Efficacy and safety evaluation of artificial intelligence-identified antimicrobial peptides for use against avian pathogenic *Escherichia coli* in the poultry industry

Emre Demirsoy[†,‡,§,*], Teagan I. Parkin[#,*], Shaeleen E. Mihalynuk[#], Anna H. Dema[#], Lorissa Corrie[#], Marika E. Heilker[#], Haley N. Kuecks-Winger[#], Anat Yanai[‖,††], Uluc B. Birol[††], Michael McIlwee[‡‡], Kay de Wet[‡‡], Mathijs Knipscheer[‡‡], Victoria Bowes[‡‡], Vanessa Tuytel[‡‡], Liam Ritchie[‡‡], Wolfgang Köster[§§], Emil Berberov[§§], William R. Cox[††], Monica Kotkoff[‖], Vanessa C. Thompson[#], Rene L. Warren[†], Erin Fraser[##,※], Linda M.N. Hoang[‡,⁑], Fraser Hof[⁂], Fatih Birol[††], Caren C. Helbing[#], Inanc Birol[‡,§,‖,††,⸸,1]

[†] BC Cancer, Vancouver, BC V5Z 1L3, Canada
[‡] British Columbia Centre for Disease Control, Public Health Laboratory, Vancouver, BC V6Z R4R, Canada
[§] Genome Science and Technology Graduate Program, University of British Columbia, Vancouver, BC V6T 1Z4, Canada
[#] Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, V8P 5C2, Canada
[‖] Canada's Michael Smith Genome Sciences Centre at BC Cancer, Vancouver, BC V5Z 4S6, Canada
[††] Amphoraxe Life Sciences Inc., BC V6L 3C9, Canada
[‡‡] SJ Ritchie Research Farms, Abbotsford, BC V4X 2N4 Canada
[§§] Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan, SK, S7N 5E3, Canada
[##] Communicable Disease Prevention and Response, British Columbia Centre for Disease Control, Vancouver, BC V6Z R4R, Canada
[※] School of Population and Public Health, The University of British Columbia, Vancouver, BC, V6T 1Z3, Canada
[⁑] Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC V6T 1Z4, Canada
[⁂] Department of Chemistry, University of Victoria, Victoria, BC, V8P 5C2, Canada
[⸸] Department of Medical Genetics, University of British Columbia, Vancouver, BC V6T 1Z3, Canada

[1]Corresponding author: inanc.birol@ubc.ca

[*]These authors contributed equally to this work.

ABSTRACT

The overuse of antibiotics in both veterinary and human medicine has resulted in the emergence of antibiotic-resistant bacteria, prompting a search for effective alternatives. Antimicrobial peptides (AMP) are short, often cationic, peptide-based molecules with antimicrobial and immunomodulatory activity, which makes them promising alternatives to conventional antibiotics in poultry production. From a prior machine-learning-guided screen of 875 candidate AMP, 62 exhibited activity against avian pathogenic *Escherichia coli* (APEC) and low in vitro hemolytic and cytotoxic activity. We selected three lead AMP from this list (named TeRu4, TeBi1, and PeNi4), and evaluated their in vitro and in vivo efficacy, safety, and immunomodulatory potential for use in poultry farming. In animal experiments, AMP were administered via in ovo injection on day 18 of embryonic development. In APEC challenge trials, yolk sacs were inoculated with APEC post-hatch to assess early chick mortality, while in pen trials, birds were raised in a commercial production setting for 35 days. For challenged birds, TeBi1 (10 µg/egg) significantly reduced bacterial detection in the air sac and pericardium, increased body weight by 50% and reduced cytokine transcript levels by 10-30% on day 7 post hatch. In HD11 chicken macrophage-like cultured cells, TeRu4 (16 µg/mL) suppressed lipopolysaccharide (LPS)-induced pro-inflammatory cytokine transcript levels. In pen trials, TeRu4 (20 µg/egg) increased the survival probability of female birds by 4.9%, while TeBi1 (20 µg/egg) increased the survival probability of all birds by 4.4%, by day 35. Gene expression analysis revealed AMP- and sex-specific cytokine responses. In pen trials, no significant differences were observed in mean weights, feed conversion ratio (FCR), and flock uniformity on day 35. These findings demonstrate that the three selected AMP are safe antibiotic

62 alternatives that improve survival, modulate immune responses, and maintain normal growth
63 performance in broiler chickens.
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65 Key words: Antimicrobial peptide, APEC, broiler chicken, efficacy, safety
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67 **INTRODUCTION**

68 Avian pathogenic *Escherichia coli* (**APEC**) is a Gram-negative bacterium that poses a
69 severe threat to the poultry industry. APEC infections induce fatal bacterial septicemia and early
70 chick mortality (**ECM**), a significant cause of mortality and morbidity across multiple poultry
71 commodities (Cuperus et al., 2016; Allan et al., 2018; Rezaee et al., 2021; Nguyen et al., 2021;
72 Sarfraz et al., 2022; Hu et al., 2022). Antibiotics have been used to treat bacterial infections since
73 the early 20th century. In the poultry industry, antibiotics have been used as growth promoters
74 and prophylactic agents since the 1950s (Bean-Hodgins and Kiarie, 2021). However,
75 unnecessary or overuse of antibiotics has contributed to the rise of antibiotic-resistant bacteria in
76 both veterinary and human medicine (Bean-Hodgins and Kiarie, 2021; Richter et al., 2022). To
77 address this growing challenge, regulatory, veterinary, and poultry industry policy changes in
78 Canada and in other jurisdictions have been instituted to limit the use of antimicrobials for
79 growth promotion purposes and, also, to limit the use of medically important antimicrobials
80 (Bean-Hodgins and Kiarie, 2021; Wallinga et al., 2022; Schmerold et al., 2023). This has
81 resulted in fewer antimicrobial options being available to control pathogens in poultry flocks
82 (Bean-Hodgins and Kiarie, 2021). The One Health approach recognizes the linkages between
83 human, animal, and environmental health, and highlights the urgency of addressing antibiotic
84 resistance as a global crisis (Destoumieux-Garzón et al., 2018). Unfortunately, novel antibiotics
85 are not approved at a pace sufficient to combat developing antibiotic-resistant bacteria (Richter

86 et al., 2022). Moreover, the last classes of antibiotics to be discovered, including lipopeptides
87 (daptomycin) and oxazolidinones (linezolid), were identified during the 1980s (Durand et al.,
88 2019). Consequently, there is growing interest in alternative antimicrobial strategies, such as
89 antimicrobial peptides (**AMP**), to address this therapeutic gap.

90 AMP are peptide-based biomolecules composed of 5 to 100 amino acids and are typically
91 cationic (Bahar and Ren, 2013). In nature, they are produced by the innate immune system and
92 act as the first line of defense against infections in a wide variety of organisms, including
93 humans, animals, and plants (Bahar and Ren, 2013; Durand et al., 2019). Most conventional
94 antibiotics have specific cellular targets, including cellular machinery involved in DNA and
95 protein synthesis and components of bacterial cell walls (Bahar and Ren, 2013). In contrast,
96 AMP primarily cause rapid cell death by binding to anionic bacterial cell membranes and
97 disrupting their integrity, leading to cell lysis (Bahar and Ren, 2013; Durand et al., 2019). AMP
98 typically do not target eukaryotic cells, as their membranes usually have low anionic charge
99 (Bahar and Ren, 2013). However, at higher concentrations, some AMP may exhibit cytotoxic
100 effects on eukaryotic cells (Bahar and Ren, 2013). Therefore, rigorous safety evaluation of AMP
101 is essential before regulatory approval, including in vitro assays, such as hemolysis and
102 cytotoxicity tests, as well as in vivo experiments.

103 AMP exert less selective pressure on bacteria than conventional antibiotics due to their
104 nonspecific targets and diverse mechanisms of action, thus reducing the development of
105 antimicrobial resistance (Zasloff, 2002; Hancock and Sahl, 2006; Fjell et al., 2012; Richter et al.,
106 2022). In addition to their direct antimicrobial activity, some AMP demonstrate
107 immunomodulatory properties, recruiting immune cells or regulating cytokine levels to enhance
108 the host response to infection (Rima et al., 2021; Richter et al., 2022). Cytokines are important

signaling molecules that facilitate the growth, differentiation, activation of immune cells (Garcia et al., 2021). In the present study, the effect of AMP on the expression of the following cytokines was investigated: Interleukin (**IL**)-1β, IL-8, IL-6, IL-10, interferon regulator factor 4 (**IRF-4**), and interferon gamma (**IFN-*γ***). IL-1β, IL-8, and IL-6 are associated with activation of inflammation in chickens (Kogut, 2002; Garcia et al., 2021). Furthermore, IL-8 recruits heterophils to the infection site (Kogut, 2002). Conversely, IL-10, plays a role in tissue repair, angiogenesis, and attenuation of the inflammatory response and is an inducible feedback regulator of the immune response in chickens (Rothwell et al., 2004; Wu et al., 2016; Garcia et al., 2021). The roles of IRF-4 and IFN-*γ* are not well-characterized in chickens; however, there is evidence that IRF-4 plays a critical role in B cell development and maturation, similar to its mammalian counterpart (Yu et al., 2021), while IFN-*γ* generally functions to bridge the adaptive and innate immune systems (Yuk et al., 2016; Santhakumar et al., 2017).

Previously, we screened 875 AMP candidates, identified using our artificial intelligence (**AI**) based prediction tools: AMPlify (Li et al., 2022), rAMPage (Lin et al., 2022), and AMPd-Up (Li et al., 2024), and used in vitro assays to characterize their antibacterial, hemolytic, and cytotoxic properties. In this study, we selected three lead AMP (named TeBi1, TeRu4, and PeNi4) for further testing, due to their high antimicrobial activity and low toxicity. The three AMP were tested in vivo as a prophylactic treatment prior to APEC inoculation (challenge trials). Immunomodulatory effects of these AMP were tested in tissue harvested from animals involved in the challenge trials and in HD11 cells, a chicken macrophage-like cell line. Lastly, the AMP were tested for their safety in a commercial production setting (pen trials). Overall, TeBi1 and TeRu4 showed efficacy in challenge trials and cell culture while maintaining safety in commercial production settings, supporting their potential for future translational studies.

132

133 **MATERIALS AND METHODS**

134 ***In Silico Peptide Selection***

135 Candidate AMP sequences were predicted using three machine-learning-based software
136 programs developed by our team: AMPlify, rAMPage, and AMPd-Up (Li et al., 2022, 2024; Lin
137 et al., 2022). Candidate AMP were evaluated for potential cytotoxicity using tAMPer
138 (Ebrahimikondori et al., 2024), a machine-learning-based toxicity prediction tool, and those
139 predicted to be cytotoxic were excluded from further evaluation.

140 ***Peptide Synthesis***

141 All AMP for this study were purchased from GenScript (Piscataway, NJ, USA). AMP
142 purity of ≥85% was verified using analytical HPLC. For in vitro assays and APEC challenge
143 trials, AMP were synthesized in 80 µg aliquots, while AMP selected for pen trials were
144 synthesized in 125, 250, or 500 mg aliquots.

145 ***Bacterial Species and Growth.***

146 APEC strain EC317 (Allan et al., 1993) (clinical turkey isolate, obtained from VIDO-
147 InterVac, Saskatchewan, CA), was used as the bacterial target for AMP. Glycerol stocks were
148 stored at -80°C. The primary streaks were done by plating the stocks on 5% Columbia blood agar
149 (Thermo Fisher Scientific, Waltham, MA, USA) and incubating for 20-24 hours at 37°C under
150 aerobic conditions, as described by Richter et al. (Richter et al., 2022). The secondary streaks
151 were done by transferring 2-3 colonies from the primary streak on a new 5% Columbia blood
152 agar plate. Colonies from the secondary streak were used for downstream analysis. Bacterial
153 growth medium was prepared by dissolving 10.5 g Mueller Hinton Broth (Sigma-Aldrich, St.
154 Louis. MO, USA) powder in 500 ml distilled water and autoclaving for sterility.

155 ***In Vitro Lead Peptide Selection***

156 Minimum inhibitory concentrations (**MICs**) of candidate AMP were determined in vitro
157 against EC317, using ASTs as previously described (Richter et al., 2022). For the selection
158 process, candidate AMP with MICs ≤ 32 µg/mL against EC317 were designated as hit AMP and
159 selected for further in vitro testing. Following the protocol by Richter et al. (Richter et al., 2022),
160 hemolytic activities of hit AMP were evaluated in porcine red blood cells (**RBCs**). Those with
161 low hemolytic activity (HC_{50} ≥ 128 µg/mL, where HC_{50} is the AMP concentration that lyses
162 50% RBCs) were subsequently tested for cytotoxicity in human kidney HEK293 cells according
163 to the protocol described (Richter et al., 2022). AMP with CC_{50} ≥ 128 µg/mL (CC_{50}, the AMP
164 concentration that reduces cell viability to 50%) were designated as lead AMP (Table 1).

165 ***Care and Use of Animals***

166 All animal experiments were approved by the University of Saskatchewan and University
167 of Victoria Animal Care Committees (Protocols #19940213 and AE-23-012, respectively) and
168 conducted in accordance with the Canadian Council on Animal Care.

169 ***APEC Challenge Trials***

170 On day 18 of incubation (trial day -3), 50-57 fertilized Ross broiler chicken eggs per
171 treatment were injected with either 1, 5, 10, or 20 µg of AMP, or PBS control. When the birds
172 hatched on day 21 post-fertilization (trial day 0), they were neck-tagged with unique
173 identification numbers and co-housed on Biofresh bedding (Early's Farm and Garden, Saskatoon,
174 Canada), with a light:dark cycle of 23L:1D. Egg hatchability rates and average bird weights were
175 recorded for each treatment group. One day after hatch, the birds were challenged with 5×10^4
176 cfu EC317.

177 Before inoculation, APEC was cultured overnight at 37 °C in LB broth (Difco, Becton
178 Dickinson, Sparks, MD, USA) in a shaking incubator (Model No: 1570, Sheldon Manufacturing,
179 Inc., Cornelius, OR, USA) at 240 rpm. This culture was sub-cultured to a pre-determined OD_{600}
180 of 0.7, at which time the bacteria were harvested by centrifugation, resuspended in phosphate-
181 buffered saline (**PBS**) at the desired concentration, and administered to the birds by yolk sac
182 injection.

183 The birds were given food (Masterfeeds, Humboldt, SK, Canada) and water *ad libitum*.
184 Four days after hatch, approximately half of the birds (between 8-17) were euthanized. The
185 remaining birds were euthanized seven days after hatch. Birds were monitored two to four times
186 per day for symptoms, and birds reaching a humane intervention point were euthanized. Only the
187 birds euthanized on schedule on days four and seven were included in the analyses. Bird survival
188 was also tracked.

189 All analyzed birds were weighed after death, and swabs were taken aseptically from the air
190 sac and pericardium and streaked on MacConkey agar (Difco, Becton Dickinson, Sparks, MD,
191 USA) for a semi-quantitative assessment of APEC growth. Plates were scored from 0-3,
192 representing no, low, medium, and severe levels of growth, respectively. Samples from the air
193 sacs, cecal tonsils, and spleens of birds euthanized on scheduled days were preserved in
194 RNALater (Thermo Fisher Scientific) at –20 °C for analysis by quantitative PCR (**qPCR**).

195 ***Immunomodulatory Activity Assays with HD11 Cells***

196 The effects of AMP on in vitro cytokine production were assessed using an HD11 chicken
197 semi-adherent macrophage-like cell line (Obtained from VIDO-InterVac). HD11 cells were
198 cultured in RPMI medium with 1X Glutamax and 10% FBS (Thermo Fisher Scientific) at 41°C
199 (normal chicken body temperature) and 5% CO_2 and were routinely passaged every two days.

200 ***Cell Plating.*** HD11 cells were plated for experimentation between passages three to twelve.
201 Suspended cells were collected with the growth medium, and adherent cells were obtained after
202 washing with PBS and trypsinization with 0.25% Trypsin-EDTA in PBS (both from Thermo
203 Fisher Scientific). The cells were centrifuged at 200 g for seven minutes and the pellet was
204 resuspended in fresh medium. Trypan Blue (Thermo Fisher Scientific) was added to a sample of
205 cells and live cells were counted on a Hausser Scientific Bright-Line Phase Hemacytometer
206 (Fisher Scientific, Hampton, NH, USA). HD11 cells were plated at 2.5×10^4 cells per well on
207 Nunclon Delta 96-well plates (Thermo Fisher Scientific) and incubated overnight.
208 ***HD11 Experiment Overview.*** In all experiments, HD11 growth medium was used to resuspend
209 and dilute AMP and other reagents, and all incubations were carried out at 41°C and 5% CO_2.
210 All experiments included a medium-only control and a lipopolysaccharide (**LPS**)-only control
211 (final concentration of 25 ng/mL). In all experiments other than the AMP-only Screen, 8 µg/mL
212 Polymyxin B sulfate (**PB**) was used as a positive control as it is known to attenuate the LPS-
213 triggered inflammatory response (Schromm et al., 2021; Yang et al., 2024). Prior to treatment,
214 the supernatant was removed from the wells, therefore only the adherent cell monolayer was
215 used during the experiments.
216 Four experimental approaches were devised to investigate the immunomodulatory activity
217 of AMP. First, HD11 cells were treated solely with AMP (AMP-Only Screen). Second, cells
218 were treated with a range of AMP concentrations followed by treatment with LPS from *E. coli*
219 O111:B4 (Sigma-Aldrich), to test if the AMP could modulate the immune response triggered by
220 LPS (AMP Preincubation Screen). If an AMP showed immunomodulatory activity in the AMP
221 Preincubation Screen, it was then tested in the AMP Preincubation with Wash Step and the AMP
222 and LPS Coaddition experiments.

223 ***AMP-Only Screen.*** HD11 cells were treated with 100 µL 16 µg/mL PeNi4, TeBi1, or TeRu4 and
224 incubated for 6 h.
225 ***AMP Preincubation Screen.*** HD11 cells were treated with 100 µL of 1, 2, 4, 8, or 16 µg/mL
226 PeNi4, TeBi1, or TeRu4 and incubated for 3 h. After the incubation, 100 µL LPS (50 ng/mL)
227 was administered to the cells, resulting in a final concentration of 25 ng/mL LPS. The cells were
228 incubated for an additional 3 h.
229 ***AMP Preincubation with Wash Step.*** The cells were treated identically to the AMP
230 Preincubation Screen but with the addition of a wash step prior to the addition of LPS. Briefly,
231 after the first 3 h incubation, the medium (containing the AMP or PB) was removed from the
232 wells and the cell monolayer was washed three times using fresh medium. One hundred µL LPS
233 (25 ng/mL) was administered, and the cells were incubated for an additional 3 h.
234 ***AMP and LPS Coaddition.*** AMP was resuspended and diluted to prepare a 2X concentration
235 curve of 32, 16, 8, 4, and 2 µg/mL. PB and LPS were prepared at 2X concentrations of 16 µg/mL
236 and 50 ng/mL, respectively. At regular time intervals, 2X AMP or 2X PB was added in equal
237 volume to 2X LPS to yield a 1X mixture of AMP or PB, and LPS. Mixtures were vortexed for
238 one min and incubated for 30 min prior to being administered to cells at regular time intervals in
239 the order in which they were made. The cells were incubated for 3 h.
240 ***Cell Harvest and RNA Isolation.*** Supernatant was removed from HD11 cells after the final
241 incubation. Buffer RLT from the miRNeasy Micro Kit (Qiagen, Mississauga, ON, Canada) with
242 2M DL-dithiothreitol (Sigma-Aldrich) was added to lyse adherent cells at a volume of 260 µL
243 across technical replicates, with each technical replicate comprising two wells in the 96-well
244 plate. HD11 cells in lysis buffer were disrupted by pipetting up and down, vortexed, and stored

245 at -80 °C until RNA isolation. In total, three technical replicates per treatment were collected for
246 RNA isolation and qPCR analysis.

247 ***Commercial Pen Trials***

248 Each pen trial examined the efficacy against ECM and the safety of a single AMP. Cobb
249 500 or Ross 308AP broiler chicken breeds were used in pen trials (Supplementary Table S1). All
250 breeds were sourced from a commercial hatchery (Abbotsford, BC, Canada), and the specific
251 breed used in each trial is summarized in Supplementary Table S1. For pen trials, birds were
252 sourced from older breeder flocks (a different flock for each trial) and separated by sex and
253 treatment into individual mini pens following hatching.

254 AMP were solubilized in 400 mL sterile diluent (**SD**) bags, obtained from the hatchery, to
255 reach a concentration of 0.4 or 0.2 mg/mL, except in the 20µg TeRu4 trial where PBS was used
256 instead. An AMP-free SD bag was used for control. The AMP and control solutions were then
257 taken to the hatchery, for in ovo injections on day 18 post-fertilization, where 50 µL AMP or SD
258 solution was injected into the air sac of 1,680 to 2,352 eggs using the Embrex Inovoject injection
259 machine (Zoetis Inc., Parsippany, NJ, USA). After the injections, eggs were incubated for three
260 days. On the hatching day (day 0), pips, culls, late-stage embryo mortalities (deaths occurring
261 between day 15 and 21 of incubation), post-hatch mortalities, and nonviable eggs/embryos (total
262 of pips, culls, late-stage embryo mortalities, and post-hatch mortalities) were counted and
263 recorded.

264 The birds were then transferred to SJ Ritchie Research Farms (Abbotsford, BC, Canada)
265 where they were cloacally sexed by professionals and then separated by sex and treatment. A
266 total of 24 mini pens, within six blocks, were set up. The birds were weighed individually, then
267 50 birds were placed into each mini pen according to their sex and treatment, with each block

268 containing each of the four experimental groups in a random order. Birds were provided *ad*
269 *libitum* access to water and an all-vegetable, raised without antibiotic (**RWA**) feed (Trouw
270 Nutrition, Chilliwack, BC, Canada). A step-down lighting program was implemented: from day
271 0 to day 21, birds experienced 6 h darkness per 24 h cycle; from day 21 to 28, 4 h darkness; and
272 from day 28 to the end of the trial, 1 h darkness per day. Number of birds in each mini-pen was
273 reduced to 12 on day 10 to prevent overcrowding of the mini-pens. Birds were checked three
274 times per day throughout the trial and were weighed individually on days 10, 21, and 35. Any
275 mortality during the pen trial was noted.

276 ***qPCR Analyses***

277 Multiplex qPCR assays were designed and separately validated for three chicken tissues:
278 air sac, cecal tonsil, and spleen; and for the HD11 macrophage-like cell line. Primers and probes
279 were designed for the normalizer genes *ribosomal protein L8* (*rpl8*) and *ribosomal protein S10*
280 (*rps10*); the cytokine genes *IL-1β, IL-6, IL-8, IL-10,* and *IFN-γ;* and the gene for the transcription
281 factor *IRF-4*. The primer and probe sequences are reported in Supplementary Table S2.

282 RNA was extracted from tissues with the miRNeasy Tissue/Cells Advanced Mini Kit and
283 Qiavac vacuum manifold (Qiagen). Tissue samples were homogenized in 450 µL buffer RLT
284 reduced with DTT, loaded on the RNeasy mini spin column, washed on the Qiavac vacuum
285 manifold, and eluted in 50 µL RNAse-free water. RNA was extracted from HD11 cells using the
286 using the QIAGEN miRNeasy Tissue/Cells Advanced Micro Kit, per the manufacturer's
287 instructions, except for the final elution, where 11.2 µL RNase-free water was added to the
288 column, centrifuged, collected, added back to the column, and centrifuged again.

289 The concentration of RNA from tissue samples was measured by NanoDrop (NanoDrop
290 1000 3.8.1; Thermos Fisher Scientific) and diluted to a concentration of approximately 100

291 ng/µL before complementary DNA (**cDNA**) synthesis, while RNA from HD11 cells was used
292 neat due to the low concentration. All RNA samples were converted to cDNA using the Applied
293 Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), per the
294 manufacturer's instructions.

295 cDNA from HD11 cells and chicken tissues was diluted 1/20 after synthesis and analysed
296 by their respective qPCR assays with reaction conditions outlined in Supplementary Table S3.
297 All samples and controls were run in technical quadruplicates, and all plates included a non-
298 template (negative) control and inter-plate standard as a positive control. Standard deviations
299 between the Cycle Threshold (**Ct**) values of technical replicates were computed. If any deviation
300 was greater than 0.5 cycles, the outlier value in that set of replicates was removed. Normalizer
301 stability was verified by BestKeeper (Pfaffl et al., 2004). Mean values between technical
302 replicates were taken into the next stage of analysis. Data was normalised against the geometric
303 mean of rpl8 and rps10, and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method, as previously
304 described (Livak and Schmittgen, 2001). Final relative fold changes for each sample and
305 cytokine were determined by comparing to the relevant control: median of the PBS-treated birds
306 from day 4 (for challenge trials), median of the PBS-treated female birds (for pen trials), or the
307 median of medium-treated HD11 cells (for cell culture experiments).

308 ***Statistical Analyses***

309 Statistical analyses of the qPCR results were conducted in R version 3.6.3 (R Core Team,
310 2024). The data were tested for normality by the Shapiro–Wilk and Levene's tests. They were
311 then subjected to pairwise analyses by the Kruskal-Wallis and Wilcoxon rank-sum tests for non-
312 parametric data with a significance threshold of $\alpha = 0.1$. For the challenge and pen trials, this
313 significance cut off was selected due to the high variability of in vivo data. For the HD11

314 experiments, this threshold was appropriate as the smallest p-value achievable using a rank-sum
315 test with n = 3 was approximately 0.08.

316 The rest of the challenge trial data was analyzed in R version 4.4.0 and 4.5.1(R Core Team,
317 2024). Hatchability rates of treated eggs compared to control eggs, as well as rates of bacterial
318 detection in the birds' air sacs and pericardia, were evaluated using the Fischer's exact test.
319 Cumulative weight gain of birds euthanized on days four and seven was evaluated using a
320 Wilcoxon rank-sum test, and survival was graphed and evaluated using the log-rank test and the
321 "survminer" package (Kassambara et al., 2016). Other graphs were constructed using the
322 packages "tidyverse," "readr," "janitor," "superb," "ggsignif," "rstatix,", "DescTools", and
323 "ggpubr" (Signorell, 2014; Wickham et al., 2015b; a, 2019; Kassambara, 2016, 2019; Firke,
324 2016; Ahlmann-Eltze and Patil, 2017; Cousineau, 2021).

325 An additional analysis was conducted to compare the relative cytokine abundance in bird
326 air sacs to the air sac bacterial load scores of the same birds. The difference in cytokine
327 abundance for each bacterial level (light, moderate, severe) was compared to the cytokine
328 abundance for no growth using a Wilcoxon rank-sum test, and additionally, a correlation
329 coefficient was determined using Spearman's Rho.

330 Statistical analyses of hatchability, survival, mean weights, feed conversion ratio, and flock
331 uniformity in pen trials were conducted using R (version 4.1.3) and the packages: "survival",
332 "survminer", "openxlsx", "readxl", "tidyverse, "zoo", "car", "ggplot2", "ggsignif", and
333 "MHTdiscrete" (Fox et al., 2001; Therneau, 2001; Zeileis et al., 2004; Wickham et al., 2007,
334 2019; Schauberger and Walker, 2014; Wickham and Bryan, 2015; Kassambara et al., 2016;
335 Yalin Zhu, Wenge Guo, 2016; Ahlmann-Eltze and Patil, 2017). For pen trials, statistical
336 comparisons between treatment and control groups used Wilcoxon rank-sum tests (hatchability

337 and production parameters) and log-rank tests (survival), with production and survival analyzed
338 separately by sex. P-values were corrected for multiple comparisons using the Šidák method.
339

340 **RESULTS**

341 ***In Vitro Peptide Selection***

342 The 875 candidate AMP identified by rAMPage (Lin et al., 2022), AMPlify (Li et al.,
343 2022), and AMPd-Up (Li et al., 2024) were previously tested for antimicrobial activity and
344 cellular toxicity in vitro (Richter et al., 2022). Of these, 331 with MICs ≤ 32 µg/mL against
345 EC317 were designated as hit AMP. Among hit AMP, 275 exhibited minimal hemolytic activity
346 (HC_{50} ≥ 128 µg/mL) against porcine RBCs. Of these, 62 also demonstrated minimal cytotoxicity
347 (CC_{50} ≥ 128 µg/mL) in HEK293 cells. These were classified as lead AMP. Three representative
348 lead AMP (TeRu4, TeBi1, and PeNi4) were selected for further experiments. TeRu4 and TeBi1
349 demonstrated lower MIC values than PeNi4. The physiochemical properties of these AMP and
350 their in vitro activities are summarized in Table 1. These AMP were evaluated in vitro using
351 HD11 cells and in vivo using APEC challenge trials and pen trials.

352 ***qPCR Primer Design and Validation***

353 The sequences of the qPCR primers and probes, reaction conditions, and assay efficiencies
354 are reported in Supplementary Tables S2-4. Primer efficiencies ranged from 93-108% in the air
355 sac, 92-107% in the cecal tonsil, 87-107% in the spleen, and 84-101% in HD11 cells.

356 ***APEC Challenge Trials***

357 To assess the prophylactic efficacy of TeBi1, TeRu4, and PeNi4 in chickens, fertilized
358 eggs were injected in ovo at day 18 of incubation with either 1, 5, 10, or 20 µg AMP, or a PBS

359 control. One day after hatch, the birds were challenged with 5×10^4 cfu APEC strain EC317 by
360 yolk sac injection.

361 Egg hatchability was between 90-98% in all treatment groups, with no significant
362 differences between treated birds and control birds (data not shown). Notably, when birds were
363 treated with 20 μg AMP (Supplementary Figure S1), treated birds appeared to die faster than
364 untreated birds within the first few days post-infection, though there were no significant
365 differences in overall survival. The spleens of birds treated with 20 μg AMP were analyzed by
366 qPCR, and some differences were observed, suggesting that the peptides were bioactive, but the
367 qPCR results were not conclusive (data not shown), and subsequent challenge trials tested the
368 AMP on a dose curve at 1, 5, or 10 μg. In the latter trials, there were no significant differences in
369 survival probability with any dose of AMP (≤10 μg) compared to control (Supplementary Figure
370 S2).

371 Birds treated with TeBi1 had significantly higher weights on day 7 than control birds
372 (Figure 1a). Birds treated with 1 or 5 μg of TeBi1 were approximately 25% heavier than control
373 birds ($p = 0.03$ and $p = 0.07$, respectively), and birds treated with 10 μg TeBi1 were
374 approximately 50% heavier than control birds ($p = 2 \times 10^{-4}$). There were no significant
375 differences in weight gain in birds treated with TeRu4 or PeNi4 (Supplementary Figure S3).

376 On day 7, birds treated with 10 μg TeBi1 had significantly lower rates of infection in both
377 the air sac ($p = 0.01$) (Figure 1b) and pericardium ($p = 0.03$) (Figure 1c). There were no
378 significant differences in bacterial detection rates in the air sacs and pericardia of birds treated
379 with TeRu4 or PeNi4 (Supplementary Figure S4).

380 Increased bacterial load in the air sac was correlated with increased levels of transcript
381 abundance of certain cytokines in the air sac (Figure 2). Across all three challenge trials, birds

382 with light, moderate or heavy levels of active APEC infection in their air sacs had significantly
383 higher relative abundance of *IL-1β* transcripts ($p = 2 \times 10^{-4}$, 1×10^{-6}, and 7×10^{-13}, respectively)
384 than birds with no bacterial growth in their air sacs (Figure 2a). They are also positively
385 correlated by Spearman's rho, with $\rho = 0.58$ and $p = 1 \times 10^{-19}$. The same trend, though weaker, is
386 observed with *IL-8*. While $\rho = 0.32$ ($p = 3 \times 10^{-6}$) overall, birds with light, moderate, or heavy
387 levels of APEC infection also had significantly higher relative abundance of *IL-8* transcripts (p =
388 0.03, 3×10^{-6}, and 5×10^{-6}, respectively) than birds with no bacterial growth in their air sacs
389 (Figure 2b).

390 In the cecal tonsil (Supplementary Figure S5), qPCR results show minimal changes due to
391 treatment with PeNi4, with only a 2-fold increase in the inflammatory cytokine *IL-8* in the 10 μg
392 group on day 4 (p = 0.1); and with TeRu4, a 2-fold decrease in *IL-8* in the 1 μg group on day 4 (p
393 = 0.1). In contrast, there are numerous changes due to treatment with TeBi1, including a 2.4-fold
394 increase in *IL-8* transcripts in the 5 μg group on day 4 (p = 0.1), and a 5-fold decrease in *IL-8*
395 transcripts in the 10 μg group on day 7 (p = 0.06). *IFN-γ* expression increased 1.4-fold (p = 0.03)
396 and 2.5-fold (p = 0.03) in the 5 and 10 μg groups, respectively, on day 4, but decreased 1.4-fold
397 (p = 0.03) and 1.1-fold (p = 0.09) in the 1 and 10 μg groups, respectively, on day 7. With respect
398 to *IL-1β,* on day 7, the TeBi1 5 μg group showed a 5-fold decrease (p = 0.07). For the anti-
399 inflammatory transcript *IL-10,* there is a 2.5-fold decrease (p = 0.07) with 5 μg TeBi1 on day 7.
400 Finally, TeBi1 treatment was also associated with changes in *IRF-4* transcripts: the 10 μg group
401 was associated with a 1.7-fold increase on day 4 (p = 0.04), and the 5 μg group was associated
402 with a 1.5-fold increase on day 7 (p = 0.1).

403 In the spleen (Supplementary Figure S6), fewer responses are observed. For TeRu4, there
404 is a 2-fold decrease in *IL-8* transcripts on day 7 (p = 0.1) after treatment with 1 μg. For PeNi4,

405 there is a 5-fold increase in *IL-10* transcripts on day 7 (p = 0.09) after treatment with 5 µg. For
406 TeBi1, while there are no differences in the inflammatory cytokines *IFN-γ, IL1β,* or *IL-8*, there is
407 a 3-fold increase in *IL-10* transcripts on day 4 (p = 0.03) after treatment with 10 µg. There are
408 also multiple increases in *IRF-4* transcripts: on day 4, a 2-fold increase after treatment with 1 µg
409 (p = 0.1), a 3-fold increase after treatment with 5 µg (p = 0.1); and on day 7, a 1.3-fold decrease
410 after treatment with 1 µg (p = 0.1).

411 In the air sac (Figure 3), all observed changes are on day 7, after treatment with 10 µg
412 TeBi1 relative to the PBS control. There is a 10-fold decrease in *IFN-γ* transcripts (p = 0.1), a 3-
413 fold decrease in *IL-1β* transcripts (p = 0.07), and a 5-fold decrease in *IL-8* transcripts (p = 0.02).
414 Additionally, there is a 50-fold decrease in *IL-10* transcripts (p = 0.1), and a 10-fold decrease in
415 *IRF-4* transcripts (p = 0.1).

416 ***HD11 Experiments***

417 In addition to challenge trials, the ability of TeRu4, TeBi1, and PeNi4 to influence the
418 cellular immune response was tested in an HD11 cell model system. In all HD11 experiments
419 (n=21), the LPS induction control resulted in a wide-ranging, but consistent increase in relative
420 fold change compared to the medium control for *IL-1β* (14 - 677), *IL-8* (8 - 249), and *IL-6* (55 -
421 3,284) (Figure 4 and Supplementary Figures S7 and S8). Therefore, all experimental
422 manipulations with AMP were compared with their experimentally paired LPS controls. The
423 induction of cytokine transcripts by LPS was significantly attenuated by PB in all but a single
424 experiment (n=15), with fold change decreases compared to LPS alone ranging from 6-166 for
425 *IL-1β*, 2-34 for *IL-8*, and 11-335 for *IL-6* (Figure 4 and Supplementary Figure S8). While only
426 one representative graph is shown for each experiment, the preincubation, preincubation with
427 wash step, and coaddition experiments were performed in biological triplicate, and the AMP-

428 only experiments in biological duplicate. Only data consistently reproduced across all replicates
429 are highlighted, unless otherwise stated.
430 ***Induction of Inflammatory Cytokines in HD11 cells by TeRu4, TeBi1, and PeNi4.*** To assess
431 the ability of AMP alone to induce cytokine production in HD11 cells, cells were incubated with
432 16 µg/mL of each AMP or 8 µg/mL PB. Treatment with TeRu4 resulted in a 2-fold increase in
433 *IL-1β* (in one of two biological replicates) and *IL-8,* while TeBi1 lead to a 4-fold decrease in *IL-6*
434 (Supplementary Figure S7). Treatment with PeNi4 did not significantly alter the abundance of
435 any of the three cytokines, while TeBi1 had no significant effect on *IL-1β* and *IL-8,* and TeRu4
436 had no significant effect on *IL-6* (Supplementary Figure S7). Treatment with PB alone did not
437 significantly or consistently change *IL-1β, IL-8*, and *IL-6* levels (Supplementary Figure S7).
438 ***Preincubation Screen of TeRu4, TeBi1, and PeNi4.*** To examine if AMP affect HD11 response
439 to LPS, cells were preincubated with AMP or PB (positive control), followed by treatment with
440 LPS. Here and in subsequent experiments, medium only and LPS only served as negative and
441 positive controls, respectively. A significant 2.8-fold decrease was observed for *IL-1β* and *IL-6*
442 following preincubation with 8 µg/mL TeRu4 compared to the LPS control (Supplementary
443 Figure S8). Preincubation with 16 µg/mL TeRu4 led to a significant reduction in *IL-1β, IL-8*, and
444 *IL-6* levels by 13.6-fold, 2.9-fold, and 22.0-fold, respectively, compared to LPS only control
445 (Supplementary Figure S8). Similarly, treatment with 8 µg/mL PB significantly decreased *IL-1β*,
446 *IL-8* (in 2 of 3 biological replicates), and *IL-6* abundance relative to LPS alone by 8.8-fold, 3.4-
447 fold, and 20.4-fold, respectively (Supplementary Figure S8). While a significant 2.8-fold
448 increase was observed for *IL-8* at 4 µg/mL TeRu4 compared to the LPS control, this observation
449 was only seen in two of three biological replicates (Supplementary Figure S8). Preincubation
450 with TeBi1 and PeNi4 did not consistently induce a significant change in *IL-1β, IL-6*, and *IL-8*

451 transcript levels compared to the LPS control across biological replicates (Supplementary Figure
452 S8). The attenuation of LPS-induced responses of *IL-1β, IL-8*, and *IL-6* transcripts after TeRu4
453 preincubation prompted further investigation via the subsequent experiments to provide some
454 insight into possible mechanisms of action.

455 ***Preincubation Experiment with Wash Step.*** The effects on HD11 cells of transient exposure to
456 TeRu4, followed by its removal and the addition of LPS, was examined. A significant decrease
457 in *IL-1β, IL-8,* and *IL-6* transcripts relative to the LPS control remained after preincubation with
458 8 µg/mL PB (21-, 5-, and 80-fold respectively) and 16 µg/mL TeRu4 (3-, 2-, and 4-fold,
459 respectively) (Figure 4). Preincubation with 8 µg/mL TeRu4 induced a significant decrease in
460 *IL-1β* levels by 2.0-fold and *IL-6* by 1.9-fold in two of three biological replicates relative to the
461 LPS only control (Figure 4). Unlike in the previous preincubation experiment (without a wash
462 step), treatment with 1 and 2 µg/mL TeRu4 followed by the wash step, frequently (but not
463 consistently in all biological replicates) resulted in a significant increase in *IL-1β* (1.3- and 1.7-
464 fold respectively) and *IL-8* (1.3- and 1.6-fold respectively) transcript levels relative to the LPS
465 only control (Figure 4). The significant increase in *IL-6* (2-fold for relative to LPS only) at these
466 low concentrations was consistent across all three biological replicates (Figure 4).

467 ***Coincubation of TeRu4 and LPS.*** The impact of co-incubating TeRu4 and LPS prior to treating
468 HD11 cells on cytokine transcript expression was assessed. Coaddition of LPS with either PB or
469 TeRu4 (at concentrations ranging from 4 to 16 µg/mL) resulted in significant decreases in all
470 cytokine transcripts compared to the LPS control across all three biological replicates (Figure
471 10). Specifically, co-treatment with PB and LPS led to reductions of 101-fold for *IL-1β*, 27-fold
472 for *IL-8*, and 260-fold for *IL-6* (Figure 4). Similarly, co-treatment with 16 µg/mL TeRu4 and
473 LPS decreased *IL-1β* abundance by 75-fold, 20-fold for *IL-8*, and 409-fold for *IL-6* (Figure 4).

474 LPS and 8 µg/mL TeRu4 reduced *IL-1β* transcripts by 97-fold, *IL-8* by 23-fold, and *IL-6* by 368-
475 fold compared to LPS alone (Figure 4). LPS and 4 µg/mL TeRu4 resulted in reductions of 15-
476 fold for *IL-1β*, 6-fold for *IL-8*, and 47-fold for *IL-6* compared to LPS alone (Figure 4). For *IL-6*,
477 4-16 µg/mL TeRu4 repressed the LPS induction so much that it was indistinguishable from the
478 medium only negative control (observed in two of three replicates at 4 µg/mL) (Figure 4). Like
479 what was observed in the previous experiment, 1 and 2 µg/mL of TeRu4 resulted in significantly
480 increased transcript abundance in *IL-1β* (2-fold), *IL-8* (2-fold), and *IL-6* (3-fold) relative to the
481 LPS control, but unlike the previous experiment, these results were consistent across three
482 biological replicates (Figure 4).

483 ***Commercial Pen Trials***

484 Pen trials were designed to test the efficacy against ECM and the safety of treating
485 chickens with TeBi1, TeRu4, and PeNi4 in a commercial production setting. Late-stage viability
486 (viable embryos from day 15 of incubation until placement in pens) was between 88 and 98% in
487 all pen trials. No statistically significant differences were detected between the treatment and
488 control groups (Supplementary Figure S9). There were no significant differences between the
489 treatment and control groups for post-hatch mortality, culls, pips, and late embryo mortality.

490 For the first 10 days, there were no statistical differences between the survival probability
491 of males and females (Supplementary Figure S10). Thus, the sexes were combined for
492 subsequent analyses. No significant differences in survival probability were detected between
493 treatment and control groups for any AMP during the first 10 days of the pen trials, indicating
494 that the tested AMP had no measurable impact on ECM (Supplementary Figure S11). However,
495 for the full duration of the pen trials (35 days), sex-specific differences in survival probabilities
496 were observed for TeRu4 at 20 µg and TeBi1 at 10 µg (Supplementary Figure S12). For these

497 two pen trials, the sexes were kept separate for analysis (Figure 5, Supplementary Figure S13).
498 Notably, TeRu4 at 20 µg significantly increased the survival probability of female birds
499 compared to the controls by approximately 4.9 % by day 35 ($p = 0.04$). Moreover, TeBi1 at 20
500 µg significantly increased the survival probability of the birds compared to the control by
501 approximately 4.4% by day 35 ($p = 0.02$). There was no other difference in survival probabilities
502 between the treatment and control groups by day 35. In the 10 µg TeRu4 and TeBi1 trials,
503 survival curves for both sexes closely overlapped (Supplementary Figure S13).
504 Significant differences in mean weight between treatment and control groups were
505 observed only in the 20 µg TeBi1 trial (Supplementary Figure S14). Notably, on day 0, mean
506 weights in the treatment groups were approximately 2.8% lower in males and 2.4% lower in
507 females compared to controls ($p = 0.02$ and 0.03). However, on day 10, no significant difference
508 in mean weights were observed. Moreover, in the 10 µg TeRu4 trial, numerous birds of both
509 sexes exhibited lower body weights on days 21 and 35, particularly in the treatment group,
510 although this difference was not statistically significant. This observation may suggest
511 subclinical effects of TeRu4 at lower doses, high biological variability among individual birds,
512 or environmental effects, all of which warrant further investigation. By day 35, no significant
513 differences in mean weight were detected (Supplementary Figure S14).
514 No statistically significant differences were detected between FCR of the treatment and
515 control groups on day 35, suggesting that the tested AMP did not exhibit measurable growth-
516 promoting properties (Supplementary Figure S15). Of interest, there were occasions where FCR
517 values were below 1 (Supplementary Figure S15). One was observed between days 0 and 10, in
518 the 10 µg TeBi1 trial (FCR = 0.997). Further, in the 20 µg TeRu4 trial, two FCR values were
519 below 1 (FCR = 0.923 and 0.961) in the second interval, between days 11 and 21. Overall, the

520 mean FCR values were consistently the lowest in the first interval, and the highest in the last
521 interval, between days 21 and 35, for both sexes. These findings suggest that AMP did not
522 significantly alter FCR under the tested conditions, and observations during the first interval of
523 certain pen trials warrant further investigation.

524 Coefficient of variation (**CV**) percentages were calculated as a measure of flock
525 uniformity. There was no significant difference in the CV percentages of the treatment and
526 control groups on day 35 (Supplementary Figure S16). Among males, on day 10 in the 20 µg
527 TeBi1 trial, the treatment group had an approximately 2.8% lower CV compared to the control
528 group, but this difference did not reach statistical significance ($p = 0.07$) (Supplementary Figure
529 S16). Moreover, the birds in the 10 µg TeRu4 trial exhibited greater variability in flock
530 uniformity on days 21 and 35, with CV values exhibiting greater range than those in the control,
531 consistent with the presence of birds with low body weight.

532 The qPCR results were analyzed with sexes separated, and significant differences are
533 reported for both comparisons between birds in the same sex group that received different
534 treatments, and for birds in the same treatment group with differences between sexes. As there
535 were some significant differences in transcript abundance between male and female birds in the
536 same treatment groups, the results are shown with sexes separated in Figure 6.

537 After treatment with 10 µg of TeRu4, female birds had a 1.8-fold increase ($p = 0.1$) in *IFN-*
538 *γ* transcripts relative to (female) control birds, and in that trial, male controls birds had a 1.2-fold
539 increase ($p = 0.1$) in *IRF-4* transcripts relative to female control birds. After treatment with 20 µg
540 of TeRu4, male birds had a 1.3-fold increase ($p = 0.08$) in *IRF-4* transcripts relative to control
541 birds.

542 After treatment with 20 µg PeNi4, female birds had a 1.4-fold increase (p = 0.02) in *IRF-4*
543 transcripts relative to control birds. Additionally, in that trial, male control birds had a 1.3-fold
544 increase (p = 0.01) in *IRF-4* transcripts and a 1.6-fold increase (p = 0.04) in *IL-8* transcripts
545 relative to female control birds.

546 When considering the sexes separately, there were no significant differences in transcript
547 abundance in birds treated with 10 µg TeBi1 (Figure 6). As there were no significant differences
548 between sexes in this trial, qPCR results from that trial were also combined (Supplementary
549 Figure S17). Here, a 1.2-fold increase (p = 0.1) in *IL-1β* transcripts was observed. Examination
550 of the transcript abundances in birds treated with 20 µg TeBi1 revealed numerous differences
551 relative to PBS controls.

552 After treatment with 20 µg TeBi1, male birds had a 2-fold decrease in *IFN-γ* and *IRF-4*
553 transcripts (p = 0.02 for both), and a 3-fold decrease in *IL-10* and *IL-8* transcripts (p = 0.03 and
554 0.02, respectively) relative to control birds. In that trial, male control birds had higher abundance
555 of all tested transcripts than female control birds: 2-fold more *IFN-γ* transcripts (p = 0.1), 3-fold
556 more *IL-10* transcripts (p = 0.06), 1.7-fold more *IL-1β* transcripts (p = 0.008), 3-fold more *IL-8*
557 transcripts (p = 0.01), and 2-fold more *IRF-4* transcripts (p = 0.1). Additionally, male birds
558 treated with 20 µg TeBi1 had significantly lower abundance of certain transcripts than female
559 birds in the same treatment group, a 1.3-fold decrease in both *IFN-γ* and *IRF-4* transcripts (p =
560 0.09 for both).

561

562 **DISCUSSION**

563 This study demonstrates that antimicrobial peptides can improve early health outcomes in
564 broiler chickens while maintaining production performance under commercial conditions. Across

565 complementary in vivo and in vitro assays, two of the three lead AMP, TeBi1 and TeRu4,
566 showed efficacy and immunomodulatory activity consistent with their intended roles as
567 antibiotic alternatives for preventing APEC-associated disease. Here, AI-assisted discovery is
568 relevant for enabling large-scale candidate generation and triage that would be impractical by
569 hand. All outcome data from the in vivo and in vitro assays were analyzed using nonparametric
570 methods, Kruskal–Wallis for omnibus comparisons and Wilcoxon rank-sum for pairwise
571 contrasts. Given the inherent variability of in vivo data and the exploratory nature of this study, a
572 significance threshold of $\alpha = 0.1$ was used, as specified in the methods.

573 First, in controlled challenge trials, pre-hatch administration of TeBi1 was associated
574 with reduced post-hatch bacterial burden and improved early growth. At 10 µg per egg, TeBi1
575 significantly lowered culture detection of APEC in the air sac and pericardium by day 7 and
576 yielded an approximate 50% increase in body weight relative to controls at the same time point.
577 These findings support a direct antibacterial contribution in vivo and align with TeBi1's
578 favorable in vitro profile (Lin et al., 2022). Concordant decreases in pro-inflammatory cytokine
579 transcripts in air sac tissue at day 7 (Figure 3) further suggest that limiting bacterial load
580 mitigates downstream inflammatory responses. In contrast, TeRu4 and PeNi4 did not
581 reproducibly reduce organ-specific bacterial detection in the challenge setting at tested doses up
582 to 10 µg, indicating peptide-specific differences in in vivo antibacterial activity despite their
583 performance in in vitro experiments. Consistent with this, positive correlations between air sac
584 bacterial loads and *IL-1β* ($\rho = 0.58$) and *IL-8* ($\rho = 0.32$) expression (Figure 2) both support the
585 validity of the infection model and indicate that AMP-mediated reductions in bacterial burden
586 translate to dampened inflammatory signaling.

587 Second, the HD11 macrophage-like cell experiments demonstrate the potential for TeRu4
588 to attenuate the effects of LPS. Preincubation with TeRu4 and its coaddition with LPS elicited
589 robust and dose-dependent attenuation of *IL-1β*, *IL-6*, and *IL-8* transcripts, with effects at higher
590 exposures of TeRu4 that were comparable in direction and magnitude to PB. While the
591 persistence of attenuation throughout the experiments (Figure 4) may indicate that TeRu4 can
592 interact both with LPS and with HD11 cells to mitigate inflammation, further study is required to
593 confirm these mechanisms. Notably, a biphasic pattern was observed: attenuation of *IL-1β, IL-6,*
594 and *IL-8* at higher exposures of TeRu4, but mild potentiation at sub-threshold doses. Taken
595 together, these data support an immunomodulatory profile for TeRu4 that could help contain
596 excessive inflammation induced by LPS, even when direct antibacterial effects are limited in
597 vivo.

598 Third, pen trials provide critical evidence of field-relevant safety and performance. In ovo
599 administration of TeBi1 or TeRu4 at 10 or 20 µg per egg did not adversely affect hatchability,
600 FCR, or flock uniformity through day 35. Importantly, survival probabilities improved modestly
601 but significantly in two settings: a 4.9% increase in females with 20 µg TeRu4 and a 4.4%
602 increase in all birds with 20 µg TeBi1 by day 35. Although effect sizes were small, they are
603 operationally meaningful at commercial scale and were achieved without detectable adverse
604 effects in growth or efficiency. These findings support a favorable benefit-risk profile for both
605 peptides when delivered through standard in ovo automation.

606 Together, the in vivo and in vitro data outline complementary roles for these leads. TeBi1
607 appears to act primarily through direct antibacterial activity in the immediate post-hatch period,
608 reducing tissue colonization and the associated inflammatory response. TeRu4 shows consistent
609 anti-inflammatory activity in a macrophage model and over 35 days translates to a survival

610 advantage in females at higher dose, suggesting that immunomodulation can contribute to
611 resilience under farm conditions. These sex-specific responses may reflect differences in immune
612 system maturation rates or hormone-immune interactions, as male broilers typically show
613 different growth and immune development patterns than females (Leitner et al., 1989; Younis et
614 al., 2023). The patterns observed in both survival and cytokine transcript baselines warrant
615 additional investigation into sex-dependent pharmacodynamics (**PD**), immune set-points, and
616 potential interactions with breeder age and genetics.

617 While the results presented here are encouraging, this study also has several limitations.
618 First, the challenge trials used a single APEC strain and a prophylactic in ovo regimen. Broader
619 pathogen panels, additional exposure routes, and therapeutic or peri-hatch dosing schedules
620 should be evaluated to define the generalizability and optimal use cases. Second, although this
621 study shows safety and performance neutrality in pens, pharmacokinetics (**PK**), peptide stability
622 in the embryonic and early post-hatch milieu, and residue depletion profiles remain to be
623 established. Third, gene expression endpoints were measured at discrete time points and in a
624 single cell line or selected tissues. High-resolution temporal profiling, including protein-level
625 cytokine measurements and cellular phenotyping in multiple cell lines and tissues, would
626 sharpen mechanistic inference and help explain biphasic responses at lower TeRu4
627 concentrations. Further, some FCR values below 1.0 in early trial periods likely reflect
628 measurement artifacts related to initial hydration status rather than true feed conversion,
629 highlighting the importance of standardized weighing protocols in early post-hatch periods.
630 Finally, while the analyses used conservative non-parametric tests and multiple-comparison
631 corrections, some findings were near the chosen α threshold of 0.1, reflecting expected
632 variability in vivo and underscoring the need for replication across flocks and sites.

633 The results have practical implications for poultry health programs. The in ovo delivery
634 method offers multiple advantages: it integrates cleanly into existing hatchery workflows, avoids
635 post-hatch handling, and ensures uniform exposure across flocks. This delivery approach,
636 combined with a demonstrated safety profile, minimizes barriers to commercial adoption. From
637 an economic perspective, even modest survival improvements translate to substantial numbers at
638 commercial scale, while early bacterial control reduces the need for subsequent therapeutic
639 interventions. The complementary mechanisms of TeBi1 (direct antibacterial) and TeRu4
640 (immunomodulatory) suggest potential for combination strategies that leverage both activities.
641 Future work should benchmark these AMP against current animal husbandry standards and
642 evaluate alternative delivery formats, such as water supplementation during high-risk periods, to
643 optimize protection throughout the production cycle.
644 In conclusion, AI-discovered AMP TeBi1 and TeRu4 show complementary efficacy and
645 safety signals in broilers when administered in ovo. TeBi1 reduces early APEC burden and
646 inflammation and improves early growth, whereas TeRu4 attenuates macrophage inflammatory
647 responses and improves survival under commercial conditions without compromising
648 performance. These findings support advancement to dose-optimization, PK and residue studies,
649 expanded pathogen panels, and multi-site validation, with an eye toward integrated antimicrobial
650 stewardship in poultry production.
651

652 **DECLARATION OF COMPETING INTEREST**

653 Inanc Birol is a founder, Chief Scientific Officer, and equity holder of Amphoraxe Life
654 Sciences Inc., which has licensed intellectual property related to antimicrobial peptides described
655 in this manuscript. The following authors are named inventors on related intellectual property:

Caren C. Helbing, Erin Fraser, Fraser Hof, Lorissa Corrie, René L. Warren, Vanessa C. Thompson, Anat Yanai, Inanc Birol, and Linda M. N. Hoang. Monica Kotkoff is also a stakeholder on the IP. Emre Demirsoy, Teagan I. Parkin, and Anna H. Dema received internship funding from Amphoraxe through Mitacs. Amphoraxe contributed to aspects of the work, including study design for selected experiments, resource allocation, collaborative experimental activities, internship support, and participation in manuscript preparation. The authors retained control over data collection, analysis, interpretation, and the decision to submit the manuscript for publication. All other authors declare no competing interests.

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830 **TABLES**

831 **Table 1.** Physiochemical properties and in vitro activities of three lead antimicrobial peptides
832 (AMP) TeRu4, TeBi1, and PeNi4. Listed for each AMP are their sequence length, net charge,
833 and amino acid sequence. Each AMP was synthesized as the C-terminal carboxylic acid. In vitro
834 activities include the antimicrobial activity against avian pathogenic *E. coli* (APEC) EC317
835 (minimum inhibitory concentration, MIC), the hemolytic activity against porcine red blood cells
836 (RBCs) (hemolytic concentration 50%, HC50), and cytotoxicity towards HEK293 human cells
837 (cytotoxic concentration 50%, CC50).

AMP	Sequence length	Net charge (+)	Amino acid sequence	EC317 MIC (µg/mL)	RBC HC50 (µg/mL)	HEK293 CC50 (µg/mL)
TeRu4	30	8	SWLSKSVKKLVNKKNYTRLE KLAKKKLFNE	1.3	>128	>128
TeBi1	23	6	KIKIPWGKVKDFLVGGMKAV GKK	1.75	>128	128
PeNi4	15	3	GLLGKVLGVGKKVLC	32	>128	>128

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844 **FIGURES**

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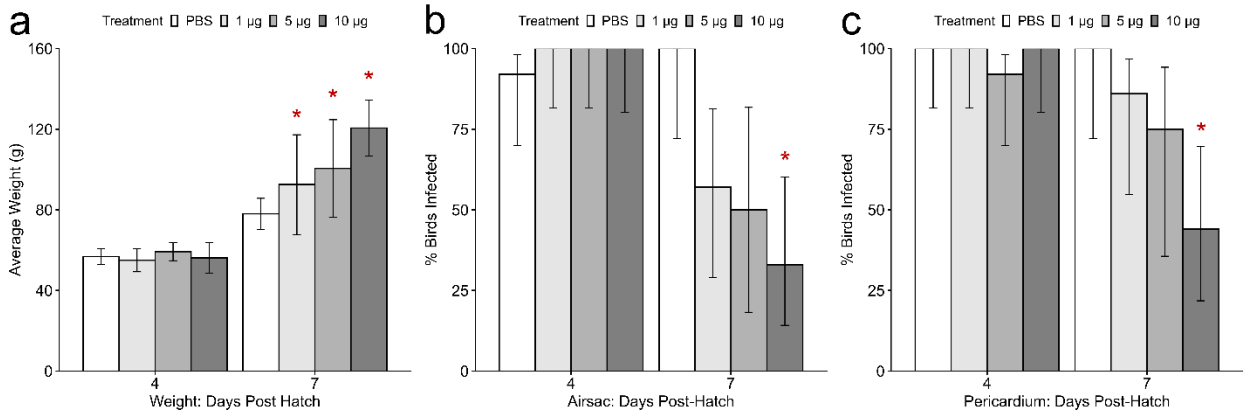

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847 **Figure 1**: a) Weight gain of TeBi1 treated birds between day 4 and 7. Birds, euthanized on
848 schedule, were weighed individually, and values shown are median ± median absolute deviation
849 (MAD). On day 7, birds treated with 1 or 5 µg TeBi1 were approximately 25% heavier than
850 control birds, and birds treated with 10 µg TeBi1 were approximately 50% heavier than control
851 birds. Significance ($p \leq 0.05$) relative to the phosphate buffered saline (PBS) control is indicated
852 by an asterisk. b/c) Percent of TeBi1 treated birds infected by avian pathogenic *E. coli* (APEC) at
853 on day 4 and 7 post-hatch in the b) air sacs and c) pericardia. Birds were euthanized as scheduled
854 and aseptic swabs were taken from the tissues and streaked onto MacConkey agar. The
855 percentage of birds in each treatment group that resulted in bacterial growth is shown ± a 90%
856 Wilson confidence interval. Each treatment group was compared to the PBS control by Fisher's
857 Exact Test. Significance ($p \leq 0.05$) relative to the PBS control is indicated by an asterisk. On day
858 7, there were significantly fewer birds with detectable bacteria in the 10 µg TeBi1 treatment
859 group than the control group, in both tissues.

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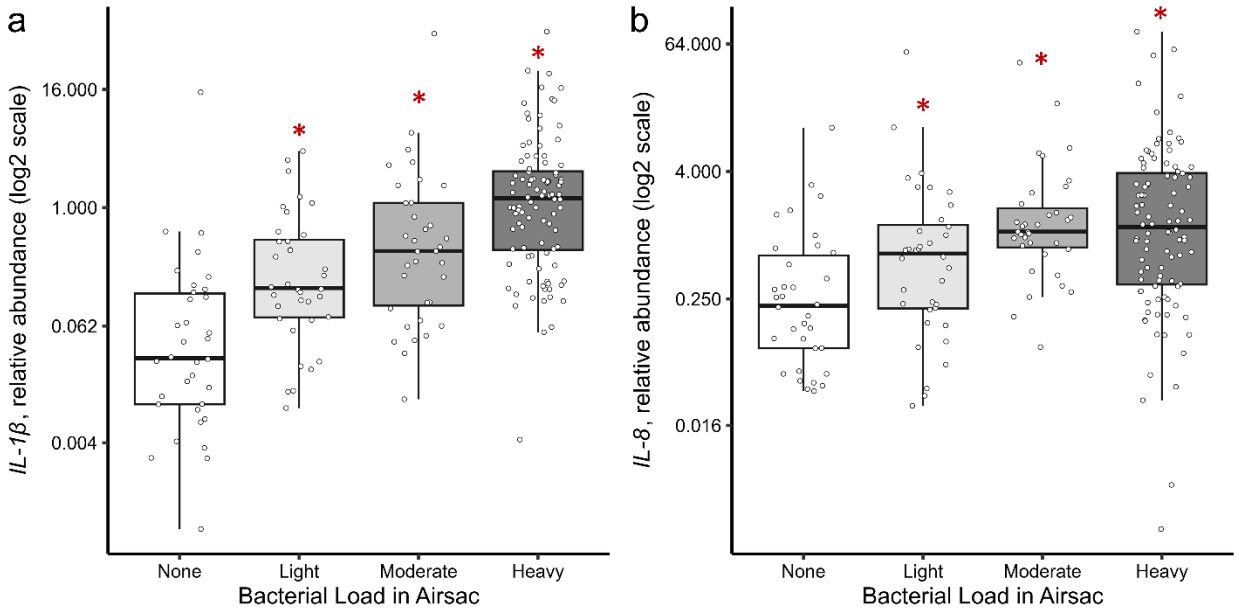

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866 **Figure 2.** Relative transcript abundance of a) *Interleukin (IL)-1β* and b) *IL-8* in the air sacs of

867 birds treated with ≤10 µg TeRu4, TeBi1, or PeNi4. Each data point represents the air sac of a

868 single bird. The light, moderate, and heavy infection groups were compared to the non-infected

869 group (none) using the Wilcoxon rank-sum test. Significance ($p \leq 0.05$) relative to non-infected

870 group is indicated by an asterisk.

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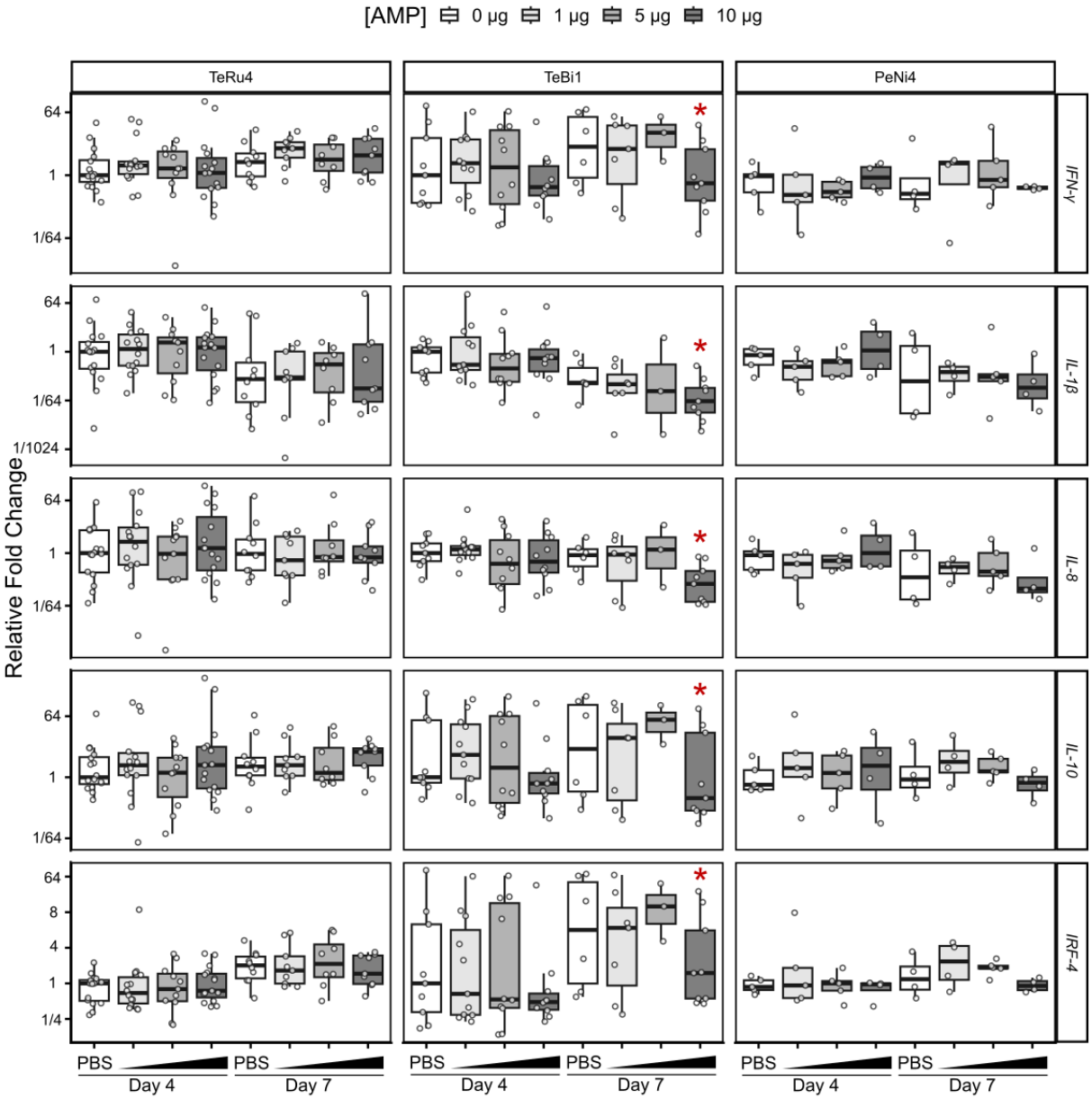

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873 **Figure 3.** Relative fold change values of cytokine transcripts in the air sac of avian pathogenic *E.*

874 *coli* (APEC) challenged birds treated before hatch with 1, 5, or 10 µg antimicrobial peptide

875 (AMP). Birds were euthanized on day 4 or 7 after hatch, and all significance comparisons are

876 relative to the phosphate-buffered saline (PBS) on their respective days. Asterisks indicate

877 significance at $p \leq 0.1$. *IFN*, interferon; *IL,* interleukin; *IRF*, interferon regulatory factor.

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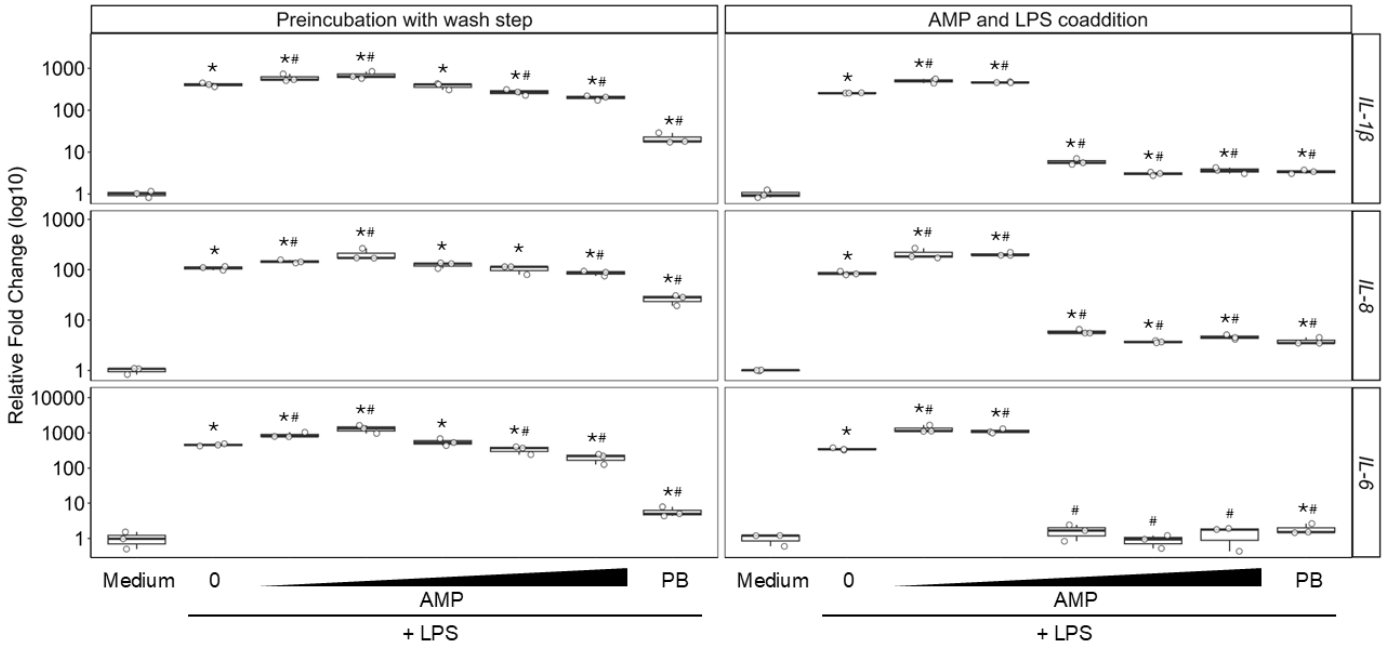

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880 **Figure 4.** Relative fold change of *Interleukin (IL)-1β*, *IL-8*, and *IL-6* from HD11 cells as
881 determined by qPCR in the "preincubation with wash step" (left column) and "AMP and LPS
882 coaddition" experiments (right column). In the preincubation with wash step experiment, cells
883 were incubated for 3 h with medium, a range of 2-fold serially diluted antimicrobial peptide
884 (AMP; 1 to 16 µg/mL TeRu4), or 8 µg/mL polymyxin B sulfate (PB), followed by three washes
885 prior to the addition of 25 ng/mL lipopolysaccharide (LPS) for an additional 3 h. In the
886 coaddition experiment, cells were incubated for 3 h with medium, or with 25 ng/mL LPS mixed
887 with a range of 2-fold serially diluted AMP (1 to 16 µg/mL TeRu4), or 8 µg/mL PB. A
888 significant difference from the medium control is indicated by "*", and "#" indicates a
889 significant difference from LPS treatment (p-value < 0.1, Mann-Whitney-U test). Open circles
890 represent the fold change of one technical replicate relative to the medium control. The
891 horizontal lines represent the median of the three technical replicates within each treatment

condition, the boxes show the interquartile range, and the vertical whiskers represent the minimum and maximum values. Shown are representative experiments of three biological replicates.

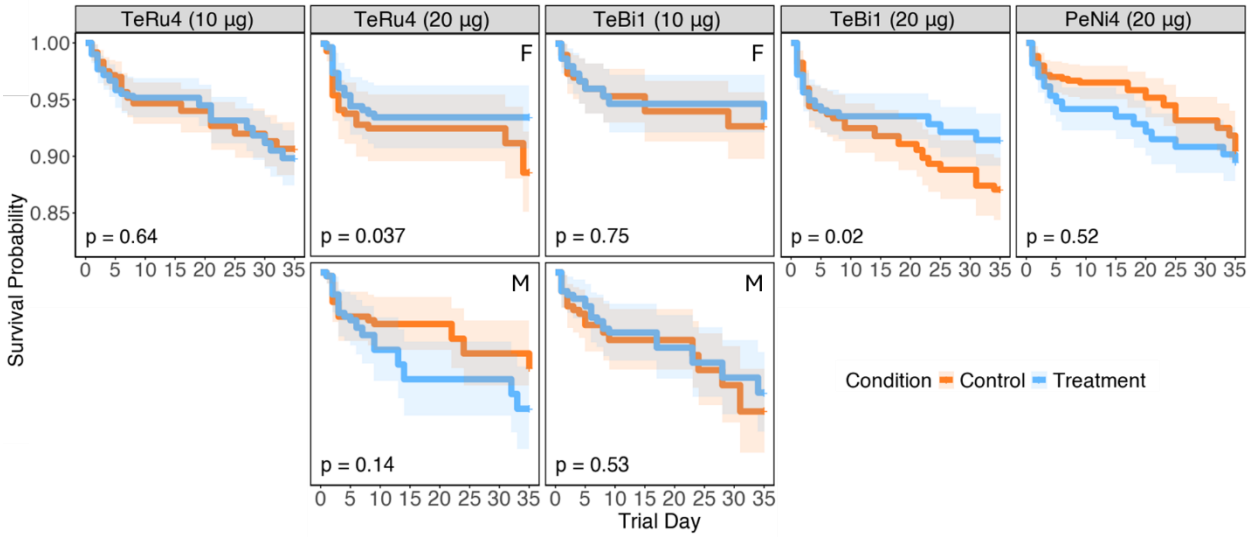

Figure 5. Kaplan-Meier curves depicting the survival probabilities of experimental groups during pen trials across different antimicrobial peptides (AMP) and dosages. Columns represent AMP at specific dosages and each AMP–dosage combination corresponds to a separate pen trial. Sex-specific differences in survival probabilities were observed in the TeRu4 (20 µg) and TeBi1 (10 µg) trials. For these trials, males and females were analyzed separately, and each panel is labeled with "F" for females or "M" for males in the top right corner to indicate the sex of the birds included in the analysis. P-values, as determined by Log-rank tests, are displayed in the bottom-left corner of each panel.

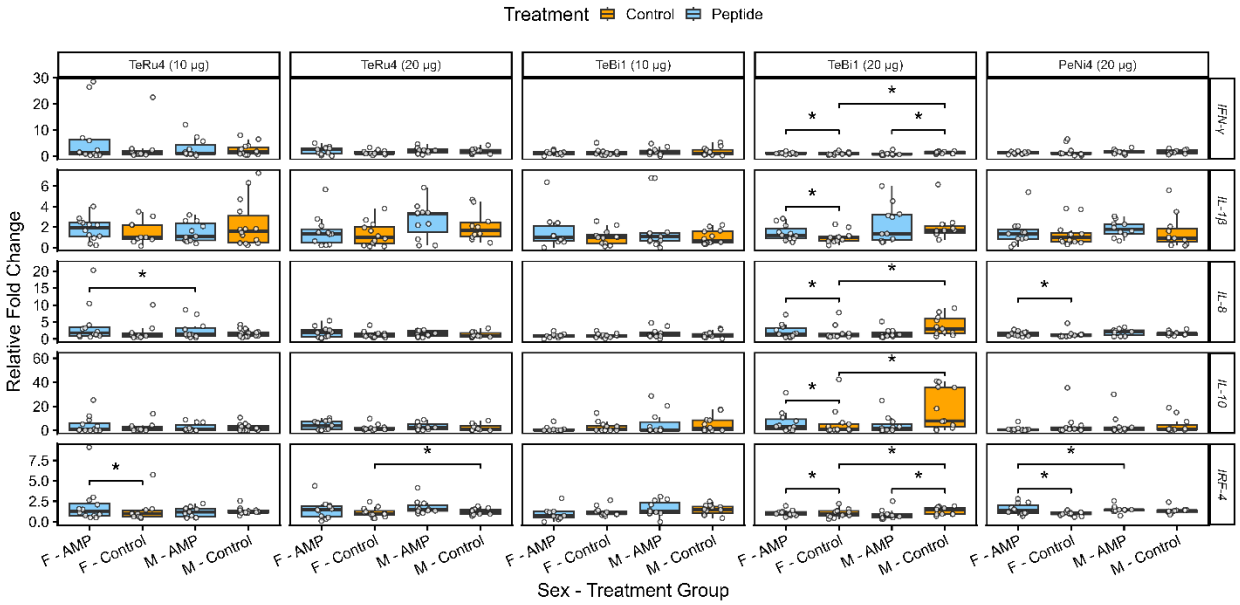

Figure 6. Relative fold change values of cytokine transcripts in the spleens of pen trial birds (no pathogen challenge) treated with 10 or 20 µg TeRu4, TeBi1, or PeNi4. Birds were euthanized 10 days after hatch. Asterisks indicate significance at $p \leq 0.1$. F, female birds; M, male birds. Additional details are available in the Figure 5 legend.