify that hyphae and conidia were removed from the solution, the spore solution was investigated by phase contrast microscopy. Spores were quantified using a Helber chamber 0.02 mm (Ehartnack; Berlin, Germany) by counting four large squares of 5 × 5 small squares for each sample. The concentration of *Aspergillus niger* spores was determined to be on average 1.1×10^8 spores per ml PBS, whereas the *Penicillium decumbens* spore solution contained on average 5.5×10^7 spores per ml PBS.

Inoculation of Lentil Seeds

For inoculation of lentil (*Lens culinaris*) seeds, 1 g of seeds was placed in a 15 ml centrifuge tube before adding 1 ml of the accordingly diluted bacterial overnight culture or spore solution, respectively. The seeds were inoculated by placing the tube for 5 min on a plate rotator and subsequently dried by placing in a sterile petri dish under a laminar flow bench for 4 h. During the drying process, the seeds were shaken once after 2 h of drying. After the drying process, the seeds were transferred to sterile 15 ml centrifuge tubes. For low initial inoculation level experiments, overnight cultures from *E. coli* B174, *L. monocytogenes*, *S. aureus* and *Salmonella* Typhimurium were further diluted by a factor of 1:1000.

Treatment of Seeds in the Diffuse Coplanar Surface Barrier Discharge

Seed samples were treated in an atmospheric pressure DCSBD developed by Robust Plasma Systems (RPS400; Roplass s.r.o., Brno, Czechia), which is described in detail elsewhere (Šimor et al., 2002; Černák et al., 2011; Homola et al., 2012). The DCSBD consists of a high number of various microdischarges and creates a homogenous plasma layer with approximately effective 0.3 mm thickness on the top of the planar dielectric barrier which is a 96 mm × 230 mm alumina ceramic plate. The maximum nominal power directed into the plasma discharge was 400 W, which corresponded to maximum plasma intensity. Ozone and UV were measured by optical emission spectroscopy using an optical fiber (QP400-3-SR-BX, Ocean Optics) connected to a spectrometer (USB2000+XR1-ES).

Prior to the inactivation experiments, all parts in contact with seeds were sterilized using 70% ethanol. All experiments were conducted applying the DCSBD system in its original configuration at maximum power using ambient air as the operating gas. Static treatment was performed by placing dry lentil seeds equally distributed on the surface of the dielectric plate of the RPS400. After ignition of the plasma, the intensity was gradually increased to maximum by gradually adjusting the rotation swivel to 10 over a period of 30 s. The same procedure was repeated in reverse for turning off the plasma. Each side of the lentil seed was treated separately by flipping the lentil seeds after halftime using sterile tweezers. Thus, the total plasma treatment time for lentils corresponds to the total time spent on the plasma field. After plasma treatment, the seeds were placed in sterile 15 ml centrifuge tubes. For statistical purposes, all experiments were performed at least in triplicate. In each experiment, a control without plasma treatment was analyzed in parallel to calculate the logarithmic reduction of treated samples.

Determination of Logarithmic Reduction

Logarithmic reduction of treated samples was determined by quantification of numbers of microorganisms (CFU/g seeds) after treatment using the standard plate count method. For this purpose, 9 ml PBS were added to 1 g of plasma treated seeds and 1 g of the untreated control. After transferring the seeds into stomacher bags with filter (Separator 400, Grade Products LTD; Coalville, United Kingdom), the samples were homogenized using a stomacher (Smasher®, AES chemunex; Geneva, Switzerland). A decimal dilution series in PBS was performed, before plating 100 µL of the suitable dilutions on regular (83 mm) selective agar plates. Selective media were used for *E. coli* (Chromocult Agar; Biolife Italiana, Italy), *Listeria monocytogenes* (Oxford Agar; Biolife Italiana, Italy), *Salmonella* Typhimurium (XLD Agar; Biolife Italiana, Italy), and *Staphylococcus aureus* (Baird-Parker Agar; Biolife Italiana, Italy). The dilution factor was dependent on the initial concentration and the treatment time. For low initial inoculation experiments, 1000 µl were plated on large (136 mm) agar plates to quantify microorganisms at the dilution of 10^{-1}. After incubation of the agar plates, the number of colonies per plate was determined for regular agar plates containing 10 – 300 colonies and for

large agar plates containing 10 – 500 colonies. An average was calculated from plates of two adjacent dilutions whenever possible. These values were used to calculate an average of colony forming units (CFU) per g of seeds. In order to determine and compare the performance of the DCSBD treatment, the logarithmic reduction was calculated as an indicator by how many $\log_{10}$ units the initial number of bacteria has been reduced by the treatment.

Statistical Analyses

All experiments were performed at least three times. For statistical analyses of plating results, duplicates were averaged first, and mean as well as standard deviation was calculated from the three independent experimental results. As a reference, three control samples, which experienced identical sample preparation as the plasma treated seeds, were analyzed to determine the initial levels of microbial contamination. Finally, the logarithmic reduction in CFU is expressed by the difference in decimal logarithm of CFU/g for plasma treated seeds and the CFU/g determined for the untreated control samples.

Evaluation of Germination Properties of Lentil Seeds After Plasma Treatment

The assessment of seed germination was performed with untreated control seeds and plasma treated seeds by the Agroscope, Seed Quality Laboratory, Zurich, Switzerland. Prior to the seed analysis, the statistically significant sample size of 66 seeds was calculated and the analysis was performed in triplicate. In brief, seeds were placed in pairs into pleated paper, soaked in 40 mL of tap water and kept at a germination temperature of 20°C at 85% humidity in a climate chamber with an 8 h day with light (Lux = 4000) and 16 h night cycle. Seeds and seedlings were inspected after 7 days. The presence of a root indicated normal germination, whereas in the case of an absence of a root, the seeds were classified as dead.

Characterization of Microbial Inactivation Mechanisms

The antimicrobial effect of plasma could be the result of multiple inactivation mechanisms such as UV, temperature, electrons, ion bombardment and reactive species. In order to study the impact of plasma in more detail, an experimental setup was chosen that facilitated the separate investigation of UV and other plasma components. Plasma treatment of liquid suspensions of *E. coli* ATCC 8739 grown to exponential phase was performed in µ-Slide 2-well uncoated slides (ibidi; Planegg, Germany) in the RPS400 system. For selective UV treatment experiments, the slides were covered with a lid to prevent access of ozone and other reactive species to the *E. coli* cells. After 180 s treatment, aliquots of the bacterial suspensions were mixed with propidium iodide (Live/Dead BacLight Bacterial Viability Kit L13152, ThermoFischer Scientific) to determine membrane integrity or 50 mM 5-cyano-2,3-ditolyltetrazolium chloride (CTC; Fluka) for evaluation of metabolic activity. After incubation at room temperature, images of the samples were recorded using a confocal laser scanning microscope (Leica Microsystems GmbH; Wetzlar, Germany) equipped with a HCX PL FLUOTAR 100.0 1.30 oil objective. Analysis of images was performed using ImageJ Software (version 1.6.0, National Institutes of Health, United States). The percentage of bacteria showing fluorescence after plasma treatment was quantified from 10 randomly recorded images of each sample.

Atomic Force Microscopy

For AFM analysis of plasma treated bacteria, 10 µl of an *E. coli* B174 overnight culture were transferred on a microscopic glass slide (76 mm × 26 mm × 1 mm; Menzel, Germany) and distributed on an area of approximately 40 × 20 mm using a cover slip before drying at room temperature for 15 min. CAPP treatment was performed on the DCSBD system for 60 s at 100% intensity by placing the microscopic slide with the side containing the bacteria facing the planar dielectric barrier. A distance of approximately 0.3 mm to the surface of the planar dielectric barrier of the DCSBD was adjusted by placing cover slips (18 mm × 18 mm, #1; Menzel, Germany) under both ends of the microscopic slide. For visualization and comparison of the surface of identical bacterial cells prior to and after plasma treatment, images were recorded from identical positions of the glass slide by AFM using Dimension FastScan scanning probe microscope (Bruker, United States) operating in tapping mode under ambient conditions. For further evaluation of AFM images, analysis of surface roughness was carried out and a height distribution data analysis was performed for viable, untreated *E. coli* cells and *E. coli* cells after 1 min CAPP treatment on the DCSBD system. Quantification of viable counts on glass slides was performed using standard plate count method. For this purpose, glass slides were transferred to 50 ml Falcon tubes containing 40 ml PBS and 20 g of glass beads (2 mm; VWR) and vortexed (3 × 15 s) before a decimal dilution series in PBS was performed. Plating of 100 µL of the suitable dilutions was performed on regular (83 mm) selective agar plates for *E. coli* (Chromocult Agar; Biolife Italiana, Italy). For statistical purposes, all experiments were performed at least in triplicate. In each experiment, a control (no plasma treatment) was analyzed in parallel to calculate the logarithmic reduction of the treated samples.

RESULTS

Inactivation of *E. coli* on Lentil Seeds

In the first experiments, non-pathogenic *Escherichia coli* strains were used to establish the inoculation and inactivation procedure for plasma treatment of seeds in the DCSBD. In preliminary studies, we observed that a high portion of *E. coli* cells do not survive the drying process after artificial inoculation of samples. Therefore, we used the heat-tolerant strain *Escherichia coli* B174, due its higher resistance to desiccation as an important feature for the inoculation and drying procedure of seeds. In addition to high initial inoculation of seeds (5.0×10^9 CFU/g) with *E. coli* B174, also low initial inoculation (1.6×10^5

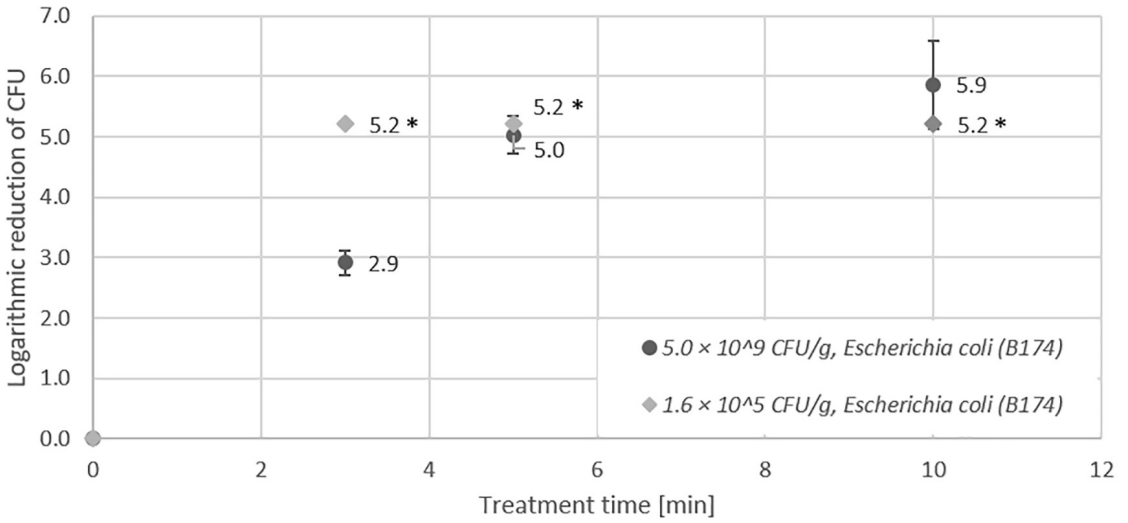

FIGURE 1 | Effect of CAPP treatment of *E. coli* B174 on lentil seeds. Inoculation was performed either as high initial inoculation (5.0×10^9 CFU/g) or low initial inoculation (1.6×10^5 CFU/g) prior to treatment of seeds for 3, 5, or 10 min in the DCSBD. Logarithmic reductions were represented as log10 CFU/g ± standard deviation. The limit of detection was 10 CFU/sample for low initial inoculation and 100 CFU/sample for high initial inoculation. Logarithmic reduction below the limit of detection is indicated by an asterisk.

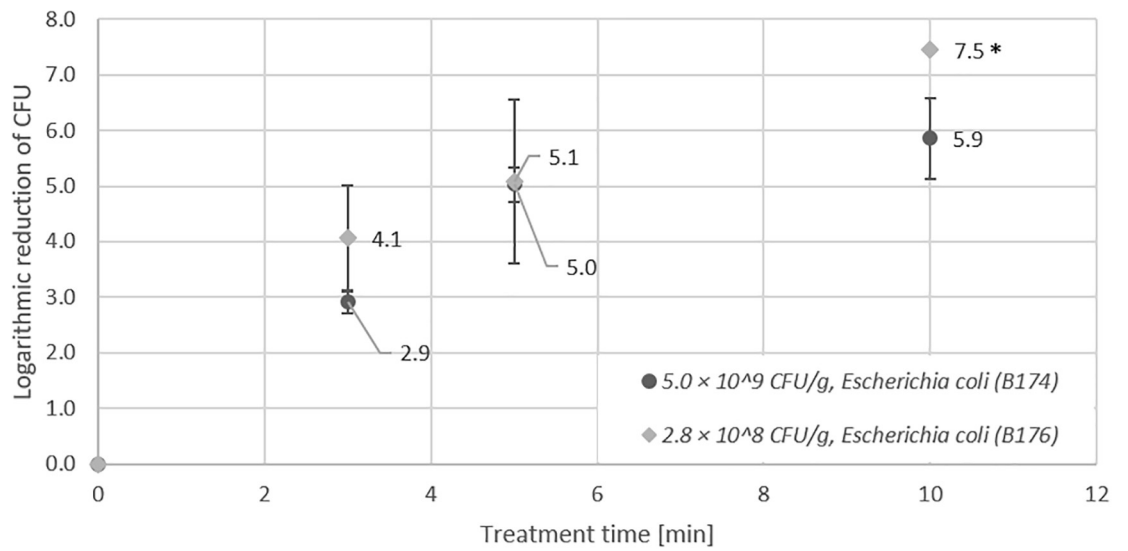

FIGURE 2 | Effect of CAPP treatment of strains *E. coli* B174 and *E. coli B176* on lentil seeds. For both *E. coli* strains a high initial inoculation of lentil seeds was performed prior to treatment of seeds for 3, 5, and 10 min in the DCSBD. Logarithmic reductions were represented as log10 CFU/g ± standard deviation. The limit of detection was 100 CFU/g. Logarithmic reduction below the limit of detection is indicated by an asterisk.

CFU/g) was performed to simulate more realistic contamination levels. For low initial inoculation samples, the detection limit (indicated by an asterisk) was already reached after 3 min (5.2 log CFU/g reduction), whereas for high initial inoculation, 5 min treatment was necessary to obtain an identical log reduction (**Figure 1**). The second *E. coli* strain (B176) used for CAPP inactivation experiments was a non-pathogenic surrogate for enterohemorrhagic *Escherichia coli* (EHEC). The maximum reduction determined for *E. coli* B176 with an initial inoculation of 2.8×10^8 CFU/g was compared to the results obtained for *E. coli* B174. As shown in **Figure 2**, the EHEC surrogate revealed a higher logarithmic reduction compared to *E. coli* B174.

Control of Foodborne Pathogens on Lentil Seeds

A major objective of this study was the determination of the inactivation efficiency for foodborne pathogens that are particularly relevant for sprout production because they are

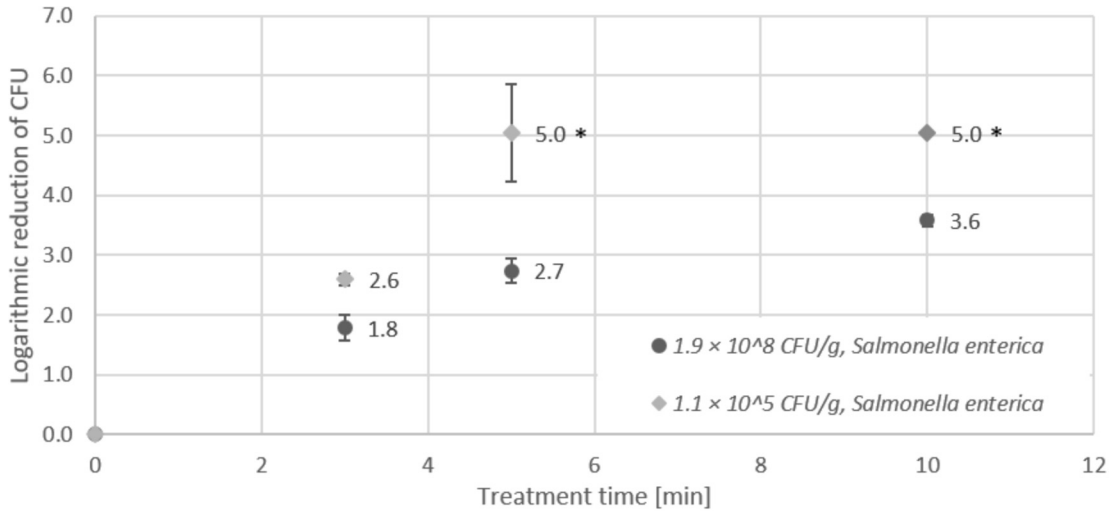

FIGURE 3 | Effect of CAPP treatment of *Salmonella enterica* on lentil seeds. Inoculation was performed either as high initial inoculation (1.9×10^8 CFU/g) or low initial inoculation (1.1×10^5 CFU/g) prior to treatment of seeds for 3, 5 or 10 min in the DCSBD. Logarithmic reductions were represented as log10 CFU/g ± standard deviation. The limit of detection was 10 CFU/g for low initial inoculation and 100 CFU/g for high initial inoculation. Logarithmic reduction below the limit of detection is indicated by an asterisk.

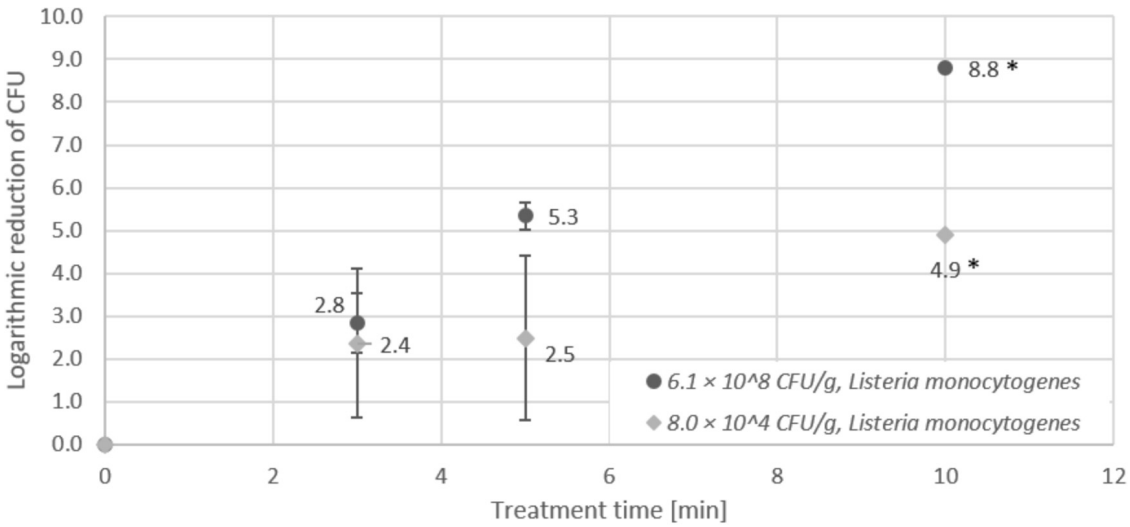

FIGURE 4 | Effect of CAPP treatment of *Listeria monocytogenes* on lentil seeds. Inoculation was performed either as high initial inoculation (6.1×10^8 CFU/g) or low initial inoculation (8.0×10^4 CFU/g) prior to treatment of seeds for 3, 5, or 10 min in the DCSBD. Logarithmic reductions were represented as log10 CFU/g ± standard deviation. The limit of detection was 10 CFU/g for low initial inoculation and 100 CFU/g for high initial inoculation. Logarithmic reduction below the limit of detection is indicated by an asterisk.

frequently linked to foodborne illnesses after the consumption of sprouts. In addition to the EHEC surrogate *E. coli* B176, lentil seeds were artificially inoculated with *Salmonella* Typhimurium, *Listeria monocytogenes* and *Staphylococcus aureus* and treated with CAPP in the DCSBD system. Also for these pathogens, the logarithmic reduction was determined for high (10^8 – 10^9 CFU/g) and low (10^4 – 10^5 CFU/g) initial inoculation on seeds.

Figure 3 shows that similar to *E. coli*, *Salmonella* Typhimurium revealed a much higher inactivation efficiency on seeds with low initial inoculation (5.0 log after 5 min) compared to the results for seeds with high initial inoculation (3.6 log after 10 min).

Plasma treatment of seeds artificially contaminated with high (6.1×10^8 CFU/g) and low (8×10^4 CFU/g) numbers of *Listeria monocytogenes* (**Figure 4**) revealed the opposite trend. Logarithmic reduction (5.3 log after 5 min) was significantly higher for high than for low initial inoculation (2.5 log after 5 min). However, in both cases the detection limit was reached after 10 min treatment time, which corresponded to a maximum logarithmic reduction of 8.8 log for *Listeria monocytogenes*.

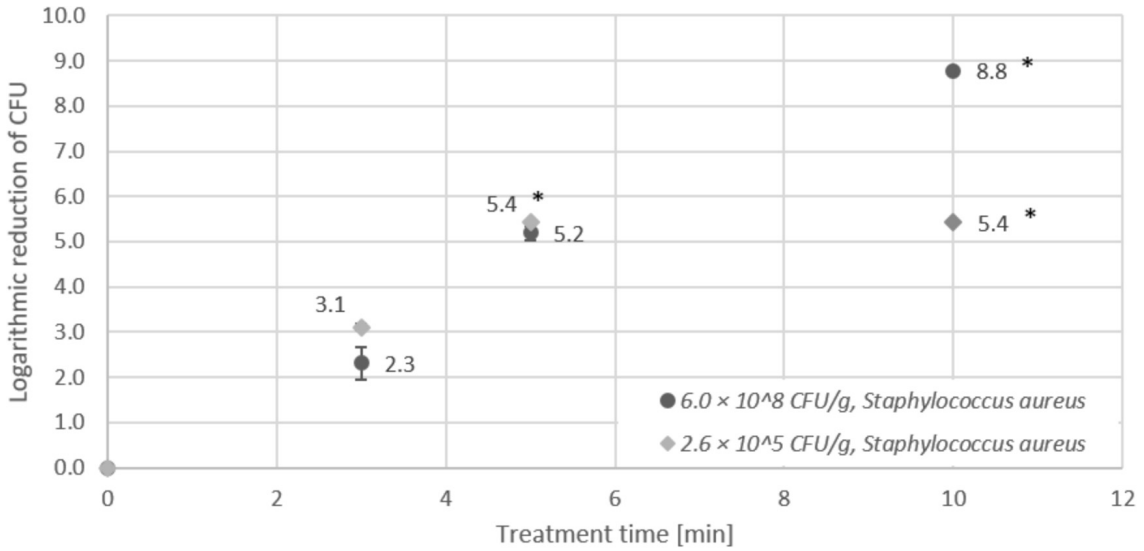

FIGURE 5 | Effect of CAPP treatment of *Staphylococcus aureus* on lentil seeds. Inoculation was performed either as high initial inoculation (6.0×10^8 CFU/g) or low initial inoculation (2.6×10^5 CFU/g) prior to treatment of seeds for 3, 5, or 10 min in the DCSBD. Logarithmic reductions were represented as log10 CFU/g ± standard deviation. The limit of detection was 10 CFU/g for low initial inoculation and 100 CFU/g for high initial inoculation. Logarithmic reduction below the limit of detection is indicated by an asterisk.

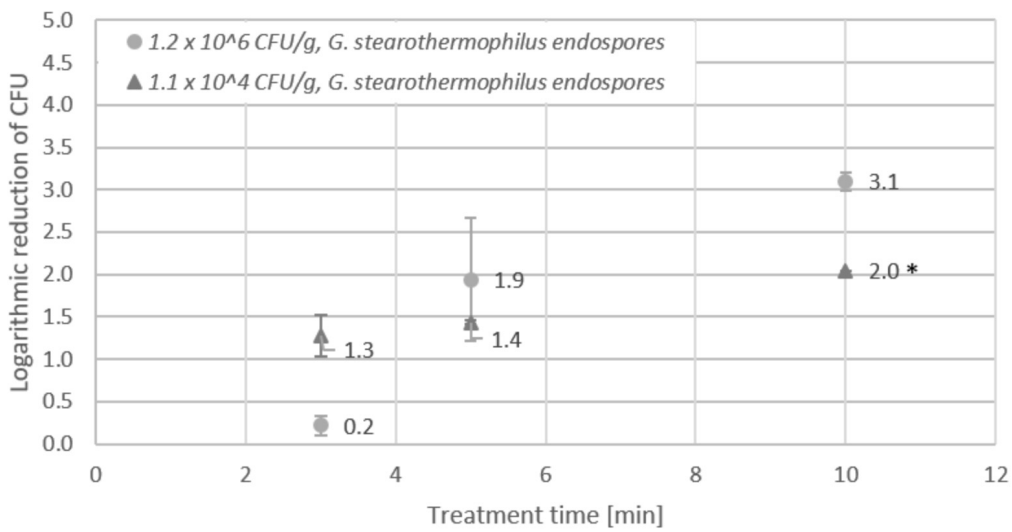

FIGURE 6 | Effect of CAPP treatment of *Geobacillus stearothermophilus* endospores on lentil seeds. Inoculation was performed either as high initial inoculation (1.2×10^6 CFU/g) or low initial inoculation (1.1×10^4 CFU/g) prior to treatment of seeds for 3, 5, or 10 min in the DCSBD. Logarithmic reductions were represented as log10 CFU/g ± standard deviation. The limit of detection for endospores was 100 CFU/g. Logarithmic reduction below the limit of detection is indicated by an asterisk.

In contrast to the results obtained for *Listeria monocytogenes* and *Salmonella* Typhimurium, CAPP treatment of *Staphylococcus aureus* on lentil seeds (**Figure 5**) revealed almost identical logarithmic reductions for high (6×10^8 CFU/g) and low (2.6×10^5 CFU/g) initial contamination of seeds. As obvious from **Figure 5**, the detection limit (<100 CFU/g) for low initial inoculation was already reached after 5 min and 5.4 log CFU/g reduction, whereas for high initial inoculation, the detection limit was reached after 10 min treatment time after a logarithmic reduction of 8.8 log CFU/g.

Inactivation of *Geobacillus stearothermophilus* Endospores on Lentil Seeds

Inactivation of *Geobacillus stearothermophilus* endospores on lentil seeds was investigated using a high initial inoculation of

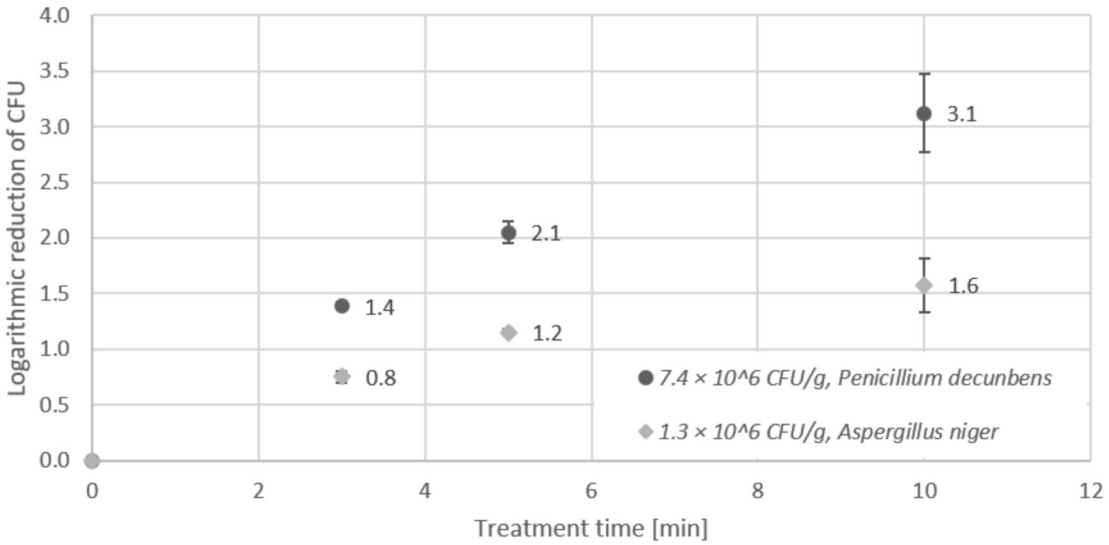

FIGURE 7 | Effect of CAPP treatment of mold spores on lentil seeds. Inoculation was performed either with 7.4×10^6 CFU/g *Penicillium decumbens* spores or 1.3×10^6 CFU/g *Aspergillus niger* spores, prior to treatment of seeds for 3, 5, and 10 min in the DCSBD. Logarithmic reductions were represented as log10 CFU/g ± standard deviation. The limit of detection was 100 CFU/g.

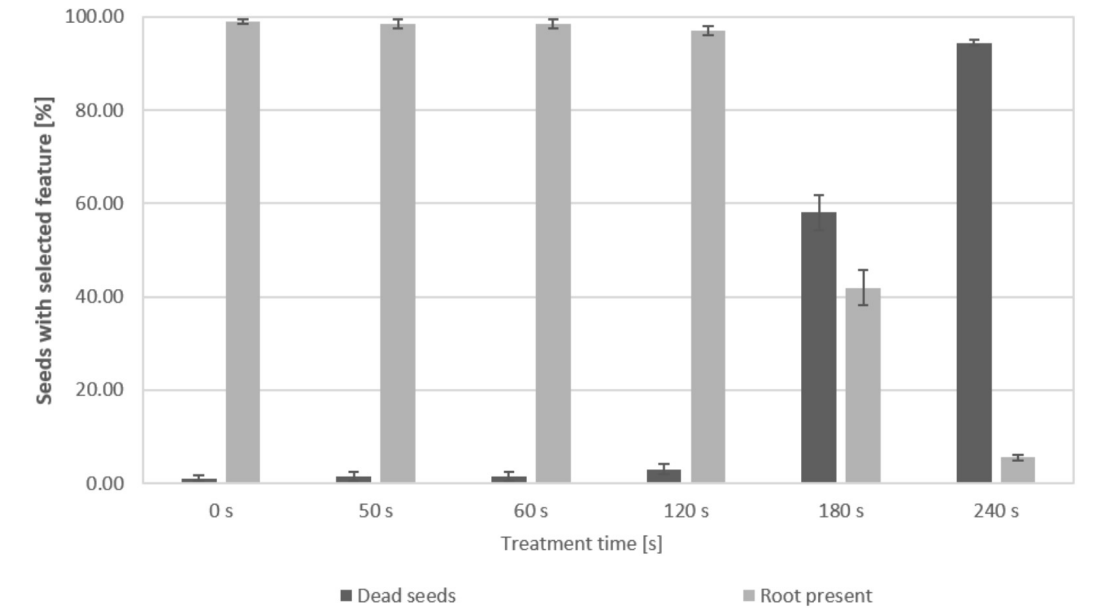

FIGURE 8 | Quantitative assessment of the germination capacity of lentil seedlings after CAPP treatment in the DCSBD. The percentage of dead seeds or germinated seeds, showing the presence of a root, was determined for untreated seeds (0 s) and seeds after increasing plasma exposure times. Error bars express the standard deviation of triplicates for each data point in a 66 seed sample.

1.2×10^6 CFU/g and a low initial inoculation 1.1×10^4 CFU/g prior to treatment of seeds for 3, 5, or 10 min in the DCSBD. After 3 min treatment, seeds with low initial inoculation revealed a logarithmic reduction of 1.3 log, whereas the logarithmic reduction seeds with high initial inoculation was only 0.2 log (**Figure 6**). Prolonged treatment of 10 min resulted in an increase of the logarithmic reduction to 3.1 log for seeds with high initial inoculation. For seeds with low initial inoculation, a logarithmic reduction of 2 log was obtained, which represented the detection limit (<100 CFU/g) for low initial inoculation.

Inactivation of Fungal Spores on Lentil Seeds

For inactivation of fungal spores, lentil seeds were contaminated with a spore solution prepared either from *Aspergillus niger* or

Penicillium decumbens. Determination of spore numbers in a microscopic counting chamber revealed 5.5×10^7 spores/ml for the spore solution from *Penicillium decumbens*, and 1.1×10^8 spores/ml for *Aspergillus niger*. After inoculation and drying of the seeds, the recovery was 7.4×10^6 CFU/g for *Penicillium decumbens* and 1.3×10^6 CFU/g for *Aspergillus niger*. In contrast to the inactivation of bacterial pathogens, the logarithmic reduction determined for the fungal spore contaminations was significantly lower and the detection limit (<100 CFU/g) was not reached, even after 10 min treatment in the DCSBD (**Figure 7**). Inactivation of *Penicillium decumbens* spores on lentil seeds revealed a maximum logarithmic reduction of 3.1 log CFU/g after 10 min treatment, whereas *Aspergillus niger* spores showed a maximum reduction of 1.6 log CFU/g after 10 min.

Influence of Plasma Treatment on the Germination Capacity of Seeds

The germination capacity was defined as the percentage of lentil seedlings with the presence of a root after 7 days of germination. **Figure 8** shows the results obtained for untreated control seeds and seeds treated with an increasing duration of CAPP in the DCSBD. While 120 s treatment time still resulted in 90% germination, the germination capacity dropped to 42% after 180 s exposure of seeds to CAPP. After 240 s CAPP treatment, 95% of the seeds did not germinate.

Characterization of Mechanisms of Microbial Inactivation by Plasma

Due to the complexity of the plasma multiple components such as UV, temperature, electrons, ion bombardment and reactive species are known to contribute to the antimicrobial effect. In order to improve the understanding of the mechanisms that contribute to the inactivation of microorganisms, an experimental setup was used to selectively investigate the impact of UV and other plasma components on *E. coli* in comparison to untreated bacteria and bacteria treated by whole plasma. After the different treatment regimes, the viability of the bacteria was investigated by performing a CTC stain as an indicator of an intact metabolic activity of the bacterial cells, while a PI stain was used as an indicator of the membrane integrity of the bacteria. **Table 2** shows that whole plasma treatment resulted in 81% reduction of viable cells as indicated by a positive CTC stain for 19% of treated cells. Accordingly, 73% of plasma treated cells showed a positive PI stain, which indicated the loss of membrane integrity. Using the experimental setup to shield from UV, only 10 % of bacterial cells showed a PI signal, whereas 74% of treated bacteria were still viable, as indicated by the CTC signal. The lack of CTC signals from *E. coli* cells after selective UV treatment indicated complete inactivation of the bacteria, which was confirmed by a positive PI stain for 99% of the bacteria treated by this experimental setup.

TABLE 2 | Percentage of *E. coli* cells showing a fluorescence signal after treatment.

Stain	No treatment	Full plasma	UV	Plasma without UV
CTC-stain	95.2% ± 1.8	19.3% ± 3.2	n.d.	74.1% ± 12.1
PI-stain	0.7% ± 0.7	72.7% ± 12.4	98.6% ± 2.3	10.2% ± 6.4

n.d., no signal detected.

Atomic Force Microscopy Analysis of Plasma Treated Bacteria

Atomic force microscopy was carried out to investigate whether CAPP treatment results in visible changes of the bacterial cell morphology. In order to compare the cell surface topology of identical *E. coli* B174 cells, images were taken from identical positions of the glass slides carrying the immobilized bacteria prior to and after CAPP treatment on the DCSBD system (**Figures 9A,B**). Treatment of bacteria on the glass slides for 1 min resulted in the reduction of viable *E. coli* below the detection limit, which corresponds to 5 log reduction. In particular, the 3D images shown in **Figures 9D,E** revealed visible changes in the topography of the cell surface after CAPP treatment, which suggests that there is physical damage to the cell envelope. This observation is further corroborated by the analysis of the height of the bacterial cells deduced from the AFM imaging before and after CAPP treatment (**Figure 9C**), which revealed a 20 nm decrease in the average height of *E. coli* cells after treatment.

DISCUSSION

A major goal of this study was to evaluate CAPP generated by a DCSBD system for its suitability as a decontamination technique for seeds used for sprout production. For this reason, the inactivation efficiency for CAPP treatment of relevant foodborne pathogens artificially inoculated on lentil seeds was investigated on a DCSBD system. Besides microbial decontamination, the germination capacity of seeds was assessed after CAPP treatment since it is an important feature for sprout production (NACMCF, 1999). In order to simulate more realistic contamination levels for bacterial pathogens on seeds, a low initial inoculation (10^4 – 10^5 CFU/g seeds) was applied in addition to a high initial inoculation (10^8 – 10^9 CFU/g seeds), which was necessary to determine the respective maximal logarithmic reduction. The results from the decontamination experiments performed in this study demonstrated an overall high efficiency of CAPP for the inactivation of bacterial pathogens. A maximum logarithmic reduction of 8.8 log after 10 min CAPP treatment was observed for seeds with high initial inoculation of the gram-positive bacteria *Listeria monocytogenes* and *Staphylococcus aureus*. The highest logarithmic reduction of 5.2 log after a shorter CAPP treatment of 3 min was obtained for gram-negative *E. coli*, whereas lentils inoculated with endospores of *Geobacillus stearothermophilus* revealed only low logarithmic reductions between 1 and 2 log after 5 min treatment time.

The results obtained in this study correspond well to the treatment of other granular foodstuffs in similar DCSBD systems. The CAPP treatment of black pepper resulted in logarithmic reductions of >5 log for *E. coli* and *Salmonella*, whereas endospores revealed about 2 log reduction

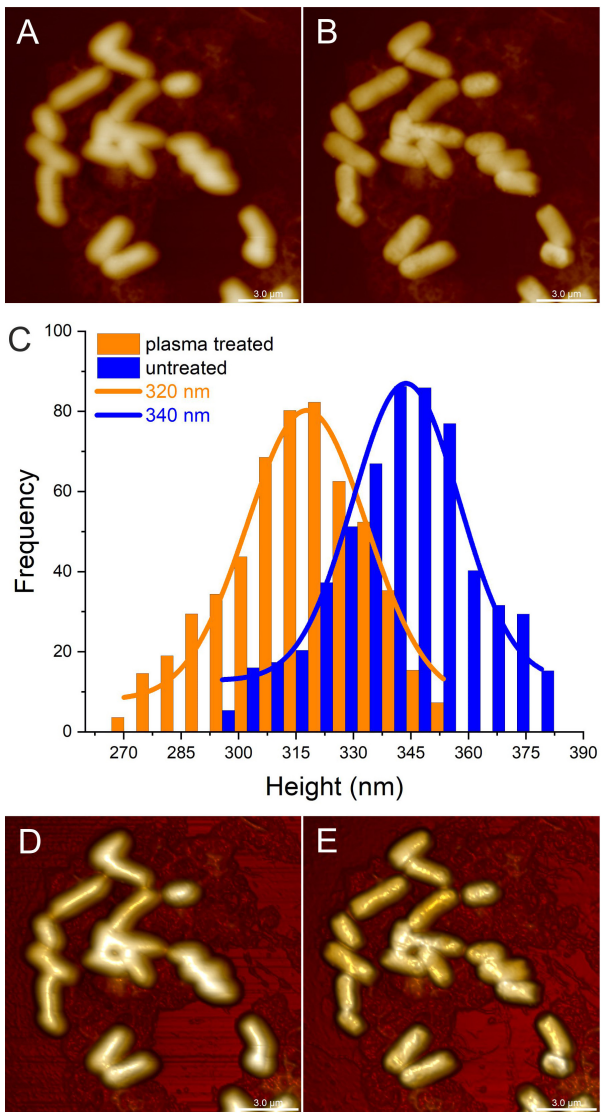

FIGURE 9 | Atomic force microscopy (AFM) imaging of identical *Escherichia coli* cells on a microscopic slide before and after cold atmospheric pressure plasma (CAPP) treatment in the DCSBD system. The upper panel of images represents 2D scans from untreated *E. coli* cells **(A)** and after 1 min **(B)** CAPP treatment as directly generated by AFM. Plot C shows the respective height distribution for untreated *E. coli* cells (blue columns) and *E. coli* cells after 1 min CAPP treatment (orange columns), where the frequency represents the number of *E. coli* cells with a certain height. In comparison to the average height of 340 nm determined for untreated *E. coli* cells (blue line), the average height of *E. coli* cells after 1 min CAPP treatment was decreased by 20 nm resulting in an average height of 320 nm (orange line). The 3-dimensional scans from untreated *E. coli* cells **(D)** and cells after 1 min CAPP treatment **(E)** allow better visualization of the surface of the *E. coli* cells and revealed an increase in the roughness of the cell surface after plasma-treatment. All scale bars represent 3 µm.

(Mosovska et al., 2018), and the inactivation of *Salmonella* Enteritidis PT30 on the surface of unpeeled almonds by CAPP treatment achieved >5 log reduction of *E. coli* after 15 min treatment of unshelled almonds (Hertwig et al., 2017).

The investigation of the impact of high and low initial inoculation numbers of bacteria on the inactivation efficiency revealed controversial results, which showed a higher logarithmic reduction for treatment of seeds with low initial inoculation for *E. coli* and *Salmonella* Typhimurium (**Figures 1**, **2**). In contrast, higher logarithmic reduction was observed for high initial inoculation with *Listeria monocytogenes* (**Figure 3**), whereas logarithmic reduction of *Staphylococcus aureus* was not dependent on the initial number of bacteria on the seeds. In a study on the effect of cell surface loading and phase of growth in cold atmospheric gas plasma inactivation of *Escherichia coli* K12, varying numbers of *E. coli* deposited on the surface of membrane filters were exposed to the plume from a cold atmospheric gas plasma (Yu et al., 2006). The authors observed a decrease in reduction by 1 log for higher cell numbers and explained this observation by stacking of bacteria on top of each other in case of high cell numbers (e.g., 9.5×10^7 CFU/ml). Similar results were reported by another study on the application of atmospheric-pressure glow discharges (APGD) for the inactivation of *Bacillus subtilis* endospores, which showed that high microbial loading may lead to a stacking structure that acts as a protective shield against the APGD treatment (Deng et al., 2005). This is in accordance with the CAPP treatment of *Geobacillus stearothermophilus* endospores on lentil seeds in this study, at least for a short treatment time of 3 min. Furthermore, Fernandez et al. (2012) demonstrated that increasing concentrations of *Pseudomonas fluorescens* cells added to a *Salmonella* population of 10^5 CFU/filter resulted in an exponential decrease in the rate of killing of the *Salmonella* cells. Fluorescence microscopy of the bacteria on polycarbonate membrane filters showed that, unlike single dispersed cells observed at low cell densities, at higher cell densities bacteria were present in a multilayered structure (Fernandez et al., 2012). This stacking phenomenon could explain the reduced inactivation by the plasma, since the top layer may present a physical barrier that protects underlying cells. Since in this study comparable numbers of bacteria were applied for high initial inoculation experiments, it is likely that stacking may have contributed to the lower logarithmic reduction observed for high numbers of *E. coli* and *Salmonella* Typhimurium. A potential explanation for the observation that the stacking effect was less pronounced for *Staphylococcus aureus* might be the fact that staphylococci are much smaller cells with only about half the areal footprint of gram-negative rods. The smaller cells may provide less shielding against the plasma treatment and therefore, the shape and the size of bacterial cells may play a role in the stacking phenomenon, rather than subtle species-dependent cell characteristics. However, although *Listeria* rods are smaller cells compared to rods of *E. coli* and *Salmonella,* this difference hardly explains the contrary effect observed for high inoculation with *Listeria* and *E. coli*.

Due to the known differences in the cell wall structure, it is generally believed that gram-negative bacteria are more sensitive to CAPP than gram-positive bacteria (Hertwig et al., 2018). Consequently, several studies report higher susceptibility of gram-negative bacteria to cold atmospheric air plasma, leading to a more efficient reduction of gram-negative bacteria such as *Salmonella enterica* and *Escherichia coli* compared to

gram-positive bacteria such as *Listeria monocytogenes* (Fröhling et al., 2012; Ziuzina et al., 2014). However, this assumption is not supported by the results from this study, which did not indicate a more efficient inactivation of gram-negative bacterial species in general. The logarithmic reduction determined for the gram-positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes* was comparable to gram-negative *Escherichia coli*, whereas gram-negative *Salmonella* Typhimurium turned out to be the least susceptible bacteria tested in this study, at least for high initial inoculation values. This finding is in accordance with the results from a study on the inactivation of foodborne pathogens using atmospheric uniform glow discharge plasma that did not observe differences in the inactivation efficiency for gram-positive and gram-negative bacteria (Kayes et al., 2007). The investigation of cold atmospheric air plasma sterilization against spores and other microorganisms of clinical interest revealed also no indication of a more efficient inactivation of gram-negative bacteria (Klampfl et al., 2012). The effect of 30 s of plasma treatment varied among all tested gram-negative and gram-positive bacteria from about 4–6 log CFU/g reduction. However, fungal cells like *Candida albicans* generally turned out to be more resistant than bacterial cells, and bacterial endospores turned out to be less susceptible than *C. albicans*. This is in accordance to the results from this study, which revealed a higher inactivation efficiency for vegetative cells of bacteria than for fungi and bacterial endospores, which revealed a similar logarithmic reduction using the DCSBD system. Further support for the observed differences in the susceptibility of different microorganisms to CAPP is provided by a study of Lee and colleagues on the sterilization of bacteria, yeast, and bacterial endospores by CAPP, who reported a similar trend of a decreasing efficiency from bacteria to yeast and bacterial endospores (Lee et al., 2006). However, they did not observe a correlation of the inactivation of *B. subtilis* spores with the initial inoculation concentration like it was reported by the aforementioned study from Deng et al. (2005).

Besides the different plasma sterilization systems used for treatments, the observed differences in the efficiency of treatment might be explained by the different substrates that carried the microorganisms, which was flat nitrocellulose membranes compared to lentil seeds used in this study. In the study of Ziuzina et al. (2014), artificially inoculated tomatoes and strawberries were treated with CAPP. The influence of substrate shape and properties on the efficacy of plasma inactivation was already shown in a previous study investigating plasma inactivation of bacterial endospores on wheat grains and polymeric model substrates in a dielectric barrier discharge (Butscher et al., 2016a). Furthermore, it was also shown that moisture content is an important parameter in CAPP treatment that directly impacts the efficiency of CAPP decontamination. Water can trigger a liquid chemistry and may affect the stability of plasma generated bactericidal species, such as reactive oxygen and nitrogen species. On the other hand, it can lead to quenching of the plasma which results in a decrease in plasma intensity as the energy is redirected to the rotational and vibrational excitation of molecules instead of ionization (van Gils et al., 2013; Butscher et al., 2016a).

The lower inactivation efficiency of CAPP treatment for fungi compared to bacteria reported by other studies (Lee et al., 2006; Klampfl et al., 2012) is also consistent with the results from this study. With maximum logarithmic reductions of 1.6 log for *Aspergillus niger* and 3.1 log for *Penicillium decumbens* spores on lentil seeds, the treatment efficiency was significantly lower compared to the inactivation of bacteria, indicating that fungal spores appear to be more resistant to non-thermal plasma. Compared to *Penicillium decumbens,* the values for logarithmic reduction of *Aspergillus niger* spores were approximately cut in half (**Figure 7**). This observation is controversial to the results from another study on the inactivation of *Aspergillus* spp. and *Penicillium* spp. on seeds using low pressure cold plasma (LPCP), which revealed no significant differences in the inactivation of the two mold species (Selcuk et al., 2008). However, the reported logarithmic reductions of 1 log CFU/g after 5 min and 1.5 log CFU/g after 10 min treatment reported by Selcuk et al. (2008) were in the same low range as in our study for *Aspergillus niger* (**Figure 7**). Higher resistance of fungal spores to cold plasma compared to bacterial cells is also reported by a recent review on the potential of cold plasma for safe and sustainable food production (Bourke et al., 2018). However, a previously published study on the decontamination of *Aspergillus flavus* and *Aspergillus parasiticus* spores on hazelnuts in an atmospheric pressure fluidized bed plasma reactor revealed significantly higher reductions with 4.5 log CFU/g for *A. flavus* and 4.2 log CFU/g for *A. parasiticus* after 5 min treatment time (Dasan et al., 2016). In contrast to previous studies on the inactivation of fungal spores, more recent work of Zahoranová and colleagues on the effect of CAPP treatment on maize and wheat seeds revealed complete inactivation of 10^6 spores/g of seeds for *A. flavus* and *A. alternata* after 5 min CAPP treatment (Zahoranová, 2016, 2018). Furthermore, these studies confirmed the observation that *Aspergillus* spp. seem to be less susceptible to CAPP treatment than other fungal genera like *Fusarium* or *Penicillium*. The results from these studies demonstrate that although fungal spores seem to be less susceptible to cold plasma treatment than bacteria, the CAPP technology might be adapted accordingly to succeed also in the decontamination of difficult-to-treat target organisms. This was also shown by the successful application of CAPP treatment for the inactivation of bacterial endospores (Butscher et al., 2016b).

In the present study, the heat and desiccation resistant *E. coli* strain B174 strain revealed a slightly lower logarithmic reduction compared to the EHEC surrogate *E. coli* B176. Laroussi and Leipold (2004) concluded that heat has only a small effect on the inactivation of bacteria while reactive oxygen and nitrogen species play the more important role for CAPP treatment in dielectric barrier discharge (DBDs) (Laroussi and Leipold, 2004). However, in a previous study using an in-house developed DBD device for CAPP treatment of seeds (Butscher et al., 2016a), temperature measurements revealed that the environment in which the samples are treated can reach temperatures that may already contribute to an inactivation of microorganisms and at the same time negatively impact the germination capacity of seeds. Furthermore, it was shown that in a DBD plasma, the flow rate of the work gas correlates to the gas temperature,

meaning a higher gas flow rate results in an overall cooler plasma (Butscher et al., 2016b). The treatment of seeds artificially inoculated with *E. coli* in the DBD system resulted in logarithmic reductions between 1 and 3 log CFU/g for different types of seeds, with a maximum logarithmic reduction of 3.4 log CFU/g for cress seeds after 10 min treatment time (Butscher et al., 2016a). Thus, the higher reduction rate of 10^5 CFU/g after 3 min treatment determined for *E. coli* on lentil seeds in this study suggest that the DCSBD system generated a stronger plasma. The assumption that the DCSBD system used in this study produced a stronger CAPP is further supported by the observation of a stronger impact of the CAPP treatment in the DCSBD on the germination capacity of treated seeds. In contrast to a higher germination capacity observed after 3 and 5 min treatment in the DBD plasma (Butscher et al., 2016a), the germination capacity of seeds decreased already after 3 min treatment in the DCSBD plasma applied in this study. A recent study on the application of CAPP for treatment of cucumber and pepper seeds demonstrated that short treatment times preserved or even increased the germination capacity of seeds, whereas for longer treatment times the germination capacity was negatively influenced by heating, ozone and UV radiation produced by the plasma (Štěpánová et al., 2018). High temperatures may also contribute to the observed decrease in the germination capacity after 3 min CAPP treatment in this study. Hence, measures to prevent overheating of seeds, e.g., by mixing on the dielectric plate of the DCSBD, either mechanically or by an airstream should counteract low germination percentage of seeds. Also cooling of the alumina ceramic dielectric plate on which the plasma is generated in future experiments might provide a potential solution for minimizing the detrimental impact of prolonged CAPP treatment on the germination capacity of seeds.

Although we achieved the inactivation of bacteria, endospores, and fungal spores while preserving the germination capacity of seeds when treatment parameters were optimized, the inactivation mechanisms are still poorly understood and this is mainly attributed to the complexity of plasma. CAPP is a reactive cocktail of different components generated by the plasma such as UV photons, charged particles, radicals and other reactive nitrogen, oxygen and hydrogen species, which can act individually and/or synergistically to cause detrimental effects on microorganisms (Hertwig et al., 2018). However, the precise mechanisms of cold atmospheric plasma treatment and the contribution of the single components to the inactivation of microorganisms is still controversially discussed in literature (Lerouge et al., 2000; Boudam et al., 2006; Hertwig et al., 2018).

In order to simplify the investigation of plasma inactivation mechanisms, Schneider and colleagues developed an experimental setup to separate VUV photons from the reactive particles in a plasma jet with Helium gas flow, where neutral particles were pushed through a side channel, allowing only the VUV photons to continue through the direct channel (Schneider et al., 2011). Under the conditions tested, VUV and UV photons alone had only a weak impact on *E. coli* monolayers on agar plates. In contrast, ROS-only and combined treatment revealed that the cells were most probably inactivated by ozone at larger distances from the jet and by a combined effect of ozone, atomic oxygen, and some other possible impurities in the region close to the jet axis. This assumption is further supported by results from a study of Laroussi and Leipold (2004), in which emission spectroscopy and gas detection was used to evaluate important plasma inactivation factors such as UV radiation and reactive species. The measurements indicated that for non-equilibrium, atmospheric pressure air plasmas oxygen-based and nitrogen-based reactive species play the most important role in the inactivation process (Laroussi and Leipold, 2004). Furthermore, microscopic characterization of CAPP inactivation of individual bacterial spores suggested that ROS were the CAPP component causing the most spore killing, with UV-A photons and charged particles being of lesser importance (Wang et al., 2016). To facilitate the selective investigation of the impact of UV and other plasma components (e.g., ozone) on *E. coli* cells, an experimental setup was used to compare their effect with untreated bacteria and bacteria treated by whole plasma in preliminary experiments. For this reason, ozone and UV were measured by optical emission spectroscopy. The results obtained from the separate treatment are summarized in **Table 2**. Plasma treatment, while shielded from UV, resulted in an inactivation of 26% of the bacterial cells, indicated by the absence of a CTC signal for metabolic activity. Propidium iodide staining of the respective samples revealed a damaged membrane in 10% of treated bacteria. Considering that there was a physical barrier between the plasma and bacterial liquid suspension, our study resulted in an indirect treatment of the bacteria and thus, the distance to the sample likely played a role. With a larger distance, there were probably less reactive species that could reach the bacteria in this setup, whereas UV had a shorter distance to travel in order to reach the sample. Furthermore, although it is commonly known that UV has an effect on DNA, it has been shown that UV affects the membrane integrity in bacteria as well (McKenzie et al., 2016). This might explain the high percentage of bacteria stained by PI after selective UV treatment. On the other hand, an experimental study on the inactivation of bacteria on the surface of agarose gels using dc corona discharge showed that neither UV radiation, ozone or H_2O_2 nor other neutral active species alone produced by the corona discharge had an effect on the viability of the bacteria (Dobrynin et al., 2011). Therefore, the strong impact of UV observed in this study (**Table 2**) might have been favored by the experimental setup and the substrate used in these preliminary experiments. For the selective investigation of UV treatment, the bacteria were applied as liquid suspensions in µ-Slide 2-well uncoated slides covered with a lid to prevent access of ozone and other reactive species. Compared to the more realistic setup of inoculation of bacteria on the seed surface, where the bacteria may be protected from UV radiation by hiding in fissures and cracks on the seed surface (Butscher et al., 2016a), the bacteria in the liquid suspension were readily accessible by the generated UV radiation. This high inactivation was also observed in another study where the treatment of *E. coli* in liquid media inside a sealed package with a dielectric barrier discharge atmospheric cold plasma (DBD-ACP) resulted in the inactivation of high concentrations of *E. coli* in seconds (Ziuzina et al., 2013). Furthermore, in a humid environment, bacterial cells are much more susceptible

to elevated temperatures compared to dry conditions, where higher temperatures are less detrimental. This is supported by several studies that investigated dry heat inactivation of bacteria (Beuchat and Scouten, 2002; Bang et al., 2011; Choi et al., 2016; Hong and Kang, 2016). Thus, the experimental setup used for the investigation of isolated CAPP components in this study might have caused an increase in the temperature of the liquid suspension that contributed to the inactivation of the bacteria by membrane damage, as suggested by the high number of bacteria showing a positive PI stain. Additionally, it would be expected that UV would have a different effect on bacterial cells in a liquid suspension compared to the monolayer of bacterial cells on the surface of an agar plate used by the aforementioned study of Schneider et al. (2011). Consequently, further experiments using an improved setup will be necessary in the future to determine the contribution of the different plasma components to the inactivation of microorganisms during CAPP treatment. Not only would an improved setup be needed to better understand the inactivation mechanisms but also the use of different bacteria since the vulnerable cell envelope of gram-negative bacteria might have favored this effect. In a recent study on the inactivation efficiency of in-package high-voltage atmospheric cold plasma (HVACP) and the role of intracellular ROS (Han et al., 2016), the authors reported differences in the mechanisms of inactivation by HVACP observed for different bacterial species. Reactive species were found to either react primarily with the cell envelope or to damage intracellular components. While the inactivation of *E. coli* was mainly due to cell leakage after damage of the cytoplasmic membrane and only low-level DNA damage, the inactivation of *Staphylococcus aureus* cells was primarily due to intracellular damage with significantly higher levels of intracellular ROS observed. However, for both bacteria studied, an increasing treatment time had a positive effect on the intracellular ROS levels generated (Han et al., 2016). Likewise, it was recently shown for *Aspergillus flavus* that CAPP treatment has an impact on cell surface structures, cell wall, and plasma membrane, inflicting injury on hyphal cells that causes oxidative stress and finally cell death at higher CAPP doses (Simoncicova et al., 2018). The results indicated that plasma treatment resulted in accumulation of intracellular ROS and showed that plasma-generated oxidants attack lipids and cause an increase in cell membrane permeability. Furthermore, DNA fragmentation occurred in plasma treated cells.

For further evaluation of the inactivation mechanisms of CAPP on *E. coli*, identical cells were then analyzed by AFM, before and after CAPP treatment. The resulting AFM images revealed distinct morphological alterations in the cell surface topology after plasma exposure as shown for treated *E. coli* cells in the 2D and 3D AFM images (**Figure 9**). In particular the comparison of 3D AFM images from *E. coli* cells before treatment (**Figure 9D**) and after treatment (**Figure 9E**) revealed a rough surface with indentations and swellings all-around the cell surface. The 3D AFM images indicate that the cell surface of the bacteria is considerably affected by the CAPP treatment, showing areas of physical damage distributed over the whole surface. A possible interpretation of this observation might be the partial rupture of the cell wall and release of cytoplasmic content to the outside. This observation was further corroborated by the analysis of the height distribution of *E. coli* cells before and after treatment as calculated from the AFM image data (**Figure 9C**), which revealed a decrease in the average height for *E. coli* cells after CAPP treatment. The observed impact of CAPP treatment on the morphology of the bacterial cells was comparable to other investigations on the impact of plasma treatment on bacteria (Galvin et al., 2013; Cahill et al., 2014). Furthermore, the determination of viable counts after CAPP treatment done in parallel revealed a 5 log reduction after 60 s CAPP treatment, indicating that the bacterial cells recorded after CAPP treatment were dead cells. However, even though the results from the AFM analysis indicate that damage of the cell surface leads to killing of the bacteria, it cannot be undoubtedly concluded that the observed damages are causative for the inactivation of the bacteria. Using Raman spectroscopy and phase-contrast microscopy to characterize CAPP inactivation of bacterial endospores, it was observed that damages observed by microscopy may appear to occur only after spores are already dead due to other causes (Wang et al., 2016). Therefore, the precise mechanisms whereby the spores are killed by CAPP treatment still remains unclear.

Overall, the results from this study indicated that CAPP treatment has the potential for efficient reduction of microorganisms on the seed surface, while preserving the germination properties of seeds, at least for moderate treatment times. The characterization of the inactivation mechanisms by preliminary experiments combining a setup for the separate investigation of CAPP components and confocal laser scanning microscopy analysis indicated that UV radiation is an important component of CAPP, while the application of AFM demonstrated that physical damage of the bacterial cell envelope occurs as a consequence of CAPP treatment of bacterial cells. However, it is important to note that the results obtained in this study provide only preliminary evidence for the role of selective plasma components to the inactivation of microorganisms with CAPP treatment, but the detailed mechanisms of how killing occurs in microorganisms is still not completely clear.

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) recommends hypochlorite treatment for seeds used for sprouting (NACMCF, 1999). In a study on the decontamination of alfalfa sprouts artificially inoculated with approximately 10^8 CFU/g *E. coli* by a combined hypochlorite and lactic acid treatment, a reduction of 4 – 6 log CFU/g was achieved after 15 – 25 min treatment, while retaining a high germination capacity of the seeds (Lang et al., 2000). Using CAPP in this study, it was possible to achieve a similar log reduction after a much shorter treatment time of 3 min. However, as discussed by a recent review (Bourke et al., 2018), an improved antimicrobial efficiency due to an increase in treatment duration or input power usually results in an increase of the negative impact on the germination capacity of seeds, which is the most important feature for sprout production. Furthermore, before implementing on an industrial scale, another remaining challenge is the construction of systems that guarantee uniform plasma treatment during continuous processing of seeds. In conclusion, CAPP technology is still under development and

has a great potential for further optimization that will allow to maintain the germination capacity of seeds, while providing an effective surface decontamination that contributes to safe and sustainable sprout production.

AUTHOR CONTRIBUTIONS

DB, PvR, and MS were responsible for the study conception. MS and DB conceived the experimental design. AW and JB were responsible for data acquisition. DK and AB-M performed sprouting analyses. JA performed the AFM analyses. AW, JB, DB, GO, and MS analyzed and interpreted the data. AW and MS drafted the manuscript. All authors provided critical revisions and approved the manuscript.

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Inhibition or Stimulation of Ochratoxin A Synthesis on Inoculated Barley Triggered by Diffuse Coplanar Surface Barrier Discharge Plasma

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Ochratoxin A (OTA) is one of the most abundant food-contaminating mycotoxins. Besides their high toxicity, mycotoxins are highly stable to physical, chemical or biological detoxification. Therefore, the treatment with cold atmospheric plasma could be one approach to reduce the amount of mycotoxins in different products. The aim of this study was to determine the influence of cold atmospheric plasma on the inactivation of *Aspergillus niger* and *Penicillium verrucosum* inoculated on barley and their production of OTA. Inoculated barley was treated with plasma generated by dry air, CO_2 or $CO_2 + O_2$ for 1 or 3 min and stored for up to two weeks at 9, 25, or 37°C. Three minutes of air plasma treatment effectively significantly reduced the total mold count of both microorganisms by 2.5–3 log cycles. The production of OTA from *A. niger* was only low, therefore the treatment effect was indistinguishable. The treatment of *P. verrucosum* on barley after an incubation of five days using a $CO_2 + O_2$ plasma resulted in a reduction of the OTA content from 49.0 (untreated) to 27.5 (1 min) and 23.8 ng/g (3 min), respectively. In contrast, CO_2 plasma caused an increase of the OTA amount from 49.0 (untreated) to 55.8 (1 min) and 72.9 ng/g (3 min). Finally, the use of air plasma resulted likewise in a decrease of the OTA concentration from 56.9 (untreated) to 25.7 (1 min) and 20.2 ng/g (3 min), respectively. Reducing the incubation time before the treatment to 24 h caused in contrast an increase of the OTA content from 3.1 (untreated) to 29.1 (1 min) and 20.7 ng/g (3 min). Due to the high standard deviation, these changes were not significant, but the tendencies were clearly visible, showing the strong impact of the plasma gas on the OTA production. The results show, that even if the total mold count was reduced, under certain conditions the OTA amount was yet enhanced, probably due to a stress reaction of the mold. Concluding, the plasma gas and incubation conditions have to be considered to allow a successful inactivation of molds and in particular their toxic metabolites.

Keywords: mycotoxin, ochratoxin A, *Aspergillus niger, Penicillium verrucosum*, cold atmospheric plasma, mold inhibition

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INTRODUCTION

When crop is not stored properly after harvest, especially when moisture content and temperature are too high, different molds like, e.g., *Aspergillus niger* and *Penicillium verrucosum* can grow on its surface. Both molds belong to the family Trichocomaceae. Family members are saprobes with aggressive colonization strategies, adaptable to extreme environmental conditions. They are cosmopolitan in distribution, ubiquitous in soil, and common associated with decaying plant and food material. *Aspergillus niger* (*A. niger*) is one of the most common species of the genus *Aspergillus* and is a frequent contaminant of food. The mold grows at temperatures between 6 and 47°C, with a temperature optimum between 35 and 37°C. The spores do not survive damp cold at −22°C or less. The mold tolerates pH ranges from 1.5 to 9.8 and is therefore able to exist in both strongly acidic and basic environments. Some strains of *A. niger* can produce the mycotoxin ochratoxin A (Abarca et al., 1994). *Penicillium verrucosum* (*P. verrucosum*) belongs to the genus *Penicillium* and has important implications in food, specifically for grains and other cereal crops on which it grows predominantly in Northern Europe. It has a white mycelium with green conidia, which have the ability to germinate at temperatures between 0 and 31°C with a temperature optimum between 21 and 23°C (Domsch et al., 1980). At temperatures between 10 and 25°C and a water activity (a_w) of around 0.95, ochratoxin A (OTA) synthesis of some *P. verrucosum* strains occur (Lund and Frisvad, 2003; Cairns-Fuller et al., 2005).

Due to its chemical stability against heat and during industrial food processing, OTA is one of the most abundant food-contaminating mycotoxins. It is a naturally occurring mycotoxin and a secondary metabolite of toxigenic species of *Aspergillus* and *Penicillium* molds, e.g., *A. niger* or *P. verrucosum*. It is present in different geographical regions and contaminates cereals such as wheat, maize, rye, barley, and oats under preharvest and postharvest conditions. It also occurs in peanuts, coffee beans, bread, rice, and dried fruits (Kuiper-Goodman and Scott, 1989; Al-Anati and Petzinger, 2006). When ingested in the organism, OTA can be found in various tissues, with particularly high accumulation in the kidney. As a result, the compound has predominantly nephrotoxic, but also hepatotoxic, immunotoxic and possibly neurotoxic properties. It has also been classified as probably carcinogenic for humans (Kuiper-Goodman and Scott, 1989; IARC Monographs, 1993). The exact mechanism that leads to toxicity is not yet fully understood. Due to its high toxicity, the maximum level of OTA in different foods is regulated, e.g., in unprocessed cereals to 5 μg/kg (FAO, 2006).

In general, mycotoxins including OTA are highly stable to physical, chemical or biological detoxification. For example, the thermal stability of OTA depends on the water content. When heating dry milled wheat, the reduction in OTA content only occurred at high temperatures and long exposure times (e.g., 50% reduction at 150°C and 200 min), while the reduction was slightly faster with moistened material; however, complete destruction of OTA was not achieved even at 200 or 250°C, respectively (Boudra et al., 1995). Even a gamma irradiation (20 kGy) of feed contaminated with OTA resulted only in a reduction of 36–47% (Refai et al., 1996). A treatment of barley with 5% ammonia for 96 h at 70°C resulted in a 90% reduction, but feeding the barley showed only a slight improvement in pig performance over contaminated cereals (Madsen et al., 1983). Mixing of contaminated barley with 3.5% NaOH for 30 min reduced the OTA amount from approximately 650 to 20–60 μg/kg (Richter et al., 1997), but the OTA reaction with NaOH was reversible (Valenta and Richter, 1998).

A relatively new method for a product-protecting decontamination of contaminated food could be a cold plasma treatment at atmospheric pressure, which is mostly of interest for the food industries due to the used moderate conditions (Misra et al., 2011). The types of plasma generation contain the corona discharge, radio-frequency plasmas, the gliding arc discharge and dielectric barrier discharges. The inactivation of microorganisms is based on different mechanisms. Resulting reactive oxygen- and nitrogen-based species have a direct oxidative effect on the outer cell surface ('etching'), provoking lesions which are not reparable fast enough. Additionally, UV irradiation can directly damage the genetic material of the microorganisms. Furthermore, intrinsic photodesorption by UV photons may occur, destroying chemical cell bonds and releasing volatile compounds of intrinsic atoms of the microorganism (Moisan et al., 2001).

For several years, plasma applications were used for sterilization of food and other products (Moisan et al., 2001; Laroussi, 2005; Niemira, 2012). Plasma was also found to be effective in reducing molds on food surfaces (Hojnik et al., 2017). The treatment of, e.g., maize grains contaminated with *A. flavus* und *A. parasiticus* spores resulted in a decrease of 5.5 and 5.2 log CFU/g after 5 min in a non-thermal atmospheric pressure fluidized bed plasma system with air as plasma gas (Dasan et al., 2016). Moreover, plasma has also the potential to inactivate different mycotoxins, like, e.g., aflatoxin B1, deoxynivalenol (DON) and nivalenol, that were degraded after 5 s of treatment with a microwave-induced argon plasma system at atmospheric pressure (Park et al., 2007). Using ambient air as plasma gas, deoxynivalenol, zearalenone, enniatins, fumonisin B1, T2 toxin, and sterigmatocystin were completely degraded within 60 s. Zearalenone, enniatin B, fumonisin B1, and sterigmatocystin were additionally embedded in mold cultures on rice to investigate the matrix effects. For zearalenone and sterigmatocystin, the degradation rates were slowed down, but after 60 s, nearly the full amount was removed. For enniatin B and fumonisin B1 instead, nearly half of the mycotoxins remained intact after 60 s (ten Bosch et al., 2017). On malting barley, DON was reduced to ca. 82% of the initial value and trichothecene (T-2) decreased to 40% of the initial content after 4 min treatment with a gliding arc discharge at atmospheric pressure (Kříž et al., 2015).

However, only one study considered the effect of plasma on the actual production of mycotoxins by molds. Ouf et al. (2015) contaminated date palm fruits with *A. niger* and treated them i. a. for 7.5 min with a double atmospheric pressure cold argon plasma, which resulted in a complete reduction of OTA after 10 days at 25°C. However, as argon is a noble gas and therefore relatively expensive and the treatment time with 7.5 min quite long, in this study different cheaper plasma gasses were tried

to have a similar effect. Therefore, in this study, barley was inoculated with *A. niger* and *P. verrucosum*, then treated with cold plasma generated by dry air, CO_2 and 80% CO_2 + 20% O_2 and stored for up to two weeks to observe the influence on the inactivation of molds and their production of OTA on barley.

MATERIALS AND METHODS

Inoculation of Barley

The experiments were performed with barley of the variety 'Grace 1250' (IREKS, Kulmbach, Germany). The barley had an initial moisture content of 10.5–11% and was sterilized in an autoclave at 134°C for 20 min.

As molds *Aspergillus niger* (DSM 22593) and *Penicillium verrucosum* (provided by Max Rubner-Institut, Karlsruhe, Germany) were used. They were spread on Potato-Dextrose-Agar (PDA) and grown for five days at 37°C (*A. niger*) or seven days at 25°C (*P. verrucosum*), respectively.

For the inoculation of the barley, five agar pieces including mycelium (cut out with a cork borer Ø = 1 cm) were mixed with 3 mL purified water to get a mycelium suspension. 1.9 mL of this suspension was used to inoculate 10 g of autoclaved barley in a 50 mL tube resulting in a final moisture content of 25–28%, corresponding to a_w values of 0.949–0.968 in the barley, which was adequate for the used molds to induce the production of mycotoxins (Fleurat-Lessard, 2017). Over the storage period, the barley was not moistened again and therefore, moisture content probably decreased. After homogeneous mixing for 1 h in an overhead shaker, the barley was spread evenly distributed into a petri dish and incubated at 37°C (*A. niger*) or 25°C (*P. verrucosum*) overnight in an incubator. On the next day, the plasma treatment was performed.

In addition, for one series of experiments with *A. niger* using plasma generated with air, a spore suspension was used for inoculation of the barley. For the preparation of the spore suspension, 10 mL of ringer solution (Merck KGaA, Darmstadt, Germany) was spread on the PDA plate, overgrown with *A. niger*. Subsequently, the surface was rubbed by an inoculation loop to detach the mycelium including spores. The resulting suspension was filtered afterward through a gauze bandage to remove pieces of mycelium. The concentration of the spore suspension was counted using the THOMA chamber and varied between 5.59 and 5.78 log spores/g barley.

Furthermore, for another series of experiments with *P. verrucosum* using plasma generated with air, CO_2 or 80% CO_2 + 20% O_2, the incubation time of the inoculated barley before the plasma treatment was extended to five days to allow an adaption of the mold to the barley.

For a better overview, the performance of the experiments is described in **Table 1**.

Plasma Source and Treatment

A diffuse coplanar surface barrier discharge (DCSBD) 400 plasma source (CEPLANT, R&D Centre for Low-Cost Plasma and Nanotechnology Surface Modifications, Masaryk University, Brno, Czechia) was used for the plasma treatment of the barley. The plasma equipment is described in detail by Hertwig et al. (2017). The treatment was performed in a reaction chamber with one bottom and one top DCSBD plate, the last one being adjustable in height. Between the plates, a plastic mesh with a distance of 1.5 cm to the plates was placed, where 20 g of barley were evenly spread. Plasma was only generated in the top plate; the power input was set to 350 W. For the plasma generation, dry air, CO_2 or 80% CO_2 + 20% O_2, regulated by a gas flow controller (Multi Gas Controller 647C, MKS Instruments, Andover, MA, United States), were used as process gasses with a gas flow of 10 sL/min. After flushing the reaction chamber with the respective process gas, plasma treatment for 1 or 3 min occurred, followed by another flushing step to remove the generated residues. The experiments were performed in triplicates. After plasma treatment, the inoculated barley was stored at 9°C (*P. verrucosum* and *A. niger*), 25°C (*P. verrucosum*) or 37°C (*A. niger*) for up to two weeks.

Microbiological Analysis

For microbiological analysis of mold count on the inoculated barley, 5 g of barley were transferred into a sterile flask. A 1:10 dilution was prepared using peptone salt solution (DIN EN ISO 6887-1:1999) and the mixture was shaken for 30 min at 180 rpm (TR-125, Infors AG, Bottmingen, Switzerland) to allow a removal of molds from the barley. Subsequently, the samples were serially diluted in Rotilabo®-microtest plates (96er U-profile, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) using peptone salt solution. 100 µl of each dilution were spread on Potato-Dextrose-Agar (AppliChem GmbH, Darmstadt, Germany) in duplicates. After growth at 25°C (*P. verrucosum*) or 37°C (*A. niger*) for up to 72 h, the number of colony forming units (CFU/g) was

TABLE 1 | Performance of the realized experiments with all varied parameters.

Strain	Inoculation method	Incubation time before plasma treatment	Incubation temperature	Plasma treatment	Storage time after plasma treatment	Storage temperature	Measured parameters
A. niger	Mycelium suspension	24 h	37°C	Dry air; CO_2; CO_2+O_2	1 week	37°C	OTA
A. niger	Mycelium + spore suspension	24 h	37°C	Dry air	2 weeks	9°C	OTA; CFU/g
P. verrucosum	Mycelium suspension	110 h	25°C	Dry air; CO_2; CO_2+O_2	2 weeks	25°C	OTA
P. verrucosum	Mycelium suspension	24 h	25°C	Dry air	2 weeks	9°C	OTA; CFU/g

determined to obtain the aerobic viable mold count of the barley. The lower detection limit of the plate count analyses was 100 CFU/g. The total mold counts for different conditions were compared using Welch's unequal variances *t*-test. *P*-values below 0.05 were considered statistically significant.

HPLC Analysis

For the extraction of ochratoxin A, ca. 2 g of ground (45 s, Superior PCML-2013A, Harvest Industry Limited, Guangzhou, China) barley was mixed with 10 mL acetonitrile (80%, VWR International GmbH, Dresden, Germany) with glacial acetic acid (1%, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for 60 min in an overhead shaker (Stuart® Rotator STR4, Bibby Scientific Ltd., Staffordshire, United Kingdom). Afterward, the samples were centrifuged (Centrifuge 5810 R, Eppendorf AG, Hamburg, Germany) at 3100 × *g* for 10 min and the supernatant was filtered (1 µm). A mixture of 5 mL filtrate and 35 mL aqua dest was cleaned up on an immunoaffinity column (OchraTest WB, Ruttmann GmbH, Hamburg, Germany). After rinsing with 5 mL aqua dest, OTA was eluted with 1.5 mL acetonitrile (100%) + glacial acetic acid (1%) and evaporated at 60°C in an evaporator (Concentrator Plus, Eppendorf AG, Hamburg, Germany) to dryness. The residue was redissolved in 0.2 mL acetonitrile (70%) + glacial acetic acid (1%), which was also used for preparation of the OTA-standard for calibration of the HPLC. The used HPLC unit (Nexera^{TM} HPLC, Shimadzu Deutschland GmbH, Duisburg, Germany) was equipped with a pump (LC-30 AD), an auto sampler (SIL-30 AC), a degasser (DGU- 20A5), a column oven (CTO 20 AC), a fraction collector (FRC-10A), a fluorescence detector (FLD, RF-20A XS) and LabSolution software. The column was a reverse-phase column (EC 250/4.6 Nucleodur Sphinx RP, 3 µm, Macherey-Nagel, Düren, Germany) with a reverse-phase pre-column (XBridge BEH Shield RP 18, 3.5 µm, Waters GmbH, Eschborn, Germany) and the fluorescence detector was adjusted to $\lambda_{ex}/\lambda_{em}$ = 333/460 nm. The calibration curve for OTA ranged from 1 to 100 ng/mL (r^2 = 0.999) and the injection volume of the samples was 10 µL. The limit of detection was 0.5 ng/mL and the limit of quantification was 1 ng/mL. The method was adapted according to DIN EN (14132):2009 (2009). The OTA amounts for different conditions were compared using Welch's unequal variances *t*-test. *P*-values below 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Plasma Treatment of Barley, Inoculated by *Aspergillus niger*

After inoculation of barley with a mycelium suspension of *Aspergillus niger* followed by a plasma treatment with air, CO_2 or 80% CO_2 + 20% O_2 as process gasses for 1 or 3 min, the amount of produced OTA directly and after storage at 37°C for one week was measured using HPLC. This storage temperature was chosen, because it is the optimal growth temperature of *A. niger*. The measured amounts were all under 0.36 ng/g, one week after the treatment they had decreased to <0.25 ng/g (**Figure 1**). One exception was the plasma treatment using CO_2 as process

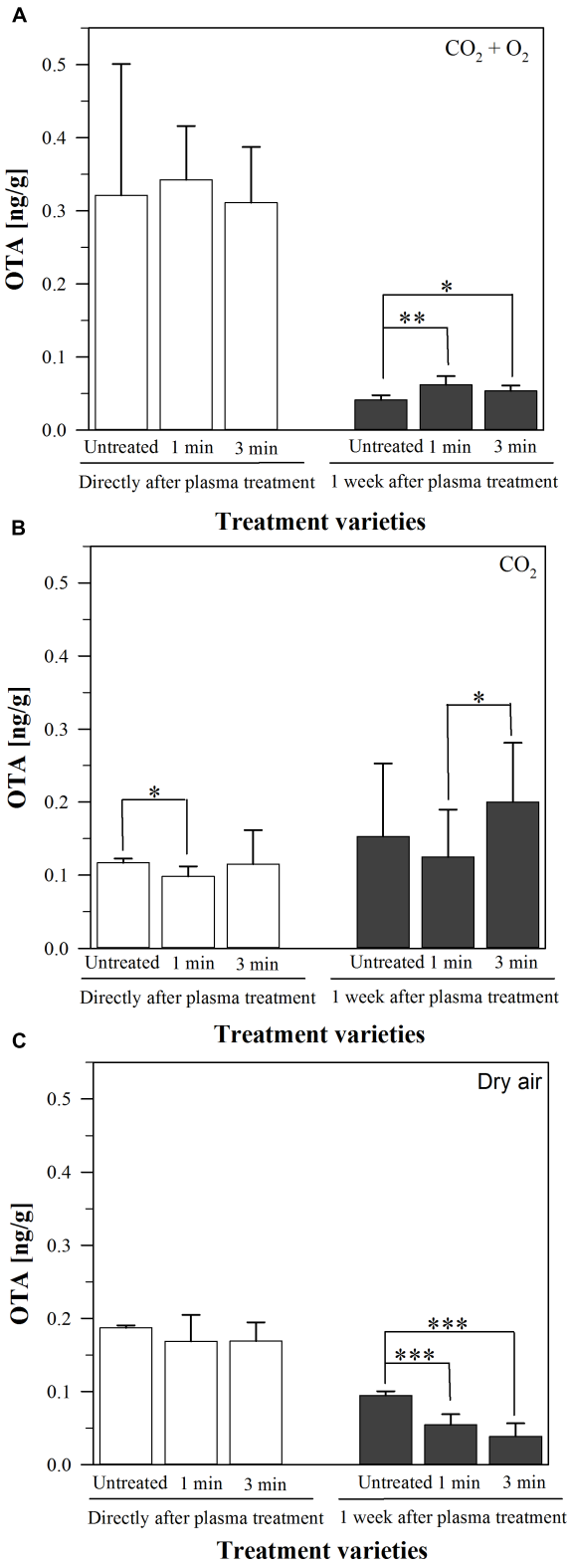

FIGURE 1 | Amount of ochratoxin A (OTA, [ng/g]) **(A–C)** on barley, inoculated by a mycelium suspension of *A. niger*, directly or one week after plasma treatment for 1 or 3 min or without any treatment, stored at 37°C. Significance levels *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

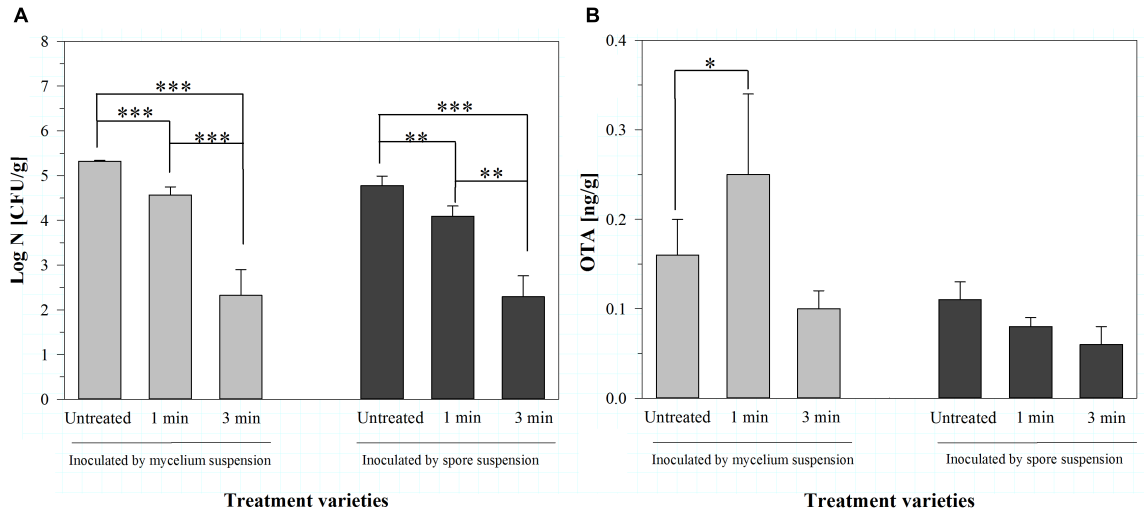

FIGURE 2 | Total mold count [log CFU/g] **(A)** and amount of ochratoxin A (OTA, [ng/g]) **(B)** on barley, inoculated with *A. niger* as mycelium or spore suspension, two weeks after air plasma treatment for 1 or 3 min or without any treatment, stored at 9°C. Significance levels $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

gas for 3 min, where the OTA concentration slightly increased after one week from 0.15 ± 0.1 ng/g in the untreated grains to 0.20 ± 0.08 ng/g after 3 min plasma. However, this increase was not significant; and in general, these low amounts cannot be attributed alone to the plasma treatment since the untreated barley showed similar values.

Even if the concentration of OTA in general was low, significant inactivation rates were achieved using dry air as process gas. Therefore, the experiments were repeated using only dry air as process gas, but with two different inoculation methods (mycelium and spore suspension) and a lower storage temperature of 9°C. This storage temperature was chosen to investigate the effect of a lower temperature on the growth of the molds and the production of the mycotoxins, because the latter can be higher at lower temperatures (Sherwood and Peberdy, 1974). Furthermore, the success of the inoculation and the effect of the plasma treatment on the mold were controlled by analyzing the total mold count of the barley two weeks after the plasma treatment, because there was no mycelium visible at this storage temperature (**Figure 2**).

The inoculation with a mycelium suspension resulted in a total mold count of 5.32 ± 0.02 log CFU/g. The treatment with air plasma for 1 min significantly reduced the total mold count to 4.57 ± 0.18 log CFU/g and the treatment for 3 min lowered it to 2.32 ± 0.58 log CFU/g. The corresponding amounts of OTA were 0.16 ± 0.04 ng/g for the untreated barley, 0.25 ± 0.09 ng/g after 1 min plasma and 0.10 ± 0.02 ng/g after 3 min plasma treatment, showing no significant difference. Summarizing, the treatment with plasma for 1 min resulted in a slight, but not significant increase of the OTA amount, compared to the untreated barley. Prolonging the time of plasma treatment led in contrast to a slight, but also not significant decrease of OTA in the barley (**Figure 2**).

The inoculation of the barley with a spore suspension resulted in slightly lower total mold counts compared to the inoculation by mycelium suspension (**Figure 2A**). However, the air plasma treatment caused a similar, significant reduction [from 4.78 ± 0.21 log CFU/g (untreated) to 4.09 ± 0.23 log CFU/g (1 min plasma) and 2.29 ± 0.47 log CFU/g (3 min plasma)] of the total mold count. According to the lower total mold count, also the amount of OTA in the barley was slightly lower (0.11 ± 0.02 ng/g). Treatment with plasma for 1 min caused a slight decrease to 0.08 ± 0.01 ng/g, and 3 min of plasma treatment nearly halved the OTA amount in the barley (**Figure 2B**). However, the OTA amounts in the barley produced by *A. niger* showed no significant differences after the treatments. In general, the OTA contents were again low, as in consequence the validity of the plasma treatment regarding the influence on the OTA production at inoculated barley.

Comprising, the reduction of the total mold count after air plasma treatment for 1 min was 0.75 log CFU/g (mycelium suspension) or 0.69 log CFU/g (spore suspension), respectively. The plasma treatment for 3 min caused a decrease of 3 log CFU/g (mycelium suspension) or 2.49 log CFU/g (spore suspension), respectively. It is assumed that the total mold counts were even lower directly after plasma treatment, because during 2 weeks of storage the microorganisms would obviously have more time to grow. This reduction of the total mold counts was in good accordance with other results. Using a low pressure cold plasma prototype unit with air as process gas, *Aspergillus* spp. on seeds was reduced by around one log cycle after 5 min (Selcuk et al., 2008). *Aspergillus parasiticus* on hazelnuts was reduced by around 2.5 log cycles after 3 min treatment by an atmospheric pressure fluidized bed plasma using dry air as process gas (Dasan et al., 2017). Many reactive oxygen and nitrogen species like, e.g., nitrous gasses and also UV photons are generated in air plasmas (Laroussi and Leipold, 2004; Laroussi, 2005; Hertwig et al., 2017). The resulting oxidative effects lead to strong damages on fatty acids and proteins in the cell membranes and on the genetic

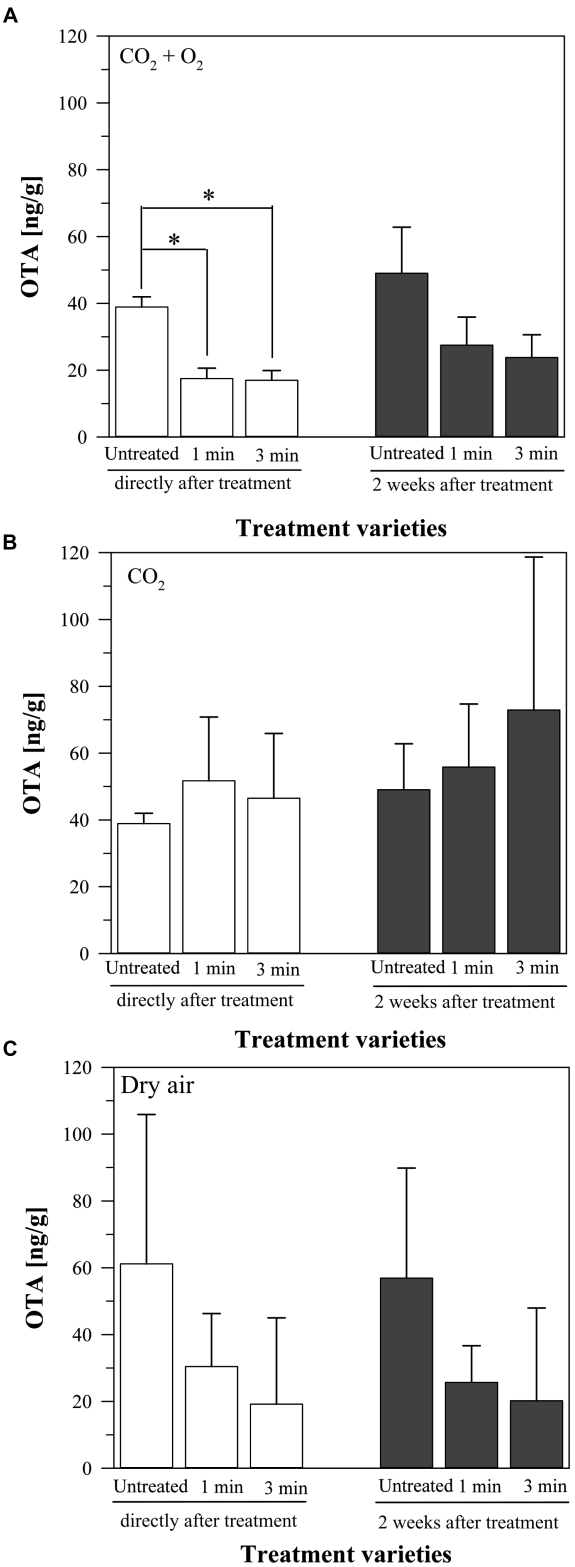

FIGURE 3 | Amount of ochratoxin A (OTA) on barley, inoculated with a mycelium suspension of *P. verrucosum*, directly or two weeks after plasma treatment for 1 or 3 min or without any treatment, stored at 25°C. Process gasses: $CO_2 + O_2$ **(A)**, CO_2 **(B)**, and dry air **(C)**. Significance level $*p < 0.05$.

material (Laroussi and Leipold, 2004; Boudam et al., 2006). Additionally, erosion of the microorganisms through intrinsic photodesorption and etching occurs (Moisan et al., 2001).

Plasma Treatment of Barley, Inoculated by *Penicillium verrucosum*

Penicillium verrucosum was used for inoculation of the barley to see the effect of the plasma treatment on another mold also producing OTA as mycotoxin. Barley was inoculated with *P. verrucosum*, incubated for 5 days, treated by plasma generated with different process gasses for 1 or 3 min and stored at 25°C for two weeks. This temperature was the optimal growth temperature for the mold. The incubation time of *P. verrucosum* on the barley was longer in order to achieve a certain amount of mycotoxins on the samples to allow the investigation of mycotoxin inhibition by cold plasma treatment.

When using a mixture of $CO_2 + O_2$ as plasma process gas, the production of OTA on the inoculated barley was reduced compared to the untreated grains (38.9 ± 3.1 ng/g, **Figure 3A**). Plasma treatment for 1 min significantly reduced OTA to 17.5 ± 3.1 ng/g and for 3 min to 17 ± 2.9 ng/g. Two weeks after treatment the amount of OTA slightly increased in all cases. The untreated grains showed now an OTA content of 49 ± 13.8 ng/g. After 1 min plasma treatment, the OTA amount increased to 27.5 ± 8.4 ng/g and after 3 min plasma to 23.8 ± 6.8 ng/g. Compared to the untreated barley, the OTA content was therefore still reduced to around the half, albeit this decrease was not significant due to the high standard deviation.

In contrast, the use of only CO_2 as plasma process gas had a different effect on the OTA production (**Figure 3B**). Compared to the untreated barley (38.9 ± 3.1 ng/g OTA), the plasma treatment for 1 min resulted in an increase (51.7 ± 19.1 ng/g OTA), which was a little lower after 3 min plasma treatment (46.5 ± 19.4 ng/g OTA). After 2 weeks of storage, the OTA content increased again in all varieties. The OTA amount of the untreated grains was now 49 ± 13.8 ng/g, the OTA content of the 1 min plasma treated grains was 55.8 ± 18.9 ng/g, and the one of the 3 min plasma treated barley was 72.9 ± 45.8 ng/g. Compared to the untreated barley, the treatment varieties caused all an increase of the OTA production of *P. verrucosum*. However, this increase was not statistically significant due to the high standard deviation of the results.

The treatment with air plasma caused again a decrease of the OTA amounts. At the untreated barley, the OTA content was 61.2 ± 44.7 ng/g, compared to 30.4 ± 15.9 ng/g after 1 min plasma and 19.2 ± 25.8 ng/g after 3 min plasma, analyzed directly after treatment (**Figure 3C**). Two weeks later, the values were nearly the same. The OTA amounts in the untreated grains slightly decreased to 56.9 ± 33 ng/g, like in the 1 min plasma treated barley (to 25.7 ± 11 ng/g OTA). The values for the 3 min treatment slightly increased to 20.2 ± 27.7 ng/g OTA. However, the standard deviation of the results was again too high to reveal a significant difference.

In dielectric barrier discharges generated with air or oxygen, especially ozone and reactive oxygen species are formed (Eliasson et al., 1987; Kalghatgi et al., 2012; Kogelschatz, 2012).

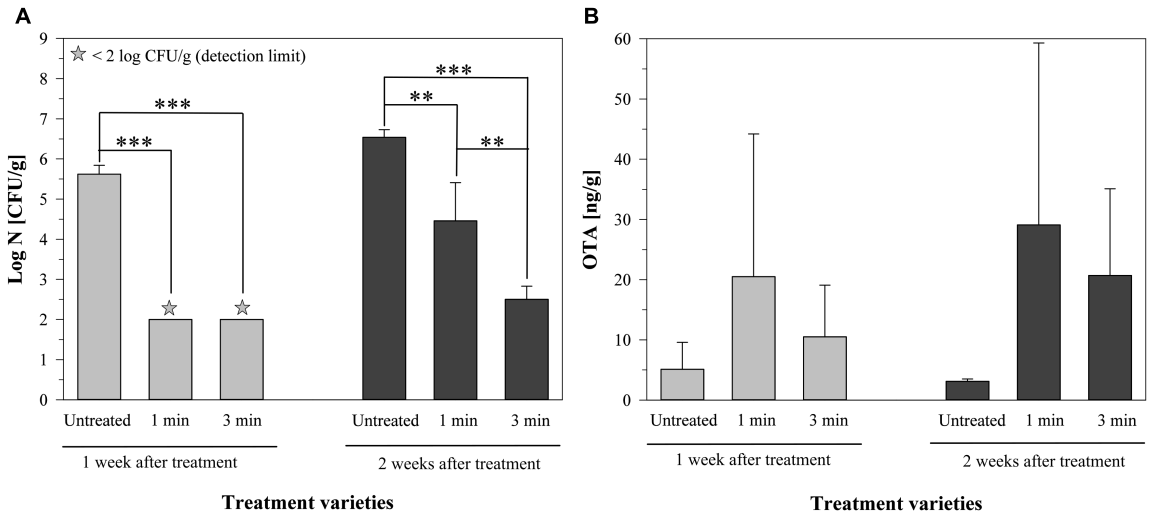

FIGURE 4 | Total mold content **(A)** and amount of ochratoxin A **(B)** on barley, inoculated with a mycelium suspension of *P. verrucosum*, 1 and 2 weeks after air plasma treatment for 1 or 3 min or without any treatment, stored at 9°C. Significance levels $^{**}p < 0.01$; $^{***}p < 0.001$.

In particular, the formation of ozone in plasmas generated with O_2 as process gas is 10-fold higher than in CO_2 plasmas (Hertwig et al., 2017). Ozone has a high antimicrobial potential due to the occurring oxidation of cell components like polyunsaturated fatty acids, enzymes and proteins (Victorin, 1992). The high amount of ozone in the plasmas generated by $CO_2 + O_2$ and dry air led therefore probably to a stronger inactivation of *P. verrucosum*, resulting in a reduced ability to produce OTA. Additionally, ozone has a direct inactivating effect on the pre-existing mycotoxins by causing chemical modifications leading to a reduced biological activity (Tiwari et al., 2010). Due to the long incubation time before the plasma treatment, the mold produced a high amount of OTA, which was then attackable by the ozone. In the case of using only CO_2 as process gas, the formation of ozone was lower, probably leading to a minor damage of the mold and the pre-existing OTA.

In another experiment, the incubation time before the plasma treatment was shortened to investigate the influence of plasma treatment directly on the production process of mycotoxins. Additionally, the total mold count of the inoculated barley was recorded in order to determine the direct effect of the plasma treatment on *P. verrucosum*. Therefore, barley was inoculated with *P. verrucosum*, incubated for 24 h, treated with plasma generated by dry air for 1 or 3 min and stored for one or two weeks at 9°C. The lower temperature could be an additional stress factor for the mold that may affect the mycotoxin production.

The total mold count of the untreated barley was 5.62 ± 0.22 log CFU/g after one week, increasing to 6.54 ± 0.19 log CFU/g after two weeks of storage (**Figure 4A**). The air plasma treatment for 1 min led to a decrease of the total mold count under the detection limit of 2 log CFU/g after one week of storage, followed by another increase to 4.46 ± 0.95 log CFU/g after two weeks. A longer plasma treatment for 3 min resulted in a reduction below the detection limit after one week and a total mold count of 2.50 ± 0.33 log CFU/g after two weeks of storage (**Figure 4A**).

Looking at the respective amounts of OTA, the untreated grains had a concentration of 5.1 ± 4.5 ng/g after one week of storage, which was slightly lower (3.1 ± 0.4 ng/g) after two weeks (**Figure 4B**). One minute of air plasma resulted in an OTA content of 20.5 ± 23.7 ng/g or 29.1 ± 30.2 ng/g after one or two weeks, respectively. The plasma treatment for 3 min led to an increase to 10.5 ± 8.6 ng/g after one week and to 20.7 ± 14.4 ng/g after two weeks of storage. The standard deviation was relatively high and therefore the differences were not significant, but it was still visible, that the amount of OTA increased after the treatment with air plasma, even if *P. verrucosum* was inactivated to some extent by the treatments (**Figure 4**), which implies that the plasma treatment with air has led to a triggering of the mycotoxin production.

In other studies, the spores of *Penicillium expansum* were reduced by corona discharge plasma for approx. one log CFU/mL after 120 min (Ye et al., 2012). Electron micrographs revealed noticeable defects in the morphology and internal sub-structure of the spores, leading to the inactivation. Spores in general are highly resistant against all kinds of disinfection, which also explains the long inactivation time. On dried filefish fillets, *P. citrinum* was reduced by 1 log CFU/g after 10 min of cold oxygen plasma treatment (Park and Ha, 2014). *Penicillium* spp. on grain was likewise decreased for 1 log cycle after 5 min of air plasma treatment (Selcuk et al., 2008).

Other research showed that the use of neutralized electrolyzed water reduced the Fusarium microbial count on wheat, but partially increased the amount of produced deoxynivalenol (Audenaert et al., 2012). This was explained by the effect of reactive oxygen species (in this case hydrogen peroxide). In plasma generated by air as process gas, the formation of reactive oxygen species is higher than in plasmas generated by O_2 and CO_2, where instead a higher amount of ozone is released (Hertwig et al., 2017). Also hydrogen peroxide in particular was found to be formed in dielectric barrier discharges generated

by air (Kalghatgi et al., 2012). Therefore, it is assumed, that the exposure of *P. verrucosum* to the reactive oxygen and nitrogen species generated by the plasma constituted a stressor, which resulted in a higher production of OTA. The plasma treatment for 3 min caused certainly more damage to the molds; therefore the amount of OTA was lower than after plasma treatment for only 1 min. Furthermore, the incubation time before the treatment was only 24 h, therefore the amount of OTA produced by *P. verrucosum* on the barley was lower and consequently less of the mycotoxin could be inactivated directly through the plasma. Additionally, the low storage temperature could increase the mycotoxin production by a simultaneous retardation of the mold growth (Sherwood and Peberdy, 1974).

In general, the variance of the produced OTA amounts was unexpectedly high, leading to the fact, that most results regarding the plasma effect on the OTA production of *P. verrucosum* were not significant. A reason therefore could be an inhomogeneous plasma treatment, although the distribution on the plasma plate appeared even. However, this assumption is contradicted by the low standard deviations of the total mold counts, leading to significant mold reductions. Another explanation could lie in the nature of the mold, provoking an unequal OTA production under stress conditions. However, the tendencies were clearly visible, leading to the implication that the process gas of the plasma treatment has a strong impact on the production of the mycotoxins. It can be assumed that these contradictory results would also appear with other plasma gasses and sources. This has to be considered when using plasma treatment for inactivation of molds, particularly for a large-scale industrial application. Dielectric barrier discharges principally have a promising design for scaling-up, allowing continuous processing approaches generally used in food industry. However, to realize the retention of the long-lived reactive species in a continuous process, which would lead to lower treatment times, will be one of the challenges in the future (Cullen et al., 2017).

CONCLUSION

Three minutes treatment of barley inoculated with *A. niger* and *P. verrucosum* using plasma generated by dry air effectively reduced the total mold count by 2.5–3 log cycles after two weeks. However, since the hazardous aspect of these molds is their ability to produce mycotoxins, it is very important to consider the effect of plasma treatment also on the production of mycotoxins. Here, the various process gasses led to contradictory results. In the case of $CO_2 + O_2$ plasma, the OTA production of *P. verrucosum* was clearly reduced. When using CO_2 as process gas, the OTA amount was nearly the same directly after the treatment but was increased, although not significantly, after two weeks of storage. Finally, the use of dry air for plasma generation resulted in a decreased OTA concentration when the incubation time before the treatment was five days and in an increased OTA amount when the barley was incubated only 24 h before the treatment. Therefore, the conditions including the incubation time and the process gas have to be taken into account when using plasma treatment to reduce the mold and mycotoxin concentration on grains and to avoid a stimulation of mycotoxin production. Additionally, it must always be carried out a combined examination of molds and their metabolite mycotoxin in order to correctly evaluate the success of the inactivation process.

AUTHOR CONTRIBUTIONS

JD conceived and designed the experiments, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. OS conceived and designed the experiments, contributed reagents, material, analysis tools, or data, and proofread the manuscript. AR performed the experiments. PD analyzed and interpreted the data. AF conceived and designed the experiments, analyzed and interpreted the data, and proofread the manuscript.

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Flow Cytometric Assessment of the Morphological and Physiological Changes of *Listeria monocytogenes* and *Escherichia coli* in Response to Natural Antimicrobial Exposure

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Essential oils (EOs) or their components represent one of the most promising natural, safe, and feasible alternatives to prevent the growth of food-borne pathogens like *Listeria monocytogenes* and *Escherichia coli* in food matrices. Although antimicrobial properties of EOs and their components are well-documented, limited and fragmented information is available on the changes induced by these compounds, even at sub-lethal concentrations, in the physiological properties of microbial cells. The aim of this study was to explore the morpho-physiological changes of *L. monocytogenes* Scott A and *E. coli* MG 1655 induced after 1 h exposure to different sub-lethal and lethal concentrations of citral, carvacrol, (E)-2-hexenal, and thyme EO. For this purpose, different cell viability parameters such as membrane integrity, esterase activity, and cytoplasmic cell membrane potential were measured by flow cytometry. Flow cytometric data revealed specific response patterns in relation to the strain, the natural antimicrobial and its concentrations. Both the target microbial strains showed an increased cell membrane permeabilization without a loss of esterase activity and cell membrane potential with increasing citral, carvacrol and thyme EO concentrations. By contrast, (E)-2-hexenal did not significantly affect the measured physiological properties of *L. monocytogenes* Scott A and *E. coli* MG 1655. The used approach allowed identifying the most effective natural antimicrobials in relation to the microbial target.

Keywords: food safety, non-thermal treatment, pathogens, stress response, membrane permeabilization

INTRODUCTION

Consumer's demand for minimally processed and ready-to-eat foods with a reduced content of synthetic preservatives has stimulated the research of alternative preservation strategies. Essential oils (EOs) or their components represent one of the most promising natural feasible alternatives to improve food safety, shelf-life and quality. Recognized as safe from international food authorities they are traditionally used in food industry as flavor and taste enhancers (Newberne et al., 2000).

Their antimicrobial activity and the wide action spectra against several pathogenic and spoilage microorganisms are well-documented and several reviews are available (Burt, 2004; Hyldgaard et al., 2012; Tongnuanchan and Benjakul, 2014; Patel, 2015; Pandey et al., 2017). A wide literature documented their application as natural preservatives also in different food matrices, such as meat (Fratianni et al., 2010; Barbosa et al., 2015; Radha Krishnan et al., 2015), dairy products (Amatiste et al., 2014; Ehsani et al., 2016; Ben Jemaa et al., 2017), minimally processed fruits and vegetables (Patrignani et al., 2015; Siroli et al., 2015b,c) and beverages (Kiskó and Roller, 2005; Chueca et al., 2016). Among the natural antimicrobials, thyme EO, and some components of citrus and officinal EOs, such as citral, carvacrol, and (E)-2-hexenal, are very promising alternatives to traditional preservatives (Ivanovic et al., 2012). In fact, they are widely reported to be able to improve safety and shelf-life of several foods even when used at concentrations lower than their minimal bactericidal concentrations. In fact, their application in foods is subordinated to the usage of concentrations not detrimental for the product sensory properties (Zanini et al., 2014a,b; Silva-Angulo et al., 2015). In general, the cell wall, the cytoplasmic membrane and membrane proteins have been considered as the main targets of EOs and their components (Burt, 2004). However, the EO action mechanisms are reported to vary according to the natural antimicrobial and its concentration, the target species or strain, the food matrix, the storage conditions, etc. (Valero and Francés, 2006; Somolinos et al., 2008, 2010; Patrignani et al., 2013). As described by Burt (2004) and Hyldgaard et al. (2012), the primary site of toxic action of terpenes like citral and carvacrol is represented by the cell membrane due to their hydrophobic properties.

As reported by Somolinos et al. (2010), the exposure to different citral concentrations caused a disruption of the cytoplasmic cell membrane of *Escherichia coli* BJ4 and BJ4L1. A similar permeabilization effect was also observed for different *Listeria monocytogenes* strains. As reported by Zanini et al. (2014a,b), the exposure to citral and carvacrol increased the permeabilization of the cytoplasmic cell membrane and potentiated the activity of various antibiotics even at sub-lethal concentrations.

Additionally, the cytosol coagulation and the depletion of the microbial cell proton-motive force have been identified as action mechanisms of EOs (Burt, 2004; Hyldgaard et al., 2012; Patel, 2015). Aldehydes such as hexanal and (E)-2-hexenal are antimicrobials produced by plants and vegetable tissues which are damaged by biotic or abiotic stresses throughout the lipoxygenase pathway to prevent and/or inhibit the growth of plant pathogens (Lanciotti et al., 2004). It has been demonstrated that such tissue show a noticeable activity against several yeasts, molds, Gram-positive and Gram-negative bacterial strains of food interest (Nakamura and Hatanaka, 2002; Trombetta et al., 2002; Zhang et al., 2017) both in model and real food systems (Lanciotti et al., 1999, 2003; Siroli et al., 2014). As reported by Patrignani et al. (2008), (E)-2-hexenal acts as a surfactant and permeates by passive diffusion across the plasma membrane of many microorganisms. After reaching the cytoplasm, the α,β-unsaturated aldehyde is able to react with different nucleophilic groups (Kubo and Fujita, 2001; Lanciotti et al., 2004). Moreover, (E)-2-hexenal may cause cytoplasm coagulation as a result of thiol containing enzyme inhibition (Aiemsaard et al., 2011). The antimicrobial properties of thyme EO depend on its chemical composition and the target microorganism (Kim et al., 1995; Nevas et al., 2004; Sienkiewicz et al., 2011; Picone et al., 2013; Boskovic et al., 2015; Siroli et al., 2015a,c; Swamy et al., 2016).

Thyme EO is constituted of numerous different compounds, but its antimicrobial activity is mainly attributed to carvacrol and thymol. Thymol is structurally similar to carvacrol and they share their cellular targets. Studies have shown that thymol interacts with cell membrane permeability, leading to a depletion of membrane potential, cellular uptake of ethidium bromide, and leakage of potassium ions, ATP, and carboxyfluorescein (Helander et al., 1998; Lambert et al., 2001; Xu et al., 2008). Although literature regarding the action mechanisms of citral, carvacrol, (E)-2-hexenal, and thyme EO has been dramatically increased in the last years, the knowledge on their mechanisms on *L. monocytogenes* and *E. coli* is still fragmentary since it is affected by several factors such as concentration, strains, cell physiological state, treatment conditions, microbial interaction with exposure systems, etc.

In addition, antimicrobial activity of EOs and their components are not attributable to a specific mechanism but to the actions toward several cell targets. Moreover, for EOs a holistic approach should be considered, since synergistic actions among present components greatly affects their antimicrobial activities also at very low concentrations (Caccioni et al., 1997), and, consequently, the understanding of their action mechanisms becomes more complex. In addition, a heterogeneity in microbial population resistance to stress is reported to occur as a monomodal Gaussian with a narrow or broad distribution, or as a multimodal distribution comprising subpopulations of similar or vastly different numbers of individuals (Dhar and McKinney, 2007). However, the literature on the behavior of *L. monocytogenes* and *E. coli* cell populations exposed to natural antimicrobials is still scarce (Burt, 2004; Bakkali et al., 2008; Hyldgaard et al., 2012).

Flow cytometry represents a reliable and fast tool in food microbiology for the measurements of the changes on physiological single cell properties. By the use of the appropriate fluorescent dyes, it is possible to classify cells into three different categories: metabolically active, intact, or permeabilized cell mixtures (Hewitt and Nebe-Von-Caron, 2004; Johnson et al., 2013). Most common fluorescent dyes used in flow cytometry are fluorescent immune-conjugates and probes for fluorescence *in situ* hybridization and nucleic acid stains. In addition, several probes capable of measuring the membrane potential as well as cell enzymatic activity, viability, organelles, phagocytosis, development, and other properties are available (Haugland, 1994). Various authors demonstrated the suitability of flow cytometry to study the microbial cell responses even after the exposure to sub-lethal stress conditions (Luscher et al., 2004; Ananta et al., 2005;

Berney et al., 2007; Mathys et al., 2007; Sunny-Roberts and Knorr, 2008; Da Silveira and Abee, 2009; Mols et al., 2010; Fröhling et al., 2012; Tamburini et al., 2013; Fröhling and Schlüter, 2015). In fact, this technique provides several information on the whole cell population and its changes during the exposure to stresses and the following recovery during storage. The knowledge of the behavior of the different sub-populations after exposure to natural antimicrobials is fundamental for their further application at industrial level as alternative to traditional preservatives, also to avoid resistance phenomena.

In this framework, the main aim was to investigate the potential of flow cytometry to study the changes of morphological and physiological properties of the food-borne pathogen *L. monocytogenes* Scott A and the indicator strain *E. coli* MG 1655, after 1 h exposure to different sub-lethal and lethal concentrations of citral, carvacrol, (E)-2-hexenal and thyme EO in order to clarify their specific action mechanisms and the responses of the whole cell population. For these purposes, different cell viability parameters, such as membrane integrity, esterase activity and cytoplasmic cell membrane potential were measured by flow cytometry.

MATERIALS AND METHODS

Natural Antimicrobials

Some EOs (citral, carvacrol, and (E)-2-hexenal) used in these experiments were purchased from Sigma-Aldrich (Milan, Italy) while thyme EO was obtained from Flora s.r.l. (Pisa, Italy). Before conducting the experiments, EOs were properly diluted using absolute ethanol (Sigma-Aldrich, Milan, Italy) to prepare 100X EO stock solutions.

Bacterial Strains

Listeria monocytogenes Scott A and *E. coli* MG 1655 were stored as glass bead cultures at −80°C for long-term preservation. To acclimatize cultures to the experimental conditions, one glass bead of each strain was given to 5 ml of Brain Heart Infusion broth (BHI) (Thermo-fisher, Milan, Italy) and incubated for 24 h without shaking at 37°C. After growth, cells were sub-cultured at 37°C for 24 h in BHI broth.

Exposure to Natural Antimicrobials

In each assay, 250 mL of fresh BHI broth was inoculated with 2.5 mL of bacteria suspension (corresponding to 1% of the final volume) to reach a 4 log CFU/mL concentration and incubated without stirring at 37°C. The growth was monitored by measuring the optical density (OD) at λ = 600 nm. For *L. monocytogenes* Scott A the exposure was performed in the middle of the exponential growth phase while for *E. coli* MG 1655 the exposure was performed in the stationary growth phase (OD = 2, λ = 600 nm). For both microbial strains, 200 μL of natural antimicrobial hydro alcoholic stock solutions was added to 20 mL of liquid cultures in order to obtain the concentrations reported in **Tables 1**, **2**. Cultures were incubated for 1 h at 37°C.

For *L. monocytogenes* Scott A the natural antimicrobial operative concentrations were determined according to the minimum inhibitory concentration standard method (MIC) as previously described by Siroli et al. (2015a,b). For each selected natural antimicrobial, *L. monocytogenes* cells were exposed to three sub-lethal concentrations corresponding to the 1/5, 1/3, 1/2 of the MIC value as well as to the MIC values (**Table 1**). For *E. coli* MG 1655, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the selected natural antimicrobials were determined in preliminary studies according to the protocol described by Siroli et al. (2015a,b) (data not shown). *E. coli* MG 1655 cells were exposed to different natural antimicrobials in sub-lethal concentrations (corresponding to 1/5, 1/3, 1/2 of the MIC values) as well as to the MIC and MBC values (**Table 2**). After exposure, the total viable cell count of *L. monocytogenes* Scott A and *E. coli* MG 1655 was immediately performed. Afterward, bacterial cells were harvested by centrifugation at 3214 × *g* and 4°C for 15 min, resuspended in 250 μL of phosphate buffered saline (PBS, 50 mM) and centrifuged at 7000 × *g* and 4°C for 5 min. For the subsequent staining procedures and flow cytometric analysis, *L. monocytogenes* pellets were resuspended in 100 μL (50 mM) PBS, while *E. coli* samples were resuspended in 100 μL (50 mM) Tris buffer. For both microorganisms the final cell density of each sample was about 10^9 cells/mL.

Total Viable Cell Count

The total viable cell count of bacteria after exposure to EOs or their components was determined by plate count methods in duplicate. Samples were serially diluted in microtest plates (96er U-profile, Carl Roth GmbH & Co KG, Germany) using physiological saline buffer (9 g/L NaCl) as dilution solution. 100 μL of each dilution was spread on BHI agar (Thermo Fisher Scientific, Ltd., Milan, Italy) and the

TABLE 1 | Essential oils, their components, and relative concentrations, used for the treatments of *L. monocytogenes Scott A.*

Natural antimicrobial	Concentration tested (mg/L)
Citral	50[1], 85, 125, 250 mg/L[1]
Carvacrol	20[1], 35, 50, 100 mg/L[1]
(E)-2-hexenal	150[1], 250, 400, 800 mg/L[1]
Thyme essential oil	40[1], 70, 100, 200 mg/L[1]

[1]MIC value tested for Listeria monocytogenes Scott A.

TABLE 2 | Essential oils, their components, and relative concentrations, used for the treatments of *Escherichia coli* MG 1655.

Natural antimicrobial	Concentration tested (mg/L)
Citral	200, 330, 500, 1000[1], 3000 mg/L[2]
Carvacrol	25, 40, 60, 120[1], 250 mg/L[2]
(E)-2-hexenal	80, 135, 200, 400[1], 425 mg/L[2]
Thyme essential oil	50, 86, 125, 250[1], 300 mg/L[2]

[1]MIC value tested for Escherichia coli MG 1655; [2]MBC value tested for Escherichia coli MG 1655.

growth (colony forming units) was evaluated after 24 h at 37°C.

Flow Cytometric Analysis

All experiments were performed using a CyFlow ML flow cytometer (Sysmex Partec GmbH, Görlitz, Germany) equipped, among others, with a 50 mW blue solid state laser emitting at a wavelength of 488 nm. A photomultiplier with a band pass filter of 536 ± 20 nm was used to collect fluorescence data of thiazole orange (TO), carboxyfluorescein (cF), and green $DiOC_2(3)$, while the fluorescence of propidium iodide and red $DiOC_2(3)$ was recorded in the photomultiplier with a band pass filter of 620 ± 11 nm. To correct the overlap of one dye's emission into another dye's detector, fluorescence signal compensation was performed. Data obtained from each photomultiplier channels were collected as logarithmic signals and analyzed using the FloMax software 3.0 (Sysmex Partec GmbH, Görlitz, Germany). For each sample, one hundred thousand events were measured at a flow rate of approximately 3000 events/sec. According to Fröhling et al. (2012), the density plots obtained by flow cytometric analyses were divided into four regions. Each region is associated with cells revealing different physiological or morphological properties. The average of the percentage values obtained from three density plots was calculated and illustrated as diagrams where the treatment concentration and the percentage of fluorescent cells is displayed by the x-axis and the y-axis, respectively. The different cell parameters investigated during this experimentation were: cell membrane integrity (TO-PI staining), cell membrane potential ($DiOC_2(3)$ staining), and cell membrane integrity and esterase activity (cF-PI staining) as indicator of the microbial population viability. Staining procedures were performed as described earlier by Fröhling and Schlüter (2015) with some adaptations to the bacterial species used.

Membrane Integrity

Cell membrane integrity was evaluated after the treatments using a combination of thiazole orange (TO) and propidium iodide (PI) dyes (Sigma-Aldrich KGaA, Darmstadt, Germany). Staining procedures were performed in the dark with some variations depending on the microorganisms tested.

For *L. monocytogenes,* 20 μL of resuspended pellets was diluted in PBS (50 mM) to a cell density of approximately 10^6 cells/mL. Thiazole orange (0.2 μM) was added to the samples and incubated for 10 min at room temperature. After incubation, 30 μM propidium iodide was added and samples were analyzed after 5 min.

As described above, *E. coli* resuspended pellets were diluted in Tris buffer (50 mM) to a cell density of approximately 10^6 cells/mL. Thiazole orange was added to a final concentration of 2.5 μM and samples were incubated for 15 min at room temperature. Propidium iodide staining was performed as described for *L. monocytogenes* samples.

Esterase Activity and Membrane Permeabilization

The cell esterase activity and the membrane integrity were evaluated after the exposure to natural antimicrobials using 5(6)-carboxyfluorescein diacetate mixed isomers (cFDA) (Sigma-Aldrich, KGaA, Darmstadt, Germany) and PI. A volume of 60 μL of concentrated *L. monocytogenes* samples were stained with an equal volume of cFDA (200 μM) stock solution to obtain a final cFDA concentration of 100 μM, incubated at 37°C in a water bath for 5 min and centrifuged at 7000 × *g* and 4°C for 5 min. Cell pellets were diluted 1:1 in PBS (50 mM). The PI staining procedure was performed as previously described. The cFDA staining procedure for *E. coli* followed the protocol described for *L. monocytogenes* with some differences. Cells were incubated with 833 μM cFDA (in 50 mM Tris) at 37°C in a water bath for 45 min and then centrifuged at 7000 × *g* and 4°C for 5 min. The pellets were resuspended in 60 μL Tris (50 mM) and stained with 30 μM PI. Samples were analyzed after 10 min of incubation.

Membrane Potential

The membrane potential of bacteria cells was measured using 3,3′-diethyloxacarbocyanine iodide [$DiOC_2(3)$] provided by Sigma-Aldrich, Germany. The protocol applied was in agreement with Novo et al. (1999) and Fröhling and Schlüter (2015) with further modifications. *L. monocytogenes* suspensions were diluted in 50 mM PBS containing 10 mM D-Glucose and 30 μM $DiOC_2(3)$ and then incubated for 15 min at room temperature in the dark. Afterward the suspension was centrifuged at 7000 × *g* and 4°C for 5 min and the pellet was resuspended in 1 mL PBS (50 mM). *E. coli* staining procedure required a different staining buffer due to the higher complexity of the outer and inner cell membrane. First, samples were suspended in 10 mM D-Glucose, 30 μM $DiOC_2(3)$ and EDTA (0.5 mM) Tris buffer (50 mM). After the centrifugation and the incubation time as previously described, *E. coli* samples were resuspended in 1 mL of Tris (50 mM). After the staining procedure samples were immediately analyzed to detect shifts in the cell membrane potential. The ratio of the mean red to the mean green $DiOC_2(3)$-fluorescence channel value was calculated to investigate changes in the membrane potential. Due to the chosen cytometer settings the red/green $DiOC_2(3)$-fluorescence ratio of depolarized cells was ≤1. It was assumed that the red/green ratio of untreated cells represents the relative membrane potential of intact cells (Novo et al., 1999). A reduction of the red/green ratio reflects the loss of cell membrane potential.

Statistical Analysis

Statistical analyses were performed to evaluate significant differences between samples using the R software (R Core Development Team , 2017). One way-ANOVA with Tukey test with a significance level of 0.05 was used.

RESULTS

Treatment Effects on Total Viable Count of *Listeria monocytogenes* Scott A

The exposure of *L. monocytogenes* cells to the natural antimicrobials at different concentrations was performed in the middle of the exponential growth phase (OD = 0.4; λ = 600 nm). In all trials, the cell loads before the treatments

TABLE 3 | Total viable counts of *Listeria monocytogenes* Scott A after 1 h exposure to different concentrations of Citral **(A)** and Carvacrol **(B)**.

A	log CFU/mL		*SD*	B	log CFU/mL		*SD*
Untreated control	8.79	±	0.10[a]	Untreated control	8.81	±	0.17[a]
EtOH 1%	8.95	±	0.23[a]	EtOH 1%	8.7	±	0.18[a]
Citral 50 mg/L	8.86	±	0.11[a]	Carvacrol 20 mg/L	8.75	±	0.23[a]
Citral 85 mg/L	8.59	±	0.10[a]	Carvacrol 35 mg/L	8.79	±	0.09[a]
Citral 125 mg/L	8.88	±	0.14[a]	Carvacrol 50 mg/L	8.79	±	0.16[a]
Citral 250 mg/L (MIC)	8.69	±	0.08[a]	Carvacrol 100 mg/L (MIC)	7.59	±	0.14[b]

Different letters indicate that data are significantly different ($p < 0.05$).

TABLE 4 | Total viable counts of *Listeria monocytogenes* Scott A after 1 h exposure to different concentrations of (E)-2-hexenal **(C)** and Thyme EO **(D)**.

C	log CFU/mL		*SD*	D	log CFU/mL		*SD*
Untreated control	8.87	±	0.22[a]	Untreated control	8.71	±	0.17[a]
EtOH 1%	8.99	±	0.10[a]	EtOH 1%	8.59	±	0.14[a]
(E)-2-Hexenal 150 mg/L	8.99	±	0.23[a]	Thyme EO 40 mg/L	8.72	±	0.07[a]
(E)-2-Hexenal 250 mg/L	9.13	±	0.11[a]	Thyme EO 70 mg/L	8.69	±	0.28[a]
(E)-2-Hexenal 400 mg/L	9.00	±	0.09[a]	Thyme EO 100 mg/L	7.45	±	0.72[b]
(E)-2-Hexenal 800 mg/L (MIC)	8.49	±	0.10[a]	Thyme EO 200 mg/L (MIC)	5.23	±	0.15[c]

Different letters indicate that data are significantly different ($p < 0.05$).

were about 8.8 log CFU/mL (**Tables 3**, **4**). After 1 h exposure, the untreated controls and the samples exposed to 1% ethanol showed the same cell counts. Only the 1 h exposure to carvacrol and thyme EO resulted in a significant reduction of the total viable counts at the highest concentration tested (**Tables 3**, **4**). More specifically, the exposure to 100 mg/L of carvacrol which is corresponding to the MIC value, reduced the viable cell load by one logarithmic cycle (7.59 log CFU/mL) (**Table 3B**). A more severe effect was observed after the exposure to thyme EO, reducing the viable counts to 7.45 log CFU/mL and 5.23 CFU/mL after the exposure to 100 and 200 mg/L, respectively (**Table 4D**). In contrast, 1 h exposure to citral and (E)-2-hexenal produced no significant effect on cell loads of *L. monocytogenes* Scott A cells (**Tables 3A**, **4C**).

Treatment Effects on Total Viable Count of *Escherichia coli* MG 1655

Escherichia coli MG 1655 samples were exposed to EO or their bioactive compounds at the beginning of the stationary growth phase (OD = 2; λ = 600 nm). The cell loads of *E. coli* before the exposure to the natural antimicrobials ranged between 8.5 and 9.0 log CFU/mL (**Tables 5**, **6**). Analogously to *L. monocytogenes*, no differences on the cell loads were highlighted between the untreated controls and the samples exposed to 1% EtOH (**Tables 5**, **6**). The highest effect on *E. coli* was observed after the exposure to citral, even at lowest concentration tested. The exposure to 200 mg/L reduced the viable cell load to 7.22 log CFU/mL. Increasing concentrations reduced the total viable counts to values ranging between 6.60 and 6.12 CFU/mL. The exposure to the citral MBC concentration caused a reduction of cell loads below the detection limit (**Table 5A**). Also thyme EO and carvacrol treatments significantly decreased the total viable cell loads. A reduction of three logarithmic cycles were observed after the exposure to carvacrol MBC concentration (250 mg/L), while an exposure to thyme EO MIC and MBC concentrations reduced cell load to 6.52 and 5.59 log CFU/mL, respectively (**Tables 5B**, **6D**). (E)-2-hexenal exposure did not affect the cell loads of *E. coli* (**Table 6C**).

Treatment Effects on the Membrane Integrity of *Listeria monocytogenes* Scott A

The exposure of *L. monocytogenes* Scott A to different natural antimicrobials caused an augment of the fractions with cells having slightly permeabilized cell membranes (PI + TO stained cells) and permeabilized cell membranes (PI stained cells). The distribution of stained cells varied according to different treatments and concentrations used. The percentage of *L. monocytogenes* Scott A with intact cell membranes remained almost constant (above 80%) after exposure to 1% ethanol in all the performed trials (**Figure 1**). The different concentrations of citral (**Figure 1A**) increased the percentages of cells with slightly permeabilized cell membranes. The magnitude of the damaging effect rose with increasing citral concentrations. In fact, at the highest concentration (250 mg/L) 60% of stained cells showed a slightly permeabilized cell membrane. A value of about 3% cells with permeabilized cell membrane was observed in all the conditions independently on the severity of chemical stress applied (citral concentration). A similar pattern was observed for carvacrol (**Figure 1B**). All concentrations tested induced a significant reduction in the population with intact cell membranes. In particular,

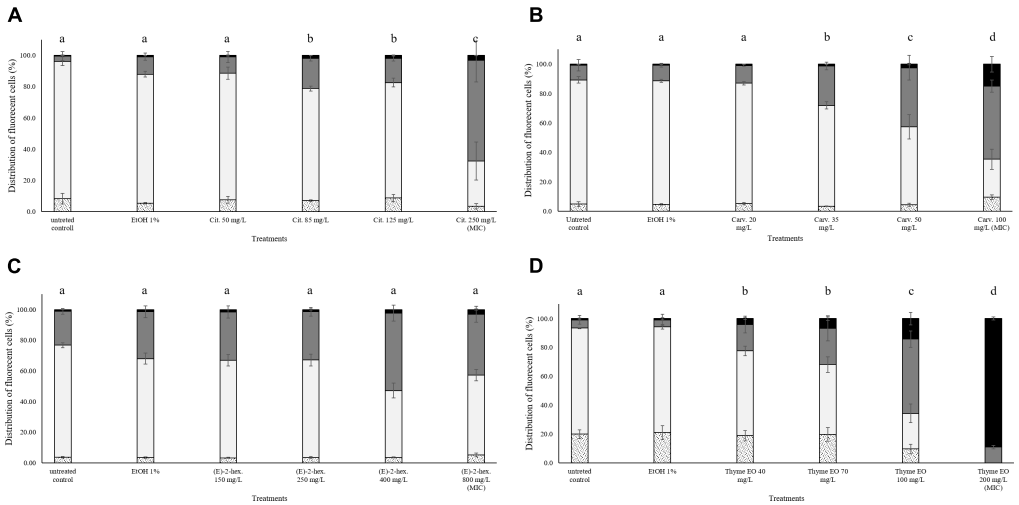

FIGURE 1 | Membrane integrity of *Listeria monocytogenes* Scott A after 1 h exposure to different concentrations of natural antimicrobials: Citral **(A)**, Carvacrol **(B)**, (E)-(2)-hexenal **(C)**, and thyme EO **(D)**. White bars with dots represent cell fragments or unstained cells; White bars represent cells with intact cell membrane; Gray bars represent cells with slight cell membrane permeabilization; Black bars represent cells with (complete) cell membrane permeabilization. Different letters indicate that data are significantly different ($p < 0.05$).

while in the control samples the population with intact cell membranes was about 80%, this value decreased to 25% in the samples exposed to 100 mg/L carvacrol (MIC value). Simultaneously, the percentage of cells with slightly permeabilized cell membranes increased from 12 to 50% augmenting the carvacrol concentrations. The exposure to the MIC carvacrol concentration also raised the percentage of cells with permeabilized cell membranes up to 15%. Compared to the untreated control, the exposure to (E)-2-hexenal had no significant effect on the cell membrane integrity

TABLE 5 | Total viable count of *Escherichia coli* MG 1655 after 1 h exposure to Citral **(A)** and Carvacrol **(B)**.

A	log		*SD*	B	log		*SD*
	CFU/mL				**CFU/mL**		
Untreated control	8.97	±	0.24[a]	Untreated control	8.9	±	0.16[a]
EtOH 1%	8.96	±	0.15[a]	EtOH 1%	8.98	±	0.21[a]
Citral 200 mg/L	7.22	±	0.20[b]	Carvacrol 25 mg/L	8.65	±	0.09[a]
Citral 330 mg/L	6.6	±	0.13[c]	Carvacrol 40 mg/L	9.02	±	0.11[a]
Citral 500 mg/L	6.28	±	0.18[c]	Carvacrol 60 mg/L	8.75	±	0.25[a]
Citral 1000 mg/L (MIC)	6.12	±	0.20[d]	Carvacrol 125 mg/L (MIC)	8.79	±	0.22[b]
Citral 3000 mg/L (MBC)	-*			Carvacrol 250 (MBC)	4.85	±	0.47[c]

**Detection limit 1 log CFU/mL.*
Different letters indicate that data are significantly different ($p < 0.05$).

TABLE 6 | Total viable count of Escherichia *coli* MG 1655 after 1 h exposure to (E)-2-hexenal **(C)** and Thyme EO **(D)**.

C	log		*SD*	D	log		*SD*
	CFU/mL				**CFU/mL**		
Untreated control	8.88	±	0.26[a]	Untreated control	8.76	±	0.24[a]
EtOH 1%	9	±	0.23[a]	EtOH 1%	8.63	±	0.22[a]
(E)-2-Hex. 80 mg/L	8.93	±	0.20[a]	Thyme EO 50 mg/L	8.53	±	0.32[a]
(E)-2-Hex. 135 mg/L	8.78	±	0.20[a]	Thyme EO 85 mg/L	8.63	±	0.35[a]
(E)-2-Hex. 200 mg/L	8.89	±	0.22[a]	Thyme EO 125 mg/L	8.86	±	0.53[a]
(E)-2-Hex. 400 mg/L (MIC)	8.83	±	0.09[a]	Thyme EO 250 mg/L (MIC)	6.52	±	0.04[c]
(E)-2-Hex. 425 mg/L (MBC)	8.64	±	0.23[a]	Thyme EO 300 mg/L (MBC)	5.59	±	0.26[d]

Different letters indicate that data are significantly different ($p < 0.05$).

of *L. monocytogenes* Scott A (**Figure 1C**). Thyme EO had the highest effect on the cell membrane integrity compared to the other natural antimicrobials tested. The percentage of slightly permeabilized and completely permeabilized cells increased with the treatment concentrations, and the exposure to 200 mg/L of thyme EO (MIC concentration) induced a complete membrane permeabilization in more than 90% of the cell population (**Figure 1D**). Except for Thyme EO exposure, the percentage values of unstained cells/cell fragments were lower than 3% independently on the antimicrobial and its concentration (**Figure 1**).

Treatment Effects on the Esterase Activity and Membrane Integrity of *Listeria monocytogenes* Scott A

No effects on the esterase activity of *L. monocytogenes* cells were evidenced after the exposure to natural antimicrobials or their bioactive compounds. As shown in **Figure 2**, the percentage of fluorescent cells with intact cell membranes and esterase activity was constantly above 80% with the only one exception of thyme EO 200 mg/L exposure. The MIC thyme EO exposure caused a complete permeabilization of the cells, however, these cells still showed esterase activity (**Figure 2D**).

Treatment Effects on the Cell Membrane Potential of *Listeria monocytogenes* Scott A

The measurement of the relative membrane potential using 3,3′-Diethyloxacarbocyanine iodide $DiOC_2(3)$ showed no significant differences between untreated and treated cells of *L. monocytogenes* Scott A. In fact, red/green ratios were always lower than the value 1 independently on the treatments and concentrations used (**Figure 3**).

Treatment Effects on Membrane Integrity of *Escherichia coli* MG 1655

In all trials, the percentage of *E. coli* stained only with TO (cells with intact cell membrane) remained almost constant (above 80%) after the exposure to 1% ethanol (**Figure 4**). Citral determined a significant effect on cell membrane integrity even at sub-lethal concentrations (**Figure 4A**). After exposure to 200–300 mg/L citral, the percentages of cells with slightly permeabilized cell membranes were higher than 80%. No cells with intact cell membrane were found after the exposure to citral MIC and MBC values (**Figure 4A**). Minor impacts on *E. coli* cell membrane integrity were evidenced after the exposure to carvacrol and (E)-2-hexenal, independently on their sub-lethal concentrations (**Figures 4B,C**). Only the exposure to carvacrol inhibition and bactericidal concentrations (120–250 mg/L) triggered cell membrane permeabilization (**Figure 4B**). The effect of thyme EO on the cell membrane was dependent on the applied concentration. The fluorescence signal of intact cells decreased (80–20%) with an increase of the treatment concentration, while the percentage of cells with permeabilized cell membranes increased simultaneously (**Figure 4D**).

Treatment Effects on Esterase Activity and Membrane Integrity of *Escherichia* coli MG 1655

The exposure to citral induced cell membrane permeabilization of *E. coli* without a loss of esterase activity. The percentage of fluorescent cells with permeabilized cell membranes but still existing esterase activity was higher than 80% independent of the natural antimicrobial concentrations. Only the exposure to bactericidal concentrations (1000 and 3000 mg/L citral) increased (10%) the amounts of the populations with permeabilized cell membranes and without esterase activity (**Figure 5A**). Minor impacts on *E. coli* cell membrane integrity and esterase activity were evidenced after the exposure to carvacrol and (E)-2-hexenal. *E. coli* samples treated with the bactericidal concentrations (120 and 250 mg/L) of carvacrol showed a cell membrane permeabilization without a loss of esterase activity (**Figure 5B**). The exposure to (E)-2-hexenal increased the percentage of cell fragments or unstained cells, independently on the concentration used. They represented about the 15% of the whole population in all the conditions tested (**Figure 5C**). The effects of thyme EO on the cell membrane and esterase activity were related to the concentration tested. The fluorescence signal of the intact cells decreased (80–30%) with the exposure to the sub-lethal concentration tested while the percentage of cell populations with permeabilized cell membranes and esterase activity increased (**Figure 5D**). A significant loss in the cell esterase activity was only observed after the exposure to 250 and 300 mg/L thyme EO.

Treatment Effects on Cell Membrane Potential of *Escherichia coli* MG 1655

The measurement of the relative membrane potential using $DiOC_2(3)$ showed that the untreated *E. coli* cells had a red/green ratio of 1.69 before the exposure to citral (**Figure 6A**). The value was reduced below 1 independently of the concentration used, suggesting the capability of citral to depolarize the cell membrane of *E. coli*. A membrane depolarization was also observed for the MIC and MBC values of thyme EO (**Figure 6D**). No cell membrane depolarization was observed after the exposure to carvacrol and (E)-2-hexenal (**Figures 6B,C**) but (E)-2-hexenal induced a concentration dependent reduction of the cell membrane potential.

DISCUSSION

As described by many authors, EOs and their bioactive compounds are characterized by an antimicrobial activity both *in vitro* and in real food systems. Although their antimicrobial properties are well-documented, only limited information is available about their mechanisms of action on *E. coli* and *L. monocytogenes* (Lambert et al., 2001; Burt, 2004; Bakkali et al., 2008; Xu et al., 2008; Hyldgaard et al., 2012; Picone et al., 2013). Additionally, the literature on the responses of

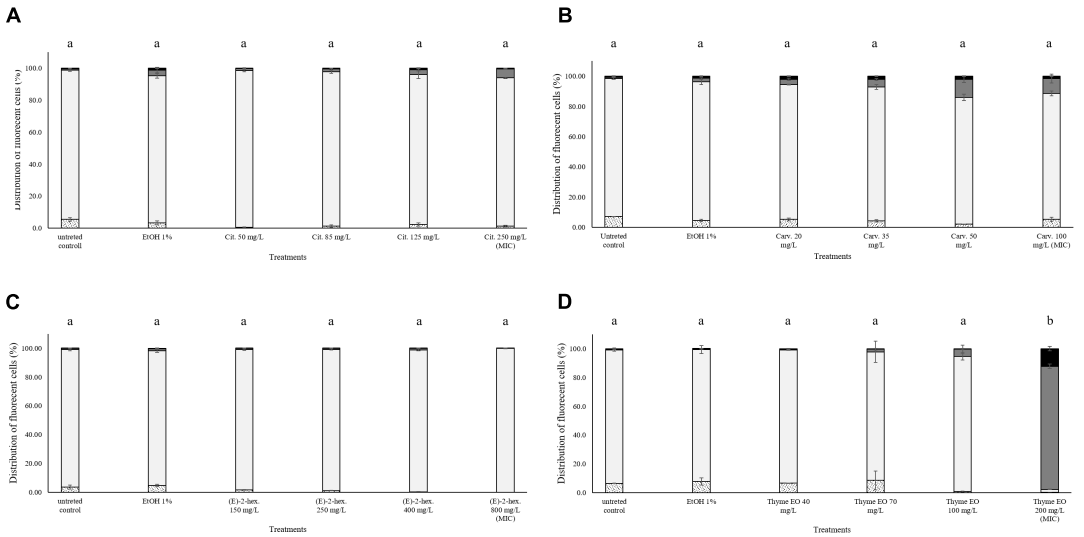

FIGURE 2 | Esterase activity and membrane integrity of *L. monocytogenes* Scott A after 1 h exposure to natural antimicrobials: Citral **(A)**, Carvacrol **(B)**, (E)-(2)-hexenal **(C)**, and thyme EO **(D)**. White bars with dots represent cell fragments or unstained cells; White bars represent cells with intact cell membrane and esterase activity; Gray bars represent cell membrane permeabilization but still existing esterase activity; Black bars represent cells with cell membrane permeabilization but without esterase activity. Different letters indicate that data are significantly different ($p < 0.05$).

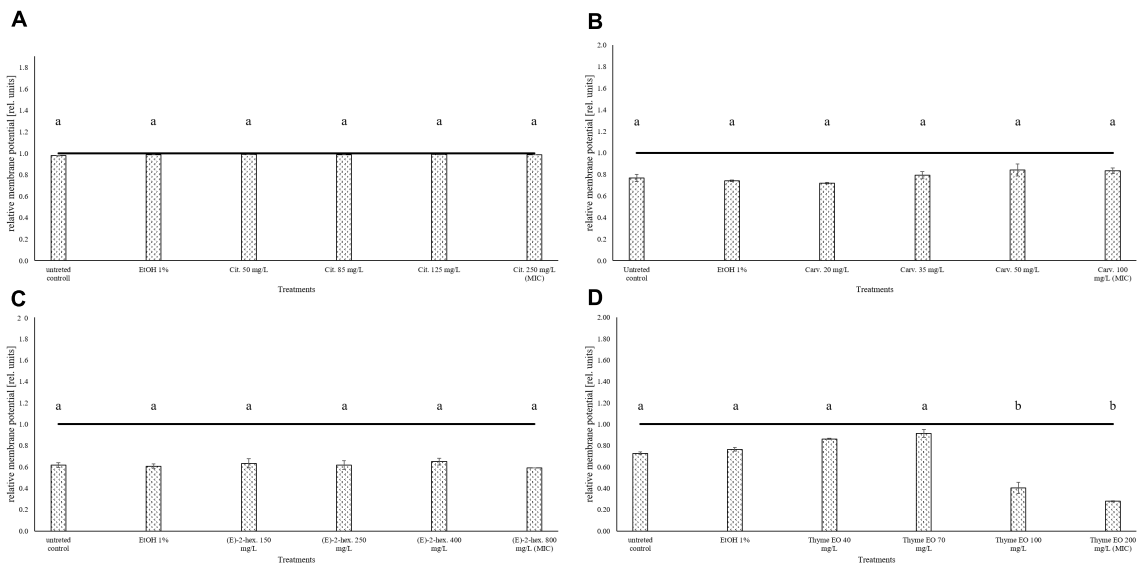

FIGURE 3 | Relative membrane potential of *L. monocytogenes* Scott A, expressed as red/green ratio of $DiOC_2(3)$-fluorescence intensity, after 1 h exposure to natural antimicrobials: Citral **(A)**, Carvacrol **(B)**, (E)-(2)-hexenal **(C)**, and thyme EO **(D)**. The black line represents the threshold between polarized (ratio > 1) and depolarized (ratio < 1) cells. Different letters indicate that data are significantly different ($p < 0.05$).

the whole cell populations of the selected species to EOs is still scarce (Xu et al., 2008). However, cell wall, membrane and energetic pathways are generally considered as the main microbial cell targets of EOs (Burt, 2004; Bakkali et al., 2008; Hyldgaard et al., 2012). In this framework multiparametric flow cytometric analyses were performed in order to assess the effects of citral, carvacrol, (E)-2-hexenal and thyme EO on the whole microbial populations of the two target microorganisms. Consequently, their effects on membrane integrity, esterase activity and cell membrane potential were investigated. Flow cytometric data of *L. monocytogenes* Scott A and *E. coli* MG 1655 populations after 1 h stress exposure revealed specific response patterns in relation to the natural antimicrobials and their concentrations. Concerning the membrane permeabilization, the percentage of permeabilized cells raised with the antimicrobial concentration applied, with the only exception for (E)-2-hexenal. The membrane integrity was analyzed using dye exclusion methods with thiazole orange (TO) and the divalent propidium

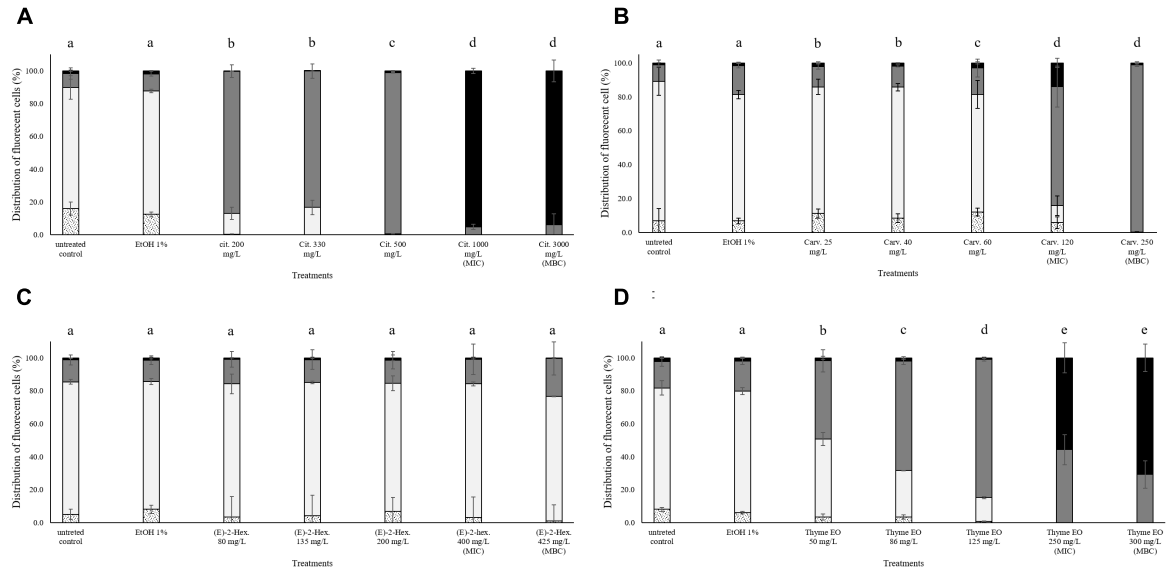

FIGURE 4 | Membrane integrity of *Escherichia coli* MG 1655 after 1 h exposure to different concentrations of natural antimicrobials: Citral **(A)**, Carvacrol **(B)**, (E)-(2)-hexenal **(C)**, and thyme EO **(D)**. White bars with dots represent cell fragments or unstained cells; White bars represent cells with intact cell membranes; Gray bars represent cells with slightly permeabilized cell membranes; Black bars represent cells with (complete) with permeabilized cell membranes. Different letters indicate that data are significantly different ($p < 0.05$).

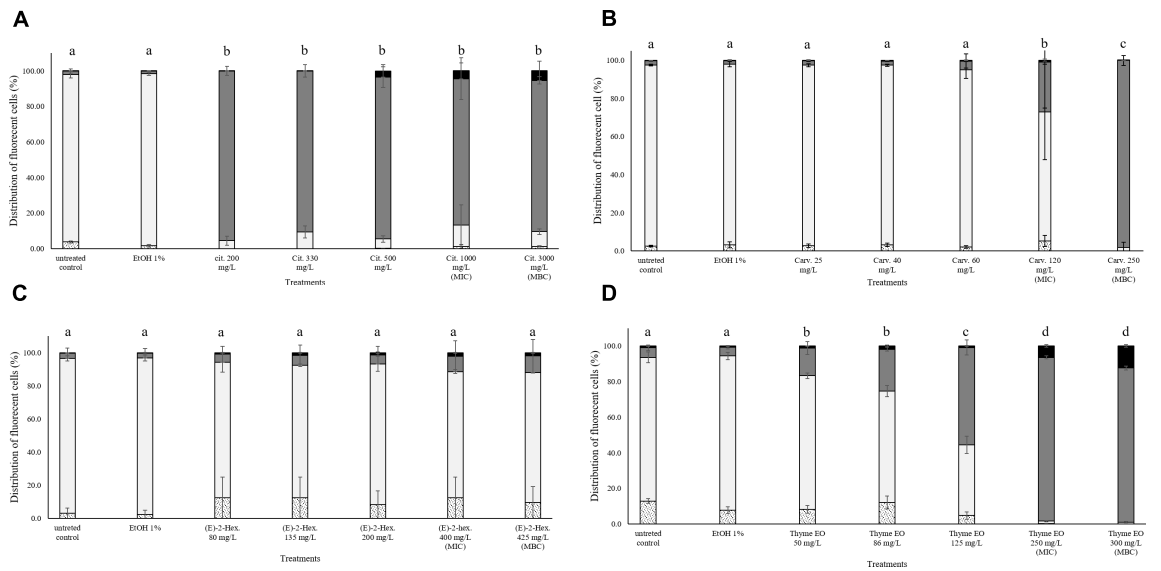

FIGURE 5 | Esterase activity and membrane integrity of *E. coli* MG 1655 after 1 h exposure to natural antimicrobials: Citral **(A)**, Carvacrol **(B)**, (E)-(2)-hexenal **(C)**, and thyme EO **(D)**. White bars with dots represent cell fragments or unstained cells; White bars represent cells with intact cell membrane and esterase activity; Gray bars represent cell membrane permeabilization but still existing esterase activity; Black bars represent cells with cell membrane permeabilization but without esterase activity. Different letters indicate that data are significantly different ($p < 0.05$).

iodide (PI) as dyes. In fact, TO is able to pass thought lipidic bilayers and to stain both DNA or RNA while PI, due to multiple charges, can only react with nucleic acids when membrane is disrupted or permeabilized (Kim et al., 2009; Díaz et al., 2010). Both target strains showed an increased cell membrane permeabilization with an increase of citral, carvacrol, and thyme EO concentrations. However, *E. coli* reacted sensitively to all the natural antimicrobials. In fact, they induced more severe membrane permeabilization and cell load reductions of *E. coli* in comparison to *L. monocytogenes*. The most effective antimicrobial against *E. coli* was citral. Actually, *E. coli* showed the highest cell load reduction and membrane permeabilization after the exposure to citral. A wide literature shows that the outer membrane of Gram-negative bacteria, which acts as a barrier

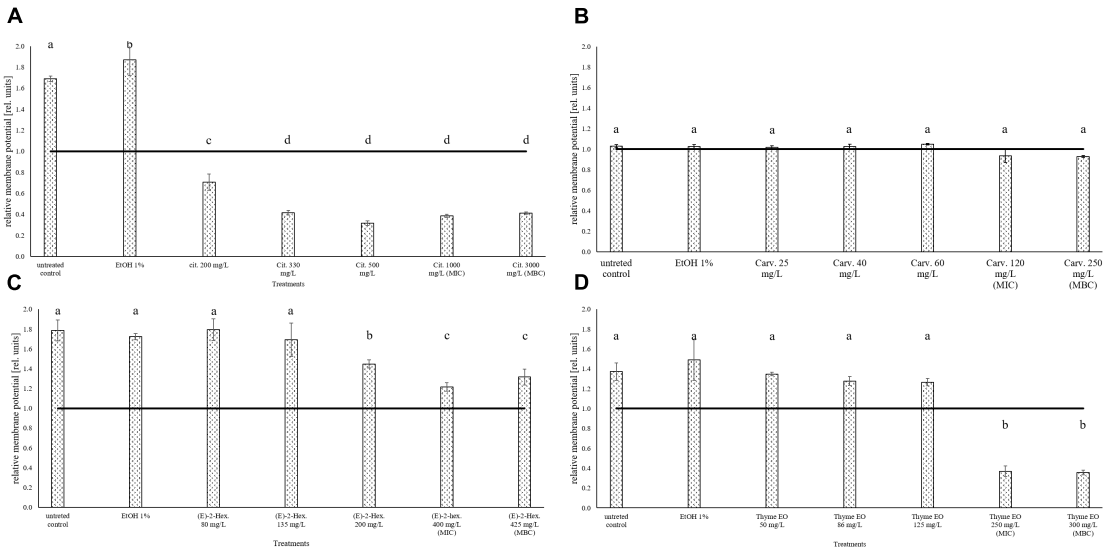

FIGURE 6 | Relative membrane potential of *E. coli* MG 1655, expressed as red/green ratio of $DiOC_2(3)$-fluorescence intensity, after 1 h exposure to natural antimicrobials: Citral **(A)**, Carvacrol **(B)**, (E)-(2)-hexenal **(C)**, and thyme EO **(D)**. The black line represent the threshold between polarized (ratio > 1) and depolarized (ratio < 1) cells. Different letters indicate that data are significantly different ($p < 0.05$).

against macromolecules and hydrophobic substances, increase their resistance to several antimicrobials including many EO (Nikaido and Vaara, 1985; Helander et al., 1997). However, also Somolinos et al. (2008) demonstrated that citral was more effective on *E. coli* J1 than on *L. monocytogenes* NCTC11994 under different experimental conditions, and especially at pH 7. These authors, using fluorescence microscopy and propidium iodide as dye, demonstrated that citral disrupted the outer cell envelope of *E. coli,* forming pores and permitting the cytoplasm entrance of molecules with a size of 660 Da. To destabilize the lipopolysaccharide layer of the outer membrane, the use of several chelating agents, such as EDTA, citric acid, and other substances, as well as high pressure homogenization have been proposed (Cutter and Siragusa, 1995; Helander et al., 1997; Vannini et al., 2004; Patrignani et al., 2010).

Different authors reported that citral and other low molecular mass ketons are sufficiently hydrophilic to pass throughout porin proteins to the deeper parts of Gram-negative bacteria without destabilization of the outer membrane (Helander et al., 1997; Lanciotti et al., 2003; Belletti et al., 2004; Belletti et al., 2008).

Carvacrol and thyme EO significantly reduced *E. coli* cell loads but only when used at MIC and MBC values. Only these concentrations induced a significant membrane permeabilization of *E. coli* cells. These data are in agreement with those of Xu et al. (2008) obtained by flow cytometry after exposure of *E. coli* AS1 90 to 200 mg/L of carvacrol and thymol, the major constituent of thyme EO. These authors showed cell membrane permeabilization processes associated with significant reductions of the cell loads (Xu et al., 2008). Also Gill and Holley (2006), using confocal laser scanning microscopy, showed a clear membrane disruption of *E. coli* O157:H7 after 10 min exposure to eugenol and carvacrol used at concentrations able to reduce the pathogen viability of about 8 log cycles.

A concentration dependent permeabilization process was also evidenced for *L. monocytogenes* Scott A after exposure to citral, carvacrol, and thyme EO. No literature is available on the effects of such antimicrobials on *L. monocytogenes* membrane permeabilization. However, Ultee et al. (1999) showed for Gram-positive *Bacillus cereus* that carvacrol caused an increased membrane permeability to cations such as H^+ and K^+. Also, no significant *L. monocytogenes* cell load reductions were highlighted for citral, independent of the concentration used. Somolinos et al. (2008) also showed a scarce sensitiveness of *L. monocytogenes* ATCC19114 serotype 4a to citral. Compared to citral, carvacrol and thyme EO were more effective on *L. monocytogenes*. However, significant cell load reductions were only observed using the MIC value of carvacrol and thyme EO concentrations. Friedman et al. (2002) described how carvacrol and thymol had the highest effect on the cell loads of *L. monocytogenes* RM2199 and RM2388 compared to other 23 EO constituents using a microplate assay. The effects of the natural antimicrobials on the esterase activity of *L. monocytogenes* and *E. coli* was measured because it is considered as reliable way to evaluate the cell damages induced after several antimicrobial treatments (Díaz et al., 2010; Surowsky et al., 2014; Fröhling and Schlüter, 2015; Hong et al., 2015; Combarros et al., 2016; Meng et al., 2016). In fact, carboxyfluorescein diacetate (cFDA), the fluorescent dye used to measure esterase activity, is a lipophilic non-fluorescent compound which is hydrolyzed in the cytoplasm to the fluorescent carboxyfluorescein (cF) by unspecific esterases. According to several literature data only cells with integer membrane and active intracellular enzymes remain fluorescent (Haugland, 1999; Hoefel et al., 2003; Fröhling and Schlüter, 2015; Reineke et al., 2015). However, the data obtained in our experimental conditions showed for both target strains that this enzymatic activity was not affected by the exposure to

the antimicrobials used, independently on their concentrations. This data seems in disagreement with literature showing that membrane permeabilization is generally associated to the losses of esterase activity (Hayouni et al., 2008; Paparella et al., 2008; Xu et al., 2008). A decrease of esterase activity which was associated to membrane permeabilization was observed also in *L. monocytogenes* and *E. coli* exposed to EOs or their components (Xu et al., 2008). However the literature data evidenced losses of esterase activity only when lethal antimicrobial concentrations were used. For example, Paparella et al. (2008) showed by flow cytometric analyses that *L. monocytogenes* ATCC19114 serotype 4a had significant reductions of both esterase activity and cell viability after 1 h exposure to emulsified cinnamon, oregano, or thyme EOs (Paparella et al., 2008). However, these Authors tested the effects of emulsions having EO concentrations ranging between 0.02 and 0.5%. These concentrations are generally lethal concentrations for *L. monocytogenes* independently on the exposure conditions. However, they are not compatible with any usage in food systems due to the low sensory thresholds of the natural antimicrobials tested. In our experimental conditions, probably due to the use of concentrations significantly lower to those tested in the literature, the amount of cFDA hydrolyzed into cF after the exposure to natural antimicrobials remained constant, independently on the cell membrane permeabilization degree and the microorganism considered. In addition, the membrane potential of *L. monocytogenes* was not affected by the 1 h exposure to the natural antimicrobials considered. The membrane potential of cells is considered as fundamental in numerous processes of the live cell physiology and it is strongly related to bacterial viability (Novo et al., 1999). In fact, according to the literature, only living cells are able to maintain membrane potential (Díaz et al., 2010). In our experimental conditions, also at MIC values, the antimicrobials tested did not affect the membrane potential of *L. monocytogenes* compared to the control ones. However, also at MIC values of the tested antimicrobials, *L. monocytogenes* cells showed only a slight presence or even a complete absence of permeabilization of the cell membrane. By contrast, *E. coli* compared to *L. monocytogenes*, showed with a higher sensitiveness to almost all the antimicrobials considered, showed also cell membrane depolarization depending on the antimicrobial used and its concentration. A complete depolarization (red/green ratio below 1) of *E. coli* cells was observed for all citral concentrations tested and thyme EO MIC and MBC values. Also Kim and Kang (2017) observed that the cell membrane potential of *E. coli* O157:H7 was significantly reduced after exposure to citral and thymol combined with an ohmic heating treatment, both in model and real food systems.

In our experimental conditions, also (E)-2-hexenal exposure caused a reduction of relative membrane potential of the *E. coli* population. In contrast, no effect on the membrane potential of *E. coli* was evidenced after carvacrol treatments. Different studies confirmed that Gram-negative bacteria, due the outer membranes, were characterized by a higher resistance to carvacrol (Kokoska et al., 2002; Okoh et al., 2010). The reduction of membrane potential after antibacterial treatments is considered fundamental for pathogenic species, since viable but not culturable cells are still able to cause diseases (Fröhling and Schlüter, 2015).

In general, the data obtained indicated that sub-lethal treatments had minor impacts on *L. monocytogenes* compared to *E. coli*. In fact, all the antimicrobial tested induced only a slight cell membrane permeabilization (with the exception of (E)-2-hexenal) of *L. monocytogenes*, while only the exposure to carvacrol and thyme EO MIC values reduces the cultivability and significant cell load reductions were observed.

Escherichia coli was more sensitive to all the antimicrobials considered, not only in terms of cultivability but also in terms of membrane permeability and membrane potential. However, also for this strain, the antimicrobials used were unable to cause irreversible damages. In fact, the percentage of unstained cells of fragments remained constant and below the 3% independently on the strain, natural antimicrobial and the concentration used. As reported by Booyens and Thantsha (2014) during an antimicrobial treatment, the increase of the unstained population is related to a severe cell lysis or to a decreased staining accuracy due to formation of conglomerates. In addition, the increase of the unstained fraction is reported to be related with highly permeabilized or lysed cells unable to grow (Fröhling and Schlüter, 2015). The higher effect on membrane permeabilization of TO and PI staining compared to cF and PI dyes can be due to the fact that the effect of the last dyes was measured after a longer time span following the exposure to antimicrobials. It is presumable that the longer incubation after the stress exposure permitted the activation of microbial response mechanisms involving cell membrane repair. A wide literature shows that a precocious modification of cell membrane composition is pivotal for the microbial adaptation and survival in harsh conditions and sub-lethal stresses in order to maintain the proper fluidity, functionality, and permeability (Guerzoni et al., 2001; Gianotti et al., 2009; Mendoza, 2014; Serrazanetti et al., 2015; Siroli et al., 2015a). In fact, the immediate activation of the response mechanisms involving membrane modification was demonstrated both in fungi and bacteria to face even short time (lower that 100 min) physical-chemical stresses, including the exposure to natural antimicrobials (Guerzoni et al., 2001; Di Pasqua et al., 2006; Patrignani et al., 2008; Gianotti et al., 2009; Mendoza, 2014; Serrazanetti et al., 2015).

CONCLUSION

The flow cytometry approach allowed to understand the targets of sub-lethal concentrations of citral, carvacrol, (E)-2-hexenal and thyme EO to *L. monocytogenes* and *E. coli* cells. The data showed that the membrane permeabilization is a common action mechanism of the antimicrobials considered on both strains. In contrast, they showed that esterase activity was not affected independent of strain, antimicrobial and its concentration. The approach used revealed that some antimicrobials such as citral, carvacrol and thyme EO were more effective against the Gram-negative strain used. These results are particularly important since Gram-negative bacteria are more

resistant to many antimicrobials. However, the multiparameter data obtained showed that the natural antimicrobials and the concentrations used also caused reversible damages on *E. coli* MG 1655, the most sensitive strain tested, since the percentage of cell fragments remained constant even when the MIC values were used and when the membrane was depolarized. These data suggests that the levels used of citral, carvacrol, and thyme EO can be used as preservatives to control the growth of *L. monocytogenes* and *E. coli* only in combinations with other hurdles. In fact, concentrations capable of having lethal effects are incompatible with the food sensory features due to their low sensory threshold. Consequently, the detailed knowledge of the action mechanisms of natural antimicrobials considered in relation to the others hurdles applied is mandatory for their implementation at industrial level as preservation strategies. However, the implementation processes should be optimized in relation to the food matrices and production processes.

AUTHOR CONTRIBUTIONS

GB and FP contributed equally to the manuscript in the realization of lab trials. LS contributed to the statistical analysis. RL, OS, and AF contributed to the manuscript writing.

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Biochemical Components Associated With Microbial Community Shift During the Pile-Fermentation of Primary Dark Tea

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Primary dark tea is used as raw material for compressed dark tea, such as Fu brick tea, Hei brick tea, Hua brick tea, and Qianliang tea. Pile-fermentation is the key process for the formation of the characteristic properties of primary dark tea, during which the microorganism plays an important role. In this study, the changes of major chemical compounds, enzyme activities, microbial diversity, and their correlations were explored during the pile-fermentation process. Our chemical and enzymatic analysis showed that the contents of the major compounds were decreased, while the activities of polyphenol oxidase, cellulase, and pectinase were increased during this process, except peroxidase activity that could not be generated from microbial communities in primary dark tea. The genera *Cyberlindnera*, *Aspergillus*, *Uwebraunia*, and *Unclassified Pleosporales* of fungus and *Klebsiella*, *Lactobacillus* of bacteria were predominant in the early stage of the process, but only *Cyberlindnera* and *Klebsiella* were still dominated in the late stage and maintained a relatively constant until the end of the process. The amino acid was identified as the important abiotic factor in shaping the microbial community structure of primary dark tea ecosystem. Network analysis revealed that the microbial taxa were grouped into five modules and seven keystone taxa were identified. Most of the dominant genera were mainly distributed into module III, which indicated that this module was important for the pile-fermentation process of primary dark tea. In addition, bidirectional orthogonal partial least squares (O2PLS) analysis revealed that the fungi made more contributions to the formation of the characteristic properties of primary dark tea than bacteria during the pile-fermentation process. Furthermore, 10 microbial genera including *Cyberlindnera*, *Aspergillus*, *Eurotium*, *Uwebraunia*, *Debaryomyces*, *Lophiostoma*, *Peltaster*, *Klebsiella*, *Aurantimonas*, and

Methylobacterium were identified as core functional genera for the pile-fermentation of primary dark tea. This study provides useful information for improving our understanding on the formation mechanism of the characteristic properties of primary dark tea during the pile-fermentation process.

Keywords: primary dark tea, food fermentation, microbial community, biochemical components, Illumina MiSeq sequencing

INTRODUCTION

Dark tea is a unique kind of microbial fermented teas, which is native to many regions of China, including Hunan, Yunnan, and Hubei provinces (Zhang et al., 2013). The manufacturing process of dark tea can be divided into primary processing stage and reprocessing stage. During the primary processing stage, primary dark tea was produced and was mainly used as material for compressed dark tea, including Fu brick tea, Hei brick tea, Hua brick tea, and Qiangliang tea. The manufacturing process of primary dark tea covers the following steps, harvesting fresh tea leaves, fixing, rolling, pile-fermentation, and drying processes (Li et al., 2017). In the above processes, the pile-fermentation, a kind of solid-state fermentation, is the key process in the production of primary dark tea. The pile-fermentation process of primary dark tea is characterized by the growth and succession of microbial communities, and the microorganism plays a key role in the formation of the characteristic properties of primary dark tea during the pile-fermentation process (Wang et al., 1991a).

A comprehensive study of microbial community succession could help to understand that how the sensory quality of primary dark tea was formed during the pile-fermentation process. Some kinds of microbial genera, including *Candida* sp., *Aspergillus niger*, and *Penicillium* in fungal community and *Non-spore bacteria*, *Bacillus*, and *Coccus* in bacterial community, were identified as the dominant microbial genera by the culture-dependent method during the pile-fermentation process of primary dark tea (Wen and Liu, 1991). However, the culture-dependent method is of limited value for identifying the microbial communities due to the limitation of cultural media (Li et al., 2011). Hence, the culture-independent methods, including terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), and high-throughput sequencing technology (i.e., Illumina MiSeq sequencing) that use direct analysis of the diversity of ribosomal RNA genes with no culture step (Amann and Ludwig, 2000), were introduced for identifying the structure of microbial communities in compressed dark tea, such as Fu brick tea and Pu-erh tea (Abe et al., 2008; Xu et al., 2011; Zhao et al., 2013; Fu et al., 2016; Li et al., 2017). The genera *Aspergillus*, *Blastobotrys*, *Bacillus*, and *Enterobacteriaceae* were the major microbial communities in Pu-erh tea (Abe et al., 2008; Zhao et al., 2013), and *Aspergillus*, *Cyberlindnera*, *Candida*, *Penicillium*, *Eurotium*, *Beauveria*, *Debaryomyces*, *Pestalotiopsis*, *Pichia*, *Rhizomucor*, and *Verticillium* were the dominant microbial communities in Fu brick tea by PCR-DGGE (Xu et al., 2011) and high-throughput sequencing technology (Li et al., 2017).

During the pile-fermentation process, some new transformation products were generated in tea leaves. Our previous study showed that the power of substance transformation in tea leaves were mainly derived from the microbial enzymes, which were secreted from the microorganism during the pile-fermentation process of primary dark tea (Wang et al., 1991a). These transformations resulted in the formation of the characteristic properties of primary dark tea. The microbial metabolism, which produce the secreted enzymes such as polyphenol oxidase (PPO), cellulase (CEL), and pectinase (PEC), thereby providing the effective biochemical power to oxidation of catechins, decomposition of cellulose, and hydrolyses of pectin and proteins. The microbial enzymatic action played an important role in the formation of the characteristics of primary dark tea (Liu et al., 1991). During the pile-fermentation process, the total contents of polyphenol, catechin, and soluble carbohydrate were decreased, and the catechin composition varied irregularly (Wang et al., 1991c). The content of total amino acid (AA) was decreased, especially theanine and glutamic acid were decreased significantly; by contrast, the content of indispensable AAs, such as lusine, phenylalanine, leucine, isoleucine, methionine, and valine, were increased remarkably. And the levels of caffeine, theobromine, and theophylline were not significantly variated (Wang et al., 1991b).

The succession of microbial community in primary dark tea during pile-fermentation process results in a dynamic taste and aroma of primary dark tea. Variation of major chemical compounds and enzyme activities in fermented tea has been studied (Liu et al., 1991; Wang et al., 1991b,c). The culturable microorganisms have also been identified in primary dark tea during the pile-fermentation process (Wen and Liu, 1991). However, the associations among microbiota and chemical compounds or enzyme activities, and so far the core functional microbiota were not clarified in primary dark tea. Thus, the assembly and dynamics of microbial community were characterized by Illumina MiSeq sequencing during the pile-fermentation of primary dark tea. The changes of major chemical compounds content and enzyme activity were also investigated during this process. Based on these results, the relationship between microbiota and chemical compounds or enzyme activities was investigated by bidirectional orthogonal partial least squares (O2PLS). Finally, the core functional microorganisms were identified by comparison of the comprehensive importance of microorganisms correlated with the formation of characteristic properties of primary dark tea. The knowledge provide a new insight for better understanding on the mechanism of pile-fermentation in primary dark tea.

MATERIALS AND METHODS

Tea Leaf Samples and Process Characterization

Sampling of primary dark tea was carried out in Ba Jiao tea factory (Yiyang, Hunan province, China). The manufacturing process of primary dark tea was performed as described by DB43/T660-2011 with a little modification (Liu et al., 2011). Briefly, mature tea leaves (one bud and three to six leaves) were plucked from medium and sprinkled with water then fixing at 280–320°C. The fixing leaves rolled twice by machine. Then, the rolling leaves (about 930 kg) were piled 1.1 m high, with ambient temperature about 28°C and moisture content about 78% in pile-fermentation apparatus for 12 h. Auto drying machine was used for drying the fermentation leaves at 120–130°C twice or drying in the sun. Fifteen samples were collected from three independent batches of primary dark tea at different time of pile-fermentation process: pile-fermentation 0-, 3-, 6-, 9-, and 12-h stages. The samples were packaged in sterile polyethylene bags, transported to the laboratory, and stored at −80°C until required.

Chemical Compounds and Enzyme Activity Analysis

The content of water extract (WE) was determined according to the National Standard GB/T 8305-2013 (Tea-Determination of water extracts content) (Zhou et al., 2013). Total polyphenols (TP) and flavonoids (FLA) content were determined by a colorimetric method, as described previously (Li et al., 2013). Free AA content was determined according to the National Standard GB/T 8314-2013 (Tea-Determination of free amino acids content) (Xu et al., 2013). Soluble sugar (SS) content was determined according to sulfuric acid–anthrone colorimetric method, as described previously (DuBois et al., 1956). All analyses were performed three times.

Extraction of enzyme extracts was based on our previous method with slight modifications (Teng et al., 2017). 1.25-g tea leaves were ground with 0.75-g polyvinylpolypyrrolidone (PVPP) in a mortar on ice. The crude enzyme was extracted with 25-mL citrate buffer (pH 5.6) on ice for 12 h. Subsequently, the mixture was centrifuged at 15,000 × *g* for 30 min at 10°C, and the clear supernatant was collected and filled to 25 mL with citrate buffer. The clear supernatant was used in all enzyme assays. The activities of PPO, peroxidase (POD), CEL, and PEC were determined according to previous studies (Wood and Bhat, 1988; Huang, 1997; Biz et al., 2014). All analyses was performed three times. The detail approaches were described in Supplementary Material 1.

DNA Extraction and Sequencing

Each tea sample (5 g) was mixed with sterile water (25 mL), stirred thoroughly, filtered through three layers of coarse sterile gauze to remove large particles, and centrifuged at 10,000 × *g* for 10 min at 4°C. The pellets were used for genomic DNA extraction. DNA from the sample was extracted using PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, United States), according to the manufacturer's protocols. The duplicate samples were pooled together for one high-throughput sequencing. The V4 region of 16S was amplified using the primer pair 515F (5′-GTGCCAGCMGCCGCGG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Xiong et al., 2012; Fan et al., 2014); the ITS rRNA gene was amplified using the primer pair ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (Mukherjee et al., 2014). The forward and reverse primers were tagged with adapter, pad, and linker sequences. The reaction mixture (50 μL) contained 25 μL of Taq PCR Master Mix (QIAGEN), 0.2 μM of each primer, and 10 ng DNA templates. Thermal cycling consisted of initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 54°C (ITS) and 52°C (16S) for 45 s, elongation at 72°C for 50 s, and finally elongation at 72°C for 10 min. Negative control was treated similarly with the exclusion of template DNA and failed to produce visible PCR products. For each sample, three independent reactions were performed. PCR products were mixed in equimolar ratios and mixture PCR products were purified with GeneJET Gel Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, United States) following manufacturer's recommendations. The library quality was assessed on the Qubit@2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina MiSeq platform using paired-end sequencing by Novogene Inc. (Beijing, China).

Data Processing and Statistical Analysis

Raw reads files were demultiplexed, quality filtered, and analyzed using the Quantitative Insights Into Microbial Ecology (QIIME, v1.7.0) pipeline (Caporaso et al., 2010). In brief, reads with less than length 220 bp and ambiguous bases were discarded. Chimeras were removed using UCHIME as implemented in QIIME. The effective tags were binned into operational taxonomic units (OTUs) by UCLUST based on 97% pairwise identity (Edgar et al., 2011). The most abundant sequence from each OTU was selected to represent the OUT. OTUs were classified taxonomically using a QIIME-based wrapper of the Ribosomal Database Project (RDP) classifier against UNITE (User-friendly Nordic ITS Ectomycorrhiza Database) and INSD (International Nucleotide Sequence Databank) database (Caporaso et al., 2010). All identified OTU sequences assigned to chloroplast and mitochondrion origins were removed from the dataset.

The alpha diversity (Shannon index), richness (Chao1 index), Good's coverage, and rarefaction curve were performed by Mothur (Schloss et al., 2011). In addition, rank abundance curves, principal components analysis (PCA) and non-metric multidimensional scaling (NMDS) were generated by using R package vegan (version 3.4.0[1]). CANOCO software (version 4.5[2]) was used to conduct multivariate analysis using detrended correspondence analysis (DCA) and redundancy analysis (RDA). One-way ANOVA test, Pearson's correlation coefficients, and *P*-values were calculated with IBM SPSS 19.0.[3] All results were

[1]https://mirrors.tuna.tsinghua.edu.cn/CRAN/

[2]http://www.canoco5.com/index.php/resources

[3]https://www.ibm.com/analytics/us/en/technology/spss/

presented as the mean value ± SD. Differences between groups were declared significant at $P < 0.05$.

Co-occurrence Network Analysis and O2PLS Analysis

The co-occurrence patterns of microbial community in the pile-fermentation process of primary dark tea were constructed and analyzed using the online Molecular Ecological Network Analyses Pipeline (MENAP[4]; Deng et al., 2012). The OTUs, which presented in at least six samples, were used for pairwise Pearson correlation calculation, for which a proper threshold was identified based on the random matrix theory (RMT) approach. Network topological properties were also calculated using MENAP. O2PLS analysis was performed using the SIMCA-P 14 (Version 14.1.0.2047), according to the previous study (Wang et al., 2016). The X-matrix was designed as the microbiota dataset, and the Y-matrix was designed as the chemical compounds and enzyme activities datasets. Finally, the networks and the correlation among microbiota, chemical compounds, and enzyme activities were visualized using the Cytoscape software (Version 3.5.1).

RESULTS

Major Chemical Compounds Along With the Time Gradient in the Pile-Fermentation of Primary Dark Tea

The contents of major chemical compounds, including WE, TP, FLA, AA, and SS, were analyzed during the pile-fermentation process of primary dark tea. As shown in **Figure 1**, all the contents of major compounds were significantly decreased after the pile-fermentation process ($P < 0.05$). The concentrations of WE and AA were significantly decreased beginning at 6-h stage, 5.43 and 47.90% decrease was observed at 12-h stage compared with 0-h stage, respectively. The levels of TP and FLA were slightly decreased from 0-h stage to 6-h stage and sharply decreased at 9-h stage until the end of process, where 31.68 and 18.84% decrease was observed. A slightly increased content of SS was found from 0-h stage to 3-h stage, but a 44.24% decrease of SS was also observed at the end of process (**Figure 1**).

Change of Enzyme Activity During the Pile-Fermentation of Primary Dark Tea

Several enzyme activities, including PPO, POD, CEL, and PEC, were also detected during the pile-fermentation of primary dark tea (**Figure 2**). The activities of PPO, CEL, and PEC were significantly increased after the fermentation process, but the POD activity was significantly decreased in this process. The PPO activity was significantly increased from 0-h stage (not detected) to 0.92 ± 0.27 U at 6-h stage and remained stable until the end of process. And the levels of CEL and PEC activities were markedly increased from 0-h stage (not detected) to 0.22 ± 0.03 and 0.21 ± 0.05 U at 3-h stage then slightly increased until the end of process. But the POD activity was significantly decreased by 74.56% after pile-fermentation process.

Microbial Community Diversity During the Pile-Fermentation Process

In total, 1,401,447 valid fungal sequences and 418,889 valid bacterial sequences across all samples clustered into 297 fungal OTUs and 77 bacterial OTUs after trimming and filtration at 97% sequence identify. The values of Good's coverage were >99% for all sequences from five groups (**Supplementary Table S1**). The rarefaction curves for all samples almost reached the saturation phase, suggesting that few new microorganisms would possibly be identified by increasing the sequencing depth, and the majority of microorganisms for the samples had already been captured in the current analysis (**Supplementary Figure S1**). The Shannon index indicated that the diversity of fungus and bacteria was significantly decreased from 0-h stage to 6-h stage and maintained relatively constant until the end of the process. The Chao1 index indicated that the richness of fungus and bacteria was slightly decreased during the pile-fermentation process, but no significant difference was observed at the end of the process (**Figure 3**). In addition, the rank abundance curves of fungus and bacteria also showed that a slightly decreased richness and evenness of fungus and bacteria after the pile-fermentation process of primary dark tea (**Supplementary Figure S2**).

To assess the microbial β-diversity, the PCA and NMDS analyses were employed to investigate the overall difference among bacterial and fungal community structure during the pile-fermentation process of primary dark tea. The results indicated that all samples could be separated into two groups with based on Euclidean distance and Bray–Curtis distance, including group I (0-h stage) and group II (3-h stage-12-h stage; **Figure 4**).

Dynamic Shift of Microbial Community During the Pile-Fermentation Process of Primary Dark Tea

For microbial diversity and succession, 4 phyla, 53 classes, 47 orders, 216 families, and 153 genera in fungal community and 5 phyla, 9 classes, 20 orders, 35 families, and 55 genera in bacterial community were identified during the pile-fermentation process of primary dark tea. At the genus level of fungal composition, the four most dominant fungal genera, including *Cyberlindnera*, *Uwebraunia*, *Aspergillus*, and *Unclassified Pleosporales* constituted 89.91–99.47% of all sequences. *Cyberlindnera* dominated the whole fermentation process, with the relative abundance dramatically increased from the initial stage (0-h stage) 32.63–95.52% in 12-h stage. However, *Uwebraunia* dramatically decreased from 16.37% in 0-h stage to 1.64% in 12-h stage. *Aspergillus* and *Unclassified Pleosporales* accounted for 11.57 and 29.35% of all sequences in 0-h stage and was significantly decreased to 0.33% in 3-h stage and 5.18% in 6-h stage, then remained relative stable until the end of the pile-fermentation process. Changes in some small proportions in the fungal groups *Fusarium* (1.45–0.45%), *Eurotium* (1.44–0.31%), and *Peyronellaea* (1.11–0.23%) were also observed during the pile-fermentation process (**Figures 5A,C,E**). For the bacterial

[4]http://129.15.40.240/mena/

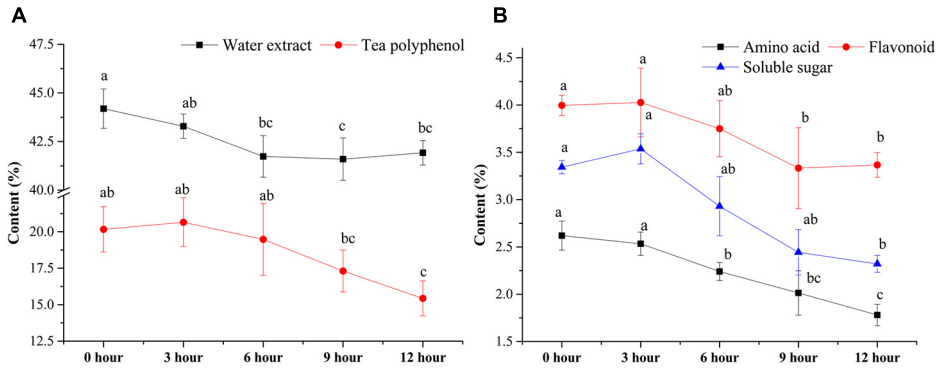

FIGURE 1 | Changes in the major chemical compounds during the pile-fermentation process of primary dark tea. Water extract and tea polyphenol **(A)**, amino acid, flavonoid, and soluble sugar **(B)**.

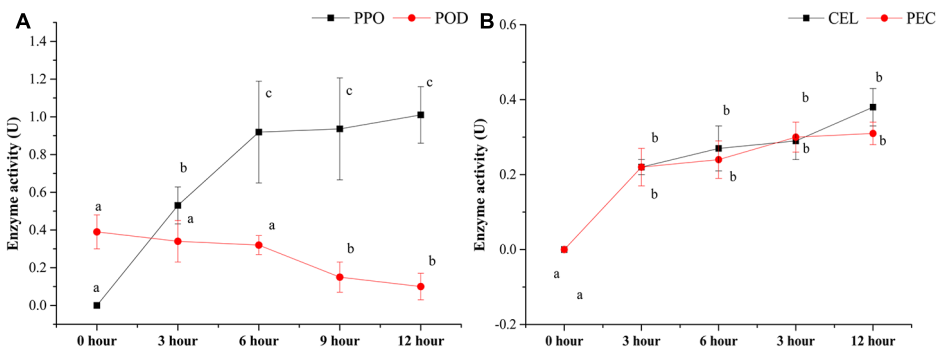

FIGURE 2 | Changes in the enzyme activities during the pile-fermentation process of primary dark tea. PPO, polyphenol oxidase; POD, peroxidase **(A)**; CEL, cellulase; PEC, pectinase **(B)**.

composition, the dominant genera *Klebsiella* and *Lactobacillus* accounted for 88.87–99.48% during the pile-fermentation process of primary dark tea. *Klebsiella* dominated the whole fermentation process, with the relative abundance gradually increased from the initial stage (0-h stage) 81.99–99.06% in 12-h stage. However, *Lactobacillus* dramatically decreased from 6.88% in 0-h stage to 0.41% in 12-h stage. Variations in some small proportions in the bacterial groups *Kluyvera* (3.31–0.29%), *Methylobacterium* (2.02–0.04%), and *Aurantimonas* (1.44–0.02%) were also observed during the pile-fermentation process of primary dark tea (**Figures 5B,D,F**).

Interaction Network Between Microbial Communities

To understand the interactions and connectivity within microbial communities during the pile-fermentation process of primary dark tea, we identified molecular ecological networks (MENs) using a novel RMT-based approach (Deng et al., 2012). In RMT analysis, the similarity threshold was determined to be 0.79. A total of 339 pairs of significant and robust correlations (edges) were identified from 124 nodes (28 nodes for bacteria and 96 nodes for fungus) (**Figure 6**). Specifically, 267 pairs of positive correlations and 72 pairs of negative correlations were identified in the network. Moreover, the value of modularity index for network was 0.446 (>0.4), suggesting the network has a modular structure (Newman, 2006) and the network global properties were showed in **Supplementary Table S2**.

All nodes in the network were assigned to seven phyla, including four bacterial phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*) and three fungal phyla (*Ascomycota*, *Basidiomycota*, and *Unclassified Fungi*); the individual node's properties were shown in **Supplementary Table S3**. All nodes could be grouped into five modules and the module compositions were shown in **Supplementary Figure S3** at the genus level. The majority of nodes (117, 94.35%) were peripherals with most of their links inside their own modules during the pile-fermentation process of primary dark tea. A total of four nodes were identified as connectors, which were derived from *Staninwardia* (phyla *Ascomycota*), *Saccharata* (phyla *Ascomycota*), and two *Unclassified Fungi* nodes. Three nodes were identified as module hubs, which were derived from *Aeromonas* (phyla *Proteobacteria*), *Acidovorax* (phyla *Proteobacteria*), and *Unclassified Pleosporales* (phyla *Ascomycota*) (**Supplementary Figure S4**). These keystone species served as gatekeepers in the ecological functions of the microbial community, implying that they may play critical roles in maintaining the stability of structure and function of ecological communities during the pile-fermentation of primary dark tea.

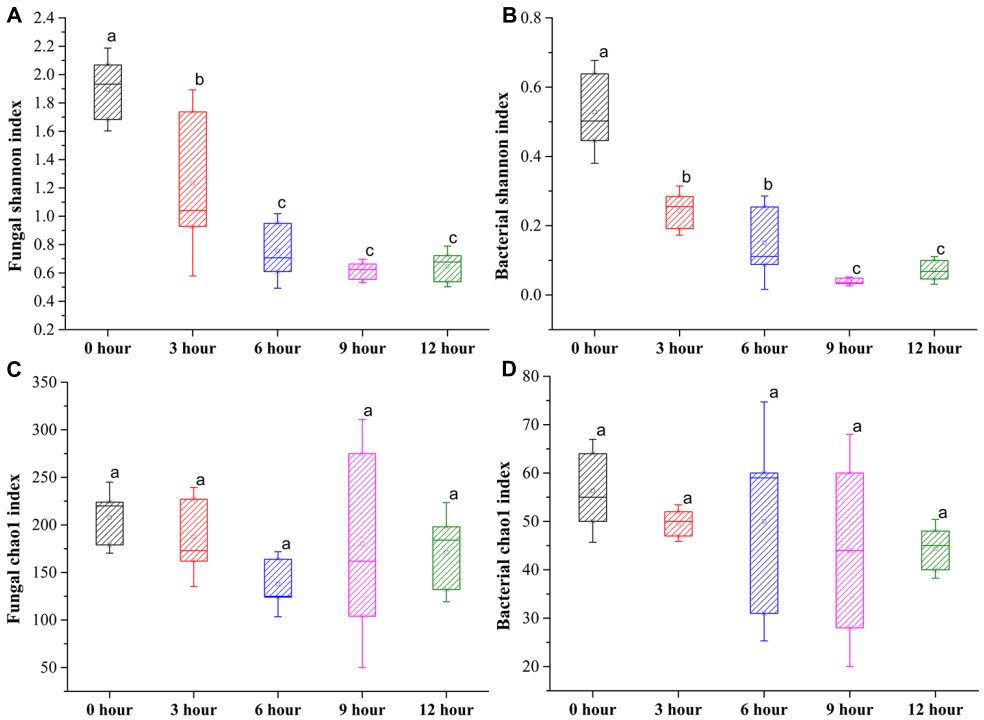

FIGURE 3 | Changes in Shannon and Chao1 index of fungal **(A,C)** and bacterial **(B,D)** community during the pile-fermentation process of primary dark tea.

Relationship Among Microbiota, Chemical Compounds, and Enzyme Activities

The effects of major chemical compounds on the structure of microbial community during the pile-fermentation process of primary dark tea were evaluated by the DCA and RDA. The result of DCA indicated that the gradient length of the axis was less than 3 SD (lengths of gradient 1.543, 0.409, 0.353, 0.861). Therefore, the linear model of RDA was considered to be the appropriate ordination method for direct gradient analysis. As shown in **Figure 7**, the pattern of microbial communities exhibited a continuous succession along with the time gradient during the pile-fermentation process. A sharply change from 0-h stage to 3-h stage and a gradual variation occurred in from 3-h stage to 9-h stage, then a greater variation occurred in the transitions from 9-h stage to 12-h stage. RDA revealed that there was a significant relationship between the structure of microbial community and major chemical component factors ($P = 0.014$). All five chemical component factors (i.e., WE, TP, AA, FLA, and SS) explained 74.5% ($P < 0.05$) of the total variability in the microbial community. The results of Monte Carlo permutation tests showed that the variability of the microbial composition was strongly related to AA ($P = 0.026$). The first ordination RDA axis (axis 1), which was strongly related with AA and SS, explained 72.6% of the variability in the microbial composition. The second ordination RDA axis (axis 2) was mainly related to WE and explained 1.1% of the total variability in the microbial community.

Bidirectional orthogonal partial least squares method was used to analyze the associations among microbiota, chemical compounds, and enzyme activities during the pile-fermentation process of primary dark tea. It was shown R_2 and Q_2 of the model was 0.938 and 0.518, respectively, thereby suggesting that O2PLS method was well fitted for analysis and prediction. Three latent variables were identified, with the microbiota (X dataset)-predictive structures accounting for 80.8% of the total variation in the X dataset, the chemical compounds and enzyme activities (Y dataset)-predictive structures accounting for 99.9% of the total variation in the Y dataset. According to the values of predictive component variable importance in the projection (VIP_{pred}) and the correlation coefficient between microbiota and chemical compounds or enzyme activities, 123 OTUs ($VIP_{pred} > 1.0$ and $|r| > 0.6$), including 19 bacterial OTUs and 104 fungal OTUs, had important effects on chemical compounds and enzyme activities (**Figure 8A**). In total, 106 OTUs including 13 bacterial OTUs and 93 fungal OTUs were correlated with chemical compounds. And 83 OTUs including 17 bacterial OTUs and 66 fungal OTUs were correlated with enzyme activities. These results suggested that the fungus were more important for primary dark tea production than bacteria.

In order to identify the core functional microorganisms in the pile-fermentation process of primary dark tea, several conditions should be considered: (a) detected stably in pile-fermentation process; (b) the VIP_{pred} value must be greater than 1; (c) the correlation coefficient must be greater than 0.7; (d) the number of microbe highly correlated with ($|r| > 0.7$) chemical compounds and enzyme activity was greater than 1. Based on

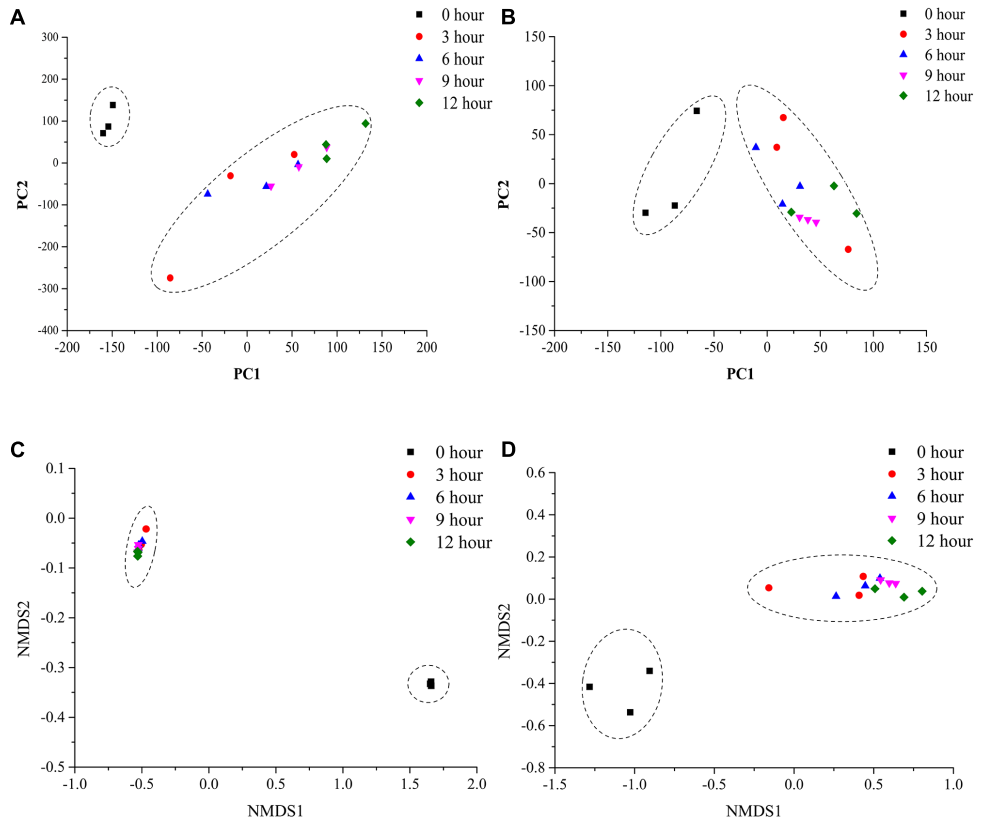

FIGURE 4 | PCA and NMDS analysis of microbial communities in sample during the pile-fermentation process of primary dark tea. Fungal PCA **(A)**, bacterial PCA **(B)**, fungal NMDS **(C)**, and bacterial NMDS **(D)**.

these, 13 OTUs including *Klebsiella* (bOTU2), *Aurantimonas* (bOTU1), and *Methylobacterium* (bOTU26) of bacterial genera and *Cyberlindnera* (fOTU176, fOTU26, and fOTU9), *Aspergillus* (fOTU24), *Uwebraunia* (fOTU1 and fOTU8), *Debaryomyces* (fOTU33), *Eurotium* (fOTU193), *Lophiostoma* (fOTU19), and *Peltaster* (fOTU32) of fungal genera were identified as core functional microorganisms for the pile-fermentation process of primary dark tea. As shown in **Figure 8B**, *Aurantimonas* was mainly responsible for the changes of CEL, WE, and PPO. *Klebsiella* was mainly responsible for WE, PPO, PEC, and CEL. *Methylobacterium* was mainly responsible for AA, SS, PPO, PEC, and CEL. *Cyberlindnera* was mainly responsible for AA, SS, FLA, PPO, PEC, and CEL. *Uwebraunia* was mainly responsible for WE, FLA, SS, PPO, PEC, and CEL. *Peltaster* was mainly responsible for PPO, PEC, and CEL. *Lophiostoma* was mainly responsible for AA, SS, FLA, and CEL. *Eurotium* was mainly responsible for PEC and CEL. *Aspergillus* was mainly responsible for PPO, PEC, and CEL.

DISCUSSION

Diverse and rich communities of bacteria and fungi with potential health benefits have been identified from various fermented food and beverages (Bourdichon et al., 2012). Microbiota inhabiting in tea leaves is of great importance for the distinctive biochemical characteristics of primary dark tea. To the best of our knowledge, the present study is the first work to extensively investigate the structural differentiation of microbial communities by Illumina MiSeq sequencing and linked with the major chemical compounds or enzyme activity during pile-fermentation process of primary dark tea.

Our chemical and enzymatic analysis showed that all major chemical compounds content were decreased, while the activities of PPO, CEL, and PEC were increased during the pile-fermentation process of primary dark tea, except POD. Previous studies revealed that the decrease of chemical compounds mainly resulted from enzymatic reaction of secretive enzyme of microbial communities during the fermentation process (Wang et al., 1991a). The activities of PPO, CEL, and PEC sourced from tea leave were inactivated by high temperature in fixing process. Very low activity of POD was remained after the fixing process, which is a kind of hyper-thermoresistant enzyme and could not be secreted by microbial communities during the pile-fermentation of primary dark tea (Liu et al., 1991). The activities of PPO, CEL, and PEC were increased along with the growth of microbial communities and provided the effective biochemical power to the biotransformation of chemical compounds in the tea leaves during the pile-fermentation process. The catechin was oxidation by PPO, which resulted in

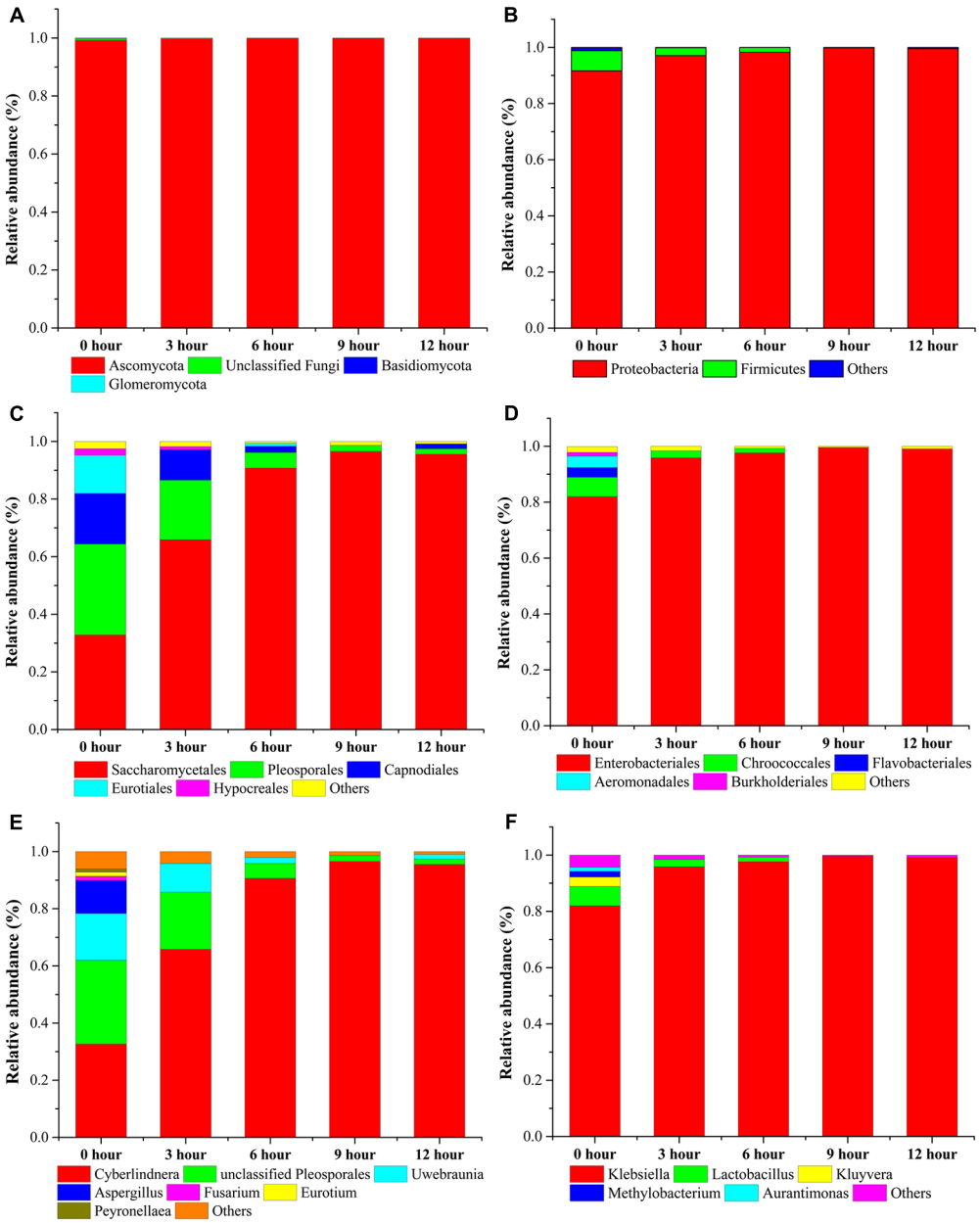

FIGURE 5 | Microbial taxonomic compositions showing the microbial successions at phylum: **(A)** fungus, **(B)** bacteria; order: **(C)** fungus, **(D)** bacteria; and genus: **(E)** fungus, **(F)** bacteria level during pile-fermentation process of primary dark tea. The taxonomic abundance <1% were classified into "others."

the decrease of TP content during the process. The decrease of SS maybe resulted from the growth of microorganisms, which was used as the most important carbon source for microbial communities (Wang et al., 1991c). The decreased of AA was also used as the mainly nitrogen source for the growth of microorganisms (Wang et al., 1991b). The genera *Cyberlindnera*, *Aspergillus*, *Uwebraunia*, and *Unclassified Pleosporales* of fungus and *Klebsiella* and *Lactobacillus* of bacteria were predominant at 0-h stage of process, but after 3-h stage and 6-h stage, only *Klebsiella* and *Cyberlindnera* were still dominated in fungal and bacterial community, and maintained a relatively constant until to the end of pile-fermentation process. This shift pattern of microbial communities might be because of the environment changes in the tea pile during fermentation process. Our RDA result also revealed that AA was the important factor shaping the microbial structure. AA, caffeine, theobromine, and theophylline were the major nitrogenous compounds of primary dark tea, but caffeine, theobromine, and theophylline were heterocyclic compounds, which could hardly be used by microorganisms (Wang et al., 1991b). Therefore, AA is the most important nitrogen source of microorganisms during the pile-fermentation process of primary dark tea. Co-occurrence analysis showed

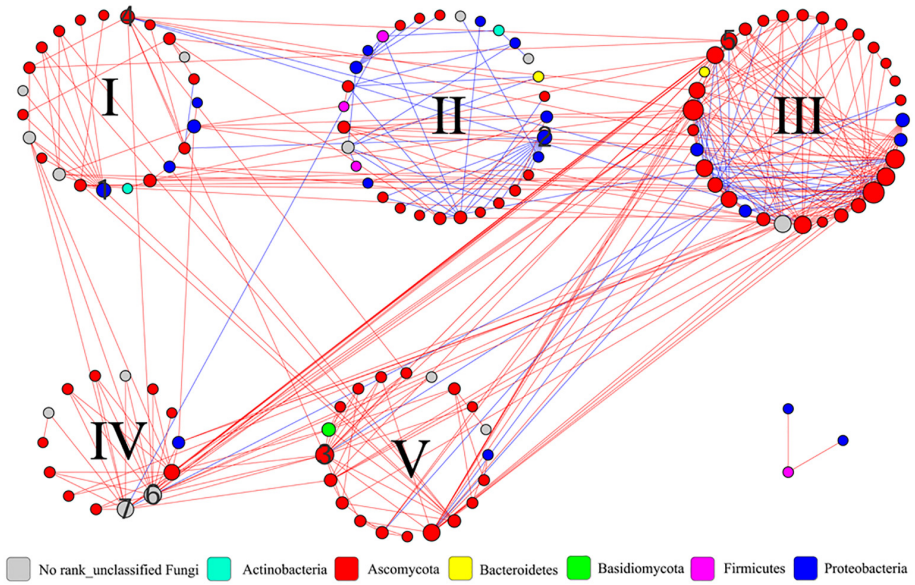

FIGURE 6 | Interaction networks in the microbial communities of primary dark tea. The nodes of network were group into five modules. The co-occurring networks are colored by phylum. The size of each node is proportion to the number of connections (that is, degree), and the red edge indicates a positive interaction and the blue edge indicated a negative interaction between two nodes. The numbers are indicated as follows: 1∼3 the module hubs bOTU8, bOTU21, and fOTU7. 4∼7 the connectors fOTU21, fOTU30, fOTU58, and fOTU162. I∼V indicated the number of modules.

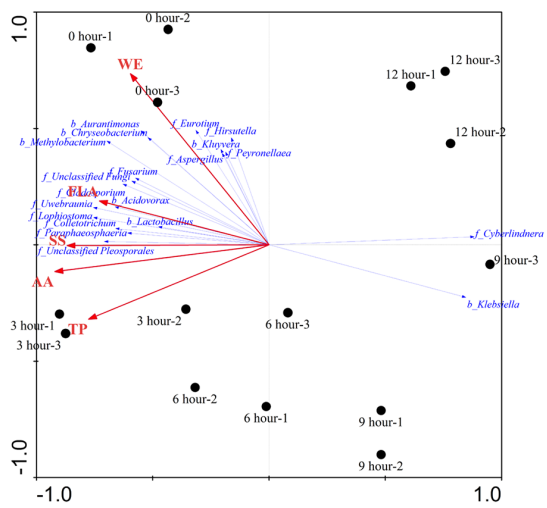

FIGURE 7 | Redundancy analysis (RDA) showing the relationship between microbial structure and major chemical compounds concentrations during the pile-fermentation process of primary dark tea. The red arrow indicated the compounds variances; the blue arrow indicated the important genera of microbial communities (relative abundance >0.01%). Filled circles indicated the samples during the pile-fermentation of primary dark tea. WE, water extract; TP, tea polyphenol; AA, amino acid; FLA, flavonoid; SS, soluble sugar.

that the OTUs were grouped into five modules, which might perform different functions during the pile-fermentation process of primary dark tea. In this study, most of the dominant genera were distributed in module III (**Supplementary Figure S3**), such as *Cyberlindnera*, *Aspergillus*, and *Uwebraunia* of fungal communities and *Klebsiella* of bacterial community, which might be mainly related to carbon and nitrogen metabolism during pile-fermentation process (Bekatorou et al., 2006; Du et al., 2006; Cho et al., 2012).

Bidirectional orthogonal partial least squares approach was used to integrate the microbiota, chemical compounds, and enzyme activities dataset in order to dig into the association among them in primary dark tea during pile-fermentation process. The results suggested that fungus were more important for primary dark tea production than bacteria. In addition, *Cyberlindnera*, *Aspergillus*, *Debaryomyces*, *Uwebraunia*, *Eurotium*, *Lophiostoma*, and *Peltaster* of fungal genera and *Klebsiella*, *Aurantimonas*, and *Methylobacterium* of bacterial genera were identified as the core microorganisms during the pile-fermentation process of primary dark tea.

The fungal genus *Cyberlindnera* contains 23 recognized teleomorphic species and 12 *Candida* species (Chang et al., 2012), in which the *Cyberlindnera jadinii* was applicated in the production of papaya wine (Pinrou et al., 2010) and prevented the growth of spoilage yeasts in cheese (Liu and Tsao, 2009). This specie is a close relative of *Candida utilis* (also referred to as *Torula* yeast), which was cultivated on economical waste liquors and used in the food and feed industries as a nutritious, well-tasting, and safe source of single-cell protein. *Cyberlindnera* (*Williopsis*) *saturnus* was reported that had the ability for producing significant amounts of sweetener xylitol from xylose and corn cob hydrolysate (Kamat et al., 2013). Moreover, some species of *Cyberlindnera*, which had a high capability of tannin tolerance, were also isolated from

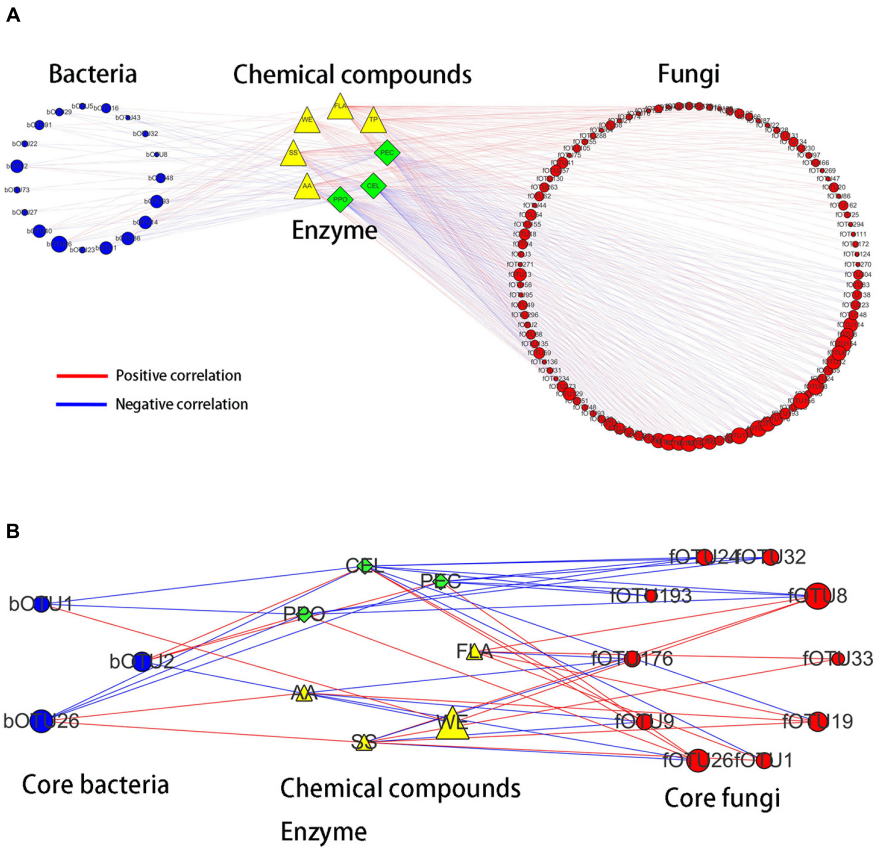

FIGURE 8 | Correlation analyses among microbiota, chemical compounds, enzyme activities **(A)** and the core microorganisms **(B)** in primary dark tea during pile-fermentation process.

Miang (a fermented food product prepared from tea leaves) (Kanpiengjai et al., 2016). *Aspergillus* is the most important economically fungal genus (Samson et al., 2014), and many species within this genus are used in biotechnology for the production of various metabolites in many food fermentations (Bourdichon et al., 2012), such as soy sauce (Wicklow et al., 2007), miso (Harayama and Yasuhira, 1994), grape pomace (Botella et al., 2007), millet spirits (Takaya et al., 2007), and katsuobushi (Manabe, 2001). In all these cases, *Aspergillus* species which produce and secrete a variety of enzymes including α-amylases, glucoamylases, CELs, PECs, xylanases and other hemicellulase, and proteases, play an important role in the improvement of taste by decomposing proteins and/or lipids and producing unique flavors (Ward et al., 2005). *Eurotium*, which adopted the newly established principle "one fungus, one name" (Norvell, 2011), species formerly included in the genus *Eurotium* are displayed with their *Aspergillus* name (Samson et al., 2014), such as the dominant fungi *Eurotium cristatum* in Fu brick tea, which is called *Aspergillus cristatum* now (Ge et al., 2016; Li et al., 2017). Several indole alkaloids and indole diketopiperazine alkaloid were identified from the culture extract of *Aspergillus cristatum* and these compounds were proved to possess brine shrimp lethality, antibacterial activity against *E. coli*, radical scavenging activity against DPPH radicals, and exhibited marginal attenuation of 3T3L1 pre-adipocytes (Du et al., 2012, 2017; Zou et al., 2014). And non-mycotoxigenic strains of *Aspergillus repens* and *Aspergillus rubrum* are used as starter cultures in the manufacture of the traditional fermented food *katsuobushi*, from bonito (*Katsuwonus pelamis*) (Manabe, 2001). *Debaryomyces* contains 19 species (Ainsworth, 2014), in which the species *Debaryomyces hansenii* (anamorph *Candida famata*) is a significant species in foods and play an important role for the food industry, as it is used for surface ripening of cheese and meat products, over-production of riboflavin (vitamin B2), bioconversion of xylose into the sweetener xylitol, and potential synthesis of arabinitol and pyruvic acid (Satyanarayana and Kunze, 2009). And its enzyme activities improve tea quality by producing the sweet substance xylitol, vitamins, and other organic acids in Fu brick tea (Xu et al., 2011). *Uwebraunia* is a kind of plant pathogenic fungi, which was a synonym of *Dissoconium* (Crous et al., 2006), but the genus have not been well described, and the understanding of its roles in pile-fermentation systems of primary dark tea is limited.

The bacterial genus *Klebsiella* have shown an unbeatable production performance of 2,3-butanediol by fermentation from a wide range of substrates, including pentoses (xylose and arabinose), hexoses (glucose, mannose, and galactose), and disaccharides (sucrose, lactose, cellobiose) (Du et al., 2006;

Cho et al., 2012). *Klebsiella* was also found in the fermentations of Pu-erh tea, soy sauce, and other fermented foods (Puerari et al., 2015; Zhao et al., 2015; Qin et al., 2016; Yang et al., 2017).

CONCLUSION

In conclusion, a complex microbial community, inhabiting the primary dark tea ecosystem, plays important roles in the formation of quality of primary dark tea during the pile-fermentation process. The dynamic changes of major chemical compounds, enzyme activities, and microbial community structure, as well as the relationships among them, were clarified during the pile-fermentation process of primary dark tea. The results indicated that the fungi made more contributions to the formation of the characteristic properties of primary dark tea than bacteria during the pile-fermentation process. And 10 core functional microbial genera were identified in primary dark tea. These findings improved our understanding on the formation mechanism of the characteristic properties of primary dark tea during the pile-fermentation process.

AUTHOR CONTRIBUTIONS

QL, LX, ZL, and JH designed the study. QL conducted the experiment, analyzed the data, and drafted the manuscript. SC and YOL prepared the sample and conducted the chemical and enzyme analysis. YUL helped to revise the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01509/full#supplementary-material

FIGURE S1 | Rarefaction curves of fungal OTUs **(A)** and bacterial OTUs **(B)** derived from primary dark tea.

FIGURE S2 | Rank abundance curve of fungal OTUs **(A)** and bacterial OTUs **(B)** derived from primary dark tea.

FIGURE S3 | Microbial community composition of each co-occurrence network module.

FIGURE S4 | ZP plot showing distribution of OTUs based on their module-based topological roles.

TABLE S1 | Comparison of microbial diversity estimation and coverage at 97% similarity from the sequencing analysis.

TABLE S2 | Topological properties of network during the pile-fermentation process of primary dark tea.

TABLE S3 | Individual node's properties of network during the pile-fermentation process of primary dark tea.

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