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Excretory-secretory products from adult helminth *Nippostrongylus brasiliensis* have *in vitro* bactericidal activity

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Abstract

Introduction. Intestinal helminths and microbiota share the same anatomical niche during infection and are likely to interact either directly or indirectly. Whether intestinal helminths employ bactericidal strategies that influence their microbial environment is not completely understood.

Hypothesis. In the present study, the hypothesis that the adult hookworm *Nippostrongylus brasiliensis* produces molecules that impair bacterial growth *in vitro*, is tested.

Aim. To investigate the *in vitro* bactericidal activity of *Nippostrongylus brasiliensis* against commensal and pathogenic bacteria.

Methodology. The bactericidal effect of somatic extract and excretory-secretory products of adult *Nippostrongylus brasiliensis* on Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Klebsiella pneumoniae*) bacteria was assessed using growth assays. Minimum inhibitory concentration and minimum bactericidal concentration assays were performed using excretory-secretory products released from the pathogen.

Results. Broad-spectrum *in vitro* bactericidal activity in excretory-secretory products, but not somatic extract of adult *Nippostrongylus brasiliensis* was detected. The bactericidal activity of excretory-secretory products was concentration-dependent, maintained after heat treatment, and preserved after repeated freezing and thawing.

Conclusion. The results of this study demonstrate that helminths such as *Nippostrongylus brasiliensis* release molecules via their excretory-secretory pathway that have broad-spectrum bactericidal activity. The mechanisms responsible for this bactericidal activity remain to be determined and further studies aimed at isolating and identifying active bactericidal molecules are needed.

DATA SUMMARY

The data underlying the findings have been included in the manuscript. If requested, the authors agree to provide copies of the original data.

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Keywords: bactericidal activity; intestinal helminth; microbiota; *Nippostrongylus brasiliensis*.

Abbreviations: ESP, excretory-secretory products; *Nb*, *Nippostrongylus brasiliensis*.

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INTRODUCTION

Intestinal helminths are among the most prevalent parasitic infections. Globally more than 1.5 billion humans are infected, most of whom reside in developing tropical countries across sub-Saharan Africa, the Americas, China, and East Asia. Socio-economic factors including poverty, overcrowding, inadequate water supplies, poor hygienic practices and sanitation exacerbate the risk and spread of infection [1]. The three main clinically relevant intestinal helminths are roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*), and hookworms (*Necator americanus* and *Ancylostoma duodenale*) [1, 2].

The mammalian intestine comprises a host of different microbial populations, including species of bacteria, viruses and fungi that are collectively termed microbiota [3]. Given that intestinal helminths and microbiota share the same environmental niche, they are likely to interact with each other with potential implications for the host, intestinal helminths, and microbiota [4]. For example, intestinal helminth infections are associated with alterations in the abundance and composition of host microbiota [5–11]. In turn, helminth-induced changes to microbiota can influence host immunity [3].

It is also well established that intestinal helminths can employ potent strategies to modulate host immunity [12]. Firstly, intestinal helminths release an array of molecules collectively termed excretory-secretory products (ESP) that can modulate host innate and adaptive immune mechanisms, and secondly, they induce the generation, expansion and activation of immunoregulatory cells, including T cells and alternatively activated macrophages [13]. Intestinal helminths also produce bactericidal factors [14–18]. Given that they inhabit a microbe-rich environment, it is logical that intestinal helminths employ antimicrobial mechanisms to help establish themselves in their niches. The potential of helminths to persist within the gut for months or years means that they need to modulate the anatomical niches they occupy and reduce local competition from other organisms for these gut niches. One potential way to achieve this is through the continuous release of ESP that can kill bacteria. If so, such molecules could enhance the parasites' longevity in their hosts, and other bystander effects on host immunity [3]. However, whether intestinal helminths interact directly or indirectly with bacterial communities of the intestinal microbiota, and how helminths shape their microbial environment is not completely understood. Interactions between helminths and bacteria in the intestine can be consequential; for example the intestinal helminth *Trichuris muris* requires direct interactions with intestinal bacteria to facilitate successful egg hatching [19].

The current study examines whether the rodent hookworm *Nippostrongylus brasiliensis* (*Nb*), which is closely related to the human hookworms *Necator americanus* and *Ancylostoma duodenale* produces factors that can kill bacteria [20]. To do this, we investigated whether somatic extract, generated from whole adult *Nb*, and ESP released from adult *Nb* exhibit *in vitro* bactericidal activity against different Gram-positive and Gram-negative bacteria that form part of the intestinal microbiota and/or have pathogenic potential. Exploring the interaction between intestinal helminths and intestinal microbiota may help deepen our understanding of the mechanisms by which they interact, and offer new antimicrobial products or pathways through which they act.

METHODS

Preparation of *Nb* adult worms

Adult *Nb* worms were obtained from infected rats housed at the Department of Life Sciences, Imperial College, London, United Kingdom. Worms were retrieved as previously described [21]. After sacrificing rats, adult worms were collected from the anterior half of the small intestine, into a muslin bag immersed in warm saline (37°C) in a Baermann collection apparatus. Worms were washed five times in sterile saline followed by five washes in Roswell Park Memorial Institute (RPMI) 1640 media containing an antibiotic cocktail of 100 µg ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. Worms were then incubated in RPMI 1640 media containing 1 mg ml⁻¹ gentamicin for 20 min at room temperature. Thereafter, worms were washed five times in RPMI 1640 media containing an antibiotic cocktail of 100 µg ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. Worms were allowed to settle by gravity for 1 to 2 min between each wash step. Worms were counted by counting two 10 µl aliquots from a 10 ml suspension. A total of 2000 worms were cultured at a density of approximately 100 worms/ml in 20 ml of RPMI 1640 media supplemented with glutamine, 1% glucose, 100 µg ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin at 37°C, 5% carbon dioxide (CO₂).

Preparation of *Nb* ESP

Nb ESP were collected from adult worms as previously described [21]. Worms were extensively washed five times in sterile saline followed by five times in RPMI 1640 with an antibiotic cocktail containing 100 µg ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin, before being cultured in RPMI 1640 media supplemented with glutamine, 1% glucose, 100 µg ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin at 37°C, 5% CO₂. After 24 h of culture, the spent media was replaced with an equal amount of fresh media and worms were maintained in culture for 7 days. ESP-rich media was collected every 48 h. ESP secreted within the first 24 h were kept aside and were not used in these experiments, as these contain rat proteolytic enzymes [21]. The collected ESP was pooled, dialysed against sterile phosphate buffered saline (PBS) and concentrated by Amicon filtration using a 3 kDa cut-off membrane at 4°C. Protein concentration of ESP was determined with the Bradford assay as previously described [22] and ranged from 2.4 to 2.9 mg ml⁻¹. Each batch of ESP was stored at -80°C until required.

Heat-inactivation of *Nb* ESP

To assess the thermal stability of *Nb* ESP, the ESP were heat-treated by boiling at 95°C for 15 min.

Freeze-thaw cycles of *Nb* ESP

To further determine the stability of *Nb* ESP, the ESP were subjected to freezing and thawing. Here ESP samples were kept frozen at -80°C and allowed to thaw to room temperature before analysis. This was repeated a minimum of three times per batch of ESP.

Preparation of somatic extract

Adult *Nb* worms were killed by freezing and then homogenized in PBS kept on ice, using a sonicator. The homogenized products were harvested by centrifugation and filter sterilized using a 0.22 µm filter. Protein concentration of somatic extract was determined by Bradford assay as previously described [22] and ranged from 2.4 to 2.9 mg ml⁻¹. Extracts were stored at -80°C until required.

Bacterial strains

Staphylococcus aureus (*S. aureus*) UAMS 1182, *Escherichia coli* (*E. coli*) O9:H4 Strain HS, *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) SL1344 and *Klebsiella pneumoniae* (*K. pneumoniae*) NCTC 418 were obtained from the strain collection at the Institute of Microbiology and Infection, University of Birmingham, United Kingdom.

Bacterial growth assays for assessment of bactericidal activity of *Nb* ESP and somatic extract

For each strain, 150 µl of mid-log phase bacterial culture, adjusted to an absorbance reading of 0.025, was dispensed into the clear sterile wells of a 96-well round-bottom microplate and challenged with 20 µl (equivalent to 340 µg ml⁻¹ concentration) of either *Nb* somatic extract or ESP. Positive control wells of bacteria cultured without *Nb* somatic extract or ESP were included in each microplate. Negative control (broth only) wells were included to confirm media sterility for the duration of the incubation period. The plates were sealed with a gas-permeable membrane and then shaken at 500 r.p.m. in the BMG Clariostar microplate reader at 37°C for 17 cycles followed by absorbance measurements. Absorbance of cultures was measured at 600 nm over 16 h. All testing was performed in triplicates and on at least two separate occasions.

Bacterial growth assays co-cultured with varying concentrations of *Nb* ESP

To determine if the bactericidal activity of *Nb* ESP was concentration-dependent, the growth of *S. aureus* UAMS 1182 and *S. Typhimurium* SL1344 was assessed at ESP concentrations of 85 µg ml⁻¹ (5 µl), 170 µg ml⁻¹ (10 µl) and 340 µg ml⁻¹ (20 µl). Bacterial growth assays were set up as described above.

Determination of Minimum Inhibitory Concentration (MIC)

A broth microdilution method in 96-well microtitre plates was used to quantitatively measure the *in vitro* bactericidal activity of *Nb* ESP against *S. aureus* UAMS 1182 and *S. Typhimurium* SL1344. A previously described method [16] was adapted as follows: serial twofold dilutions of ESP (at an initial concentration of 362.5 µg ml⁻¹) were prepared in 50 µl volumes of Iso-Sensitest Broth. Each well was then inoculated with 50 µl of test microorganism suspended in Iso-Sensitest Broth at a starting inoculum of approximately 1 × 10⁵–1 × 10⁶ c.f.u. ml⁻¹, prepared from an overnight broth culture of early log-phase organisms. Positive control (bacterial culture only) and negative control (broth only) wells were included for each test microorganism to confirm appropriate microbial growth and media sterility for the duration of the incubation period, respectively. The microtitre plate was sealed with a gas-permeable membrane and a lid and then incubated at 37°C in the static incubator for 18 h. Turbidity indicated growth of the microorganism and the MIC was assessed as the lowest concentration of ESP that resulted in no visible turbidity after 18 h of incubation. The MIC was expressed in micrograms per millilitre.

Determination of Minimum Bactericidal Concentration (MBC)

A ten microlitre of sample was subcultured onto sterile (ESP-free) nutrient agar plates from each well showing diminished or no turbidity in the MIC assay. Cell counts (c.f.u. per microlitre) were determined after 18 h of incubation using the Miles and Misra method [23]. Briefly, ten-fold dilutions of the cultures were prepared and three replicate drops of each dilution were dropped onto the surface of nutrient agar plates. Three plates per culture were prepared. After the drops had dried, the plates were incubated for 18 h at 37°C. The number of countable colonies that grew in each of the inoculated areas was recorded. The MBC corresponded to the lowest concentration of *Nb* ESP that showed no growth after subculturing.

Statistical analysis

Prism Graph-pad version five was used to analyse the data. Data are expressed as the mean ± standard error of mean (SEM); and comparison between the different test cultures was carried out using area under the curve (AUC) measurements; bacterial

doubling time (Dt) measurements; and two-way repeated analysis ANOVA. Significance was accepted at $P < 0.05$. Values $P < 0.001$ (***) were used to emphasise results that had greater statistical significance.

RESULTS AND DISCUSSION

To determine whether components derived from adult *Nb* have bactericidal effects, growth assays were performed using *Nb* somatic extract and ESP, and Gram-positive (*S. aureus* UAMS 1182) and Gram-negative (*E. coli* O9:H4 Strain HS, *S. Typhimurium* SL1344 and *K. pneumoniae* NCTC 418) bacteria. The growth assays showed that *Nb* ESP displayed bactericidal activity against all four species of bacteria at a concentration of $340 \mu\text{g ml}^{-1}$. Somatic extract at an equivalent concentration did not display bactericidal activity, which may indicate processing into active component(s) on secretion. Interestingly, when treated with *Nb* somatic extract, the area under curve (AUC) for all Gram-negative bacteria increased when compared to positive controls. However, the AUC for *S. aureus* decreased when treated with *Nb* somatic extract. When treated with *Nb* ESP, the AUC for all species of bacteria decreased when compared to the positive controls. In addition, when treated with $340 \mu\text{g ml}^{-1}$ of *Nb* ESP, the doubling time (Dt) of all species of bacteria was extended when compared to the positive controls. When treated with $340 \mu\text{g ml}^{-1}$ of *Nb* ESP, the Dt for *E. coli* O9:H4 Strain HS was 3.73 h (positive control: 2.37 h); *S. aureus* UAMS 1182 was 3.16 h (positive control: 2.16 h); *K. pneumoniae* NCTC 418 was 4.77 h (positive control: 2.38 h) and *S. Typhimurium* SL1344 was 4.10 h (positive control: 2.58 h). Therefore, when treated with *Nb* ESP, all species of bacteria showed decreased growth when compared to the positive controls. In all experiments, a significant difference in the growth kinetics of bacteria co-cultured with ESP, bacteria co-cultured with somatic extract and controls was observed ($P < 0.0001$) (Fig. 1). Previous studies have examined the bactericidal activity of ESP derived from intestinal helminths [16–18], and it was observed that the concentration of *Nb* ESP used here was higher than typically reported in those studies. Differences in helminth species and the bacterial strains used across the different studies may contribute to the differences in helminth bactericidal efficacies that were observed between studies. For example, using radial diffusion assays, Rausch *et al.* [18] demonstrated that $5 \mu\text{g}$ native ESP/well derived from *Heligmosomoides polygyrus* inhibited the growth of *E. coli*, *S. Typhimurium*, *E. faecium* and *S. aureus* [18]. Similarly, Abner *et al.* [16] demonstrated that ESP of *Trichuris suis* inhibited the growth of *Campylobacter jejuni* at concentrations 80, 60, 40, and $20 \mu\text{g}$ total protein/disc, and produced growth inhibition zones of 27, 25, 18, and 13 mm, respectively [16]. The use of different techniques to assess bactericidal activity may also account for the differences in the effective bactericidal concentrations observed in this and other studies. For example, Midha *et al.* [17] and Rausch *et al.* [18]

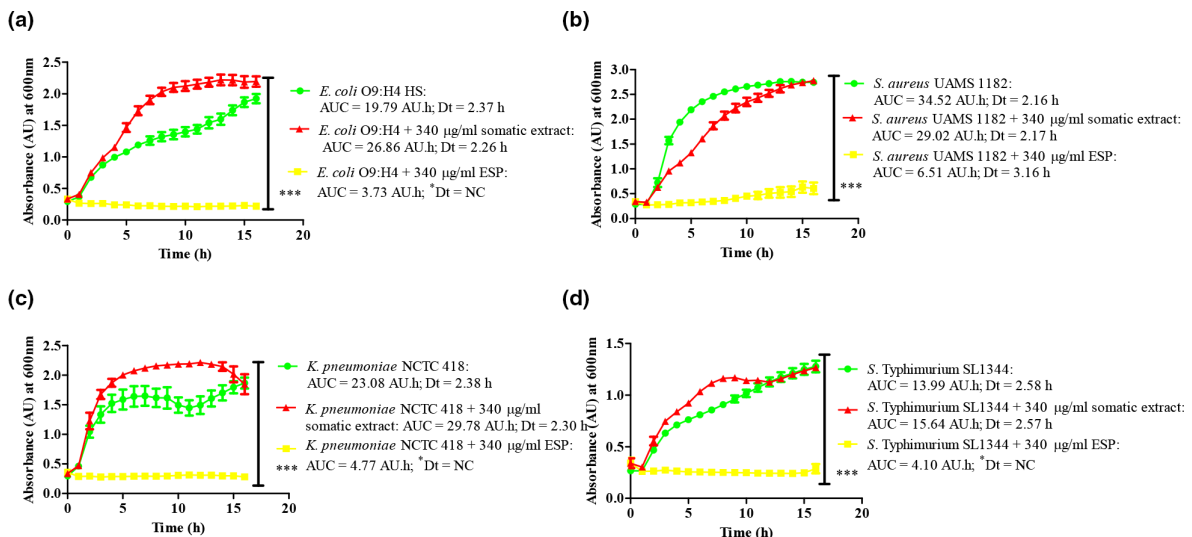


Fig. 1. Excretory-secretory products (ESP) of *Nippostrongylus brasiliensis* (*Nb*) display *in vitro* bactericidal activity. Growth assays for *E. coli* O9:H4 Strain HS (a), *S. aureus* UAMS 1182 (b), *K. pneumoniae* NCTC 418 (c) and *S. Typhimurium* SL1344 (d) were performed in the presence and absence of *Nb* somatic extract and ESP. For each strain, $150 \mu\text{l}$ of bacterial culture was challenged with $20 \mu\text{l}$ (equivalent to $340 \mu\text{g ml}^{-1}$) of either somatic extract (red) or ESP (yellow). Control wells of bacteria cultured without ESP or somatic extract were included (green). Plates were incubated at 37°C and shaken at 500 r.p.m. in the BMG Clariostar microplate reader. Absorbance of cultures was measured at 600 nm over 16 h. Data are expressed as the mean \pm SEM; comparison between different treatments was carried out using two-way repeated analysis ANOVA; area under curve (AUC) was calculated; bacterial doubling time (Dt) was calculated; *Dt=NC: doubling time not calculated because bacterial growth did not exceed baseline and AUC was used as a quantitative proxy for Dt; $n=6$ technical replicates and data representative of two independent experiments. Bacterial growth was inhibited in the presence of ESP at a concentration of $340 \mu\text{g ml}^{-1}$, but not in the presence of somatic extract. In all experiments there was a significant difference in growth kinetics of cultures with ESP, cultures with somatic extract and controls (***) ($P < 0.0001$).

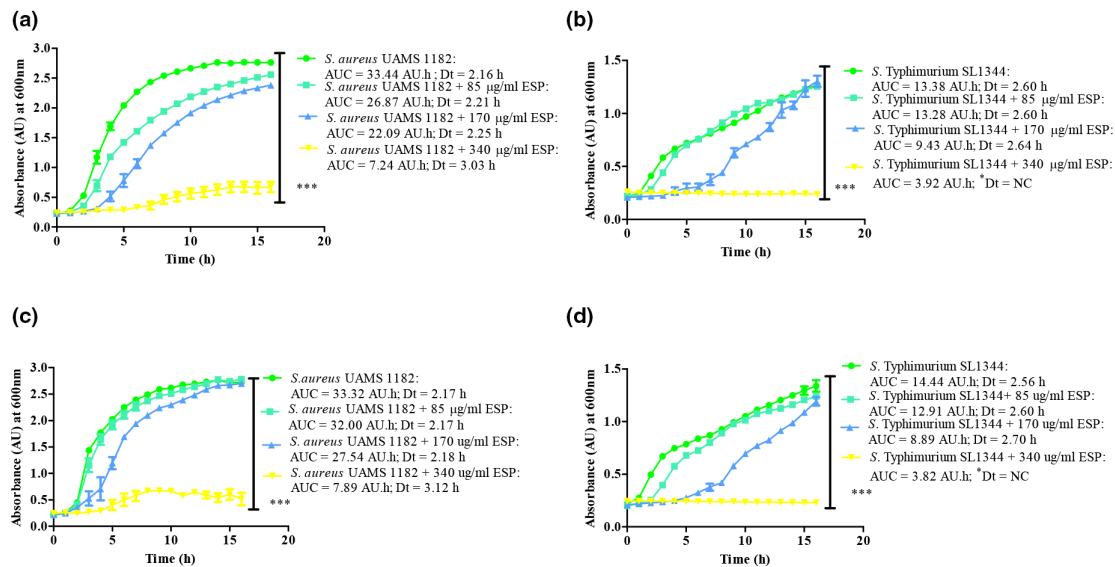


Fig. 2. Bactericidal activity of *Nippostrongylus brasiliensis* excretory-secretory products (ESP) is concentration-dependent and maintained after heat treatment at 95°C. The growth of *S. aureus* UAMS 1182 and *S. Typhimurium* SL1344 was assessed at varying concentrations of ESP (a, b) and heat-treated ESP (c, d). For each strain, 150 µl of bacterial culture was challenged with 85 µg ml⁻¹ (teal), 170 µg ml⁻¹ (blue) or 340 µg ml⁻¹ (yellow) of ESP. Control wells of bacteria cultured without ESP were included (green). Plates were incubated at 37°C and shaken at 500 r.p.m. in the BMG Clariostar microplate reader. Absorbance of cultures was measured at 600 nm over 16 h. Data are expressed as the mean ± SEM; comparison between different treatments was carried out using two way repeated analysis ANOVA; area under curve (AUC) was calculated; bacterial doubling time (Dt) was calculated; *Dt=NC: doubling time not calculated because bacterial growth did not exceed baseline and AUC was used as a quantitative proxy for Dt; n=6 technical replicates and data representative of two independent experiments. There was a significant difference in growth kinetics of cultures with ESP/heat-treated ESP and controls ***P<0.0001.

used radial diffusion assays in their studies [17, 18]. In addition to radial diffusion assays, Abner *et al.* [16] used MIC and MBC assays to assess bactericidal activity of *Trichuris suis* ESP. The MICs of *Trichuris suis* ESP against different strains of *Campylobacter* spp. ranged from 1:32 to 1:512 (dilution concentrations) and corresponded to antibacterial titres of 32–512 AU/ml [16]. To investigate the potency of the effects of *Nb* ESP, we used MIC and MBC assays to assess bactericidal activity against two bacterial species, *S. aureus* and *S. Typhimurium*. Here, the MIC of *Nb* ESP against both *S. aureus* UAMS 1182 and *S. Typhimurium* SL1344 were determined to be 181.3 µg ml⁻¹. The MBC of ESP for *S. aureus* UAMS 1182 and *S. Typhimurium* SL1344 was 181.3 µg ml⁻¹ and 362.5 µg ml⁻¹, respectively. Moreover, the active molecule(s) may only be a small component of the total ESP and so the actual MIC and MBC for the active components may be substantially lower. The bactericidal activity of *Nb* ESP against *S. aureus* UAMS 1182 and *S. Typhimurium* SL1344 was concentration-dependent, although interestingly even at the lowest concentration tested some activity against *S. aureus* was detectable (Fig. 2). Finally, whether the bactericidal activity of the ESP was sensitive to heat-treatment or freeze-thaw cycles was assessed and even after exposure to both stresses activity was maintained (Fig. 2). The heat stable antimicrobial activity from *Ascaris suum* originally described by Kato [15] was determined to be due to a defensin-like antimicrobial peptide (AMP) [24], subsequently discovered as a family of related peptides expressed by many nematode species, including hookworms [25, 26]. Abner *et al.* [16] demonstrated that bactericidal activity in the ESP derived from the porcine intestinal helminth *Trichuris suis*, was concentration-dependent, heat stable and resistant to digestion with pronase E and trypsin [16]. More recently, broad-spectrum bactericidal activity in the ESP of L4-stage larvae and adult *Ascaris suum* [17] and *Heligmosomoides polygyrus* [18] have also been demonstrated. In addition, adult *Ascaris suum* ESP was shown to interfere with biofilm formation by *E. coli* and caused bacterial agglutination [17]. Moreover, authors Midha *et al.* identified helminth proteins and peptides with known and predicted antimicrobial functions in the ESP and body fluid of *Ascaris suum*. These included galectins, C-type lectin domain-containing (CTLD) proteins, AMPs, lysozyme and cystatin [17]. Similarly, Rausch *et al.* [18] observed differential gene expression of factors with putative antimicrobial defence functions, such as chitinase and lysozyme, in *Heligmosomoides polygyrus* isolated from germ-free versus conventional mice [18]. Additionally, helminth defence molecules with potential immunomodulatory and antimicrobial properties have previously been described [27]. These studies, together with observations in the current study, demonstrate that intestinal helminths produce various antimicrobial factors that may influence bacterial microbiota in their environments. The ability to produce not only a repertoire of immunomodulatory molecules but also antimicrobial factors would enable intestinal helminths to successfully establish themselves in a microbe-rich environment. The current study has some limitations within which its findings must be interpreted. We did not perform time-kill assays in the present study and a more

comprehensive assessment of this may elucidate more features and understanding of *Nb* ESP bactericidal activity. To expand on the present findings, our follow-up experiments will include: (i) comprehensive analyses of the *Nb* ESP to characterise and identify candidate molecules with bactericidal activity; (ii) assessment of the bactericidal and/or bacteriostatic properties of *Nb* ESP using time-kill assays; (iii) the effect of protease treatment on *Nb* ESP bactericidal activity; and (iv) evaluation of the potential antiviral and antimycobacterial activities of *Nb* ESP.

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Author contributions

R.P.: Data curation, formal analysis, investigation, methodology, writing – original draft. Z.L.M.K.: Funding acquisition, project administration, supervision, writing – review and editing. C.I.: Supervision, writing – review and editing. M.E.S., R.B.: Resources, writing – review and editing. W.G.C.H., I.H., P.N., A.J.N., R.S., A.F.C.: Writing – review and editing. M.K.O'S.: Conceptualisation, resources, supervision, writing – review and editing.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Ethical statement

Ethical approval for this study was obtained from the Biomedical Research Ethics Committee at the University of KwaZulu Natal, South Africa (approval number BREC/00000516/2019). Approval for the use of *Nb* ESP and somatic extract and selected bacterial strains was obtained from the University of Birmingham, United Kingdom (approval number AG 18_44).

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