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2 **Structural and functional analyses of nematode-derived antimicrobial peptides support**
3 **the occurrence of direct mechanisms of worm-microbiota interactions**

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24 **ABSTRACT**

25 The complex relationships between gastrointestinal (GI) nematodes and the host gut microbiota
26 have been implicated in key aspects of helminth disease and infection outcomes. Nevertheless,
27 the direct and indirect mechanisms governing these interactions are, thus far, largely unknown.
28 In this proof-of-concept study, we demonstrate that the excretory-secretory products (ESPs)
29 and extracellular vesicles (EVs) of key GI nematodes contain peptides that, when
30 recombinantly expressed, exert antimicrobial activity *in vitro* against *Bacillus subtilis*. In
31 particular, using time-lapse microfluidics microscopy, we demonstrate that exposure of *B.*
32 *subtilis* to a recombinant saposin-domain containing peptide from the 'brown stomach worm',
33 *Teladorsagia circumcincta*, and a metridin-like ShK toxin from the 'barber's pole worm',
34 *Haemonchus contortus*, results in cell lysis and significantly reduced growth rates. Data from
35 this study support the hypothesis that GI nematodes may modulate the composition of the
36 vertebrate gut microbiota directly *via* the secretion of antimicrobial peptides, and pave the way
37 for future investigations aimed at deciphering the impact of such changes on the
38 pathophysiology of GI helminth infection and disease.

39 **1. Introduction**

40 Antimicrobial peptides (AMPs) are a ubiquitous and diverse group of peptides, typically less
41 than 100 amino acid residues in length, whose cationic and amphipathic properties confer
42 broad-spectrum antimicrobial activity against both prokaryotes and eukaryotes [1]. In
43 particular, the antibacterial activity of AMPs is underpinned by several mechanisms, ranging
44 from interference with the biosynthesis and processing of microbial DNA, RNA and proteins,
45 to the disruption of membrane integrity through the formation of pores and/or channels that
46 causes leakage of cellular components, or cell death by osmotic shock [2]. To date, over 3500
47 AMPs have been described from a wide range of vertebrate and invertebrate organisms, the
48 latter including insects, crustaceans, spiders, molluscs, and free-living and parasitic worms
49 (i.e., helminths) [3-5].

50 Helminths are amongst the most prevalent infectious agents of humans and animals
51 worldwide [6]. Currently, around 1.5 billion people are estimated to be infected by helminths,
52 causing over 3 million disability-adjusted life years (DALYs) annually, particularly in
53 developing regions of the globe [6,7]. Moreover, in Europe alone, helminths of livestock are
54 estimated to cost the food industry around €1.8 billion/year due to animal death, productivity
55 losses (e.g., reduced weight gain), and costs associated with veterinary intervention [8]. Current
56 strategies of helminth control in both humans and livestock rely heavily on the administration
57 of anthelmintics in mass drug administration (MDA) or in targeted strategic treatment (TST)
58 programmes, respectively [9,10]. However, resistance to all classes of anthelmintic compounds
59 is now widespread in helminths of livestock, and the reliance on a relatively small number of
60 anthelmintics in MDA programmes makes the emergence of drug-resistant human parasites
61 both likely and concerning [11]. Thus, over the last decades, efforts have been directed towards
62 achieving a better understanding of the fundamental biology of helminths and their interactions

63 with their vertebrate hosts, with the ultimate goal of identifying novel targets for the
64 development of sustainable strategies of parasite control [12].

65 One emerging area of interest in host-parasite interaction research is the elucidation of
66 the surrounding crosstalk between helminths, particularly nematodes of the vertebrate
67 gastrointestinal (GI) tract and the resident gut microbiota/-me [13-15]. The latter is defined as
68 *"a diverse consortium of bacteria, archaea, fungi, protozoa, viruses* [the microbiota]*, and their*
69 *collective genome* [the microbiome]" [16], that play several key roles in host physiology,
70 including nutrient absorption and metabolism, energy homeostasis, immune system
71 development and defence against pathogens. Over the last decade, several studies, conducted
72 in both humans and animals, under experimental and natural conditions of GI helminth
73 infections, have consistently reported significant associations between worm colonisation and
74 changes in the composition and/or function of the host gut microbiota [17-19]. However, the
75 mechanisms governing worm-microbiota interactions remain, thus far, unclear [20]. Until
76 recently, the vast majority of host-parasite interaction studies have focussed on the ability of
77 helminths to communicate with and modulate the immune system of the host, particularly
78 through the activity of their excretory/secretory products (ESPs) [21,22]. Intriguingly, recent
79 evidence points towards a role of helminth ESPs in worm-microbiota interactions, namely by
80 exerting antimicrobial activity [5,23,24]. For instance, ESPs of the swine roundworm, *Ascaris*
81 *suum,* have been shown to exert significant bactericidal activity against *Escherichia coli*,
82 *Staphylococcus aureus,* and *Salmonella typhimurium*, and to disrupt biofilm formations of *E.*
83 *coli in vitro* [5]. Indeed, *A. suum* ESPs contain several antimicrobial molecules, including
84 cecropin-P2 [25], *A. suum* antibacterial factor (ASABF), lysozymes, as well as C-type lectin
85 domain-containing proteins [5], that can disrupt bacterial membranes to cause cell lysis, or
86 promote lethal agglutination of bacterial cells. More recently, we also demonstrated that *in*
87 *vitro* exposure of *E. coli* to ESPs from the 'brown stomach worm', *Teladorsagia circumcincta*,

88 an abomasal parasite of small ruminants, results in significant reductions of colony forming
89 units over a 3-hour period [24]. Proteomics analyses of *T. circumcincta* ESPs, coupled with
90 bioinformatics screening of amino acid sequence data, identified several putative AMPs,
91 including a histone H4-like protein, an *Ancylostoma*-secreted protein (ASP)-like protein, and
92 an invertebrate-specific lysozyme-like enzyme known as destabilase [24]. Previously, we
93 hypothesised that the antimicrobial properties of ESPs from *T. circumcincta*, as well as of ESPs
94 from other GI helminths of humans and animals, may be attributed to the activity of secreted
95 AMPs, free and/or encapsulated in secreted extracellular vesicles (EVs) [24]. Nevertheless,
96 experimental evidence is essential to design follow-up mechanistic investigations aimed to
97 translate fundamental knowledge of helminth-host-microbiota relationships into novel
98 therapeutic approaches. Thus, in this study, we (i) mined publicly available ESP and EV
99 proteomics sequence data for a range of GI nematodes of public health and veterinary
100 importance and identified putative AMPs *via* targeted bioinformatics analyses; (ii)
101 characterised structural and biochemical properties of selected AMP candidates; (iii) produced
102 these candidates using heterologous expression in yeast; (iv) tested the resulting recombinant
103 AMPs *in vitro* against representative Gram-negative and Gram-positive bacteria; and (v)
104 performed microfluidics-based time-lapse microscopy to gather insights into their possible
105 mode(s) of action. Of the putative AMPs identified across publicly available ESPs and/or EV
106 proteomics data from eight GI nematodes, two (i.e., a saposin domain-containing peptide from
107 *T. circumcincta* EVs and a metridin ShK toxin domain-containing peptide from ESPs of the
108 'barber's pole worm', *Haemonchus contortus*) exerted significant antibacterial activity against
109 the Gram-positive *Bacillus subtilis*.
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2. Methods

2.1 Literature search

Iterative literature searches were carried out to compile a list of publications linked to proteomics datasets for GI nematode ESPs and/or EVs. In particular, Google Scholar (https://scholar.google.com/) and PubMed (https://pubmed.ncbi.nlm.nih.gov/) were interrogated using the search term "Gastrointestinal nematode excretory secretory products". Several secondary searches were performed for each database targeting helminth species associated with qualitative and/or quantitative alterations in vertebrate gut microbiota composition according to published literature, e.g., "*Ascaris suum* excretory secretory products", and "*Haemonchus contortus* excretory secretory products". All searches were completed on or before 4th July 2023. From each search, articles that included fully accessible links to corresponding proteomics datasets, and/or up-to-date accession numbers for sequences identified during proteomics analysis, were compiled. For articles referring to the analysis of nematode ESP and/or EV proteomics data that were however inaccessible (unpublished or unobtainable), the corresponding author was contacted by email in an attempt to retrieve this information. A list of articles containing accession numbers linked to proteomics datasets was compiled. Accession numbers were subsequently extracted, uploaded onto the UniProt ID Mapping tool (https://www.uniprot.org/id-mapping; [26]), and corresponding amino acid sequences were retrieved. In instances where accession numbers could not be assigned to corresponding sequences using the UniProt ID Mapping tool, individual accession numbers were manually entered in the search string of the specific database cited in relevant publications. Accession numbers that could not be assigned a protein sequence using either of the aforementioned methods were excluded from downstream analyses. Finally, all protein sequences and corresponding accession numbers were saved in FASTA format and subjected

138 to AMP prediction analyses as described below. For *T. circumcincta*, the original proteomics
139 datafiles obtained by Tzelos *et al.* [27] were used to interrogate a comprehensive in-house
140 dataset of Iso-Seq transcripts from third- and fourth-stage larvae (L3 and L4, respectively) and
141 adult male and female worms (data available from Mendeley data at DOI:
142 10.17632/hzrcpjh6y7.1). ESP and EV sequences identified using this approach were assigned
143 InterPro identifiers (InterPro IDs) and Gene Ontology (GO) terms using OmicsBox
144 (https://www.biobam.com/omicsbox) [28].

145

146 *2.2 Antimicrobial peptide and protein prediction*

147 All amino acid sequences collated as described above were screened for the occurrence of
148 features indicative of likely antimicrobial activity [24]. Firstly, sequences were individually
149 used to query the Collection of Anti-Microbial Peptides ($CAMP_{R3}$) database
150 (http://www.camp.bicnirrh.res.in/) [29]. Significant similarities between helminth amino acid
151 sequences and AMPs in the $CAMP_{R3}$ database were identified using the in-built BLASTp tool
152 with the following criteria: matrix = BLOSUM62; alignment = ungapped; E-value
153 threshold = $1E^{-5}$. In instances where a given query sequence shared significant similarity to
154 multiple $CAMP_{R3}$ sequences, only the top scoring hit was retained. Secondly, all GI helminth
155 ESP- and EV-associated amino acid sequences were subjected to ampir analysis
156 (https://ampir.marine-omics.net/). Ampir utilizes a supervised statistical machine learning
157 approach to assign a probability score (ranging from 0 to 1, where 1 indicates the highest
158 probability of antimicrobial activity), based on the physico-chemical properties of the amino
159 acid patterns associated with each sequence. For each ESP- and EV-associated amino acid
160 sequence, AMP prediction was performed using two vector machine classification models, i.e.,
161 “precursor” (best suited for analysis of full-length proteins) and “mature” (for sequences
162 representing the final AMP after post-translational processing) [30]. The probability scores

generated by each classification model were recorded; sequences returning ampir scores of > 0.7 were retained as ‘sequences of interest’. Finally, MultiPep (https://github.com/scheelelab/MultiPep) was used to predict the antimicrobial activity of each GI helminth ESP- and EV-associated amino acid sequence; MultiPep applies convolutional neural networks to assign each peptide to one of twenty bioactivity classes according to their intrinsic amino acid patterns [31]. Within each bioactivity class, each sequence is assigned a probability score ranging from 0 to 1, where 1 corresponds to the highest likelihood of accurately identifying a given query sequence as belonging to the respective bioactivity class. Since MultiPep screening is limited to sequences <200 amino acids in length, sequences that did not meet this criterion were excluded from this analysis, while suitable amino acid sequences were formatted according to MultiPep requirements; the ‘MultiPep_predict.py’ Python script was executed as instructed by the software developer. For each of these sequences, bioactivity class scores were recorded; sequences with scores > 0.7 for either ‘antimicrobial’ or ‘antibacterial’ classes were retained as ‘sequences of interest’ [24].

GI nematode ESP- and EV-associated amino acid sequences that were marked as sequences of interest using one or more antimicrobial activity prediction tools, and/or had been previously reported as exerting antimicrobial activity according to published literature, were compiled into a list of putative helminth-derived AMPs to be subjected to downstream analyses.

2.3 Peptide modelling

Computational structure prediction was performed for amino acid sequences with the highest probability of antimicrobial activity using Robetta (https://robetta.bakerlab.org/). Briefly, following removal of signal peptides, tertiary peptide models were generated using RoseTTAFold [32]. For each predicted model, the RoseTTAFold assigns a structure

188 confidence score between 0 (low confidence) and 1 (high confidence). The highest confidence-
189 scoring model was selected for downstream computational analyses and, for these models, the
190 Protein Data Bank (PDB) coordinate file was obtained. The spatial arrangement of the
191 predicted antimicrobial model at the bacterial membrane was simulated using the PPM 3.0
192 server (https://opm.phar.umich.edu/ppm_server3) in the Orientations of Proteins in
193 Membranes (OPM) database (https://opm.phar.umich.edu/) [33] with the following
194 parameters: (i) type of membrane = Gram-negative/-positive lipid bilayer membrane; (ii) allow
195 curvature = no; (iii) include heteroatoms = no. PPM 3.0 outputs were saved, including the
196 predicted position and orientation of the peptide of interest in the bacterial membrane, as well
197 as the specific amino acid residues putatively involved in protein-membrane interactions (when
198 applicable). The three-dimensional coordinates of the peptide-membrane assembly were
199 downloaded as a PBD file and uploaded onto CHARMM-GUI (https://www.charmm-gui.org/)
200 [34], where the bacterial membrane was rebuilt and subsequently visualised using ChimeraX
201 (https://www.rbvi.ucsf.edu/chimerax/) [35].

202 The surface electrostatics of predicted peptide structures were analysed using the
203 APBS-PDB2PQR software suite (https://server.poissonboltzmann.org/) [36]. First, predicted
204 peptide model PDB coordinates were converted into PQR files using PDB2PQR with a PARSE
205 forcefield [37]. The PQR files were subsequently uploaded to the Adaptive Poisson-Boltzmann
206 Solver (APBS), and electrostatic surface values were generated. The output files were
207 visualised on ChimeraX [35]. ChimeraX was also applied to the visualisation of any intra-
208 peptide disulphide bonds and calculation of disulphide bond length. Thiol groups of a pair of
209 cysteines within 2.0 Å and 3.0 Å were considered to likely form disulphide bonds [38]. For
210 each peptide, the isoelectric point (pI), % amino acid composition, and overall charge at pH
211 7.4 were calculated using the Prot pi tool (https://www.protpi.ch/Calculator/ProteinTool). The
212 probability of each peptide of interest forming homodimers was assessed using the Alphafold

213 structure prediction tool [39] in combination with ColabFold [40], within ChimeraX [35].
214 Briefly, within ChimeraX, each putative helminth AMP sequence was used to query the
215 Alphafold database, using default settings. For each query sequence, Alphafold generates five
216 potential models, automatically selecting the 'best' model (i.e. that with highest confidence)
217 based on predicted local distance difference test (pLDDT) scores and Domain position
218 confidence (PAE) values. For each sequence of interest, the 'best' predicted homodimer model
219 was rendered in ChimeraX, with predicted pseudobonds (defined as "*a connection other than*
220 *a covalent bond, such as a hydrogen bond, metal coordination bond*" by ChimeraX) between
221 pairs of amino acid residues, ≥ 5Å apart, on separate peptide subunits. Pseudobonds are
222 coloured according to PAE values.
223
224 *2.4 Peptide expression*
225 Recombinant expression of selected peptides of interest was carried out using the
226 methilotrophic yeast *K. phaffii* (previously known as *Pichia pastoris*) as the heterologous host
227 system. In particular, following signal peptide removal, the codon usage of each sequence was
228 optimised for expression in *K. phaffii* with the Genescript codon optimization tool [41] and the
229 synthetised DNA sequence was ligated into the vector pPICZα A (Invitrogene), encoding
230 zeocin resistance and an alpha secretion peptide from the α-mating factor from *Saccharomyces*
231 *cerevisiae*, with an N-terminal His-tag and Tobacco Etch Virus (TEV) protease cleavage site.
232 The vectors were electro-transformed into freshly prepared electrocompetent *K. phaffii*
233 KM71H cells that were subsequently grown on YPDS plates (20 g/L peptone, 10 g/L yeast
234 extract, 100 mL/L dextrose 20% (w/v), 182.2 g/L sorbitol, 20g/L agar) containing 1,000 μg/mL
235 zeocin over 72 h at 30 °C. Single colonies were selected and replated onto YPDS plates
236 containing 1,000 μg/mL zeocin. Individual colonies were inoculated into 10 mL of YPD (20
237 g/L peptone, 10 g/L yeast extract, 100 mL/L dextrose 20% (w/v) in a 250 mL baffled flask and

238 grown overnight at 30 °C with 250 rpm shaking. A 10 mL culture was used to inoculate 1 L of
239 buffered glycerol complex medium (BMGY, 10 g/L yeast extract, 20 g/L peptone, 0.1 M
240 potassium phosphate pH 6.0, 1.34% (w/v) YNB, 0.04 µg/mL biotin, 1% (v/v) glycerol) in a 5
241 L baffled flask and grown overnight at 30 °C with 250 rpm shaking, until the culture reached
242 an OD_{600} between 2 and 6 as recommended by the manufacturer [42]. The cells were harvested
243 by centrifugation at 3,000 x g for 5 minutes at room temperature and subsequently resuspended
244 into 200 mL of buffered methanol complex medium (BMMY, 10 g/L yeast extract, 20 g/L
245 peptone, 0.1 M potassium phosphate pH 6.0, 1.34% (w/v) YNB, 0.04 µg/mL biotin, 0.5% (v/v)
246 methanol) in a 1 L baffled flask, covered with 2 layers of sterile cheesecloth and incubated at
247 30 °C with constant shaking at 250 rpm. The culture was incubated for a total of 72 hours, with
248 100% methanol added to a final dilution of 0.5% (v/v) every 24 hours. The cells were then
249 pelleted by centrifugation at 3,000 x g for 5 minutes at room temperature, the supernatant was
250 collected, sterilised by filtration with 0.2 µm filter (Millipore), and stored at 4 °C until further
251 use.

252 Successfully expressed peptides were purified from the supernatant using Ni-NTA
253 affinity chromatography in a gravity column setup, in which the Ni-NTA resin specifically
254 binds to the N-terminal His-tag on the recombinant peptide. Briefly, the filtered supernatant
255 was dialyzed against wash buffer (50 mM TRIS-HCl, pH 8.0, 300 mM NaCl, 20 mM
256 imidazole) and mixed with 3 mL of HIS-Select® Nickel Affinity Gel resin (ThermoFisher)
257 equilibrated in the same buffer. After 1 h of incubation at room temperature, the resin was
258 loaded onto a column, the flow-through was collected, and the resin was washed with wash
259 buffer (50 mM TRIS-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole), until the A_{280} of the
260 column eluent reached < 0.05. The recombinant peptides were eluted with 5 column volumes
261 of elution buffer (50 mM TRIS-HCl, pH 8.0, 300 mM NaCl, 400 mM imidazole) in 1 mL
262 fractions. Subsequently, eluates containing peptides according to their A_{280} were pooled and

263 dialysed at 4 °C against 1 L of PBS (pH 7.4) for at least 24 hours. The dialysis buffer was
264 refreshed 3 times during the dialysis process to ensure complete removal of the elution buffer.
265 The concentration of each peptide was quantified using the Beer-Lambert law, with the
266 absorbance reading taken at 280 nm and adjusted using the theoretical molar extinction
267 coefficient of the peptide (https://web.expasy.org/protparam/) [43]. At each step of the
268 purification process, peptide purity was verified using precast 4-12% Novex Bis- TRIS sodium
269 dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (ThermoFisher) and
270 stained with colloidal Coomassie Brilliant Blue (Sigma).
271 For Western blotting, protein lysates were first separated on an SDS-PAGE gel.
272 Following separation, peptides were electrophoretically transferred to an Immobilon P
273 membrane (Millipore). Once the transfer was completed, the membrane was blocked using 3%
274 milk in TBS containing 0.1% Tween-20 (TBS-T) for 1 hour at room temperature with gentle
275 agitation. After blocking, the membrane was incubated with a primary anti-6His antibody
276 (MA1-135, Invitrogen), diluted to a concentration of 1:250 in the blocking solution. This
277 incubation was carried out overnight at 4 °C with gentle shaking. The following day, the
278 primary antibody solution was discarded, and the membrane was washed three times for 10
279 minutes each using TBS-T. After the washes, the membrane was exposed to the secondary
280 horse anti-mouse IgG (H+L) antibody conjugated to horseradish peroxidase (HRP) (PI-2000,
281 Vector Laboratories), diluted to a 1:400 ratio in the blocking solution, for 1 hour at room
282 temperature with gentle agitation. Then, the membrane was again washed three times with
283 TBS-T for 10 minutes. Finally, peptide bands were visualised using an Enhanced
284 Chemiluminescence (ECL) system (Pierce, Thermofisher) and captured using a Biorad
285 BioImager.
286
287 *2.5 Antimicrobial activity assays*

The bactericidal and/or bacteriostatic activity of each of the recombinantly expressed peptides was assessed against representative Gram-positive and -negative bacterial strains, i.e., *E. coli* BW25113 wild-type, *Pseudomonas aeruginosa* PA14*, B. subtilis* JH642*,* and *S. aureus* NCTC 12493 using a colony counting method [24]. Briefly, each bacterial strain was grown on a LB agar plate, and a single colony was selected from each plate and inoculated into 10 mL of sterile LB broth overnight at 37 °C with shaking at 250 rpm. Thereafter, each culture was diluted to OD_{600} = 0.165 (approximately 1.5 x 10^8 CFU/ml), and subsequently to 1:5,000. 10 µL of the 1:5,000 bacterial culture was added to the wells of a 96-well plate along with 90 µL of LB broth. Each recombinant peptide was added to the wells containing bacterial colonies to a final concentration of 100 µg/mL in a final well volume of 200 µL. 100 µg/mL of pexiganan (for *E. coli*, *P. aeruginosa,* and *B. subtilis*; [44]) and 100 µg/mL vancomycin (for *S. aureus;* [45]) were included as positive controls. Negative controls consisted of 10 µL of the 1:5,000 bacteria culture and 100 µL PBS (pH 7.4). Each experiment was conducted in triplicate. The 96-well plate was incubated at 37 °C with shaking at 250 rpm for 210 minutes. The Miles and Misra colony counting method was used to measure antibacterial activity, as previously described [24,46]. After incubation, a 10 µL aliquot was retrieved from each active well, diluted 1:10, 1:100 and, 1:1,000 using sterile filtered PBS, plated onto LB agar plates, allowed to dry completely at 25 °C, and finally incubated at 37 °C for 18 hours. For each of the three replicates included for each condition, colony counts were performed from the LB agar plates containing the least diluted bacteria that yielded distinct colonies. The number of bacteria colonies formed on negative control plates was also counted. The following formula was applied to calculate the number of colony-forming units (CFU)/mL in each replicate: CFU/mL = (number of colonies × 20 × dilution factor) x 5. GraphPad Prism version 5.01 was used to compare the CFU/mL values in the presence of recombinant peptide with the CFU/mL values of the negative control, and bactericidal activity was determined using one-way analysis of variance

313 (ANOVA) with Tukey's multiple comparison test. A reduction in CFU/mL values at $P \leq 0.05$
314 was considered statistically significant.
315
316 *2.6 Microfluidics device fabrication and cell loading*
317 Fabrication of the mother-machine microfluidic platform was achieved using
318 polydimethylsiloxane (PDMS) base elastomer and curing agent, which were combined and
319 mixed at a ratio of 10:1. To eliminate air bubbles, the mixture underwent a degassing process
320 in a desiccator for 30 minutes. The degassed mixture was uniformly spread over a silicon wafer,
321 which had microfluidic design features pre-fabricated using laser lithography. A secondary
322 degassing step was implemented to ensure the removal of any remaining bubbles. The PDMS
323 was baked at 95 °C for 1 hour. Individual mother-machine microfluidic chips were cut from
324 the PDMS mould, and holes for inlets and outlets were created using a 0.75 mm biopsy
325 puncher. Subsequently, the PDMS chips and coverslips were cleaned. The PDMS chips were
326 submerged in isopropanol and sonicated for 30 minutes, then dried using an air gun and baked
327 at 95 °C for 30 minutes. The PDMS chips were sonicated again in deionized (DI) water for 30
328 minutes. The coverslips were first sonicated in 1 M potassium hydroxide for 20 minutes, and
329 then in DI water for another 20 minutes. The clean coverslips were dried with an air gun and
330 incubated for 30 minutes at 95 °C. 24 hours prior to the experiments, the feature side of the
331 cleaned PDMS chip was bonded to a clean coverslip using plasma treatment (2 minutes at 35
332 W and air pressure set to 0.2-0.3 mbar). Post-bonding, the devices were baked for 1 hour at 95
333 °C.
334 Immediately before the experiments, the devices were passivated and cleaned by
335 flowing LB + pluronic (0.8% of a 0.1 g/mL stock) through the microfluidic lanes using a pair
336 of gel-loading tips. To load the cells into the microfluidic device, 1 mL of overnight bacterial
337 culture was concentrated by centrifugation at 1,000 x g for 3 minutes and then resuspended in

338 50 µL LB media. A small amount of resuspended high-density cells was aspirated with special
339 gel-loading pipette tips and loaded into the lanes of the mother-machine devices through the
340 inlets. The loaded chips were centrifuged at 1,000 x g for 1 minute to place the cells into the
341 narrow cell-trenches orthogonal to the flow lane. Subsequently, fresh LB was flown through
342 the device to clear the feeding lane and provide nutrients to the cells in the trenches.
343
344 *2.7 Timelapse microscopy of antimicrobial effects on cells*
345 Timelapse microscopy and effects of selected recombinantly expressed helminth AMPs on
346 selected bacteria was achieved using a Nikon ECLIPSE Ti2 with a 40x 0.95NA objective lens
347 and a 1.5x post-objective magnification. The images were acquired using a Hamamatsu
348 ORCA-Fusion Digital CMOS camera with a pixel size of 6.5 µm x 6.5 µm. Samples were
349 illuminated with a brightfield light source using the phase-contrast setup. The mother-machine
350 was fixed onto the stage inside an incubator maintained at 37 °C with a continuous supply of
351 fresh autoclaved LB media and LB media containing individual recombinant AMPs through
352 silicone tubing. The outlet was connected to a waste bottle. Focal drift from minor thermal
353 fluctuations were eliminated by the Nikon Perfect Focus System (PFS), and data acquisition
354 was controlled by the Nikon elements software. This allowed time-lapse imaging of multiple
355 fields of view (FOVs). The cells were imaged using phase contrast mode every 1 minute with
356 100 millisecond exposure per frame, and PFS turned on throughout the experiment.
357
358 *2.8 Image processing and single-cell data analysis*
359 Image data was saved in ND2 format and subsequently converted to individual TIFF
360 snapshots for each FOV using custom Python scripts. The images were registered in time-series
361 to compensate for any stage drift caused by minor thermal fluctuations, then extracted into
362 single-trench images using custom-designed image-processing pipelines, as described in

363 earlier work [47]. Cells were detected and outlined from the single-trench images using the
364 deep-learning image-segmentation algorithm called Omnipose [48], which was retrained with
365 synthetic images of cells in microfluidic trenches generated using the virtual microscopy
366 platform SyMBac [47]. A custom-designed lineage tracking program
367 (https://github.com/erezli/MMLineageTracking) was used to track the cell lineages over time.
368 Cell size timeseries data was extracted from each tracked lineage and growth rate of individual
369 cells and lysis frequency was computed from these data .
370
371 **3. Results**
372 *3.1 Literature search and* in silico *AMP predictions*
373 A total of 3,870 accession numbers were retrieved from 12 publications describing proteomics
374 datasets of ESPs from eight GI parasitic nematodes, i.e. *A. suum, H. contortus,*
375 *Heligmosomoides polygyrus, Nippostrongylus brasiliensis, Strongyloides ratti, T.*
376 *circumcincta, Toxocara canis,* and *Trichuris suis* (not shown). Of these, 3,749 were
377 successfully matched with their respective protein sequences (Table S1). A search for
378 proteomics datasets generated from EVs of GI parasitic nematodes yielded a total of 850
379 accession numbers from *A. suum, H. polygyrus, N. brasiliensis,* and *T. circumcincta*, all of
380 which were successfully matched with their respective protein sequences (Table S1).
381 Complete lists of ESP- and EV-associated protein sequences and corresponding Gene
382 Ontology (GO) and InterPro annotations, together with putative antimicrobial activity scores
383 (based on $CAMP_{R3}$ BLASTp, ampir and Multipep analyses, see Materials and Methods) are
384 provided in Table S2. Of the 3,749 ESP-associated protein sequences subjected to downstream
385 analyses, three were selected for further characterisation based on high antimicrobial activity
386 prediction scores, i.e., a metridin ShK toxin domain-containing peptide from *H. contortus*
387 (*H*met), a histone H2A from *T. canis* (*Tc*his), and a destabilase from *T. circumcincta* (*T*des)

388 (Table S2). *H*met is composed of 84 amino acid residues with a signal peptide from residue 1-
389 20. The protein includes a ShK domain-like region (residues 45 to 84) and a region with
390 membrane-binding function (residues 24 to 84) (Table S2). No significant similarity between
391 *H*met and known AMPs in the $CAMP_{R3}$ database was identified. Nevertheless, *H*met was
392 classified as a putative AMP by both ampir (score = 0.996) and Multipep (0.746) (Table 1).
393 *Tc*his (126 amino acid residues) displayed significant sequence similarity to a known
394 antibacterial histone H2A derived from the whiteleg shrimp *Litopenaeus vannamei* (E-value:
395 $4E^{-75}$) and was classified as a putative AMP by ampir (0.992) and Multipep (0.958) (Table 1;
396 Table S2). *T*des (139 amino acid residues) belongs to the invertebrate-type lysozyme protein
397 family and shares significant similarity to a destabilase from the Eastern oyster, *Crassostrea*
398 *virginica* (E-value: $4E^{-39}$). This protein contains a N-terminal signal peptide and a membrane-
399 binding region that includes the destabilase/lysozyme domains (residues 18 to 139) (Table S2).
400 *T*des was assigned an ampir score of 0.923 and a Multipep score of 0.440 (Table 1).
401 Targeted analyses of 850 EV-associated sequences yielded two proteins of interest,
402 both originating from *T. circumcincta*, i.e., a cysteine-rich secretory protein (*T*scp) and a
403 saposin-b domain containing peptide (*T*sap). *T*scp (166 amino acid residues) contains a
404 predicted signal peptide (residues 1 to 18) and a membrane-binding region (19 to 166),
405 including a '*C*ysteine-rich secretory proteins, *A*ntigen 5, and *P*athogenesis-related 1 proteins'
406 (CAP) domain (31 to 166) (Table S2). *T*scp was assigned an ampir score of 0.732 (Table 1).
407 *T*sap (104 amino acids) shared significant sequence similarity to a known AMP from the free-
408 living nematode *Caenorhabditis elegans* (i.e., caenopore 5; E-value: $2E^{-9}$) (Table S2). *T*sap
409 was assigned ampir and MultiPep scores of 0.864 and 0.942, respectively (Table 1).
410
411 *3.2 Peptide structure prediction and characterisation*

412 The tertiary structures of *H*met, *Tc*his, *T*des, *T*sap, and *T*scp were predicted using Robetta
413 RoseTTAFold, with corresponding confidence scores of 0.65, 0.77, 0.89, 0.87, and 0.76,
414 respectively. Each model exhibits low predicted B-factor values, indicative of well-defined
415 structures (Fig. S1). The *H*met model contains three disulphide bonds within the second and
416 third α-helices and the C-terminus, respectively (Fig. S2a) and an overall positive charge
417 distribution [+3.6 at pH 7.4 (pI = 8.5)] (Fig. 1a). The *H*met homodimer was predicted with a
418 high confidence, showing the formation of multiple predicted pseudobonds, with low PAE
419 values, between each chain (Fig. S3). The *Tc*his model lacks disulphide bonds and is dominated
420 by a net positive charge [+13.3 at pH 7.4 (pI = 10.6)], primarily distributed across the N- and
421 C-terminal loops and on the two α-helices that flank the central 25-amino acid long α-helix
422 (Fig. 1b). Several high confidence predicted pseudobonds were identified between *Tc*his chains
423 during assessment of homodimer formation (Fig. S3). The *T*des model contains seven
424 disulphide bonds (Fig. S2b) and a net negative charge [-3.1 at pH 7.4 (pI = 6.3)]; however,
425 electrostatic surface potential analysis predicts that the opposing faces of *T*des may be
426 oppositely charged (Fig. 1c). *T*des homodimer prediction was associated with pseudobonds
427 with low confidence (i.e., >10 Å) and therefore considered unlikely to form homodimers (Fig.
428 S3). The *T*sap model contains three disulphide bonds between C13 – C87, C16 – C81, and C42
429 – C56 (Supplementary Fig. S2c), and a net charge of -4.1 at pH 7.4 (pI = 5.3); electrostatic
430 surface potential analysis (Fig. 1d), suggests that the surface of *T*sap is dominated by a net
431 negative charge. Few pseudobonds with high confidence were predicted to occur between two
432 *T*sap chains (Fig. S5). The *T*scp model contains one disulphide bond (Fig. S2d) and a
433 predominantly positively charged surface [+0.98, pH 7.4, pI = 7.8] (Fig. 1e). *T*scp is unlikely
434 to form homodimers, as suggested by high PAE values associated with the predicted
435 pseudobonds within the homodimer complex (Fig. S3). To simulate potential interactions
436 between candidate AMPs and the bacterial membrane, the association and arrangement

437 between each model and Gram-negative/positive lipid bilayers was predicted using the
438 Positioning of Proteins in Membranes 3.0 (PPM 3.0) server
439 (https://opm.phar.umich.edu/ppm_server3). All peptide models interacted with and became
440 embedded in the bacterial membrane, albeit no membrane penetration was predicted (Fig. S4).
441

442 *3.3 Peptide expression and antimicrobial assays*

443 While all five 'peptides of interest' were selected for recombinant expression, two (i.e., *H*met
444 and *T*sap), were successfully expressed in *Komagataella phaffii* using standard expression
445 conditions (Fig. S5a). Western blot analysis using monoclonal anti-histidine antibodies
446 confirmed the presence of the poly-histidine tag on both *H*met and *T*sap. These were therefore
447 purified using Ni-NTA agarose (Fig. S5b). Final concentrations of recombinant *H*met and *T*sap
448 were 0.45 mg/mL and 0.62 mg/mL, respectively. At 100 µg/mL, neither *H*met nor *T*sap exerted
449 a significant inhibitory effect on the growth of *E. coli*, *P. aeruginosa,* or *S. aureus* (Fig. 2).
450 Conversely, exposure of *B. subtilis* to 100 µg/mL of either *H*met and *T*sap resulted in
451 significant bacterial growth reductions, i.e., 90.0% and 57.7% CFU/mL respectively, when
452 compared to negative controls (Fig. 2).
453

454 *3.4 Antimicrobial activity of* H*met and* T*sap revealed by microfluidics time-lapse microscopy*

455 The effect of *H*met and *T*sap exposure on the physiology of individual *B. subtilis* cells was
456 observed using time-resolved imaging of cells loaded in microfluidic devices. The 'mother-
457 machine' microfluidic device design was selected as it enables the tracking of individual cell
458 lineages under consistent growth conditions for extended time durations [49,50]. These devices
459 consist of fluidic lanes (Fig. 3a) with designated inlets and outlets for the continuous flow of
460 growth media and treatment solutions. Five-hundred cell trenches, each measuring 75 µm in
461 length and 1 µm in width, were positioned perpendicular to the flow lanes, such that a single

462 line of cells occupied each trench. The trenches were constructed as dead-ends, ensuring that
463 the cells at the dead-end remained in place throughout the experiment, while their offspring
464 were removed from the open end (Fig. 3a). This setup allows cell lineages to be tracked within
465 an environment that remains constant over time and is consistent across all trenches, ensuring
466 reliable comparison of cell lysis events across trenches and over time. Additionally, the shallow
467 side trenches adjacent to each main trench facilitate the diffusion of media and exposure of
468 target cells to putative AMPs [51]. Phase-contrast imaging was used to acquire movies of
469 single-cell growth dynamics before, during, and after treatment (see Fig. S6 and Fig. S7 for
470 *H*met and *T*sap, respectively). A representative image of cells within a trench is shown in Fig.
471 3b. We used a deep-learning image segmentation model (Omnipose [48]) trained with synthetic
472 data generated from the virtual microscopy platform SyMBac [47] to identify and outline
473 individual cells from the image data. A custom-designed lineage-tracking algorithm
474 (https://github.com/erezli/MMLineageTracking) was used track cells across frames and cell
475 size timeseries were extracted from these tracks to quantify growth rate and lysis rates of
476 individual cells (Fig. S8 and Fig. S9, respectively).
477 To assess the effect of *H*met and *T*sap exposure on *B. subtilis* cell physiology, a
478 comparison of cell lysis rates and growth rates in two distinct fluidic lanes (treatment and
479 control) was conducted. In the first lane, individual recombinant AMPs were administered for
480 a duration of three hours, while cells in the other fluidic lane were treated with LB mixed with
481 50% PBS. An illustrative timeline of this experiment is provided in Fig. 3d. We observed that
482 both *H*met and *T*sap caused up to 10-15% decrease in cell growth rates during the 3-h treatment.
483 The effect of the *H*met was stronger, and the results were consistent between replicates (Fig.
484 3f). Comparisons of the number of cell lysis events between the two flow lanes revealed a
485 distinct pattern; in particular, exposure of *B. subtilis* to *H*met was associated with a significantly
486 increased frequency of cell lysis following treatment (Fig. 3g). However, the observed lysis

487 frequencies estimated from this single-cell data were too low to account for the results from
488 the bulk colony count treatment data. We developed a simple computational model to assess
489 the population-level impacts of growth inhibition and lysis rate (Fig. S10) and found that the
490 effect of growth inhibition explains the decrease in colony counts observed in the bulk
491 experiments.
492
493 **4. Discussion**
494 In this study, we mined available proteomics data from ESPs and EVs of GI nematodes of
495 substantial veterinary and public health importance to identify and characterise putative AMPs,
496 and applied *in vitro* techniques to study one of the potential mechanisms governing worm-
497 microbiota interactions in the vertebrate GI tract. Using predictive algorithms, we shortlisted
498 five putative AMPs from the 'brown stomach worm', *T. circumcincta* (i.e., *T*sap, *T*des, and
499 *T*scp), the 'barber's pole worm', *H. contortus* (i.e., *H*met), and the zoonotic canine roundworm
500 *T. canis* (i.e., *Tc*his), two of which (i.e., *T*sap and *H*met) were successfully expressed in
501 recombinant form and shown to exert significant antibacterial activity against *B. subtilis*.
502 *T*sap belongs to a diverse family of saposin-like proteins (SAPLIPs), each sharing a
503 conserved core structure, the 'saposin fold'. This fold is a characteristic bundle of four or five
504 α-helices, stabilised through the formation of three conserved disulphide bonds, that is central
505 to the activity of these molecules [52,53]. Variations in the amino acid sequences of SAPLIPs
506 confer these proteins diverse functions, including but not limited to, roles in the activation of
507 sphingolipid hydrolases, lipid antigen presentation, and processing of apoptotic bodies [54]. Of
508 note, SAPLIPs have been shown to act as potent antimicrobials, as demonstrated by the ability
509 of amoebapores from *Entamoeba histolytica,* human granulysin, and porcine NK-lysin to
510 disrupt the outer membranes of parasites, bacteria, and fungi [54]. *T*sap shares significant
511 sequence identity to a caenopore from *C. elegans*. Caenopores are known antimicrobials,

512 exerting significant bactericidal activity against both Gram-positive and Gram-negative
513 bacteria, such as *Bacillus megaterium* (caenopore-1, -5 and -12), *Bacillus thuringiensis*
514 (caenopore-12), and *E. coli* (caenopore-5) [53]. *H*met contains four α-helices stabilised through
515 the formation of three conserved disulphide bonds. Although no antimicrobial activity has thus
516 far been documented for parasitic nematode-derived metridin ShK toxin domain-containing
517 proteins, a study by O'Rourke *et al.* [55] reported that, in *C. elegans*, four metridin-like ShK
518 toxin domain-containing proteins were expressed in response to infection by *Microbacterium*
519 *nematophilum*. In a similar study, *C. elegans* genes encoding ShK toxins were upregulated in
520 response to *P. aeruginosa* infection [56]. In addition, a known ShK toxin domain-containing
521 AMP from the mesoglea of the jellyfish, *Aurelia aurita*, i.e., aurelin, was shown exert
522 antibacterial activity against *Listeria monocytogenes* and *E. coli* [57].

523 While recombinant *T*sap and *H*met exerted significant antibacterial activity against *B.*
524 *subtilis*, no effect was observed against *E. coli*, *P. aeruginosa,* or *S. aureus*. This observation
525 may be linked to inherent differences between the membrane structures of each of these
526 bacterial strains. Indeed, the killing of Gram-negative bacteria involves the disruption of both
527 outer and cytoplasmic membrane, and the inability to permeabilize or disrupt the outer
528 membrane results in loss of antimicrobial activity [58]. For Gram-positive bacteria, differences
529 between the membrane composition of *B. subtilis* and *S. aureus* may be responsible for the
530 observed discrepancy between killing activities. Indeed, while the membrane of *B. subtilis*
531 contains glycans with an average strand length of 500 disaccharide units, *S. aureus* glycans are
532 short (~5−10 disaccharide units) and feature an extremely high degree of peptide cross-links
533 that confer substantial rigidity to the membrane of this bacterium [59]. Additional studies
534 examining the antimicrobial properties of nematode-derived AMPs against an expanded panel
535 of bacterial targets are necessary to clarify whether any bactericidal/bacteriostatic activity is
536 dependent upon bacterial membrane structure. For example, the membrane of *Streptococcus*

537 *pneumoniae* features glycans with an average length of 50 subunits, thus making it a potentially
538 useful intermediary between *B. subtilis* and *S. aureus* [60].
539 While the exact antibacterial mechanism of action cannot be determined for either *H*met
540 nor *T*sap, *B. subtilis* exposure to each AMP was associated with statistically increased
541 frequency of cell lysis during microfluidics time-lapse microscopy. Nevertheless, these lysis
542 frequencies were too low to account for the substantial reduction of *B. subtilis* CFUs during
543 the colony counting experiments. Instead, analysis of simulated *B. subtilis* cultures, using the
544 lysis frequencies and growth rates observed during the microfluidics experiments, suggested
545 that such diminished growth rates are likely responsible for the significantly reduced *B. subtilis*
546 CFUs recorded during the colony counting experiments. However, it is important to note that
547 the effects observed in the single-cell microfluidic assay are likely to be weaker than those in
548 the bulk colony counting assay due to inherent differences between experimental setups.
549 Indeed, during the microfluidics experiment, *H*met and *T*sap are likely to preferentially bind to
550 cells near the trench inlet and thus quickly become depleted, as evidenced by the decrease in
551 growth inhibition effect along the trench; conversely, in the colony counting assay, whole
552 bacterial colonies are exposed to the presence of the AMPs in equal measure. Nevertheless, we
553 are tempted to speculate that *H*met and *T*sap exert their antibacterial activity *via* interaction
554 with and disruption of the bacterial membrane, as evidenced by the increased lysis events
555 observed during treatment. In the future, techniques such as atomic force microscopy and/or
556 electrochemical scanning tunnelling microscopy could be applied to clarify the mechanism of
557 action of these AMPs [61], as testified by the application of such techniques to visualise and
558 characterise the mechanism of bacterial membrane disruption caused by alamethicin, a
559 channel-forming peptide [61].
560 In our previous study, we showed that adult *T. circumcincta* ESPs exert significant
561 antibacterial activity against *E. coli* [24], a finding that could not be replicated in our current

562 investigation on *H*met and *T*sap. While it is plausible that these AMPs may not be active against
563 *E. coli*, it is also possible that this discrepancy may be linked to substantial differences between
564 *in vitro* and 'real-world' conditions. For instance, the abomasa of sheep and goats naturally
565 infected by *T. circumcincta* and *H. contortus* often harbour tens of thousands of worms, each
566 continuously producing antimicrobial-containing ESPs [62,63]. Thus, the hypothesis that *T*sap
567 and/or *H*met may exert antimicrobial activity at higher concentrations than those tested in our
568 study, and/or act synergistically with other nematode ESP components to enact bactericidal
569 activity against selected bacterial species (e.g., *E. coli*), cannot be excluded. In addition, it is
570 worth noting that optimum caenopore activity is observed under acidic pH conditions (= 5.2;
571 [64]). Interestingly, the pH of the abomasum of small ruminants, in which both *T. circumcincta*
572 and *H. contortus* reside, increases substantially during GI helminth infection (i.e., from ~2.9 to
573 4.5 – 5.5) [13,65]. Thus, future investigations should focus on adapting experimental
574 parameters to closely resemble the conditions occurring over the course of natural infections
575 by these nematodes. It should also be noted that one or more of the remaining shortlisted
576 peptides that we were unable to express in the current study (i.e., *Tc*his, *T*scp, and *T*des) could
577 account for the bactericidal activity of *T. circumcincta* ESPs against *E. coli* observed during
578 our previous study [24], and/or *P. aeruginosa,* or *S. aureus*. Indeed, histone H2A from
579 *Oncorhynchus mykiss* has been shown to possess potent antibacterial activity against
580 *Aerococcus äiridans*, *B. subtilis,* and *Micrococcus luteus* [66], while human histone H4 exerts
581 antimicrobial activity against *S. aureus* and *Propionibacterium acnes* [67]. In *C. elegans*, genes
582 encoding SCPs have also been shown to be upregulated during infections by several bacterial
583 pathogens [55]. Finally, destabilases exert antibacterial activity by enzymatically cleaving a
584 glycosidic linkage within the peptidoglycan of bacterial cell walls [68]. Further optimisation of
585 experimental conditions, such as culture temperature, methanol concentration, and vector
586 feeding strategy, is required to achieve expression of *Tc*his, *T*scp, and *T*des [69].

587 Our data support the notion that GI helminth ESPs and secreted EVs may communicate
588 with and modulate the composition of their microbial surroundings [70]. In our previous study,
589 *T. circumcincta* infection was associated with significant changes in the ovine GI microbiota,
590 and it was observed that increased abundances of Bacilli, including bacteria from the genera
591 *Turicibacter, Izemoplasmatales* and the XIII AD3011 group, were strongly associated with the
592 faecal microbiota of uninfected lambs rather than that of *T. circumcincta*-infected animals [70].
593 Nevertheless, infections were equally associated with abundance reductions and expansions of
594 several other taxa less related to *B. subtilis* [70]. However, the antimicrobial activity of
595 helminth ESPs/EVs likely represents only one of several (direct and indirect) mechanisms
596 governing worm-microbiota interactions. Indeed, multiple other factors, including but not
597 limited to, physical and biochemical changes in GI environment in response to parasite
598 colonisation, nutrient availability, and host immune responses, are likely to greatly contribute
599 to pathogen-microbiota crosstalk [20]. In addition, fundamental changes in the GI microbial
600 ecosystem due to worm establishment may trigger a complex network of bacteria-bacteria
601 interactions, via e.g., quorum sensing, biofilm formation, release of bacterial EVs, and dynamic
602 competition for nutrients [71], that are difficult to investigate using reductionist systems such
603 as the one applied in this study. Future studies may exploit holistic technologies to explore the
604 effect of recombinant helminth AMPs on whole microbial ecosystems. For instance, in a recent
605 experiment, the antimicrobial activity of EVs from the rumen fluke, *Calicophoron daubneyi*,
606 was assessed in an *in vitro* rumen model consisting of rumen fluid and anaerobic incubation
607 medium [72,73]. Furthermore, the application of ovine epithelial organoids to studies of worm-
608 microbiota crosstalk holds substantial promise for the identification of host components that
609 may contribute to this complex network of interactions [74]. Of note, the transcriptional
610 profiles of ovine abomasal and ileal organoids were demonstrated to largely resemble those of
611 the respective native tissues and remained stable after at least five serial passages [74].

612 Crucially, both types of organoids were successfully challenged with and infected by *T.* 613 *circumcincta* L3s and, separately, by *Salmonella enterica serovar* Typhimurium [74], thus 614 providing a solid basis for co-culture experiments aimed to investigate helminth-microbiota 615 interactions under controlled conditions.

616 A recent analysis of publicly available sequencing data from a range of parasitic 617 helminths identified over 16,000 putative AMPs encoded by the genomes of 127 worm species 618 [75]. Building upon our findings and those by Irvine *et al*. [75], in depth structural and 619 functional characterisations of helminth-derived AMPs may provide clarity on mechanisms of 620 worm-microbiota interactions *in vivo*, and thus pave the way toward the development of novel 621 and sustainable approaches to control parasitic diseases amid the ever-increasing threat of 622 anthelmintic resistance. For instance, the rational manipulation of the ruminant gut microbiota 623 (e.g. *via* the administration of pre- and/or probiotics and/or dietary supplements) may serve to 624 counteract the effects that helminth AMPs exert on the overall gut microbial make-up, possibly 625 enhancing host resistance to colonisation by gastrointestinal helminths [76].

626

627 **5. Conclusion**

628 In this study, we investigated a yet untapped repertoire of AMPs (and antimicrobial proteins) 629 in the ESPs and EVs of several GI helminths. Our experiments show that GI helminths may 630 directly modulate their immediate microbial surroundings through the secretion of AMPs with 631 cell lysis activity. However, experiments conducted *in vivo* or *ex vivo* (e.g. through exposure 632 of whole gut inocula through one or multiple recombinant helminth AMPs) will be necessary 633 in order to validate our hypothesis. Moreover, is highly likely that worm-microbiota crosstalk 634 also occurs through a complex network of direct and indirect mechanisms that act 635 synergistically to enhance parasite survival in a hostile environment. Unravelling the 636 complexities of helminth-host microbiome relationships may lead to a better understanding of

637 helminth biology and to the discovery and development of novel and sustainable parasite

638 control strategies.

639 **REFERENCES**

640 1. Ramos R, Moreira S, Rodrigues A, Gama M, Domingues L (2013) Recombinant
641 expression and purification of the antimicrobial peptide magainin-2. Biotechnol Prog 29:
642 17–22. https://doi.org/10.1002/btpr.1650.

643 2. Benfield AH, Henriques ST (2020) Mode-of-action of antimicrobial peptides: membrane
644 disruption vs. intracellular mechanisms. Front Med Technol 2: 610997.
645 https://doi.org/10.3389/fmedt.2020.610997.

646 3. Dierking K, Yang W, Schulenburg H (2016) Antimicrobial effectors in the nematode
647 *Caenorhabditis elegans*: an outgroup to the Arthropoda. Philos Trans R Soc Lond B Biol
648 Sci 371: 20150299. https://doi.org/10.1098/rstb.2015.0299.

649 4. Wang G, Li X, Wang Z (2016) APD3: the antimicrobial peptide database as a tool for
650 research and education. Nucleic Acids Res 44: D1087–D1093.
651 https://doi.org/10.1093/nar/gkv1278.

652 5. Midha A, Janek K, Niewienda A, Henklein P, Guenther S, et al. (2018) The intestinal
653 roundworm *Ascaris suum* releases antimicrobial factors which interfere with bacterial
654 growth and biofilm formation. Front Cell Infect Microbiol 8: 271.
655 https://doi.org/10.3389/fcimb.2018.00271.

656 6. World Health Organization. *Soil-transmitted helminth infections*.
657 https://www.who.int/news-room/fact-sheets/detail/soil-transmitted-helminth-infections
658 (2023)

659 7. Montresor A, Mwinzi P, Mupfasoni D, Garba A (2022) Reduction in DALYs lost due to
660 soil-transmitted helminthiases and schistosomiasis from 2000 to 2019 is parallel to the

661 increase in coverage of the global control programmes. PLoS Negl Trop Dis 16: e0010575.
662 https://doi.org/10.1371/journal.pntd.0010575.

663 8. Charlier J, Rinaldi L, Musella V, Ploeger HW, Chartier C, et al. (2020) Initial assessment
664 of the economic burden of major parasitic helminth infections to the ruminant livestock
665 industry in Europe. Prev Vet Med 182: 105103.
666 https://doi.org/10.1016/j.prevetmed.2020.105103.

667 9. Vercruysse J, Charlier J, Van Dijk J, Morgan ER, Geary T, et al. (2018) Control of helminth
668 ruminant infections by 2030. Parasitology 145: 1655–1664.
669 https://doi.org/10.1017/S003118201700227X.

670 10. Pilotte N, Manuel M, Walson JL, Ajjampur SSR, (2022) Community-wide mass drug
671 administration for soil-transmitted helminths – risk of drug resistance and mitigation
672 strategies. Front Trop Dis 3: 155. https://doi.org/10.3389/fitd.2022.897155.

673 11. Geerts S, Gryseels B (2000) Drug resistance in human helminths: current situation and
674 lessons from livestock. Clin Microbiol Rev 13: 207–222.
675 https://doi.org/10.1128/cmr.13.2.2.

676 12. Fissiha W, Kinde MZ, (2021) Anthelmintic resistance and its mechanism: a review. Infect
677 Drug Resist 14: 5403–5410. https://doi.org/10.2147/IDR.S332378.

678 13. Li RW, Li W, Sun J, Yu P, Baldwin RL, et al. (2016) The effect of helminth infection on
679 the microbial composition and structure of the caprine abomasal microbiome. Sci Rep 6:
680 20606. https://doi.org/10.1038/srep20606.

681 14. Jenkins TP, Rathnayaka Y, Perera PK, Peachey LE, Nolan MJ, et al. (2017) Infections by
682 human gastrointestinal helminths are associated with changes in faecal microbiota diversity
683 and composition. PLoS One 12: e0184719. https://doi.org/10.1371/journal.pone.0184719.

684 15. Cortés A, Wills J, Su X, Hewitt RE, Robertson J, et al. (2020) Infection with the sheep
685 gastrointestinal nematode *Teladorsagia circumcincta* increases luminal pathobionts.
686 Microbiome 8: 60 (2020). https://doi.org/10.1186/s40168-020-00818-9.

687 16. Barko PC, McMichael MA, Swanson KS, Williams DA (2018) The gastrointestinal
688 microbiome: A Review. J Vet Intern Med 32: 9–25. https://doi.org/10.1111/jvim.14875.

689 17. Rosa BA, Supali T, Gankpala L, Djuardi Y, Sartono E, et al. (2018) Differential human gut
690 microbiome assemblages during soil-transmitted helminth infections in Indonesia and
691 Liberia. Microbiome 6: 33. https://doi.org/10.1186/s40168-018-0416-5.

692 18. Llinás-Caballero K, Caraballo L (2022) Helminths and bacterial microbiota: the
693 interactions of two of humans' "old friends". Int J Mol Sci 23: 13358.
694 https://doi.org/10.3390/ijms232113358.

695 19. Rooney J, Northcote HM, Williams TL, Cortés A, Cantacessi C, et al. (2022) Parasitic
696 helminths and the host microbiome – a missing 'extracellular vesicle-sized' link? Trends
697 Parasitol 38: 737–747. https://doi.org/10.1016/j.pt.2022.06.003.

698 20. Brosschot TP, Reynolds LA, (2018) The impact of a helminth-modified microbiome on
699 host immunity. Mucosal Immunol 11: 1039–1046. https://doi.org/10.1038/s41385-018-
700 0008-5.

701 21. McNeilly TN, Nisbet AJ, (2014) Immune modulation by helminth parasites of ruminants:
702 implications for vaccine development and host immune competence. Parasite 21: 51.
703 https://doi.org/10.1051/parasite/2014051.

704 22. Maizels RM, Smits HH, McSorley HJ, (2018) Modulation of host immunity by helminths:
705 the expanding repertoire of parasite effector molecules. Immunity 49: 801–818.
706 https://doi.org/10.1016/j.immuni.2018.10.016.

707 23. Rausch S, Midha A, Kuhring M, Affinass N, Radonic A, et al. (2018) Parasitic nematodes
708 exert antimicrobial activity and benefit from microbiota-driven support for host immune
709 regulation. Front Immunol 9: 2282. https://doi.org/10.3389/fimmu.2018.02282.

710 24. Rooney J, Williams TL, Northcote HM, Frankl FEK, Price DRG, et al. (2022) Excretory-
711 secretory products from the brown stomach worm, *Teladorsagia circumcincta*, exert
712 antimicrobial activity in in vitro growth assays. Parasit Vectors 15: 354.
713 https://doi.org/10.1186/s13071-022-05443-z.

714 25. Hansen EP, Fromm B, Andersen SD, Marcilla A, Andersen KL, et al. (2019) Exploration
715 of extracellular vesicles from *Ascaris suum* provides evidence of parasite-host cross talk. J
716 Extracell Vesicles 8: 1578116. https://doi.org/10.1080/20013078.2019.1578116.

717 26. The UniProt Consortium. UniProt: the universal protein knowledgebase in 2021. Nucleic
718 Acids Res 49: D480–D489 (2021). https://doi.org/10.1093/nar/gkaa1100.

719 27. Tzelos T, Matthews JB, Buck AH, Simbari F, Frew D, et al. (2016) A preliminary
720 proteomic characterisation of extracellular vesicles released by the ovine parasitic
721 nematode, *Teladorsagia circumcincta*. Vet Parasitol 221: 84–92.
722 https://doi.org/10.1016/j.vetpar.2016.03.008.

723 28. Götz S, García-Gómez JM, Terol J, Williams TD, Nagaraj SH, et al. (2018) High-
724 throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids
725 Res 36: 3420–3435. https://doi.org/10.1093/nar/gkn176.

726 29. Waghu FH, Barai RS, Gurung P, Idicula-Thomas S, (2016) CAMPR3: a database on
727 sequences, structures and signatures of antimicrobial peptides. Nucleic Acids Res 44:
728 D1094–D1097. https://doi.org/10.1093/nar/gkv1051.

729 30. Fingerhut LCHW, Miller DJ, Strugnell JM, Daly NL, Cooke IR, (2020) ampir: an R
730 package for fast genome-wide prediction of antimicrobial peptides. Bioinformatics 36,
731 5262–5263 (2020). https://doi.org/10.1093/bioinformatics/btaa653.

732 31. Grønning AGB, Kacprowski T, Schéele C, (2021) MultiPep: a hierarchical deep learning
733 approach for multi-label classification of peptide bioactivities. Biol Methods Protoc 6:
734 bpab021. https://doi.org/10.1093/biomethods/bpab021.

735 32. Baek M, DiMaio F, Anishchenko I, Dauparas J, Ovchinnikov S, et al. (2021) Accurate
736 prediction of protein structures and interactions using a three-track neural network. Science
737 373: 871–876. https://doi.org/10.1126/science.abj8754.

738 33. Lomize AL, Todd SC, Pogozheva ID, (2022) Spatial arrangement of proteins in planar and
739 curved membranes by PPM 3.0. Protein Sci 31: 209–220.
740 https://doi.org/10.1002/pro.4219.

741 34. Jo S, Kim T, Iyer VG, Im W, (2008) CHARMM-GUI: A web-based graphical user
742 interface for CHARMM. J Comput Chem 29: 1859–1865.
743 https://doi.org/10.1002/jcc.20945.

744 35. Pettersen EF, Goddard TD, Huang CC, Meng EC, Couch GS, et al. (2021) UCSF
745 ChimeraX: Structure visualization for researchers, educators, and developers. Protein Sci
746 30: 70–82. https://doi.org/10.1002/pro.3943.

747 36. Jurrus E, Engel D, Star K, Monson K, Brandi J, et al. (2018) Improvements to the APBS
748 biomolecular solvation software suite. Protein Sci 27: 112-128.
749 https://doi.org/10.1002/pro.3280.
750

751 37. Dolinsky TJ, Czodrowski P, Li H, Nielsen JE, Jensen JH, et al. (2007) PDB2PQR:
752 expanding and upgrading automated preparation of biomolecular structures for molecular
753 simulations. Nucleic Acids Res 35: W522–W525. https://doi.org/10.1093/nar/gkm276.

754 38. Sun M, Wang Y, Zhang Q, Xia Y, Ge W, et al. (2017) Prediction of reversible disulphide
755 based on features from local structural signatures. BMC Genomics 18: 279.
756 https://doi.org/10.1186/s12864-017-3668-8.

757 39. Varadi M, Anyango S, Deshpande M, Nair S, Natassia C, et al. (2021) AlphaFold protein
758 structure database: massively expanding the structural coverage of protein-sequence space
759 with high-accuracy models. Nucleic Acids Res 50: D439–D444.
760 https://doi.org/10.1093/nar/gkab1061.

761 40. Mirdita M, Schütze K, Moriwaki Y, Heo L, Ovchinnikov S, et al. (2022) ColabFold:
762 making protein folding accessible to all. Nat Methods 19: 679–682.
763 https://doi.org/10.1038/s41592-022-01488-1.

764 41. Gensmart (2023) *Codon Optimization Tool GenScript*.
765 https://www.genscript.com/gensmart-free-gene-codon-optimization.html.

766 42. Invitrogen (2010) *EasySelect Pichia Expression Kit*.
767 https://www.thermofisher.com/order/catalog/product/K174001.

768 43. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, et al. (2005) Protein
769 Identification and Analysis Tools on the Expasy Server. In: Walker JM, editors. The
770 Proteomics Protocols Handbook. Springer Protocols Handbooks. Humana Press. pp. 571-
771 607. https://doi.org/10.1385/1-59259-890-0:571.

772 44. Ge Y, MacDonald DL, Holroyd KJ, Thornsberry C, Wexler H, et al. (1999) *In vitro*
773 antibacterial properties of pexiganan, an analog of magainin. Antimicrob Agents
774 Chemother 43: 782–788. https://doi.org/10.1128/aac.43.4.782.

775 45. Okwu MU, Olley M, Akpoka AO, Izevbuwa OE, (2019) Methicillin-resistant
776 *Staphylococcus aureus* (MRSA) and anti-MRSA activities of extracts of some medicinal
777 plants: A brief review. AIMS Microbiol 5: 117–137.
778 https://doi.org/10.3934/microbiol.2019.2.117.

779 46. Hiemstra TF, Charles PD, Gracia T, Hester SS, Gatto L, et al. (2014) Human urinary
780 exosomes as innate immune effectors. J Am Soc Nephrol 25: 2017–2027.
781 https://doi.org/10.1681/ASN.2013101066.

782 47. Hardo G, Noka M, Bakshi S, (2022) Synthetic Micrographs of Bacteria (SyMBac) allows
783 accurate segmentation of bacterial cells using deep neural networks. BMC Biol 20: 263.
784 https://doi.org/10.1186/s12915-022-01453-6.

785 48. Cutler K, Stringer C, Lo TW, Rappez L, Stroustrup N, et al. (2022) Omnipose: a high-
786 precision morphology-independent solution for bacterial cell segmentation. Nat Methods
787 19: 1438–1448. https://doi.org/10.1038/s41592-022-01639-4.

788 49. Bakshi S, Leoncini E, Baker C, Cañas-Duarte SJ, Okumus B, et al. (2021) Tracking
789 bacterial lineages in complex and dynamic environments with applications for growth
790 control and persistence. Nat Microbiol 6: 783–791. https://doi.org/10.1038/s41564-021-
791 00900-4.

792 50. Hardo G, Bakshi S, (2021) Challenges of analysing stochastic gene expression in bacteria
793 using single-cell time-lapse experiments. Essays Biochem 65: 67–79.
794 https://doi.org/10.1042/EBC20200015.

795 51. Norman TM, Lord ND, Paulsson J, Losick R, (2013) Memory and modularity in cell-fate
796 decision making. Nature 503: 481–486. https://doi.org/10.1038/nature12804.

797 52. Willis C, Wang CK, Osman A, Simon A, Pickering D, et al. (2011) Insights into the
798 membrane interactions of the saposin-like proteins Na-SLP-1 and Ac-SLP-1 from human
799 and dog hookworm. PLoS One 6: e25369. https://doi.org/10.1371/journal.pone.0025369.

800 53. Bruno R, Maresca M, Canaan S, Cavalier JF, Mabrouk K, et al. (2019) Worms'
801 antimicrobial peptides. Mar Drugs, 17: 512. https://doi.org/10.3390/md17090512.

802 54. Darmoise A, Maschmeyer P, Winau F, (2010) The immunological functions of saposins.
803 Adv Immunol 105: 25–62. https://doi.org/10.1016/S0065-2776(10)05002-9.

804 55. O'Rourke D, Baban D, Demidova M, Mott R, Hodgkin J, (2006) Genomic clusters,
805 putative pathogen recognition molecules, and antimicrobial genes are induced by infection
806 of *C. elegans* with *M. nematophilum*. Genome Res 16: 1005–1016.
807 https://doi.org/10.1101/gr.50823006.

808 56. Troemel ER, Chu SW, Reinke V, Lee SS, Ausubel FM, et al. (2006) p38 MAPK regulates
809 expression of immune response genes and contributes to longevity in *C. elegans*. PLoS
810 Genet 2: e183. https://doi.org/10.1371/journal.pgen.0020183.

811 57. Ovchinnikova TV, Balandin SV, Aleshina GM, Tagaev AA, Leonova YF, et al. (2006)
812 Aurelin, a novel antimicrobial peptide from jellyfish *Aurelia aurita* with structural features
813 of defensins and channel-blocking toxins. Biochem Biophys Res Commun 348: 514–523.
814 https://doi.org/10.1016/j.bbrc.2006.07.078.

815 58. Li J, Koh JJ, Liu S, Lakshminarayanan R, Verma CS, et al. (2017) Membrane active
816 antimicrobial peptides: translating mechanistic insights to design. Front Neurosci 11: 73.
817 https://doi.org/10.3389/fnins.2017.00073.

818 59. Kern T, Giffard M, Hediger S, Amoroso A, Giustini C, et al. (2010) Dynamics
819 characterization of fully hydrated bacterial cell walls by solid-state nmr: evidence for
820 cooperative binding of metal ions. J Am Chem Soc 132: 10911–10919.
821 https://doi.org/10.1021/ja104533w.

822 60. Vollmer W, Massidda O, Tomasz A, (2019) The cell wall of *Streptococcus pneumoniae*.
823 Microbiol Spectr 7. https://doi.org/10.1128/microbiolspec.gpp3-0018-2

824 61. Pieta P, Mirza J, Lipkowski J, (2012) Direct visualization of the alamethicin pore formed
825 in a planar phospholipid matrix. Proc Natl Acad Sci U S A 109: 21223–21227.
826 https://doi.org/10.1073/pnas.1201559110.

827 62. Craig BH, Pilkington JG, Pemberton JM, (2006) Gastrointestinal nematode species
828 burdens and host mortality in a feral sheep population. Parasitology 133: 485–496.
829 https://doi.org/10.1017/S0031182006000618.

830 63. Flay KJ, Hill FI, Muguiro DH, (2022) *Haemonchus contortus* infection in pasture-based
831 sheep production systems, with a focus on the pathogenesis of anaemia and changes in
832 haematological parameters. Animals (Basel) 12: 1238.
833 https://doi.org/10.3390/ani12101238.

834 64. Roeder T, Stanisak M, Gelhaus C, Bruchhaus I, Grötzinger J, et al. (2009) Caenopores are
835 antimicrobial peptides in the nematode *Caenorhabditis elegans* instrumental in nutrition
836 and immunity. Dev Comp Immunol 34: 203–209.
837 https://doi.org/10.1016/j.dci.2009.09.010.

838 65. Van Winden SCL, Müller KE, Kuiper R, Noordhuizen JPTM, (2002) Studies on the pH
839 value of abomasal contents in dairy cows during the first 3 weeks after calving. J Vet Med
840 A Physiol Pathol Clin Med 49: 157–160. https://doi.org/10.1046/j.1439-
841 0442.2002.00429.x.

842 66. Fernandes JMO, Kemp GD, Molle MG, Smith VJ, (2002) Anti-microbial properties of
843 histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*. Biochem J 368:
844 611–620. https://doi.org/10.1042/bj20020980.

845 67. Lee DY, Huang CM, Nakatsuji T, Thiboutot D, Kang SA, et al. (2009) Histone H4 is a
846 major component of the antimicrobial action of human sebocytes. J Invest Dermatol 129:
847 2489–2496. https://doi.org/10.1038/jid.2009.106.

848 68. Manuvera VA, Kurdyumov AS, Filonova KA, Lazarev VN, (2015) Generation of
849 recombinant destabilase-lysozyme from medicinal leeches in three different expression
850 systems. Protein Expr Purif 116: 50–58. https://doi.org/10.1016/j.pep.2015.08.012.

851 69. Ang RP, Teoh LS, Chan MK, Miswan N, Khoo BY, (2016) Comparing the expression of
852 human DNA topoisomerase I in KM71H and X33 strains of *Pichia pastoris*. Electron J
853 Biotechnol 21: 9–17. https://doi.org/10.1016/j.ejbt.2016.01.007.

854 70. Rooney J, Cortés A, Scotti R, Price DRG, Bartley Y, et al. (2021) Vaccination against the
855 brown stomach worm, *Teladorsagia circumcincta*, followed by parasite challenge, induces
856 inconsistent modifications in gut microbiota composition of lambs. Parasit Vectors 14:
857 189. https://doi.org/10.1186/s13071-021-04688-4.

858 71. Kim JH, Lee J, Park J, Gho YS, (2015) Gram-negative and Gram-positive bacterial
859 extracellular vesicles. Semin Cell Dev Biol 40: 97–104.
860 https://doi.org/10.1016/j.semcdb.2015.02.006.

861 72. Huws S, Mayorga OL, Theodorou MK, Kim EJ, Cookson AH, et al. (2013) Differential
862 colonization of plant parts by the rumen microbiota is likely to be due to different forage
863 chemistries. J Microb Biochem Technol 6: 80-86. https://doi.org/10.4172/1948-
864 5948.1000126.

865 73. Allen NR, Taylor-Mew AR, Wilkinson TJ, Huws S, Phillips H, et al. (2021) Modulation
866 of rumen microbes through extracellular vesicle released by the rumen fluke *Calicophoron*
867 *daubneyi*. Front Cell Infect Microbiol 11: 263. https://doi.org/10.3389/fcimb.2021.661830.

868 74. Smith D, Price DRG, Burrells A, Faber MN, Hildersley KA, et al. (2021) The development
869 of ovine gastric and intestinal organoids for studying ruminant host-pathogen interactions.
870 Front Cell Infect Microbiol 11: 733811. https://doi.org/10.3389/fcimb.2021.733811.

871 75. Irvine A, McKenzie D, McCoy CJ, Graham RLJ, Graham C, et al. (2023) Novel integrated
872 computational AMP discovery approaches highlight diversity in the helminth AMP
873 repertoire. PLoS Pathog 19: e1011508. https://doi.org/10.1371/journal.ppat.1011508.

874 76. Cortés A, Rooney J, Bartley DJ, Nisbet AJ, Cantacessi C, (2020) Helminths, hosts, and
875 their microbiota: new avenues for managing gastrointestinal helminthiases in ruminants.
876 Expert Rev Anti Infect Ther, *18*: 977–985.
877 https://doi.org/10.1080/14787210.2020.1782188.

878 77. Wang T, Van Steendam K, Dhaenens M, Vlaminck J, Deforce D, et al. (2013) Proteomic
879 analysis of the excretory-secretory products from larval stages of *Ascaris suum* reveals
880 high abundance of glycosyl hydrolases. PLoS Negl Trop Dis 7: e2467.
881 https://doi.org/10.1371/journal.pntd.0002467.

882 78. Chehayeb JF, Robertson AP, Martin RJ, Geary TG, (2014) Proteomic analysis of adult
883 *Ascaris suum* fluid compartments and secretory products. PLoS Negl Trop Dis 8: e2939.
884 https://doi.org/10.1371/journal.pntd.0002939.

885 79. Wang T, Ma G, Ang CS, Korhonen PK, Koehler AV, et al. (2019) High throughput LC-
886 MS/MS-based proteomic analysis of excretory-secretory products from short-term in vitro
887 culture of *Haemonchus contortus*. J Proteomics. 204: 103375.
888 https://doi.org/10.1016/j.jprot.2019.05.003.

889 80. Maruszewska-Cheruiyot M, Szewczak L, Krawczak-Wójcik K, Głaczyńska M, Donskow-
890 Łysoniewska K, (2021) The production of excretory-secretory molecules from
891 *Heligmosomoides polygyrus bakeri* fourth stage larvae varies between mixed and single
892 sex cultures. *Parasit. Vector* 14: 106. https://doi.org/10.1186/s13071-021-04613-9.

893 81. Sotillo J, Sanchez-Flores A, Cantacessi C, Harcus Y, Pickering D, *et al.* (2014) Secreted
894 proteomes of different developmental stages of the gastrointestinal nematode
895 *Nippostrongylus brasiliensis*. *Mol Cell Proteomics* 13: 2736–2751.
896 https://doi.org/10.1074/mcp.M114.038950.

897 82. Soblik H, Younis AE, Mitreva M, Renard BY, Kirchner M, et al. (2011) Life cycle stage-
898 resolved proteomic analysis of the excretome/secretome from *Strongyloides ratti*—
899 identification of stage-specific proteases. Mol Cell Proteomics 10: M111.010157.
900 https://doi.org/10.1074/mcp.M111.010157.

901 83. Sperotto RL, Kremer FS, Aires Berne ME, Costa de Avila LF, da Silva Pinto L et al. (2017)
902 Proteomic analysis of *Toxocara canis* excretory and secretory (TES) proteins. Mol
903 Biochem Parasitol 211: 39–47. https://doi.org/10.1016/j.molbiopara.2016.09.002.

904 84. da Silva MB, Urrego A JR, Oviedo Y, Cooper PJ, Pacheco LGC, et al. (2018) The somatic
905 proteins of *Toxocara canis* larvae and excretory-secretory products revealed by
906 proteomics. Vet Parasitol 259: 25–34. https://doi.org/10.1016/j.vetpar.2018.06.015.

907 85. Leroux LP, Nasr M, Valanparambil R, Tam M, Rosa BA, et al. (2018) Analysis of the
908 *Trichuris suis* excretory/secretory proteins as a function of life cycle stage and their
909 immunomodulatory properties. Sci Rep 8: 15921. https://doi.org/10.1038/s41598-018-
910 34174-4.

911 86. Buck AH, Coakley G, Simbari F, McSorley HJ, Quintana JF, et al. (2014) Exosomes
912 secreted by nematode parasites transfer small RNAs to mammalian cells and modulate
913 innate immunity. Nat Commun 1: 5488. https://doi.org/10.1038/ncomms6488.

914 87. Eichenberger RM, Ryan S, Jones L, Buitrago G, Polster R, et al. (2018) Hookworm secreted
915 extracellular vesicles interact with host cells and prevent inducible colitis in mice. Front
916 Immunol 9: 850. https://doi.org/10.3389/fimmu.2018.00850.

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Not applicable.

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Competing interests

The authors have declared that no competing interests exist.

966 **Table 1. Top-scoring putative antimicrobial proteins identified in available proteomics datasets from excretory-secretory products (ESPs)**
967 **and extracellular vesicles (EVs) from gastrointestinal helminth species.** Putative antimicrobial activity was assigned based on comparative
968 analyses with sequence data available from the Collection of Anti-Microbial Peptides ($CAMP_{R3}$) database, as well as according to antimicrobial
969 protein and peptide (AMP) prediction algorithms (ampir and MultiPep). For each sequence, helminth species of origin, corresponding Uniprot
970 accession number, NCBI/Uniprot protein description and top CAMP BLAST hit are provided, as well as ampir and Multipep
971 antimicrobial/antibacterial prediction scores.
972

Sequence_ID	Species	Accession	Description	CAMP BLAST hit	ampir	Multipep – antimicrobial	Multipep – antibacterial
***H*met**	*Haemonchus contortus*	A0A7I4Y4R3	Metridin-ShK toxin	NA	0.996	0.746	0.215
***T*chis**	*Toxocara canis*	A0A0B2V2B5	Histone 2A	Histone H2A	0.992	0.325	0.958
***T*des**	*Teladorsagia circumcincta*	A0A2G9TVU4	Destabilase (Lysozyme)	Lysozyme 3	0.923	0.440	0.049
***T*sap**	*Teladorsagia circumcincta*	transcript/25448 (iso-seq)	Saposin-b	Caenopore-5	0.864	0.943	0.279
***T*scp**	*Teladorsagia circumcincta*	A0A2G9TU46	SCP-like protein	NA	0.732	0.003	0.000

973 **Figure Captions**
974

975 **Fig. 1. Predicted tertiary structures and electrostatic surfaces of selected nematode-**
976 **derived antimicrobial peptides.** (a) *Haemonchus contortus* metridin ShK toxin domain-
977 containing peptide (*H*met), (b) *Toxocara canis* histone H2A (*Tc*his), (c) *Teladorsagia*
978 *circumcincta* destabilase (*T*des), (d) *T. circumcincta* saposin-b domain containing peptide
979 (*T*sap), and (e) *T. circumcincta* cysteine- rich protein (*T*scp). Robetta Tertiary structures were
980 predicted using RoseTTAFold and represented as ribbon structures (top). For each structure,
981 predicted electrostatic surfaces were calculated using the APBS software at pH 7.0 (bottom).
982 Red and blue indicate negative and positive charge, respectively. Each predicted structure is
983 shown as four images, each representing a 90° turn. Visualisation was achieved using
984 ChimeraX.
985

986 **Fig. 2**. **Bacterial growth assays.** *Escherichia coli*, *Pseudomonas aeruginosa, Bacillus subtilis*,
987 and *Staphylococcus aureus* were cultured in the presence of *Teladorsagia circumcincta*
988 saposin-b domain containing peptide (*T*sap) and *Haemonchus contortus* metridin ShK toxin
989 domain-containing peptide (*H*met) (100 µg/mL), as well as of negative (i.e., PBS) and positive
990 controls [i.e., pexiganan (for *E. coli, P. aeruginosa*, and *B. subtilis*) and vancomycin (for *S.*
991 *aureus*)] for 210 minutes. Following incubation, colony counts were performed and used to
992 calculate the total CFU/mL in the respective cultures. Note that exposure to pexiganan and
993 vancomycin resulted in complete elimination of bacterial colonies. Data are representative of
994 four independent experiments. Individual datapoints reflecting biological replicates for each
995 experiment are represented as coloured dots and error bars represent standard error of the mean.
996 ***$P \leq 0.001$.
997

998 **Fig. 3. Time-lapse microfluidics assay.** (a) Schematic diagram illustrating the modified
999 Mother Machine device featuring side trenches for facilitating diffusive reach of *Teladorsagia*
1000 *circumcincta* saposin-b domain containing peptide (*T*sap) and *Haemonchus contortus* metridin
1001 ShK toxin domain-containing peptide (*H*met). The inset at the bottom demonstrates the
1002 placement of cell trenches alongside the flow lane, while the inset on the right provides a
1003 magnified view of the structure of individual cell trenches containing cells. (b) Example phase-
1004 contrast image depicting cells within a trench (1 μm wide), with visible side trenches (7 μm
1005 wide) shown in light grey. (c) Image segmentation of cells in phase-contrast using a machine-
1006 learning algorithm (Omnipose) trained with synthetic training data generated by the virtual
1007 microscopy platform SyMBac. (d) Schematic timeline outlining the experiment: growth media
1008 containing each *T*sap and *H*met were introduced after 90 minutes of pre-treatment growth in
1009 fresh LB, followed by a return to growth media (LB) without AMPs after 180 minutes of
1010 treatment. (e) Average single-cell growth rates under different treatments and the control, with
1011 the treatment interval highlighted in purple. Highlighted regions around each line represents
1012 the standard deviation for their respective treatment conditions. (f) Average growth rates
1013 measured between 250 and 300 minutes from three replicates, with individual values depicted
1014 as circles. (g) Average and individual lysis frequencies of bacterial lineages during treatment,
1015 as observed in three experiments. $*P \leq 0.05$.

1016

1017 **Supporting Information**

1018 **Table S1. Available proteomics sequence data of gastrointestinal nematode excretory-**
1019 **secretory products (ESPs) and extracellular vesicles (EVs) at the time of**
1020 **writing.** Available ESP and EV proteomics datasets from selected gastrointestinal nematode
1021 species were retrieved and subjected to targeted bioinformatics analyses for the identification
1022 of putative antimicrobial proteins and peptides. For each species, life cycle stage and numbers

1023 of proteins/peptides described in the corresponding publications, as well as the numbers of
1024 proteins/peptides that could be successfully retrieved, are provided.
1025 Abbreviations. L3 = third-stage larva; L4 = fourth-stage larva.
1026
1027 **Table S2. Putative antimicrobial proteins identified in available proteomics datasets from**
1028 **excretory-secretory products (ESPs) and extracellular vesicles (EVs) from**
1029 **gastrointestinal nematode species.** List of proteins retrieved from available proteomics
1030 analyses of gastrointestinal nematode ESPs/EVs. For each sequence, the following information
1031 is provided: SequenceID/Accession (Used in original source), species, gene ontology IDs,
1032 InterProScan IDs, top CAMP BLAST hit, and relevant information (Accession numer, Score,
1033 Evalue, Identities (%), Positives (%)), antimicrobial probability prediction scores (using the
1034 ampir 'precursor' and 'mature' classification models and MultiPep antimicrobial and
1035 antibacterial scores), and complete protein sequence.
1036
1037 **Fig. S1. Predicted structures of selected nematode-derived antimicrobial peptides.** (a)
1038 *Haemonchus contortus* metridin ShK toxin domain-containing peptide (*H*met), (b) *Toxocara*
1039 *canis* histone H2A (*Tc*his), (c) *Teladorsagia circumcincta* destabilase (*T*des), (d) *T.*
1040 *circumcincta* saposin-b domain containing peptide (*T*sap), and (e) *T. circumcincta* cysteine-
1041 rich protein (*T*scp). B-factor values ($Å^2$) were generated per residue using the Robetta
1042 RoseTTAFold software and visualised using ChimeraX. Dark blue residues are indicative of
1043 low B-factor values and thus high accuracy of the model. Dark red residues are indicative of
1044 high B-factor values and thus low confidence scores. The range of B-factor values ($Å^2$)
1045 calculated for each model, from lowest to highest, is provided under each predicted structure.
1046

1047 **Fig. S2. Predicted disulphide bonds within selected nematode-derived antimicrobial**
1048 **peptide models.** Predicted disulphide bonds within (a) *Haemonchus contortus* metridin ShK
1049 toxin domain-containing peptide (*H*met), (b) *Teladorsagia circumcincta* destabilase (*T*des), (c)
1050 *T. circumcincta* saposin-b domain containing peptide (*T*sap), and (d) *T. circumcincta* cysteine-
1051 rich protein (*T*scp). α-helices in cyan, β-sheets in red, loop regions in purple and cysteine
1052 residues in gold (with position labelled). Predicted disulphide bonds are indicated with yellow
1053 dashed lines. Disulphide bonds were predicted, measured, and visualised within ChimeraX.
1054
1055 **Fig. S3. Predicted formation of homodimers of selected nematode-derived antimicrobial**
1056 **peptides.** (a) *Haemonchus contortus* metridin ShK toxin domain-containing peptide (*H*met),
1057 (b) *Toxocara canis* histone H2A (*Tc*his), (c) *Teladorsagia circumcincta* destabilase (*T*des), (d)
1058 *T. circumcincta* saposin-b domain containing peptide (*T*sap), and (e) *T. circumcincta* cysteine-
1059 rich protein (*T*scp). Alphafold Colabfold was utilised to predict the likelihood of each peptide
1060 forming a homodimer. These predictions are visualised in ChimeraX in a globular form (left)
1061 which details the orientation of peptide chains interlocking in the homodimer complex, and as
1062 ribbons (right), where interactions between peptide chains are represented by 'pseudobonds'.
1063 These are coloured according to their predicted alignment error (Å) values, ranging from 0 –
1064 20 Å as indicated in the key. In both depictions, separate peptide chains of the predicted
1065 homodimer complex are represented in cyan and red, respectively.
1066
1067 **Fig. S4. Predicted orientation of selected nematode-derived antimicrobial peptides in a**
1068 **generalised bacterial lipid bilayer.** (a) *Haemonchus contortus* metridin ShK toxin domain-
1069 containing peptide (*H*met), (b) *Toxocara canis* histone H2A (*Tc*his), (c) *Teladorsagia*
1070 *circumcincta* destabilase (*T*des), (d) *T. circumcincta* saposin-b domain containing peptide
1071 (*T*sap), and (e) *T. circumcincta* cysteine- rich protein (*T*scp). Each predicted interaction

1072 between putative AMP and lipid bilayer is illustrated in four images, each representing a 90°
1073 turn. Peptides in navy blue, phosphate heads of the phospholipid within the lipid bilayer in red
1074 and fatty acids of the phospholipids in beige. The interaction between each protein model and
1075 a generalised bacterial lipid bilayer was calculated using the OPM PPM, rebuilt in CHARMM-
1076 GUI and visualised in ChimeraX.
1077
1078 **Fig. S5. Expression and purification of selected nematode-derived antimicrobial peptides.**
1079 (a) Expression of recombinant *T*sap (left) and *H*met (right) as detected in the supernatant of
1080 *Komagataella phaffii* (formerly known as *Pichia pastoris*) culture media. The purification of
1081 recombinant proteins from the supernatant was achieved using Ni-NTA agarose, which binds
1082 the N-terminal His-tag on the target protein. Each step of the purification process was
1083 visualised through SDS-PAGE. SN; Supernatant, FT; Flowthrough, W1; Wash 1, W2; Wash
1084 2, E1; Elution 1, E2; Elution 2, E3; Elution 3, L; Molecular weight ladder. (b) Purified *H*met
1085 and *T*sap were probed for presence of the N-terminal His-tag using primary mouse anti-6His
1086 tag antibody, and secondary horse anti-mouse peroxidase antibody at a dilution of 1:250 and
1087 1:400, respectively. The His-tag was detected in both purified protein products.
1088
1089 **Fig. S6**. Representative movie of single-cell growth dynamics before, during, and after
1090 exposure of *Bacillus subtilis* to *Haemonchus contortus* metridin ShK toxin domain-containing
1091 peptide (*H*met).
1092
1093 **Fig. S7**. Representative movie of single-cell growth dynamics before, during, and after
1094 exposure of *Bacillus subtilis* to *Teladorsagia circumcincta* saposin-b domain containing
1095 peptide (*T*sap).
1096

1097 **Fig. S8. Single-cell segmentation and lineage tracking.** (a) Phase-contrast images of cells
1098 were segmented using the machine-learning model Omnipose, trained with synthetic data from
1099 SyMBac. The segmentation process produces binary masks of individual cells (bottom left),
1100 that are ranked based on their positioning within each trench (with the mother cell darkest at
1101 the top). Subsequently, a custom-designed lineage tracking algorithm
1102 (https://github.com/erezli/MMLineageTracking) is employed to identify and label the tracks of
1103 individual cells accordingly (bottom right). Lineage connections between different cells are
1104 overlaid onto the raw phase-contrast image for illustrative purposes (top right).
1105
1106 **Fig. S9. Single cell growth and lysis analysis.** (a) A time series of individual cell lengths is
1107 estimated from their respective masks. The length of individual cells sharply decreases at the
1108 point of division and grows exponentially between division points. Consequently, the
1109 logarithm of cell length exhibits linear growth between division points, and its slope is utilised
1110 to calculate the elongation rate (exponential growth rate) of individual cells. Lysis events are
1111 identified when a lineage is abruptly truncated.
1112
1113 **Fig. S10.** (a) Population growth of four simulated cultures are compared for 210 minutes at
1114 different growth rates and lysis frequencies. The initial conditions (number of cells) and growth
1115 duration were set to match the bulk antimicrobial activity assays described in the main text.
1116 The growth rate and lysis frequencies were selected based on the single-cell AMP experiments.
1117 The impact of lysis appears to be significantly weaker than that of growth reduction in
1118 determining the final density of cells. (b) The final population cell counts after 210 minutes for
1119 different growth rates and lysis frequencies are compared. At reduced growth rates of 0.039
1120 and 0.030 (s-1), a 2-fold and 8-fold reduction in cell count, respectively, are observed. In
1121 contrast, the frequency of lysis events has a less significant impact on the final cell count. (c)

1122 The antimicrobial peptides (AMPs) diffuse from the trench inlet on the right. (d) Growth rates

1123 are significantly reduced near the open end of the trench, likely because cells closer to the open

1124 end absorb more AMPs before the latter becomes depleted.