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Susceptibility of *Clostridium difficile* to the food preservatives sodium nitrite, sodium nitrate and sodium metabisulphite

Su-Chen Lim [a], Niki F. Foster [a, b], Thomas V. Riley [a, b, *]

[a] Microbiology & Immunology, School of Pathology and Laboratory Medicine, The University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands 6009, Western Australia, Australia
[b] Department of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands 6009, Western Australia, Australia

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ABSTRACT

Clostridium difficile is an important enteric pathogen of humans and food animals. Recently it has been isolated from retail foods with prevalences up to 42%, prompting concern that contaminated foods may be one of the reasons for increased community-acquired *C. difficile* infection (CA-CDI). A number of studies have examined the prevalence of *C. difficile* in raw meats and fresh vegetables; however, fewer studies have examined the prevalence of *C. difficile* in ready-to-eat meat. The aim of this study was to investigate the *in vitro* susceptibility of 11 *C. difficile* isolates of food animal and retail food origins to food preservatives commonly used in ready-to-eat meats. The broth microdilution method was used to determine the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) for sodium nitrite, sodium nitrate and sodium metabisulphite against *C. difficile*. Checkerboard assays were used to investigate the combined effect of sodium nitrite and sodium nitrate, commonly used in combination in meats. Modal MIC values for sodium nitrite, sodium nitrate and sodium metabisulphite were 250 μg/ml, >4000 μg/ml and 1000 μg/ml, respectively. No bactericidal activity was observed for all three food preservatives. The checkerboard assays showed indifferent interaction between sodium nitrite and sodium nitrate. This study demonstrated that *C. difficile* can survive in the presence of food preservatives at concentrations higher than the current maximum permitted levels allowed in ready-to-eat meats. The possibility of retail ready-to-eat meats contaminated with *C. difficile* acting as a source of CDI needs to be investigated.

1. Introduction

Clostridium difficile is an anaerobic, spore-forming, Gram-positive bacillus widely found in soil, water and the gastrointestinal tracts of food animals and humans. It causes mild to severe diarrhea and, occasionally, the more serious pseudomembranous colitis and toxic megacolon in humans. Since 2000, there has been a global increase in *C. difficile* infection (CDI) with heightened severity and mortality, and a rise in community-acquired infection (CA-CDI) in individuals without traditional risk factors of old age or antibiotic usage [1–5]. This has been mainly due to the emergence of so-called hypervirulent strains of *C. difficile*, particularly PCR-ribotypes 027 and 078, that produce binary toxin (CDT) in addition to toxins A and B.

Recently, *C. difficile* has been found in retail meats, seafoods and vegetables with prevalences up to 42% [6–12]. *C. difficile* of the same ribotype has been found in foods, food animals and humans [13]. In Canada, Weese et al. (2009) found *C. difficile* ribotype 078, common in food animals and a cause of disease in humans, in ground meats and poultry [14,15]. In Scotland, ready-to-eat salads were contaminated with *C. difficile* ribotypes 017 and 001; both are common clinical isolates in Scotland and Europe [16–18]. These findings have led to growing concern that retail foods contaminated with *C. difficile* may be one of the reasons for the increased incidence of CDI, particularly in the community. Despite the potential for foodborne transmission of *C. difficile*, there are only a small number of studies that have looked at the prevalence of *C. difficile* in foods, most of which focused on raw meats and fresh vegetables. To our knowledge, only two studies have investigated the prevalence of *C. difficile* in raw sausages and only one study has

* Corresponding author. Microbiology & Immunology, School of Pathology and Laboratory Medicine, The University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands 6009, Western Australia, Australia.
E-mail address: thomas.riley@uwa.edu.au (T.V. Riley).

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investigated the prevalence of *C. difficile* in ready-to-eat meats such as cured, fermented, smoked or cooked meat [7,19].

Commercially produced ready-to-eat meats including non-heat treated meats often contain food preservatives to prevent spoilage and extend shelf life. Sodium nitrite (E250) and nitrate (E251) are food preservatives widely used in combination in ready-to-eat meats. Nitrite helps to develop flavor, reacts with myoglobin to produce nitrosylhaemochrome which gives the characteristic pink colour of processed meat and inhibits the growth of spoilage and pathogenic bacteria such as *Clostridium botulinum* [20]. Nitrate acts as a reservoir for nitrite production through the enzyme nitrate reductase produced by microorganisms such as staphylococci and lactobacilli. Sodium metabisulphite (E223) is a food preservative used in raw sausages. The use of food preservatives in foods is strictly regulated for food safety reasons. In Australia and New Zealand, the use of nitrite, nitrate and metabisulphite in meat products is restricted to 125 ppm (mg/kg), 500 ppm and 500 ppm, respectively [21,22]. In the USA, the use of nitrite and nitrate in meat products is not allowed to exceed 200 ppm and 500 ppm, respectively [23], while the European Union set a maximum of 150 ppm nitrate and nitrite added to dry-fermented sausages and 250 ppm nitrate in long-cured products when no nitrite is added [24].

The aim of this study was to investigate the *in vitro* susceptibility of *C. difficile* to three food preservatives commonly use in commercially produced ready-to-eat meats: sodium nitrite (E250), sodium nitrate (E251) and sodium metabisulphite (E223), to determine if commercially produced ready-to-eat meats may pose a risk for CDI. Checkerboard assays were used to investigate the combined effect of sodium nitrite and sodium nitrate.

2. Methods and materials

2.1. Strains and growth conditions

Eleven *C. difficile* isolates were used in this study (Table 1). Isolates were selected to represent a range of sources of *C. difficile* having been isolated from retail foods and food animals. All isolates were routinely cultured on pre-reduced supplemented Brucella agar (Becton, Dickinson and company) and blood agar under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2) at 37 °C in an anaerobic chamber (Don Whitley Scientific) for 24–48 h.

2.2. Food preservatives

The following food preservatives were used in this study: sodium nitrite (Thermo Fisher, Australia), sodium nitrate (BDH, Australia) and sodium metabisulphite (BDH, Australia). All preservative solutions were made fresh before use by dissolving pure substance in sterile distilled water.

2.3. Broth microdilution assay

To determine the susceptibility of *C. difficile* to food preservatives, broth microdilution assays were performed according to the Clinical and Laboratory Standards Institute methodology [25]. Briefly, a series of two-fold dilutions of each preservative with final concentrations ranging from 0 to 4, 000 μg/ml was made in a 96-well microtitre plate in pre-reduced supplemented Brucella broth. Suspensions of test organisms cultured on supplemented Brucella agar were adjusted to 0.5 McFarland in 0.85% saline, and then diluted in supplemented Brucella broth to correspond to a final inoculum concentration of approximately 1.0×10^6 cfu/ml. The final inoculum concentration was confirmed by viable counts. Spores constituted approximately 15% of cells, determined by a Schaffer/Fulton spore stain viewed under a light microscope.

After 24 h incubation, minimum inhibitory concentrations (MICs) were determined visually as the lowest concentration of food preservative resulting in an optically clear microtitre well. Minimum bactericidal concentrations (MBCs) were determined by sub-culturing 10 μl from each well of the microtitre plate at 24 h, spot inoculating onto ChromID agar (bioMérieux) and incubated anaerobically for 24 h at 37 °C. After incubation, colonies were counted and compared to the counts of original inoculum. The MBC was determined as the lowest concentration of food preservative resulting in ≥99.9% death of the inoculum [26]. Purity checks on inoculum suspensions were performed by subculturing an aliquot from the inoculated well that contained the lowest concentration of food preservative after serial dilutions, onto two blood agar plates for simultaneous incubation both aerobically and anaerobically. A vancomycin control was included to ensure testing quality. The MIC values obtained for vancomycin needed to fall within the acceptable range of 0.5–4 μg/ml as indicated by the CLSI guidelines. To ensure the methodology was valid to quantify the inhibitory effect of food preservatives, *Staphylococcus aureus* ATCC 29213 was used as a positive control and testing performed according to CLSI guidelines [26,27]. The MIC and MBC values of sodium metabisulphite against *S. aureus* ATCC 29213 needed to be ≤ 512 μg/ml as reported by Frank and Patel [28]. All isolates were tested on at least three separate occasions and modal MICs and MBCs were determined.

2.4. Checkerboard assay

Serial two-fold dilutions of 8000 μg/ml sodium nitrite were made in a vertical orientation in a 96-well microtitre plate using

Table 1
Origin and molecular characteristics of *C. difficile* isolates used in this study.

C. difficile isolates	Origin	Ribotype	Toxin profile	Reference
ATCC 700057	–	UK 038	A–B–CDT–	
Foods origin				
Cd1001	Pork meat, Canada	E	A+B+CDT–	[14]
Cd1002	Ground beef, Canada	UK 027	A+B+CDT+	[14]
Cd1006	Ground beef, Canada	UK 078	A+B+CDT+	[14]
Cd1009	Chicken meat, Canada	UK 078	A+B+CDT+	[15]
Cd1017	Ground beef, Canada	C (UK 251)	A+B+CDT+	[14]
Cd1106	Chicken meat, Canada	UK 014	A+B+CDT–	
Food animals origin				
AI35	Piglets, Australia	UK 237	A–B+CDT+	[39]
AI204	Calves, Australia	UK 033	A–B–CDT+	[40]
AI218	Calves, Australia	UK 127	A+B+CDT+	[40]
AI273	Calves, Australia	UK 126	A+B+CDT+	[40]

100 μl of sterile distilled water. Nine doubling dilutions of 16,000 μg/ml sodium nitrate, at four times the intended final concentration, were prepared using sterile distilled water. Fifty-microliter aliquots of each sodium nitrate dilution were added in a horizontal orientation so that the plate contained various concentration combinations of the two preservatives. Growth from 24 to 48 h Brucella agar cultures of the test organisms was adjusted to 2.0 McFarland in saline, and then diluted in four times concentrated supplemented Brucella broth to correspond to a final inoculum concentration of approximately 1.0×10^6 cfu/ml. Each well was inoculated with 50 μl of inoculum and incubated anaerobically at 37 °C for 24–48 h. Purity checks and a vancomycin control were included to ensure testing quality as described above. Three independent replicates were performed, modal MICs were determined.

The fractional inhibitory concentration (FIC) was calculated by dividing the MIC of sodium nitrite and sodium nitrate in combination by the MIC of sodium nitrite or sodium nitrate alone. The FIC index (FICI) was obtained by adding both FICs. When the FICI was ≤0.5, synergy was indicated; an FICI of >0.5 to 4.0 was defined as an indifferent interaction; and antagonism was defined as an FICI of >4.0 [29].

3. Results and discussion

The MICs and MBCs of sodium nitrite, sodium nitrate and sodium metabisulphite against the control *S. aureus* ATCC 29213 were >4000 μg/ml, >4000 μg/ml and 500 μg/ml, respectively. The MICs and MBCs of sodium nitrite, sodium nitrate and sodium metabisulphite against *C. difficile* are shown in Table 2. Sodium nitrite inhibited the growth of all tested *C. difficile* isolates at 125–500 μg/ml with an average of 250 μg/ml which is higher than the maximum permitted level allowed in ready-to-eat meats in the United States (200 ppm), Europe (150 ppm), Australia (125 ppm) and New Zealand (125 ppm). This raises possible food safety concerns as many commercially produced ready-to-eat meats are also non-heat treated cured or fermented meats. While there are no published data on the effects of food preservatives against *C. difficile* in broth or in meats, these MICs were similar to those reported for *Clostridium perfringens* [30]. Labbe and Duncan found 200–400 μg/ml of nitrite was required to inhibit spore outgrowth at pH 6. The inhibitory mechanisms of nitrite are not well understood, but nitrite inhibits microbes more effectively at low pH suggesting that the antimicrobial action is likely associated with the generation of nitric oxide or nitrous acid [30]. Studies on *Clostridium sporogenes* and *C. perfringens* spores showed that nitrite prevented vegetative cell division and inhibited the emergence of vegetative cells from spores while allowing germination to occur [30,31].

Our results suggest that nitrites can inhibit *C. difficile* growth but are unable to kill the organism. This is consistent with a study on *C. botulinum* spores where *C. botulinum* was recovered from artificially seeded, commercially formulated and processed sausages after storage at 8 °C for 5 weeks regardless of the concentration of nitrite added, with the highest *C. botulinum* counts detected in nitrite-free products [32].

Sodium nitrate had no measurable effect on the growth of *C. difficile* at the maximum permitted levels (Table 2). Even at 4000 μg/ml, which is at least eight times the concentration normally used in commercially produced ready-to-eat meats, the preservative failed to inhibit *C. difficile* growth. This is consistent with a previous *in vitro* study where nitrate had no effect on spore germination and outgrowth of *C. sporogenes* NCA 3679 [31].

Sodium metabisulphite, a food preservative used in raw sausages, inhibited the growth of *C. difficile* at 1000 μg/ml (Table 2) which is higher than the allowed level of 500 ppm in Australia. No bactericidal activity against *C. difficile* was observed at the highest concentration tested of 4000 μg/ml. There are no published data on the effect of sodium metabisulphite on spore-forming bacteria.

Checkerboard assays of sodium nitrate and sodium nitrite gave a FICI in the range of 1.5–3, indicating an indifferent interaction between the preservatives (Table 3). However, this is unlikely to be representative of the combined effects of these preservatives in meat products. In meat matrices, other commensal microorganisms that are present reduce nitrate to nitrite [33], altering their relative concentrations and therefore their inhibitory ability. In the presence of these microorganisms, nitrate might show some effect on the growth of *C. difficile* as it has on delaying botulinum toxin production [34,35].

Although the reported MICs of food preservatives are higher than their maximum permitted levels allowed in commercially produced ready-to-eat meats, it is doubtful that *C. difficile* would be able to grow in ready-to-eat meats when stored at the recommended temperature of 5 °C. This would require the meats to be stored at abusive temperatures for a long period of time. Nevertheless the recent findings of *C. difficile* in raw meats with prevalences ranging up to 42% [6–10] are a cause for concern as these may be used to make ready-to-eat meats, especially non-heat treated ready-to-eat meats such as cured or fermented products. The possibility of ready-to-eat meats contaminated with *C. difficile* needs to be investigated given that *C. difficile* can survive and grow in the presence of food preservatives commonly used in meat

Table 2
Susceptibility of *C. difficile* to food preservatives.

C. difficile isolates	Sodium nitrite		Sodium nitrate		Sodium metabisulphite	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
ATCC 700057	250	1000	>4000	>4000	1000	2000[a] (1000 – >4000)
Cd1001	125[a] *(15.33–250)*	250[a] *(62.5 – 1000)*	2000	>4000	1000	1000
Cd1002	500	>4000	>4000	>4000	1000	>4000
Cd1006	500	>4000	>4000	>4000	1000	>4000
Cd1009	250[a] *(125–500)*	>4000	>4000	>4000	1000	>4000
Cd1017	125[a] *(31.25–250)*	>4000	>4000	>4000	500	>4000
Cd1106	250[a] *(12–500)*	>4000	>4000	>4000	1000	>4000
AI35	500	>4000	>4000	>4000	1000	>4000
AI204	250[a] *(125–500)*	>4000	>4000	>4000	1000	>4000
AI218	125	>4000	>4000	>4000	1000	>4000
AI273	250[a] *(125–500)*	>4000	>4000	>4000	1000	>4000
Mode	**250**	**>4000**	**>4000**	**>4000**	**1000**	**>4000**

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.
The values are the mode from at least three separate experiments.
[a] Median (range) was reported when independent replicates yielded different MICs, hence no mode could be determined.

Table 3
In vitro activities of sodium nitrate and sodium nitrite individually and in combination against *C. difficile*.

C. difficile isolates	MIC (μg/ml)			FICI	Interactive category
	Sodium nitrate	Sodium nitrite	Sodium nitrate-sodium nitrite combination		
ATCC 700057	>4000	125	>4000/125	2	Indifferent
Cd1001	>4000	125	>4000/125	2	Indifferent
Cd1002	>4000	125	>4000/125	2	Indifferent
Cd1006	>4000	250	>4000/250	2	Indifferent
Cd1009	>4000	250	>4000/250	2	Indifferent
Cd1017	>4000	125	>4000/250	3	Indifferent
Cd1106	>4000	250	>4000/250	2	Indifferent
AI35	>4000	500	>4000/500	2	Indifferent
AI204	>4000	500	>4000/250	1.5	Indifferent
AI218	>4000	250	>4000/250	2	Indifferent
AI273	>4000	500	>4000/500	2	Indifferent

products. There is potential for foodborne transmission of *C. difficile* through commercially produced ready-to-eat meats as has been reported for other *Clostridia* spp [36–38].

A limitation of this study is that it used *C. difficile* cultures which at the time of testing contained approximately 15% of spores. Further research is required to study the effect of food preservatives on *C. difficile* spores and vegetative cells separately. The impact of pH, temperature and other commensal microorganisms, possibly through the use of meat matrices, also needs exploring.

This study represents the first to investigate the effect of food preservatives on *C. difficile*. Although the relevance of contaminated food in the epidemiology of CDI is not yet known, the ability of *C. difficile* to survive in the presence of food preservatives indicates a need to investigate the prevalence of *C. difficile* in commercially produced ready-to-eat meats as they may serve as a source of infection for people in hospitals and the community.

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