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An Inactivated Bacterium (Paraprobiotic) Expressing *Bacillus thuringiensis* Cry5B as a Therapeutic for *Ascaris* and *Parascaris* spp. Infections in Large Animals

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An inactivated bacterium (paraprobiotic) expressing *Bacillus thuringiensis* Cry5B as a therapeutic for *Ascaris* and *Parascaris* spp. infections in large animals

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ABSTRACT

Ascaris and *Parascaris* are important parasites in the family Ascarididae, large, ubiquitous intestinal-dwelling nematodes infecting all classes of vertebrates. Parasitic nematode drug resistance in veterinary medicine and drug recalcitrance in human medicine are increasing worldwide, with few if any new therapeutic classes on the horizon. Some of these parasites are zoonotic, e.g., *Ascaris* is passed from humans to pigs and vice versa. The development of new therapies against this family of parasites would have major implications for both human and livestock health. Here we tested the therapeutic ability of a paraprobiotic or dead probiotic that expresses the *Bacillus thuringiensis* Cry5B protein with known anthelmintic properties, against zoonotic *Ascaris suum* and *Parascaris* spp. This paraprobiotic, known as IBaCC, intoxicated *A. suum* larvae *in vitro* and was highly effective *in vivo* against intestinal *A. suum* infections in a new mouse model for this parasite. Fermentation was scaled up to 350 l to treat pigs and horses. Single dose Cry5B IBaCC nearly completely cleared *A. suum* infections in pigs. Furthermore, single dose Cry5B IBaCC drove fecal egg counts in *Parascaris*-infected foals to zero, showing at least parity with, and potential superiority to, current efficacy of anthelmintics used against this parasite. Cry5B IBaCC therefore represents a new, paraprobiotic One Health approach towards targeting Ascarididae that is safe, effective, massively scalable, stable, and useful in human and veterinary medicine in both the developed and developing regions of the world.

1. Introduction

Ascaris lumbricoides or the large intestinal roundworm is the most common nematode parasite of humans, infecting approximately 1 billion people in mostly tropical and subtropical countries [1,2]. These

gastrointestinal nematode (GIN) parasites live in the small intestine, reaching more than 30 cm in length. Recent nuclear genome studies indicated that *A. lumbricoides* and *Ascaris suum*, the ubiquitous and important *Ascaris* parasite of pigs, are highly interbred [3]. Indeed, *A. suum* is a zoonosis that can express as a haplotype of *A. lumbricoides*,

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with each parasite species able to infect both pigs and humans [3–8]. In the same family (Ascarididae) and closely related to these *Ascaris* parasites is *Parascaris* spp., the omnipresent parasite of foals and young horses around the world that has developed multi-drug resistance, with increasing concern [9]. *Ascaris* and *Ascaris*-related parasites are important components of the “One Health” concept [3,10,11].

Infestation in humans with *Ascaris* is responsible for an estimated loss of 1–1.5 million disability adjusted life-years (DALYs) and for 60,000–200,000 deaths yearly due to obstruction of the digestive tract and hepatobiliary and pancreatic ascariasis, the true impact of which has likely been underestimated [1,2,12]. Infection rates can be very high in places, e.g., reaching >50% prevalence in six states in India covering nearly 30% of the total population of India and > 44% prevalence in a systematic meta-analysis of studies conducted in Nigerian children [13,14]. Acute symptoms due to adult parasites inhabiting the gastrointestinal (GI) tract include abdominal pain, bloating, nausea, vomiting, anorexia, and intermittent diarrhea (with heavy loads leading to serious complications and even death). However, the more significant impact is in children who are more susceptible to these parasites. The consequences of long-term infestation includes malabsorption of nutrients, protein energy malnutrition, and reduced food intake, leading to growth retardation, negative impacts on cognitive development, school absenteeism, and reduced economic productivity [1,2,8,15].

Although single dose anthelmintic cure rates against *A. lumbricoides* are reported to be >90% [8], there are numerous reports in the literature of deficiencies in treating this parasite with benzimidazoles (anthelmintic of choice for Mass Drug Administration or MDA). These difficulties include lower than expected cure rates with albendazole in Africa and Asia (0%, 19%, 41%, and 56% in four separate studies; [16–19]) and strong persistence of this parasite in African, Asian, and Central American countries despite consistent annual deworming campaigns spanning multiple years [20–25]. Known benzimidazole resistance alleles have been detected in natural populations of *Ascaris*, although there is evidence that resistance/recalcitrance may occur without these alleles [19,21,26]. Although ivermectin can also be used against this parasite, annual MDA incorporating both albendazole and ivermectin has failed to eliminate it, resulting in only modest prevalence reductions [27].

As noted above, ascarids closely related to *A. lumbricoides* are also common and important parasites of livestock and companion animals. *Ascaris suum* is a common production and health problem in pigs worldwide because of poor feed utilization and liver condemnation at slaughter where different stringencies of management fail to eliminate infection rates even in large global producing countries [28]. Recent *A. suum* prevalence surveys in the United States are lacking but routine deworming strategies are used in >77% of production facilities for breeding age pigs and at >17% and > 14% for nursery and grower/finisher pigs, respectively [29], indicating a need for practical and effective anthelmintic formulations in the market-place. *Parascaris* spp. are the most important parasites of foals and young horses around the world [9]. The parasites are truly ubiquitous and virtually every foal should be considered exposed. Equine ascarids cause small intestinal impaction when a cluster of worms block the intestinal lumen. This is a painful and life-threatening condition, and even with successful abdominal surgery, the prognosis for long-term survival is guarded to poor [9]. This is concerning as *Parascaris* spp. are found widely resistant to currently available anthelmintic drug classes, most notably to the most widely used drug class, the macrocyclic lactones [30]. The pharmaceutical industry has developed and introduced very few new anthelmintic drug classes and none for equine use since the early 1980s, and research exploring alternative treatment modalities is urgently needed. There is real concern that *Ascaris* spp. could also (independently) develop widespread drug resistance that is now observed in closely related *Parascaris* spp. Taken together, these findings all point to the urgency and importance of developing new anthelmintic formulations for treating *Ascaris* and *Parascaris* parasites of humans, livestock,

and companion animals.

Continuing human MDA campaigns and veterinary parasite control strategies would therefore benefit substantially with the addition of new anthelmintics against ascarid parasites with the following requirements: highly and broadly potent against this family of helminths, and safe, inexpensive, and massively scalable for MDA. We have recently described an active pharmaceutical ingredient (API) called IBaCC (see below) containing the *Bacillus thuringiensis* (Bt) Crystal (Cry) protein Cry5B that is effective against human hookworm infections in rodents and against *Haemonchus contortus* infections in sheep and that meets all the requirements for MDA [31,32]. Cry5B itself was shown to be active against *A. suum* in pigs when delivered as a live spore-crystal lysate (SCL) [33]. However, delivery of SCL or of live bacteria in general is not ideal for MDA because of an unstable shelf life, environmental concerns, potential safety/regulatory issues, and development of resistance via suboptimal dosing [31,34–38]. Here, for the first time, we describe and test Cry5B IBaCC against *Ascaris* infection in pigs and Cry5B itself (as IBaCC) for the first time against *Parascaris* infection in horses.

2. Materials and methods

2.1. Animal ethics and approvals

All protocols in the study were approved by the University of Kentucky Institutional Animal Care and Use Committee (Protocol 2015–2078) for the equine studies and the USDA Beltsville - Institutional Animal Care and Use Committee #18–008, #18–029 and #17–019 for use of mice and pigs, respectively, and Institutional Biosafety Committee #271.

2.2. Preparation of Cry5B IBaCC and in vitro studies

Cry5B IBaCC was prepared as described [31]. Briefly, spo0A-*B. thuringiensis* cells expressing Cry5B from a vegetative promoter were cultured in 3 × LB for 48 h, resuspended in 1/2 volume, treated with essential oil, and then washed. All Cry5B IBaCC used in this study was prepared at the Synthetic Biomanufacturing Facility, Utah State University, at a scale of 350 l. Cry5B concentration was quantitated relative to BSA standards by SDS PAGE densitometry.

Ascaris suum fourth staged larval (L4) parasites were isolated from the small intestines of B6/STAT6KO mice ~12 days post-inoculation (P. I.) and shipped overnight to the Aroian laboratory from the Urban laboratory in Beltsville, MD. The next day, the parasites were subjected to *in vitro* testing with Cry5B IBaCC at two doses and buffer control using the same protocol for setting up and scoring *Ascaris* larval *in vitro* [33,39]. In each experiment, each condition contained two wells with five larvae per well. Simply, larvae that moved either on their own or upon touching with a needle were scored as alive and larvae that failed to move even after touching were scored as dead [33,39]. The experiment was repeated twice with two different batches of larvae and two different batches of Cry5B IBaCC. A separate experiment was performed in which IBa (Inactivated *Bacterium* with empty vector, identical and identically processed to IBaCC in every way except that the vector does not contain a Cry5B insert) was matched in optical density (OD₆₀₀) to 100 µg/mL IBaCC and tested against *A. suum* L4 parasites in two independent experiments ($n = 20$ larvae total). Incubation with IBa resulted in 100% viability (20/20) of *A. suum* L4 up to and including 96 h incubation.

2.3. Mouse *Ascaris* in vivo experiment

Breeding pairs of C57BL/6(B6)-STAT6KO were originally procured from the Jackson Laboratories, Bar Harbor, ME and bred at the USDA/ARS/Beltsville Agricultural Research Center in Beltsville, MD. Groups of five mice each containing both males and females were inoculated *per os* with 10,000 infective *A. suum* eggs and 12 days later were 1) untreated,

2) treated *per os* with IBaCC containing Cry5B at 6 mg/kg body weight, or 3) treated *per os* with IBaCC containing Cry5B at 20 mg/kg body weight. Those in group 3 received a second dose of IBaCC containing Cry5B at 20 mg/kg body weight on the next day. *A. suum* L4 were isolated from the small intestines six days later by opening the small intestine with scissors and placing them above a tea strainer submerged in 100 ml of phosphate-buffered saline (PBS) maintained for 3 h at 37°C in a water bath. The L4 falling to the bottom of the beaker containing the PBS were then placed in a line-grated rectangular culture dish and counted using a dissecting microscope; larvae were identified morphologically as L4 [40]. These mice were also inoculated with infective *Heligmosomoides polygyrus bakeri* third-stage larvae and infective *Trichuris muris* eggs to test Cry5B IBaCC efficacy against multiple nematode infections in a single mouse host. The results of IBaCC treatment on these other parasites will be presented in a separate report.

2.4. Pig *Ascaris* *in vivo* experiment

Experimental pigs were obtained from the facility at the Beltsville Agricultural Research Center, Beltsville, MD and were housed and managed as previously described [33]. The pigs were mixed sex and approximately 8 weeks of age with each group containing six pigs at the time of *per os* inoculation with 10,000 infective *A. suum* eggs [33]. Pigs were treated *per os* 12 days after parasite egg inoculation with a single dose of IBaCC at 30 mg/kg Cry5B body weight (treated group) or a vehicle control (control group) and euthanized four days later to recover *A. suum* L4 from the small intestine [33]. The L4 isolated from an agar-gel matrix of intestinal contents were counted using a dissecting microscope to determine the number of morphologically identified L4 [40] per pig.

2.5. Horse *Parascaris* spp. experiment

Ten horse foals born on the University of Kentucky Research Farm in 2019 were enrolled in the study. The foals were exposed to parasites on contaminated pasture to become naturally infected with *Parascaris* spp. and did not receive anthelmintic treatment prior to the study. From three months of age, the foals were examined for ascarid fecal egg counts determined in triplicate with the Mini-FLOTAC method as previously described [41]. Once foals exceeded 100 ascarid eggs per gram (EPG), they were ranked by egg count magnitude and blocked into pairs of two. From these blocks, foals were randomly allocated to either treatment or control group. Foals in the treatment group were administered a single dose of Cry5B IBaCC at 30 mg/kg *via* nasogastric tube. Foals in the control group were kept untreated. Egg counts were determined on the day of treatment and weekly for four weeks post treatment.

2.6. Statistical methods

Prism v.8 was used for all graphs and comparisons. The comparison of fecal egg counts in the foal study was carried out using two-way analysis of variance (mixed-effects analysis Time x Treatment). For the *in vivo* mouse experiment, one-way analysis of variance (ANOVA) with Dunnett's post-test was used relative to water control. For comparisons including just two groups in the pig study and the end of the horse study, a one-tailed Mann-Whitney (non-parametric) test was used with the assumption that treatment reduced parasite burdens or fecal egg counts. For the comparison of fecal egg counts at the beginning of the horse study, a two-tailed Mann-Whitney test was used to test for equivalence of starting fecal egg counts.

3. Results

3.1. Cry5B IBaCC intoxicates *A. suum* intestinal fourth larval stage *in vitro*

Bt Cry5B is a potent anthelmintic protein that has been studied *in vivo* against hookworms in rodents/dogs and *Ascaris* infections in pigs delivered as spore crystal lysates or as protein isolated from spore crystal lysates, neither of which are compatible with scale up technologies or broadly effective drug distribution strategies [31,33,42–46]. IBaCC or Inactivated Bacterium with Cytosolic Crystal is an API form compatible with MDA in which Bt Cry protein crystals are produced in the cytosol of asporogenous Bt, that is subsequently killed or inactivated by incubation in food-grade essential oil [31]. IBaCC is effective against hookworm infections in rodents and *Haemonchus* infections in ruminants (Li et al. 2020; Sanders et al. 2020). A dead probiotic with therapeutic benefit is known as a paraprotibiotic [37].

How IBaCC would interact with ascarids, which have different mouth parts, feeding patterns, food sources, and behaviors than hookworms, was unknown. IBaCC containing Cry5B (or Cry5B IBaCC) was initially tested against intestinal *A. suum* fourth stage larvae (L4s) using a new protocol for isolation from immune deficient mice and incubated *in vitro* with IBaCC containing two different Cry5B doses. Visible changes in the L4 treated with Cry5B IBaCC relative to buffer control were seen (e.g., paler coloration, degenerated intestinal appearance, rigidity in shape, and reduced movement), indicating clear intoxication of *A. suum* by Cry5B IBaCC (Fig. 1A). Intoxication was quantitatively evident at 10 µg/mL and more strongly at 100 µg/mL Cry5B content, with near complete death/immobilization seen at 100 µg/mL (~700 nMolar) at 48 h (Fig. 1B). As has been shown with all other parasitic nematodes tested [31,47], intoxication of *A. suum* by Cry5B IBaCC was dependent upon Cry5B protein since incubation of parasites in the presence of IBa control lacking Cry5B but otherwise identical to IBaCC resulted in no intoxication (detailed in Materials and Methods). These *in vitro* data indicated that Cry5B IBaCC, but not the inactivated bacterium *B. thuringiensis* with empty vector (IBa), has potential to impact *A. suum* infections *in vivo*.

3.2. Cry5B IBaCC has a significant impact on intestinal *A. suum* intestinal infections in mice and pigs

Before testing in large animals, we tested Cry5B IBaCC against intestinal *A. suum* infections in mice. We have found that C57BL/6(B6)-STAT6KO mice support the development of *A. suum* to the L4 intestinal stage, at which point they are expelled (~day 20 post-inoculation or P.I.). B6/STAT6KO mice were inoculated *per os* with *A. suum* eggs. Twelve days P.I., the mice ($n = 5$ per group) were treated with either water (control), IBaCC at 6 mg Cry5B/kg by body weight, or IBaCC at 20 mg Cry5B/kg body weight. Those mice that received 20 mg/kg received a second 20 mg/kg body weight dose day 13 P.I. This double 20 mg/kg dose mimics what was done in the published pig study using Cry5B SCL [33]. Five days later small intestinal worm burdens were determined. Treatment with two 20 mg/kg doses resulted in a statistically significant 92.5% reduction in intestinal worm burdens relative to control (Fig. 2), similar to what was seen in pigs using SCL and the same dosing regimen [33]. Treatment using a single 6 mg/kg body weight of Cry5B in IBaCC also resulted in a significant 62% reduction in intestinal *A. suum* L4 burdens (Fig. 2). These results in mice with a TH2-immune deficient background further reinforce previous findings that Cry5B efficacy is due to a direct impact on parasites and is not dependent upon an intact immune response [44].

Based on *in vitro* data and *in vivo* efficacy in the mouse model, Cry5B IBaCC production was scaled up to 350 l at a contract manufacturing organization (CMO) for studies in large animals [31]. Twelve pigs were inoculated *per os* with *A. suum* eggs. Based on success with single dosing in mice, a single-dose treatment trial was performed. Twelve days P.I.,

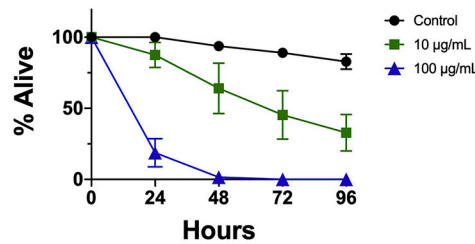
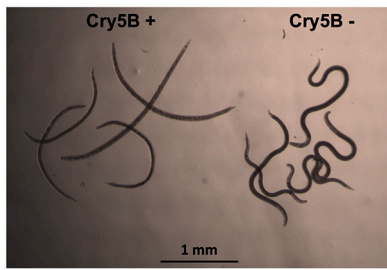


Fig. 1. Cry5B IBaCC intoxicates *A. suum* in vitro. A) Image of *A. suum* fourth-stage larvae (L4) in the presence of 100 µg/mL Cry5B in IBaCC or absence of Cry5B IBaCC. Intoxicated larvae were straightened and immobile. Healthy larvae had multiple bends and were motile. Images were taken at 48 h. B) Percent of *A. suum* larvae alive over time in vitro at two doses of Cry5B in IBaCC relative to control (buffer only). Shown is the average and standard error of two independent experiments with two wells per experiment (5 larvae per well).

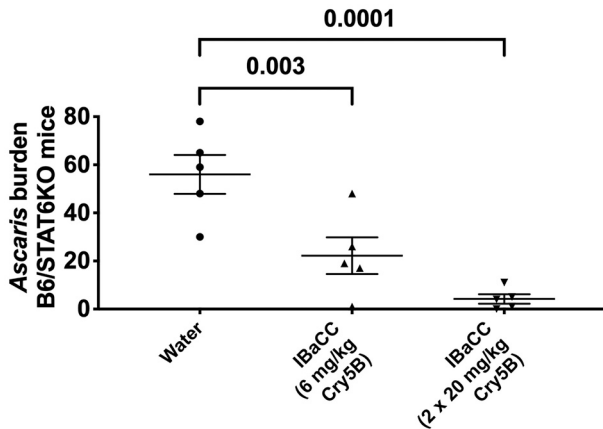


Fig. 2. Cry5B IBaCC is efficacious against *A. suum* L4 intestinal stage in mice. Shown are the parasite burdens from B6/STAT6KO mice inoculated with infective *A. suum* eggs and treated with water, single dose Cry5B IBaCC (6 mg/kg body weight), or double dose Cry5B IBaCC (2 × 20 mg/kg body weight). Statistical comparisons of treated groups relative to water control are shown. Long bar for each treatment is mean; short bars indicate standard error.

six pigs were left untreated and six pigs were given *per os* a single dose of IBaCC at a dose of 30 mg Cry5B/kg body weight. Four days later, intestinal worm counts were determined for all pigs. As shown (Fig. 3), single dose Cry5B IBaCC resulted in a statistically significant reduction and near complete clearance of *A. suum* L4 from the pig intestine (96% reduction relative to control).

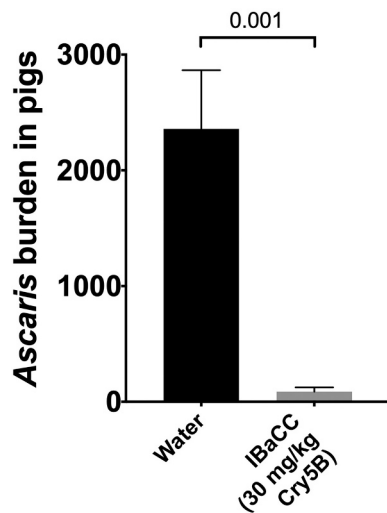


Fig. 3. Cry5B IBaCC is highly effective against *A. suum* L4 intestinal stage infections in pigs. Shown are the average parasite burdens in pigs given water or single dose Cry5B IBaCC at 30 mg/kg body weight. Error bars indicate standard error.

3.3. Cry5B IBaCC clears fecal egg counts in horses infected with the related parasite *Parascaris* spp

Parascaris infection is deleterious to equines (foals) and is phylogenetically related to *A. suum* and *A. lumbricoides* [48,49]. As scaled-up IBaCC effectively cleared *A. suum* infections in pigs at a single dose of 30 mg Cry5B/kg body weight, we similarly tested a single dose of IBaCC at 30 mg Cry5B/kg body weight against adult *Parascaris* spp. infections in naturally infected foals.

Ten foals were selected *via* rolling enrollment in control ($n = 4$) and Cry5B IBaCC treatment arms ($n = 6$). Each foal in the treatment arm was administered *via* nasogastric tube a single dose of 30 mg/kg Cry5B in IBaCC produced at 350 l scale by a CMO. Fecal egg counts were determined on the day of treatment and then once weekly for 4 weeks. The results are shown in Fig. 4. Fecal egg counts in foals from both the control and treatment arms were statistically similar at the beginning of the study, with mean ascarid egg counts of 916 and 673 eggs per gram of feces, respectively. Fecal egg counts persisted in all foals in the control group up until and including day 14 and in three out of four foals throughout the entire duration of the study. In contrast, fecal egg counts went to zero in all Cry5B IBaCC-treated foals at the first sample taken one-week post-treatment and stayed at zero for all treated foals throughout the study. Two-way analysis of variance showed a statistically significant difference between control and treatment arms. A significant difference in fecal egg counts at each time point post-treatment was also seen (shown for the final time point; Fig. 4). These data indicated that Cry5B IBaCC had a dramatic impact on *Parascaris* infections in foals.

4. Discussion

Ascarid parasites are some of the most common and damaging

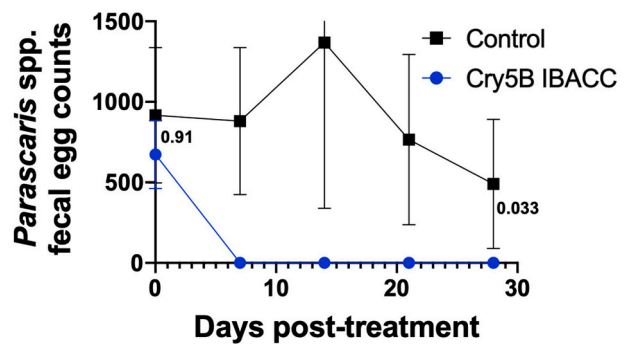


Fig. 4. Cry5B IBaCC is highly effective against *Parascaris* spp. Infections in foals. Shown are the average fecal egg counts over time; error bars indicate standard error. Day 0 fecal egg counts were taken before the first treatment. The difference between fecal egg counts between control and foals treated with IBaCC at 30 mg/kg Cry5B body weight based on two-way analysis of variance was significant ($P = 0.0074$). P values shown indicate the comparison between fecal egg counts in treated and control groups before treatment (day 0; $P = 0.91$) and at the last data point taken (day 28; $P = 0.033$).

parasites of humans and animals on earth. In veterinary medicine, the recalcitrance of *Parascaris* to treatment and development to resistance is well documented. Although *Ascaris* infections in humans are considered well managed with current therapeutics, a detailed look at the efficacy data and the propensity for livestock to develop anthelmintic resistance from intensive drug treatment is foreboding for MDA strategies in humans (see Introduction). Therefore, new therapies against this class of parasite in the pipeline are urgently needed and would play an important role in promoting One Health. However, to be therapeutically deployable in both human and veterinary medicine in the developed and developing world, such therapies need to be safe, broadly active against this class of parasite, inexpensive, and massively scalable. We are unaware of any new drugs that meet these criteria that are in the commercial pipeline.

Here we demonstrated that Cry5B IBaCC, a recombinant paraprobiotic (dead bacterial ghost) containing an active Bt Cry protein crystal, has great promise to fulfill this void. Single-dose Cry5B IBaCC is effective against two ascarid species *in vivo* in three different mammalian hosts. In mice and pigs, Cry5B IBaCC was effective at near complete elimination of *A. suum* parasite burdens in the small intestine. In horses, Cry5B IBaCC completely eliminated parasite fecal egg counts. Important future studies in horses would include determination of intestinal parasite burdens in control versus Cry5B IBaCC-treated foals. Because *A. suum* and *A. lumbricoides* are very closely related parasites and because the pig GI tract is considered a good model for the human GI tract, the data suggest that Cry5B IBaCC will be highly effective against *A. lumbricoides* infections in humans. Important future studies would also include testing this hypothesis.

Cry5B IBaCC production was scaled up for these large animal studies to 350 l scale at a CMO. Crystal proteins as part of live spore crystal lysates are massively and cheaply produced around the world to control insect pests, paving the way for comparable production levels of Cry5B IBaCC. However, the therapeutic application of live spore crystal lysates or live bacteria in general is not desirable for humans and other animals because of potential safety issues, environmental contamination, replication in soil and intestines leading to increased risk of parasite resistance, instability of live bacteria on the shelf, and the relationship between *B. thuringiensis* and *Bacillus cereus* [31]. Cry5B IBaCC alleviates all these disadvantages while retaining all the advantages of scalability and safety of Cry proteins themselves. The production process of Cry5B IBaCC itself is simple and transferable [31]. Moreover, a pilot toxicology study in hamsters demonstrated that five consecutive daily doses of IBaCC at 200 mg Cry5B/kg body weight was completely safe and non-toxic based on no deviation from normal histopathology and blood chemistry [31]. Good laboratory practice (GLP) toxicological studies with larger-sized study groups and clinical-grade materials are planned for the near future.

Notably, the development of anthelmintic resistance to Cry5B was found to be relatively more difficult than to a benzimidazole and a nicotinic acetylcholine receptor (nAChR) agonist based on forward genetic screens in *Caenorhabditis elegans* [50]. Furthermore, nematodes resistant to nAChR agonist anthelmintics were hyper-susceptible to Cry5B, and Cry5B and nAChR agonists (e.g., pyrantel and tribendimidine) were synergistically more efficacious *in vitro* and *in vivo* against free-living and parasitic nematodes [43,50]. These properties (hyper-susceptibility and synergy) are predictive of combination drug therapies recalcitrant to the development of resistance.

5. Conclusions

Our data indicate that Cry5B IBaCC can make a significant contribution to the long-term, safe, and effective control of ascarid parasites of humans and other animals. The scalability, adaptability, safety, and ease of manufacture of Cry5B IBaCC make it ideally suited for promoting One Health against ascarid parasites equally in humans and livestock in developed and developing countries. The research reported here more

generally points towards the suitability and great potential of paraprobiotics to treat pathogens of humans and livestock, that is also likely to include ascarids of companion animals such as dogs and cats. Development of a common delivery system of paraprobiotic formulations for cures for both humans and animals promotes the concept of One Health.

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CRedit authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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