Carvacrol reduces *Clostridium difficile* sporulation and spore outgrowth *in vitro*

Shankumar Mooyottu, Genevieve Flock and Kumar Venkitanarayanan*

**Abstract**

**Purpose.** *Clostridium difficile* is an anaerobic spore-forming pathogen that causes a serious toxin-mediated enteric disease in humans. Therapeutic agents that are capable of reducing *C. difficile* spore production could significantly minimize the transmission and relapse of *C. difficile* infections. This study investigated the efficacy of a food-grade, plant-derived compound, carvacrol (CR), in reducing *C. difficile* spore production, germination and spore outgrowth.

**Methodology.** Two hyper-virulent *C. difficile* isolates (ATCC BAA 1870 and 1805) were grown with or without a sub-inhibitory concentration (SIC) of CR. Total viable counts and heat-resistant spore counts were determined at different time intervals. Moreover, spores and vegetative cells were visualized using phase-contrast microscopy. To determine the effect of CR on *C. difficile* germination and spore outgrowth, *C. difficile* spores were seeded in germination medium with or without the SIC and MIC of CR, and spore germination and spore outgrowth were measured by recording optical density at 600 nm. The effect of CR on *C. difficile* sporulation genes was also investigated using real-time qPCR.

**Results.** Carvacrol significantly reduced sporulation in *C. difficile* and down-regulated critical genes involved in spore production (*P*<0.05). The SIC or MIC of CR did not inhibit *C. difficile* spore germination; however, the MIC of CR completely inhibited spore outgrowth.

**Conclusion.** The results suggest that CR could potentially be used to control *C. difficile* by reducing spore production and outgrowth.

**INTRODUCTION**

*Clostridium difficile* is a spore-forming anaerobic bacterium that causes a toxin-mediated enteric disease in humans [1]. *C. difficile* predominantly affects long-term hospital inpatients and the elderly undergoing prolonged antibiotic therapy [2]. Recent epidemiological data have shown that *C. difficile* has surpassed methicillin-resistant *Staphylococcus aureus* as the most commonly acquired hospital infection [3]. Prolonged antibiotic therapy results in the disruption of the normal enteric microflora, causing *C. difficile* spore germination and pathogen colonization in the intestine, with subsequent production of toxins [4]. Although toxins are the major virulence factors responsible for the pathogenesis of *C. difficile* infection, spore formation, germination and spore outgrowth in *C. difficile* are critical components of *C. difficile* infection (CDI) transmission and relapse [5]. Sporulation is a critical virulence factor in *C. difficile* pathogenesis. *C. difficile* spores, being survival structures that can withstand unfavourable physical, chemical and metabolic conditions, play an important role in bacterial persistence in the environment, transmission to new susceptible individuals and relapse in temporarily recovered patients [6]. This leads to germination, proliferation and colonization of *C. difficile* in the gut following prolonged antibiotic treatment that disrupts the normal gut flora, which in turn causes new infections or relapse of CDI [7, 8]. Relapse of CDI is reported in 25% of patients undergoing anti-*C. difficile* therapy [9]. Spore production in *C. difficile* is regulated by several genes, including *spo0A*, which is the master regulator of *Spo0A*, together with associated kinases and different sigma factors, such as *sigH* [10–12]. Highly resistant *C. difficile* spores shed in the faeces contaminate hospitals and healthcare facilities, which can cause infection though the faecal–oral route, reinfection or recurrence of CDI in patients following germination, and spore outgrowth [2, 13]. Therefore, agents reducing *C. difficile* sporulation and spore outgrowth in the human gastrointestinal tract could effectively control *C. difficile* infection, transmission and relapse [14, 15].

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**Abbreviations:** BHIS, brain heart infusion-supplemented; CR, carvacrol; MIC, minimum inhibitory concentration; SIC, sub-inhibitory concentration; TVC, total viable count.
Despite the fact that exposure to broad-spectrum antibiotics predisposes patients to CDI by disrupting the normal gut flora [16, 17], antibiotics still constitute the chief treatment strategy against *C. difficile*. In addition, the emergence of antibiotic resistance in hypervirulent strains of *C. difficile* is documented [18, 19]. Therefore, alternative therapeutic agents that can attenuate *C. difficile* virulence without disrupting the normal gut flora represent a viable control approach against the pathogen. Carvacrol (CR) is a monoterpeneid phenol present in oregano and thyme oils. Various pharmacological effects of CR, including antimicrobial and anti-inflammatory properties, have been demonstrated [20]. Our previous study indicated that CR effectively inhibited *C. difficile* toxin production in vitro, without deleteriously affecting the major normal gastrointestinal flora in humans [21].

The objective of this study was to investigate the potential of CR to inhibit spore production, spore germination and spore outgrowth in vitro, which are critical in the spread and relapse of *C. difficile* infection, and to delineate the potential mechanism(s) behind its effect.

**METHODS**

**Bacterial strains and culture conditions**

Two hypervirulent *C. difficile* isolates (ATCC BAA 1805, and 1870) were grown in brain heart infusion broth (BHI) supplemented with 5% yeast extract (Difco, Sparks, MD, USA) in a Whitley A35 anaerobic work station (Microbiology International, Frederick, MD, USA) in the presence of 80% N₂, 10% CO₂, and 10% H₂ at 37°C for 24 h. The bacterial population was determined by plating 0.1 ml portions of appropriate dilutions on BHI agar and *Clostridium difficile* moxalactam norfloxacin (CDMN) agar (Oxoid, Lenexa, KS, USA) supplemented with 5% horse blood, under strict anaerobic conditions at 37°C for 24 h. The cultures were sedimented by centrifugation (3600 g, 15 min, 4°C), and the pellet was washed twice and resuspended in sterile PBS (pH 7.3), and then used as the inoculum.

**Clostridium difficile spore preparation**

*C. difficile* spores were prepared as previously described, with slight modifications [22]. Briefly, single colonies of ATCC BAA 1870 and 1805 were separately inoculated into BHIS broth (BHI plus cysteine) and cultured overnight at 37°C under anaerobic conditions. A 150 µl aliquot of overnight culture was spread onto BHI agar (Oxoid) in six-well plates and cultured anaerobically for 10 days at 37°C in a Whitley A35 anaerobic work station (Don Whitley Scientific, Shipley, West Yorkshire, UK) to allow sporulation. After 10 days, spores were harvested from the wells by flooding with 2 ml ice-cold sterile water. The spore suspension was heat-treated at 60°C for 20 min to kill any vegetative cells, and washed six times in distilled water by centrifuging at 16,000 g for 5 min. Spore suspensions were examined for purity by phase-contrast microscopy before storage at −20°C prior to use.

**Carvacrol and determination of SIC and MIC on *C. difficile***

The sub-inhibitory concentration (SIC) of CR (≥98% purity, Sigma Aldrich, St Louis, MO, USA) against *C. difficile* was determined, as previously described [23]. Approximately 5.0 log c.f.u. *C. difficile* (ATCC BAA 1870 and 1805) was inoculated separately in tissue-culture plates containing 2 ml of BHI, followed by the addition of 1 to 10 µl of CR with an increment of 0.5 µl. The plates were then incubated in an anaerobic chamber incubator at 37°C for 24 h with 80% N₂, 10% H₂ and 10% CO₂, and bacterial growth was monitored by determining optical density at 600 nm (OD₆₀₀). The lowest concentration of CR that inhibited bacterial growth was selected as the MIC, and the two highest CR concentrations below the MIC that did not inhibit bacterial growth after 24 h of incubation were selected as the SICs for this study.

**Effect of CR on *C. difficile* growth and sporulation**

The effect of CR on *C. difficile* growth and sporulation was determined according to a previously published protocol [24]. Briefly, BHI with or without the SICs of CR was inoculated (5 log c.f.u. ml⁻¹) separately with each *C. difficile* isolate, and incubated at 37°C for 96 h anaerobically as before. Samples were withdrawn at different time intervals (24, 48, 72 and 96 h) for quantification of heat-resistant spores (survivors of incubation at 60°C for 20 min) and total viable count (TVC) by serially diluting each sample in PBS and plating on BHIS agar supplemented with 0.1% taurocholate. In addition, sporulation in BHIS at the specified time intervals was visualized under phase-contrast microscopy [25]. A 10 µl aliquot of the culture from different treatment groups was loaded onto a microscopy slide, air-dried and visualized under 1.5×40 magnification.

**Effect of SIC and MIC of CR on *C. difficile* spore germination and outgrowth**

To study the effect of CR on *C. difficile* spore germination, 100 µl suspension containing 10⁵ spores ml⁻¹ was added to the wells of a 12-well plate containing 1.9 ml of pre-warmed, pre-reduced BHI supplemented with 0.1% sodium taurocholate (Sigma-Aldrich, St Louis, MO, USA) inside an anaerobic workstation. The plates were closed inside the workstation with lids and sealed with a sealant. Brain–heart infusion without taurocholate and spore suspensions or medium replaced with distilled water were included as controls. A well with resazurin 0.1 mg ml⁻¹ was included for monitoring anaerobiosis in the plates during reading. Optical density at 600 nm was recorded using a Synergy plate reader (Biotek, Winooski, VT, USA) at 37°C over a 24 h time period, with readings taken at 10 min intervals, and was expressed as a percentage of the initial OD₆₀₀ (t₀). Spore germination was measured as the initial loss of OD₆₀₀ and spore outgrowth was measured by recording the increase in OD₆₀₀ following spore germination, as described previously [26, 27].
Real-time quantitative PCR (RT-qPCR)
To determine the effect of CR on the C. difficile genes involved in spore production, total RNA was isolated from early stationary-phase cultures grown with and without the SIC of CR [28]. The culture supernatant was harvested by centrifugation at 3000 g for 10 min at 4 °C. The bacterial pellet was resuspended in RNAwiz solution (Ambion, Austin, TX, USA), flash frozen in liquid nitrogen and stored at −80 °C. Total RNA extraction was performed using the Ambion RiboPure Bacteria RNA kit according to the manufacturer’s instructions, followed by DNase I digestion using Turbo DNase I (Ambion, Austin, TX, USA). The RNA obtained after each DNase I digestion was purified further using the Qiagen RNeasy RNA column purification kit (Qiagen, Germantown, MD, USA). The cDNA was synthesized using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). RT-qPCR analysis was performed using published primers for genes associated with sporulation (Spo0A, SpoIIA, SpoIIID, SpoIIIR, SigH and Cd2492) [12, 24] and normalized against 16S rRNA gene expression. The level of candidate gene expression between treated and control samples was determined using the 2−ΔΔCT method and expressed as relative fold change [29].

Statistical analysis
All experiments had duplicate samples, and the study was repeated three times. The data were analysed using one-way ANOVA. Differences between means were considered to be significant at P<0.05.

RESULTS
Effect of CR on C. difficile growth and sporulation
The MIC of CR against C. difficile was 1.2 mM. The two highest SICs of CR were found to be 0.9 mM and 0.6 mM. These two concentrations of CR did not inhibit the growth of C. difficile isolates after 24 h incubation at 37 °C. The effect of SICs of CR on C. difficile sporulation is shown in (Fig. 1a, b). In both C. difficile isolates, a significant decrease in spore counts was observed after 48 and 72 h of incubation in CR-treated samples compared to control (P<0.05). In C. difficile BAA 1870, CR at 0.6 mM and 0.9 mM resulted in −1.5 log c.f.u. ml−1 and 2.0 log c.f.u. ml−1 reduction in spore counts at 48 and 72 h of incubation, respectively. At 96 h, although spore counts in 0.6 mM CR-treated samples grew back by −1.0 log c.f.u. ml−1, CR treatment at 0.9 mM reduced the spore count −3 log c.f.u. ml−1 (Fig. 1a). However, the differences in total viable counts (plated without heat treatment) between CR-treated samples and untreated control were minimal (P>0.05). Similar results were also observed in C. difficile BAA 1805 (Fig. 1b), where −1 log c.f.u. ml−1 reduction in spore count was observed in 0.6 mM CR-treated cultures at 48, 72 and 96 h. Similarly, in the presence of 0.9 mM CR, a 2 log reduction in spore counts was observed consistently from 48 h through 96 h. Moreover, the differences in total viable counts between CR-treated and control samples were less than 1.0 log c.f.u. ml−1 throughout the sampling times (P>0.05). The results from the sporation experiments were consistent with the phase-contrast microscopy results for CR-treated and untreated C. difficile cultures (Fig. 2). In the untreated control, more phase-bright spores and fewer vegetative bacteria populations were observed throughout the microscopic field. Conversely, in CR-treated C. difficile culture, more vegetative bacteria, fewer phase-bright spores and a few phase-dark spores were observed after 48 h of incubation.

Effect of CR on C. difficile sporulation genes
To determine the effect of CR on C. difficile sporulation, RT-qPCR was performed. The results showed that CR significantly down-regulated the transcription of C. difficile sporulation genes, where the expression of spo0A was down-regulated by 22-fold in BAA 1870 and 13-fold in BAA 1805, and that of spoIIIA was down-regulated by 23-fold and 25-fold in BAA 1870 and BAA 1805, respectively (Table 1). In addition, the transcription of spoIIIR was decreased by seven-fold and eight-fold, and that of spoIIID by five-fold and seven-fold, in C. difficile BAA 1870 and 1805, respectively (P<0.05). Similarly, sigH was down-regulated by 19-fold and 26-fold in C. difficile BAA 1870 and BAA 1805, respectively (P<0.05). Likewise, the expression of CD2492 was also reduced by 14-fold and sevenfold in C. difficile BAA 1870 and BAA 1805 isolates, respectively (P<0.05).

DISCUSSION
The results from this study suggest that CR, a plant-derived, food-grade compound that has previously been shown to exert an anti-toxigenic effect in C. difficile can significantly reduce C. difficile spore production and spore outgrowth in
vitro. The SICs of CR significantly reduced spore production by *C. difficile* at 48, 72 and 96 h of incubation (*P*<0.05). The reduction in sporulation in CR-treated samples was determined by the dilution and plating method, and also visualized using phase-contrast microscopy. Interestingly, no reduction in the total number of viable cells was observed in CR-treated *C. difficile* cultures compared to the untreated control. These results indicate that CR was able to specifically inhibit the formation of spores from the vegetative cells in broth culture. A slight increase in the TVC of CR-treated cultures could be due to the presence of a higher proportion of vegetative cells, which have a superior detection limit on plating compared to spores [30]. Since the SICs of CR were used in the sporulation experiments, the decrease observed in *C. difficile* spore production in CR-treated samples was not due to inhibition of bacterial growth by CR, but it could be attributed to their potential ability to reduce the transcription of virulence genes associated with spore production. In concurrence with this, the gene expression studies indicated that CR significantly
down-regulated critical genes responsible for *C. difficile* sporulation. In the germination experiments, the results revealed that the SIC of CR (0.6 mM) significantly reduced *C. difficile* spore outgrowth (outgrowth of vegetative cells from the newly germinated spores) compared to the controls (*P*<0.05), whereas CR at the MIC (1.2 mM) completely inhibited the spore outgrowth. These results suggest that vegetative cells from newly germinated spores are more sensitive to CR compared to vegetative *C. difficile* cells grown in broth.

A potential alternative strategy for controlling microbial infections is the use of anti-virulence drugs, which target the reduction of bacterial virulence rather than the inhibition of bacterial growth [31], thereby presenting less selective pressure for the development of bacterial antimicrobial resistance [32–34]. The results of the current study indicate that CR inhibited sporulation in *C. difficile*, which aids in bacterial virulence, especially towards CDI transmission and relapse. Moreover, prior research conducted in our laboratory revealed that the SICs of CR inhibited the production of *C. difficile* toxins, which represent another important virulence factor in the pathogen, without inhibiting the normal gut bacteria [21]. Other researchers also reported that CR exerted no adverse effects on endogenous bacterial populations, including *Lactobacilli* and *Bifidobacteria* in pigs and poultry [35]. To conclude, the results from this study suggest the potential of CR for controlling *C. difficile* by reducing spore production and outgrowth, thereby warranting follow-up *in vivo* studies to confirm the findings.

**Table 1.** Effect of carvacrol (CR) on *C. difficile* ATCC BAA 1870 and 1805 sporulation genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>BAA 1870</th>
<th>BAA 1805</th>
</tr>
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<tbody>
<tr>
<td><em>SpoIIA</em></td>
<td>$-22.4\pm3.6$</td>
<td>$-13.4\pm5.7$</td>
</tr>
<tr>
<td><em>SpoIIA</em></td>
<td>$-23.7\pm6.0$</td>
<td>$-25.7\pm13.1$</td>
</tr>
<tr>
<td><em>SpoIID</em></td>
<td>$-5.7\pm2.4$</td>
<td>$-7.7\pm2.9$</td>
</tr>
<tr>
<td><em>SpoIIIR</em></td>
<td>$-7.39\pm2.2$</td>
<td>$-8.8\pm4.6$</td>
</tr>
<tr>
<td><em>SigH</em></td>
<td>$-19.5\pm9.2$</td>
<td>$-26.0\pm12.6$</td>
</tr>
<tr>
<td><em>Cd2492</em></td>
<td>$-14.4\pm3.8$</td>
<td>$-7.5\pm1.2$</td>
</tr>
</tbody>
</table>

*Fold change in gene expression (mean±SE) relative to control; control had a basal level of expression of unity. All treatments significantly differed from the controls (*P*<0.05).*
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The authors declare that there are no conflicts of interest.

References