International Journal of Food Microbiology 277 (2018) 26–33

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Inactivation of *Clostridium perfringens* spores adhered onto stainless steel surface by agents used in a clean-in-place procedure

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ARTICLE INFO

Keywords:
Clostridium perfringens
Spore
Inactivation
Food contact surface
Hydrophobicity

ABSTRACT

Enterotoxigenic *Clostridium perfringens,* a leading foodborne pathogen can be cross-contaminated from food processing stainless steel (SS) surfaces to the finished food products. This is mostly due to the high resistance of *C. perfringens* spores adhered onto SS surfaces to various disinfectants commonly used in food industries. In this study, we aimed to investigate the survivability and adherence of *C. perfringens* spores onto SS surfaces and then validate the effectiveness of a simulated Clean-in-Place (CIP) regime on inactivation of spores adhered onto SS surfaces. Our results demonstrated that, 1) *C. perfringens* spores adhered firmly onto SS surfaces and survived for at-least 48 h, unlike their vegetative cells who died within 30 min, after aerobic incubation at refrigerated and ambient temperatures; 2) Spores exhibited higher levels of hydrophobicity than vegetative cells, suggesting a correlation between cell surface hydrophobicity and adhesion to solid surfaces; 3) Intact spores were more hydrophobic than the decoated spores, suggesting a positive role of spore coat components on spores' hydrophobicity and thus adhesion onto SS surfaces; and finally 4) The CIP regime (NaOH + HNO_3) successfully inactivated *C. perfringens* spores adhered onto SS surfaces, and most of the effect of CIP regime appeared to be due to the NaOH. Collectively, our current findings may well contribute towards developing a strategy to control cross-contamination of *C. perfringens* spores into food products, which should help reducing the risk of *C. perfringens*-associated food poisoning outbreaks.

1. Introduction

Clostridium perfringens is a Gram-positive, anaerobic, rod-shaped bacterium causing a wide variety of gastrointestinal (GI) diseases in humans and animals (McClane et al., 2013). *C. perfringens* can be classified into five toxino-types (A through E) based on the production of four major toxins (α, β, ε, and ι toxins) (McClane et al., 2013; Petit et al., 1999). However, approximately 5% of *C. perfringens* type A strains are able to produce *C. perfringens* enterotoxin (CPE), which is an important virulence factor for most cases of *C. perfringens* type A food poisoning (FP) and non-food-borne (NFB) GI diseases (Sarker et al., 1999). CPE-encoding gene (*cpe*) can be either chromosomal- or a plasmid- borne. The chromosomal *cpe* isolates (C-*cpe*) are generally associated with FP, whereas the plasmid-borne *cpe* isolates (P-*cpe*) are associated with NFB GI diseases (Collie and McClane, 1998; Sarker et al., 2000). However, some studies suggested that P-*cpe* isolates also can be a causative agent for FP (Grant et al., 2008; Lahti et al., 2008; Lindstrom et al., 2011). Spores of *C. perfringens* type A isolates exhibit a high resistance to various treatments such as heat, osmotic stress, chemicals, prolonged frozen storage, and high pressure processing (Li and McClane, 2006a, 2006b; Paredes-Sabja et al., 2007; Paredes-Sabja et al., 2008a; Raju et al., 2006; Sarker et al., 2000). These resistance properties allow spores to survive against various preservative approaches applied in the food industry and resume growth via germination once the favorable conditions are achieved (McClane et al., 2013; Paredes-Sabja et al., 2008b).

Adherence of microorganisms onto SS surfaces in food manufacturing plants could act as a source of product contamination, eventually leading to the occurrence of foodborne disease outbreaks. It was estimated that approximately 15% of reported cases of *C. perfringens* FP resulted from cross-contamination of *C. perfringens* spores adhered onto SS surfaces into food products (McClane et al., 2013). Bacterial adherence to surfaces has been related to cell surface hydrophobicity and relative surface charge, as well as the presence of particular cell surface structures (Faille et al., 2002; Faille et al., 2007; Peng et al., 2001; van Loosdrecht et al., 1987; Wiencek et al., 1990). Some reports suggest

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https://doi.org/10.1016/j.ijfoodmicro.2018.04.016
Received 16 November 2017; Received in revised form 27 February 2018; Accepted 10 April 2018
Available online 12 April 2018
0168-1605/

that the presence of spore structures such as the outer coat or exosporium in *Bacillus cereus* group and *Clostridium difficile* can further enhance the adhesion process to solid surfaces (Faille et al., 2007; Joshi et al., 2012; Tauveron et al., 2006). The adherence of *Bacillus* spores and *Staphylococcus epidermidis* to solid surfaces is correlated with hydrophobicity and cell-surface negative charges (Gilbert et al., 1991; Koshikawa et al., 1989; Ronner et al., 1990). Moreover, the spores of some *Clostridium* species are highly hydrophobic and have the ability to adhere firmly on surfaces encountered in manufacturing plants (Craven and Blankenship, 1987; Wiencek et al., 1990). These spores' characteristics can lead to the cross-contamination of pathogenic bacteria from contaminated food contact surfaces into finished products during food processing and handling (André et al., 2012; Kusumaningrum et al., 2003). Adhered pathogenic bacteria on surfaces are more resistant than planktonic cells to various disinfectants used in the food industry and could serve as a continuous source of product contamination affecting their quality, shelf-life, and safety of the consumer (Andrade et al., 1998; André et al., 2012; Frank and Koffi, 1990; Kreske et al., 2006; LeChevallier et al., 1988). A recent study on *C. perfringens* type A demonstrated that commonly used disinfecting agents showed limited inhibitory effect against *C. perfringens* spores adhered onto SS surfaces (Udompijitkul et al., 2013).

Food processing industries successfully use clean-in-place (CIP) procedure to clean and disinfect the surface of large and fixed equipment without disassembling. The general CIP regime involves cleaning with alkaline solution (NaOH) followed by acid solution (HNO_3) in order to control bacterial contamination and remove organic and inorganic residues (Bremer et al., 2006; Stanga, 2010). However, the effectiveness of CIP regime depends on a number of factors, i.e., the concentration of cleaning solutions, treatment duration, temperature of the solutions, the characteristics of the surface being cleaned, and the bacterial species (Boulange-Petermann et al., 2004; Dufour et al., 2004; Lelievre et al., 2001; Stewart et al., 1996). Although the effectiveness of CIP regime had been evaluated against *Bacillus* spores adhered onto SS surfaces (Blel et al., 2007; Lelievre et al., 2001), no study on the application of CIP regime against *C. perfringens* spores adhered onto SS surfaces is available. Consequently, our current study demonstrated that the spores of *C. perfringens* clinical isolates survived and adhered firmly onto SS surfaces and these adhered spores could be eliminated from SS surfaces by CIP regime.

2. Material and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study included four C-*cpe* isolates (SM101, E13, NCTC10239, and NCTC8239) and two P-*cpe* isolates (F4969 and NB16) (Sarker et al., 2000; Collie and McClane, 1998). All isolates were maintained at −20 °C in a cooked meat medium (Difco, BD Diagnostic Systems, Sparks, MD, USA). Each strain was revived by inoculating 0.1 ml of cooked meat culture into 10 ml fluid thioglycollate medium (FTG) (Difco), and incubating at 37 °C overnight. Vegetative cell cultures of *C. perfringens* were grown in TGY (3% trypticase, 2% glucose, 1% yeast extract, and 0.1% L-cysteine) broth (Kokai-Kun et al., 1994).

2.2. Spore preparation and purification

Sporulating cultures of *C. perfringens* were prepared by using the previously described method (Akhtar et al., 2008; Paredes-Sabja et al., 2008b). Briefly, 0.1 ml of *C. perfringens* stock cultures were inoculated into 10 ml FTG and grown overnight at 37 °C. Then 0.4 ml of the FTG cultures were transferred to a fresh 10 ml FTG medium and incubated for 8 to 12 h at 37 °C. Later 0.4 ml of actively growing cultures were then inoculated into 10 ml of freshly-prepared Duncan Strong (DS) sporulation medium (Duncan and Strong, 1968) and incubated for 24 h at 37 °C. Spore formation in DS culture was confirmed by phase-contrast microscopy (Leica DMLS, Leica microsystems, Wetzlar, Germany). The preparation of large amounts of *C. perfringens* spores was accomplished by scaling up the aforementioned procedure. Spores were purified by repeated washing with sterile distilled water through centrifuging (8000 rpm, 15 min) at 4 °C until spore suspension was > 99% free of vegetative cells, cell debris, and germinated spores as confirmed by phase-contrast microscope. The purified free spores were suspended in sterile distilled water and adjusted to a final optical density at 600 nm (OD_{600}) of ~6 using Smartspec™ 3000 Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) and stored at −20 °C until used (Paredes-Sabja et al., 2008b).

2.3. SS surface preparation and adhesion of spores onto SS chips

SS chips (300 series, no. 4 finish) were purchased and prepared as 2 × 3 in. size from The Home Depot (Corvallis, OR, USA) for adhesion and survival experiments. Prior to use, the surface of each SS chip was cleaned with 1% (*w/V*) Alconox® (VWR International, West Chester, PA, USA) followed by rinsing with distilled water, drying, and then wrapping individually with aluminum foil. The SS chips were sterilized by autoclaving for 20 min at 121 °C and stored at room temperature (RT) until used (Udompijitkul et al., 2013). Purified dormant spore or vegetative cell suspensions of *C. perfringens* at OD_{600} of ~6 were prepared in a total volume of 0.1 ml. Spore suspensions were heat-activated for 10 min at 80 °C or 75 °C for C-*cpe* and P-*cpe* spores, respectively, and then cooled in a water bath at room temperature for 5 min. Our previous study demonstrated that spores of C-*cpe* and P-*cpe* isolates germinated better upon heat activation at 80 °C or 75 °C, respectively (Paredes-Sabja et al., 2008b). Spore- or cell-adhesion to SS chips were assessed by inoculating 0.1 ml of heat-activated spores or vegetative cells onto sterilized SS chips and spread with a sterile bent glass rod (Udompijitkul et al., 2013). All chips were contaminated under Class II biosafety cabinet (Labconco, Kansas, MO, USA) and dried for 1 h to promote adherence. For every trial of adhesion experiment, two chips were included. After drying, SS chips were placed in sterile plastic bags and stored under aerobic conditions at both RT (20 ± 2 °C) and refrigeration temperature (4 ± 1 °C).

The number of total viable cells and non-germinated spores was determined after 0, 1, 3, 6, 10, 24 or 48 h storage at both temperatures. The contaminated chips were aseptically transferred to sterile petri dishes and the surface of every individual chip was entirely dried by swabbing with 4 sterile cotton swabs (Puritan Medical Products Company LLC, Guilford, ME, USA) and soaked in 10 ml of 25 mM Na_2HPO_4 (pH 7.5) prior to vigorously mixed with a vortex mixer (Vortex Genie2, Model G-560, Scientific Industries Inc., NY, USA) for 1 min (Ortega et al., 2010). One ml-aliquot of the spore suspension was then subjected to heat shock at 75 °C for 20 min in order to enumerate population of remaining non-germinated dormant spores and another 1 ml without heat shock to determine the population of total count of dormant spores and germinated spores. The number of viable *C. perfringens* cells was assessed by serially diluting aliquots from swabs, plating onto Brain Heart Infusion (BHI) agar (Difco), and counting colonies after 24-h anaerobic incubation at 37 °C.

2.4. Scanning electron microscopy

Contaminated SS chips were prepared as described in Section 2.3. The chips were sputter coated with gold palladium at a 10–15 nm thickness and imaged on a Quanta Dual Beam Scanning Electron Microscope (FEI Co.) at the Oregon State University Electron Microscopy Facility (Corvallis, OR, USA).

2.5. Attachment of spores onto SS surfaces

A set of chips were artificially contaminated as described in Section