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Administration of Probiotic Kefir to Mice with *Clostridium difficile* Infection Exacerbates Disease

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Abstract

Lifeway® kefir, a fermented milk product containing 12 probiotic organisms, is reported to show promise as an alternative to fecal microbiota transplantation for recurrent *Clostridium difficile* infection (CDI). We employed a murine CDI model to study the probiotic protective mechanisms and unexpectedly determined that kefir drastically increased disease severity. Our results emphasize the need for further independent clinical testing of kefir as alternative therapy in recurrent CDI.

Keywords

Clostridium difficile; toxin; kefir; probiotic; fecal microbiota transplantation

A continuous rise in *Clostridium difficile* infection (CDI) coupled with the increased threat of antimicrobial resistance and limited antibiotic treatment options for this pathogen has generated a great deal of interest in alternative therapy [1–8]. CDI occurs when the normal gastrointestinal (GI) microbiota is disrupted; typically by antibiotic treatment - one of the major risk factors for contracting CDI. When conventional antibiotic therapy fails to resolve CDI, fecal microbiota transplantation (FMT) is an effective therapy that restores the normal microbial balance and combats CDI. While FMT is the most effective treatment option for patients suffering from recurrent episodes of CDI [9, 10], it is considered an investigational therapy by the US Food and Drug Administration and may not be covered by third-party payers [11]. Additionally, a recent case report highlighting new-onset obesity in a patient

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receiving stool from an overweight donor [12] raises concerns about the long-term health effects of FMT. Due to costs related to the lack of insurance coverage, unavailability of treatment providers and/or other confounding health factors, FMT is not always a viable option for CDI patients. As a result, alternative prevention and treatment options are currently being investigated.

Lifeway® kefir (www.kefir.com) is a fermented dairy product containing 12 live probiotic organisms that has recently been described as a successful, low-cost, non-invasive alternative to FMT [13, 14]. In 2009 [13] and 2014 [14], Bakken reported that the majority (84%) of patients diagnosed with recurrent CDI (rCDI) given Staggered and Tapered Antibiotic Withdrawal (STAW) therapy with Lifeway® kefir supplementation saw complete resolution of symptoms. The STAW treatment consisted of a 6 week staggered and tapered metronidazole or vancomycin regimen accompanied by a minimum of three Lifeway® kefir drinks each day and for 2 months following the 6 week STAW therapy. These reports demonstrate that supplementation of extended antibiotic therapy with a cocktail of beneficial microbes is as efficacious as FMT in adult patients with rCDI. To date, these results make this one of the most promising probiotic therapies for treating severe, recurring CDI.

Our objective was to demonstrate the efficacy of Lifeway® kefir probiotics in an established mouse model of CDI [15] to begin to unravel mechanisms of how the beneficial microbes protect against disease. Lifeway® kefir products contain 7–10 billion colony forming units of 12 different probiotic organisms (listed in Table 1), many of which are intrinsically resistant to antimicrobials used to treat rCDI. A meta-analysis (*data not shown*) of published microbiome studies [16–21] with samples from patients with antibiotic associated diarrhea or CDI showed that antibiotic treatment results in an expansion of GI lactic acid bacteria, presumably due to the intrinsic antibiotic resistance of these organisms which allows them to persist in this environment. While there are relative expansions of GI lactic acid bacteria in patients receiving antibiotics, operational taxonomic units (OTUs) closely related to the probiotic organisms found in Lifeway® kefir are not always present in patients with or without CDI (Table 1). Bakken's studies indicate that providing them in the diet of patients with rCDI is effective at ameliorating disease recurrence.

In line with the Bakken study [14], we delivered multiple doses of kefir daily to mice infected with *C. difficile*. Eight-week-old C57BL/6 female mice were purchased from Jackson Laboratories and were administered antibiotics and probiotics as outlined in Figure 1. All protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. CDI induction in mice was carried out as previously described [15, 22], with minor modifications. Mice (n= 5/group) were challenged orally with 1×10^6 *C. difficile* VPI 10463 (ATCC 43255) spores [23, 24]. To determine the effects of Lifeway® kefir on disease progression in mice with CDI, animals were gavaged with 200 μL of either kefir or 1X phosphate buffered saline (PBS) prior to and during CDI (Figure 1). Stool specimens were collected and the health of each animal scored daily. Health scoring was completed by a single individual (A.B.) over the course of the experiment and consisted of numerical values (0–3; higher numbers being reflective of greater disease) for body condition, appearance, GI disease, natural behavior and provoked behavior. Moribund animals were euthanized and surviving animals were euthanized at day seven.

C. difficile challenge resulted in weight loss (Figure 2A) and declining health (Figure 2B) for all groups within 2–3 days post infection (dpi). Animals receiving kefir three times daily in the presence of *C. difficile* infection exhibited the greatest weight loss and health decline with all animals in this group becoming moribund by 3 dpi (Figures 2B & 2C). Infected control animals receiving PBS twice daily became ill, but not moribund and all animals in this group began to recover by 4 dpi. Of the infected animals receiving kefir twice daily, one became moribund on 3 dpi with the remaining animals recovering by 4 dpi. The non-infected group (Kefir 3x Ctrl) was monitored to 4 dpi and did not experience weight loss or health decline due to thrice daily kefir gavages in the absence of *C. difficile* demonstrating that disease was not caused by the probiotic itself.

Concentrations of *C. difficile* in stool were determined to establish whether increased pathogen concentrations were responsible for the severity of disease observed in the clinically recommended kefir treatment group. Microbial DNA was isolated from stool specimens [25, 26] and relative *C. difficile* content was quantified by qPCR using primers specific to the *C. difficile* 16S rDNA gene [27]. Quantities of *C. difficile* 16S rDNA copies are normalized by total DNA concentration and data are represented in Figure 2D. In all groups, relative *C. difficile* concentrations increased following challenge at day 0 as expected. The mean relative concentration of *C. difficile* in both kefir treated groups was greater than the PBS group at 1 dpi (Figure 2D), however these differences are not significant (p value > 0.05) and do not explain the increased disease severity observed in the groups receiving daily kefir. Futhermore, toxin levels in stool measured by TcdA and TcdB ELISA were not dramatically elevated in infected animals receiving kefir (*data not shown*). Therefore, greater disease severity in animals receiving kefir was not a result of increased *C. difficile* bacterial load or toxicity. Increased host susceptibility to *C. difficile* is a potential disease outcome associated with Kefir supplementation in mice, possibly mediated by inhibiting protective innate immune responses [28, 29].

The results of this study were unexpected considering Bakken reported no adverse effects in CDI patients that participated in either Lifeway® kefir study [13, 14]. A limitation of our study includes the use of a gold standard lab-adapted high toxin producing *C. difficile* strain (VPI). Kefir may be more effective at preventing infection with clinical isolates of *C. difficile.* While we show that disease severity is not a result of greater *C. difficile* concentrations or toxicity, it is possible that kefir increased host sensitivity in these animals pointing to potential species differences. However, organisms isolated from kefir are reported to be beneficial in several mouse models, including CDI where mice treated orally with Kefir-derived *Lactobacillus kefiri* showed no adverse effects [30]. A mouse model of giardiasis showed that oral administration of kefir reduced infection with *Giradia intestinalis* by activating a variety of humoral and cellular immune responses typically downregulated during parasitic infection [31]. In a mouse model of non-alcoholic fatty liver disease (NAFLD), kefir was administered to mice orally for four weeks and improved symptoms of NAFLD by inhibiting the lipogenesis pathway [32]. Kefir has even shown protective effects against x-ray irradiation-induced intestinal damage in mice [33]. In each of these cases, either kefir or kefir-derived microbes were administered orally to mice without adverse effects.

Our results demonstrate the difficulty in studying translational therapies in animal models. While Bakken has provided solid evidence to support kefir as a viable supplement to STAW therapy in patients with recurrent CDI, kefir was not protective in this animal study. Our results highlight the need for repeated clinical trials using kefir in larger and independent patient cohorts. Due to the escalating incidence and mortality rates associated with CDI and its unique relationship with antibiotic treatment, the Centers for Disease Control and Prevention (CDC) have classified *C. difficile* as an urgent public health threat [2]. Limited treatment options [9] and recurrence rates upward of 35% [6, 7, 9, 34–37] emphasize the importance of finding alternative treatment and prevention options for CDI. Despite the results of a 2013 Cochrane review reporting probiotics to be both safe and effective for preventing CDI [38], clinical communities are presently working from outdated 2010 SHEA/IDSA guidelines [39] that are not supportive of using probiotics as prevention strategies to CDI. There is an urgent need supported by clinical data [4, 13, 14, 38, 40] to continue striving for better model systems that will drive research progress of alternative therapies like probiotics for CDI prevention towards translation.

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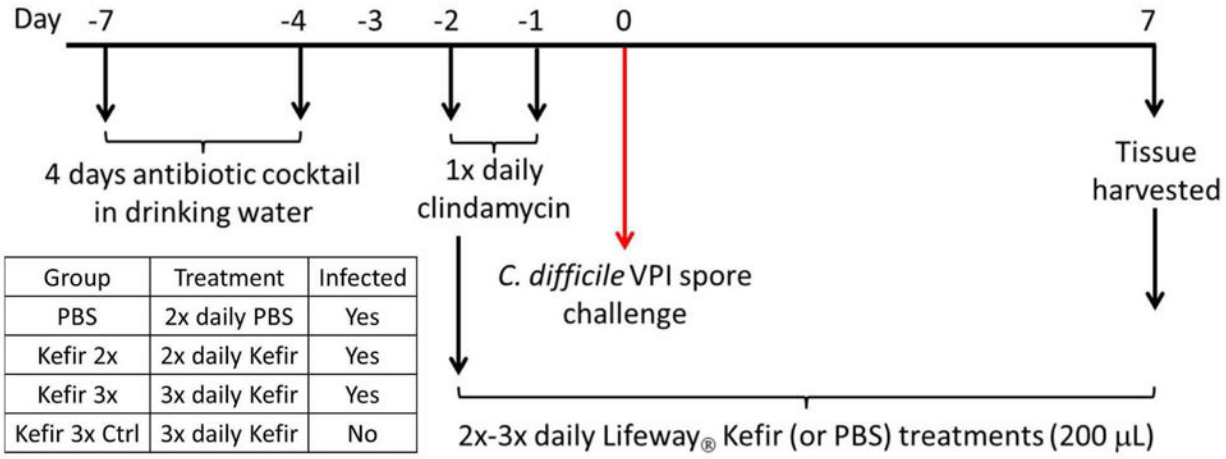
Research Highlights

- Lifeway Kefir probiotic supplementation exacerbates *C. difficile* disease in mice.
- Probiotic-induced disease is unrelated to *C. difficile* burden or toxin production.
- Profound host-differences in probiotic efficacy highlight the need for additional clinical trials.



Group	Treatment	Infected
PBS	2x daily PBS	Yes
Kefir 2x	2x daily Kefir	Yes
Kefir 3x	3x daily Kefir	Yes
Kefir 3x Ctrl	3x daily Kefir	No

Figure 1.

Experimental design of kefir supplementation prior to and after challenge with 1×10^6 *C. difficile* VPI spores. All groups (n=5/group) received antibiotic cocktail in drinking water, followed by 2 days of single IP clindamycin injections. The PBS, Kefir 2x, and Kefir 3x groups received a subsequent challenge of *C. difficile* VPI spores resulting in a uniform and severe clinical illness. The Kefir 3x Ctrl group was not infected with *C. difficile* and was observed up to day 4.

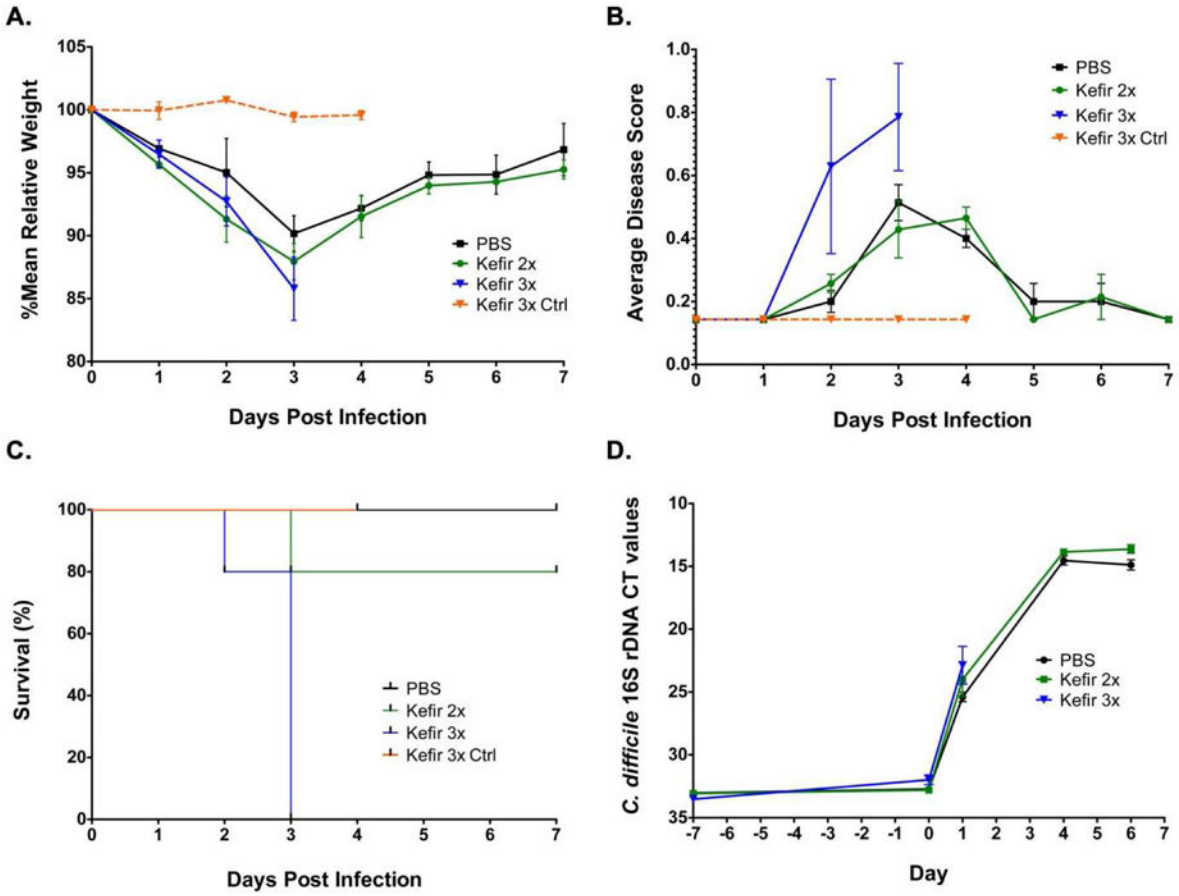


Figure 2.

Supplementation with kefir does not protect against CDI in mice. (A) Mean relative weights of surviving mice based on the weight at day 0; (B) Average disease scores for groups post infection and the non-infected group; (C) Kaplan-Meier survival plot of mice treated with PBS or kefir; (D) Concentrations of *C. difficile* in infected animals as determined by *C. difficile* 16S rDNA specific qPCR, normalized to DNA concentration. Panels A, B, and D represent means ± standard error, and a repeat measure of ANOVA with a Tukey post hoc analysis revealed no significant difference (p-value > 0.05) between groups in panel A (weights) or D (quantity of *C. difficile*). The log-ranked Mantel-Cox test revealed significant differences (p-value = 0.0001) in survival between infected PBS controls and infected animals receiving kefir three times daily (panel C).

Table 1

Presence of OTUs closely related to Lifeway® Kefir probiotic organisms in healthy subjects and CDI patients as determined by 16S rDNA sequencing from multiple clinical studies.

	B. breve	*B. lactis*	*B. longum*	*L. acidophilus*	*L. casei*	*L. lactis*	*L. plantarum*	*L. reuteri*	*L. rhamnosus*	*Leuconostoc cremoris*	*S. diacetylactis*	*Saccharomyces florentinus*
Healthy			•	•	•			•	•			N/A
CDI	•		•	•	•			•	•			N/A