Probiotic product enhances susceptibility of mice to cryptosporidiosis

Running title: Probiotics and cryptosporidiosis

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Abstract

Cryptosporidiosis, a leading etiology of infant diarrhea, is caused by apicomplexan parasites classified in the genus *Cryptosporidium*. The lack of effective drugs is motivating research to develop alternative treatments. To this aim, the impact of probiotics on the course of cryptosporidiosis was investigated. The native intestinal microbiota of specific pathogen-free immunosuppressed mice was initially depleted with orally administered antibiotics. A commercially available probiotic product intended for human consumption was subsequently added to the drinking water. Mice were infected with *Cryptosporidium parvum* oocysts. On average, mice treated with probiotic developed a more severe infection. The probiotics significantly altered the fecal microbiota, but no direct association between ingestion of probiotic bacteria and their abundance in fecal microbiota was observed. These results suggest that probiotics indirectly altered the intestinal microenvironment or the intestinal epithelium in a way that favors proliferation of *C. parvum*.

Importance

The results show that *C. parvum* responds to changes in the intestinal microenvironment induced by a nutritional supplement. This outcome paves the way for research to identify nutritional interventions aimed at limiting the impact of cryptosporidiosis.

KEYWORDS: *Cryptosporidium*, cryptosporidiosis, probiotics, fecal microbiota

INTRODUCTION

In humans, cryptosporidiosis is an enteric infection caused mostly by two species of *Cryptosporidium* parasites, *C. parvum* and *C. hominis*. Transmission occurs when infectious oocysts are ingested, either with contaminated food and water (1, 2), by fecal-oral contact and possibly by inhalation (3). Recent surveys have revealed the high prevalence of cryptosporidiosis among infants living in developing nations, where it causes substantial morbidity and mortality in infants less than 2 years of age (4). The treatment of cryptosporidiosis is limited to supportive care since no effective drugs are available. As no vaccines are available either, hygiene and water sanitation to reduce transmission remain the most effective preventative approaches.

Using experimental mouse infections, we previously showed that cryptosporidiosis changes the gut microbiota (5). Given the importance of the microbiota to the physiology of the gut, here we investigated whether a reverse effect, of the microbiota on the parasite, could be demonstrated. We reasoned that the unmet need for anti-*Cryptosporidium* drugs could be alleviated by probiotics or dietary supplements. This hypothesis does not necessarily imply that the microbial community of the gut directly impacts the parasite. Indeed, the transient nature of *Cryptosporidium* extracellular stages limits the interaction between the resident microbiota and extracellular cryptosporidial life stages, including sporozoites, merozoites and gametes. It is conceivable that the microbiota impacts parasite proliferation by modulating epithelial integrity, impacting the protective mucus layer, or stimulating innate and acquired immune cells.

The literature on the impact of the intestinal microbiota on cryptosporidiosis is sparse. A few studies have investigated the impact of *Cryptosporidium* parasites on the gut microbiota, but the effect of the gut environment on the course of the infection is not understood and underlying

mechanisms are unknown. Using germ-free severe combined immune deficiency (SCID) mice compared to SCID mice colonized with intestinal microbes, Harp et al. showed that normal intestinal microbiota delayed the onset of *C. parvum* oocyst excretion by several weeks (6). These authors also showed that resistance of mice to *C. parvum* can be increased by transferring intestinal mucosa from resistant animals to susceptible infant mice (7). A protective role of the gut microbiota against cryptosporidiosis was also observed in neonatal mice (8, 9). This research found that gut microbiota synergized with poly(I:C) to elicit a protective intestinal immunity against *C. parvum*. A study on the effect of inosine monophosphate dehydrogenase inhibitors in *C. parvum* infected mice detected an increase in *C. parvum* virulence in response to the drug. This effect was attributed to an alteration of the intestinal microbiota (10).

Research on the effect of probiotics using animal models of other infectious diseases has generated diverging results. A mouse model of rotavirus infection was used to show that administration of *Lactobacillus reuteri* reduced the duration of diarrhea (11). Similarly, and consistent with what has been observed in human trials, probiotics administered to mice had a mitigating impact on colitis induced by *Citrobacter rodentium* (12). The significant public health impact of nosocomial *Clostridium difficile* infection has generated a large body of research, including experiments in mice aimed at testing the benefit of fecal transplant (13) and defined probiotics (14-16). Only a few studies report a worse outcome with probiotic treatment. Working with the cichlid fish tilapia, Liu et al. found that a 14-day treatment with probiotics made fishes more susceptible to infection with *Aeromonas hydrophila* after the treatment was discontinued (17). More relevant to the present study, research with mice showed that supplementation of diet with kefir exacerbated the outcome *Clostridium difficile* infection (18). Indicating that a harmful

effect of probiotics is an unusual observation, no other studies in rodent or mammalian models demonstrating increased susceptibility to infection appear to have been published.

We previously reported a significant impact of cryptosporidiosis on the profile of the bacterial intestinal microbiota (5). Replicated experiments with two *C. parvum* isolates comprising two infected and two control groups of mice revealed that the intestinal microbiota of infected animals differed from that of uninfected animals, regardless of the *C. parvum* isolate. A taxonomic analysis of bacterial taxa highlighted two unclassified Bacteroidetes Operational Taxonomic Units (OTUs), Prevotellaceae and Porphyromonadaceae as overrepresented in the feces of infected mice, whereas OTUs most over-represented in uninfected mice were classified as Porphyromonadaceae and one unclassified Bacteroidetes OTU.

Probiotics are typically defined as microorganisms, mostly bacteria, that are consumed with the goal of improving health (19). Examples of gastro-intestinal conditions which have been the focus of human clinical trials of probiotics treatment include inflammatory bowel diseases, antibiotics-associated diarrhea following *C. difficile* infection, peptic ulcer associated with *Helicobacter pylori* infection and giardiasis (20). With an eye on developing alternative treatments for cryptosporidiosis, the experiments described here were aimed at assessing whether probiotics can influence the course of cryptosporidiosis. Against expectations, we found that mice consuming probiotics developed a more severe infection. Although the goal of this research is to find treatments that mitigate cryptosporidiosis, the results are significant because they show that *C. parvum* proliferation responds to relatively minor changes in the intestinal microbiota. These observations open the way for targeted editing of the intestinal microbiota (21-23) as a low-cost approach to reducing the impact of these parasites.

RESULTS

Probiotic increases severity of infection. To test whether the probiotic product impacts the severity of *C. parvum* infection, fecal oocyst output was measured by flow cytometry (FCM) as described above. In experiment 1, a total of 92 oocyst concentration values were acquired from 16 mice and six timepoints over a 15-day period. In experiment 2, 79 datapoints were obtained from the same number of mice and in experiment 3 12 timepoints for 4 groups generated 48 FCM values (Fig. 1, Table 1). In experiment 1, mice which received probiotic excreted a significantly higher concentration of oocysts. A mean oocyst output of 76,463 oocysts/g feces (n =44) was measured against a mean of 26,732 oocysts/g for the control mice (n=48). The difference between treatments was highly significant (Mann-Whitney U=541, $p<0.001$). An analogous significant probiotic effect was obtained for experiment 2; $mean_{probiotic}$=378,736 oocysts/g, $n_{probiotic}$=37, $mean_{control}$=68,778 oocysts/g, $n_{control}$=42; U=269.5, $p<0.001$). Similarly, in experiment 3, the priobiotics effect was significant (Kruskal-Wallis 1-Way Analysis of Variance (ANOVA) on ranks, p=0.001). Fig. 1 shows the pattern of normalized oocyst output for the three experiments plotted on a log scale.

Probiotic treatment significantly impacts fecal microbiota. To assess whether probiotic treatment impacted the fecal microbiota, pairwise weighted UniFrac distances (24) between sequence data from 64 experiment 1 fecal samples were visualized on a Principal Coordinate Analysis (PCoA) plot (Fig. 2). Samples collected starting on day 5 of probiotic treatment (day 4 post-infection (PI)) until day 16 of treatment (day 15 PI) were included. The number of samples in this analysis is smaller than shown in Fig. 1, because not all fecal samples were sequenced. Consistent with an impact of probiotic consumption on the profile of the intestinal microbiota,

131 this analysis revealed a non-overlapping distribution of datapoints according to treatment.
132 ANOSIM (25) was used to check the significance of the treatment effect. The test returned a
133 highly significant *R* value of 0.305 ($p<10^{-5}$). Analogous results were obtained for experiment 2
134 based on 55 samples collected starting on day 10 after probiotic treatment was initiated (day 9
135 PI) until day 20 (day 19 PI). As for experiment 1, clustering by treatment was significant (R =
136 0.210, $p<10^{-5}$). In experiment 3, microbiota clustering by treatment was also significant (R=0.09,
137 n=34, p=0.002).
138
139 **Bacterial α-diversity does not correlate with oocyst output.** Having detected an impact of
140 probiotic consumption on oocyst output and on the fecal microbiota, the FCM and small-subunit
141 (16S) ribosomal RNA data were analyzed jointly to identify possible associations between
142 bacterial microbiota profile and severity of cryptosporidiosis. These analyses included all
143 samples for which 16S and FCM data were acquired. A total of 44 samples each were included
144 in experiment 1 and 53 in experiment 2. As dysbiosis is typically characterized by low bacterial
145 diversity and is often associated with increased susceptibility to enteric infections (26), the first
146 global analysis examined the correlation between microbiota α-diversity (within-sample
147 diversity) and total oocyst output (Fig. 3). Regardless whether data were pooled by experiment,
148 or samples from probiotic-treated and control mice were analyzed separately, the correlation
149 between α-diversity and oocyst output was very low, explaining 9% of oocyst count at most.
150 These analyses indicate that the gut of mice excreting large numbers of oocysts, in general, is not
151 populated with less diverse microbiota.
152 Redundancy Analysis (RDA) was used to assess whether fecal oocyst concentration
153 significantly correlated with microbiota profile and identifying bacterial OTUs correlating in

154 relative abundance with oocyst output. In experiment 1, a Monte Carlo test with 44 samples
155 collected over the same time period as shown in Fig. 1 and 1000 permutations indicated a
156 significant correlation between oocyst concentration and OTU profile (pseudo-F=1.0, p=0.014).
157 This effect remained significant (F=2.7, p=0.024) after accounting for treatment, i.e., defining
158 treatment (±probiotic) as covariate, or accounting for "mouse" (defining "mouse" as covariate;
159 pseudo-F=3.8, $p=6x10^{-5}$). The analogous test in experiment 2 also returned a significant pseudo-
160 F ratio of 4.2 (n=44, p=0.0016). If removing the effect of treatment or "mouse" by defining these
161 variables as covariates, the association remains significant (pseudo-F=2.6; p=0.015 and pseudo-
162 F=5.2; $p=3x10^{-4}$, respectively). These and subsequent analyses were not performed for
163 experiment 3 because samples from mice in the same group were pooled.
164 Reasoning that the microbiota at the time of infection could be important for subsequent
165 parasite proliferation, we analyzed whether the microbiota in treated and control mice differed on
166 the day of infection, i.e., 1 and 2 days after microbiota administration was initiated in experiment
167 1 and 2, respectively. In both experiments, a signficant correlation between probiotic treatment
168 and composition of the bacterial fecal microbiota was observed (experiment 1 pseudo-F=5.3,
169 p=0.028; experiment 2 pseudo-F=3.5, p=0.055). The permutation test is explained in Materials
170 and Methods.
171
172 **High abundance of facultative anaerobes in severe infections.** Having identified a significant
173 correlation between fecal oocyst concentration and OTU profile, the taxonomic make-up of the
174 fecal microbiota was examined in more detail. First we used program LEfSe (27) to identify
175 OTUs which significantly define the difference between samples containing high and low oocyst
176 concentration. This analysis was based on 20 samples for each experiment, 10 samples with the

highest oocyst concentration and 10 samples with the lowest concentration collected from the entire experiment. In experiment 1, 7/10 samples in the high-oocyst group originated from mice treated with probiotics and 7/10 samples in the low-oocyst group came from control mice (Chi-square=3.2, p=0.07). In experiment 2, 9/10 samples in the high-oocyst group originated from treated mice and 8/10 samples in the low-oocyst group originated from control mice (Chi-square=9.9, p=0.002). Feces from highly infected animals were characterized by a high Proteobacteria abundance, whereas feces from lightly infected animals were significantly enriched for Firmicutes. As observed for the impact of the probiotic on the severity of cryptosporidiosis (Fig. 1) and on the global microbiota profile (Fig. 2), a similar shift towards higher Proteobacteria abundance was observed in the two experiments (Fig. 4).

A second taxonomy analysis aimed at identifying OTUs enriched in highly infected animals was performed with RDA (28). For experiment 1, in the 10 OTUs which best correlated in relative abundance with oocyst concentration, 61% of the sequences were classified as *Lactobacillus*, 24% as *Proteus* and 14% as *Enterococcus*. In the 10 OTUs which most negatively correlated with oocyst concentration, as seen in with LEfSe, *Blautia* was the most abundant classification (61% of reads), followed by Clostridiaceae (27%), Lachnospiraceae (10%), *Romboutsia* (8%) and unclassified Firmicutes. For experiment 2, the identical analysis of the 10 OTUs which best correlated in relative abundance with oocyst concentration, 92% of sequences originated from Enterobacteriaceae and 8% from Firmicutes. In the 10 OTUs with lowest oocyst concentration 70% of sequences were classified as *Lactobacillus*, 21% as *Turicibacter*, 6% as unclassified Lactobacillales and 1% as Stenotrophomonas (phylum Proteobacteria) (Table S1). The results obtained with RDA are thus is close agreement with the LEfSe results shown in Fig. 4.

200

201 **Severity of infection correlates with fecal microbiota profile.** Fig. 3 illustrates an important
202 difference between the experiments; mean fecal oocyst concentration across all time points,
203 treatments and mice in experiment 2 was 2.4 x 10^5 oocysts/g (n=79, SD=3.4 x 10^5), or about 5
204 times higher than in experiment 1 (mean=5.0 x 10^4 oocysts/g, n=92, SD=8.7 x 10^4) and >8 times
205 higher than experiment 3 (mean=2.8 x 10^4, n=48, SD=4.6 x 10^4). Oocyst output between
206 experiments was significantly different (Kruskal-Wallis ANOVA on ranks, H=34.3, 2 d.f.,
207 $p<0.001$). Pairwise comparisons between experiment 1 and 2, and experiment 3 and 2 were
208 signficant according to Dunn's multiple comparisons test, whereas experiment 1 and 3 were not.
209 It is likely that these differences was caused by the dexamethasone administration route: drinking
210 water only in experiment 1 and 3, vs. drinking water followed by subcutaneous injection in
211 experiment 2 (table 1). The more severe infection in experiment 2 represents an unplanned
212 opportunity to further assess the impact of the infection on the gut environment. If heavier
213 infections cause an increase in Proteobacteria relative abundance, as suggested by LEfSe
214 analysis (Fig. 4) and by the RDA described above, one would expect a higher proportion of
215 Proteobacteria in experiment 2 samples originating from severely infected mice, which is exactly
216 what was observed (Fig. 4 and RDA results). Further, severe cryptosporidiosis can be expected,
217 based on research on human patients suffering from other enteric infections (29-31), to lead to a
218 change in bacterial microbiota towards populations enriched for facultative anaerobes, possibly
219 resulting in loss of diversity. To test this hypothesis, mean pairwise weighted UniFrac distances
220 between microbiota from heavily infected samples were compared to distances between lightly
221 infected samples. In experiment 1, mean β-diversity (between-sample diversity) between the 10
222 high-oocyst samples was 0.536 (45 pairwise distances, SD=0.142), whereas between low-output

223 samples mean β-diversity was 0.590 (45 pairwise distances, SD=0.121; Mann-Whitney
224 T=1819.0, p=0.07). In experiment 2, the mean β-diversity values are 0.312 (45 pairwise
225 distances, SD=0.117) and 0.411 (45 pairwise distances, SD=0.137) for the 10 samples with the
226 highest and lowest oocysts concentration, respectively (Mann-Whitney U=590, p<0.001).
227 Although for experiment 1 the effect is not significant, together these results are consistent with
228 the model postulated above; i.e. that severe infection leads to a convergence of the microbiota
229 towards a population rich in Proteobacteria and low in Firmicutes.
230
231 **Loss of microbiota functional diversity in heavily infected mice.** To extend the observed
232 taxonomic differences between severely and lightly infected mice to the metagenome, program
233 PICRUSt (32) was used to infer microbiota function from OTU profiles. Given the more severe
234 infection in experiment 2, metagenome analyses are only reported for this experiment. PICRUSt
235 identified 39 KEGG level 2 categories in the combined metagenome. A PCoA based on pairwise
236 SSR distance between KEGG abundance values normalized across KEGG categories revealed a
237 tight clustering of samples with high oocyst concentration relative to the samples with lower
238 oocyst concentration (Fig. S1). This visual assessment was tested by comparing pairwise
239 distances between KEGG profiles. For the ten samples with the lowest oocyst concentration the
240 distance averaged 69.9 (SD=47.6) and for the same number of samples with the highest
241 concentration 54.8 (SD=41.41), which is statistically not significant (Mann-Whitney Rank Sum
242 test, p=0.121). If only eight samples with highest and lowest oocyst concentration were tested
243 (28 pairwise distance values for each group), the distances between the high concentration
244 samples are significantly smaller (Mann-Whitney Rank Sum test p=0.015). As for the taxonomy

analysis described above, we conclude from these results that proliferation of *C. parvum* leads to a convergence of the inferred bacterial metagenome.

As described above for the taxonomy analysis, LEfSe was used to identify KEGG pathways which differ significantly in abundance between the 10 samples from highly infected and the same number of samples from lightly infected mice. Underscoring the difference at the metagenome level between fecal samples from severely and mildly infected mice, 22 of 39 KEGG level 2 pathways were significantly different between the two groups (Table S2). In comparison to the severely infected mice, the microbiota of mild infections was characterized by a high abundance of pathways related to replication, such as carbohydrate, amino acid and nucleic acid metabolism. These results extend the taxonomy presented in Fig. 4, suggesting that the mouse dysbiotic cryptosporidiosis metagenome is selected for other functions than bacterial replication.

DISCUSSION

In a comprehensive review of the literature, Kristensen et al. (33) found a small number of publications describing randomized controlled probiotics trials which included the characterization of fecal microbiota. The surprising conclusion of this survey is that no publications reported a significant change in the microbiota based on OTU richness, evenness or diversity analysis. As no uninfected controls were included in our experiments, the probiotics' impact on the microbiota can only be observed in the initial phase of the experiment, as described above for day 1 and 2 post-initiation of the probiotics treatment. The increase in facultative aerobes later in the infection, likely represents the effect of *C. parvum* multiplication in the intestinal epithelium, rather than a direct impact of probiotics.

As we did not observe a significant increase in probiotic bacteria in the feces of treated mice, we postulate that some of the bacterial or prebiotic ingredients present in the probiotic product induced changes in the mouse intestinal environment favoring the proliferation of *C. parvum*. Proliferation of the parasite then leads to extensive secondary modifications of the microbiota as shown in Fig. 4. An impact of the prebiotics present in the product, i.e., Acacia gum, larch gum, oligosaccharides and L-glutamine, on the microbiota cannot be excluded. Elucidating the mechanism by which probiotic administration promotes proliferation of *C. parvum* will require testing of individual probiotic species or defined combinations of species and/or prebiotics (34) and metabolomics analysis to identify mediators of the probiotics effect. This research is of primary importance to enable targeted manipulations of the microbiota aimed at limiting proliferation of *Cryptosporidium* parasites. Zhu et al. (21) describe methods to "edit" the gut microbiota, in this case by inhibiting the multiplication of facultative anaerobes. An analogous approach could be used to investigate the causal link between parasite proliferation and dysbiosis. Although mice infected with *C. parvum* do not develop diarrhea, the fecal microbiota from heavily infected animals in our experiments resembles the fecal microbiota of humans suffering from cholera diarrhea (29-31) or diarrhea of other etiologies (31). This observation is significant because it indicates that neither the actual pathogen nor diarrhea are important to induce dysbiosis. A characteristic of many intestinal pathologies of infectious or other causes is an increase in the proportion of Gammaproteobacteria (35). Although exceptions to this trend have been reported (36), a shift towards facultative anaerobes reflecting increased permeability of the gut epithelium is a hallmark of infectious (29, 30, 37, 38), inflammatory (39-41) and other intestinal pathologies (42). The abundance of Gammaproteobacteria in the distal gut of mice heavily infected with *C. parvum* indicates a shift in the luminal oxygen gradient (43),

likely a consequence of epithelium erosion and villus atrophy (44-46). These observations raise the question whether *Cryptosporidium* proliferation responds to oxygen concentration in the gut lumen. Selective inhibition or promotion of oxygen-consuming bacteria (21) to temporarily raise or deplete luminal O_2 (22) could potentially be investigated to assess the response of *C. parvum* and explore dietary interventions to mitigate the severity and duration of cryptosporidiosis.

Few studies have reported on the effect of diet on *C. parvum* infection. Liu et al. (47) found that protein deficiency increases the concentration of *C. parvum* DNA in feces. However, in this study the difference between normal and protein-deficient animals was reported at 20 h post-infection. Since *C. parvum* is not known to complete its life cycle in less than 72 h (48), these results are difficult to interpret. Another study found a positive effect of pomegranate extract on cryptosporidiosis in calves (49). The authors report that calves fed milk supplemented with extract excreted fewer oocysts. The very limited range of the literature on the effects of diet on cryptosporidiosis illustrates the need for additional research, particularly basic research on mechanistic aspects of parasite-microbiota interaction.

Although the results across experiments are consistent, differences were also noticed. Most notably, average oocyst output in experiment 2 was higher, indicative of a more severe infection. Corroborating the model discussed above, more severe infection was associated with higher relative abundance of Gammaproteobacteria (Fig. 4). The reason for the difference between severity of infection is hard to determine, but the different route of dexamethasone administration in experiment 2 mentioned above could have contributed to this outcome. Differences in fecal oocyst output between co-housed mice was also observed in experiment 1 and 2 (Fig. 1). As described in Materials and Methods, in these experiments animals from a same cage were housed individually only for 16 h three times a week for collection of feces, but were

314 otherwise housed together in two cages per treatment or 4 cages for each experiment. The
315 difference in oocyst output and microbiota profile between cagemates is difficult to explain
316 given the close contact between animals. This phenomenon justifies mice to be sampled
317 individually, rather than sampling by group as is common practice, and is the reason why
318 experiment 3 samples were not subjected to a full set of analyses as for experiment 1 and 2.
319
320 **Conclusions.** In the absence of effective drugs to control cryptosporidiosis, a search for
321 alternative treatments is warranted. However, in most cases we do not know how microbiota
322 perturbation, whether induced by diet, pro-, anti- or prebiotics, affects enteric pathogens and
323 *Cryptosporidium* parasites in particular. Identifying specific mechanisms impacting pathogen
324 virulence in response to diet may enable the development of targeted microbiota editing
325 measures to mitigate the severity of cryptosporidiosis. Methods designed to detect changes in the
326 metabolome (50) will be needed to supplement taxonomic analyses based on 16S amplicon
327 sequencing. Lastly, enhancing the value of the rodent cryptosporidiosis model, the observed shift
328 towards facultative anaerobes in the infected gut indicates common pathogenic changes in the
329 human and rodent intestine in response to enteric infections.
330
331 **MATERIALS AND METHODS**
332 **Parasites.** Oocysts from *C. parvum* isolate MD (51) were used in experiment 1 and 3, whereas
333 mice in experiment 2 were infected with *C. parvum* isolate TU114 (52). MD is a zoonotic
334 isolate. TU114 belong to the anthroponotic subgroup characterized by a IIc GP60 genotype (53).
335 Oocysts were purified from mouse feces on gradients of Nycodenz (Alere Technologies, Oslo,

Norway) as described (54). The age of the oocysts was 13, 110 and 62 days for experiment 1 and 2 and 3, respectively.

Mouse experiments. To test the effect of a commercially available probiotic, three experiments were conducted in mice. In each experiment, herein referred to as experiment 1, experiment 2 and experiment 3, respectively, 16 female CD-1 mice approximately 6 weeks of age were used. Upon delivery, each mouse was individually tagged and randomly assigned to one of four groups of four mice. Mice were immunosuppressed by adding dexamethasone 21-phosphate disodium (Sigma, cat no. D1169) to the drinking water at a concentration of 16 mg/l (55) starting on the day of arrival, defined here as day -7 PI, i.e., 7 days prior to infection. To deplete the native intestinal microbiota, vancomycin and streptomycin were added to the drinking water at a concentration of 500 mg/l and 5 g/l, respectively, starting on day -6 PI. Metronidazole at a dose of 20 mg/kg was given daily by gavage starting on day -6 PI. The antibiotic treatment was discontinued on day -2 PI. Starting on day -1 PI, the drinking water was supplemented with 1.3 g of probiotic added to 500 ml water. This product contains 15 bacterial strains belonging to the genera *Bifidobacter* (4 species), *Lactobacillus* (9 species) and *Streptococcus thermophilus*. In addition to bacteria, 1 g of product contains 8 mg Acacia gum, 540 mg Larch gum, 115 mg galactooligosaccharide, 212 mg L-glutamine and 150 IU vitamin D3. When dissolved into 500 ml of water, the final concentration of these ingredients was 20 µg/ml, 1.4 mg/ml, 0.3 mg/ml, 0.55 mg/ml and 0.4 IU/ml, respectively. The product is flavorless. Drinking water with dexamethasone and probiotic was replaced every 3 days. Mice were orally infected on day 0 PI with approximately 5 x 10^4 *C. parvum* oocysts. In experiment 1, to compensate for increased water uptake in the two groups receiving probiotic, the concentration of dexamethasone

359 phosphate in the water of the two treated groups was reduced to 10 mg/l starting on day 10 PI. In
360 experiment 2, to avoid possible differences in dexamethasone uptake with drinking water, the
361 drug (Dexamethasone; Sigma, cat no. D1756) was given only subcutaneously every second day
362 starting on day 2 PI, following 9 days of dexamethasone administration in the water (Table 1). A
363 volume of 100 µl of 10 mg/ml dexamethasone suspension was injected alternatively into the
364 right and left side of the abdomen. In experiment 3, 16 mg/L dexamethasone at was added to the
365 drinking water or all (n=4) groups of mice. To obtain fecal pellets for microbiota analysis, mice
366 were individually transferred to a 1-liter plastic beaker and pellets collected upon defecation. In
367 experiment 3, feces were collected by group, not by mouse. Pellets were stored at -20°C. To
368 collect feces for oocyst enumeration, mice were individually transferred overnight to collection
369 cages fitted with a wire bottom. Feces were collected from these cages were stored at 4°C. In the
370 morning, mice were returned to their respective group cage, such that they were housed
371 individually for 14-16 h on the days when feces for oocyst enumeration were collected. While in
372 conventional cages, the mice were always housed with the same cagemates.
373 Animal experiments adhered to the NIH Guide for the Care and Use of Laboratory
374 Animals and were approved by the Tufts University Animal Care and Use Committee.
375
376 **Enumeration of oocysts**. Feces collected overnight were weighted, diluted 1:5 in distilled water
377 and homogenized with a vortex. To remove debris, fecal homogenates were filtered through 100
378 µm cell strainers (Corning cat. no. 431752) by centrifugation at 1300 x g. The filtrates were
379 homogenized and volumes of 1 ml centrifuged at 6700 x g for 5 min. The supernatant was
380 discarded and the pellet was suspended in 500 µl of PBS supplemented with 10% fetal bovine
381 serum. A volume of 20 µl of this suspension was transferred to a 1.5 ml microcentrifuge tube and

20 µl of a 1:5 dilution of monoclonal antibody 5F10 cell culture supernatant. This antibody reacts specifically with the *Cryptosporidium* oocyst wall without binding to other parasite antigens (Sheoran, unpublished observation). Samples were incubated for 30 min at room temperature. Following incubation with primary antibody, the samples were centrifuged for 10 min at 6700 x g, the pellet washed once in 500 µl of PBS with 10% FBS and incubated with 20 µl of secondary antibody (Alexa Fluor 488 goat IgG anti-mouse) for 30 min at room temperature. After incubation, 500 µl of PBS was added and the samples washed once in PBS. For each experiment five samples were randomly selected for replication. Replication involved processing and labelling 5 separate aliquots originating from each strained and washed sample. Labelled samples were analyzed by flow cytometry using a Becton Dickinson Accuri C6 cytometer. Distance matrices were calculated in GenAlEx 6.5 (56) based on the pairwise difference between oocyst concentrations. Specifically, the distance between sample A containing an oocyst concentration of x_a/g feces and sample B containing a concentration of x_b/g feces was calculated as $(x_a - x_b)^2$. The accuracy of the flow cytometry oocyst enumeration method was evaluated by correlating the counts against the *Cryptosporidium* 18S ribosomal RNA gene copy number estimated using real-time PCR with published primers (57). This analysis based on five randomly chosen experiment 1 samples generated a correlation coefficient of 69%.

Microbiota analysis. DNA was extracted from 10 mg of feces collected individually from each mouse. DNA was extracted in a QIACube instrument using the QIAamp PowerFecal DNA kit (QIAGEN, cat. 12830-50) according to the manufacturer's protocol. DNA was eluted in 50 µl of elution buffer and stored at -20°C. A previously described PCR protocol to prepare 16S V1V2 amplicons libraries for high-throughput sequencing was used (5). The only deviation from this

405 procedure was the downstream primer; instead of canonical primer 338R we used primer Bac R
406 V2 short (GTTCAGACGTGTGCTCTTCCGATCtgctgcctcccgtaggagt), where the lowercase
407 characters are equivalent to the conserved 338R sequence (58). One microliter of primary PCR
408 reaction was subjected to a secondary PCR to incorporate a 6-nucleotide barcode using to each
409 sample. The secondary amplification was as described (5), except that downstream primer
410 *CAAGCAGAAGACGGCATACGAGAT*nnnnnnGTGACTGGAGTTCAGACGTGTGCTCTTCC
411 was used. The italicized nucleotides represent the Illumina adaptor and the lowercase characters
412 the barcode unique to each sample. To assess the quality of the PCRs, a portion of the
413 amplification product was electrophoresed on 1.5% agarose and visualized using GelRed™
414 (Biotium). The concentration of each final amplicon was measured in a Qbit spectrophotomer
415 and up to 80 amplicons pooled at approximately equal concentration. The pooled library was size
416 selected with a Pippin HT library size selection system (Sage BioScience). Libraries were
417 sequenced in an Illumina MiSeq sequencer at Tufts University Genomics core (tucf.org) using
418 single-end 300-nucleotide strategy. To control for technical variation introduced during library
419 preparation and sequencing, each library included two replicates of two randomly chosen
420 samples. Replication involved the processing of duplicate fecal samples processed, amplified and
421 barcoded individually.
422
423 **Bioinformatics**. FASTQ formatted sequences were processed using programs found in mothur
424 (59) essentially as described (5). Briefly, random subsamples of 5000 sequences per sample were
425 processed. This procedure is not expected to bias the analysis (60) since the number of sequences
426 per barcode was relatively constant. The mean number of sequences per sample in experiment 1
427 was 1.03×10^5 (SD=2.44×10^4). In experiment 2 the average number of reads was 1.01×10^5

(SD=3.22 x 10^4), representing a coefficient of variation of 0.23 and 0.31, respectively. V1V2 sequences were trimmed to 200 nucleotides to eliminate 3′ sequence with a mean Phred quality score <30. Sequences were aligned and sequences with the following properties were removed: sequences that did not align, sequences with ambiguous base calls and sequences with homopolymers >8 nt. To further reduce the number of putative sequence errors, program *pre.cluster* was used to merge unique sequences differing by one nucleotide position with the majority sequence (61). This sequence curation protocol removed 15,748 from 400,000 experiment 1 sequences (3.9%) and 21,546 from 355,000 experiment 2 sequences (6.0%) sequences. Pairwise UniFrac phylogenetic distances (24) between samples were calculated in *mothur*. Analysis of Similarity (ANOSIM) (25) was used to test the significance of clustering by treatment. Program *anosim* was run in *mothur* using a weighted UniFrac distance matrix as input. Operational Taxonomic Units (OTUs) were obtained using program *cluster*, also found in *mothur*, using the OptiClust clustering method (62) and a distance cut-off of 3%.

Linear Discriminant Analysis as implemented in program LEfSe (27) was used to identify statistically significant differences in OTU abundance profiles between two groups of samples defined, independently of treatment, as heavily and lightly infected. Heavy and light infection was defined on the basis of fecal oocyst concentration determined by flow cytometry as described above. In the experiment-wide LefSe analyses, the 10 samples with the highest oocyst concentration and the 10 samples with the lowest oocyst concentration were selected. When analyzing subsamples of probiotic and control mice separately, the five samples with the highest oocyst concentration and the same number of samples with the lowest oocyst concentration were tested using LEfSe (27).

RDA was used to test the significance of association between OTU profile and oocyst concentration or between KEGG function profile and oocyst concentration. The program was run in CANOCO (28). The pseudo-F statistic was calculated by Monte Carlo permutation of samples between treatment groups. OTU abundance values for the 100 most abundant OTUs, or KEGG functions (n=39) inferred with metagenome prediction tool PICRUSt (32) served as dependent variables. Oocyst concentration determined by flow cytometry as described above served as the independent variable. Pairwise distance between KEGG profiles was calculated as described above for oocyst concentration, except that the squared difference was summed over all KEGG categories. This metric is equivalent to the square of the Euclidean distance.

Sequence data accession number. Raw sequence data were deposited in the ENA Sequence Read Archive under study accession number PRJEB25162 and PRJEB25164, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material may be found at SUPPLEMENTAL FILE 1.

ACKNOWLEDGEMENTS:

Lucas Vinicius Shigaki de Matos, Olga Douvropoulou and Kevin Huynh performed preliminary experiments that led to the design of the research presented here. Support from the NIAID (5R21AI125891) is gratefully acknowledged.

BCMO and GW designed the experiments and analyzed the data. BCMO performed the experiments. GW wrote the manuscript.

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FIGURE LEGENDS

FIG 1 Effect of probiotic on severity of *C. parvum* cryptosporidiosis in immune-suppressed mice. The graphs show oocyst counts (log) expressed as oocysts/g feces for three independent experiments. In experiments 1 and 2, mice were sampled individually. Values represent mean of 4 mice. Error bars show SD. In experiment 3, each group of 4 mice was sampled together. Error bars in experiment 3 show SD based on five replicate flow cytometry counts of selected samples.

FIG 2. Impact of probiotic on the fecal microbiome of *C. parvum* infected mice. Principal Coordinate Analysis was used to display weighted UniFrac distances between pairs of fecal microbiome samples. Experiment 1 analysis (left) includes data from 64 fecal samples collected from day 5 of treatment (day 4 PI) until day 16 of treatment (day 15 PI) from individual mice. For experiment 2, 55 samples from individual mice were analyzed. Each datapoint represents one sample, color-coded according to treatment and group as shown in Fig. 1. Matching triangle

symbols indicate replicate analyses of the same fecal samples. Experiment 3 data are not shown because group-wise sample collection resulted in small number of datapoints.

FIG 3 Oocyst output is unrelated to diversity of bacterial microbiome. Oocyst output normalized for feces volume was plotted against Shannon diversity for 44 samples collected on three days which were analyzed for both properties in experiment 1 and 53 samples from experiment 2. Color indicates experimental group as described in Fig. 1. Samples were collected from individual mice. Due to the small number of datapoints, experiment 3 was not analyzed.

FIG 4 Taxonomy of fecal microbiome from heavily and lightly infected mice shows that heavy infections are associated with increased abundance of Proteobacteria. For each experiment, bacterial taxa significantly associated with severity of infection were identified using LEfSe (24) in a comparison of ten samples with the highest (high) and lowest (low) oocyst concentration; twenty samples were included for each experiment. Color indicates phylum and color intensity genus or highest taxonomic level (family, order etc.) identified; green Proteobacteria; red, Firmicutes; blue, Actinobacteria.

Table 1. Summary of experiments

Experiment	Isolate	Groups[a]	Dexamethasone treatment	Oocyst age	Feces collection	Onset of oocyst shedding (day PI)
1	MD	4	in water; 10 mg/l and 16 mg/l	13	by mouse	4
2	TU114	4	in water day -7 to day 1 PI; subcut. day 2 PI onward	110	by mouse	6
3	MD	4	in water 16 mg/l	62	by group	4

[a] 2 treated and 2 control groups

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