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Inactivation of *Clostridium difficile* spore outgrowth by synergistic effects of nisin and lysozyme

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Title: Inactivation of *Clostridium difficile* spore outgrowth by synergistic effects of nisin and lysozyme

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Abstract

Inactivating *Clostridium difficile* spores is difficult, as they are resistant to heat, chemicals, and antimicrobials. However, this note describes inactivation of *C. difficile* spore outgrowth by incubation in a solution containing a germinant (1% w/v sodium taurocholate), co-germinants (1% w/v tryptose and 1% w/v NaCl), and natural antimicrobials (20 nM nisin and 0.2 mM lysozyme). *C. difficile* spores were resistant to nisin and lysozyme but became susceptible during germination and outgrowth triggered and promoted by sodium taurocholate, tryptose, and NaCl. The degree of inactivation of germinated and outgrowing *C. difficile* spores by both nisin and lysozyme was greater than the sum of that by nisin and lysozyme individually, suggesting synergistic inactivation of *C. difficile* spores. The germinant, co-germinants, and natural antimicrobials used in this study are safe for human contact and consumption. Therefore, these findings will facilitate the development of a safe and effective method to inactivate *C. difficile* spore.

Keywords: *Clostridium difficile*, lysozyme, nisin, spore, synergistic inactivation

Clostridium difficile, a Gram-positive and spore-forming obligate anaerobe, is the causative agent of *C. difficile*-associated colitis (CDAC), which manifests as clinical symptoms ranging from asymptomatic intestinal colonization to diarrhea, colitis, pseudomembranous colitis, and death (Ghose 2013). *C. difficile* is acquired nosocomially, and the organism is resistant to multiple antibiotics (Carroll and Bartlett 2011). *C. difficile* enters a dormant spore state in environments unfavorable for survival. *C. difficile* spores are commonly found on bed commodes and stainless steel surfaces previously occupied by *C. difficile*-infected patients (Weber et al. 2010). Transmission of *C. difficile* is generally mediated via contact with surfaces in healthcare facilities (including rooms, beds, bed commodes, rectal thermometers, and medical devices) used by *C. difficile*-infected patients, particularly in hospitals (Weber et al. 2010). Thus, removing *C. difficile* spores on contaminated surfaces is crucial for preventing transmission of *C. difficile* (Weber et al. 2010).

Nisin, a peptide antibiotic with activity against Gram-positive bacteria, effectively inhibits *C. difficile*; indeed, its *in-vitro* activity against *C. difficile* in the vegetative form is eightfold greater than those of vancomycin and metronidazole, primary antibiotics used for the treatment of CDAC (Bartoloni et al. 2004). Nisin is a candidate agent for the treatment of CDAC (Ali et al. 2013). A synergistic effect of the nisin and lysozyme combination on the inhibition of vegetative *C. difficile* has been reported (Chai et al. 2015). However, inactivating *C. difficile* spores is difficult because of their thick polymer cortex and outer proteinaceous coat, which render the spores resistant to heat, chemicals, and antimicrobials (Permpoonpattana et al. 2011).

C. difficile spores in the intestines germinate and outgrow to vegetative cells in response to germination triggers (germinants), primarily bile salts and specific amino acids, particularly if the gut microbiome is disrupted by administration of antibiotics (Poutanen and Simor 2004;

Theriot and Young 2015; Wilson 1983). *C. difficile* spores can be induced to germinate and outgrow *in-vitro* by sodium taurocholate and glycine or histidine (Ramirez et al. 2010; Sorg and Sonenshein 2008). *C. difficile* spores during germination and outgrowth are susceptible to ultraviolet (UV) irradiation and nisin (Nerandzic and Donskey 2013). Induction of spore germination and outgrowth and inactivation of the spores using non-toxic substances may be a practical strategy for the development of a liquid surface sanitizer to reduce the *C. difficile* spore burden in healthcare facilities and on the skin of healthcare workers.

This note describes the induction of germination and outgrowth of *C. difficile* spore as well as effective inactivation of the spore using substances that are safe for human contact and consumption. Germination of *C. difficile* spores is triggered by sodium taurocholate and promoted by specific amino acids (Nerandzic and Donskey 2013; Ramirez et al. 2010; Sorg and Sonenshein 2008). However, purified amino acids were used in previous investigations of *C. difficile* spore germination. The high cost of purified amino acids has hampered the development of a method to inactivate *C. difficile* spores that can be applied in healthcare facilities. We evaluated the ability of protein hydrolysates (peptone, yeast extract, tryptone, and tryptose) to promote spore germination and outgrowth. We also investigated the effects of NaCl concentration on the germination and outgrowth of *C. difficile* spores triggered by sodium taurocholate, as it has been reported that osmotic stress affects the germination of bacterial spores, including those of *Bacillus* and *Clostridium* species (Nerandzic and Donskey 2013; Preston and Douthit 1984). The findings of the aforementioned investigations were applied to formulate a solution that would induce germination and outgrowth of *C. difficile* spores (GO solution). Inactivation of *C. difficile* spore outgrowth by nisin and lysozyme was investigated by enumerating *C. difficile* spores surviving in GO solution containing nisin and lysozyme.

C. difficile ATCC 9689 (PCR ribotype 001) was purchased from the Korean Collection for Type Cultures and cultured overnight in brain–heart infusion broths (BHI broth; OXOID) at 37°C under anaerobic conditions (80% N₂, 10% H₂, and 10% CO₂) in an anaerobic chamber (Don Whitley Scientific). For sporulation, 1 mL of freshly prepared *C. difficile* ATCC 9689 culture was added to each of 6 BHI agar plates and incubated for 4 days at 37°C under anaerobic conditions. *C. difficile* spores were collected by flooding the plates with 10 mL of ice-cold distilled water (DW). *C. difficile* spores collected in 15 mL centrifuge tubes were stored overnight at 4°C. The spore suspension was centrifuged at 4000 × g, supernatant was discarded, and spore pellets were resuspended in 10 mL of ice-cold DW. This was repeated three times, and then the spore suspension was stored at 4°C. Media used to culture *C. difficile* and prepare *C. difficile* spores were maintained under anaerobic conditions at least overnight. The spore suspension was heated at 60°C for 20 min to inactivate vegetative *C. difficile* prior to use.

Solutions composed of 1% w/v sodium taurocholate (Sigma-Aldrich) with various amendments were prepared in DW and then inoculated with *C. difficile* spores. These amendments were: 1) 1% w/v protein hydrolysate [peptone (Bacteriological Peptone L37; Oxoid), yeast extract (Yeast Extract L21; Oxoid), tryptone (Tryptone L42; Oxoid) or tryptose (Tryptose L47)]; 2) 0.2, 0.4, 0.6, 0.8, or 1.0% w/v tryptose; 3) 0.1% w/v tryptose as well as 0.25, 0.5, 1, 5, 10, or 15% w/v NaCl; and 4) 0.2, 0.4, 0.6, 0.8, or 1.0% w/v tryptose along with 1% w/v NaCl; see Fig. 1A, B, C, and D, respectively. Twenty milliliter of the solution inoculated with *C. difficile* spores was placed in a 50-mL beaker, and incubated for 2 h under an aerobic atmosphere at room temperature to explore the possibility of enhancing germination and outgrowth of *C. difficile* spores under ambient environmental conditions. After incubation for 2 h, 1 mL of the solution was transferred to a 1.5 mL microtube and centrifuged at 10000 × g for 2 min to collect

spores. Collected spores were resuspended in 1 mL DW, heated at 60°C for 20 min, diluted with 0.1% peptone water, and plated on TBHI agar [BHI agar containing 5 mg mL⁻¹ yeast extract (Oxoid), 0.1% L-cysteine (Sigma-Aldrich), and 0.1% sodium taurocholate (Sigma-Aldrich)]. The TBHI agar plates were incubated for 24 h at 37°C under anaerobic conditions, and then *C. difficile* spores were counted (N). The logarithmic difference between N at 0 min (N_0) and at 2 h (N_{2h}) was regarded as the level of germinated and outgrowing *C. difficile* spores (LO ; $LO = \log N_0 - \log N_{2h}$). Because the logarithmic value of N_0 varied from 4 to 5 log CFU mL⁻¹, the LO was divided by the logarithmic value of N_0 , and the relative level of LO (RLO ; $RLO = LO / \log N_0$) was used to evaluate the effects of protein hydrolysates on the germination and outgrowth of *C. difficile* spores and to formulate the GO solution.

Inactivation of *C. difficile* spore outgrowth by nisin and lysozyme was investigated by enumerating surviving *C. difficile* spores during incubation in GO solution containing nisin and lysozyme. Solutions composed of 1% w/v sodium taurocholate, 1% tryptose, and 1% NaCl with various amendments were prepared in DW. These amendments were: 1) 20, 40, 60, 80, or 100 nM nisin (nisin A; Sigma-Aldrich); 2) 0.2, 0.4, 0.6, 0.8, or 1.0 mM lysozyme (Sigma-Aldrich); and 3) 0.2 mM lysozyme, and 5, 10, 15, or 20 nM nisin; see Figs. 1, 2, and 3, respectively. Twenty milliliter of the solution inoculated with *C. difficile* spores was placed in a 50-mL beaker and incubated for 2 h under aerobic conditions at room temperature. During the incubation, 1 mL of the solution was transferred to a 1.5 mL microtube every 30 min and centrifuged at 10000 × g for 2 min. Pelleted *C. difficile* spores were resuspended in 1 mL of DW to wash out nisin and lysozyme and centrifuged at 10000 × g for 2 min; this procedure was repeated twice. Collected *C. difficile* spores were resuspended in 1 mL DW and cultured on TBHI agar plates, and surviving *C. difficile* spores (N^s) were counted after incubating the TBHI agar plates for 24 h at 37°C

under anaerobic conditions. The logarithmic value of N^s in GO solution containing nisin or lysozyme or both nisin and lysozyme at 0 min ($\log N_0^s$) varied from 4 to 5 log CFU mL⁻¹. Hence, the logarithmic value of N^s at a certain time ($\log N_t^s$) was divided by $\log N_0^s$, which represented the relative level of surviving spores (RLS ; $RLS = \log N_t^s / \log N_0^s$). All tests described above were carried out in triplicate, and the results are presented as means and standard deviations. The germination and outgrowth of *C. difficile* spores data were subjected to one-way analysis of variance, and P-values < 0.05 were considered to indicate significance.

Sodium taurocholate alone did not induce germination and outgrowth of *C. difficile* spores; another carbon source, such as protein hydrolysate, was required (Fig. 1A). *C. difficile* spores exposed to sodium taurocholate responded differently to peptone, yeast extract, tryptone, and tryptose (Fig. 1A). A greater number of *C. difficile* spores germinated and outgrew in the presence of 1% w/v sodium taurocholate and 1% w/v tryptose than in the presence of the other protein hydrolysates, as indicated by the greater RLO value (Fig. 1A). According to the manufacturer's product information, peptone and tryptose were derived from enzymatic hydrolysis of meat, tryptone from enzymatic hydrolysis of milk protein, and yeast extract from yeast. Thus the amino acid composition of each protein hydrolysate is different. Tryptose contains abundant glycine and alanine compared to peptone, yeast extract, and tryptone (manufacturer's product information). Alanine and glycine act as co-germinants for *C. difficile* spores and promote *C. difficile* spore germination more effectively than any other amino acids (Howerton et al. 2011). Therefore, the greater RLO values observed in the presence of tryptose relative to other protein hydrolysates might be due to the greater abundance of glycine and alanine in this hydrolysate (Fig. 1A). The RLO value increased as the tryptose concentration increased from 0 to 0.8% w/v, but there was no increment in the RLO value as the tryptose

concentration increased from 0.8 to 1.0% w/v (Fig. 1B). The germination and outgrowth of *C. difficile* spores triggered and promoted by sodium taurocholate and tryptose were stimulated by NaCl (Fig. 1C). The *RLO* value increased with the NaCl concentration from 0 to 1% w/v in the presence of 1% w/v sodium taurocholate and 0.1% w/v tryptose (Fig. 1C). However, the *RLO* values decreased as the NaCl concentration increased from 1 to 15% w/v (Fig. 1C). In the presence of 1% w/v sodium taurocholate and 1% w/v NaCl, *C. difficile* spore germination and outgrowth increased as the tryptose concentration increased from 0 to 0.8% w/v (Fig. 1D). Indeed, all *C. difficile* spores germinated and outgrew in solutions containing 1% w/v sodium taurocholate, 1% w/v NaCl, and 0.8 and 1.0% w/v tryptose, as indicated by an *RLO* value of 1 (Fig. 1D). These *RLO* values (Fig. 1) led to the formulation of a GO solution composed of 1% w/v sodium taurocholate, 1% tryptose, and 1% NaCl in DW.

C. difficile in a vegetative state is susceptible to nisin and lysozyme, the combination of which exerts synergistic effects (Chai et al. 2015). *C. difficile* spores were not inactivated by either nisin or lysozyme if these treatments were used without GO solution (data not shown). However, *C. difficile* spores that were germinated and outgrew in GO solution were susceptible to nisin and lysozyme, which resulted in the decrease in *RLS* values (Fig. 2A and B). Inactivation of *C. difficile* spore outgrowth by nisin and lysozyme was dependent on their concentrations; the *RLS* values decreased with increasing nisin and lysozyme concentrations (Fig. 2A and B). Germinated and outgrowing *C. difficile* spores were likely more sensitive to nisin than to lysozyme, as inactivation occurred at a lower concentration of nisin than of lysozyme. After incubation for 2 h, the *RLS* values of 100 nM nisin and 1 mM lysozyme were 0.12 and 0.13, respectively (Fig. 2A and B). The difference between the dose of nisin and that of lysozyme required to inactivate *C. difficile* spore outgrowth might be due to differences in their antimicrobial mechanisms. Both

nisin and lysozyme exhibit antimicrobial activity, especially toward Gram-positive bacteria, including *Clostridium* spp. (Chai et al. 2015; Chung and Hancock 2000). Nisin is transported across the peptidoglycan layer and incorporated into the cytoplasmic membrane of Gram-positive bacteria, resulting in leakage of cytoplasmic contents (AlKhatib et al. 2014). In contrast, lysozyme hydrolyzes the glycosidic bonds between *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) in the peptidoglycan layer encasing the cytoplasmic membrane of Gram-positive bacteria, resulting in lysis (Kirby 2001).

C. difficile spore outgrowth was synergistically inactivated by the combination of nisin and lysozyme (Fig. 2C). The decreases in the *RLS* values of the 0.2 mM lysozyme and 20 nM nisin treatments after 2 h of incubation were only 0.06 and 0.21, respectively (*RLS* values, 0.94 and 0.79, respectively) (Fig. 2A and B). However, *RLS* values were markedly lower in the presence of both lysozyme and nisin in GO solution than in the presence of only lysozyme and nisin (Fig. 2C). Notably, no viable *C. difficile* spores were detected after 2 h of incubation in GO solution containing both 20 nM nisin and 0.2 mM lysozyme (*RLS* value, 0) (Fig. 2C). The decrease in the *RLS* value after 2 h of incubation of *C. difficile* spores in GO solution containing 20 nM nisin and 0.2 mM lysozyme was greater than the sum of the decrease in the *RLS* values in the presence of 20 nM nisin and 0.2 mM lysozyme alone (Fig. 2C). Lysozyme likely hydrolyzes the NAG-NAM linkages of the peptidoglycan layer of *C. difficile* spores that were germinated and outgrew in GO solution, which possibly facilitated the transport of nisin across the peptidoglycan layer to the cytoplasmic membrane, thus resulting in a synergistic effect on *C. difficile* spore outgrowth. Synergistic antimicrobial effects of the nisin and lysozyme combination on pathogens in food and on food packaging have been reported by others (Bhatia and Bharti 2015; Lucera et al. 2012).

In this study, *C. difficile* spores were inactivated by a solution containing 1% w/v sodium taurocholate, 1% w/v tryptose, 1% w/v NaCl, 20 nM nisin, and 0.2 mM lysozyme, without additional treatment. Because sodium taurocholate, tryptose, NaCl, nisin, and lysozyme have been proven safe for human consumption (United States. Food and Drug Administration 2016), this note suggests the feasibility of developing a safe and effective method of reducing the *C. difficile* spore burden. However, there should follow a study for the inactivation of *C. difficile* spore deposited on actual surfaces of healthcare facilities using the GO solution containing nisin and lysozyme.

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Fig. 1. The relative level of germinated and outgrowing *C. difficile* spores (*RLO*) after 2 h of incubation in solutions containing: (A) 1% w/v sodium taurocholate alone (control), 1% w/v sodium taurocholate and peptone, yeast extract, tryptone, or tryptose (1% w/v); (B) 1% w/v sodium taurocholate and tryptose (0, 0.2, 0.4, 0.6, 0.8, or 1.0% w/v); (C) 1% w/v sodium taurocholate, tryptose (0.1% w/v), and NaCl (0, 0.25, 0.5, 1, 5, 10, or 15% w/v); or (D) 1% w/v sodium taurocholate, tryptose (0, 0.2, 0.4, 0.6, 0.8, or 1.0% w/v), and NaCl (1% w/v). The *RLO* ($RLO = LO / \log N_0$) was calculated using the level of germinated and outgrowing *C. difficile* spores (*LO*; $LO = \log N_0 - \log N_{2h}$) and the numbers of *C. difficile* spores before (N_0) and after 2 h of incubation (N_{2h}). Data represented by the same letter are not significantly different at $P < 0.05$.

Fig. 2. The relative level of surviving *C. difficile* spore (*RLS*) during incubation in GO solution containing: (A) nisin (0, 20, 40, 60, 80, or 100 nM); (B) lysozyme (0, 0.2, 0.4, 0.6, 0.8, or 1.0 mM); and (C) both nisin (0, 5, 10, 15, or 20 nM) and lysozyme (0.2 mM). GO solution was composed of sodium taurocholate (1% w/v), tryptose (1% w/v), and NaCl (1% w/v). The *RLS* ($RLS = \log N_t^s / \log N_0^s$) was calculated using the number of *C. difficile* spores at 0 min (N_0^s) and a certain time (N_t^s).