UNIVERSIDAD DE COSTA RICA
SISTEMA DE ESTUDIOS DE POSGRADO

DESINFECCIÓN DE SUPERFICIES CONTAMINADAS CON ESPORAS DE *Clostridioides difficile* CON DISOLUCIONES ACTIVADAS ELECTROQUÍMICAMENTE

Tesis sometida a la consideración de la Comisión del Programa de Estudios de Posgrado en Biología para optar al grado y título de Maestría Académica en Biología.

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Ciudad Universitaria Rodrigo Facio

2019

DEDICATORIA

A mis padres, a mis hermanos,
a mi novia Marian y a mi familia.

AGRADECIMIENTOS

El autor desea dejar constancia de su agradecimiento a las siguientes personas e instituciones, que de alguna u otra forma colaboraron para la culminación de este proyecto:

A mis padres Alejandro Medaglia Chaverri y Chris Mata Quirós, quienes siempre me han apoyado e impulsado al éxito en mi vida.

A mis hermanos Mariella y Fabrizzio, quienes han sido mis acompañantes en cada una de las actividades que he emprendido a lo largo de estos años.

Al Dr. César Rodríguez Sánchez por sus provechosos consejos y por todo el apoyo, atención y compromiso demostrado hacia mi persona durante el desarrollo de este trabajo.

A la M.Sc. Ethel Sánchez Chacón por acceder a fungir como mi asesor en este proyecto, así como por todas las sugerencias y consejos que me brindó en términos de procesamiento de muestras.

Al Dr. rer. nat. Ricardo Starbird Pérez por acceder a fungir como mi asesor en este proyecto, así como por todo el tiempo y atención que dedicó para atender cualquier duda que tuve.

Al Centro de Investigación en Enfermedades Tropicales y el Laboratorio de Investigación en Bacteriología Anaerobia de la Universidad de Costa Rica, por permitirme el uso de sus instalaciones, equipos y reactivos para el desarrollo de este trabajo. Así como a todos sus funcionarios quienes me ayudaron de manera atenta en cada consulta que tuve.

Al Laboratorio Institucional de Microscopía del Instituto Tecnológico de Costa Rica por el apoyo recibido, así como por permitirme hacer uso de sus instalaciones y equipos para culminar el proyecto.

"Esta tesis fue aceptada por la Comisión del Programa de Estudios de Posgrado en Biología de la Universidad de Costa Rica como requisito parcial para optar al grado y título de Maestría Académica en Biología"

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RESUMEN

Clostridioides difficile es el principal agente causal de diarrea nosocomial. Esta bacteria se diferencia bajo ciertas condiciones adversas en endosporas, las cuales son difíciles de controlar debido a su alta resistencia a múltiples agentes desinfectantes. Agentes oxidantes fuertes y compuestos derivados del cloro (p.ej. hipoclorito de sodio (NaOCl), aldehídos y ácido peracético) han demostrado ser efectivos en la eliminación de esporas de *C. difficile* en suspensión y en superficies. Sin embargo, su uso se asocia a numerosas desventajas, como corrosión de superficies, mal olor, irritación de ojos, piel y mucosas, entre otras. Estas características indeseables están ausentes en las llamadas disoluciones electroquímicamente activadas (ECAS), las cuales muestran actividad bactericida y esporicida, bajos costos de producción y almacenamiento, capacidad de ser preparadas *in situ*, y alta compatibilidad ambiental. En esta investigación se comparó la eficacia de ECAS derivadas de NaCl (0.19% w/v NaOCl, pH=9.6-10.3), cloro comercial (2.83% w/v NaOCl, pH=5.6) e isocianurato de sodio (NaDCC, pH=6.8) para inactivar esporas de diversas cepas de *C. difficile* sobre superficies inanimadas de acuerdo con la prueba estándar cuantitativa aplicada US EPA MB-21-03. Los valores de reducción logarítmica (VRL) obtenidos fueron estadísticamente diferentes entre los tratamientos ($F_{3,40}$=76.09, p<0.0001), las cepas ($F_{9,40}$=16.42, p<0.0001) y la su interacción ($F_{27,40}$=4.43, p<0.0001). Para efectos de una potencial futura aplicación en campo, las ECAS mostraron VRLs similares o mejores (0.40-5.56) que los obtenidos con NaOCl (0.12-5.50) o NaDCC (0.10-5.12). Análisis por TEM revelaron que la morfología de esporas expuestas a ECAS, NaOCl y NaDCC es similar, pero distinta a la de esporas no tratadas. En este contexto, no se observó una disrupción evidente de la ultraestructura de las esporas, por lo que es probable que el daño causado por los desinfectantes probados ocurra a nivel funcional. Sin importar la cepa ni el tiempo de activación de las ECAS, un análisis factorial confirmó que 15 minutos de exposición son suficientes para reducir al menos en 5 $\log_{10}$ los recuentos en plato de esporas de la cepa más tolerante ensayada. Estos resultados posicionan a las ECAS derivadas de NaCl como una alternativa prometedora para la desinfección de esporas de *C. difficile* en superficies ambientales duras no porosas y dispositivos médicos

ABSTRACT

Clostridioides difficile is the causal agent of nosocomial diarrhea and arises as an important cause of community-acquired diarrhea. Under adverse conditions, this bacterium differentiates into endospores, which are resistance structures with high tolerance to multiple disinfectant agents and therefore troublesome to control. Strong oxidizing agents and chlorine-derived compounds, such as hypochlorite (ClO^{-1}), sodium dichloroisocyanurate (NaDCC), glutaraldehyde, o-phthalaldehyde, hydrogen peroxide and peracetic acid, have proven effective in the inactivation of spores of *C. difficile*. However, their use is associated with numerous drawbacks, including corrosion of surfaces, bad odor, and irritation of eyes, skin and mucous membranes, among others. These undesired characteristics are not present in the so-called electrochemically activated solutions (ECAS), which show bactericidal and sporicidal activity, low production and storage costs, possibility to be prepared *in situ*, and high environmental compatibility. In this study, the efficacy of NaCl-derived ECAS (0.18% w/v NaOCl, pH=9.6-10.3), commercial chlorine (2.83% w/v NaOCl, pH=5.6) and NaDCC (pH=6.8) to inactivate spores of various strains of *C. difficile* on inanimate surfaces was compared using the US EPA MO-21-03 standard quantitative test. The logarithmic reduction values (LR) recorded were significantly different between treatments ($F_{3,40}$ = 76.09, p<0.0001), strains ($F_{9,40}$=16.42, p<0.0001) and the interaction of both factors ($F_{27,40}$=4.43, p<0.0001). For the purposes of a potential future field application, ECAS showed similar or better LR values (0.40-5.56) than NaOCl (0.12-5.50) or NaDCC (0.10-5.12). Transmission Electron Microscopy (TEM) analyses revealed that the morphology of spores exposed to ECAS, NaOCl and NaDCC was similar but distinct to that of untreated spores. No evident ultrastructural disruptions were seen, so the damage likely occurs at a functional level. Regardless of the strain or the ECAS activation time, a factorial design confirmed that 15 minutes of exposure time are sufficient to reduce spore plate counts from the most tolerant strain assayed in at least 5 $\log_{10}$ units. These results highlight NaCl-derived ECAS as a promising alternative for the disinfection of *C. difficile* spores on hard non-porous environmental surfaces and medical devices.

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LISTA DE ABREVIACIONES

ECAS: Electrochemically activated solutions

CDI: *Clostridioides difficile* infection

ORP: Oxidation-reduction potential

NaCl: Sodium chloride

NaOCl: Sodium hypochlorite

NaDCC: Sodium dichloroisocyanurate

MLST: Multi-locus sequence typing

TSA: Trypticase soy agar

TEM: Transmission electron microscopy

PBS: Phosphate buffer saline

ANOVA: Analysis of variance

LR: Logarithmic reduction

CFU: Colony forming unit

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Capítulo I. Traditional and new disinfection methods of *C. difficile* vegetative cells and spores on hospital surfaces

Introduction

Clostridioides difficile is an anaerobic, endospore-forming, Gram-positive bacterium that has been isolated from human and animal feces, soils and sediments, and surfaces or personnel in healthcare facilities, among other sources (Barbut & Petit, 2001; Levinson, 2004). Under certain conditions, including the use of antibiotics, gut colonization with *C. difficile* may progress into *C. difficile* infections (CDI), which are nowadays a leading cause of nosocomial infectious diarrhea (Dworkin & Falkow, 2006; Blossom & McDonald, 2007). In 2011 about half a million CDI cases and 30,000 deaths attributed to CDI were documented only in the United States (Lessa et al., 2015), where CDI expenditures have been estimated to reach $433-793 million every year (Ghantoji, Sail, Lairson, DuPont, & Garey, 2010).

Bacterial endospores are resistance structures. They are composed of a core with DNA, RNA, and enzymes, an inner membrane, a peptidoglycan-based cortex region, a coat demarcated by an outer membrane, and, in some strains, an exosporium with hair-like projections (Lawley et al., 2009; Paredes-Sabja, Shen, & Sorg, 2014). These structures are characterized by a low water content, high levels of dipicolinic acid, and their DNA is bound to small acid soluble proteins that prevent the formation of thymine dimers induced by UV radiation (Driks, 2002; Paredes-Sabja et al., 2014). Therefore, they are highly resistant to heat, chemical and physical agents (Weber, Rutala, Miller, Huslage, & Sickbert-Bennett, 2010), and can persist in the environment (e.g. floor, walls, and tables) for as far as five months (Lautenbach, Woeltje, & Malani, 2010; Kavaler & Alexander, 2014; Lessa F et al., 2015).

Spores are critical for the acquisition and spread of *C. difficile* in hospitals (Wyllie, Hyams, & Kay, 2011). Traditional disinfection methods for the control of nosocomial pathogens in healthcare facilities include manual cleaning and the use of chemical agents (Zhang & Gamage, 2010; Schneider, 2013). However, these practices are often ineffective because:

i) they are restricted to accessible surfaces, ii) many disinfectants are incompatible with certain materials and/or toxic for humans, and iii) many disinfectants do not inactivate spores (Maclean et al., 2015).

In this review we compare the efficiency of traditional methods for the inactivation of *C. difficile* spores in the hospital environment and present modern alternative agents to limit the spread and thereby control this emerging pathogen.

Methodology

A search of peer-reviewed journal articles or book chapters in English or Spanish published between 1993 and 2016 was performed using the Web of Science database. This time span was selected because most advances in *C. difficile* disinfection methods were published during this time window. The following search were used: '*Clostridium difficile* infection*',* '*Clostridium difficile* disinfection', '*Clostridium difficile* spores', '*Clostridioides difficile spores',* '*Clostridioides difficile* disinfection', 'chlorhexidine', 'chlorine-based compounds', 'aldehydes', 'peroxygens', 'hydrogen peroxide vapor', 'electrochemically activated solutions', 'atmospheric pressure plasma', and 'photocatalytic disinfection'. The analysis was restricted to publications that report results of *C. difficile* disinfection tests. Moreover, due to the heterogeneity of experimental conditions applied across the studies, only logarithmic reductions and exposure times were compared.

Results and Discussion

Traditional methods

Guanidines, chlorine-releasing compounds, aldehydes, and peroxides have been used to sanitize hands, sterilize instruments, or disinfect surfaces contaminated with *C. difficile* spores (Table 1). On the other hand, alcohol-based hand-rub solutions are not effective against *C. difficile* spores and it has been hypothesized that they may have contributed to

increasing the incidence of CDI (Siqueira et al., 2007; Weinstein, Milstone, Passaretti, & Perl, 2008).

Table 1. Commonly used agents for the disinfection of *C. difficile* in healthcare facilities.

Agent	Recommended use	Recommended dose	Sporicidal	Reference
Chlorhexidine	Hand cleaning	4% (m/v)	No	(Bettin, Clabots, Mathie, Willard, & Gerding, 2007)
Sodium hypochlorite	Surface disinfection	10% (v/v)	Yes	(Orenstein, Aronhalt, McManus, & Fedraw, 2011)
Sodium dichloroisocyanurate	Surface disinfection	1000 ppm	Yes	(Ascenzi, 1995)
Acidified sodium chlorite	Surface disinfection	200 ppm	Yes	(Goda et al., 2017)
Glutaraldehyde	Instrument sterilization	2% (v/v)	Yes	(Wullt, Odenholt, & Walder, 2003)
***Ortho*-phthalaldehyde**	Instrument sterilization	0.55% (v/v)	Yes	(William Rutala & Weber, 2001)
Hydrogen peroxide	Instrument sterilization and surface disinfection	3-6% (v/v)	Yes	(Alfa et al., 2010)
Peracetic acid	Surface disinfection	0.26-0.35% (v/v)	Yes	(Block, 2004)

Chlorhexidine

Chlorhexidine (CHX) is a cationic bis guanidine with broad activity against Gram-positive and Gram-negative bacteria, yeasts, and some enveloped viruses. It shows low toxicity to humans; hence it is one of the most common active ingredients of antiseptics, particularly in hand washing and oral products (Mcdonnell, Russell, & Block, 1999; Siqueira et al., 2007; Weinstein et al., 2008; Shen, Stojicic, & Haapasalo, 2011). CHX is a strong alkali, practically insoluble in water. In disinfectant formulations, CHX is present as water-soluble salt forms such as chlorhexidine diacetate, chlorhexidine digluconate, or chlorhexidine dihydrochloride (Karpiński & Szkaradkiewicz, 2015). At low concentrations, CHX exerts a bacteriostatic effect due to protein cross-linking (Gomes et al., 2001). At high concentrations, instead, CHX exhibits a bactericidal effect by adsorbing onto the negative groups of the cell wall, causing leakage of cellular components. Though CHX lacks sporicidal activity, Nerandzic & Donskey (Nerandzic & Donskey, 2015) showed that *C. difficile* spores immersed in a CHX solution become susceptible to heat killing at 80 ºC. Other studies have suggested that the use of CHX in hospital bathing can decrease CDI by killing *C. difficile* vegetative cells and through inhibition of spore germination (Rupp et al., 2012).

Chlorine-based compounds

Chlorine-releasing formulations for disinfection purposes often contain sodium hypochlorite (NaOCl) or sodium dichloroisocyanurate (NaDDC). These compounds possess antimicrobial and sporicidal activity and their disinfection efficiency depends on the release of free available chlorine in the form of hypochlorous acid (HOCl) in aqueous solutions (Fraise, 1999; Clasen & Edmondson, 2006; Pfafflin & Ziegler, 2006).

Hypochlorous acid (HOCl) is a strong oxidizing agent that reacts with proteins (Pattison & Davies, 2001, 2005; Pattison, Hawkins, & Davies, 2007) and free amino acids (Stadtman & Levine, 2003) by the formation of monochloramides and dichloramides with exposed amide functional groups of amino acids (Pullar, Vissers, & Winterbourn, 2000). Hypochlorous acid (HOCl) penetrates membranes by passive diffusion; hence most of its antimicrobial activity is in function of its concentration (Fukuzaki, 2006).

Sodium hypochlorite (NaOCl), also known as household bleach, is often commercialized as a 5.25% w/v solution with pH 12-13 (Greenberg, 2003). It shows activity against microbial spores (de Almeida et al., 2005) but is easily inactivated by organic matter (Kearns, Freeman, & Lightfoot, 1995). Orenstein et al. (2011) demonstrated that daily cleaning of hospital surfaces with chlorine-releasing agents reduced significantly the incidence rates of CDI and increased the time between hospital-acquired cases of CDI from 8 to 80 days. (Barbut et al. (2009) obtained up to 4.33 $\log_{10}$ CFU reduction of *C. difficile* spores with 20 minutes of exposure to a 0.5% NaOCl solution.

Sodium dichloroisocyanurate (NaDCC) is used for surface decontamination or in industrial sanitizing products. It is often used as an alternative to sodium hypochlorite (NaOCl), because it is more stable and less susceptible to inactivation by organic material (Ascenzi, 1995). Although, it is not as effective against *C. difficile* spores as NaOCl, it shows activity against spores from this pathogen (Bloomfield & Arthur, 1992). This activity is more strongly influenced by concentration rather than by contact time (Ungurs et al., 2011).

Sodium chlorite is a strong oxidizing agent linked to antimicrobial effects (Lu, Luo, Turner, & Feng, 2007). It is approved by the Food and Drug Administration (FDA) for disinfection in poultry and red meat processing plants, and of raw agricultural commodities (Federal Register, 1998, 1999). Studies by Kobayashi, Iwashita, & Suzuki (1989) showed that acidification of a sodium chlorite solution increases its antimicrobial properties. Goda et al. (2017) proved the sporicidal effect of a weakly acidified chlorite acid water (WACAW) against *C. difficile*, with >3 log reductions in presence of organic matter (0.5% polypeptone) after 1 minute of contact time at a concentration of 200 ppm.

Aldehydes

The activity of the aldehydes is defined by the presence of a carbonyl group. One of the simplest aldehydes is acrolein, which is very active but also toxic, thus alternatives such as formaldehyde, glutaraldehyde, and *ortho*-phthalaldehyde have been used in disinfection (Ascenzi, 1995).

Glutaraldehyde (GTA) is a dialdehyde used for cold sterilization of medical equipment. It is usually commercialized as a 50% acidic aqueous solution and exhibits several advantages, including a broad spectrum of antimicrobial activity, rapid inactivation of microorganisms, and activity in the presence of organic matter (Leung, 2001; W Rutala, Gergen, & Weber, 1993; Simons et al., 2000; Ünal et al., 2006). It is a surface-acting disinfectant that crosslinks external proteins with a concomitant loss of function (Baba et al., 2002; McDonnell & Burke, 2011). GTA solutions are very effective against *C. difficile* vegetative cells and spores (Russell, 1999). For instance, GTA-based sterilization of *C. difficile* spores with a 2% v/v solution for 30 minutes has led to a 4-log reduction (Wullt et al., 2003). However, GTA must be activated prior to use because their sporicidal activity is only achieved at alkaline pH (Fraud, Maillard, & Russell, 2001). Unfortunately, it is linked to adverse effects such as irritation and sensitization of the eyes, skin, and respiratory tract (Takigawa & Endo, 2006; Vonberg et al., 2008).

Ortho-phthalaldehyde (OPA) is an aromatic dialdehyde with both antimicrobial and sporicidal activity that has been reported as an alternative to GTA in high-level disinfection (Fraud et al., 2001; Cabrera-Martinez, Setlow, & Setlow, 2002). OPA, which is usually found as a pale-blue solution containing 0.55% of the active compound, does not require activation prior to its use and has excellent stability over a wide range of pH (Pala & Moscato, 2013). It has shown activity against both Gram-positive and Gram-negative bacteria and is not inactivated by organic matter (Babb & Bradley, 1999; Bridier et al., 2011). Its antimicrobial activity is due to strong cross-linking with primary amines of proteins (Simões et al., 2003, 2006). Simons et al. (2000) reported that OPA is less reactive for nucleophilic addition reactions than aliphatic aldehydes such as GTA, leading to a less efficient reaction with proteins. However, the lipophilic nature of OPA promotes its transport through lipids and compensates its lower cross-linking efficiency. The activity of OPA against *C. difficile* has not been widely studied (Barah, 2013). Nevertheless, a study conducted by Gonçalves et al. (2013) evidenced OPA efficacy of 100% for the inactivation of *C. difficile* spores within 3 minutes of exposure time, yet, no information regarding the logarithmic density of the initial inoculum was reported in this research.

Peroxygens

Peroxygens include relatively simple biocides such as hydrogen peroxide (H_2O_2) and peracetic acid (PAA). These compounds have been used for antiseptic and disinfecting purposes on account of their capacity to oxidize proteins, lipids, and nucleic acids (McDonnell, 2007).

H_2O_2 is commonly used as a high-level disinfectant for the disinfection and sanitization of medical equipment in healthcare facilities (William Rutala & Weber, 2004; Brudzynski et al., 2011). It is marketed as a liquid containing 3-6% v/v of the active compound (Andersen et al., 2006). H_2O_2 may also promote the oxidation and removal of organic matter present on surfaces (Ksibi, 2006). Its antimicrobial activity is due to the formation of hydroxyl radicals (•OH) (Labas et al., 2008) that disrupt membrane integrity and damage membrane proteins by the oxidation of amino acids such as histidine, methionine, cysteine, and phenylalanine, leading to protein malfunction (Coyle & Puttfarcken, 1993; Labas et al., 2008). Hydroxyl radicals may also cause lipid peroxidation, leading to structural alteration of cellular membranes (Dix & Aikens, 1993) as well as oxidation of nucleic acids, particularly at guanine residues (Hofer et al., 2005). Some microorganisms are more difficult to inactivate with H_2O_2 due to intrinsic catalase and peroxidase activities, which convert H_2O_2 to water (Finkel & Holbrook, 2000; Otter & French, 2009). Logarithmic reduction rates as high as 6 $\log_{10}$ can be achieved through a 10 min exposure of *C. difficile* spores to H_2O_2 (Pérez, Springthorpe, & Sattar, 2005). Congruently, Alfa et al. (2010) achieved 3 $\log_{10}$ reductions within one minute of exposure to a 7% v/v H_2O_2 formulation, establishing H_2O_2 as an alternative for killing *C. difficile* spores on bathroom surfaces. Although H_2O_2 is an effective disinfectant agent, its application is affected by the fact that the rooms requiring disinfection must be empty of patients and personnel (Rutala & Weber, 2011; Rutala, Gergen, & Weber, 2012).

PAA consists of an equilibrium solution of peracetic acid, acetic acid, H_2O_2 and water usually found in concentrations ranging from 12 to 15% v/v (Watada et al., 2005; Lindler, Lebeda, & Korch, 2007; Boyce et al., 2008). It is a strong oxidizing agent used as a high-level disinfectant due to its broad-spectrum antimicrobial effect, even in the presence of organic matter. Its biocidal activity is associated with its undissociated acid, which acts by

oxidation of sulfhydryl (-SH) and sulfur (S-S) bonds of proteins, such as those present in microbial spores.(Liberti, López, & Notarnicola, 1999; W Rutala & Weber, 1999; Santoro et al., 2007). PAA inactivates catalase; an enzyme that detoxifies free hydroxyl radicals (Mcdonnell et al., 1999). Wullt et al. (2003) achieved a 4 $\log_{10}$ reduction of *C. difficile* spores within 5 minutes of exposure time to a 0.26% PAA solution and it showed to be more toxic to *C. difficile* spores on stainless steel surfaces than NaDCC (Block, 2004). PAA formulations are slightly corrosive (Bielanski, 2005), hence they are not suitable for high-frequency application in healthcare facilities. Moreover, their sporicidal concentration causes skin and eye damage (Boyce & Pittet, 2002; Wheeldon et al., 2008; Edmonds et al., 2013).

Alternative methods

Hydrogen peroxide vapor

Hydrogen peroxide vapor (HPV) is an emerging technology for surface sterilization and disinfection which according to the Center for Chemical Process Safety (2010) is safer and easier to contain than chlorine dioxide and ethylene oxide. It degrades into water and oxygen after its application, is effective in low-temperature sterilization, and maintains its properties at high temperatures (Lindler et al., 2007). Boyce et al. (2008) achieved a considerable reduction of *C. difficile* in hospital rooms that were decontaminated with HPV at a concentration of hydrogen peroxide of 30% and between 3-4 hours of exposure time per patient room, as well as a reduction in the incidence of CDI during the intervention period. More recently, it has been shown that *C. difficile* spores with logarithmic densities as high as 6.4 log CFU can be inactivated with HPV in 90 minutes exposure time (Otter & French, 2009). HPV has been shown to be highly effective at lower concentrations than liquid H_2O_2, with the benefit of breaking down into harmless components, However, it holds certain disadvantages such as that rooms need to be sealed and that its levels must be closely monitored to allow workers and patients re-entry. Finally, HPV disinfection costs are considerably high (Boyce et al., 2008).

Electrochemically activated solutions (ECAS)

ECAS are produced through electrolysis of a diluted salt solution (electrolyte) by application of direct current. This treatment leads to the conversion of the electrolyte into an activated 'metastable' state in which its chemical reactivity is increased (Oxidation-reduction potential (ORP) from +800 mV to +1200 mV) (Thorn, Lee, & Robinson, 2011; Garg & Garg, 2013). The electrochemical chamber usually consists of two submerged electrodes separated by a central compartment (O'Donnell et al., 2009; Liato et al., 2015). The electrolysis process involves two different phenomena, namely, reduction reactions on the negative electrode ("cathode") and oxidation reactions on the positive electrode ("anode") (Aider et al., 2012). When the mineral component is NaCl, the solution obtained at the negative electrode exhibits detergent properties, with NaOH as principal component, and the solution obtained at the positive electrode consists of a mixture of oxidants (e.g hypochlorous acid). According to Thorn et al. (2011), outer and inner cell membranes are likely primary targets for ECAS, where they cause permeabilization, alteration of protein structure, and ultimately cell lysis. Since NaCl-derived ECAS are mainly composed of hypochlorite, hydrochloric acid, and hypochlorous acid, their high effectivity is associated with their ORP, which generates osmotic unbalance, damaging membranes (Helme et al., 2010; Thorn et al., 2011). Robinson et al. (2010) reached a 5 $\log_{10}$ reduction in the recovery of *C. difficile* spores after a 20 seconds exposure to an ECAS that was derived from NaCl and had an ORP of approximately +1170 mV. ECAS can be generated on-site in the required quantities at low expenses, reducing the operating costs associated with transport, storage of oxidizing agents, as well as environmental impacts. Nevertheless, they cannot be stored for long periods of time and its effectiveness relies on the electrochemical cell capabilities (Thorn et al., 2011).

Photocatalytic disinfection with TiO_2 nanomaterials

Photocatalysis is a process in which a chemical reaction is accelerated by the action of light and a catalyst such as TiO_2, ZnO, CdS, and Fe_2O_3 (Taicheng An, Zhao, & Wong, 2016). According to Foster et al. (2011) TiO_2 nanocomposites can act as a disinfectant because

they absorb photons to form thereby reactive oxygen species that disturb cell membrane phospholipids, lipoproteins, and nucleic acids, leading to function loss and eventually cell death. TiO_2 nanocomposites can be conjugated with other compounds to increase their antibacterial and sporicidal properties, for example, Krishna et al. (2005) showed that multi-wall carbon nanotubes coated with titanium dioxide were more effective and required lower disinfection times for disinfection of *Bacillus cereus* spores than titanium dioxide nanopowders. With regard to *C. difficile*, Dunlop et al. (2010) obtained 2 $\log_{10}$ reductions of *C. difficile* spores in 5 hours exposure time using TiO_2 thin films. TiO_2 nanocomposites can be synthesized at low cost. Moreover, their products have low toxicity and high photostability (Taicheng An et al., 2016). However, no standard methods for disinfectant testing have been designed so far, making it difficult to compare results across studies (Foster et al., 2011).

Atmospheric-pressure plasma (APP) discharge

Plasma, considered as the fourth state of matter, is an ionized gas with ions, electrons, and uncharged particles such as atoms, molecules, and radicals (Dunlop et al., 2010). There is thermal and cold plasma (CP), and in the latter, the electrons are at a hotter temperature than the heavy particles, which remain at room temperature. So-called CP jets have been used for disinfection purposes (Mai-Prochnow, Murphy, Mclean, Kong, & Ken, 2014). According to Vatansever (Vatansever et al., 2013), microorganism inactivation by CP occurs through direct permeabilization of membranes, oxidative damage to proteins, and chemical damage of nucleic acids. APP application holds promising advantages, such as a targeted disinfection in short periods of time (Mai-Prochnow et al., 2014; Vatansever et al., 2013). Nonetheless, not many studies have confirmed the disinfection potential of APP or have characterized plasma, its active species, and phototoxic side reactions (Mai-Prochnow et al., 2014). A study by Claro et al. (Claro, 2015) demonstrated the effectiveness of CP against *C. difficile* spores, with a time-dependent activity and a 2.69 $\log_{10}$ reduction with a 90-seconds exposure time.

Table 2 summarizes features of selected disinfection agents for *C. difficile*.

Table 2. Summary of the principal characteristics of each disinfection study on the reduction of *C. difficile.*

Disinfectant	Concentration	Exposure time	Test method	Contamination conditions	Log reduction achieved	Reference
Sodium hypochlorite	0.5%	20 min	Spore-carrier test	Clean	4.33	(Barbut et al., 2009)
Sodium dichloroisocyanurate	6000 ppm	2 min	Spore-carrier test	Dirty	2.39	(Ungurs et al., 2011)
Sodium chlorite	200 ppm	1 min	Solution	Dirty	>3	(Goda et al., 2017)
Glutaraldehyde	2%	30 min	Solution	Clean	4.1	(Wullt et al., 2003)
Ortho-phthalaldehyde	0.55%	3 min	Spore-carrier test	Clean	n.r.	(Gonçalves et al., 2013)
Hydrogen peroxide	7%	10 min	Spore-carrier test	Dirty	6	(Pérez et al., 2005)
Peracetic acid	0.26%	5 min	Solution	Clean	4	(Wullt et al., 2003)
Hydrogen peroxide vapor	n.r.	30 min	Spore-carrier test	Dirty	6.4	(Otter & French, 2009)

ECAS	n.r.	20 sec	Solution	Clean	5	(G. Robinson et al., 2010)
Titanium dioxide	-	5 hours	Surface	Clean	2	(Dunlop et al., 2010)
Atmospheric-pressure plasma discharge	-	90 sec	Spore-carrier test	Dirty	2.69	(Claro et al., 2015)

n.r: not reported.

Conclusion

We compared the sporicidal activity of chemical agents of diverse nature against *C. difficile* spores. The traditional methods and alternative disinfection methods revised can achieve similar reduction levels (2.39 -6 log units vs. 2-6.4 log units) and require comparable exposure times (5-30 min vs. 1.5-90 min) (Pérez et al., 2005; Barbut et al., 2009; Otter & French, 2009; Claro et al., 2015). Therefore, it is not possible to discriminate them on the basis of efficacy. Unlike the modern alternative disinfectants, the traditional methods require specific activation conditions, are easily inactivated by organic matter, show high toxicity, and in some cases, are hard to implement in hospitals (e.g. rooms must be emptied for very long periods). Moreover, the modern alternative disinfectants can be cheaper and/or more practical and may overcome the resistance developed by some strains to, for instance, chlorine-based compounds (Cherchi & Gu, 2011). However, some of them are linked to poorly understood properties and therefore require further investigation.

Capítulo II. Desinfección de superficies contaminadas con esporas de *Clostridioides difficile* utilizando disoluciones activadas electroquímicamente

Introduction

Clostridioides difficile is an important causal agent of diarrhea in hospitals and the community (Lydyard et al., 2009; Harold & Dupont, 2019). This illness is triggered by a destabilization of the gut microbiota and its functioning, often due to antibiotic consumption (Ramirez, Liggins, & Abel-Santos, 2010; Feuerstadt, 2015). According to Garey et al. (2008), the number of cases and the severity of *C. difficile* infections (CDI) is increasing worldwide. Moreover, CDI are associated with high morbidity (Kwon, Olsen, & Dubberke, 2015) and high recurrence rates (McFarland, Elmer, & Surawicz, 2002), hence they are linked to a significant economic burden for healthcare systems and negatively impact the productivity of a country.

A crucial step in the pathogenesis of CDI is the oral ingestion of endospores, which show structural and physiological properties that render them high resistance to physical agents, such as radiation or high temperatures, and to most chemical agents commonly used in hospital disinfection (Pankey, 2000). Furthermore, spores are able to survive and maintain their germinative potential on inanimate surfaces for long periods of time, acting as reservoirs for the infection of personnel and patients in the hospital environment (Lautenbach et al., 2010).

Chlorhexidine (Bettin et al., 2007), glutaraldehyde (Dyas & Das, 1985), peracetic acid (Bridier et al., 2011), hydrogen peroxide (Labas et al., 2008), and chlorine-derived compounds (A. Fraise, 1999) are the most common chemical agents used in hospitals and healthcare centers for disinfection of inanimate surfaces contaminated with *C. difficile*. However, their application is linked to certain disadvantages, including long exposure times, erratic efficiency, high toxicity, and damage to hospital infrastructure (Rutala et al., 1993; Thorn et al., 2011; William Rutala et al., 2012).

Based on their accessibility, low cost, broad spectrum of antimicrobial activity, and the possibility of on-site preparation, different electrochemically activated solutions (ECAS) have emerged as an alternative for traditional disinfection methods (Helme et al., 2010;

Thorn et al., 2011). They are produced by electrolysis of a dissolved salt, with the consequent generation of strong oxidizing and reducing agents and free radicals in a metastable state that retains its biocidal activity for days or even months (Thorn et al., 2011).

ECAS are usually classified according to its pH into acidic, neutral or alkaline ECAS. Robinson et al. (2010) successfully killed vegetative cells of *Staphylococcus aureus* and *Pseudomonas aeruginosa* as well as spores of *C. difficile* and *Bacillus atrophaeus* using acidic ECAS. Likewise, Helme et al. (2010) reported a high killing activity of acidic, neutral, and alkaline ECAS with a NaOCl concentration of as low as 0.006% against various strains of *B. cereus*, *Candida albicans*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella oxytoca*, *P. aeruginosa*, and *S. aureus.*

In the case of NaCl-derived ECAS, the oxidation reactions that occur on the anode yield the formation of chlorine species, which react with water to form HOCl and HCl in an acidic solution (Barry-Ryan, 2012). On the cathode, by contrast, hydrogen and antioxidant compounds in an alkaline solution are produced (Thorn et al., 2011). The physicochemical properties of the ECAS vary depending on the voltage and amperage used in their synthesis; hence it is relevant to characterize the process and the end product of the electrolysis (Robinson, Thorn, & Reynolds, 2012).

Robinson et al. (2010) showed that acidic ECAS inactivate *C. difficile* vegetative cells and spores in exposure periods of 30 seconds. However, their ECAS characterization was limited to pH and oxidation-reduction potential (ORP) and the disinfection method was applied directly to spore suspensions. Aiming to test the potential application of ECAS for *C. difficile* disinfection on healthcare facilities, we compared the logarithmic reduction of *C. difficile* spores on contaminated inanimate surfaces caused by a NaCl-derived ECAS and two chlorine-derived compounds widely used in hospitals (5000 ppm sodium hypochlorite, NaOCl and 1000 ppm) sodium dichloroisocyanurate, NaDCC).

Materials and Methods

Chemicals

All chemicals were analytical grade and used without further purification. Platinum foil, sodium chloride (NaCl), sodium hypochlorite (NaOCl), sodium dichloroisocyanurate

(NaDCC), and sodium taurocholate were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Brucella Agar with hemin and vitamin K1 was purchased from Beckton, Dickinson & Company (Franklin Lakes, New Jersey, USA).

Bacterial strains and culture conditions

All experiments were performed with reference *C. difficile* strains or WGS-typed field isolates (LIBA) from MLST Clades 1 to 5: 630 (Clade 1), LIBA-6276 (Clade 1), LIBA-5758 (Clade 2), LIBA-5757 (Clade 2), LIBA-6507 (Clade 3), LIBA-7110 (Clade 3), M68 (Clade 4), LIBA-7719 (Clade 4), M120 (Clade 5), and LIBA-7854 (Clade 5). When required, bacteria were subcultivated in TYT broth (3% w/v Bacto tryptone, 2% w/v yeast extract, and 0.1% w/v sodium thioglycolate, pH 6.8). All incubations were done at 37ºC into an anaerobic chamber (Bactron, Shel Lab, Cornelius, Oregon, USA) with a controlled atmosphere composed of 90% N_2, 5% CO_2 and 5% H_2.

Spore isolation

The spore isolation procedure followed was based on protocols published by Paredes-Sabja et al. (2008) and Fraise et al. (2015). In detail, liquid cultures containing vegetative cells were inoculated onto TSA plates (1.5% pancreatic digest of casein, 0.5% peptic digest of soybean meal, and 0.5% NaCl) and incubated for 120 h at 37ºC. The resulting biomass was thereafter resuspended in PBS (0.01 M, pH 7.4) and spores were separated from vegetative cells by centrifugation in a Histodenz™ gradient. To remove Histodenz™ traces and promote spore maduration, spore pellets were washed with PBS, resuspended into PBS + BSA 1% w/v, and stored at 4ºC for 15 days. Prior to their use, the purity and number of viable spores in the suspensions were determined through cultivation on Brucella Agar plates supplemented with 0.5% sodium taurocholate for 120 h at 37ºC. In addition, all spore suspensions were observed under a light microscope to confirm the absence of vegetative cells.

ECAS synthesis

Fifty ml of a 10% NaCl solution was placed into an electrochemical chamber equipped with platinum electrodes (Sigma Aldrich, St. Louis, Missouri, USA) and lacking a membrane between the anode and the cathode. A current of 3 A and a voltage of 20 V was

applied for 30 minutes using an Analog DC Power Supply (Goldstar, GP-303, Yeouido-dong, Seoul, South Korea).

Chlorine quantification

Total and available chlorine in ECAS, household bleach (3.15% w/v in label), and NaDCC (Sigma Aldrich, St. Louis, Missouri, USA) were quantified by titration with the standard procedure ASTM D 2022 (ASTM International, 2003) and sodium thiosulphate 0.1 M ($Na_2S_2O_3$, Sigma Aldrich, St. Louis, Missouri, USA). This procedure was done by triplicate. A 10% NaCl solution (Sigma Aldrich, St. Louis, Missouri, USA) was used as a standard for quality control purposes. Titration results were reported as geometric means with 95% confidence intervals.

Voltammetry analyses

The electrochemical behavior of the synthesized ECAS, the 10% NaCl solution used for ECAS synthesis, and a 10% NaOCl solution (Sigma Aldrich, St. Louis, Missouri, USA), was measured by triplicate with an electrochemical workstation (AUTOLAB, model: PGSTAT-302, Utrecht, Netherlands) set to measure cyclic voltammetry from -5 to 5 V at a 100 mV/s scan rate. This workstation was equipped with a platinum electrode as the working electrode (1.875 cm^2) and platinum foil as a counter electrode. (Hubler, Baygents, Chaplin, & Farrell, 2014; M. Spasojević, Krstajić, Spasojević, & Ribić-Zelenović, 2015). This series of electrochemical experiments was carried out in a 50 mL voltammetry cell, and the open circuit potential (OCP) of each solution was obtained before and after the analysis.

Sporicidal activity tests

The sporicidal activity of the synthesized ECAS, household bleach, and NaDCC was measured using the Standard Operating Procedure (SOP) MB-21-03 of the United States Environmental Protection Agency (EPA) (US EPA, 2017). Prior to disinfection, all spore suspensions were placed in a water bath at 65°C for 10 min to eliminate remaining vegetative cells (Kenters et al., 2017). Stainless-steel pieces (5x5x1 mm) were inoculated with 10 μL of spore suspensions adjusted to contain $1x10^5$ CFU/mL. These test surfaces were carefully

placed into 1.5 mL plastic microcentrifuge tubes containing 400 µL of synthesized ECAS, 5000 ppm NaOCl, or 1000 ppm NaDCC, and after 5 minutes 600 µL of ice-cold Luria Bertani (LB) broth was added as neutralizing agent. After this exposure, spores were harvested by centrifugation at 13,000 rpm for 6 min, washed with ice-cold LB broth, resuspended, and diluted for plating on Brucella Agar supplemented with 0.5% sodium taurocholate. These plates were incubated for 5 days under anaerobic conditions. Log reduction values were obtained by subtracting the CFU/mL of each disinfection treatment to the control CFU/mL count. As a control, inoculated stainless-steel pieces were exposed to phosphate buffer saline (PBS) with 0.1 w/v Tween 80 instead of disinfectant agents. These tests were done by duplicate.

Transmission Electron Microscopy (TEM)

Spores from all treatments and the control assay were processed for TEM. To this end, spores were pelleted by centrifugation and fixed using an aldehyde solution composed of 2.5% glutaraldehyde and 2% paraformaldehyde in phosphates buffer 0.1 M, pH 7.4. Pellets were first washed with phosphates buffer (0.1 M, pH 7.2) and post-fixed with 1% osmium tetroxide (OsO_4) in phosphates buffer (0.1 M, pH 7.4). Thereafter, they were washed with distilled water and dehydrated with acetone. Fixed pellets were embedded into Spurr's resin (Spurr, 1969), cured at 70 ℃ for 48 hours, cut into ultrathin sections (~80 nm), and observed under a transmission electron microscope (JEM-2100, JEOL, Tokyo, Japan).

Statistical analyses

A Two-way ANOVA test followed by Tukey multiple comparison tests were performed to determine whether the treatments and strain responses differed. $P < 0.05$ was considered to be significant. All tests were done with the GraphPad Prism 6 software. A full-factorial design was performed by duplicate on isolates LIBA 5758 (highly susceptible to disinfectants) and LIBA 5757 (more tolerant to disinfectants) for it can provide information on factor significance and be exploited for response optimization. In this regard, three levels of the factor exposure time (5, 10, and 15 min) and ECAS activation time (10, 20, and 30 min) were tested.

Results and discussion

ECAS characterization

As determined by titration, household bleach (2.97% w/v, 95% CI 2.41-3.58) contained more NaOCl than the synthesized ECAS (0.19% w/v, 95% CI 0.171-0.21). This anticipated result is caused in non-divided cells by competing oxidation and reduction reactions that lead to cell inefficiencies by oxygen generation (Abdel-Aal, Sultan, & Hussein, 1993; M. Spasojević et al., 2015). However, as the method only quantifies total chlorine and NaOCl, other unidentified oxidant agents that may have been generated during the electrolysis process could have contributed to boost the disinfectant potential of ECAS.

The disinfection tests were performed with a NaOCl concentration of 5000 ppm, which is the recommended concentration for surface disinfection to be deployed during CDI outbreaks (Barra-Carrasco & Paredes-Sabja, 2014). However, such high concentration carries several risks, including skin and mucosa irritation, corrosion of metallic surfaces, and damage of sensitive equipment. Our ECAS contained a lower NaOCl concentration than bleach, turning them into an attractive alternative for control of *C. difficile* spores on surfaces.

In agreement with a previous report (Helme et al., 2010), the pH of the ECAS ranged between 9.6 and 10.3. This alkaline pH can be attributed to the configuration of the open electrochemical cell used for the synthesis, as it does not prevent the flux of ions between the anolyte and the catholyte. In such non-balanced cells, pH increases because water electrolysis is a non-balanced process and also due to the release of chlorine during electrolysis, as shown in Eq. 1 (Bergmann & Koparal, 2005; Baniasadi, Dincer, & Naterer, 2013).

$$2\ Cl^- + 2\ H_2O \rightarrow H_2 + Cl_2 + 2\ OH^- \quad (1)$$

In this regard, as the NaCl concentration is increased, the current spent on chlorine and hypochlorite generation also increases, at oxygen evolution expenses (El-Ashtoukhy, Amin, & Abdelwahab, 2009). This process may be tuned by using ruthenium oxide- (RuO_2) or titanium oxide- (TiO_2) as catalysts during the electrolysis process (Ibl & Landolt, 1967; M. D. Spasojević, Trišović, Ribić-Zelenović, & Spasojević, 2013).

Figure 1 shows the electrochemical behavior measured for each of the solutions. Most of the oxidation and reduction processes in the NaCl reaction (Fig. 1A) were observed between -2 and 2 V. Theoretically, this behavior is associated with the production of chlorine (Cl_2), hypochlorite (ClO^-), chlorite (ClO_2), chlorate (ClO_3^-), and perchlorate (ClO_4^-) [Eq. 8–10] in both the anode and the cathode as shown below (Ibl & Landolt, 1967; Krstajić, Nakić, & Spasojević, 1991; Wang & Margerum, 1994) :

On the anode: $2\ Cl^- \rightarrow Cl_2 + 2\ e$ (1.36 V) (2)

On the cathode: $2\ H_2O + 2\ e \rightarrow 2\ OH^- + 2\ H^+$ (-0.83 V) (3)

In the bulk solution: $Cl_2 + H_2O \rightarrow HClO + Cl^- + H^+$ (4)

$HClO \rightarrow H^+ + ClO^-$ (5)

The chlorine generated at the anode (Eq. 2) diffuses throughout the bulk solution and subsequently undergoes hydrolysis reactions that lead to the production of hypochlorous acid (HOCl), chloride ions, and protons (Eq. 4) (Krstajić et al., 1991; M. Spasojević et al., 2015). If the bulk solution pH is neutral or alkaline, HOCl dissociates into its ions (Eq. 5), which hold disinfectant properties (Szpyrkowicz, Cherbanski, & Kelsall, 2005; Cheng & Kelsall, 2007;). On the other hand, the electrochemical behavior of NaOCl (Fig. 1B) and the synthesized ECAS (Fig. 3C) was rather similar between -2 and 2 V, as expected for solutions enriched in HOCl (Robinson et al., 2012). Despite this similarity, the ECAS cyclic voltammogram showed some unique oxidation/reduction processes that can be attributed to the generation of products other than HOCl during the electrolysis (Cai, 2005; Chinello, Hashemi, Psaltis, & Moser, 2019).

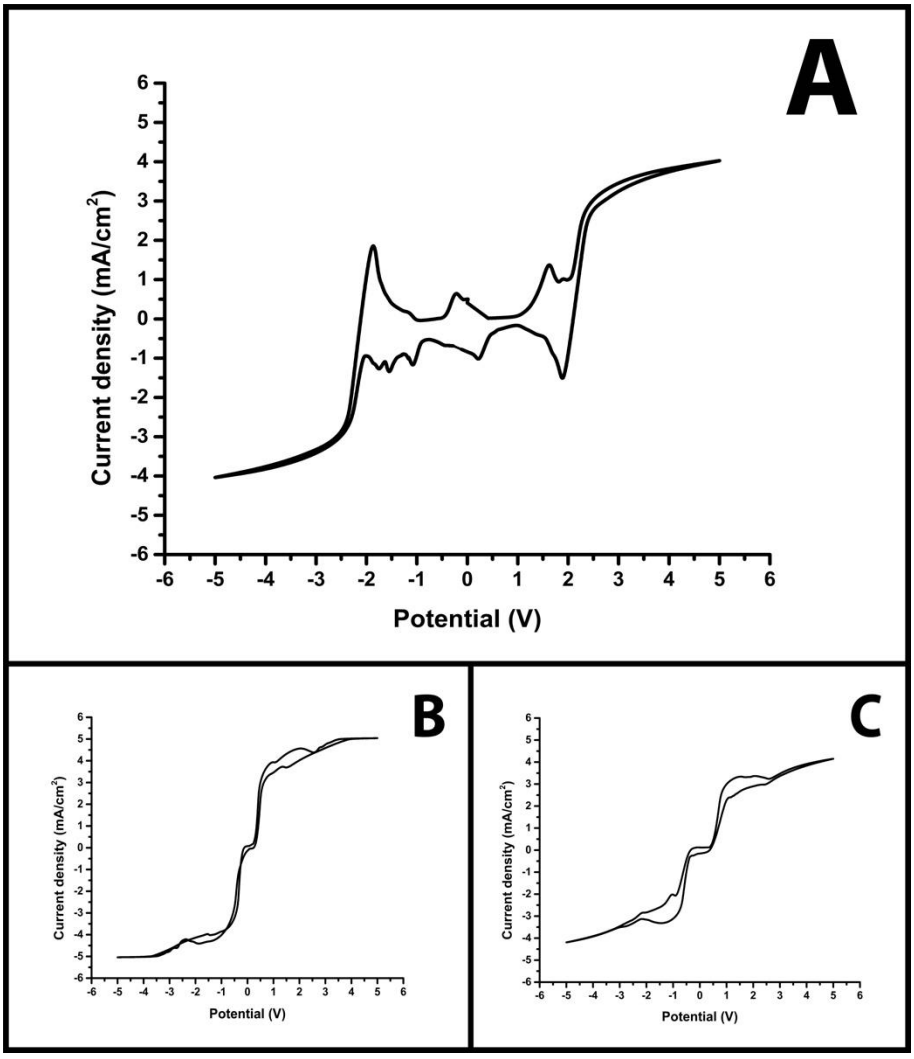


Figure 1. Cyclic voltammograms obtained for (A) sodium chloride (NaCl), (B) sodium hypochlorite (NaOCl), and (C) electrochemically activated solutions (ECAS). Measurements obtained with platinum electrodes and no reference electrode.

The open circuit potential (OCP) or zero-current potential is defined as the potential at which no appreciable current flows through the electrochemical cell because an equilibrium has been established (Bard, Faulkner, Leddy, & Zoski, 1980). The OCP of the three solutions were measured to obtain further information regarding their electrochemical behavior. The NaOCl (540 mV) and ECAS (500 mV) solutions showed a positive potential, similar to OCP previously reported for a hypochlorite/hypochlorous solution (Ordeig et al., 2005). Instead, the OCP of the NaCl system (-190 mV) had a tendency towards negative

values, but as the cyclic voltammetry was performed, it varied from -190 mV to 440/540 mV. No OCP change was recorded despite extensive cleaning of the electrodes and agitation of the solution to avoid any residual charge in the electrode surface. This result indicates that ECAS activation likely occurs at the beginning of the process.

Sporicidal activity tests and statistical analysis

We noted significant differences among the logarithmic reductions of the ten strains ($F_{9,40}$=16.42, p<0.0001), the four treatments ($F_{3,40}$=76.09, p<0.0001), and the interaction of strain and treatment ($F_{27,40}$=4.43, p<0.0001).

The highest overall logarithmic reduction was achieved with ECAS (3.22 $\log_{10}$ reduction, 95% CI, 2.00-4.43), followed by NaOCl (2.74 $\log_{10}$ reduction, 95% CI, 1.62-3.86) and NaDCC (2.02 $\log_{10}$ reduction, 95% CI, 0.91-3.12) (Figure 2). ECAS and NaOCl performed statistically equally (q_{40}=3.52); therefore ECAS could be used as an alternative to NaOCl on the disinfection of surfaces contaminated with *C. difficile* spores in healthcare facilities.

It was possible to distribute the strains in three different groups depending on their susceptibility levels to the disinfectants tested (Table 3 and Supplementary Figure 1). The first group included strains LIBA-7854 (4.23 $\log_{10}$ reduction, 95% CI=2.73-5.70) and LIBA-6507 (4.23 $\log_{10}$ reduction, 95% CI=2.76-5.69), which exhibited the higher overall susceptibility. The second group was characterized by moderately susceptible strains, such as 630 (2.91 $\log_{10}$ reduction, 95% CI=1.17-4.68), M68 (2.62 $\log_{10}$ reduction, 95% CI 1.14-4.10), M120 (2.80 $\log_{10}$ reduction, 95% CI 2.31-3.29), LIBA-7110 (2.30 $\log_{10}$ reduction, 95% CI 1.54-3.06), LIBA-6276 (3.020 $\log_{10}$ reduction, 95% CI 1.71-4.33), and LIBA-5758 (3.89 $\log_{10}$ reduction, 95% CI 1.83-5.96). Finally, the least susceptible strains were LIBA-5757 (0.33 $\log_{10}$ reduction, 95% CI 0.18-0.50) and LIBA-7719 (0.36 $\log_{10}$ reduction, 95% CI 0.17-0.55).

Table 3. Susceptibility of *C. difficile* strains to sodium hypochlorite (NaOCl), sodium dichloroisocyanurate (NaDCC) and electrochemically activated solutions (ECAS) as determined by a carrier test disinfection method.

Strain	MLST Clade	Log_{10} CFU reduction (95% confidence intervals)		
		NaOCl	NaDCC	ECAS
630[a]	1	3.50 (2.23-4.77)	1.15 (0.88-3.18)	4.08 (3.95-4.21)
LIBA 6276[a]		4.360 (4.11-4.61)	2.03 (1.90-2.16)	2.67 (1.53-3.81)
LIBA 5757[b]	2	0.41 (0.03-0.79)	0.16 (0.05-0.85)	0.42 (0.28-0.54)
LIBA 5758[ab]		4.56 (4.31-4.81)	1.56 (1.43-1.69)	5.56 (4.72-6.49)
LIBA 7110[a]	3	2.33 (0.97-5.63)	1.49 (1.11-1.87)	3.07 (2.94-3.20)
LIBA 6507[c]		4.12 (3.87-4.37)	5.12 (4.86-5.36)	3.11 (2.87-3.37)
M68[a]	4	1.85 (0.77-2.93)	1.78 (0.13-3.67)	4.24 (3.99-4.49)
LIBA 7719[b]		0.18 (0.06-0.87)	0.34 (0.27-0.40)	0.57 (0.32-0.82)
M120[a]	5	2.49 (1.01-5.18)	2.56 (1.29-3.83)	3.36 (3.11-3.61)
LIBA 7854[c]		3.60 (2.33-4.87)	3.97 (3.84-4.10)	5.12 (4.87-5.38)

*Different letters indicate statistical differences between strains

Patients with CDI may release up to $1x10^7$ spores per gram of stool (Smits, Lyras, Lacy, Wilcox, & Kuijper, 2016). Consequently, the level of contamination of surfaces in healthcare centers may reach up to 1 to 3 log_{10} CFU depending on the quantification method (Barbut, 2015). It is widely accepted that an effective disinfection method for *C. difficile* spores must achieve a > 5 log_{10} reduction in a minimum of 5 minutes (Fraise et al., 2015). Therefore, none of the disinfectants tested here can be considered appropriate for all strains. However, this interpretation might be inaccurate, as most results in the literature were obtained with spore suspensions rather than with artificially contaminated surfaces.

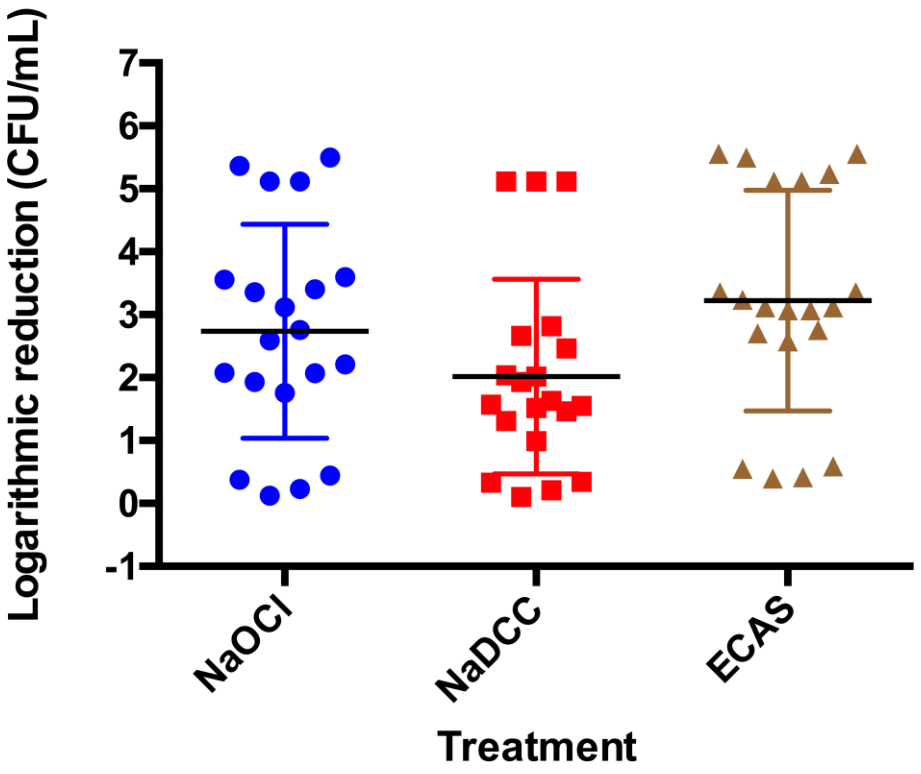


Figure 2. Logarithmic reduction of *C. difficile* spores caused by sodium hypochlorite (NaOCl, blue points), sodium dichloroisocyanurate (NaDCC, red boxes), and electro-chemically activated solutions (ECAS, brown triangles). Black lines represent the mean of the treatment.

Surface response plots were used to identify ECAS activation and exposure times that optimize the logarithmic reduction of spores from strains LIBA-5757 (highly tolerant) and LIBA-5758 (highly susceptible). Regardless of the selected activation time, 15 minutes of exposure time were sufficient to maximize the logarithmic reduction observed for LIBA-5757 (5.170 $\log_{10}$ reduction, Fig 3A). This time period does not deviate much from the minimum contact time recommended for disinfection of hospital surfaces with NaOCl, which is 10 minutes (Dubberke et al., 2008). The logarithmic reduction obtained for the susceptible strain LIBA-5758 with an exposure time of 5 minutes was maximal only when the activation time was 30 minutes (Fig. 3B). However, when the ECAS exposure time

exceeded 10 minutes, activation time seemed not to be a determining factor because all factor combinations reached the maximum disinfection value (5.490 $\log_{10}$ reduction).

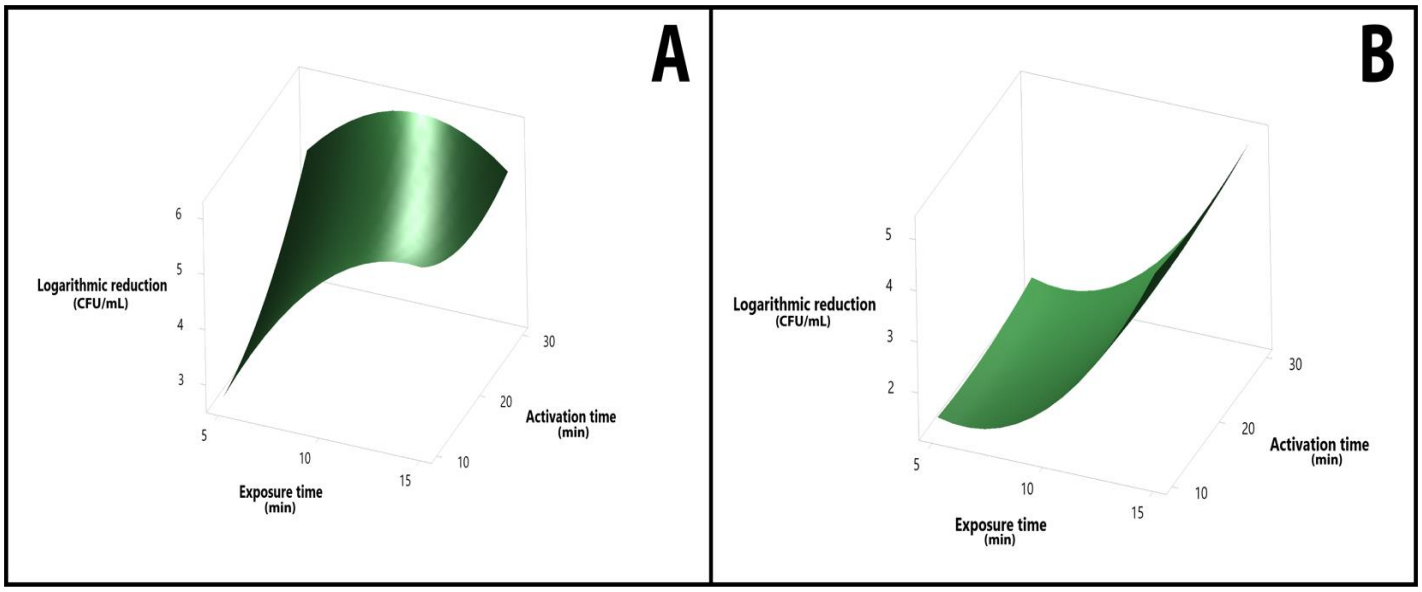
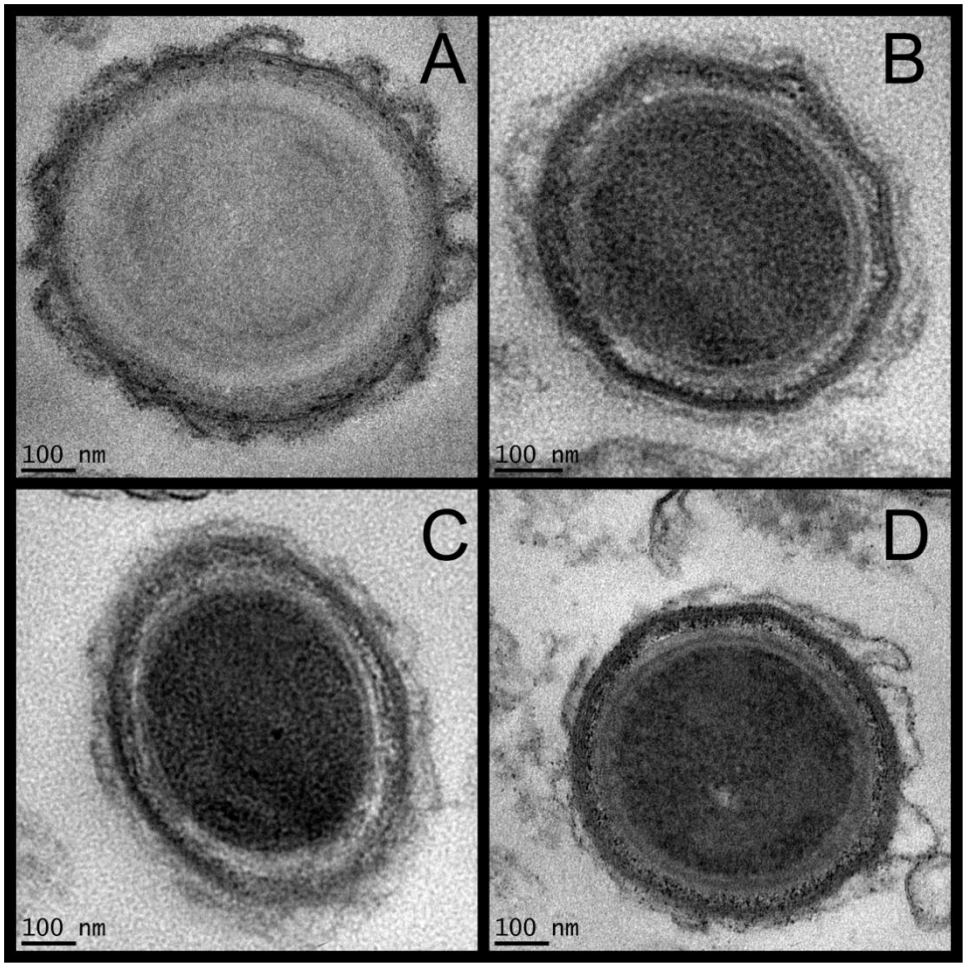


Figure 3. Logarithmic spore reduction as function of ECAS exposure and activation times. (A) LIBA-5757 (Low ECAS susceptibility) (B) LIBA-5758 (High ECAS susceptibility).

Electron microscopy

Our TEM analyses revealed only minor differences in the structure of spores exposed to the treatments. As opposed to the control sample (Fig. 5A), in which the core of the spore, the membranes, the cell wall, and the outer layers were conserved, spores exposed to ECAS (Fig. 5B) NaOCl (Fig. 5C) and NaDCC (Fig. 5D) showed a greater electron density in the core, as well as a ripple in their outermost layers. None of the treated samples showed physical ruptures in the inner and the outer layers. We therefore conclude that the ECAS likely cause functional damages previous to spore germination. According to Loshon et al.



(2001), this damage could be related to protein and fatty acid oxidation leading to permeabilization of the spore membrane and therefore loss of germinative capacity.

Figure 4. TEM micrographs of *C. difficile* LIBA-5758 exposed to ECAS (B), NaOCl (C), and NaDCC (D). An unexposed control is shown in (A).

Conclusions

Our NaCl-derived ECAS showed sporicidal activity against *C. difficile* spores, however most of the strains did not reached the required 5 $\log_{10}$ reduction required by EPA. Our ECAS performed equally than NaOCl in disinfection tests, yet the former is accessible, more environmentally friendly, and safer for its application in healthcare facilities. Considering the TEM qualitative results we suggest that the action mechanism of ECAS may be associated to functional damage rather than physical rupture of the spores. Our results

also provide novel insights in the electrochemical characterization of ECAS. In this regard, the electrochemical behavior of the ECAS solutions seems to be responsible for its increased disinfection potential. Finally, ECAS exposure time and activation time must be optimized prior to their field application, as our results suggest a high impact of those parameters on the logarithmic reduction rates of *C. difficile* on contaminated surfaces.

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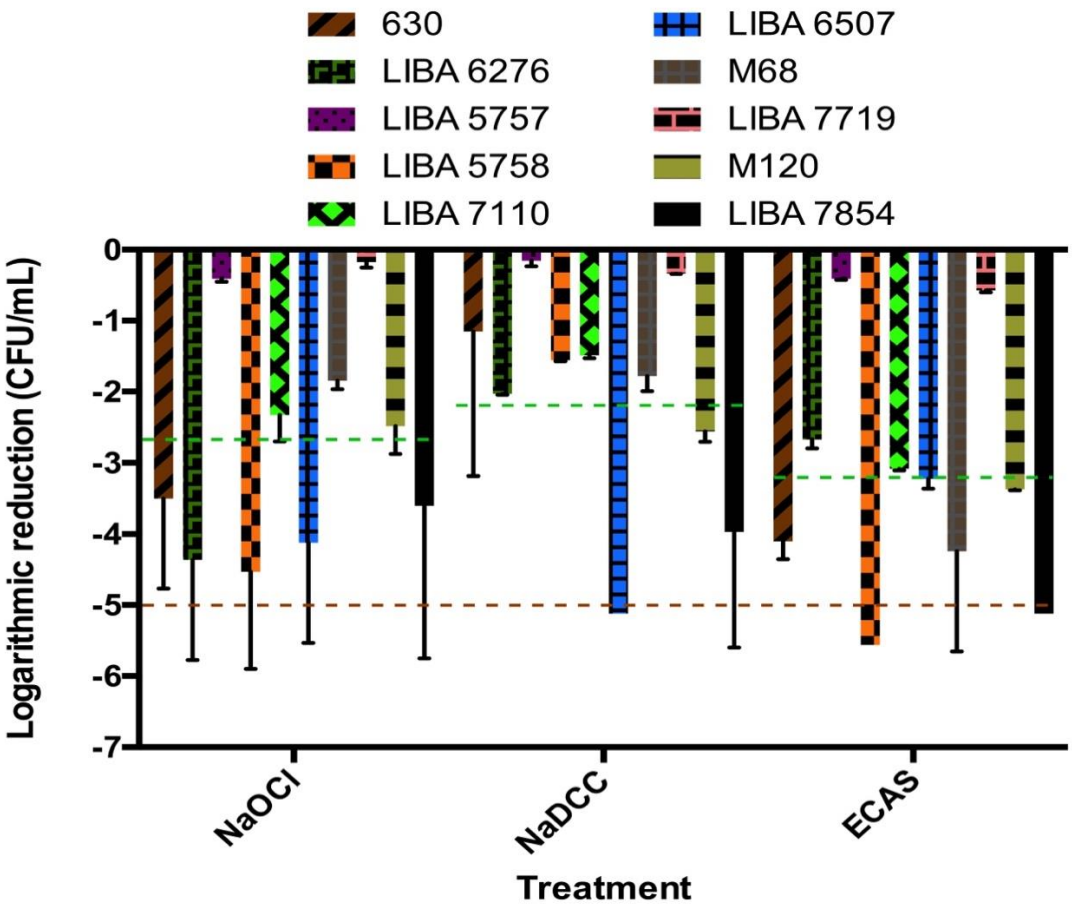
APÉNDICES



Supplementary Figure 1. Mean logarithmic reduction of *C. difficile* spores from different strains caused by sodium hypochlorite (NaOCl), sodium dichloroisocyanurate (NaDCC), and electrochemically activated solutions (ECAS). Bars represent means with standard deviation. (Green dotted line: mean for each treatment, red dotted line: 5 $\log_{10}$ reduction).