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

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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 AMPs, 62 exhibited activity against avian pathogenic *Escherichia coli* (APEC) and low in vitro hemolytic and cytotoxic activity. We selected three lead AMPs 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, AMPs 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 AMPs are safe antibiotic

62 alternatives that improve survival, modulate immune responses, and maintain normal growth
63 performance in broiler chickens.
64
65 Key words: Antimicrobial peptide, APEC, broiler chicken, efficacy, safety
66

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 new class of antibiotics, oxazolidinones, was discovered with
87 linezolid in 1987 (Durand et al., 2019). Consequently, there is growing interest in alternative
88 antimicrobial strategies, such as antimicrobial peptides (**AMP**), to address this therapeutic gap.
89 AMPs are peptide-based biomolecules composed of 5 to 100 amino acids and are
90 typically cationic (Bahar and Ren, 2013). In nature, they are produced by the innate immune
91 system and act as the first line of defense against infections in a wide variety of organisms,
92 including humans, animals, and plants (Bahar and Ren, 2013; Durand et al., 2019). Most
93 conventional antibiotics have specific cellular targets, including cellular machinery involved in
94 DNA and protein synthesis and components of bacterial cell walls (Bahar and Ren, 2013). In
95 contrast, AMPs primarily cause rapid cell death by binding to anionic bacterial cell membranes
96 and disrupting their integrity, leading to cell lysis (Bahar and Ren, 2013; Durand et al., 2019).
97 AMPs typically do not target eukaryotic cells, as their membranes usually have low anionic
98 charge (Bahar and Ren, 2013). However, at higher concentrations, some AMPs may exhibit
99 cytotoxic effects on eukaryotic cells (Bahar and Ren, 2013). Therefore, rigorous safety
100 evaluation of AMPs is essential before regulatory approval, including in vitro assays, such as
101 hemolysis and cytotoxicity tests, as well as in vivo experiments.
102 AMPs exert less selective pressure on bacteria than conventional antibiotics due to their
103 nonspecific targets and diverse mechanisms of action, thus reducing the development of
104 antimicrobial resistance (Zasloff, 2002; Hancock and Sahl, 2006; Fjell et al., 2012; Richter et al.,
105 2022). In addition to their direct antimicrobial activity, some AMPs demonstrate
106 immunomodulatory properties, recruiting immune cells or regulating cytokine levels to enhance
107 the host response to infection (Rima et al., 2021; Richter et al., 2022). Cytokines are important
108 signaling molecules that facilitate the growth, differentiation, activation of immune cells (Garcia

109 et al., 2021). In the present study, the effect of AMPs on the expression of the following
110 cytokines was investigated: Interleukin (**IL**)-1β, IL-8, IL-6, IL-10, and interferon gamma (**IFN-**
111 ***γ***), as well as the transcription factor interferon regulator factor 4 (**IRF-4**). IL-1β, IL-8, and IL-6
112 are associated with activation of inflammation in chickens (Kogut, 2002; Garcia et al., 2021).
113 Furthermore, IL-8 recruits heterophils to the infection site (Kogut, 2002). Conversely, IL-10
114 plays a role in tissue repair, angiogenesis, and attenuation of the inflammatory response and is an
115 inducible feedback regulator of the immune response in chickens (Rothwell et al., 2004; Wu et
116 al., 2016; Garcia et al., 2021). The roles of IRF-4 and IFN-*γ* are not well-characterized in
117 chickens; however, there is evidence that IRF-4 plays a critical role in B cell development and
118 maturation, similar to its mammalian counterpart (Yu et al., 2021), while IFN-*γ* generally
119 functions to bridge the adaptive and innate immune systems (Yuk et al., 2016; Santhakumar et
120 al., 2017).
121 Previously, we screened 875 AMP candidates, identified using our artificial intelligence
122 (**AI**) based prediction tools: AMPlify (Li et al., 2022), rAMPage (Lin et al., 2022), and AMPd-
123 Up (Li et al., 2024), and used in vitro assays to characterize their antibacterial, hemolytic, and
124 cytotoxic properties. In this study, we selected three lead AMPs (named TeBi1, TeRu4, and
125 PeNi4) for further testing, due to their high antimicrobial activity and low toxicity. The three
126 AMPs were tested in vivo as a prophylactic treatment prior to APEC inoculation (challenge
127 trials). Immunomodulatory effects of these AMPs were tested in tissue harvested from animals
128 involved in the challenge trials and in HD11 cells, a chicken macrophage-like cell line. Lastly,
129 the AMPs were tested for their safety in a commercial production setting (pen trials). Overall,
130 TeRu4 demonstrated activity in HD11 cell culture, while both TeBi1 and TeRu4 showed efficacy

131 in challenge trials and maintaining safety in commercial production settings, supporting their
132 potential for future translational studies.
133

134 **MATERIALS AND METHODS**

135 ***In Silico Peptide Selection***

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

141 ***Peptide Synthesis***

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

146 ***Bacterial Species and Growth.***

147 APEC strain EC317 (Allan et al., 1993) (clinical turkey isolate, obtained from VIDO-
148 InterVac, Saskatchewan, CA), was used as the bacterial target for AMPs. Glycerol stocks were
149 stored at -80°C. The primary streaks were done by plating the stocks on 5% Columbia blood agar
150 (Thermo Fisher Scientific, Waltham, MA, USA) and incubating for 20-24 hours at 37°C under
151 aerobic conditions, as described by Richter et al. (Richter et al., 2022). The secondary streaks
152 were done by transferring 2-3 colonies from the primary streak on a new 5% Columbia blood
153 agar plate. Colonies from the secondary streak were used for downstream analysis. Bacterial

154 growth medium was prepared by dissolving 10.5 g Mueller Hinton Broth (Sigma-Aldrich, St.
155 Louis. MO, USA) powder in 500 ml distilled water and autoclaving for sterility.

156 ***In Vitro Lead Peptide Selection***

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

166 ***Care and Use of Animals***

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

170 ***APEC Challenge Trials***

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

176 recorded for each treatment group. One day after hatch, the birds were challenged with 5×10^4
177 cfu EC317.

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

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

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

196 ***Immunomodulatory Activity Assays with HD11 Cells***

197 The effects of AMPs on in vitro cytokine production were assessed using an HD11 chicken
198 semi-adherent macrophage-like cell line (Obtained from VIDO-InterVac). HD11 cells were

199 cultured in RPMI medium with 1X Glutamax and 10% Fetal Bovine Serum (Thermo Fisher
200 Scientific) at 41°C (normal chicken body temperature) and 5% CO_2 and were routinely passaged
201 every two days.

202 ***Cell Plating.*** HD11 cells were plated for experimentation between passages three to twelve.
203 Suspended cells were collected with the growth medium, and adherent cells were obtained after
204 washing with PBS and trypsinization with 0.25% Trypsin-EDTA in PBS (both from Thermo
205 Fisher Scientific). The cells were centrifuged at 200 g for seven minutes and the pellet was
206 resuspended in fresh medium. Trypan Blue (Thermo Fisher Scientific) was added to a sample of
207 cells and live cells were counted on a Hausser Scientific Bright-Line Phase Hemacytometer
208 (Fisher Scientific, Hampton, NH, USA). HD11 cells were plated at 2.5×10^4 cells per well on
209 Nunclon Delta 96-well plates (Thermo Fisher Scientific) and incubated overnight.

210 ***HD11 Experiment Overview.*** In all experiments, HD11 growth medium was used to resuspend
211 and dilute AMPs and other reagents, and all incubations were carried out at 41°C and 5% CO_2.
212 All experiments included a medium-only control and a lipopolysaccharide (**LPS**)-only control
213 (final concentration of 25 ng/mL). In all experiments other than the AMP-only Screen, 8 µg/mL
214 Polymyxin B sulfate (**PB**) was used as a positive control as it is known to attenuate the LPS-
215 triggered inflammatory response (Schromm et al., 2021; Yang et al., 2024). Prior to treatment,
216 the supernatant was removed from the wells, therefore only the adherent cell monolayer was
217 used during the experiments.

218 Four experimental approaches were devised to investigate the immunomodulatory activity
219 of AMPs. First, HD11 cells were treated solely with AMPs (AMP-Only Screen). Second, cells
220 were treated with a range of AMP concentrations followed by treatment with LPS from *E. coli*
221 O111:B4 (Sigma-Aldrich), to test if the AMPs could modulate the immune response triggered by

222 LPS (AMP Preincubation Screen). If an AMP showed immunomodulatory activity in the AMP
223 Preincubation Screen, it was then tested in the AMP Preincubation with Wash Step and the AMP
224 and LPS Coaddition experiments.
225 ***AMP-Only Screen.*** HD11 cells were treated with 100 µL 16 µg/mL TeRu4, TeBi1, or PeNi4,
226 and incubated for 6 h.
227 ***AMP Preincubation Screen.*** HD11 cells were treated with 100 µL of 1, 2, 4, 8, or 16 µg/mL
228 TeRu4, TeBi1, or PeNi4, and incubated for 3 h. After the incubation, 100 µL LPS (50 ng/mL)
229 was administered to the cells, resulting in a final concentration of 25 ng/mL LPS. The cells were
230 incubated for an additional 3 h.
231 ***AMP Preincubation with Wash Step.*** The cells were treated identically to the AMP
232 Preincubation Screen but with the addition of a wash step prior to the addition of LPS. Briefly,
233 after the first 3 h incubation, the medium (containing the AMP or PB) was removed from the
234 wells and the cell monolayer was washed three times using fresh medium. One hundred µL LPS
235 (25 ng/mL) was administered, and the cells were incubated for an additional 3 h.
236 ***AMP and LPS Coaddition.*** AMP was resuspended and diluted to prepare a 2X concentration
237 curve of 32, 16, 8, 4, and 2 µg/mL. PB and LPS were prepared at 2X concentrations of 16 µg/mL
238 and 50 ng/mL, respectively. At regular time intervals, 2X AMP or 2X PB was added in equal
239 volume to 2X LPS to yield a 1X mixture of AMP or PB, and LPS. Mixtures were vortexed for
240 one min and incubated for 30 min prior to being administered to cells at regular time intervals in
241 the order in which they were made. The cells were incubated for 3 h.
242 ***Cell Harvest and RNA Isolation.*** Supernatant was removed from HD11 cells after the final
243 incubation. Buffer RLT from the miRNeasy Micro Kit (Qiagen, Mississauga, ON, Canada) with
244 2M DL-dithiothreitol (Sigma-Aldrich) was added to lyse adherent cells at a volume of 130 µL

245 per well, with each technical replicate comprising two wells in the 96-well plate. HD11 cells in
246 lysis buffer were disrupted by pipetting up and down, vortexed, and stored at -80 °C until RNA
247 isolation. In total, three technical replicates per treatment were collected for RNA isolation and
248 qPCR analysis.

249 ***Commercial Pen Trials***

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

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

266 The birds were then transferred to SJ Ritchie Research Farms (Abbotsford, BC, Canada)
267 where they were cloacally sexed by professionals and then separated by sex and treatment. A

268 total of 24 mini pens, within six blocks, were set up. The birds were weighed individually, then
269 50 birds were placed into each mini pen according to their sex and treatment, with each block
270 containing each of the four experimental groups in a random order. Birds were provided ad
271 libitum access to water and an all-vegetable, raised without antibiotic (**RWA**) feed (Trouw
272 Nutrition, Chilliwack, BC, Canada). A step-down lighting program was implemented: from day
273 0 to day 21, birds experienced 6 h darkness per 24 h cycle; from day 21 to 28, 4 h darkness; and
274 from day 28 to the end of the trial, 1 h darkness per day. Number of birds in each mini-pen was
275 reduced to 12 on day 10 to prevent overcrowding of the mini-pens. Birds were checked three
276 times per day throughout the trial and were weighed individually on days 10, 21, and 35. Any
277 mortality during the pen trial was noted.

278 ***qPCR Analyses***

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

284 RNA was extracted from tissues with the miRNeasy Tissue/Cells Advanced Mini Kit and
285 Qiavac vacuum manifold (Qiagen). Tissue samples were homogenized in 450 µL buffer RLT
286 reduced with 2M DTT, loaded on the RNeasy mini spin column, washed on the Qiavac vacuum
287 manifold, and eluted in 50 µL RNAse-free water. RNA was extracted from HD11 cells using the
288 using the QIAGEN miRNeasy Tissue/Cells Advanced Micro Kit, per the manufacturer's
289 instructions, except for the final elution, where 11.2 µL RNase-free water was added to the
290 column, centrifuged, and the eluate was added back to the column and centrifuged again.

291 The concentration of RNA from tissue samples was measured by NanoDrop (NanoDrop
292 1000 3.8.1; Thermos Fisher Scientific) and diluted to a concentration of approximately 100
293 ng/µL before complementary DNA (**cDNA**) synthesis, while RNA from HD11 cells was used
294 neat due to its low concentration. All RNA samples were converted to cDNA using the Applied
295 Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), per the
296 manufacturer's instructions.

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

310 ***Statistical Analyses***

311 Statistical analyses of the qPCR results were conducted in R version 3.6.3 (R Core Team,
312 2024). The data were tested for normality by the Shapiro–Wilk and Levene's tests. They were
313 then subjected to pairwise analyses by the Kruskal-Wallis and Wilcoxon rank-sum tests for non-

314 parametric data with a significance threshold of $\alpha = 0.1$. For the challenge and pen trials, this
315 significance cut off was selected due to the high variability of in vivo data. For the HD11
316 experiments, this threshold was appropriate as the smallest p-value achievable using a rank-sum
317 test with $n = 3$ was approximately 0.08.

318 The rest of the challenge trial data was analyzed in R version 4.4.0 and 4.5.1(R Core Team,
319 2024). Hatchability rates of treated eggs compared to control eggs, as well as rates of bacterial
320 detection in the birds' air sacs and pericardia, were evaluated using the Fischer's exact test. The
321 presence or absence of bacteria detected in the birds' air sacs and pericardia was also compared
322 to AMP dose using a Cochrane-Armitage trend analysis on both days 4 and 7. Cumulative
323 weight gain of birds euthanized on days four and seven was evaluated using a Wilcoxon rank-
324 sum test, and survival was graphed and evaluated using the log-rank test and the "survminer"
325 package (Kassambara et al., 2016). Other challenge trial graphs were constructed using the
326 packages "tidyverse," "readr," "janitor," "superb," "ggsignif," "rstatix,", "DescTools", and
327 "ggpubr" (Signorell, 2014; Wickham et al., 2015b; a, 2019; Kassambara, 2016, 2019; Firke,
328 2016; Ahlmann-Eltze and Patil, 2017; Cousineau, 2021).

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

334 Statistical analyses of hatchability, survival, mean weights, feed conversion ratio, and flock
335 uniformity in pen trials were conducted using R (version 4.1.3) and the packages: "survival",
336 "survminer", "openxlsx", "readxl", "tidyverse, "zoo", "car", "ggplot2", "ggsignif", and

337 "MHTdiscrete" (Fox et al., 2001; Therneau, 2001; Zeileis et al., 2004; Wickham et al., 2007,
338 2019; Schauberger and Walker, 2014; Wickham and Bryan, 2015; Kassambara et al., 2016;
339 Yalin Zhu, Wenge Guo, 2016; Ahlmann-Eltze and Patil, 2017). For pen trials, statistical
340 comparisons between treatment and control groups used Wilcoxon rank-sum tests (hatchability
341 and production parameters) and log-rank tests (survival), with production and survival analyzed
342 separately by sex. P-values were corrected for multiple comparisons using the Šidák method.
343

344 **RESULTS**

345 ***In Vitro Peptide Selection***

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

356 ***qPCR Primer Design and Validation***

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

360 ***APEC Challenge Trials***

361 To assess the prophylactic efficacy of TeBi1, TeRu4, and PeNi4 in chickens, fertilized
362 eggs were injected in ovo at day 18 of incubation with either 1, 5, 10, or 20 μg AMP, or a PBS
363 control. One day after hatch, the birds were challenged with 5×10^4 cfu APEC strain EC317 by
364 yolk sac injection.

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

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

380 On day 7, birds treated with 10 μg TeBi1 had significantly lower rates of infection in both
381 the air sac ($p = 0.01$) (Figure 1b) and pericardium ($p = 0.03$) (Figure 1c). Increased peptide dose
382 was also generally correlated with decreased numbers of birds with detectable bacteria in either

383 the air sac or the pericardium ($p = 0.02$ and $p = 0.008$, respectively, also on day 7). There were
384 no significant differences in bacterial detection rates in the air sacs and pericardia of birds treated
385 with TeRu4 or PeNi4 (Supplementary Figure S4), but for TeRu4-treated birds on day 4, there
386 was a slight but similar trend of decreased numbers of infected birds with increasing AMP dose
387 (air sac: $p = 0.009$, pericardium: $p = 0.1$), and for PeNi4, on day 4, in the air sac only, there was a
388 slight trend of increasing numbers of infected birds with increased AMP dose ($p = 0.1$).

389 Increased bacterial load in the air sac was correlated with increased levels of transcript
390 abundance of certain cytokines in the air sac (Figure 2). Across all three challenge trials, birds
391 with light, moderate or heavy levels of active APEC infection in their air sacs had significantly
392 higher relative abundance of *IL-1β* transcripts ($p = 2 \times 10^{-4}$, 1×10^{-6}, and 7×10^{-13}, respectively)
393 than birds with no bacterial growth in their air sacs (Figure 2a). They are also positively
394 correlated by Spearman's rho, with $\rho = 0.58$ and $p = 1 \times 10^{-19}$. The same trend, though weaker, is
395 observed with *IL-8*. While $\rho = 0.32$ ($p = 3 \times 10^{-6}$) overall, birds with light, moderate, or heavy
396 levels of APEC infection also had significantly higher relative abundance of *IL-8* transcripts ($p =$
397 0.03, 3×10^{-6}, and 5×10^{-6}, respectively) than birds with no bacterial growth in their air sacs
398 (Figure 2b).

399 In the cecal tonsil (Supplementary Figure S5), qPCR results show minimal changes due to
400 treatment with PeNi4, with only a 2-fold increase in the inflammatory cytokine *IL-8* in the 10 μg
401 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
402 $= 0.1$). In contrast, there are numerous changes due to treatment with TeBi1, including a 2.4-fold
403 increase in *IL-8* transcripts in the 5 μg group on day 4 ($p = 0.1$), and a 5-fold decrease in *IL-8*
404 transcripts in the 10 μg group on day 7 ($p = 0.06$). *IFN-γ* expression increased 1.4-fold ($p = 0.03$)
405 and 2.5-fold ($p = 0.03$) in the 5 and 10 μg groups, respectively, on day 4, but decreased 1.4-fold

406 (p = 0.03) and 1.1-fold (p = 0.09) in the 1 and 10 µg groups, respectively, on day 7. With respect
407 to *IL-1β,* on day 7, the TeBi1 5 µg group showed a 5-fold decrease (p = 0.07). For the anti-
408 inflammatory transcript *IL-10,* there is a 2.5-fold decrease (p = 0.07) with 5 µg TeBi1 on day 7.
409 Finally, TeBi1 treatment was also associated with changes in *IRF-4* transcripts: the 10 µg group
410 was associated with a 1.7-fold increase on day 4 (p = 0.04), and the 5 µg group was associated
411 with a 1.5-fold increase on day 7 (p = 0.1).

412 In the spleen (Supplementary Figure S6), fewer responses are observed. For TeRu4, there
413 is a 2-fold decrease in *IL-8* transcripts on day 7 (p = 0.1) after treatment with 1 µg. For PeNi4,
414 there is a 5-fold increase in *IL-10* transcripts on day 7 (p = 0.09) after treatment with 5 µg. For
415 TeBi1, while there are no differences in the inflammatory cytokines *IFN-γ, IL1β,* or *IL-8*, there is
416 a 3-fold increase in *IL-10* transcripts on day 4 (p = 0.03) after treatment with 10 µg. There are
417 also multiple increases in *IRF-4* transcripts: on day 4, a 2-fold increase after treatment with 1 µg
418 (p = 0.1), a 3-fold increase after treatment with 5 µg (p = 0.1); and on day 7, a 1.3-fold decrease
419 after treatment with 1 µg (p = 0.1).

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

425 ***HD11 Experiments***

426 In addition to challenge trials, the ability of TeRu4, TeBi1, and PeNi4 to influence the cellular
427 immune response was tested in an HD11 cell model system. In all HD11 experiments (n=21), the
428 LPS induction control resulted in a wide-ranging, but consistent increase in relative fold change

429 compared to the medium control for *IL-1β* (14 - 677), *IL-8* (8 - 249), and *IL-6* (55 - 3,284)
430 (Figure 4 and Supplementary Figures S7 and S8). With the exception of the AMP-only
431 experiment, all stated fold-changes are relative to the LPS induction control. The induction of
432 cytokine transcripts by LPS was significantly attenuated by PB in all but a single cytokine
433 measurement from one experiment (n=15), with fold change decreases compared to LPS alone
434 ranging from 6-166 for *IL-1β*, 2-34 for *IL-8*, and 11-335 for *IL-6* (Figure 4 and Supplementary
435 Figure S8). The preincubation, preincubation with wash step, and coaddition experiments were
436 performed in biological triplicate, and the AMP-only experiments in biological duplicate. While
437 only one representative biological replicate is shown per experiment, these selected replicates
438 capture the overall trends in fold changes observed across replicates (unless otherwise stated).
439 The fold change values stated are that of the representative replicate. ***AMP-Only Screen of***
440 ***TeRu4, TeBi1, and PeNi4.*** To assess the ability of AMPs alone to induce cytokine production in
441 HD11 cells, cells were incubated with 16 µg/mL of each AMP or 8 µg/mL PB and cytokine
442 levels were compared to a medium-only control. Treatment with TeRu4 resulted in a 2-fold
443 increase in *IL-1β* (in one of two biological replicates) and 3-fold increase in *IL-8,* while TeBi1
444 lead to a 4-fold decrease in *IL-6* (Supplementary Figure S7). Treatment with PeNi4 did not
445 significantly alter the abundance of any of the three cytokines, while TeBi1 had no significant
446 effect on *IL-1β* and *IL-8,* and TeRu4 had no significant effect on *IL-6* (Supplementary Figure
447 S7). Treatment with PB alone did not significantly or consistently change *IL-1β, IL-8*, and *IL-6*
448 levels (Supplementary Figure S7).
449 ***Preincubation Screen of TeRu4, TeBi1, and PeNi4.*** To examine if AMP affect HD11 response
450 to LPS, cells were preincubated with AMP or PB (positive control), followed by treatment with
451 LPS. Here and in subsequent experiments, medium only and LPS only served as negative and

452 positive controls, respectively. A 2-fold decrease was observed for *IL-1β* and *IL-6* following
453 preincubation with 8 µg/mL TeRu4 (Supplementary Figure S8). Preincubation with 16 µg/mL
454 TeRu4 led to a significant reduction in *IL-1β, IL-8*, and *IL-6* levels by 13-fold, 4-fold, and 28-
455 fold, respectively, (Supplementary Figure S8). Similarly, treatment with 8 µg/mL PB
456 significantly decreased *IL-1β*, *IL-8* (in 2 of 3 biological replicates), and *IL-6* abundance by 10-
457 fold, 3-fold, and 20-fold, respectively (Supplementary Figure S8). While a significant 4-fold
458 increase was observed for *IL-8* at 4 µg/mL TeRu4, this observation was only seen in two of three
459 biological replicates (Supplementary Figure S8). Preincubation with TeBi1 and PeNi4 did not
460 consistently induce a significant change in *IL-1β, IL-6*, and *IL-8* transcript levels across
461 biological replicates (Supplementary Figure S8). The attenuation of LPS-induced responses of
462 *IL-1β, IL-8*, and *IL-6* transcripts after TeRu4 preincubation prompted further investigation via the
463 subsequent experiments to provide some insight into possible mechanisms of action.
464 ***Preincubation Experiment with Wash Step.*** The effects on HD11 cells of transient exposure to
465 TeRu4, followed by its removal and the addition of LPS, was examined. A significant decrease
466 in *IL-1β, IL-8,* and *IL-6* transcripts remained after preincubation with 8 µg/mL PB (21-, 5-, and
467 80-fold respectively) and 16 µg/mL TeRu4 (2-, 1-, and 2-fold, respectively) (Figure 4).
468 Preincubation with 8 µg/mL TeRu4 induced a significant decrease in *IL-1β* levels by 2-fold and
469 *IL-6* by 1-fold in two of three biological replicates (Figure 4). Unlike in the previous
470 preincubation experiment (without a wash step), treatment with 1 and 2 µg/mL TeRu4 followed
471 by the wash step resulted in a 1- and 2-fold increase respectively in *IL-1β* and *IL-8* transcript
472 levels(but not consistently in all biological replicates) (Figure 4). For *IL-6* in this experiment,
473 treatment with 1 and 2 µg/mL TeRu4 resulted in a2-and 3-fold increase respectively across all
474 three biological replicates (Figure 4).

475 ***Coincubation of TeRu4 and LPS.*** The impact of co-incubating TeRu4 and LPS prior to treating
476 HD11 cells on cytokine transcript expression was assessed. Coaddition of LPS with either PB or
477 TeRu4 (at concentrations ranging from 4 to 16 µg/mL) resulted in significant decreases in all
478 cytokine transcripts across all three biological replicates (Figure 10). Specifically, co-treatment
479 with PB and LPS led to reductions of 75-fold for *IL-1β*, 24-fold for *IL-8*, and 226-fold for *IL-6*
480 (Figure 4). Similarly, co-treatment with 16 µg/mL TeRu4 and LPS decreased *IL-1β* abundance
481 by 70-fold, 18-fold for *IL-8*, and 191-fold for *IL-6* (Figure 4). LPS and 8 µg/mL TeRu4 reduced
482 *IL-1β* transcripts by 83-fold, *IL-8* by 23-fold, and *IL-6* by 363-fold (Figure 4). LPS and 4 µg/mL
483 TeRu4 resulted in reductions of 46-fold for *IL-1β*, 15-fold for *IL-8*, and 205-fold for *IL-6* (Figure
484 4). For *IL-6*, 4-16 µg/mL TeRu4 repressed the LPS induction so much that expression levels
485 were not significantly different from the medium only negative control (observed in two of three
486 replicates at 4 µg/mL) (Figure 4). Like what was observed in the previous experiment, 1 and 2
487 µg/mL of TeRu4 resulted in significantly increased transcript abundance in *IL-1β* (2-fold), *IL-8*
488 (2-fold), and *IL-6* (3-fold) relative to the LPS control, but unlike the previous experiment, these
489 results were consistent across three biological replicates (Figure 4).

490 ***Commercial Pen Trials***

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

497 For the first 10 days, there were no statistical differences between the survival probability
498 of males and females (Supplementary Figure S10). Thus, the sexes were combined for
499 subsequent analyses. No significant differences in survival probability were detected between
500 treatment and control groups for any AMPs during the first 10 days of the pen trials, indicating
501 that the tested AMPs had no measurable impact on ECM (Supplementary Figure S11). However,
502 for the full duration of the pen trials (35 days), sex-specific differences in survival probabilities
503 were observed for TeRu4 at 20 µg and TeBi1 at 10 µg (Supplementary Figure S12). For these
504 two pen trials, the sexes were kept separate for analysis (Figure 5, Supplementary Figure S13).
505 Notably, TeRu4 at 20 µg significantly increased the survival probability of female birds
506 compared to the controls by approximately 4.9 % by day 35 ($p = 0.04$). Moreover, TeBi1 at 20
507 µg significantly increased the survival probability of the birds compared to the control by
508 approximately 4.4% by day 35 ($p = 0.02$). There was no other difference in survival probabilities
509 between the treatment and control groups by day 35. In the 10 µg TeRu4 and TeBi1 trials,
510 survival curves for both sexes closely overlapped (Supplementary Figure S13).
511 Significant differences in mean weight between treatment and control groups were
512 observed only in the 20 µg TeBi1 trial (Supplementary Figure S14). Notably, on day 0, mean
513 weights in the treatment groups were approximately 2.8% lower in males and 2.4% lower in
514 females compared to controls ($p = 0.02$ and 0.03). However, on day 10, no significant difference
515 in mean weights were observed. Moreover, in the 10 µg TeRu4 trial, numerous birds of both
516 sexes exhibited lower body weights on days 21 and 35, particularly in the treatment group,
517 although this difference was not statistically significant. This observation may suggest
518 subclinical effects of TeRu4 at lower doses, high biological variability among individual birds,

519 or environmental effects, all of which warrant further investigation. By day 35, no significant
520 differences in mean weight were detected (Supplementary Figure S14).

521 No statistically significant differences were detected between FCR of the treatment and
522 control groups on day 35, suggesting that the tested AMPs did not exhibit measurable growth-
523 promoting properties (Supplementary Figure S15). Of interest, there were occasions where FCR
524 values were below 1 (Supplementary Figure S15). One was observed between days 0 and 10, in
525 the 10 µg TeBi1 trial (FCR = 0.997). Further, in the 20 µg TeRu4 trial, two FCR values were
526 below 1 (FCR = 0.923 and 0.961) in the second interval, between days 11 and 21. Overall, the
527 mean FCR values were consistently the lowest in the first interval, and the highest in the last
528 interval, between days 21 and 35, for both sexes. These findings suggest that AMPs did not
529 significantly alter FCR under the tested conditions, and observations during the first interval of
530 certain pen trials warrant further investigation.

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

539 The qPCR results were analyzed with sexes separated, and significant differences are
540 reported for both comparisons between birds in the same sex group that received different
541 treatments, and for birds in the same treatment group with differences between sexes. As there

542 were some significant differences in transcript abundance between male and female birds in the
543 same treatment groups, the results are shown with sexes separated in Figure 6.
544 After treatment with 10 μg of TeRu4, female birds had a 1.8-fold increase (p = 0.1) in *IFN-*
545 *γ* transcripts relative to (female) control birds, and in that trial, male controls birds had a 1.2-fold
546 increase (p = 0.1) in *IRF-4* transcripts relative to female control birds. After treatment with 20 μg
547 of TeRu4, male birds had a 1.3-fold increase (p = 0.08) in *IRF-4* transcripts relative to control
548 birds.
549 After treatment with 20 μg PeNi4, female birds had a 1.4-fold increase (p = 0.02) in *IRF-4*
550 transcripts relative to control birds. Additionally, in that trial, male control birds had a 1.3-fold
551 increase (p = 0.01) in *IRF-4* transcripts and a 1.6-fold increase (p = 0.04) in *IL-8* transcripts
552 relative to female control birds.
553 When considering the sexes separately, there were no significant differences in transcript
554 abundance in birds treated with 10 μg TeBi1 (Figure 6). As there were no significant differences
555 between sexes in this trial, qPCR results from that trial were also combined (Supplementary
556 Figure S17). Here, a 1.2-fold increase (p = 0.1) in *IL-1β* transcripts was observed. Examination
557 of the transcript abundances in birds treated with 20 μg TeBi1 revealed numerous differences
558 relative to PBS controls.
559 After treatment with 20 μg TeBi1, male birds had a 2-fold decrease in *IFN-γ* and *IRF-4*
560 transcripts (p = 0.02 for both), and a 3-fold decrease in *IL-10* and *IL-8* transcripts (p = 0.03 and
561 0.02, respectively) relative to control birds. In that trial, male control birds had higher abundance
562 of all tested transcripts than female control birds: 2-fold more *IFN-γ* transcripts (p = 0.1), 3-fold
563 more *IL-10* transcripts (p = 0.06), 1.7-fold more *IL-1β* transcripts (p = 0.008), 3-fold more *IL-8*
564 transcripts (p = 0.01), and 2-fold more *IRF-4* transcripts (p = 0.1). Additionally, male birds

565 treated with 20 μg TeBi1 had significantly lower abundance of certain transcripts than female
566 birds in the same treatment group, a 1.3-fold decrease in both *IFN-γ* and *IRF-4* transcripts ($p =$
567 0.09 for both).
568

569 **DISCUSSION**

570 This study demonstrates that antimicrobial peptides can improve early health outcomes in
571 broiler chickens while maintaining production performance under commercial conditions. Across
572 complementary in vivo and in vitro assays, two of the three lead AMPs, TeBi1 and TeRu4,
573 showed efficacy and immunomodulatory activity consistent with their intended roles as
574 antibiotic alternatives for preventing APEC-associated diseases. Here, AI-assisted discovery is
575 relevant for enabling large-scale candidate generation and triage that would be impractical by
576 hand. All outcome data from the in vivo and in vitro assays were analyzed using nonparametric
577 methods, Kruskal–Wallis for omnibus comparisons and Wilcoxon rank-sum for pairwise
578 contrasts. Given the inherent variability of in vivo data and the exploratory nature of this study, a
579 significance threshold of $\alpha = 0.1$ was used, as specified in the methods.

580 First, in controlled challenge trials, pre-hatch administration of TeBi1 was associated
581 with reduced post-hatch bacterial burden and improved early growth. At 10 μg per egg, TeBi1
582 significantly lowered culture detection of APEC in the air sac and pericardium by day 7 and
583 yielded an approximate 50% increase in body weight relative to controls at the same time point.
584 These findings support a direct antibacterial contribution in vivo and align with TeBi1's
585 favorable in vitro profile (Lin et al., 2022). Concordant decreases in pro-inflammatory cytokine
586 transcripts in air sac tissue at day 7 (Figure 3) further suggest that limiting bacterial load
587 mitigates downstream inflammatory responses. In contrast, TeRu4 and PeNi4 did not

588 reproducibly reduce organ-specific bacterial detection in the challenge setting at tested doses up
589 to 10 μg, indicating peptide-specific differences in in vivo antibacterial activity despite their
590 performance in in vitro experiments. Consistent with this, positive correlations between air sac
591 bacterial loads and *IL-1β* ($\rho = 0.58$) and *IL-8* ($\rho = 0.32$) expression (Figure 2) both support the
592 validity of the infection model and indicate that AMP-mediated reductions in bacterial burden
593 translate to dampened inflammatory signaling.

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

605 Third, pen trials provide critical evidence of field-relevant safety and performance. In ovo
606 administration of TeBi1 or TeRu4 at 10 or 20 μg per egg did not adversely affect hatchability,
607 FCR, or flock uniformity through day 35. Importantly, survival probabilities improved modestly
608 but significantly in two settings: a 4.9% increase in females with 20 μg TeRu4 and a 4.4%
609 increase in all birds with 20 μg TeBi1 by day 35. Although effect sizes were small, they are
610 operationally meaningful at commercial scale and were achieved without detectable adverse

611 effects in growth or efficiency. These findings support a favorable benefit-risk profile for both
612 peptides when delivered through standard in ovo automation.

613 Together, the in vivo and in vitro data outline complementary roles for these leads. TeBi1
614 appears to act primarily through direct antibacterial activity in the immediate post-hatch period,
615 reducing tissue colonization and the associated inflammatory response. TeRu4 shows consistent
616 anti-inflammatory activity in a macrophage model and over 35 days translates to a survival
617 advantage in females at higher dose, suggesting that immunomodulation can contribute to
618 resilience under farm conditions. These sex-specific responses may reflect differences in immune
619 system maturation rates or hormone-immune interactions, as male broilers typically show
620 different growth and immune development patterns than females (Leitner et al., 1989; Younis et
621 al., 2023). The patterns observed in both survival and cytokine transcript baselines warrant
622 additional investigation into sex-dependent pharmacodynamics (**PD**), immune set-points, and
623 potential interactions with breeder age and genetics.

624 While the results presented here are encouraging, this study also has several limitations.
625 First, the challenge trials used a single APEC strain and a prophylactic in ovo regimen. Broader
626 pathogen panels, additional exposure routes, and therapeutic or peri-hatch dosing schedules
627 should be evaluated to define the generalizability and optimal use cases. Second, although this
628 study shows safety and performance neutrality in pens, pharmacokinetics (**PK**), peptide stability
629 in the embryonic and early post-hatch milieu, and residue depletion profiles remain to be
630 established. Third, gene expression endpoints were measured at discrete time points and in a
631 single cell line or selected tissues. High-resolution temporal profiling, including protein-level
632 cytokine measurements and cellular phenotyping in multiple cell lines and tissues, would
633 sharpen mechanistic inference and help explain biphasic responses at lower TeRu4

concentrations. Further, some FCR values below 1.0 in early trial periods likely reflect measurement artifacts related to initial hydration status rather than true feed conversion, highlighting the importance of standardized weighing protocols in early post-hatch periods. Finally, while the analyses used conservative non-parametric tests and multiple-comparison corrections, some findings were near the chosen α threshold of 0.1, reflecting expected variability in vivo and underscoring the need for replication across flocks and sites.

The results have practical implications for poultry health programs. The in ovo delivery method offers multiple advantages: it integrates cleanly into existing hatchery workflows, avoids post-hatch handling, and ensures uniform exposure across flocks. This delivery approach, combined with a demonstrated safety profile, minimizes barriers to commercial adoption. From an economic perspective, even modest survival improvements translate to substantial numbers at commercial scale, while early bacterial control reduces the need for subsequent therapeutic interventions. The complementary mechanisms of TeBi1 (direct antibacterial) and TeRu4 (immunomodulatory) suggest potential for combination strategies that leverage both activities. Future work should benchmark these AMPs against current animal husbandry standards and evaluate alternative delivery formats, such as water supplementation during high-risk periods, to optimize protection throughout the production cycle.

In conclusion, AI-discovered AMPs, TeBi1 and TeRu4 show complementary efficacy and safety signals in broilers when administered in ovo. TeBi1 reduces early APEC burden and inflammation and improves early growth, whereas TeRu4 attenuates HD11 inflammatory responses and improves survival under commercial conditions without compromising performance. These findings support advancement to dose-optimization, PK and residue studies,

expanded pathogen panels, and multi-site validation, with an eye toward integrated antimicrobial stewardship in poultry production.

DECLARATION OF AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT and Gemini to improve grammar, readability, and language. After using this tool/service, the author(s) reviewed and edited the content as needed and takes full responsibility for the content of the publication.

DECLARATION OF COMPETING INTEREST

Birol I. is a founder, Chief Scientific Officer, and equity holder of Amphoraxe Life Sciences Inc., which has licensed intellectual property related to antimicrobial peptides described in this manuscript. The following authors are named inventors on related intellectual property: Helbing C.C., Fraser E., Hof F., Corrie L., Warren R.L., Thompson V.C., Yanai A., Birol I., and Hoang L.M.N. Kotkoff M. is also a stakeholder on the IP. Demirsoy E., Parkin T.I., and Dema A.H. 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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843 **TABLES**

844 **Table 1.** Physiochemical properties and in vitro activities of three lead antimicrobial peptides
845 (AMP) TeRu4, TeBi1, and PeNi4. Listed for each AMP are their sequence length, net charge,
846 and amino acid sequence. Each AMP was synthesized as the C-terminal carboxylic acid. In vitro
847 activities include the antimicrobial activity against avian pathogenic *E. coli* (APEC) EC317
848 (minimum inhibitory concentration, MIC), the hemolytic activity against porcine red blood cells
849 (RBCs) (hemolytic concentration 50%, HC50), and cytotoxicity towards HEK293 human cells
850 (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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857 **FIGURES**

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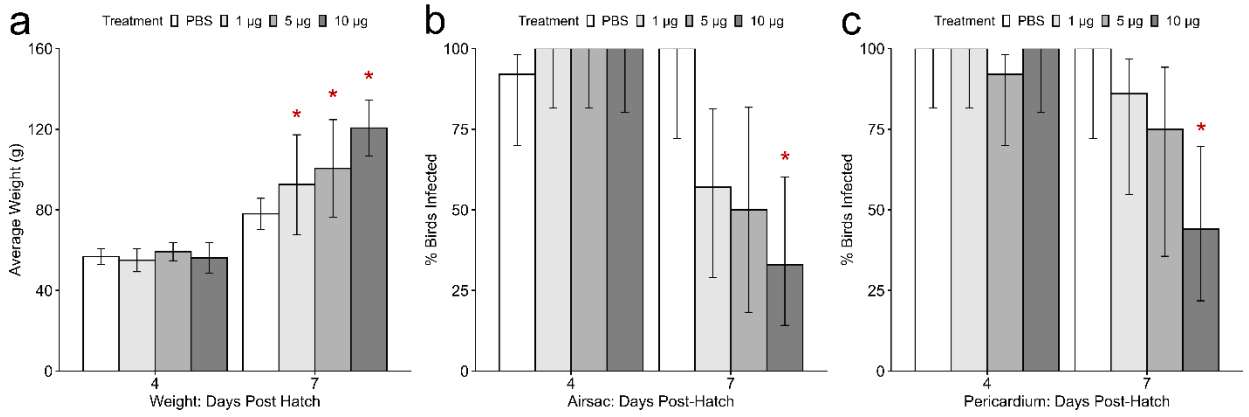

859

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

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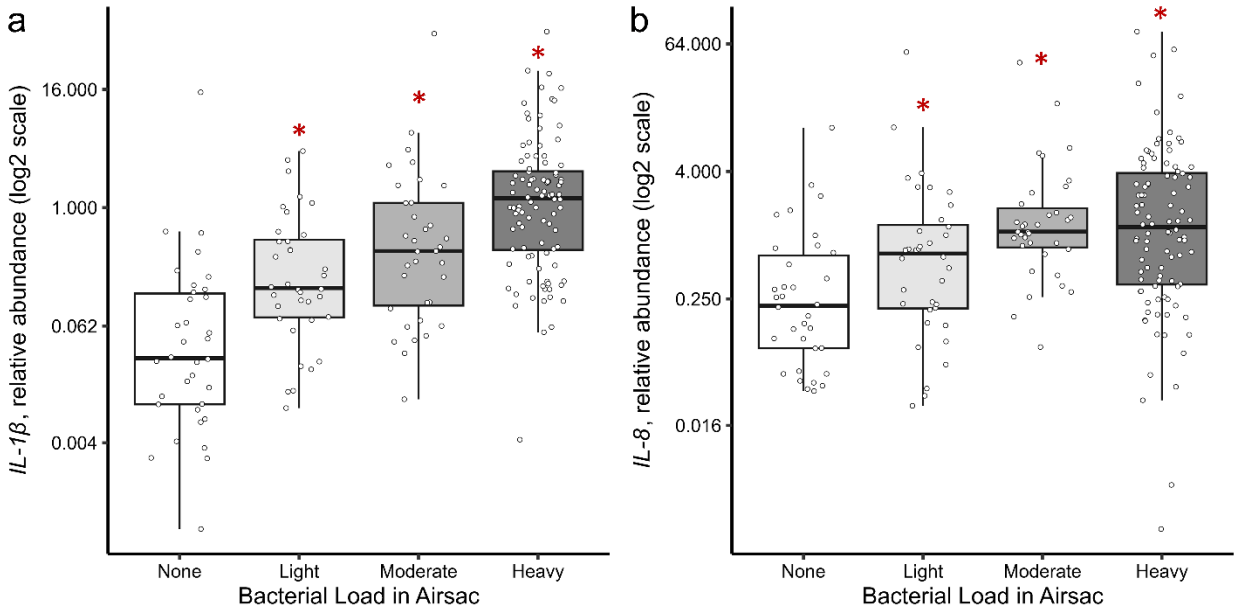

878

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

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

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

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

883 group is indicated by an asterisk.

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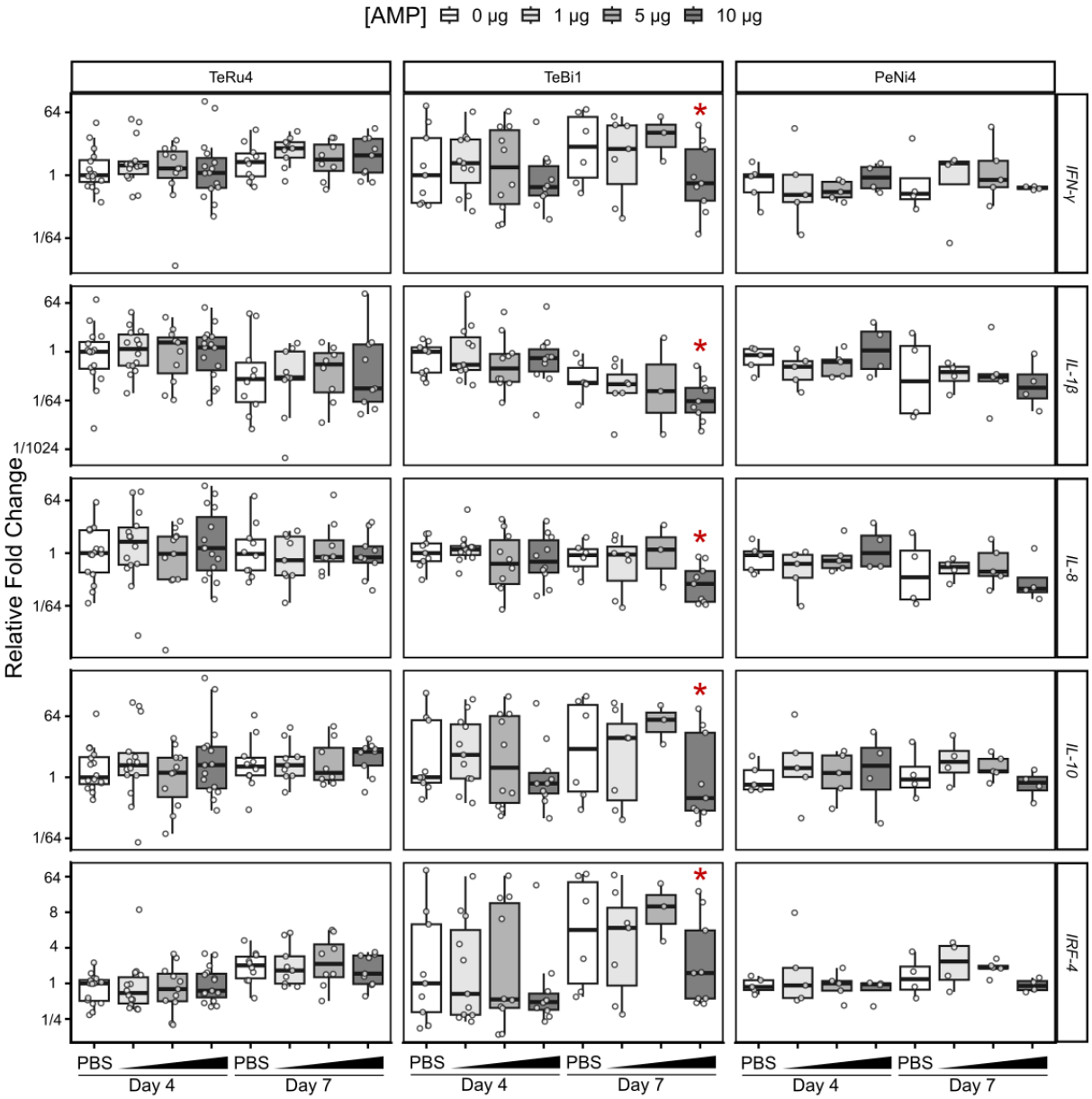

885

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

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

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

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

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

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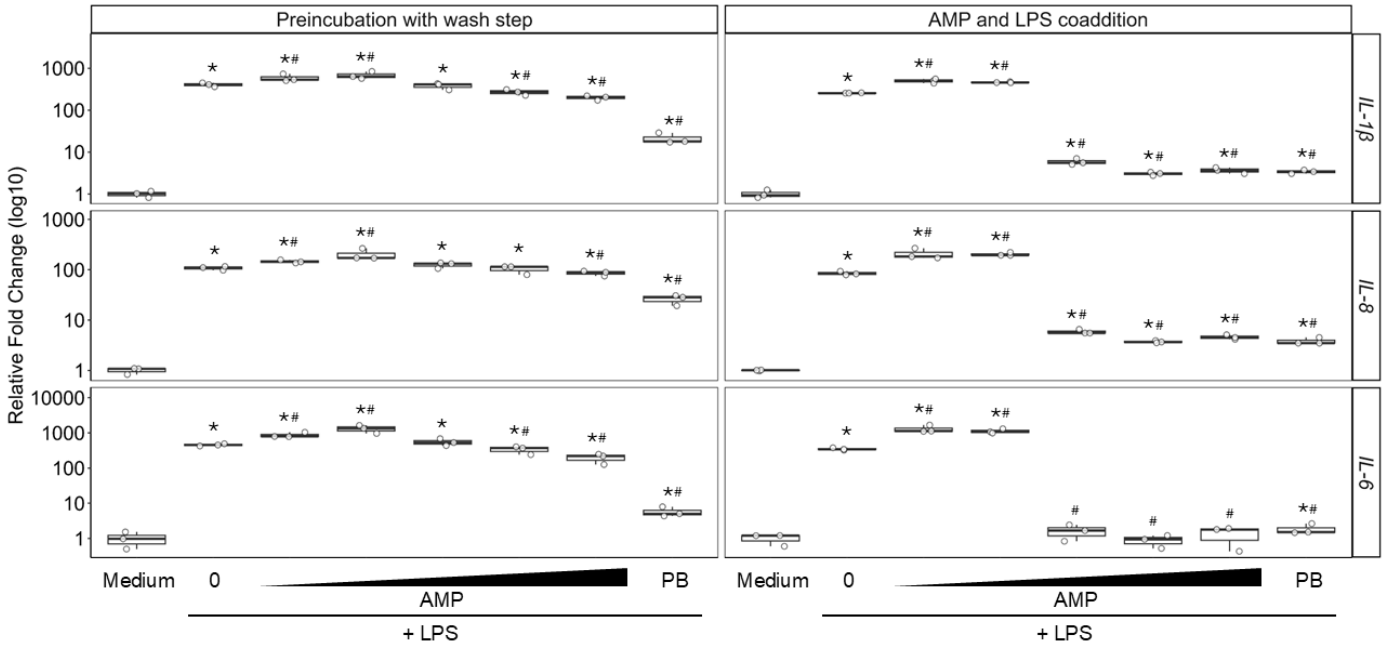

892

893 **Figure 4.** Relative fold change of *Interleukin (IL)-1β*, *IL-8*, and *IL-6* from HD11 cells as
894 determined by qPCR in the "preincubation with wash step" (left column) and "AMP and LPS
895 coaddition" experiments (right column). In the preincubation with wash step experiment, cells
896 were incubated for 3 h with medium, a range of 2-fold serially diluted antimicrobial peptide
897 (AMP; 1 to 16 µg/mL TeRu4), or 8 µg/mL polymyxin B sulfate (PB), followed by three washes
898 prior to the addition of 25 ng/mL lipopolysaccharide (LPS) for an additional 3 h. In the
899 coaddition experiment, cells were incubated for 3 h with medium, or with 25 ng/mL LPS mixed
900 with a range of 2-fold serially diluted AMP (1 to 16 µg/mL TeRu4), or 8 µg/mL PB. A
901 significant difference from the medium control is indicated by "*", and "#" indicates a
902 significant difference from LPS treatment (p-value < 0.1, Mann-Whitney-U test). Open circles
903 represent the fold change of one technical replicate relative to the medium control. The
904 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.

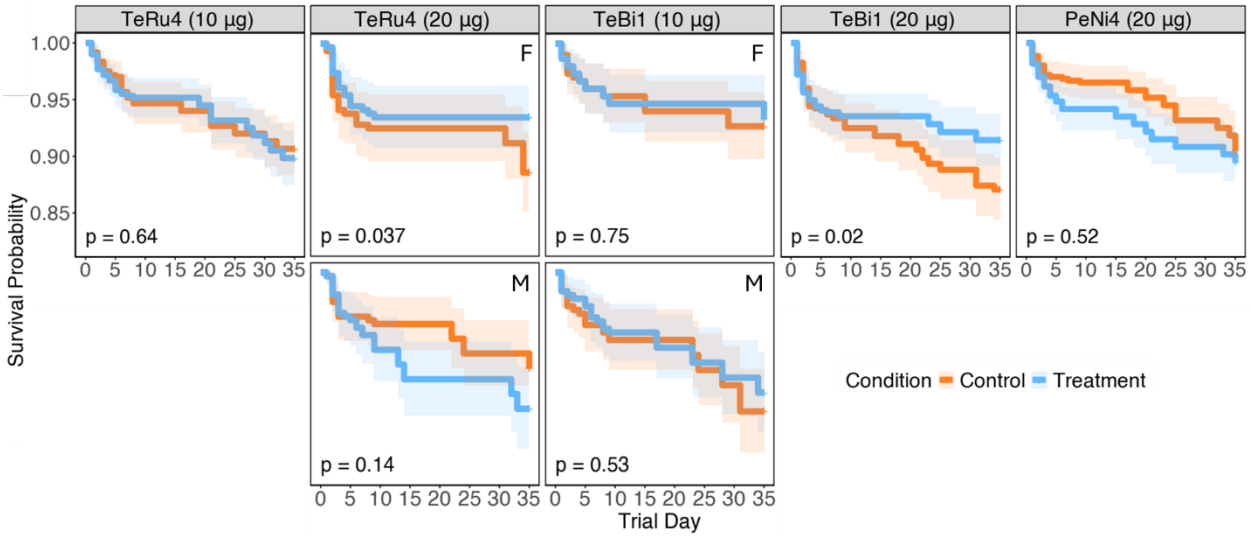

Figure 5. Kaplan-Meier curves depicting the survival probabilities of experimental groups during pen trials across different antimicrobial peptides (AMP) and dosages. Columns represent AMPs at specific dosages and each AMP–dosage combination corresponds to a separate pen trial. Sex-specific differences 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.

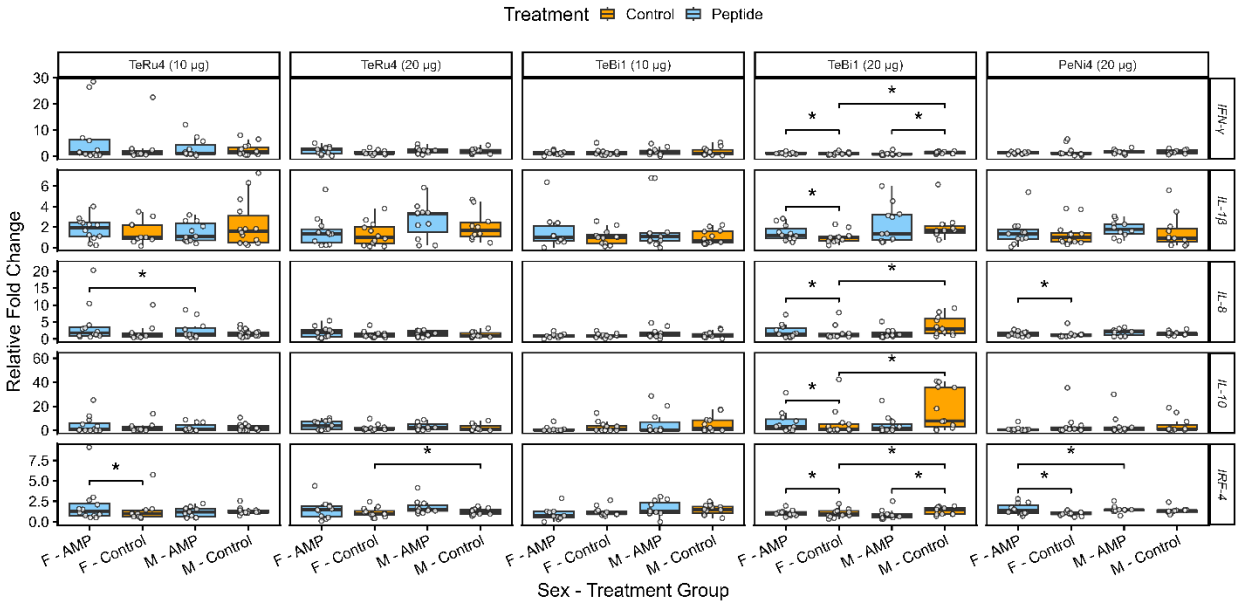

Figure 6. Relative fold change values of cytokine transcripts in the spleens of pen trial birds (no pathogen challenge) treated with 10 or 20 µg TeRu4, TeBi1, or PeNi4. Birds were euthanized 10 days after hatch. Asterisks indicate significance at $p \leq 0.1$. F, female birds; M, male birds. Additional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