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# Study of the Antibacterial Potency of Electroactivated Solutions of Calcium Lactate and Calcium Ascorbate on *Bacillus cereus* ATCC 14579 Vegetative Cells

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**ABSTRACT:** *Bacillus cereus* is a pathogenic bacterium, Gram-positive, aerobic, and facultative anaerobic that can produce spores and different toxins. It is involved in serious foodborne illnesses such as the diarrheal and emetic syndromes, depending on the ingested toxin. This work is aimed to study the potency of electroactivated solutions (EAS) of calcium lactate, calcium ascorbate, and their mixture as antibacterial agents against *B. cereus* ATCC 14579 vegetative cells. The solutions used were electroactivated under electric current intensities of 250, 500, and 750 mA for 30 min. The obtained EAS were tested in direct contact with *B. cereus* ($10^7$ CFU/mL) for different durations ranging from 5 s to 2 min. Moreover, standard lactic and ascorbic acids were tested as controls at equivalent titratable acidity as that of the corresponding electroactivated solutions. The obtained results showed that EAS exhibit high antibacterial efficacy against *B. cereus* vegetative cells. The EAS obtained after electroactivation of calcium lactate and calcium ascorbate were more efficient than those of their corresponding standard acids (lactic and ascorbic). The observed antibacterial effect of the EAS resulted in a reduction of 7 log CFU/mL after 5 s of direct contact in some specific cases. Scanning (SEM) and transmission (TEM) electron microscopic observations provided conclusive evidence of the antibacterial activity of the used EAS. These results outlined the highly antimicrobial potency of EAS against *B. cereus* vegetative cells and that they can be considered in an eventual strategy to ensure food safety, surface cleaning, as well as replacement of hazardous disinfecting chemicals.
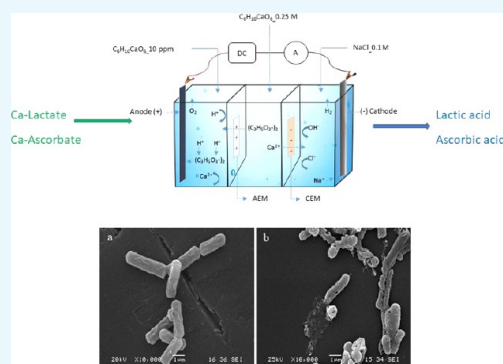
## 1. INTRODUCTION

*Bacillus cereus* (sensu lato) is a group of bacteria that roused scientific interest due to their potential to cause different illnesses.[1,2] *Bacillus cereus* can survive in temperatures ranging from 4 to 55 °C,[3,4] media water activity ($a_w$) as low as 0.92, a pH ranging from 4.3 to 9.3, an oxidation−reduction potential (ORP) varying from −100 to +300 mV, and tolerate salt concentration (NaCl) up to 10%.[3,4] Moreover, other bacterial species have been identified in this group according to their genetically 16S and 23S rRNA sequences, such as *Bacillus anthracis*, *B. cereus* (sensu stricto), *Bacillus cytotoxicus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus toyonensis*, *Bacillus thuringiensis*, and *Bacillus weihenstephanensis*.[2,5,6] *B. cereus* (sensu stricto) is a motile rod and spore-forming bacteria that can cause two specific types of foodborne diseases: the emetic (nausea and vomiting) and the diarrheal syndromes.[7] *B. cereus* is also associated with other infections, such as endocarditis, endophthalmitis, gangrene, liver failure, lung abscesses, panophthalmitis, and septicemia.[4,8,9] Moreover, many cases of nosocomial sickness and mortality involving *B. cereus* have also been reported worldwide.[2,10−12] However, the prevalence related to this pathogen varies from one country to another due to the lack of data on undocumented cases of diseases caused by *B. cereus*. According to some published statistics, *B. cereus* caused an estimated number of 63 400 episodes in the United States per year, and it was considered as the second leading cause of foodborne outbreaks in France, the third in Europe and China, and the fifth in Canada with over 36 260 estimated cases of foodborne diseases per year.[10,13−15] Because of its ability to survive and grow under both aerobic and anaerobic conditions, *B. cereus* is generally recognized as a ubiquitous microorganism that is widespread in a large variety of environments.[2,4,16]

The emetic form of the disease caused by *B. cereus*, which syndrome is closely similar to that caused by *Staphylococcus aureus* (e.g., nausea and vomiting), can occur after only 15 min to 6 h, following the consumption of food previously

contaminated with cereulide toxin having been produced during the growth of emetic (B) strains of the pathogen. Between $10^5$ and $10^8$ UFC/g of food are enough to produce an amount of cereulide (5−10 μg/kg of body weight) capable of poisoning someone.[3,7] The toxin cereulide was identified as a cyclic dodecadepsipeptide with a repeated sequence of three amino acids (D-O-Leu-D-Ala-L-O-Val-L-Val). This tripeptide whose molecular weight was estimated at 1.2 kDa, has a high structural similarity with the antibiotic valinomycin, another dodecadepsipeptide and potassium ionophore produced by *Streptomyces* spp.[17−19] The cereulide toxin is known to be highly resistant to a wide range of pH 2−11, to proteases such as pepsin or trypsin, and to thermal treatments (126 °C/90 min).[20,21] When this toxin is already secreted in foods by the pathogen, it remains hazardous to human health even when the vegetative cells would ultimately be killed during the food processing.[20,22] When the toxin reaches the intestine, it adheres to the 5-HT3 receptor and stimulates the vagus afferent nerve, thereby causing vomiting in the consumer who has ingested the contaminated food, especially rice or other starchy products.[22] Thus, it is very important to inactivate the vegetative forms of this pathogenic bacteria before it will be able to produce its toxins.

The diarrheal syndrome, very similar to the *Clostridium perfringens* syndrome, is generally observed between 8 and 16 h after the ingestion of $10^4$–$10^9$ viable cells (type A strains) in the contaminated foods (e.g., fish, meat, milk, vegetables, or other derived foodstuffs).[3,23,24] *B. cereus* secreted the diarrheal toxin in the small intestine (in vivo), which induced an increase in the proportion of liquid in the consumer's abdomen, leading to diarrhea that can sometimes be accompanied by abdominal cramps and/or pain.[4,21,23] Among the diarrheal toxins reported so far are listed two complex proteins, namely, the hemolysin BL (types B, L1, and L2) and the nonhemolytic enterotoxin (types A, B, and C).[2,25] The cytotoxin K (types CytK1 and CytK2) identified as a single protein has also been reported as a diarrheal toxin in numerous studies.[26−29] Unlike cereulide, it has been reported that diarrheal toxin has some sensitivity to heat treatments and can be inactivated/destroyed at only 60 °C during 5 min of treatment. The diarrheal toxin was known to be affected by proteolytic enzymes such as trypsin as well as acidic pH < 4.[20,21,30]

In addition to the aforementioned toxins, many other virulence factors produced by *B. cereus* have been elucidated, namely, hemolysins, phospholipases, proteases (e.g., metalloproteinases), sphingomyelinases, and others, that might be hazardous to public health.[2,31,32] Even when many strains of *B. cereus* were known to be sensitive to different antibiotics, such as chloramphenicol, gatifloxacin, gentamicin, moxifloxacin, streptomycin, or vancomycin,[33] several researchers have reported that certain clinical, environmental, or dietary strains of this pathogen having been isolated could potentially be resistant to many other antibiotics, namely, ampicillin, erythromycin, penicillin, and tetracycline, which somehow remained a health problem for consumers.[33−35]

*B. cereus* is also well known to be associated with food spoilage causing, for example, acidification, proteolysis, lipolysis, or the production of gases, which could sometimes make food unsuitable for human consumption.[4,36] To date, after the genus *Clostridium*, *Bacillus* has been recognized as the only spore-forming and toxin-producing pathogenic bacterium that could spoil vacuum-packed foods during their storage.[16,37]

Many researchers have reported the ability of *B. cereus* to adopt multiple biological survival strategies to overcome hostile conditions, such as biofilms, filaments, spores, and viable but nonculturable (VBNC) cells, which render this pathogen extremely difficult to control.[38−43] These data are not encouraging for the food processing industry, which is constantly seeking new methods to produce better quality and safer foods. Based on these substantial risks, it is crucial through this research to investigate a more efficient technology and strategy like the use of electroactivation (EA) of salts of organic acids, namely, calcium lactate ($C_6H_{10}CaO_6$), calcium ascorbate ($C_{12}H_{14}CaO_{12}$), and their mixtures to produce highly reactive and acidic electroactivated solutions (EAS) to better control this pathogen in foods and mitigate public health concern.

The electroactivation of different aqueous solutions is a nonthermal technology that was first experimented in Russia, before it was well developed in Japan and became a topic of research for scientists from many other countries, namely, Canada, United States, as well as China.[44] The Russian scientist named Bakhir was one of the pioneers to point out in 1972 that electroactivated aqueous solutions with a very low concentration of salt might have different chemical properties, in particular, a pH with characteristics ranging from acid to alkaline.[45] The electroactivation is a technology operating based on the electrolysis principles.[46,47] In an electrolysis process, a simultaneous migration of anions to the anode and cations to the cathode is observed, respectively, resulting in oxidation and reduction reactions where electrons are given or caught accordingly.[46,48] When an electroactivation reactor is connected to an electric power supply generator, certain significant physicochemical modifications (e.g., acidity, pH, ORP) can be observed in the aqueous solutions nearby the anodic and cathodic interfaces.[49] Consequently, a very acidic solution (anolyte) can be generated in the anodic section, whereas an alkaline medium (catholyte) is produced at the cathodic compartment, where properties could be monitored according to the time, current intensity, and ion-exchange membranes used to design the electroactivation reactor.[46,50,51] Because the electroactivation process could make electroactivated solutions highly reactive (metastable state) with presumptive antimicrobial properties, such solutions have been investigated by many researchers to shed light on further potential applications in several fields, especially the food industry.[49] Among the applications reported for EAS over the last years are listed inactivation of bacterial cells and spores, fungi, protozoans, and viruses,[46,48,50] sterilization of canned corn and pea,[52] development of dairy ingredients,[53] as well as the effective extraction of proteins from plant materials.[54] Despite the use of several preservatives in the food industry to fight against spoilage and pathogenic microorganisms, foodborne diseases remain a serious public health issue. Given the potential hazard posed by *B. cereus* and in light of the increase of consumers' demand for minimally processed and safer foods worldwide,[45] finding alternative methods becomes more essential. To this context, the main objective of this work was to study the effects of electroactivated solutions of calcium lactate, calcium ascorbate, and their equimolar mixture on *B. cereus* ATCC 14579 vegetative cells under model conditions to improve the safety and preservation of food products.

**Table 1. Compartments of the Electroactivation Reactor**

| experimental case | anodic compartment | central compartment | cathodic compartment |
|---|---|---|---|
| 1 | calcium lactate: 10 ppm | calcium lactate: 0.25 M | sodium chloride 0.1 M |
| 2 | calcium ascorbate: 10 ppm | calcium ascorbate: 0.25 M | sodium chloride 0.1 M |
| 3 | mixture: calcium lactate + calcium ascorbate: 10 ppm | mixture: calcium lactate + calcium ascorbate: 0.25 M | sodium chloride 0.1 M |

## 2. MATERIALS AND METHODS

**2.1. Chemicals and Materials.** The chemical products utilized for this research included the following: the sodium hydroxide solution (NaOH 1 N) was ordered from Fisher Chemical (Fair Lawn, NJ). Sodium chloride (NaCl) was purchased from VWR International Co. (Mississauga, ON, Canada). The standard anhydrous L-ascorbic acid and L-lactic acid, as well as calcium L-ascorbate dihydrate and calcium L-lactate hydrate, were obtained from Sigma-Aldrich (St. Louis, MA). The anionic- [AMI-7001] and cationic [CMI-7000]-exchange membranes used in the electroactivation reactor were bought from Membranes International Inc. (Ringwood, NJ). Also, the two anticorrosion electrodes (ruthenium−iridium-coated titanium for the anode and stainless steel food grade for the cathode) used were purchased from Qixin Titanium Co. (Baoji, Shaanxi, China).

**2.2. Bacterial Strain Preparation.** The *B. cereus* ATCC 14579 strain used in this study was supplied by the American Type Culture Collection (Manassas, VA). The cells were received frozen at −80 °C and stored as directed by the supplier. Before each use, the cells were transferred to trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI) for incubation at 30 °C for 24 h. Ten microliters of *B. cereus* suspension was taken and placed in tubes with 90 $\mu$L of sterile peptone water and mixed to obtain the desired concentration of $10^7$ CFU/mL.

**2.3. Electroactivation of Salted Solutions.** The production of electroactivated solutions (EAS) was done according to a previous protocol used by Cayemitte et al. (2021).[55] Briefly, the electroactivation reactor used was made with Plexiglas and comprised three compartments. Two anticorrosion electrodes made of ruthenium−iridium-coated titanium and stainless steel were used as anode and cathode, respectively. An anionic-exchange membrane was placed between the anodic and central sections, while a cationic-exchange membrane isolated the central compartment from the cathodic section. This EA-reactor configuration allowed the complete elimination of interference between $H^+$ and $OH^-$ ions generated at the anode and cathode interfaces, respectively. The calcium lactate, calcium ascorbate, and their equimolar mixture were dissolved in distilled water to obtain aqueous solutions with two different concentrations of 10 ppm and 0.25 M that were used to fill the anodic and central compartments, respectively. The 10 ppm solution was used in the anodic side as the lowest concentration to allow the passage of the electric current at the beginning of the EA process when this section is at its highest electrical resistance. Another sodium chloride solution of 0.1 M concentration was prepared and added to the cathodic compartment of the electroactivation reactor, as shown in Table 1. Three electric current intensities (250, 500, and 750 mA) were applied to the reactor using a direct current electric source (Circuit Specialists CSI 12001X, Tempe, FL). The EAS (anolytes) generated in the anodic compartment were collected after 10, 20, and 30 min of electroactivation for use against the

vegetative cells of *B. cereus*. All manipulations were carried out at ambient temperature (22 ± 1 °C).

**2.4. Application of EAS against *B. cereus* ATCC 14579.** To assess the antibacterial effects of electroactivated solutions (EAS), 190 $\mu$L of previously prepared EAS was mixed with 10 $\mu$L of *B. cereus* ATCC 14579 suspension ($10^7$ CFU/mL), and the bacterial cells stayed in contact with EAS for different periods of time (5, 30, and 60 s). As soon as a contact time has been reached, 100 $\mu$L of the final solution was taken (using a micropipette) and plated before incubation at 30 °C for 24 h. Subsequently, the Petri plates were observed to determine if there was bacterial growth and enumeration was carried out accordingly.

**2.5. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for Electroactivated Solutions.** *2.5.1. MIC.* The minimum inhibitory concentration (MIC) is considered as the lowest quantity of an antimicrobial agent necessary to inhibit the visible growth of a microorganism in a standard medium.[56] To achieve this, the MIC of electroactivated solutions (EAS) was determined as follows: first, the fresh EAS of calcium lactate, calcium ascorbate, and their equimolar mixture, obtained after electroactivation treatments of 10, 20, and 30 min under different current intensities (250, 500, and 750 mA), were selected. Thereafter, each EAS was serially diluted using deionized water to yield the diluted EAS solution presenting 10−90% of the initial mother solution. Microtest plates (96 well, F) from SARSTEDT (Numbrecht, Germany) were used, in which 10 $\mu$L of bacterial suspension was added to each well, followed by 190 $\mu$L of diluted EAS, and finally 100 $\mu$L of tryptic soy broth (TSB) (Becton Dickinson GmbH, Heidelberg, Germany) were also added and mixed. When all of the microplates were filled, they were placed in an Infinite F200PRO spectrophotometer for optical density (OD) monitoring (Tecan Nano Quant, Madison, WI) at 30 °C for 24 h. Each hour, the optical density (OD) measurement was performed automatically by the spectrophotometer and the data were computed and recorded for a total of 24 cycles. Considering this, the microplates with the lowest concentration of EAS and without the growth of *B. cereus* ATCC 14579 were considered as the MIC value.

*2.5.2. MBC.* The minimum bactericidal concentration (MBC) of EAS was determined to evaluate their ability to provoke microbial death at 99.9%.[57] The MBC of EAS was fixed as follows: from the microplates of the MIC, samples were collected and spread on Petri dishes with trypticase soy broth (TSB) medium overnight at 30 °C. Finally, the minimum concentration at which no bacterial growth was observed was stated as the MBC value.

**2.6. Effects of EAS versus Standard Acids on *B. cereus* ATCC 14579.** The antibacterial effect of the studied EAS was compared to that of standard solutions of lactic acid, ascorbic acid, and their equimolar mixture at equivalent titratable acidity as obtained after the EA treatment. This was based on the results obtained in the previous article of Cayemitte et al. (2021) dealing with the electroactivation of calcium lactate,

**Table 2. Effect of Direct Application of EAS on *B. cereus* ATCC 14579 Vegetative Cells[a]**

| types of EAS | intensity (mA) | EA time (min) | pH | titratable acidity (mol/L) | contact time (s) | survivor cells (log CFU/mL) |
|---|---|---|---|---|---|---|
| EA-calcium lactate | 250 | 10 | 2.74 ± 0.36 | 0.019 ± 0.000 | 5, 30, 60 | ND |
| | | 20 | 2.67 ± 0.22 | 0.029 ± 0.002 | 5, 30, 60 | ND |
| | | 30 | 2.66 ± 0.10 | 0.065 ± 0.002 | 5, 30, 60 | ND |
| | 500 | 10 | 2.64 ± 0.10 | 0.009 ± 0.001 | 5, 30, 60 | ND |
| | | 20 | 2.64 ± 0.09 | 0.032 ± 0.001 | 5, 30, 60 | ND |
| | | 30 | 2.46 ± 0.04 | 0.087 ± 0.002 | 5, 30, 60 | ND |
| | 750 | 10 | 2.64 ± 0.07 | 0.030 ± 0.002 | 5, 30, 60 | ND |
| | | 20 | 2.42 ± 0.03 | 0.052 ± 0.012 | 5, 30, 60 | ND |
| | | 30 | 2.16 ± 0.01 | 0.107 ± 0.007 | 5, 30, 60 | ND |
| EA-calcium ascorbate | 250 | 10 | 2.41 ± 0.17 | 0.009 ± 0.001 | 5, 30, 60 | ND |
| | | 20 | 2.21 ± 0.07 | 0.014 ± 0.001 | 5, 30, 60 | ND |
| | | 30 | 2.39 ± 0.03 | 0.025 ± 0.001 | 5, 30, 60 | ND |
| | 500 | 10 | 2.17 ± 0.12 | 0.012 ± 0.001 | 5, 30, 60 | ND |
| | | 20 | 2.26 ± 0.12 | 0.029 ± 0.001 | 5, 30, 60 | ND |
| | | 30 | 2.05 ± 0.07 | 0.063 ± 0.004 | 5 | 0.10 ± 0.17 |
| | | | | | 30, 60 | ND |
| | 750 | 10 | 2.05 ± 0.05 | 0.024 ± 0.002 | 5, 30, 60 | ND |
| | | 20 | 2.03 ± 0.10 | 0.051 ± 0.001 | 5, 30, 60 | ND |
| | | 30 | 1.94 ± 0.15 | 0.109 ± 0.001 | 5, 30, 60 | ND |
| EA-equimolar mixture | 250 | 10 | 2.28 ± 0.05 | 0.012 ± 0.001 | 5 | 0.58 ± 0.53 |
| | | | | | 30 | 0.55 ± 0.52 |
| | | | | | 60 | ND |
| | | 20 | 2.19 ± 0.16 | 0.036 ± 0.000 | 5, 30, 60 | ND |
| | | 30 | 2.08 ± 0.05 | 0.025 ± 0.001 | 5 | 0.20 ± 0.17 |
| | | | | | 30, 60 | ND |
| | 500 | 10 | 2.18 ± 0.02 | 0.009 ± 0.003 | 5 | 0.26 ± 0.45 |
| | | | | | 30 | 0.20 ± 0.35 |
| | | | | | 60 | ND |
| | | 20 | 2.19 ± 0.05 | 0.038 ± 0.003 | 5 | 0.26 ± 0.45 |
| | | | | | 30, 60 | ND |
| | | 30 | 2.13 ± 0.04 | 0.050 ± 0.002 | 5, 30, 60 | ND |
| | 750 | 10 | 2.34 ± 0.07 | 0.055 ± 0.001 | 5 | 0.16 ± 0.28 |
| | | | | | 30, 60 | ND |
| | | 20 | 2.29 ± 0.09 | 0.117 ± 0.002 | 5 | 0.16 ± 0.28 |
| | | | | | 30, 60 | ND |
| | | 30 | 2.08 ± 0.05 | 0.102 ± 0.001 | 5, 30, 60 | ND |

[a]EA, electroactivation; mA, milliampere; ND, not detected; and initial cell population, 7 ± 0.01 log CFU/mL.

calcium ascorbate, and their equimolar mixture. The same procedure was followed as that used for MIC and MBC determination, except for the contact time that was fixed at 5, 30, 60, and 120 s.

**2.7. Microscopic Observation of *B. cereus* ATCC 14579.** To better observe how EAS affected the vegetative cells of *B. cereus* ATCC 14579 after the treatments, the cells (control and treated) were stained using the methylene blue staining method to distinguish quickly dead from living cells under optical microscopic examination (BX51TRF, Olympus Corporation, Tokyo, Japan). To achieve this objective, a drop of water was mixed with a drop of inoculated solution on a slide using an inoculating loop, and the bacterial cells were carefully fixed by passing the slide through a flame three times. Thereafter, methylene blue (Sigma-Aldrich, Oakville, ON, Canada) was used to stain the cells for approximately 60 s. Subsequently, the smear was washed with distilled water to remove any excess stain, and iodine (Sigma-Aldrich, Oakville, ON, Canada) was added to fix the methylene blue. Finally, an ultimate washing was carried out followed by a wiping before placing the slide under microscope objective, with oil at a magnification set at 100×, for the observation of the cells.

Afterward, several pictures of untreated and treated (with EAS 750 mA, 30 min) cells were also taken to make comparisons.

The fluorescence staining method BacLight (Live/Dead Bacterial Viability Kit, L13152) was performed to investigate the viability of *B. cereus* vegetative cells after EAS (750 mA, 30 min) treatments for 30 s. Basically, the Live/Dead BacLight method is based on the assessment of the membrane integrity of bacterial cells using a staining kit.[58] The kit contains two types of fluorochromatic dyes: the SYTO9 nucleic acid stain (green fluorescent dye for living cells) and the propidium iodide (PI) (red fluorescent dye for dead cells) (Molecular Probes Life Technologies, Eugene, OR). For this purpose, the *B. cereus* cells in suspension were stained separately with SYTO9 and PI, as well as with a combination of the two dyes previously dissolved in filter-sterilized water. The volume proportion between the bacterial suspension and the dyes was 1:1, and the final concentrations of SYTO9 and PI were 6 and 30 $\mu$M, respectively. The stained bacterial suspension was incubated at room temperature in the dark for a period of 15 min. Subsequently, the observation of the staining samples was carried out using a fluorescence microscope (BX51TRF, Olympus Corporation, Tokyo, Japan), equipped with Image-
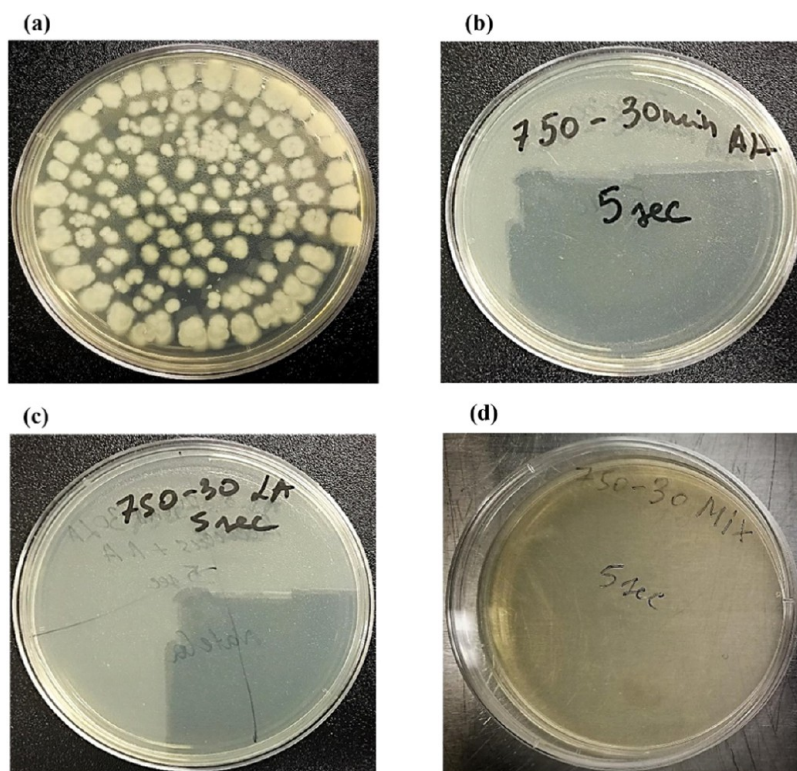
**Figure 1.** Enumeration of *Bacillus cereus* ATCC 14579 after direct contact with EAS. (a) Positive control with $10^7$ CFU/mL; (b) EAS-calcium ascorbate (750 mA, 30 min—contact time 5 s, no growth); (c) EAS-calcium lactate (750 mA, 30 min—contact time 5 s, no growth); and (d) EAS-mixture (750 mA, 30 min—contact time 5 s, no growth).

Pro Plus software (Media Cybernetics Inc., Rockville, MD) for cell enumeration. Filters (fluorescein isothiocyanate (FITC)) with excitation/emission wavelengths of 480/520 nm were used to observe live bacteria (intact cell membranes) in green fluorescence, and while filters (TXRED) with excitation/emission 560/630 nm were used to examine cells with compromised membranes in red fluorescent color.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were also carried out to evaluate the effects of EAS on vegetative cells of *B. cereus*. An earlier protocol used by El Jaam et al.[47] was adapted to prepare the samples. Vegetative cells of *B. cereus* treated with the EAS were collected and fixed with a 3% formaldehyde buffer to which 2.5% glutaraldehyde has been added and stored for 24 h at room temperature (22 ± 1 °C). The cells were washed in distilled water and postfixed with osmium tetroxide ($OsO_4$ 1%) in sodium cacodylate buffer (pH 7.3, 0.1 M) for 90 min, before being serially washed (3 × 10 min) with sodium cacodylate buffer. Afterward, serial dilutions of ethanol (50, 70, 95, 100%) were used to dehydrate the cells (2 × 10 min). Another dehydration of the cells was performed using 100% ethanol for 40 min. Then, the samples were washed again with hexamethyldisilazane twice for 30 min each time, followed by drying under a chemical fume hood before a metallization step. The prepared samples were ready for observation under SEM (JEOL JSM-6360LV JEOL Ltd., Tokyo, Japan). Regarding TEM, after the step of dehydrating the cells with 100% ethanol for 40 min, the specimens were washed (2 × 30 min) with propylene oxide, followed by Epon infiltration using propylene oxide (1:1) for 24 h, while the evaporation of propylene oxide was carried out under a chemical fume hood. Two other Epon infiltrations were performed for 24 and 3 h. After that, the cells

were placed in epoxy resin before polymerization at temperatures of 37 °C for 24 h and 60 °C for an additional 72 h. An ultramicrotome equipped with a diamond knife was used to cut the polymerized resin containing the cells into thin pieces and stained using lead citrate (0.1%) and uranyl acetate (3%) for 3 min before observation at 80 kV under TEM (JEOL JEM-1230 JEOL Ltd., Tokyo, Japan).

**2.8. Statistical Analysis.** The statistical analysis of the obtained data was carried out with the use of SPSS software (IBM SPSS Statistics 26, NY). The experiments were repeated at least three times (three replicates), and the obtained results were expressed as mean ± standard deviation. Analysis of the variance (ANOVA) including Tukey's test was performed to analyze the data with a significant difference when $p < 0.05$.

## 3. RESULTS AND DISCUSSION

**3.1. Effect of EAS against *B. cereus* ATCC 14579 by Direct Application.** For this experiment, three types of solutions (calcium lactate, calcium ascorbate, and their equimolar mixture) were electroactivated with three different current intensities (250, 500, and 750 mA) during three specific times (10, 20, and 30 min). Each electroactivated solution (EAS) was used in direct contact with the vegetative cells of *B. cereus* ATCC 14579 for 5, 30, and 60 s, followed by subsequent incubation for 24 h at 30 °C to evaluate the inhibitory efficacy of the EAS. In general, the obtained results well demonstrated that all of the EAS used could provoke a high level of inactivation (destruction) of *B. cereus* cells in a relatively very short time. The increase of the contact time seemed to lead to an increase of the antibacterial efficacy of the EAS. For example, a 100% inhibition (destruction) of *B. cereus* (≥7 $\log_{10}$ CFU/mL) was obtained after an exposure time of 60

**Table 3. MIC and MBC of the Electroactivated Solutions Applied against *B. cereus*[1]**

| types of EAS | intensity (mA) | EA time (min) | MIC (%) | MBC (%) |
|---|---|---|---|---|
| calcium lactate | 250 | 10 | 26.67 ± 5.77 | 30.00 ± 0.00 |
| | | 20 | 16.67 ± 5.77 | 30.00 ± 0.00 |
| | | 30 | 26.67 ± 5.77 | 33.33 ± 5.77 |
| | 500 | 10 | 20.00 ± 0.00 | 26.67 ± 5.77 |
| | | 20 | 20.00 ± 0.00 | 20.00 ± 0.00 |
| | | 30 | 16.67 ± 5.77 | 20.00 ± 0.00 |
| | 750 | 10 | 26.67 ± 5.77 | 30.00 ± 0.00 |
| | | 20 | 16.67 ± 5.77 | 26.67 ± 5.77 |
| | | 30 | 20.00 ± 0.00 | 20.00 ± 0.00 |
| calcium ascorbate | 250 | 10 | 30.00 ± 0.00 | 30.00 ± 0.00 |
| | | 20 | 16.67 ± 5.77 | 20.00 ± 0.00 |
| | | 30 | 16.67 ± 5.77 | 23.33 ± 5.77 |
| | 500 | 10 | 10.00 ± 0.00 | 20.00 ± 0.00 |
| | | 20 | 16.67 ± 5.77 | 20.00 ± 0.00 |
| | | 30 | 16.67 ± 5.77 | 20.00 ± 0.00 |
| | 750 | 10 | 16.67 ± 5.77 | 20.00 ± 0.00 |
| | | 20 | 16.67 ± 5.77 | 23.33 ± 5.77 |
| | | 30 | 20.00 ± 0.00 | 20.00 ± 0.00 |
| mixture | 250 | 10 | 26.67 ± 5.77 | 26.67 ± 5.77 |
| | | 20 | 26.67 ± 5.77 | 26.67 ± 5.77 |
| | | 30 | 16.67 ± 5.77 | 20.00 ± 0.00 |
| | 500 | 10 | 16.67 ± 5.77 | 20.00 ± 0.00 |
| | | 20 | 10.00 ± 0.00 | 20.00 ± 0.00 |
| | | 30 | 20.00 ± 0.00 | 20.00 ± 0.00 |
| | 750 | 10 | 20.00 ± 0.00 | 20.00 ± 0.00 |
| | | 20 | 16.67 ± 5.77 | 20.00 ± 0.00 |
| | | 30 | 20.00 ± 0.00 | 20.00 ± 0.00 |

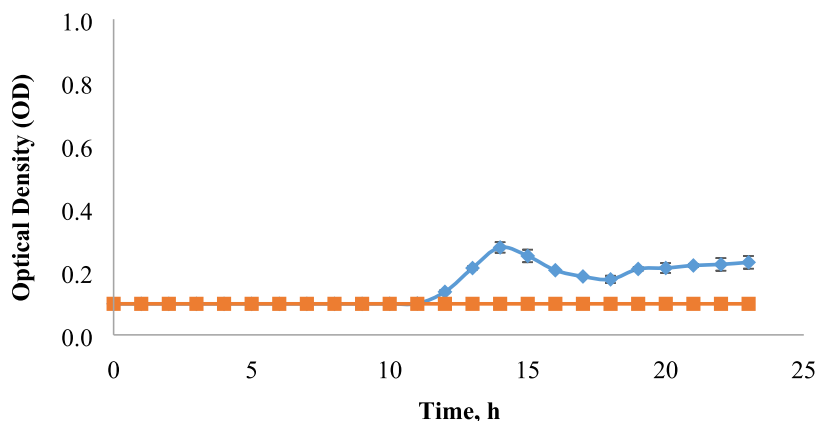[1]EA, electroactivation; EAS: electroactivated solutions; mA: milliampere; MIC, minimum inhibitory concentration; MBC, the minimum bactericidal concentration.

s with all of the EAS used, whereas limited growth was observed when certain EAS were used in contact with *B. cereus* for 5 or 30 s, as presented in Table 2. The electroactivated calcium ascorbate and calcium lactate appeared to be more potent than their mixture given the growth obtained for *B. cereus*.

For the EAS of calcium ascorbate treated at 250, 500, and 750 mA for 10, 20, and 30 min, a complete inactivation of *B. cereus* ($\geq$7 log CFU/mL) was obtained regardless of the contact time used, as shown in Figure 1, except for 500 mA, 30 min where very limited growth was observed at 5 s of exposure time (reduction $\geq$6.90 ± 0.21 log CFU/mL), a difference that was not statistically significant ($p > 0.05$). According to the results obtained with the EAS of calcium lactate, all of the exposure times gave a complete reduction ($\geq$7 log CFU/mL) of *B. cereus*. Regarding the EAS obtained following the EA of the mixture of calcium lactate and calcium ascorbate, the results showed that they exhibited a significant inhibition of *B. cereus* even if their antibacterial efficacy seemed to be weaker than the individual EAS solutions, as presented in Table 2. In all cases where growth was observed (mostly for 5 s contact time), the reduction in *B. cereus* count was always greater than 6 log CFU/mL. These slight differences can be explained by the fact that the mixture is a heterogeneous composition, which losses some bactericidal potencies at short time exposure and could probably require longer exposure time than the individual EAS used to completely inhibit (kill) *B. cereus*. Hence, it is relevant to highlight that even when some little growth was observed for some specific EAS, a previous Tukey test performed did not indicate any statistically significant difference between the pH obtained after 10, 20, and 30 min of electroactivation for all of the related EAS ($p > 0.05$). However, the difference in titratable acidity was statistically significant between 10, 20, and 30 min for the EAS-calcium lactate ($p < 0.002$). Additionally, the difference in titratable acidity was significant between 10 and 30 min ($p = 0.001$) and between 20 and 30 min ($p = 0.006$) for the EAS-calcium ascorbate. For the EAS-mixture, the difference in titratable acidity was significant only between 10 and 20 min ($p = 0.046$). Considering these results, pH and titratable acidity should be considered as essential factors in the monitoring and tackling of pathogenic microorganisms in foods. It is worthy to mention that all of the obtained reductions of the initial *B. cereus* population were higher than 4 log (99.99% reduction
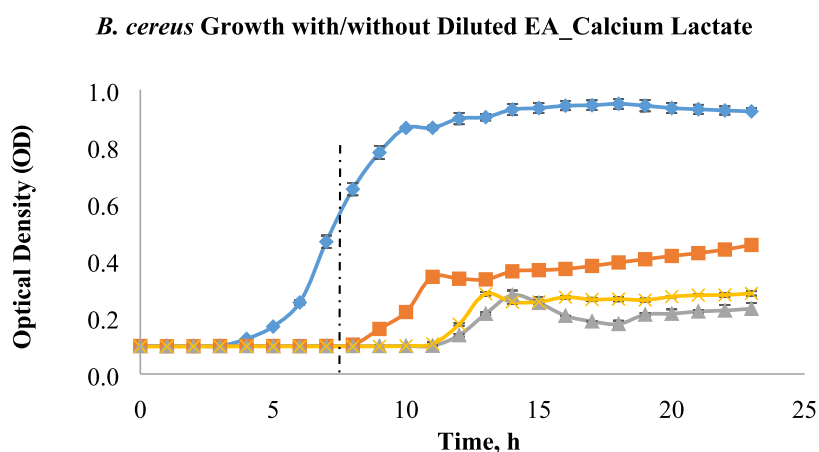


**Figure 2.** Observation of the growth of *B. cereus* ATCC 14579 by spectrophotometry (optical density). The electroactivated solutions (EAS) of calcium lactate (500 mA, 20 min) allow the growth of *B. cereus* (blue rhombus) at a dilution of 10% of the initial EAS concentration, while at a dilution of 20% (MIC) or more, no growth was observed (orange squares).

**B. cereus Growth in EA_Calcium Ascorbate**
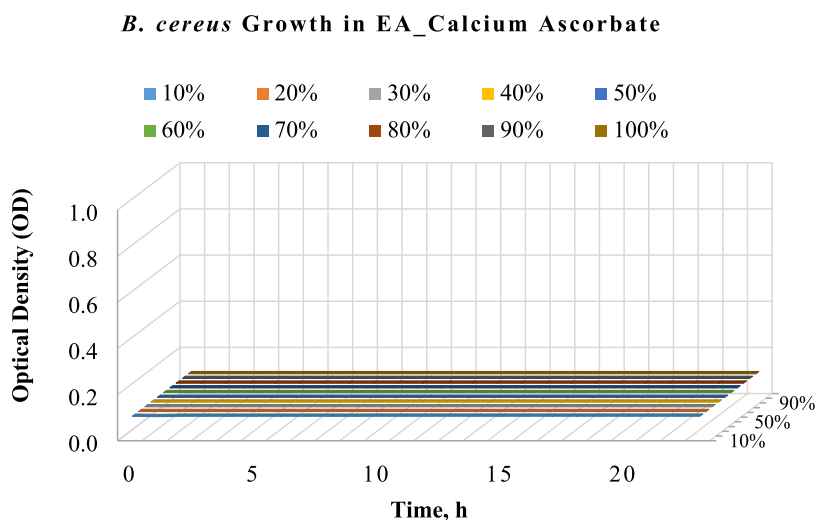


**Figure 3.** Observation of *B. cereus* ATCC 14579 growth by spectrophotometry (optical density). The curves show diluted solutions of calcium ascorbate, which were electroactivated (EA) at 500 mA, 10 min and diluted before being applied against *B. cereus*, and no growth was observed regardless of the dilution level considered.

**B. cereus Growth with/without Diluted EA_Calcium Lactate**



**Figure 4.** Observation of *B. cereus* growth by spectrophotometry (optical density). The positive control (blue rhombus) represents the standard growth of *B. cereus* after 24 h without electroactivated solutions (EAS). The other curves are solutions of calcium lactate electroactivated at 250 mA, 10 min (orange squares), 500 mA, 20 min (gray triangles), and 750 mA, 10 min (yellow crosses), which allow the growth of *B. cereus* after approximately 7.5 h when the dilution level was set at 10% of the EAS initial strength.

efficacy), which is the required reduction to satisfy the requirement for any agent to be considered as antibacterial.[59]

In many previous studies, it has already been proven that anolytes have strong bactericidal activity. The very low pH (below the minimum of 4.3 that can support *B. cereus*),[3] as well as the high concentration of titratable acidity generated during the electroactivation process, could lead to very oxidant media containing, e.g., a huge amount of dissolved oxygen and other reactive components that may potentially explain the high bactericidal effects of such EAS.[60] There have also been various research works supporting the fact that anolytes have, in general, very high oxidation−reduction potential (ORP) values, which could reach +1000 mV or more, contributing very likely to the reactivity of EAS species in the metastable state.[47,60] High ORP would clearly render these EAS unfavorable for the growth of aerobic/facultative anaerobic bacteria like *B. cereus*, which mainly support ORP from −100 to +300 mV and would necessarily cause the inactivation (destruction) of the pathogen.[4,47] Gluhchev and collaborators have reported that even when anolytes showed high-level

antimicrobial activities (e.g., antibacterial, antifungal, antiviral, etc.), their biocidal oxidant components have no toxic effects on the somatic cells of multicellular organisms (e.g., human beings).[61] These findings have provided tangible evidence on the safety side of this technology, which makes it an interesting option, especially for those who wish to use it in their facilities to combat spoilage and pathogenic bacteria without using heat at high intensity or strong disinfecting chemicals. However, it is important to mention here that oxidizing agents can have corrosive effects on certain equipment used in the food industry, which may require specific controls to avoid physicochemical damages to the equipment.

To compare with the present investigation, Liato et al.[60] studied the antimicrobial effectiveness of EAS made from potassium acetate (current intensity: 1 A, 30 min, pH = 2.2, and OPR ≥ +1100 mV) against *Listeria monocytogenes*, *Salmonella enterica*, and *S. aureus*. They reported a level reduction of more than 6 log CFU/mL for all of the bacteria tested after a contact time of 10 min with EAS.[60] These previous findings were in good agreement with those obtained
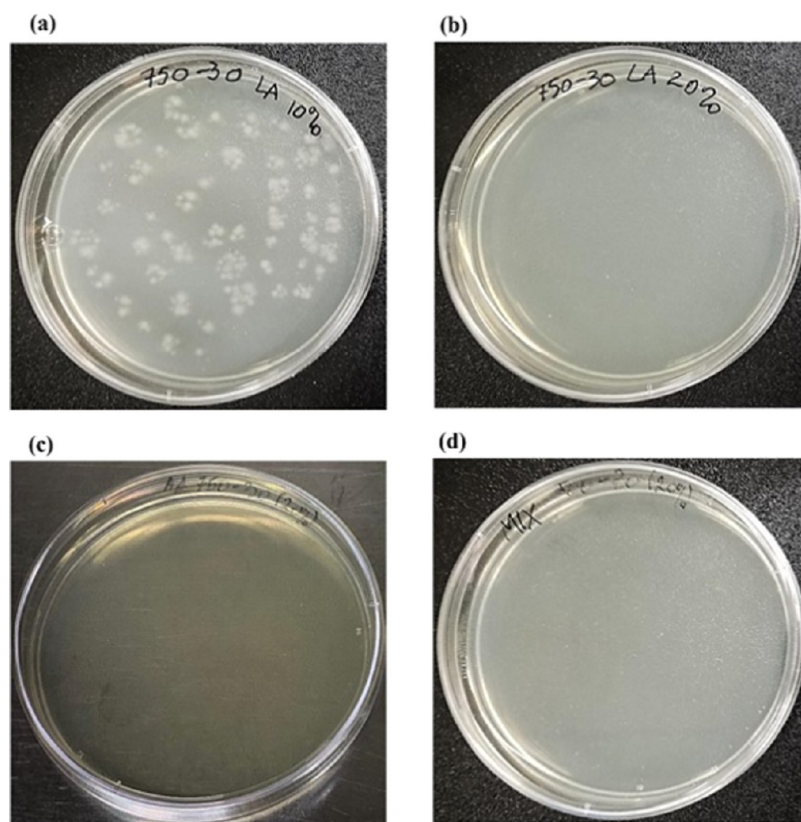
**Figure 5.** Minimum bactericidal concentration. (a) EAS-calcium lactate: 750 mA, 30 min, concentration of 10%, with growth; (b) EAS-calcium lactate: 750 mA, 30 min, concentration of 20%, no growth = MBC; (c) EAS-calcium ascorbate: 750 mA, 30 min, concentration of 20%, no growth = MBC; and (d) EAS-mixture: 500 mA, 30 min, concentration of 20%, no growth = MBC.

in our present study even when a shorter contact time between *B. cereus* and EAS was used. In previous work on the topic, Kim and collaborators have evaluated the bactericidal capacity of electrolyzed oxidizing solutions (pH = 2.6, ORP = +1160 mV, and 56 mg/L of residual chlorine) against *B. cereus* and they have obtained $3 \log_{10}$ reductions of the pathogen after an exposure time of 10 s.[62] These researchers have also discovered that the vegetative cells of *B. cereus* were more resistant than those of other bacteria investigated such as *Escherichia coli* O157:H7 or *L. monocytogenes* when treated under the same conditions.[62] Another research that also assessed the antibacterial activity of electrochemically activated solutions (NaCl) revealed that *S. aureus* and *Pseudomonas aeruginosa* could totally be inactivated after only 10 s in the anolyte.[63] These results indicate the antimicrobial potential of EAS that could be used in the food industry to produce safer foods.

**3.2. Determination of MIC and MBC for the Electro-activated Solutions.** The minimum inhibitory concentration (MIC) of EAS was determined for *B. cereus* ATCC 14579 using a spectrophotometer by monitoring (analyzing) the optical density (OD) in microplates. In general, the findings indicated that the antibacterial activity of EAS, even very diluted with deionized water (10−90%), remained strong in almost all cases. Regarding the EAS of calcium lactate, the lowest MIC value was $\geq 16.67 \pm 5.77\%$ of the EAS initial strength, as presented in Table 3. The following Figures 2 and 3 helped us to confirm the MIC values presented in Table 3 compared to positive control samples without EAS, which showed the standard growth of *B. cereus*. To illustrate, Figure 2 demonstrates the growth of *B. cereus* at 10% of the initial

strength of the EAS-calcium lactate (500 mA, 20 min), while at 20% (MIC) or greater, no growth was obtained. It is important to emphasize the following observation here: in the cases where *B. cereus* showed growth, it was not only limited but also started at approximately 7.5 h or more after being exposed to the diluted EAS, as indicated by the black dotted line in Figure 4. For the EAS of calcium ascorbate, the results demonstrated that the lowest MIC was $\geq 10.00 \pm 0.00\%$ of the EAS initial strength. Even when solutions with concentrations of less than 10% of the EAS initial force have not been evaluated on *B. cereus* in this study, but the fact that some specific diluted solutions (e.g., 500 mA, 10 min) did not show any visible growth after 24 h regardless of the dilution level considered (Figure 3), it most likely suggested that the MIC could be lower than 10% of the EAS initial strength in some cases. These results suggest that the damages caused by the EAS were too devastating to be repaired by the bacterial cells, which probably led to their death. As for the EAS-calcium lactate, it was observed in the case of the EAS-calcium ascorbate that the growth of *B. cereus* appeared mainly after 8 h or more. The screening of the bactericidal effect of the EAS-mixture displayed an MIC value $\geq 10.00 \pm 0.00\%$ of the respective EAS initial strength. As mentioned before for the EAS-calcium lactate and ascorbate, some EAS-mixture (e.g., 500 mA, 20 min) did not allow any growth whatever the dilution rate used, which presumably suggested that the MIC could be under 10% of the EAS initial force in specific cases. Also, it has been observed that the EAS-mixture produced under 250 mA, 10 min showed generally the highest MIC value, which is correlated with the used electric current intensity of 250 mA

**Table 4. Comparison of the Effects of Electroactivated Solutions (EAS) and Standard Acids against *B. cereus*[a]**

| types of EAS | intensity (mA) | EA time (min) | titratable acidity (mol/L) | contact time (s) | survivor cells (log CFU/mL) EAS | survivor cells (log CFU/mL) standard acids |
|---|---|---|---|---|---|---|
| EA-calcium lactate | 250 | 10 | 0.019 ± 0.000 | 5 | ND | 1.28 ± 0.34 |
| | | | | 30 | ND | 0.94 ± 0.40 |
| | | | | 60 | ND | 0.69 ± 0.09 |
| | | | | 120 | | 0.52 ± 0.07 |
| | | 20 | 0.029 ± 0.002 | 5 | ND | 1.12 ± 0.45 |
| | | | | 30 | ND | 0.88 ± 0.76 |
| | | | | 60 | ND | 0.68 ± 0.14 |
| | | | | 120 | | 0.49 ± 0.20 |
| | | 30 | 0.065 ± 0.002 | 5 | ND | 1.12 ± 0.07 |
| | | | | 30 | ND | 0.87 ± 0.52 |
| | | | | 60 | ND | 0.68 ± 0.25 |
| | | | | 120 | | 0.46 ± 0.15 |
| | 500 | 10 | 0.009 ± 0.001 | 5 | ND | 1.02 ± 0.41 |
| | | | | 30 | ND | 0.86 ± 0.17 |
| | | | | 60 | ND | 0.65 ± 0.37 |
| | | | | 120 | | 0.42 ± 0.10 |
| | | 20 | 0.032 ± 0.001 | 5 | ND | 0.96 ± 0.12 |
| | | | | 30 | ND | 0.84 ± 0.21 |
| | | | | 60 | ND | 0.63 ± 0.13 |
| | | | | 120 | | 0.39 ± 0.36 |
| | | 30 | 0.087 ± 0.002 | 5 | ND | 0.95 ± 0.20 |
| | | | | 30 | ND | 0.56 ± 0.49 |
| | | | | 60 | ND | 0 ± 0.00 |
| | | | | 120 | | 0.10 ± 0.17 |
| | 750 | 10 | 0.030 ± 0.002 | 5 | ND | 0.95 ± 0.10 |
| | | | | 30 | ND | 0.83 ± 0.13 |
| | | | | 60 | ND | 0.59 ± 0.26 |
| | | | | 120 | | 0.26 ± 0.24 |
| | | 20 | 0.052 ± 0.012 | 5 | ND | 0.91 ± 0.11 |
| | | | | 30 | ND | 0.59 ± 0.55 |
| | | | | 60 | ND | 0.46 ± 0.41 |
| | | | | 120 | | 0.20 ± 0.17 |
| | | 30 | 0.107 ± 0.007 | 5 | ND | 0.69 ± 0.09 |
| | | | | 30 | ND | 0.30 ± 0.00 |
| | | | | 60 | ND | 0 ± 0.00 |
| | | | | 120 | | 0 ± 0.00 |
| EA-calcium ascorbate | 250 | 10 | 0.009 ± 0.001 | 5 | ND | 1.19 ± 0.44 |
| | | | | 30 | ND | 1.02 ± 0.16 |
| | | | | 60 | ND | 0.72 ± 0.12 |
| | | | | 120 | | 0.40 ± 0.35 |
| | | 20 | 0.014 ± 0.001 | 5 | ND | 1.17 ± 0.07 |
| | | | | 30 | ND | 0.91 ± 0.24 |
| | | | | 60 | ND | 0.69 ± 0.09 |
| | | | | 120 | | 0.36 ± 0.10 |
| | | 30 | 0.025 ± 0.001 | 5 | ND | 1.14 ± 0.12 |
| | | | | 30 | ND | 0.80 ± 0.18 |
| | | | | 60 | ND | 0.67 ± 0.19 |
| | | | | 120 | | 0.36 ± 0.10 |
| | 500 | 10 | 0.012 ± 0.001 | 5 | ND | 1.12 ± 0.29 |
| | | | | 30 | ND | 0.83 ± 0.16 |
| | | | | 60 | ND | 0.66 ± 0.32 |
| | | | | 120 | | 0.33 ± 0.35 |
| | | 20 | 0.029 ± 0.001 | 5 | ND | 1.08 ± 0.24 |
| | | | | 30 | ND | 0.82 ± 0.11 |
| | | | | 60 | ND | 0.64 ± 0.30 |
| | | | | 120 | | 0.30 ± 0.30 |
| | | 30 | 0.063 ± 0.004 | 5 | 0.10 ± 0.21 | 1.08 ± 0.11 |
| | | | | 30 | ND | 0.80 ± 0.27 |
| | | | | 60 | ND | 0.63 ± 0.06 |

**Table 4. continued**

| types of EAS | intensity (mA) | EA time (min) | titratable acidity (mol/L) | contact time (s) | survivor cells (log CFU/mL) EAS | survivor cells (log CFU/mL) standard acids |
|---|---|---|---|---|---|---|
| | | | | 120 | | 0.30 ± 0.00 |
| | 750 | 10 | 0.024 ± 0.002 | 5 | ND | 1.07 ± 0.16 |
| | | | | 30 | ND | 0.77 ± 0.28 |
| | | | | 60 | ND | 0.62 ± 0.28 |
| | | | | 120 | | 0.26 ± 0.24 |
| | | 20 | 0.051 ± 0.001 | 5 | ND | 0.94 ± 0.23 |
| | | | | 30 | ND | 0.75 ± 0.25 |
| | | | | 60 | ND | 0.58 ± 0.27 |
| | | | | 120 | | 0.20 ± 0.17 |
| | | 30 | 0.109 ± 0.001 | 5 | ND | 1.09 ± 0.44 |
| | | | | 30 | ND | 0.67 ± 0.19 |
| | | | | 60 | ND | 0.23 ± 0.40 |
| | | | | 120 | | 0.10 ± 0.17 |
| EA-equimolar mixture | 250 | 10 | 0.012 ± 0.001 | 5 | 0.58 ± 0.49 | 1.29 ± 0.34 |
| | | | | 30 | 0.55 ± 0.31 | 1.01 ± 0.37 |
| | | | | 60 | ND | 0.84 ± 0.06 |
| | | | | 120 | | 0.62 ± 0.28 |
| | | 20 | 0.036 ± 0.000 | 5 | ND | 1.21 ± 0.27 |
| | | | | 30 | ND | 1.01 ± 0.24 |
| | | | | 60 | ND | 0.78 ± 0.42 |
| | | | | 120 | | 0.59 ± 0.11 |
| | | 30 | 0.025 ± 0.001 | 5 | 0.20 ± 0.21 | 1.20 ± 0.28 |
| | | | | 30 | ND | 0.98 ± 0.25 |
| | | | | 60 | ND | 0.78 ± 0.18 |
| | | | | 120 | | 0.53 ± 0.21 |
| | 500 | 10 | 0.009 ± 0.003 | 5 | 0.26 ± 0.00 | 1.20 ± 0.08 |
| | | | | 30 | 0.20 ± 0.42 | 0.91 ± 0.39 |
| | | | | 60 | ND | 0.77 ± 0.07 |
| | | | | 120 | | 0.53 ± 0.21 |
| | | 20 | 0.038 ± 0.003 | 5 | 0.26 ± 0.55 | 1.09 ± 0.35 |
| | | | | 30 | ND | 0.90 ± 0.09 |
| | | | | 60 | ND | 0.75 ± 0.05 |
| | | | | 120 | | 0.42 ± 0.10 |
| | | 30 | 0.050 ± 0.002 | 5 | ND | 1.07 ± 0.30 |
| | | | | 30 | ND | 0 ± 0.00 |
| | | | | 60 | ND | 0 ± 0.00 |
| | | | | 120 | | 0.20 ± 0.17 |
| | 750 | 10 | 0.055 ± 0.001 | 5 | 0.16 ± 0.34 | 1.06 ± 0.23 |
| | | | | 30 | ND | 0.87 ± 0.19 |
| | | | | 60 | ND | 0.69 ± 0.36 |
| | | | | 120 | | 0.36 ± 0.32 |
| | | 20 | 0.117 ± 0.002 | 5 | 0.16 ± 0.00 | 1.04 ± 0.04 |
| | | | | 30 | ND | 0.83 ± 0.16 |
| | | | | 60 | ND | 0.67 ± 0.19 |
| | | | | 120 | | 0.30 ± 0.30 |
| | | 30 | 0.102 ± 0.001 | 5 | ND | 0.94 ± 0.15 |
| | | | | 30 | ND | 0.78 ± 0.15 |
| | | | | 60 | ND | 0.56 ± 0.07 |
| | | | | 120 | | 0 ± 0.00 |

[a]EA, electroactivation; mA, milliampere; ND, not detected; and initial cells, 7 ± 0.01 log CFU/mL.

(the lowest value). These results indicate that the effect of the used electric current intensity combined with the EA duration is a highly significant parameter that must be considered when preparing the electroactivated solution that will be used as antibacterial agents (preservation brine or washing solutions).

The minimum bactericidal concentration (MBC) was determined for all of the diluted EAS applied against *B. cereus*, as presented in the previous Table 3. Two types of EAS samples (the MIC sample and a sample of the solution without growth but containing a higher concentration) were analyzed until the MBC was defined. According to the EAS-calcium lactate, the lowest MBC value was ≥20.00 ± 0.00% of the EAS initial strength, as illustrated in Figure 5b. Regarding the EAS-calcium ascorbate and the mixture, the results revealed that the lowest MBC value was ≥20.00 ± 0.00% of their respective EAS initial force, similar to that obtained for the EAS-calcium
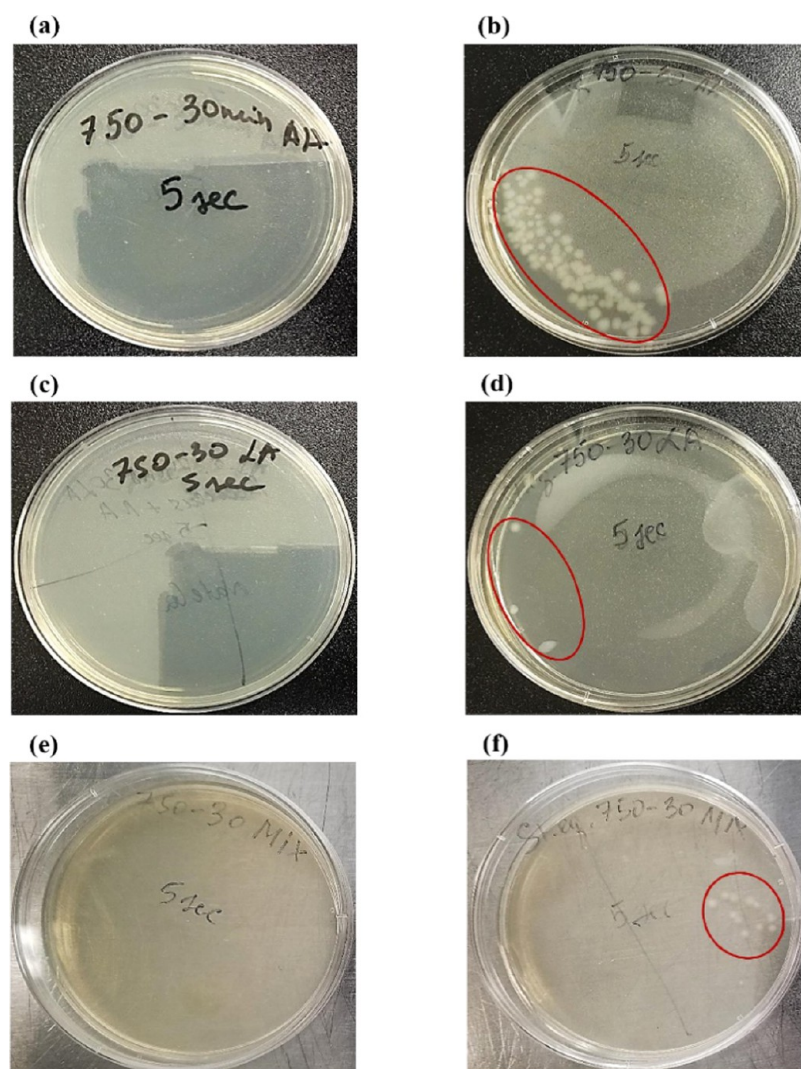
**Figure 6.** Comparison of EAS and standard acids against *B. cereus* ATCC 14579. (a) EAS-calcium ascorbate 750 mA, 30 min—contact time 5 s, no growth; (b) standard ascorbic acid—contact time 5 s, with growth in the red ring; (c) EAS-calcium lactate 750 mA, 30 min—contact time 5 s, no growth; (d) standard lactic acid—contact time 5 s, with growth in the red ring; (e) EAS-mixture 750 mA, 30 min—contact time 5 s, no growth; and (f) mixture of standard acids—contact time 5 s, with growth in the red ring.

lactate. The results also indicated that the MBC values were higher for the EAS treated under 250 mA, 10 min in comparison to those obtained for the other electric current intensities used. In an article published on the topic by French, it was reported that, generally, when the MBC value of a component is 4 times (or less) of that obtained for the MIC, it could be considered to be bactericidal, which is in good agreement with the results of our study.[56]

**3.3. Comparison of EAS and Standard Acids against *B. cereus* ATCC 14579.** The EAS prepared following electroactivation of calcium ascorbate and calcium lactate, as well as their respective standard acids (lactic and ascorbic acids), were used under the same conditions to compare their antibacterial efficacy against *B. cereus* ATCC 14579 vegetative cells. For this purpose, standard acids having the same equivalent of titratable acidity as in the EAS were prepared accordingly. In all cases, the results indicated that both EAS and standard acids possessed high antibacterial activities, resulting in significant reductions in *B. cereus* cells ($p \leq 0.05$) but at different levels. The EAS have displayed stronger destruction/inactivation effects on *B. cereus* than the standard

acids at the same equivalent titratable acidity, as shown in Table 4. By comparison, the EAS-calcium ascorbate prepared at 250, 500, and 750 mA for 10, 20, and 30 min, respectively, were more powerful than the standard ascorbic acid, which showed limited growth in almost all cases. For example, the EAS-calcium ascorbate treated under 750 mA, 30 min exhibited no growth of *B. cereus* (reduction $\geq 7$ log CFU/mL) after 5 s contact time (Figure 6a), while the standard ascorbic acid allowed growth (reduction of $5.91 \pm 0.56$ log CFU/mL), a difference that was statistically significant ($p \leq 0.05$). For the standard ascorbic acid, containing the same equivalent titratable acidity as in the EAS-calcium ascorbate produced at 250 mA, 10 min, it permitted the growth of *B. cereus* even after 120 s of exposure time, whereas the EAS did not allow any growth after only 5 s of direct contact. The standard lactic acid displayed a level of bactericidal efficacy that was lower than EAS-calcium lactate under almost all circumstances. For example, no growth was obtained after a 5 s contact time between the EAS-calcium lactate 750 mA, 30 min and *B. cereus* (Figure 6c), while the standard lactic acid exhibited a visible growth (reduction of $6.31 \pm 0.91$ log CFU/
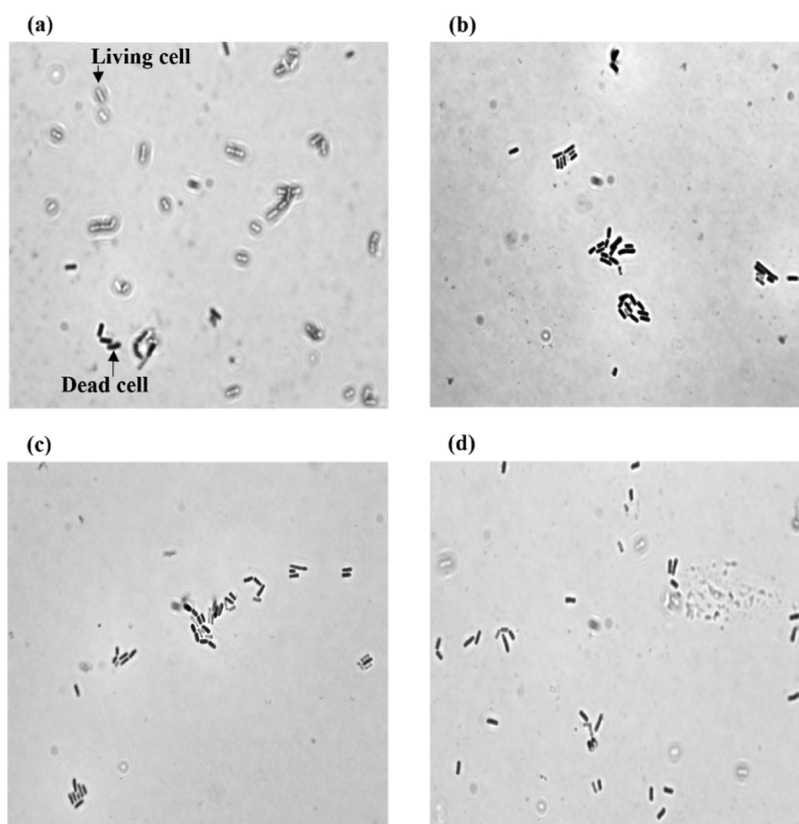
**Figure 7.** Microscopic observation of *B. cereus* ATCC 14579. (a) Initial suspension of *B. cereus*, (b) EAS-calcium lactate 750 mA, 30 min, (c) EAS-calcium ascorbate 750 mA, 30 min, and (d) EAS-mixture 750 mA, 30 min.

mL), a difference that was statistically significant ($p \leq 0.001$). After a 60 s contact time with *B. cereus*, the EAS-calcium lactate produced under 500 mA, 30 min and 750 mA, 30 min gave the same results (no growth) as for the standard lactic acid. For the mixture, the same trend was observed as that previously mentioned for the other EAS. To illustrate, the EAS-mixture generated at 750 mA, 30 min revealed a 100% destruction/inactivation of *B. cereus* after 5 s of direct contact (Figure 6e), while the mixture-standard acids allowed growth (reduction of 6.06 ± 0.85 log CFU/mL); the difference was statistically significant ($p \leq 0.001$).

These results confirmed that the electroactivation technology significantly affected the parameters of EAS and provided to them high antibacterial properties, a fact that was already reported in several scientific literatures.[46,47,51,61] An earlier study, in which standard citric acid was compared to EAS made from potassium citrate, showed that the EAS was more powerful than the standard citric acid in terms of bacterial inactivation of different pathogenic bacteria.[60] In a research paper published in 2004, Koseki and his collaborators studied the antimicrobial effectiveness of acidic electrolyzed water containing 30 ppm of free chlorine available, which was compared to that of ozonated water (5 ppm of ozone) and to sodium hypochlorite solution (150 ppm of free chlorine available) in the decontamination of cucumbers. They reported that the use of acidic electrolyzed water against targeted microorganisms (e.g., aerobic-mesophilic bacteria), naturally present on cucumbers, resulted in a reduction of 1.4 log CFU per cucumber after 10 min of contact, while ozonated water induced a reduction of only 0.7 log CFU per cucumber ($p \leq 0.05$). They also mentioned that the acidic electrolyzed water

demonstrated an even higher sanitation potential than the sodium hypochlorite solution (NaClO), which was able to reduce microorganisms by 1.2 log CFU per cucumber after 10 min of exposure.[64] Similarly, Hao and colleagues have evaluated the potential of slightly acidic electrolyzed water (SAEW) compared to the NaClO solution in the reduction of microbial contamination by *E. coli* O78 and *Bacillus subtilis* 1.1849 on fresh-cut coriander (cilantro). They reported that after 5 min of contact time, the SAEW and NaClO were able to reduce *E. coli* O78 population by 2.49 and 1.5 log CFU/g of coriander, respectively, whereas the population of *B. subtilis* 1.1849 was decreased by 1.54 and 1.89 log CFU/g of coriander after 5 min in contact with SAEW and NaClO, respectively.[65] These results are in good agreement with those obtained in our study and have demonstrated once again that EAS have a strong antibacterial power and could be an excellent alternative for washing vegetables in the agri-food industry and ensuring microbial safety in packed ready-to-eat fruits and vegetables. In the meantime, more research needs to be done on the use of these EAS in food systems.

**3.4. Microscopic Evaluation of *B. cereus* ATCC 14579.** The observation of *B. cereus* ATCC 14579 vegetative cells was carried out using an optical microscope (BX51TRF, Olympus Corporation, Tokyo, Japan) to evaluate the effects of the EAS on the bacteria by identifying living and dead cells. According to studies, when bacteria are stained with methylene blue, the stain penetrates through the cells before being reduced and decolorized due to specific transmembrane enzymes such as reductases, which are activated only in viable cells, and made them appear colorless under microscope objective.[66,67] The results showed that, from the initial suspension (positive
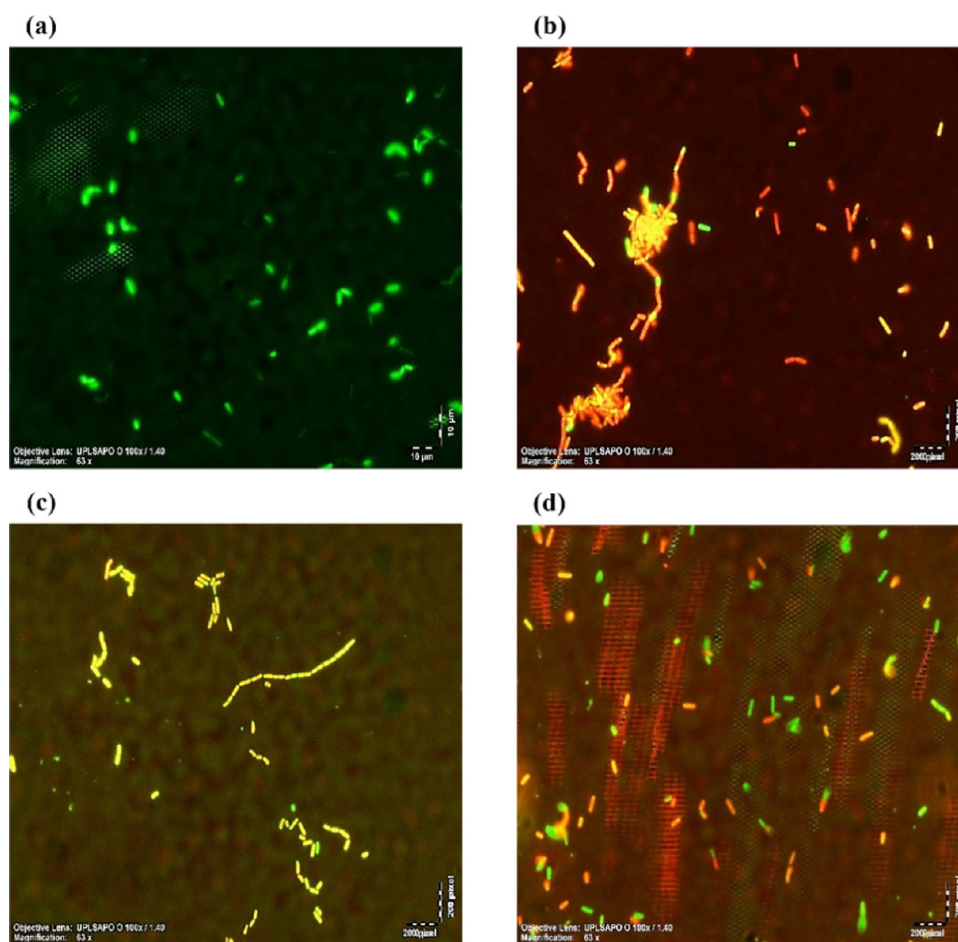
**Figure 8.** Microscopic observation of *B. cereus* cell integrity by the BacLight (Live/Dead) staining method. The green fluorescent cells are living bacteria (undamaged membranes), while the fluorescent red cells have damaged membranes. (a) Initial suspension of *B. cereus*, (b) EAS-calcium lactate 750 mA, 30 min, (c) EAS-calcium ascorbate 750 mA, 30 min, and (d) EAS-mixture 750 mA, 30 min.

control) of *B. cereus*, living cells appeared unstained (clear), as illustrated in Figure 7a. It can also be observed that the EAS-calcium lactate and EAS-calcium ascorbate were able to completely kill *B. cereus* after approximately 30 s exposure time, respectively, as shown in Figure 7b,c. Regarding the EAS-mixture, although very few living cells could be observed, most of them were also killed, as depicted in Figure 7d. These results provided more evidence on the antibacterial efficacy of these solutions, suggesting at the same time that the EAS-calcium lactate and EAS-calcium ascorbate would be more effective than the mixture for destroying *B. cereus* cells at short time. After exposure, EAS seemed to exert strong toxic effects through the cellular contents of *B. cereus*, which presumably compromised their homeostasis and physiological capacity to repair the damages and multiply.[60]

In addition to the aforementioned microscopic method, the Live/Dead BacLight staining method was also performed to examine the physiological state of *B. cereus* cells having been treated with EAS (750 mA, 30 min) for 30 s. As a matter of fact, the green fluorescent dye SYTO9 used can penetrate and stain the compromised and intact cell membranes of bacteria, while the red fluorescent propidium iodide (PI) can only pass through bacteria whose membranes are damaged and stained their genetic material (DNA).[60] The obtained results indicated that all of the EAS tested produced significant damages in the membranes of *B. cereus*, as shown in Figure 8. According to

EAS-calcium ascorbate and lactate, very few green (living) fluorescent cells have been observed (Figure 8b,c), which has demonstrated the antibacterial efficacy of these solutions. The green fluorescent cells could be an indication of inactivated/dead cells but with uncompromised membranes based on the results previously obtained for these EAS (see Table 1).[60] Also, knowing that *B. cereus* has the capacity to enter a physiological state called viable but nonculturable (VBNC) in response to unfavorable conditions (e.g., stress),[68] the green fluorescent cells observed could most likely be *B. cereus* in the VBNC state. Considering this, even when these cells could not grow on standard medium (see Table 1), the BacLight test made it possible to reveal their presence and at the same time confirm the inactivation/inhibitory power of the EAS. The mixture exhibited a lower inactivation rate (with more green fluorescent cells) compared to the EAS-calcium ascorbate and lactate (Figure 8d), which is correlated with the data previously reported in this study. In addition, a large number of cells have been observed to display yellow fluorescence color when stained with both SYTO9 and PI. According to Stiefel et al., this phenomenon can be observed in the BacLight test when both PI and SYTO9 are retained in the cells, which presumably corresponded to dead bacteria.[58] Lu and his collaborators[69] evaluated a treatment (32% Manuka-type honey) to destroy the biofilms produced by certain bacterial strains of *S. aureus*. By using SYTO9 and PI to stain their
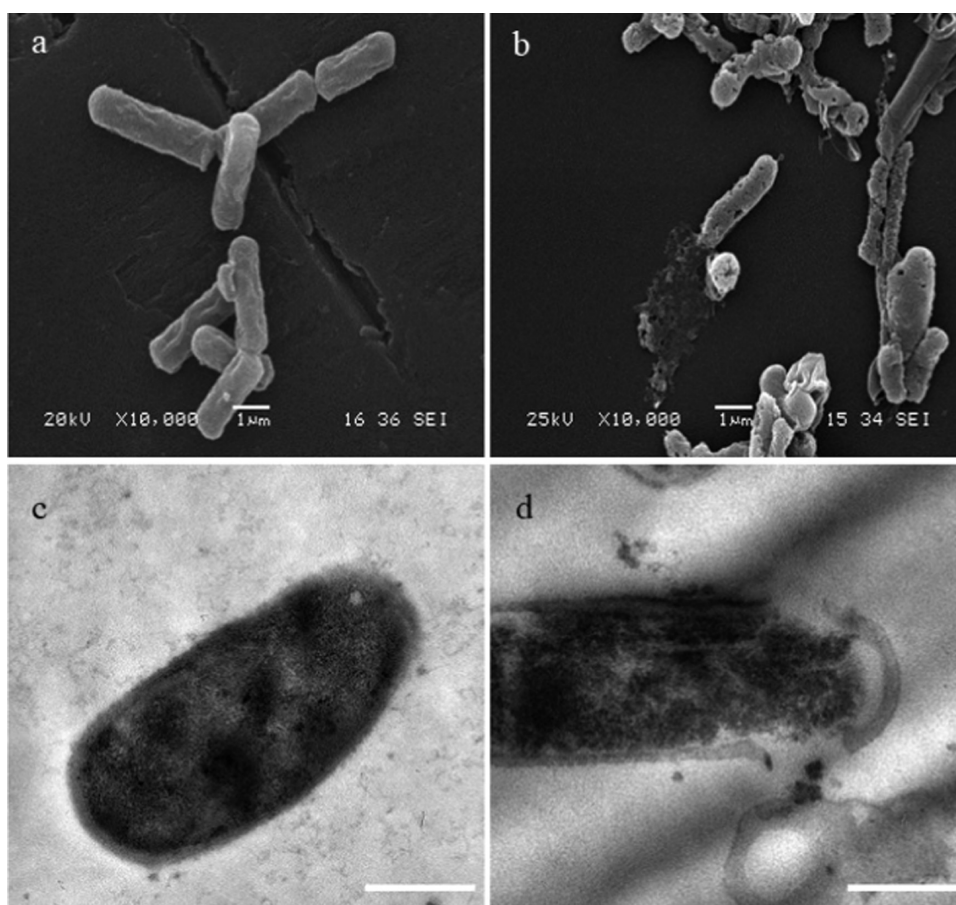
**Figure 9.** Scanning (SEM) and transmission (TEM) electron microscopy micrographs of *B. cereus* ATCC 14579 vegetative cells: (a) SEM of control untreated cells. (b) SEM of treated cells by electroactivated solutions. (c) TEM of control untreated cells. (d) TEM of treated cells by electroactivated solutions.

samples, they realized that the biofilms were mostly stained in yellow due to the retention of the two dyes in bacterial cells, which they considered to be possible dead cells.[69] Recently, Liato et al.[60] studied the antibacterial activity of EAS produced from potassium acetate, potassium citrate, and calcium lactate compared to certain commercial acids (acetic, citric, and lactic) on different pathogenic bacteria, namely, *L. mono-cytogenes*, *S. enterica*, and *S. aureus*. They used the Live/Dead BacLight staining method to assess the physiological state of the bacteria after being treated with EAS and commercial acids. They reported that the EAS-potassium acetate and citrate have significantly affected the bacterial cells and were more potent than their respective conjugate acids in terms of inactivation activity against the pathogens. They also observed that EAS produced with potassium acetate could provoke a reduction of ≥6 log CFU/mL of each tested bacterium after a 10 min contact time.[60] The results of our study are in good agreement with those obtained in the aforementioned research and provide interesting information and bases for further investigations, particularly their application in food systems. Scanning and transmission electron microscopies (Figure 9) showed that treatment with electroactivated solutions damaged the cell integrity and induced a leakage of the intracellular material by inducing pores in the cell membrane.

The results obtained from this study clearly demonstrated the antibacterial activities of EAS made from calcium lactate, calcium ascorbate, and their equimolar mixture. The use of these electroactivated solutions in direct contact with

vegetative cells of *B. cereus* showed a very high reduction level, which ranged between 4 and 7 log, which is significant and sufficient to confirm their antibacterial efficacy. These, in addition to the results obtained previously in other studies, provide a comprehensive view of the antimicrobial potential of electroactivation even when further research is needed to demonstrate the efficacy of EAS on vegetative cells of *B. cereus* in food matrices. Even though changes in the physicochemical properties of EAS (affecting, e.g., ORP, pH, titratable acidity) are commonly associated with their antimicrobial efficacy, further studies on this topic would be relevant for a better understanding of the phenomenon complexity. Given that in our study, the anolytes used did not contain any chlorinated components, it could be argued that the antimicrobial efficacy of EAS could not only be attributed to the chlorine species generated in certain EAS (e.g., EAS made with NaCl).

The problem addressed in this research article is related to the development of an efficient strategy to improve the food safety regarding the problems related to *B. cereus*. To achieve this goal, the use of electroactivated solutions made on the basis of aqueous solutions of weak organic acid and chlorine-free is a highly promising strategy. Electroactivation technology was shown to be an effective approach to convert lactate and ascorbate solutions into highly active and antimicrobial solutions. The principle of this approach is based on electrochemistry, and the originality of this study consists of using electroactivated solutions as antimicrobial agents against vegetative cells of *B. cereus* under ambient temperature for their

complete inactivation within very short direct contact time. This new approach was successfully used in eliminating this pathogenic bacterium, and the obtained results support their compatibility with a technological process.

This work enhances the knowledge on the production of electroactivated solutions derived from aqueous solutions of weak organic acids. These solutions having highly acidic and oxidative properties can be used to ensure microbial safety in the food industry. The electroactivation process creates unique conditions that modify the oxidation−reduction potential of solutions and generates a medium with high reactivity against *B. cereus*. In this study, the highly acidic condition created in the used electroactivation rector permitted highly effective electroactivation of calcium lactate and calcium ascorbate without using any catalyst. This research field is new and original, which has high potential of creating different research branches such as applied electrochemistry and applied microbiology to ensure food safety as well as to understand the mechanisms of action of electroactivation on different compounds. Moreover, this new knowledge will have applications in the food sciences and technology, in general, and in the microbial food technology, in particular. This research is focused on the development of new and highly promising antibacterial agents using already approved salts of weak organic acids. These new antimicrobial agents can easily be used in the manufacturing of different canned foods and for cleaning purposes to enhance the microbial safety in the food industry.

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