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 (AMPs) 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 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, and flock uniformity on day 35. These findings demonstrate that the three selected AMPs are safe antibiotic alternatives that improve survival, modulate immune responses, and maintain normal growth performance in broiler chickens.

Key words: Antimicrobial peptide, APEC, broiler chicken, efficacy, safety

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) is a Gram-negative bacterium that poses a severe threat to the poultry industry. APEC infections induce colibacillosis and early chick mortalities in poultry, the primary cause of mortality and morbidity in the industry (Cuperus et al., 2016; Allan et al., 2018; Rezaee et al., 2021; Nguyen et al., 2021; Sarfraz et al., 2022; Hu et al., 2022). Antibiotics have been used to treat bacterial infections since the early 20th century. In the poultry industry, antibiotics have been used as growth promoters and prophylactic agents since the 1950s (Bean-Hodgins and Kiarie, 2021). Commonly, antibiotics are added to feed or water at subtherapeutic levels to increase weight gain and feed efficiency, as well as to prevent disease outbreaks in healthy flocks (Bean-Hodgins and Kiarie, 2021). However, overuse of antibiotics has contributed to the rise of antibiotic-resistant bacteria in both veterinary and human medicine (Bean-Hodgins and Kiarie, 2021; Richter et al., 2022). Moreover, policy changes in Canada's commercial poultry industry that have mandated limits on medically important antimicrobial use has left fewer antimicrobial options to control pathogens in poultry flocks (Bean-Hodgins and Kiarie, 2021). The One Health approach recognizes the linkages between human, animal, and environmental health, and highlights the urgency of addressing antibiotic resistance as a global crisis (Destoumieux-Garzón et al., 2018). Unfortunately, novel antibiotics are not approved at a pace sufficient to combat developing antibiotic-resistant bacteria (Richter et al., 2022). Moreover, the last classes of antibiotics to be discovered, including lipopeptides (daptomycin) and oxazolidinones (linezolid), were identified during the 1980s (Durand et al.,

86 2019). Consequently, there is growing interest in alternative antimicrobial strategies, such as
87 antimicrobial peptides (AMPs), to address this therapeutic gap.
88 AMPs are peptide-based biomolecules composed of 5 to 100 amino acids and are
89 typically cationic (Bahar and Ren, 2013). In nature, they are produced by the innate immune
90 system and act as the first line of defense against infections in a wide variety of organisms,
91 including humans, animals, and plants (Bahar and Ren, 2013; Durand et al., 2019). Most
92 conventional antibiotics have specific cellular targets, including cellular machinery involved in
93 DNA and protein synthesis and components of bacterial cell walls (Bahar and Ren, 2013). In
94 contrast, AMPs primarily cause rapid cell death by binding to anionic bacterial cell membranes
95 and disrupting their integrity, leading to cell lysis (Bahar and Ren, 2013; Durand et al., 2019).
96 AMPs typically do not target eukaryotic cells, as their membranes usually have low anionic
97 charge (Bahar and Ren, 2013). However, at higher concentrations, some AMPs may exhibit
98 cytotoxic effects on eukaryotic cells (Bahar and Ren, 2013). Therefore, rigorous safety
99 evaluation of AMPs is essential before regulatory approval, including vie in vitro assays, such as
100 hemolysis and cytotoxicity tests, as well as in vivo experiments.
101 AMPs exert less selective pressure on bacteria than conventional antibiotics due to their
102 nonspecific targets and diverse mechanisms of action, thus reducing the development of
103 antimicrobial resistance (Zasloff, 2002; Hancock and Sahl, 2006; Fjell et al., 2012; Richter et al.,
104 2022). In addition to their direct antimicrobial activity, some AMPs demonstrate
105 immunomodulatory properties, recruiting immune cells or regulating cytokine levels to enhance
106 the host response to infection (Rima et al., 2021; Richter et al., 2022). Cytokines are important
107 signaling molecules that facilitate the growth, differentiation, activation of immune cells (Garcia
108 et al., 2021). Interleukin (IL)-1β, IL-8, IL-6, and IL-10, interferon regulator factor 4 (IRF-4), and

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

130 **MATERIALS AND METHODS**

131 ***In Silico Peptide Selection***

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

137 ***Peptide Synthesis***

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

142 ***Bacterial Species and Growth.***

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

152 ***In Vitro Lead Peptide Selection***

153 Minimum inhibitory concentrations (MICs) of candidate AMPs were determined in vitro
154 against EC317, using ASTs as previously described (Richter et al., 2022). For the selection

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

162 ***Care and Use of Animals***

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

166 ***APEC Challenge Trials***

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

174 Before inoculation, APEC was cultured overnight at 37 °C in LB broth (Difco, Becton
175 Dickinson, Sparks, MD, USA) in a shaking incubator (Model No: 1570, Sheldon Manufacturing,
176 Inc., Cornelius, OR, USA) at 240 rpm. This culture was sub-cultured to a pre-determined OD_{600}

177 of 0.7, at which time the bacteria were harvested by centrifugation, resuspended in PBS at the
178 desired concentration, and administered to the birds by yolk sac injection.
179 The birds were given food (Masterfeeds, Humboldt, SK, Canada) and water *ad libitum*.
180 Four days after hatch, approximately half of the birds (between 8-17) were euthanized. The
181 remaining birds were euthanized seven days after hatch. Birds were monitored two to four times
182 per day for symptoms, and birds reaching a humane intervention point were euthanized. Only the
183 birds euthanized on schedule on days four and seven were included in the analyses. Bird survival
184 was also tracked.
185 All analyzed birds were weighed after death, and swabs were taken aseptically from the air
186 sac and pericardium and streaked on MacConkey agar (Difco, Becton Dickinson, Sparks, MD,
187 USA) for a semi-quantitative assessment of APEC growth. Plates were scored from 0-3,
188 representing no, low, medium, and severe levels of growth, respectively. Samples from the air
189 sacs, cecal tonsils, and spleens of birds euthanized on scheduled days were preserved in
190 RNALater (Thermo Fisher Scientific) at –20 °C for analysis by quantitative PCR (qPCR).
191 ***Immunomodulatory Activity Assays with HD11 Cells***
192 The effects of AMPs on in vitro cytokine production were assessed using an HD11 chicken
193 semi-adherent macrophage-like cell line (Obtained from VIDO-InterVac). HD11 cells were
194 cultured in RPMI medium with 1X Glutamax and 10% FBS (Thermo Fisher Scientific) at 41°C
195 (normal chicken body temperature) and 5% CO_2 and were routinely passaged every two days.
196 ***Cell Plating.*** HD11 cells were plated for experimentation between passages three to twelve.
197 Suspended cells were collected with the growth medium, and adherent cells were obtained after
198 washing with PBS and trypsinization with 0.25% Trypsin-EDTA in PBS (both from Thermo
199 Fisher Scientific). The cells were centrifuged at 200 g for seven minutes and the pellet was

200 resuspended in fresh medium. Trypan Blue (Thermo Fisher Scientific) was added to a sample of
201 cells and live cells were counted on a Hausser Scientific Bright-Line Phase Hemacytometer
202 (Fisher Scientific, Hampton, NH, USA). HD11 cells were plated at 2.5×10^4 cells per well on
203 Nunclon Delta 96-well plates (Thermo Fisher Scientific) and incubated overnight.
204 ***HD11 Experiment Overview.*** In all experiments, HD11 growth medium was used to resuspend
205 and dilute AMPs and other reagents, and all incubations were carried out at 41°C and 5% CO_2.
206 All experiments included a medium-only control and an LPS-only control (final concentration of
207 25 ng/mL). In all experiments other than the AMP-only Screen, 8 µg/mL Polymyxin B sulfate
208 (PB) was used as a positive control as it is known to attenuate the LPS-triggered inflammatory
209 response (Schromm et al., 2021; Yang et al., 2024). Prior to treatment, the supernatant was
210 removed from the wells, therefore only the adherent cell monolayer was used during the
211 experiments.
212 Four experimental approaches were devised to investigate the immunomodulatory activity
213 of AMPs. First, HD11 cells were treated solely with AMP (AMP-Only Screen). Second, cells
214 were treated with a range of AMP concentrations followed by treatment with lipopolysaccharide
215 (LPS) from *E. coli* O111:B4 (Sigma-Aldrich), to test if the AMPs could modulate the immune
216 response triggered by LPS (AMP Preincubation Screen). If an AMP showed immunomodulatory
217 activity in the AMP Preincubation Screen, it was then tested in the AMP Preincubation with
218 Wash Step and the AMP and LPS Coaddition experiments.
219 ***AMP-Only Screen.*** HD11 cells were treated with 100 µL 16 µg/mL PeNi4, TeBi1, or TeRu4 and
220 incubated for 6 h.
221 ***AMP Preincubation Screen.*** HD11 cells were treated with 100 µL of 1, 2, 4, 8, or 16 µg/mL
222 PeNi4, TeBi1, or TeRu4 and incubated for 3 h. After the incubation, 100 µL LPS (50 ng/mL)

223 was administered to the cells, resulting in a final concentration of 25 ng/mL LPS. The cells were
224 incubated for an additional 3 h.
225 ***AMP Preincubation with Wash Step.*** The cells were treated identically to the AMP
226 Preincubation Screen but with the addition of a wash step prior to the addition of LPS. Briefly,
227 after the first 3 h incubation, the medium (containing the AMP or PB) was removed from the
228 wells and the cell monolayer was washed three times using fresh medium. One hundred µL LPS
229 (25 ng/mL) was administered, and the cells were incubated for an additional 3 h.
230 ***AMP and LPS Coaddition.*** AMP was resuspended and diluted to prepare a 2X concentration
231 curve of 32, 16, 8, 4, and 2 µg/mL. PB and LPS were prepared at 2X concentrations of 16 µg/mL
232 and 50 ng/mL, respectively. At regular time intervals, 2X AMP or 2X PB was added in equal
233 volume to 2X LPS to yield a 1X mixture of AMP or PB, and LPS. Mixtures were vortexed for
234 one min and incubated for 30 min prior to being administered to cells at regular time intervals in
235 the order in which they were made. The cells were incubated for 3 h.
236 ***Cell Harvest and RNA Isolation.*** Supernatant was removed from HD11 cells after the final
237 incubation. Buffer RLT from the miRNeasy Micro Kit (Qiagen, Mississauga, ON, Canada) with
238 2M DL-dithiothreitol (Sigma-Aldrich) was added to lyse adherent cells at a volume of 260 µL
239 across technical replicates, with each technical replicate comprising two wells in the 96-well
240 plate. HD11 cells in lysis buffer were disrupted by pipetting up and down, vortexed, and stored
241 at -80 °C until RNA isolation. In total, three technical replicates per treatment were collected for
242 RNA isolation and qPCR analysis.
243 ***Commercial Pen Trials***
244 Each pen trial examined the safety of a single AMP. Cobb 500 or Ross 308AP broiler
245 chicken breeds were used in pen trials (Supplementary Table S1). All breeds were sourced from

246 a commercial hatchery (Abbotsford, BC, Canada), and the specific breed used in each trial is
247 summarized in Supplementary Table S1. For pen trials, birds were sourced from older breeder
248 flocks (a different flock for each trial) and separated by sex and treatment into individual mini
249 pens following hatching.

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

259 The birds were then transferred to SJ Ritchie Research Farms (Abbotsford, BC, Canada)
260 where they were cloacally sexed by professionals and then separated by sex and treatment. A
261 total of 24 mini pens, within six blocks, were set up. The birds were weighed individually, then
262 50 birds were placed into each mini pen according to their sex and treatment, with each block
263 containing each of the four experimental groups in a random order. Birds were provided *ad*
264 *libitum* access to water and an all-vegetable, raised without antibiotic (RWA) feed (Trouw
265 Nutrition, Chilliwack, BC, Canada). A step-down lighting program was implemented: from day
266 0 to day 21, birds experienced 6 h darkness per 24 h cycle; from day 21 to 28, 4 h darkness; and
267 from day 28 to the end of the trial, 1 h darkness per day. Number of birds in each mini-pen was
268 reduced to 12 on day 10 to prevent overcrowding of the mini-pens. Birds were checked three

269 times per day throughout the trial and were weighed individually on days 10, 21, and 35. Any
270 mortality during the pen trial was noted.

271 ***qPCR Analyses***

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

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

286 The concentration of RNA from tissue samples was measured by NanoDrop (NanoDrop
287 1000 3.8.1; Thermos Fisher Scientific) and diluted to a concentration of approximately 100
288 ng/µL before cDNA synthesis, while RNA from HD11 cells was used neat due to the low
289 concentration. All RNA samples were converted to cDNA using the Applied Biosystems High-
290 Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), per the manufacturer's
291 instructions.

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

305 ***Statistical Analyses***

306 Statistical analyses of the qPCR results were conducted in R version 3.6.3 (R Core Team,
307 2024). The data were tested for normality by the Shapiro–Wilk and Levene's tests. They were
308 then subjected to pairwise analyses by the Kruskal-Wallis and Wilcoxon rank-sum tests for non-
309 parametric data with a significance threshold of $\alpha = 0.1$. For the challenge and pen trials, this
310 significance cut off was selected due to the high variability of in vivo data. For the HD11
311 experiments, this threshold was appropriate as the smallest p-value achievable using a rank-sum
312 test with $n = 3$ was approximately 0.08.

313 The rest of the challenge trial data was analyzed in R version 4.4.0 and 4.5.1(R Core Team,
314 2024). Hatchability rates of treated eggs compared to control eggs, as well as rates of bacterial

315 detection in the birds' air sacs and pericardia, were evaluated using the Fischer's exact test.
316 Cumulative weight gain of birds euthanized on days four and seven was evaluated using a
317 Wilcoxon rank-sum test, and survival was graphed and evaluated using the log-rank test and the
318 "survminer" package (Kassambara et al., 2016). Other graphs were constructed using the
319 packages "tidyverse," "readr," "janitor," "superb," "ggsignif," "rstatix,", "DescTools", and
320 "ggpubr" (Signorell, 2014; Wickham et al., 2015b; a, 2019; Kassambara, 2016, 2019; Firke,
321 2016; Ahlmann-Eltze and Patil, 2017; Cousineau, 2021).
322 An additional analysis was conducted to compare the relative cytokine abundance in bird
323 air sacs to the air sac bacterial load scores of the same birds. The difference in cytokine
324 abundance for each bacterial level (light, moderate, severe) was compared to the cytokine
325 abundance for no growth using a Wilcoxon rank-sum test, and additionally, a correlation
326 coefficient was determined using Spearman's Rho.
327 Statistical analyses of hatchability, survival, mean weights, feed conversion ratio (FCR),
328 and flock uniformity in pen trials were conducted using R (version 4.1.3) and the packages:
329 "survival", "survminer", "openxlsx", "readxl", "tidyverse, "zoo", "car", "ggplot2", "ggsignif",
330 and "MHTdiscrete" (Fox et al., 2001; Therneau, 2001; Zeileis et al., 2004; Wickham et al., 2007,
331 2019; Schauberger and Walker, 2014; Wickham and Bryan, 2015; Kassambara et al., 2016;
332 Yalin Zhu, Wenge Guo, 2016; Ahlmann-Eltze and Patil, 2017). For pen trials, statistical
333 comparisons between treatment and control groups used Wilcoxon rank-sum tests (hatchability
334 and production parameters) and log-rank tests (survival), with production and survival analyzed
335 separately by sex. P-values were corrected for multiple comparisons using the Šidák method.
336
337 **RESULTS**

338 ***In Vitro Peptide Selection***

339 The 875 candidate AMPs identified by rAMPage (Lin et al., 2022), AMPlify (Li et al.,
340 2022), and AMPd-Up (Li et al., 2024) were previously tested for antimicrobial activity and
341 cellular toxicity in vitro (Richter et al., 2022). Of these, 331 with MICs ≤ 32 μg/mL against
342 EC317 were designated as hit AMPs. Among hit AMPs, 275 exhibited minimal hemolytic
343 activity (HC_{50} ≥ 128 μg/mL) against porcine RBCs. Of these, 62 also demonstrated minimal
344 cytotoxicity (CC_{50} ≥ 128 μg/mL) in HEK293 cells. These were classified as lead AMPs. Three
345 representative lead AMPs (TeRu4, TeBi1, and PeNi4) were selected for further experiments.
346 TeRu4 and TeBi1 demonstrated lower MIC values than PeNi4. The physiochemical properties of
347 these AMPs and their in vitro activities are summarized in Table 1. These AMPs were evaluated
348 in vitro using HD11 cells and in vivo using APEC challenge trials and pen trials.

349 ***qPCR Primer Design and Validation***

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

353 ***APEC Challenge Trials***

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

358 Egg hatchability was between 90-98% in all treatment groups, with no significant
359 differences between treated birds and control birds (data not shown). Notably, when birds were
360 treated with 20 μg AMP (Supplementary Figure S1), treated birds appeared to die faster than

361 untreated birds within the first few days post-infection, though there were no significant
362 differences in overall survival. The spleens of birds treated with 20 μg AMP were analyzed by
363 qPCR, and some differences were observed, suggesting that the peptides were bioactive, but the
364 qPCR results were not conclusive (data not shown), and subsequent challenge trials tested the
365 AMPs on a dose curve at 1, 5, or 10 μg. In the latter trials, there were no significant differences
366 in survival probability with any dose of AMP (≤10 μg) compared to control (Supplementary
367 Figure S2).

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

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

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

384 levels of APEC infection also had significantly higher relative abundance of *IL-8* transcripts ($p =$
385 0.03, 3×10^{-6}, and 5×10^{-6}, respectively) than birds with no bacterial growth in their air sacs
386 (Figure 2b).

387 In the cecal tonsil (Supplementary Figure S5), qPCR results show minimal changes due to
388 treatment with PeNi4, with only a 2-fold increase in the inflammatory cytokine *IL-8* in the 10 μg
389 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
390 $= 0.1$). In contrast, there are numerous changes due to treatment with TeBi1, including a 2.4-fold
391 increase in *IL-8* transcripts in the 5 μg group on day 4 ($p = 0.1$), and a 5-fold decrease in *IL-8*
392 transcripts in the 10 μg group on day 7 ($p = 0.06$). *IFN-γ* expression increased 1.4-fold ($p = 0.03$)
393 and 2.5-fold ($p = 0.03$) in the 5 and 10 μg groups, respectively, on day 4, but decreased 1.4-fold
394 ($p = 0.03$) and 1.1-fold ($p = 0.09$) in the 1 and 10 μg groups, respectively, on day 7. With respect
395 to *IL-1β,* on day 7, the TeBi1 5 μg group showed a 5-fold decrease ($p = 0.07$). For the anti-
396 inflammatory transcript *IL-10*, there is a 2.5-fold decrease ($p = 0.07$) with 5 μg TeBi1 on day 7.
397 Finally, TeBi1 treatment was also associated with changes in *IRF-4* transcripts: the 10 μg group
398 was associated with a 1.7-fold increase on day 4 ($p = 0.04$), and the 5 μg group was associated
399 with a 1.5-fold increase on day 7 ($p = 0.1$).

400 In the spleen (Supplementary Figure S6), fewer responses are observed. For TeRu4, there
401 is a 2-fold decrease in IL8 transcripts on day 7 ($p = 0.1$) after treatment with 1 μg. For PeNi4,
402 there is a 5-fold increase in *IL-10* transcripts on day 7 ($p = 0.09$) after treatment with 5 μg. For
403 TeBi1, while there are no differences in the inflammatory cytokines *IFN-γ*, *IL1β*, or *IL-8*, there is
404 a 3-fold increase in *IL-10* transcripts on day 4 ($p = 0.03$) after treatment with 10 μg. There are
405 also multiple increases in *IRF-4* transcripts: on day 4, a 2-fold increase after treatment with 1 μg

406 (p = 0.1), a 3-fold increase after treatment with 5 µg (p = 0.1); and on day 7, a 1.3-fold decrease
407 after treatment with 1 µg (p = 0.1).
408 In the air sac (Figure 3), all observed changes are on day 7, after treatment with 10 µg
409 TeBi1 relative to the PBS control. There is a 10-fold decrease in *IFN-γ* transcripts (p = 0.1), a 3-
410 fold decrease in *IL-1β* transcripts (p = 0.07), and a 5-fold decrease in *IL-8* transcripts (p = 0.02).
411 Additionally, there is a 50-fold decrease in *IL-10* transcripts (p = 0.1), and a 10-fold decrease in
412 *IRF-4* transcripts (p = 0.1).
413 ***HD11 Experiments***
414 In addition to challenge trials, the ability of TeRu4, TeBi1, and PeNi4 to influence the
415 cellular immune response was tested in an HD11 cell model system. In all HD11 experiments
416 (n=21), the LPS induction control resulted in a wide-ranging, but consistent increase in relative
417 fold change (RFC) compared to the medium control for *IL-1β* (14- 677), *IL-8* (8 - 249), and *IL-6*
418 (55 - 3,284) (Supplementary Figures S6 and S7, and Figure 4). Therefore, all experimental
419 manipulations with AMPs were compared with their experimentally paired LPS controls. The
420 induction of cytokine transcripts by LPS was significantly attenuated by PB in all but a single
421 experiment (n=15), with fold change decreases compared to LPS alone ranging from 6-166 for
422 *IL-1β*, 2-34 for *IL-8*, and 11-335 for *IL-6* (Supplementary Figure S8). While only one
423 representative graph is shown for each experiment, the preincubation, preincubation with wash
424 step, and coaddition experiments were performed in biological triplicate, and the AMP-only
425 experiments in biological duplicate. Only data consistently reproduced across all replicates are
426 highlighted, unless otherwise stated.
427 ***Induction of Inflammatory Cytokines in HD11 cells by TeRu4, TeBi1, and PeNi4.*** To assess
428 the ability of AMPs alone to induce cytokine production in HD11 cells, cells were incubated

429 with 16 µg/mL of each AMP or 8 µg/mL PB. Treatment with TeRu4 resulted in a 2-fold increase
430 in *IL-1β* (in one of two biological replicates) and *IL-8*, while TeBi1 lead to a 4-fold decrease in
431 *IL-6* (Supplementary Figure S7). Treatment with PeNi4 did not significantly alter the abundance
432 of any of the three cytokines, while TeBi1 had no significant effect on *IL-1β* and *IL-8*, and
433 TeRu4 had no significant effect on *IL-6* (Supplementary Figure S7). Treatment with PB alone
434 did not significantly or consistently change *IL-1β, IL-8,* and *IL-6* levels (Supplementary Figure
435 S7).

436 ***Preincubation Screen of TeRu4, TeBi1, and PeNi4.*** To examine if AMPs affect HD11 response
437 to LPS, cells were preincubated with AMP or PB (positive control), followed by treatment with
438 LPS. Here and in subsequent experiments, medium only and LPS only served as negative and
439 positive controls, respectively. A significant 2.8-fold decrease was observed for *IL-1β* and *IL-6*
440 following preincubation with 8 µg/mL TeRu4 compared to the LPS control (Supplementary
441 Figure S8). Preincubation with 16 µg/mL TeRu4 led to a significant reduction in *IL-1β*, *IL-8*, and
442 *IL-6* levels by 13.6-fold, 2.9-fold, and 22.0-fold, respectively, compared to LPS only control
443 (Supplementary Figure S8). Similarly, treatment with 8 µg/mL PB significantly decreased *IL-1β,*
444 *IL-8* (in 2 of 3 biological replicates), and *IL-6* abundance relative to LPS alone by 8.8-fold, 3.4-
445 fold, and 20.4-fold, respectively (Supplementary Figure S8). While a significant 2.8-fold
446 increase was observed for *IL-8* at 4 µg/mL TeRu4 compared to the LPS control, this observation
447 was only seen in two of three biological replicates (Supplementary Figure S8). Preincubation
448 with TeBi1 and PeNi4 did not consistently induce a significant change in *IL-1β*, *IL-6*, and *IL-8*
449 transcript levels compared to the LPS control across biological replicates (Supplementary Figure
450 S8). The attenuation of LPS-induced responses of *IL-1β, IL-8,* and *IL-6* transcripts after TeRu4

451 preincubation prompted further investigation via the subsequent experiments to provide some
452 insight into possible mechanism of action.
453 ***Preincubation Experiment with Wash Step.*** The effects on HD11 cells of transient exposure to
454 TeRu4, followed by its removal and the addition of LPS, was examined. A significant decrease
455 in *IL-1β, IL-8,* and *IL-6* transcripts relative to the LPS control remained after preincubation with
456 8 µg/mL PB (21-, 5-, and 80-fold respectively) and 16 µg/mL TeRu4 (3-, 2-, and 4-fold,
457 respectively) (Figure 4). Preincubation with 8 µg/mL TeRu4 induced a significant decrease in
458 *IL-1β* levels by 2.0-fold and IL-6 by 1.9-fold in two of three biological replicates relative to the
459 LPS only control (Figure 4). Unlike in the previous preincubation experiment (without a wash
460 step), treatment with 1 and 2 µg/mL TeRu4 followed by the wash step, frequently (but not
461 consistently in all biological replicates) resulted in a significant increase in *IL-1β* (1.3- and 1.7-
462 fold respectively) and *IL-8* (1.3- and 1.6-fold respectively) transcript levels relative to the LPS
463 only control (Figure 4). The significant increase in *IL-6* (2-fold for relative to LPS only) at these
464 low concentrations was consistent across all three biological replicates (Figure 4).
465 ***Coincubation of TeRu4 and LPS.*** The impact of co-incubating TeRu4 and LPS prior to treating
466 HD11 cells on cytokine transcript expression was assessed. Coaddition of LPS with either PB or
467 TeRu4 (at concentrations ranging from 4 to 16 µg/mL) resulted in significant decreases in all
468 cytokine transcripts compared to the LPS control across all three biological replicates (Figure
469 10). Specifically, co-treatment with PB and LPS led to reductions of 101-fold for *IL-1β*, 27-fold
470 for *IL-8*, and 260-fold for *IL-6* (Figure 4). Similarly, co-treatment with 16 µg/mL TeRu4 and
471 LPS decreased *IL-1β* abundance by 75-fold, 20-fold for *IL-8*, and 409-fold for *IL-6* (Figure 4).
472 LPS and 8 µg/mL TeRu4 reduced *IL-1β* transcripts by 97-fold, *IL-8* by 23-fold, and *IL-6* by 368-
473 fold compared to LPS alone (Figure 4). LPS and 4 µg/mL TeRu4 resulted in reductions of 15-

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

481 ***Commercial Pen Trials***

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

488 For the first 10 days, there were no statistical differences between the survival probability
489 of males and females (Supplementary Figure S10). Thus, the sexes were combined for
490 subsequent analyses. No significant differences in survival probability were detected between
491 treatment and control groups for any AMP for the first 10 days of the pen trials (Supplementary
492 Figure S11). However, for the full duration of the pen trials (35 days), sex-specific differences in
493 survival probabilities were observed for TeRu4 at 20 µg and TeBi1 at 10 µg (Supplementary
494 Figure S12). For these two pen trials, the sexes were kept separate for analysis (Figure 5,
495 Supplementary Figure S13). Notably, TeRu4 at 20 µg significantly increased the survival
496 probability of female birds compared to the controls by approximately 4.9 % by day 35 (p =

497 0.037). Moreover, TeBi1 at 20 µg significantly increased the survival probability of the birds
498 compared to the control by approximately 4.4% by day 35 ($p = 0.02$). There was no other
499 difference in survival probabilities between the treatment and control groups by day 35. In the 10
500 µg TeRu4 and TeBi1 trials, survival curves for both sexes closely overlapped (Supplementary
501 Figure S13).

502 Significant differences in mean weight between treatment and control groups were
503 observed only in the 20 µg TeBi1 trial (Supplementary Figure S14). Notably, on day 0, mean
504 weights in the treatment groups were approximately 2.8% lower in males and 2.4% lower in
505 females compared to controls ($p = 0.017$ and 0.034). However, on day 10, no significant
506 difference in mean weights was observed. Moreover, in the 10 µg TeRu4 trial, numerous birds of
507 both sexes exhibited lower body weights on days 21 and 35, particularly in the treatment group,
508 although this difference was not statistically significant. This observation may suggest
509 subclinical effects of TeRu4 at lower doses, high biological variability among individual birds,
510 or environmental effects, all of which warrant further investigation. By day 35, no significant
511 differences in mean weight were detected (Supplementary Figure S14).

512 No statistically significant differences were detected between FCR of the treatment and
513 control groups on day 35 (Supplementary Figure S15). Of interest, there were occasions where
514 FCR values were below 1 (Supplementary Figure S15). One was observed between days 0 and
515 10, in the 10 µg TeBi1 trial (FCR = 0.997). Further, in the 20 µg TeRu4 trial, two FCR values
516 were below 1 (FCR = 0.923 and 0.961) in the second interval, between days 11 and 21. Overall,
517 the mean FCR values were consistently the lowest in the first interval, and the highest in the last
518 interval, between days 21 and 35, for both sexes. These findings suggest that while AMPs did

519 not significantly alter FCR under the tested conditions, and observations during the first interval
520 of certain pen trials warrant further investigation.

521 Coefficient of variation (CV) percentages were calculated as a measure of flock
522 uniformity. There was no significant difference in the CV percentages of the treatment and
523 control groups on day 35 (Supplementary Figure S16). Among males, on day 10 in the 20 µg
524 TeBi1 trial, the treatment group had an approximately 2.8% lower CV compared to the control
525 group, but this difference did not reach statistical significance ($p = 0.067$) (Supplementary Figure
526 S16). Moreover, the birds in the 10 µg TeRu4 trial exhibited greater variability in flock
527 uniformity on days 21 and 35, with CV values exhibiting greater range than those in the control,
528 consistent with the presence of birds with low body weight.

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

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

539 After treatment with 20 µg PeNi4, female birds had a 1.4-fold increase ($p = 0.02$) in *IRF-4*
540 transcripts relative to control birds. Additionally, in that trial, male control birds had a 1.3-fold

541 increase (p = 0.01) in *IRF-4* transcripts and a 1.6-fold increase (p = 0.04) in *IL-8* transcripts
542 relative to female control birds.

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

549 After treatment with 20 µg TeBi1, male birds had a 2-fold decrease in *IFN-γ* and *IRF-4*
550 transcripts (p = 0.02 for both), and a 3-fold decrease in *IL-10* and *IL-8* transcripts (p = 0.03 and
551 0.02, respectively) relative to control birds. In that trial, male control birds had higher abundance
552 of all tested transcripts than female control birds: 2-fold more *IFN-γ* transcripts (p = 0.1), 3-fold
553 more *IL-10* transcripts (p = 0.06), 1.7-fold more *IL-1β* transcripts (p = 0.008), 3-fold more *IL-8*
554 transcripts (p = 0.01), and 2-fold more *IRF-4* transcripts (p = 0.1). Additionally, male birds
555 treated with 20 µg TeBi1 had significantly lower abundance of certain transcripts than female
556 birds in the same treatment group, a 1.3-fold decrease in both *IFN-γ* and *IRF-4* transcripts (p =
557 0.09 for both).

558

559 **DISCUSSION**

560 This study demonstrates that antimicrobial peptides can improve early health outcomes in
561 broiler chickens while maintaining production performance under commercial conditions. Across
562 complementary in vivo and in vitro assays, two of the three lead AMPs, TeBi1 and TeRu4,
563 showed efficacy and immunomodulatory activity consistent with their intended roles as

564 antibiotic alternatives for preventing APEC-associated disease. Here, AI-assisted discovery is
565 relevant for enabling large-scale candidate generation and triage that would be impractical by
566 hand. All outcome data from the in vivo and in vitro assays were analyzed using nonparametric
567 methods, Kruskal–Wallis for omnibus comparisons and Wilcoxon rank-sum for pairwise
568 contrasts. Given the inherent variability of in vivo data and the exploratory nature of this study, a
569 significance threshold of $\alpha = 0.1$ was used, as specified in the methods.

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

584 Second, the HD11 macrophage-like cell experiments demonstrate the potential for TeRu4
585 to attenuate the effects of LPS. Preincubation with TeRu4 and its coaddition with LPS elicited
586 robust and dose-dependent attenuation of *IL-1β*, *IL-6*, and *IL-8* transcripts, with effects at higher

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

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

603 Together, the in vivo and in vitro data outline complementary roles for these leads. TeBi1
604 appears to act primarily through direct antibacterial activity in the immediate post-hatch period,
605 reducing tissue colonization and the associated inflammatory response. TeRu4 shows consistent
606 anti-inflammatory activity in a macrophage model and over 35 days translates to a survival
607 advantage in females at higher dose, suggesting that immunomodulation can contribute to
608 resilience under farm conditions. These sex-specific responses may reflect differences in immune
609 system maturation rates or hormone-immune interactions, as male broilers typically show

610 different growth and immune development patterns than females (Leitner et al., 1989; Younis et
611 al., 2023). The patterns observed in both survival and cytokine transcript baselines warrant
612 additional investigation into sex-dependent pharmacodynamics (PD), immune set-points, and
613 potential interactions with breeder age and genetics.

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

630 The results have practical implications for poultry health programs. The in ovo delivery
631 method offers multiple advantages: it integrates cleanly into existing hatchery workflows, avoids
632 post-hatch handling, and ensures uniform exposure across flocks. This delivery approach,

633 combined with a demonstrated safety profile, minimizes barriers to commercial adoption. From
634 an economic perspective, even modest survival improvements translate to substantial numbers at
635 commercial scale, while early bacterial control reduces the need for subsequent therapeutic
636 interventions. The complementary mechanisms of TeBi1 (direct antibacterial) and TeRu4
637 (immunomodulatory) suggest potential for combination strategies that leverage both activities.
638 Future work should benchmark these AMPs against current animal husbandry standards and
639 evaluate alternative delivery formats, such as water supplementation during high-risk periods, to
640 optimize protection throughout the production cycle.

641 In conclusion, AI-discovered AMPs TeBi1 and TeRu4 show complementary efficacy and
642 safety signals in broilers when administered in ovo. TeBi1 reduces early APEC burden and
643 inflammation and improves early growth, whereas TeRu4 attenuates macrophage inflammatory
644 responses and improves survival under commercial conditions without compromising
645 performance. These findings support advancement to dose-optimization, PK and residue studies,
646 expanded pathogen panels, and multi-site validation, with an eye toward integrated antimicrobial
647 stewardship in poultry production.

648

649 **DECLARATION OF COMPETING INTEREST**

650 Inanc Birol is a founder, Chief Scientific Officer, and equity holder of Amphoraxe Life
651 Sciences Inc., which has licensed intellectual property related to antimicrobial peptides described
652 in this manuscript. The following authors are named inventors on related intellectual property:
653 Caren C. Helbing, Erin Fraser, Fraser Hof, Lorissa Corrie, René L. Warren, Vanessa C.
654 Thompson, Anat Yanai, Inanc Birol, and Linda M. N. Hoang. Monica Kotkoff is also a
655 stakeholder on the IP. Emre Demirsoy, Teagan I. Parkin, and Anna H. Dema received internship

656 funding from Amphoraxe through Mitacs. Amphoraxe contributed to aspects of the work,
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658 activities, internship support, and participation in manuscript preparation. The authors retained
659 control over data collection, analysis, interpretation, and the decision to submit the manuscript
660 for publication. All other authors declare no competing interests.

661

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822 **TABLES**

823 **Table 1.** Physiochemical properties and in vitro activities of three lead antimicrobial peptides
824 (AMPs) TeRu4, TeBi1, and PeNi4. Listed for each AMP are their sequence length, net charge,
825 and amino acid sequence. In vitro activities include the antimicrobial activity against APEC
826 EC317 (minimum inhibitory concentration, MIC), the hemolytic activity against porcine red
827 blood cells (RBCs) (hemolytic concentration 50%, HC50), and cytotoxicity towards HEK293
828 human cells (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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835 **FIGURES**

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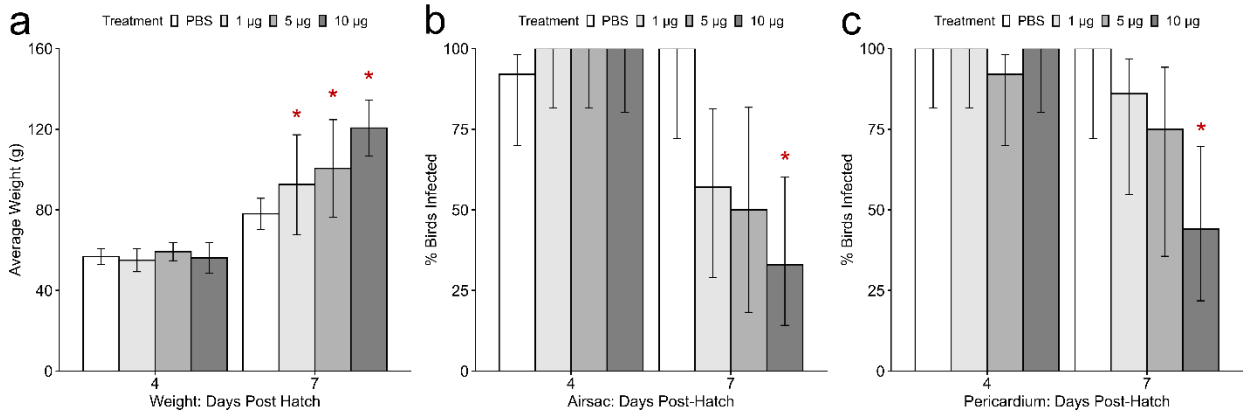

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838 **Figure 1**: a) Weight gain of TeBi1 treated birds between day 4 and 7. Birds, euthanized on
839 schedule, were weighed individually, and values shown are median ± mean absolute deviation
840 (MAD). On day 7, birds treated with 1 or 5 µg TeBi1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

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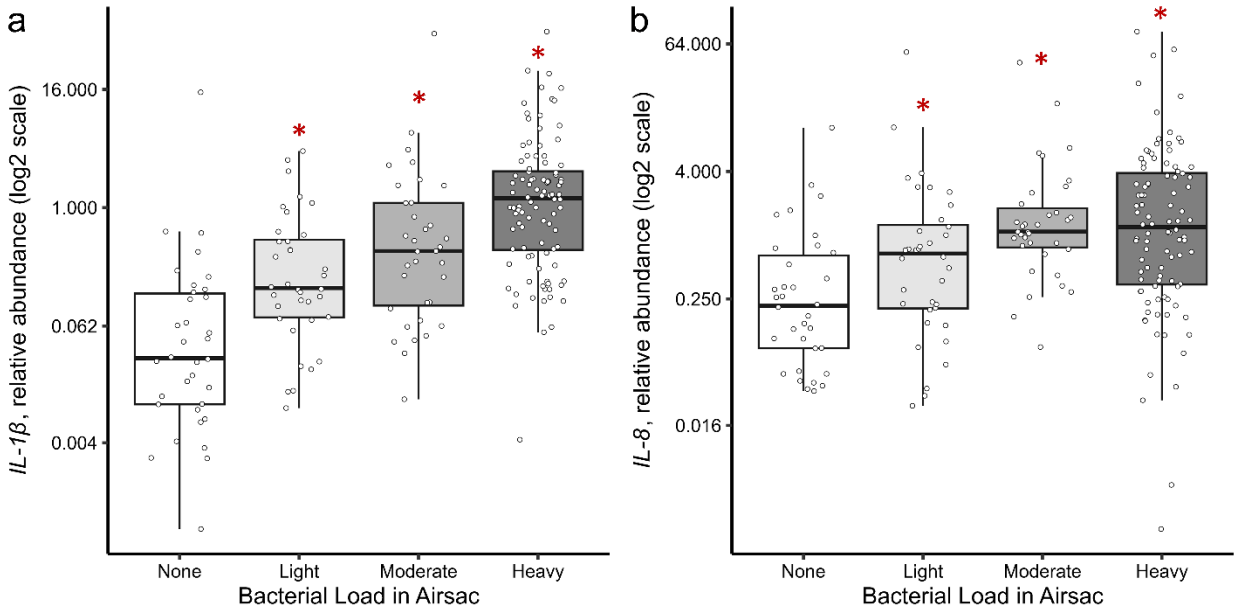

855

856 **Figure 2.** Relative transcript abundance of a) *IL-1β* and b) *IL-8* in the air sacs of birds treated

857 with ≤10 µg TeRu4, TeBi1, or PeNi4. Each data point represents the air sac of a single bird. The

858 light, moderate, and heavy infection groups were compared to the non-infected group (None)

859 using the Wilcoxon rank-sum test. Significance ($p \leq 0.05$) relative to non-infected group is

860 indicated by an asterisk.

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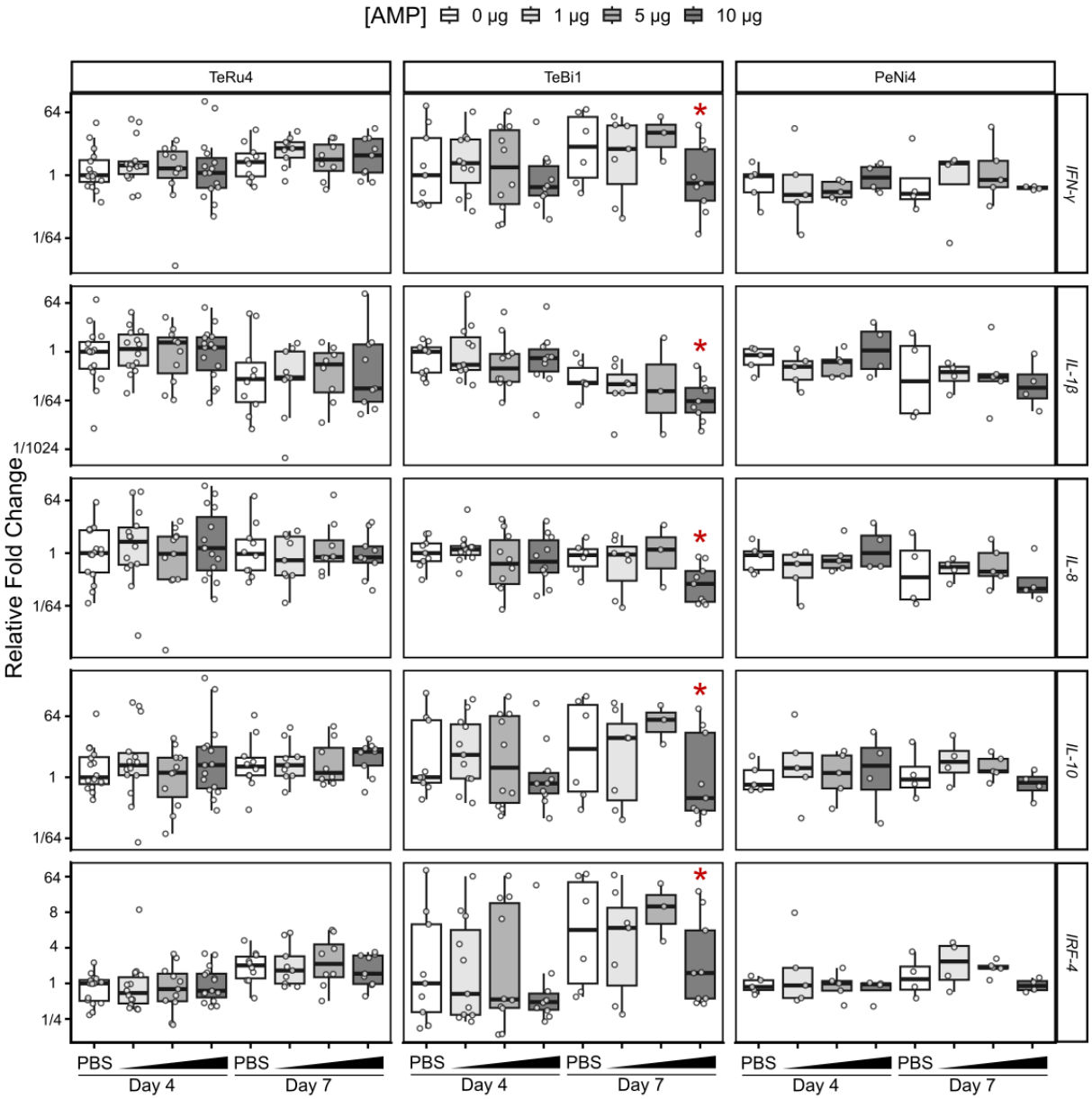
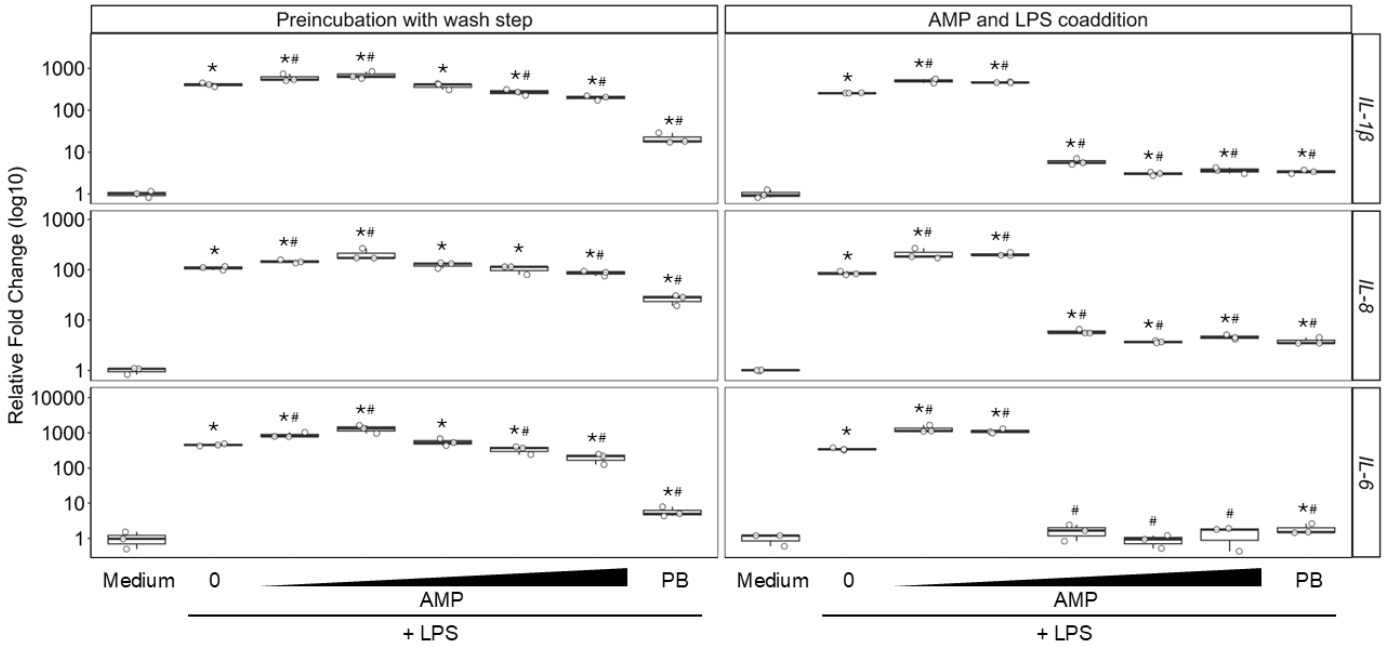


Figure 3. Relative fold change values of cytokine transcripts in the air sac of APEC challenged birds treated before hatch with 1, 5, or 10 µg AMP. Birds were euthanized on day 4 or 7 after hatch, and all values are relative to the PBS median on their respective days. Asterisks indicate significance at $p \leq 0.1$. *IFN*, interferon; *IL,* interleukin; *IRF*, interferon regulatory factor.



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869 **Figure 4.** Relative fold change of *IL-1β*, *IL-8*, and *IL-6* from HD11 cells as determined by qPCR
870 in the "preincubation with wash step" (left column) and "AMP and LPS coaddition" experiments
871 (right column). In the preincubation with wash step experiment, cells were incubated for 3 h with
872 medium, a range of 2-fold serially diluted AMP (1 to 16 µg/mL TeRu4), or 8 µg/mL polymyxin
873 B sulfate (PB), followed by three washes prior to the addition of 25 ng/mL LPS for an additional
874 3 h. In the coaddition experiment, cells were incubated for 3 h with medium, or with 25 ng/mL
875 LPS mixed with a range of 2-fold serially diluted AMP (1 to 16 µg/mL TeRu4), or 8 µg/mL
876 polymyxin B sulfate (PB). A significant difference from the medium control is indicated by "*",
877 and "#" indicates a significant difference from LPS treatment (p-value < 0.1, Mann-Whitney-U
878 test). Open circles represent the fold change of one technical replicate relative to the medium
879 control. The horizontal lines represent the median of the three technical replicates within each
880 treatment condition, the boxes show the interquartile range, and the vertical whiskers represent

881 the minimum and maximum values. Shown are representative experiments of three biological

882 replicates.

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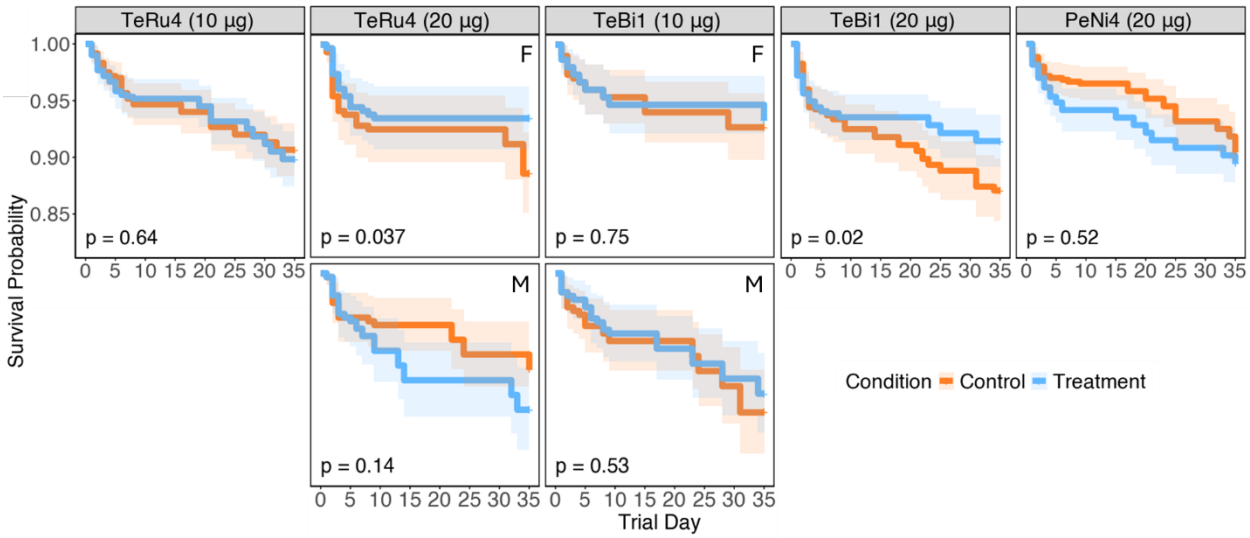

886

887 **Figure 5.** Kaplan-Meier curves depicting the survival probabilities of experimental groups

888 during pen trials across different AMPs and dosages. Columns represent AMPs at specific

889 dosages and each AMP–dosage combination corresponds to a separate pen trial. Sex-specific

890 differences in survival probabilities were observed in the TeRu4 (20 µg) and TeBi1 (10 µg)

891 trials. For these trials, males and females were analyzed separately, and each panel is labeled

892 with "F" for females or "M" for males in the top right corner to indicate the sex of the birds

893 included in the analysis. P-values, as determined by Log-rank tests, are displayed in the bottom-

894 left corner of each panel.

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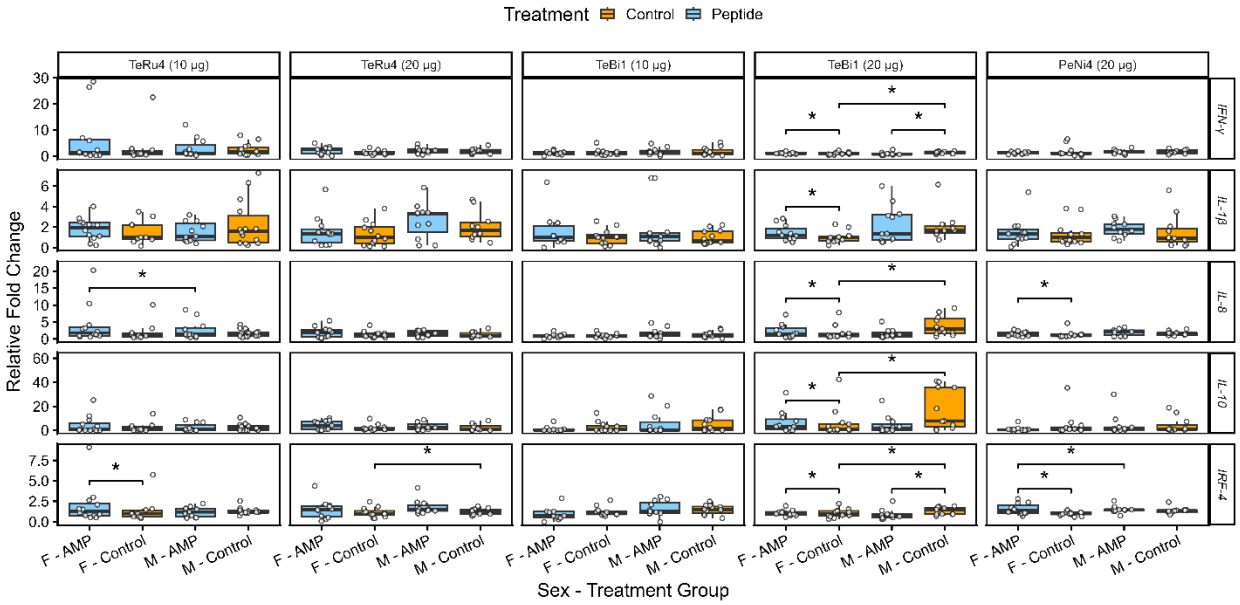

Figure 6. Relative fold change values of cytokine transcripts in the spleens of pen trial birds (no pathogen challenge) treated with 10 or 20 µg TeRu4, TeBi1, or PeNi4. Birds were euthanized 10 days after hatch. Asterisks indicate significance at $p \leq 0.1$. F, female birds; M, males birds. Additional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