



# Effect of the Synthetic Bile Salt Analog CamSA on the Hamster Model of *Clostridium difficile* Infection

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**ABSTRACT** *Clostridium difficile* infection (CDI) is the leading cause of antibiotic-associated diarrhea and has gained worldwide notoriety due to emerging hypervirulent strains and the high incidence of recurrence. We previously reported protection of mice from CDI using the antigerminant bile salt analog CamSA. Here we describe the effects of CamSA in the hamster model of CDI. CamSA treatment of hamsters showed no toxicity and did not affect the richness or diversity of gut microbiota; however, minor changes in community composition were observed. Treatment of *C. difficile*-challenged hamsters with CamSA doubled the mean time to death, compared to control hamsters. However, CamSA alone was insufficient to prevent CDI in hamsters. CamSA in conjunction with suboptimal concentrations of vancomycin led to complete protection from CDI in 70% of animals. Protected animals remained disease-free at least 30 days postchallenge and showed no signs of colonic tissue damage. In a delayed-treatment model of hamster CDI, CamSA was unable to prevent infection signs and death. These data support a putative model in which CamSA reduces the number of germinating *C. difficile* spores but does not keep all of the spores from germinating. Vancomycin halts division of any vegetative cells that are able to grow from spores that escape CamSA.

**KEYWORDS** *C. difficile*, microbiome, antigermmination, CamSA, hamster

*Clostridium difficile* is an anaerobic, spore-forming pathogen that colonizes the gut of susceptible individuals and causes *Clostridium difficile* infection (CDI) (1). CDI is recognized as the leading cause of antibiotic-associated diarrhea in nosocomial environments. CDI poses a large burden for the health care system, costing the United States >3 billion dollars annually (2). An emergent hypervirulent *C. difficile* strain (BI/NAP1/027) is associated with even more severe disease (3, 4). The morbidity and mortality rates associated with CDI have prompted the CDC to upgrade *C. difficile* to an urgent antimicrobial resistance threat (5).

*C. difficile* spores are transmitted via the fecal-oral route, and CDI most often occurs after a patient has been treated with antibiotics. In fact, most broad-spectrum antibiotics can render a person susceptible to *C. difficile* (6). Antibiotic use disrupts the natural gut microbiota that normally resists colonization by *C. difficile* (7). In this new favorable environment, *C. difficile* spores germinate; the resulting vegetative cells proliferate in the gut and release two exotoxins, toxin A and toxin B. These toxins cause injury to and inflammation of the colonic lining, resulting in disease (8, 9). Because of the intrinsic antibiotic resistance of *C. difficile* spores and the inability of current antibiotic treatments for CDI to completely clear the pathogen from the intestinal tract, nonantibiotic

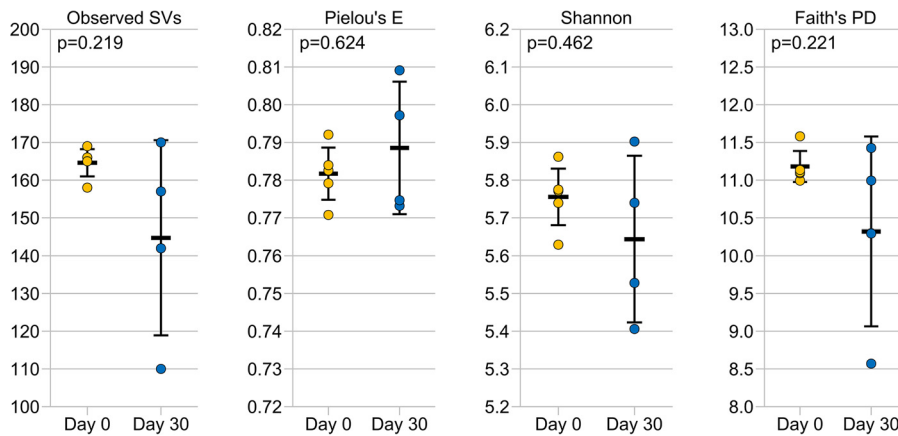
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**FIG 1** Alpha diversity metrics comparing hamster fecal microbiomes before (day 0,  $n = 5$ ) (yellow circles) and after (day 30,  $n = 4$ ) (blue circles) a 30-day CamSA treatment. The data for 1 hamster after CamSA treatment failed quality control standards and was removed from the data set. Bars represent means and 95% confidence intervals. No values were significantly different before versus after CamSA treatment (pairwise Kruskal-Wallis test,  $P > 0.05$ ). Observed SVs, observed sequence variants; Pielou's E, Pielou's evenness; Shannon, Shannon diversity index; Faith's PD, Faith's phylogenetic diversity.

strategies should be investigated (10–14). Continued antibiotic use to treat CDI prevents commensal microbiota from repopulating the gut, allowing persistent *C. difficile* spores to germinate and to reestablish disease. As a result, 25 to 30% of CDI patients experience repeated bouts of relapse (15–17).

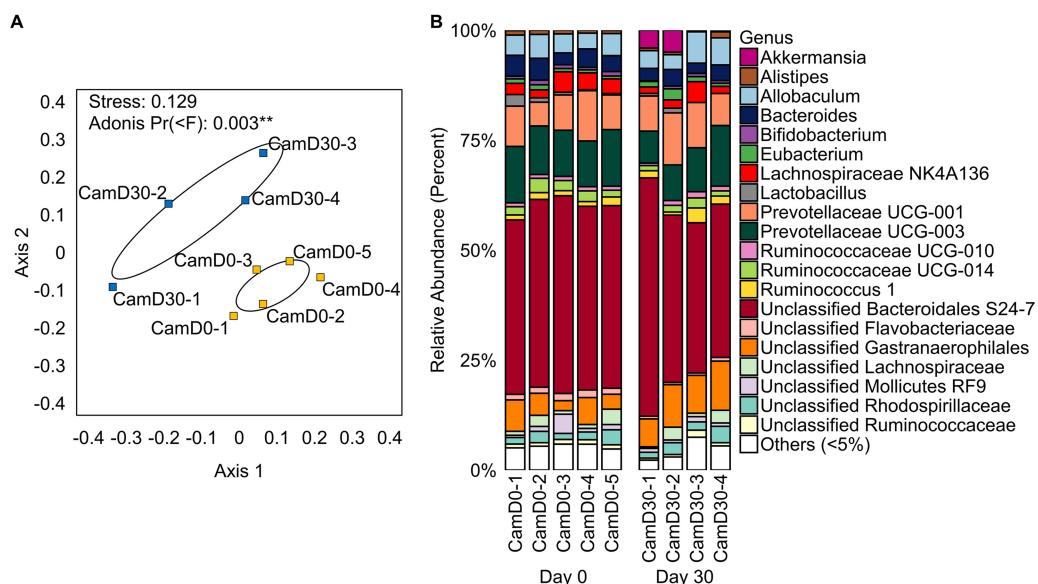
*C. difficile* spores must germinate to establish disease. The activation of dormant *C. difficile* spores requires binding of the primary bile salt taurocholate and amino acids (18, 19). We showed that a synthetic bile salt analog, CamSA, was a competitive inhibitor of taurocholate-induced *C. difficile* spore germination *in vitro* (18). Furthermore, CamSA prevents CDI in a murine model in a dose-dependent manner (20).

In the current study, we assessed the effectiveness of CamSA in the more susceptible hamster CDI model. As in mice, CamSA showed no toxicity and did not cause large-scale disruption of the hamster intestinal microbiota. CamSA alone was unable to prevent CDI in hamsters but resulted in a delay in the onset of disease. Interestingly, CamSA in combination with a suboptimal dose of vancomycin, administered at the same time as *C. difficile* spores, prevented CDI in 70% of challenged hamsters. However, CamSA-vancomycin combinations were unable to prevent CDI recurrence when vancomycin was administered 24 h postchallenge with *C. difficile* spores. These results suggest that antigermination therapies have the potential to be used in combination with current antibiotic treatments to reduce CDI progression. This study has also revealed limitations of CamSA alone as prophylaxis for CDI in hamsters.

## RESULTS

**CamSA toxicity.** Hamsters treated for 30 consecutive days with the highest CamSA dose (300 mg/kg) used in previous studies (20) showed no obvious adverse physiological or behavioral effects. Similarly, gross anatomical examination of treated animals showed no lesions or abnormalities. Microscopic examinations of colons showed no histological anomalies, and findings were indistinguishable from those for untreated animals (see Fig. S1 in the supplemental material).

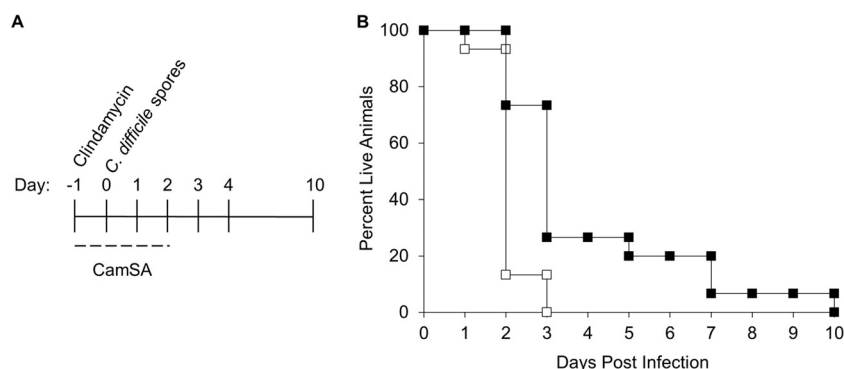
**Microbiome effects.** CamSA (300 mg/kg) treatment for 30 days did not significantly alter the richness, evenness, or diversity of the hamster intestinal microbiome (Fig. 1). However, nonmetric multidimensional scaling (NMDS) analysis based on Bray-Curtis dissimilarity, along with a corresponding Adonis analysis, showed that treatment with CamSA for 30 days led to a statistically significant change in the community composition of hamster gut microbiomes (Fig. 2A). Bar graphs showing the relative abundance of microbial taxa before and after CamSA treatment, along with a corresponding



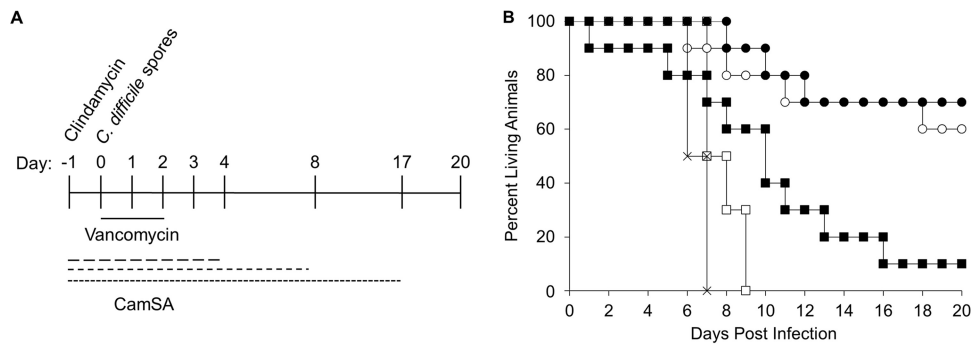
**FIG 2** (A) NMDS analysis based on Bray-Curtis dissimilarity, demonstrating that gut microbiomes were significantly different before (CamD0) (yellow squares) versus after (CamD30) (blue squares) CamSA treatment. Numbers after the hyphens refer to specific hamsters. Ellipses represent the standard errors of the mean (95% confidence). (B) Bar plot showing the relative abundance of gut microbiota at the genus level in each hamster before (day 0) and after (day 30) CamSA treatment. Uncultivated and unnamed genera are listed according to the lowest named taxonomic level, as categorized by SILVA. In cases in which the SILVA taxonomy was flagged as misclassified, the Ribosomal Database Project (RDP) taxonomy was used.

SIMPER analysis, showed these shifts to be small in comparison to broad-spectrum antibiotic treatment (Fig. 2B; also see Fig. S2 and Table S1 in the supplemental material) (5, 6, 21). Taxa that contributed most to the variance between microbiomes before and after CamSA treatment included a relative increase in *Akkermansia* (*Verrucomicrobia*) and shifts in populations of unclassified *Gastranaerophilales* (*Cyanobacteria*), *Prevotellaceae* (*Bacteroidetes*), *Lachnospiraceae* (*Firmicutes*), and *Bacteroidetes*.

**Delay of CDI onset by CamSA.** Hamsters challenged with *C. difficile* spores became symptomatic rapidly and were culled within 3 days postchallenge (Fig. 3A and B). Challenged hamsters treated with 300 mg/kg CamSA for 4 days developed CDI signs similar to those in untreated animals and were culled, but the onset was delayed up to 7 days, leading to a statistically significant difference ( $P = 0.0006$ ) in survival times



**FIG 3** Effects of CamSA on hamsters infected with *C. difficile* spores. (A) Timeline of experiments. All hamsters were treated with a single 30-mg/kg dose of clindamycin (day -1). On day 0, animals were challenged with 100 *C. difficile* spores. Animals were treated with either 0 or 300 mg/kg CamSA in DMSO for 4 consecutive days beginning at day -1. Animals were scored for signs of CDI twice daily. (B) Percent survival, presented as a Kaplan-Meier survival plot, which shows a significant difference between CamSA-treated (■) and untreated (□) animals ( $P = 0.0006$ ).



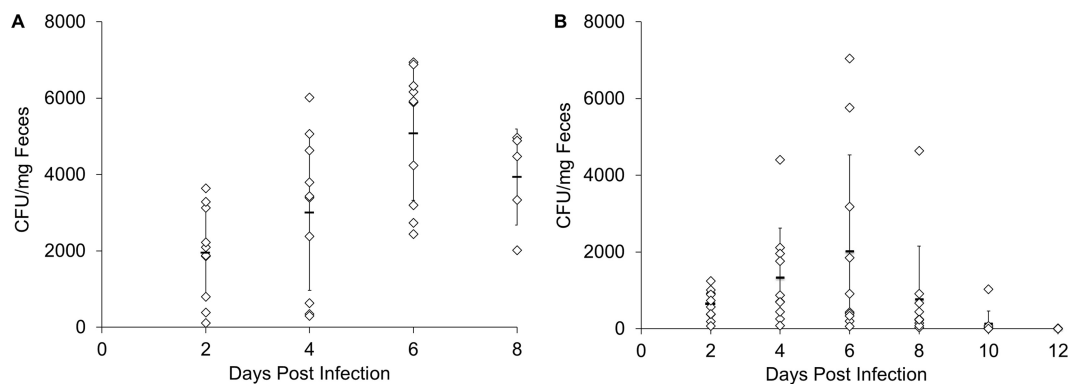
**FIG 4** Protection of hamsters from CDI with combined administration of a suboptimal vancomycin dose and CamSA. (A) Timeline of experiments. All hamsters were treated with a single 30-mg/kg dose of clindamycin (day -1). Also starting at day -1, animals were treated daily with 300 mg/kg CamSA for 0, 6, 10, or 19 consecutive days. Another group of animals was treated with 50 mg/kg CamSA for 10 consecutive days. On day 0, all animals were challenged with 100 *C. difficile* spores. Starting at day 0, all animals were also treated daily with 5 mg/kg vancomycin for 3 consecutive days. (B) Percent survival, presented as a Kaplan-Meier survival plot, which shows significant increases in animal survival rates with 300-mg/kg CamSA treatment for 6 days ( $P = 0.04$ ) (■), 10 days ( $P = 0.001$ ) (○), and 19 days ( $P = 5.18 \times 10^{-5}$ ) (●), compared to untreated animals (□). Survival of animals treated with 50 mg/kg CamSA for 10 days (×) was undistinguishable from that of untreated animals.

between CamSA-treated and untreated hamsters. Under these conditions, CamSA alone increased the mean time to death from 1.73 to 3.77 days.

**Effects of CamSA in combination with vancomycin.** To determine the effects of combining vancomycin and CamSA treatments, animals were challenged with *C. difficile* spores and treated with a suboptimal vancomycin dose (5 mg/kg) (22, 23) and CamSA (300 mg/kg) (Fig. 4A). As expected, animals treated with vancomycin alone become moribund within 9 days postchallenge. Combining vancomycin with CamSA for 19 consecutive days resulted in asymptomatic survival of 70% of the animals (Fig. 4B). Similar results were obtained when the CamSA dosage was stopped after 10 consecutive days. In contrast, limiting CamSA treatment to 6 doses resulted in survival of only 10% of infected animals. The increases in survival rates for hamsters treated with vancomycin and CamSA for 6 ( $P = 0.04$ ), 10 ( $P = 0.001$ ), or 19 ( $P = 5.18 \times 10^{-5}$ ) consecutive days were significant, compared to hamsters treated with vancomycin alone. When the CamSA dosage was reduced to 50 mg/kg for 10 days, *C. difficile*-challenged animals developed CDI signs, with scores indistinguishable from those of untreated animals.

Vancomycin-CamSA-protected animals showed intact colonic mucosal lining, with no distinct tissue damage or separation of the submucosal region, and were indistinguishable from control animals. In contrast, histological examination of animals showing signs of CDI presented extreme intestinal tissue damage, including disruption or loss of the colonic mucosal lining, loss of integrity of epithelial cells, and substantial separation of the submucosa (Fig. S3).

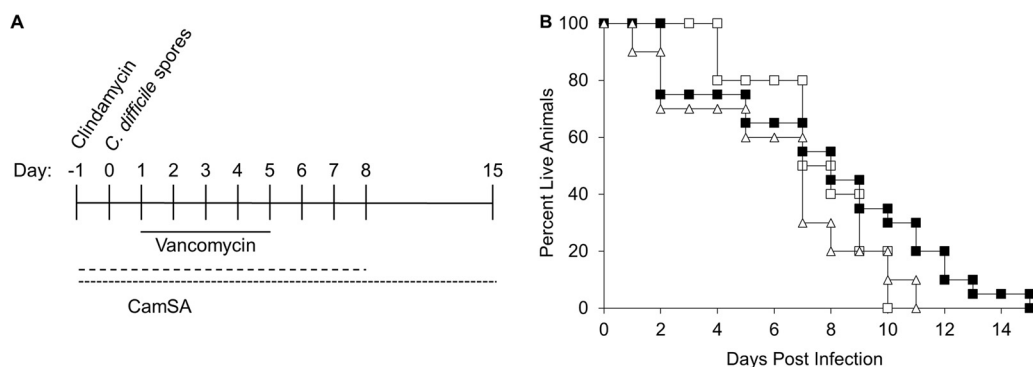
Fecal samples from challenged animals showed only *C. difficile* vegetative cells; no spores were recovered. Samples were heterogeneous with regard to *C. difficile* cells shed and the timing needed to clear *C. difficile* from the gastrointestinal tract. Most vancomycin-treated control animals showed maximum shedding between 4 and 6 days postchallenge but continued shedding until culling (Fig. 5A). CamSA-treated animals that did not develop CDI started shedding as early as 2 days and continued until 10 days postchallenge (Fig. 5B). Animals treated with only vancomycin showed a statistically significant ( $P = 0.005$ ) increase in *C. difficile* shedding over time. In contrast, shedding in vancomycin-CamSA-treated animals remained constant ( $P = 0.2$ ). These differences in shedding behavior resulted in statistically significant decreases in fecal *C. difficile* loads in vancomycin-CamSA-treated animals, compared to animals treated with only vancomycin, at days 2 ( $P = 0.005$ ), 4 ( $P = 0.042$ ), 6 ( $P = 0.005$ ), and 8 ( $P = 0.001$ ) postchallenge. Fecal samples were collected up to day 30 postchallenge; however, *C. difficile* was undetectable in fecal samples after day 12. CamSA-treated animals that



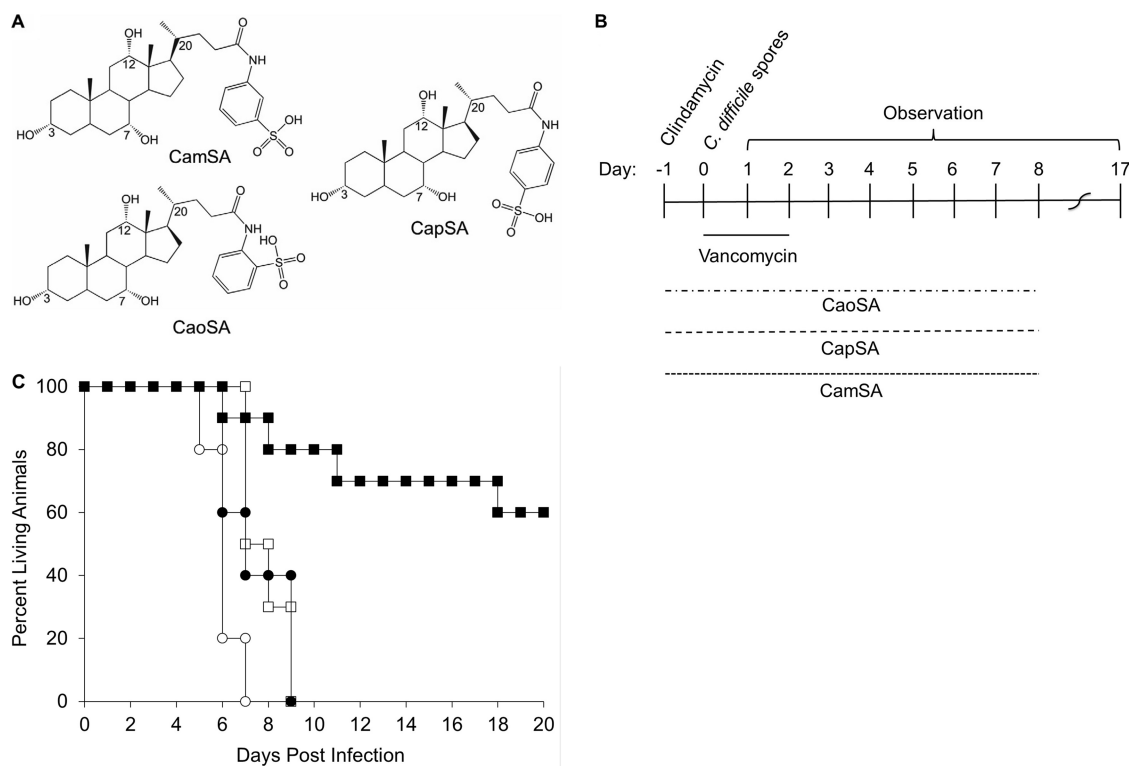
**FIG 5** Box-and-whisker plots showing total numbers of *C. difficile* in hamster feces, including spores and vegetative cells (mean  $\pm$  standard deviation). *C. difficile*-challenged animals were treated with 5 mg/kg vancomycin only (A) or with 5 mg/kg vancomycin and 300 mg/kg CamSA (B) for 19 consecutive days. Two-way mixed ANOVA was used to assess the significance of differences for groups treated with vancomycin only or CamSA plus vancomycin on days 2 ( $P = 0.005$ ), 4 ( $P = 0.042$ ), 6 ( $P = 0.005$ ), and 8 ( $P = 0.001$ ). Within groups, animals treated with vancomycin only showed significant differences in *C. difficile* CFU per milligram of feces over time ( $P = 0.005$ ), but no significant differences were found for the CamSA-vancomycin group ( $P = 0.2$ ) over time.

developed CDI shed *C. difficile* up to culling or 10 days postchallenge (data not shown). All data collected correspond to total *C. difficile* found in feces. Heat treatment of feces to obtain only *C. difficile* spores was unsuccessful. Surviving animals from all groups did not develop any CDI signs, even 30 days after the final CamSA dose.

**Effects of CamSA on CDI in hamsters with delayed vancomycin treatment.** To test for synergistic effects of antibiotic and antigermination treatments on CDI, vancomycin treatment was started 1 day after spore challenge, as described previously (24–26) (Fig. 6A). This delay in vancomycin dosing negated the synergistic effects of vancomycin-CamSA combinations (Fig. 6B). There was no statistically significant difference in survival times between hamsters treated with vancomycin and CamSA (10-day treatment,  $P = 0.67$ ; 17-day treatment,  $P = 0.25$ ) and hamsters given vancomycin alone. Even animals that were continuously administered CamSA daily developed CDI within 15 days after *C. difficile* challenge, resulting in only 17 consecutive CamSA doses. The calculated mean time to death for infected untreated animals was 7.2 days. The calculated mean times to death for animals treated with CamSA and vancomycin for 10 and 19 consecutive CamSA doses were 6.0 days and 7.5 days, respectively.



**FIG 6** Effects of delayed treatment on CDI with combined administration of a suboptimal vancomycin dose and CamSA. (A) Timeline of experiments. All hamsters were treated with a single 30-mg/kg dose of clindamycin (day  $-1$ ). Starting at day  $-1$ , animals were treated daily with 300 mg/kg CamSA for 0, 10, or 17 consecutive days. On day 0, all animals were challenged with 100 *C. difficile* spores. The beginning of vancomycin treatment was delayed for 24 h (day 1) after challenge, and treatment was continued daily for 5 consecutive days. Animals were scored for signs of CDI twice daily. (B) Percent survival, presented as a Kaplan-Meier survival plot. No difference in survival rate was conferred with 10 days of CamSA treatment ( $P = 0.67$ ) ( $\Delta$ ) or 17 days of CamSA treatment ( $P = 0.25$ ) ( $\blacksquare$ ), compared to untreated animals ( $\square$ ).



**FIG 7** Effects of CapSA and CaoSA on CDI prevention in hamsters. (A) Structures of CamSA, CapSA, and CaoSA. (B) Timeline of experiments. Animals were treated with clindamycin (day  $-1$ ) and challenged with *C. difficile* spores (day 0), as described above. Vancomycin (5 mg/kg) was administered for 3 consecutive days beginning at day 0. Starting at day  $-1$ , CaoSA, CapSA, or CamSA at 300 mg/kg was administered daily to animals for 10 consecutive days or until the animals became moribund. Animals treated with vancomycin only served as controls. (C) Percent survival, presented as a Kaplan-Meier survival plot, demonstrating that CamSA (■) offered protection ( $P = 0.001$ ), compared to untreated control animals (□), whereas CapSA (●) did not ( $P = 0.75$ ) and CaoSA (○) led to more rapid deaths ( $P = 0.001$ ).

**Effects of CamSA analogs on CDI in hamsters.** The structure of CamSA contains a sulfonic acid moiety at the *meta*-position of the aromatic ring conjugated to cholic acid (Fig. 7A). We showed previously that analogs of CamSA with the sulfonic acid moiety in either the *para*- or *ortho*-position negated inhibition of *C. difficile* spore germination *in vitro*, but *in vivo* studies were not performed (18). To test the effects of CamSA structural isomers in CDI protection, hamsters were treated with suboptimal concentrations of vancomycin and either CapSA (*para*-analog) or CaoSA (*ortho*-analog) for 10 consecutive days or until they were moribund (Fig. 7B). As shown above, CamSA-treated hamsters showed a significant increase in survival times ( $P = 0.001$ ), compared to hamsters given vancomycin alone (Fig. 7C). In contrast, CapSA did not offer protection ( $P = 0.75$ ), and CaoSA-treated animals showed decreased survival times ( $P = 0.001$ ), compared to animals administered vancomycin alone (Fig. 7C).

## DISCUSSION

*C. difficile* spore germination is required for the establishment of CDI (11) and thus can be used as a target for CDI prophylaxis. Using the antigerminal CamSA, we reported the complete prevention of CDI in mice (20, 27). In this study, we extended antigerminal therapy to the more stringent hamster model of CDI.

Reportedly, mice can be quite resistant to CDI and require an aggressive regimen of antibiotics to make them susceptible to infection (28–30). Others showed that mice can be quite susceptible to various *C. difficile* strains under different treatment strategies (21, 31, 32). Even so, mice must be challenged with a nonphysiologically large number of *C. difficile* spores. Murine CDI signs develop slowly and follow a predictable pattern (29, 33). Furthermore, in our experience, most mice recover within a few days (20, 27).

The murine model used previously to test CamSA mimics human CDI in showing gradual increases in CDI signs, and the murine relapse model has been refined to resemble human relapse more closely (28, 34).

Due to their high susceptibility to CDI, hamsters have been the standard CDI model. Hamster CDI is readily fulminant after challenge with a few dozen spores (35). These extreme symptoms are rarely seen in primary human CDI (26, 35). Indeed, hamster CDI progresses so rapidly that it more closely resembles an intoxication than a developing infection. However, suboptimal vancomycin treatment of infected hamsters results in delayed sign onset, similar to human CDI relapse (23, 35). Testing the effects of CamSA in the hamster model of CDI and CDI after delayed vancomycin treatment allowed us to determine the limits of antigermination therapy. CamSA prevented CDI in mice in a dose-dependent manner, even when mice were challenged with 1 million times more *C. difficile* spores than in the current study (20). In the more susceptible hamster model, antigermination therapy delayed the onset of disease and prevented CDI in only 70% of hamsters when administered for  $\geq 10$  doses and in the presence of a suboptimal concentration of vancomycin.

In our hands, the antigerminant CamSA has lower efficacy in protecting hamsters from *C. difficile* strain 630, compared to mice; this finding is not unexpected. Previous reports have consistently shown that CDI treatment in hamsters results in worse outcomes than in a corresponding mouse model (36–39). Furthermore, treatment efficacy is dependent on the *C. difficile* strain used in the hamster model (40). The most recently approved CDI treatment, fidaxomicin, was able to protect 100% of hamsters from *C. difficile* strain ATCC 9689 (ribotype 001) (41) and 80% of animals from *C. difficile* strain B11 (ribotype 027) but protected only 40% of animals from the same *C. difficile* strain 630 (ribotype 012) used in our experiments (25).

The role of the microbiome in preventing CDI has been used to develop probiotic treatments (42). A recent article reported that specific normal gut bacterial species, such as *Clostridium scindens*, can ameliorate CDI in mice (43). Differences in the microbiome before and after antibiotic disruption have been reported to produce changes in bile salt composition, which can affect *C. difficile* spore germination (44). Here we provide evidence, using deep sequencing of the V4 region of the 16S rRNA gene, that CamSA treatment for 30 days induced small but statistically significant changes in the gut microbiota. The small increase in *Akkermansia* is not suggestive of harmful dysbiosis. *Akkermansia* is an abundant gut inhabitant that has been linked to gut health and resistance to obesity (45, 46). The effects of minor shifts in unclassified *Gastranaerophilales*, *Prevotellaceae*, *Lachnospiraceae*, and *Bacteroidetes* species are difficult to interpret, since no close relatives of these organisms have been isolated and characterized. In addition, our study does not rule out other causes for changes in the microbiome during CamSA treatment. It is possible that changes were caused by aging, stress induced by oral gavage, or the dimethyl sulfoxide (DMSO) vehicle, rather than CamSA. Together, these data suggest that CamSA does not induce large-scale dysbiosis in the gut microbiome; however, more detailed work may be needed to understand more fully the effects of CamSA on the hamster gut microbiome.

In this study, CamSA alone was unable to prevent CDI development in hamsters even at high doses. Interestingly, CDI onset in CamSA-treated animals was delayed, suggesting that CamSA provides some antigermination effects. However, spore germination is an irreversible process and, even if CamSA inhibits the germination of most spores, residual spore germination into toxin-producing vegetative cells may be sufficient to cause colitis in hamsters. In contrast, due to their intrinsic resistance to CDI, potentially due to colonization resistance of natural microbiota, mice can tolerate the germination of a few spores without developing CDI signs (30, 42). It is important to note that in this study we were unable to test the direct effect of CamSA on spore germination in the hamsters, as was conducted in the murine study (20, 27). We were unable to recover *C. difficile* spores from fecal samples, likely due to the small number of *C. difficile* spores used for challenge (100 spores). Hypotheses and extrapolations

based on the antigermination effects of CamSA in the hamster model are based on previous studies *in vitro* and in the murine model (18, 20, 27).

Vancomycin has been shown to temporarily protect hamsters from CDI, and the onset and severity of disease can vary, depending on whether vancomycin is administered at the time of infection or 1 day following infection (23, 24, 47). Furthermore, lower concentrations of vancomycin show suboptimal protection and result in delayed CDI onset (23, 47). Treatment of *C. difficile*-challenged hamsters with low-dose vancomycin failed to prevent CDI but delayed the onset of disease. Combining 300 mg/kg CamSA with suboptimal concentrations of vancomycin administered prophylactically protected most hamsters from both immediate and delayed CDI onset. In fact, surviving animals did not develop CDI even 30 days after CamSA treatment ended. In mice, 50 mg/kg CamSA was sufficient to protect animals from CDI (20), but this same dose did not prevent CDI in hamsters when administered alone (data not shown) or in combination with suboptimal doses of vancomycin. We postulate that the two treatments work synergistically. CamSA prevents most *C. difficile* spores from germinating, as seen previously in mice (27). Indeed, animals cotreated with CamSA and vancomycin showed significant reductions in *C. difficile* loads, compared to animals treated with vancomycin alone (compare Fig. 5A and B). *C. difficile* shedding throughout CamSA-vancomycin treatment remained constant over the course of 10 days, while animals treated with only vancomycin showed an increase in shedding of *C. difficile* between day 2 and day 6. CamSA treatment may also allow the excretion of ungerminated spores, thus significantly reducing the infective load of *C. difficile* (27). The number of spores required to initiate disease in hamsters, i.e., 100 spores, is 6 orders of magnitude lower than the number needed to infect mice (27, 29, 48). The greater spore load in mice allowed easy recovery of *C. difficile* from feces, for calculation of spore versus vegetative cell excretion (20, 27). Unfortunately, in this study we were unable to separate spores from vegetative cells; therefore, *C. difficile* found in hamster feces is reported as total *C. difficile*.

Vancomycin can prevent germinated spores from proliferating in the hamster gut. Indeed, there is a strong correlation between the transit time of *C. difficile* spores in the hamster gut and the length of CamSA treatment necessary to protect animals. Animals stopped shedding *C. difficile* cells by day 10 postchallenge, and antigermination therapy must be continued for at least the same amount of time to be effective. This correlation is strengthened by the finding that delaying vancomycin treatment for 24 h (day 1) eliminated CDI protection (Fig. 6B), with all animals succumbing by day 15, compared to 70% survival in animals treated with vancomycin on day 0 (Fig. 4B). This delay in vancomycin treatment potentially allows a few spores to germinate into vegetative cells, which could start producing toxins (49). At that point, hamsters' intestines are irrevocably compromised.

CamSA is a *meta*-aminosulfonate cholate derivative that has been shown to inhibit *C. difficile* spore germination effectively *in vitro* and in mice (18, 20) and to reduce the incidence of CDI in hamsters in combination with vancomycin. In contrast, the *ortho*-isomer (CaoSA) and the *para*-isomer (CapSA) of CamSA did not inhibit *C. difficile* spore germination *in vitro* (18). However, due to the highly dynamic nature of *in vivo* testing (50), these isomers were assessed for activity in hamster CDI. As expected, both CaoSA and CapSA were ineffective in the prevention of CDI in hamsters, even when combined with vancomycin. These data support earlier hypotheses that putative *C. difficile* spore germination receptors are very selective in binding germinants and inhibitors such as CamSA (19, 51, 52).

In conclusion, CamSA is a nontoxic, highly valuable tool for the investigation of *C. difficile* spore germination in rodent models of CDI. Importantly, CamSA does not cause major changes to the gut microbiota. CamSA reduces the incidence of CDI in hamsters when administered in conjunction with low doses of vancomycin. In this study, CamSA was unable to prevent CDI recurrence in hamsters. Antigermination treatments continue to prove to be a novel method worth further exploration for the prevention of CDI and the elucidation of this disease *in vivo*.



## MATERIALS AND METHODS

**Materials.** Bile salts were purchased from Sigma-Aldrich (St. Louis, MO) or were synthesized in the Abel-Santos laboratory (18). *Clostridium difficile* selective agar (CDSA) and brain heart infusion (BHI) medium were purchased from BD Biosciences (Franklin Lakes, NJ). ChromID *C. difficile* agar was purchased from bioMérieux (Marcy l'Etoile, France), and the PRO disc kit was purchased from Remel (Lenexa, KS). *C. difficile* strain 630 (ATCC BAA-1382) was purchased from ATCC (Manassas, VA). Reagents for DNA isolation were obtained from Qiagen (product no. 51504; Qiagen, Germantown, MD). Reagents for PCR were obtained from Quantabio (product no. 2200410; Quantabio, Beverly, MA).

**Animals.** The institutional animal care and use committee at the University of Nevada, Las Vegas, approved the animal protocol (protocol R0411-266) used in this study. All experiments were performed according to the National Institutes of Health guidelines in the Guide for Care and Use of Laboratory Animals. Weaned female golden Syrian hamsters (3 to 4 weeks of age) were purchased from Harlan Laboratories (Indianapolis, IN) and Charles River (Wilmington, MA). Animals were housed individually and fed *ad libitum*. Water, food, and bedding were autoclaved prior to use. Animals were allowed to acclimate in the animal facility for 1 week. The following protocols involved animals 5 to 8 weeks of age. Animals were divided into experimental groups of 5, and each experiment was repeated at least twice, with the exception of the CamSA analog experiment (described below).

**CamSA toxicity.** Three groups of 5 hamsters each were analyzed for CamSA-mediated toxicity. The first group was treated with 135  $\mu$ l of DMSO, by oral gavage, once daily for 30 days, the second group was treated with 300 mg/kg CamSA dissolved in DMSO, by oral gavage, once daily for 30 days, and the third group received water *ad libitum*. Animals were weighed daily and observed for external signs of toxicity. Fecal samples were collected and analyzed as discussed below. On day 30, hamsters were sacrificed and their colons were removed for histological studies.

**Histology.** Colon tissue was sectioned and prepared for histological analysis as described previously (24, 53). Briefly, tissues were snap-frozen in Cryo-OCT (Thermo Scientific, Waltham, MA) and stored at  $-80^{\circ}\text{C}$ . Ultrathin sections were prepared using a Vibratome UltraPro 5000 cryostat set at 50 nm. Tissue samples were fixed for 15 min in cold acetone and stored at  $-80^{\circ}\text{C}$ . Samples were stained using the Rapid Chrome hematoxylin and eosin (H & E) kit (Thermo Scientific, Waltham, MA) and visualized by light microscopy. Colon samples were blindly evaluated for epithelial damage, integrity of the mucosal lining, and separation of the submucosa.

**Effects of CamSA on the hamster microbiome.** Fecal samples for microbiome analysis were collected aseptically, within 3 h after deposition, on day 0 and day 30 from a cohort of 5 hamsters treated with either DMSO or 300 mg/kg CamSA. Fecal samples from DMSO-treated hamsters and CamSA-treated hamsters were resuspended in Qiagen ASL buffer, flash frozen, and archived at  $-80^{\circ}\text{C}$ . DNA was harvested from freshly thawed samples using the QIAamp DNA stool minikit and was quantified using a NanoDrop 2000 spectrophotometer. Amplification and sequencing of the V4 region of 16S rRNA genes of bacteria and archaea were performed as described by Kozich et al. (54), with the following modifications: (i) to be more inclusive of several archaeal lineages, forward primer 515F was modified to contain a C or T at the 4th position from the 5' end (5'-GTGYCAGCMGCCGCGGTAA) (55), and a corresponding modification was made to the read 1 sequencing primer; (ii) 5 Prime HotMasterMix DNA polymerase was used; (iii) 33 cycles were used for PCR; and (iv) sequencing on the Illumina MiSeq platform was performed at Micro-Seq Enterprises (Las Vegas, NV). Paired-end Illumina MiSeq reads were quality filtered, aligned, and analyzed using Qiime2 (56). A single sample (CamSA treatment, day 30, hamster 5) yielded fewer than 1,000 quality sequences and was removed from analysis. The remaining reads were truncated at the first position with a PHRED score of  $<30$ , quality filtered, and clustered into sequence variants (SVs) using the q2-dada2 plugin (57) in Qiime2 version 2017.6 (56). Each SV was classified under the SILVA NR 99 reference database (version 128), using the classify-sklearn function of the q2-feature-classifier plugin (56). SVs identified as mitochondrial, chloroplast, or from an unknown domain were removed. SVs were aligned using mafft (58) through the q2-alignment plugin, with default settings. Samples were rarified to contain 13,318 features. Alpha diversity metrics (richness, Shannon diversity, Faith's phylogenetic diversity, and Pielou's evenness) were calculated using the q2-diversity plugin (56). Kruskal-Wallis tests were performed in R version 3.4.1 (56). Proportions of taxa at each taxonomic rank were calculated using the R package phyloseq version 1.20.0 (59) and were visualized using ggplot2 version 2.2.1 (60). Bray-Curtis dissimilarity, Adonis, and SIMPER analyses were performed using phyloseq and vegan version 2.4.4 (61), and results were visualized with NMDS using vegan. Adonis partitions the variance similar to analysis of variance (ANOVA) and tests whether the variation within a category is smaller or larger than the variation between categories; it calculates a pseudo- $F$  value, a  $P$  value, and a correlation coefficient ( $R^2$ ). Data categories with Adonis  $P$  values of  $<0.05$  were considered significantly different.

**Preparation of *C. difficile* spores.** Spores were purified following the method described by Howerton et al. (20). Briefly, *C. difficile* 630 was plated on BHI agar supplemented with 1% yeast extract, 0.1% L-cysteine HCl, and 0.05% sodium taurocholate, to yield single-cell clones. Individual *C. difficile* colonies were grown in BHI broth and replated to confluence. Plates were incubated anaerobically for 7 days at  $37^{\circ}\text{C}$ . Bacterial lawns were harvested by washing with ice-cold nanopure water and gentle scraping. The cell mass was pelleted and washed three times. Pellets were then centrifuged through a Histodenz gradient. Purified spores were washed and analyzed using the Shaeffer-Fulton staining method (62), to ensure  $>95\%$  pure spores.

Before infection, *C. difficile* spores were heat activated at  $68^{\circ}\text{C}$  for 30 min and washed. Purified spores were resuspended in water to an optical density at 580 nm ( $\text{OD}_{580}$ ) of 1.0. Spore aliquots were serially diluted onto supplemented BHI agar to enumerate CFU.

**Effects of CamSA on CDI.** To determine the effects of CamSA on hamster CDI, 30 animals were oro-gastrically dosed with 30 mg/kg clindamycin 1 day prior to infection (day -1). On day 0, animals were challenged with 100 *C. difficile* spores (25, 48). A test group ( $n = 15$ ) was treated daily with CamSA at 300 mg/kg, by oral gavage, for 4 consecutive days starting at day -1. An infected control group ( $n = 15$ ) was treated with DMSO, also by oral gavage. Fecal samples were collected using sterilized spatulas, at preselected time points (see below). Animals were observed twice daily and scored for CDI sign progression based on the following rubric: mild lethargy (score of 1), severe lethargy (score of 2), wet/sticky feces (score of 1), runny diarrhea (score of 2), wet tail and pink anogenital area (score of 1), wet anogenital area (score of 2), hunched posture (score of 2). Animals scoring  $\geq 5$  were considered moribund and were euthanized. Animals scoring  $\leq 1$  were indistinguishable from uninfected controls.

**Enumeration of *C. difficile* from hamster feces.** Fecal samples were weighed and homogenized in sterile water to a concentration of 0.05 mg/ml. An aliquot of the fecal suspension was heated to 68°C for 15 min. Heated and unheated fecal samples were serially diluted and plated on CDSA and ChromID *C. difficile* selective agar (63, 64). Plates were incubated anaerobically for 48 h, and CFU were counted. CFU obtained from unheated samples represent the sum of *C. difficile* vegetative cells and spores. CFU obtained from heated samples represent the number of *C. difficile* spores only. The PRO disc kit was used to confirm *C. difficile* identity (63, 65).

**Effects of coadministration of CamSA and vancomycin.** Animals were treated with 30 mg/kg clindamycin, by oral gavage, 1 day prior to challenge (day -1). On day 0, animals were challenged with 100 *C. difficile* spores, by oral gavage. Vancomycin (5 mg/kg) was administered once daily for 3 consecutive days starting at day 0, also by oral gavage (22–24). Starting at day -1, animals were treated once daily with 0 mg/kg ( $n = 10$ ) or 300 mg/kg CamSA for 6 ( $n = 10$ ), 10 ( $n = 10$ ), or 19 ( $n = 10$ ) consecutive days. Another group of animals ( $n = 4$ ) was treated with a lower dose of CamSA (50 mg/kg) for 10 consecutive days or until the animals were moribund. CamSA or DMSO (control) was administered by oral gavage. CDI signs were scored up to 30 days post-CamSA treatment. Fecal samples were analyzed as described above. Selected animals from control groups and the 19-day CamSA treatment group were necropsied after euthanasia, their gastrointestinal tracts were removed and observed for visual signs of disease, and the colons were evaluated by histological staining.

**Effects of coadministration of CamSA analogs CaoSA and CapSA on hamster CDI.** CamSA is a *meta*-aminosulfonate cholate analog. To determine whether the *para*- or *ortho*-isomers of CamSA prevent CDI in hamsters, *C. difficile*-challenged hamsters were treated with 5 mg/kg vancomycin, as described above. Starting at day -1, 300 mg/kg of the *meta*-analog CamSA ( $n = 10$ ), the *ortho*-analog CaoSA ( $n = 5$ ), or the *para*-analog CapSA ( $n = 5$ ) (18) was administered once daily by oral gavage for 10 consecutive days or until animals became moribund. CDI signs were scored as described above.

**Effects of coadministration of CamSA and vancomycin in a CDI delayed-treatment model.** Following previous models for CDI relapse (23, 24, 26, 66), clindamycin-treated hamsters were challenged with *C. difficile* spores, as described above. The beginning of vancomycin treatment (5 mg/kg) was delayed for 24 h after the challenge (day 1), and vancomycin was administered once daily for 5 days. Starting at day -1, animals were also administered 300 mg/kg CamSA once daily for 10 days ( $n = 10$ ) or 17 days ( $n = 20$ ) or until the animals became moribund. The remaining challenged animals ( $n = 10$ ) were treated with DMSO. All treatments were administered by oral gavage. CDI signs were scored as described above.

**Statistical analysis.** Microbiome data were analyzed statistically using pairwise Kruskal-Wallis tests (alpha diversity) or Adonis (beta diversity), as described in more detail above. Hamster survival was assessed by Kaplan-Meier analysis. Statistical comparisons of survival curves were calculated using a log-rank test in R. The scores for the severity of signs were analyzed as box-and-whisker plots. Data were expressed as the mean  $\pm$  standard deviation in box-and-whisker plots. Standard deviations represent at least 3 independent measures. Student's unpaired *t* test was used to determine the significance of differences of means. The data on CFU per milligram of feces were analyzed as box-and-whisker plots. A two-way mixed ANOVA was performed to assess the significance of the differences of between-group means (CFU per milligram of feces for untreated hamsters versus CamSA-treated hamsters per day) and within-group means (changes in CFU per milligram of feces over time within experimental groups). The 5 hamsters that died by day 8 in the control group were excluded from this analysis because of the criteria for the ANOVA calculation.

**Accession number(s).** Files containing the original unfiltered sequences are available from the NCBI Sequence Read Archive under project no. PRJNA376248. The following Qiime2-compatible supplemental files are available at <https://github.com/hedlundb/MACamSA>: metadata, unfiltered feature table, representative sequences, and SILVA taxonomy.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02251-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 1.0 MB.

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