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Efficacy and safety evaluation of artificial intelligence-identified antimicrobial peptides targeting avian pathogenic *Escherichia coli* in broiler chickens

Emre Demirsoy[1,2,3†], Teagan I. Parkin[4†], Shaeleen E. Mihalynuk[4], Anna H. Dema[4], Lorissa Corrie[4], Marika E. Heilker[4], Haley N. Kuecks-Winger[4], Anat Yanai[5,6], Uluc B. Birol[6], Michael McIlwee[7], Kay de Wet[7], Mathijs Knipscheer[7], Victoria Bowes[7], Vanessa Tuytel[7], Liam Ritchie[7], Wolfgang Köster[8], Emil Berberov[8], William R. Cox[6], Monica Kotkoff[5], Vanessa C. Thompson[4], René L. Warren[1], Erin Fraser[9,10], Linda M. N. Hoang[2,11], Fraser Hof[12], Fatih Birol[6], Caren C. Helbing[4] and Inanc Birol[2,3,5,6,13*]

Abstract

Background 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.

Results From a prior machine-learning-guided screen of 875 candidate AMPs against a wide bacterial panel, 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 d 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 d. For challenged birds, TeBi1 (10 µg/egg) significantly reduced culture-positive rates for APEC in the air sac and pericardium, increased body weight by 50% and reduced cytokine transcript levels by 10%–30% on d 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 d 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 d 35. By integrating high-throughput in ovo automation with large-scale commercial pen trials, this study provides a systematic translational bridge from in silico AI discovery to field-relevant poultry production interventions.

†Emre Demirsoy and Teagan I. Parkin contributed equally to this work.

*Correspondence:
Inanc Birol
inanc.birol@ubc.ca
Full list of author information is available at the end of the article

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Conclusions These findings demonstrate that TeBi1 and TeRu4 are promising antibiotic alternatives that improve survival, modulate immune responses, and maintain normal growth performance in broiler chickens in this experimental setting.

Keywords Antimicrobial peptide, APEC, Broiler chicken, Efficacy, Immunomodulation, Safety, Transcriptomics

Background

Avian pathogenic *Escherichia coli* (APEC) is a Gram-negative bacterium that poses a severe threat to the poultry industry. APEC infections induce fatal bacterial septicemia and early chick mortality (ECM), a significant cause of mortality and morbidity across multiple poultry commodities [1–6]. 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 [7]. However, misuse or overuse of antibiotics has contributed to the rise of antibiotic-resistant bacteria in both veterinary and human medicine [7, 8]. To address this challenge, regulatory, veterinary, and poultry industry policy changes in Canada and in other jurisdictions have been instituted to limit the use of antimicrobials for growth promotion purposes and, also, to limit the use of medically important antimicrobials [7, 9, 10]. This has resulted in fewer antimicrobial options being available to control pathogens in poultry flocks [7]. 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 [11]. Unfortunately, novel antibiotics are not approved at a pace sufficient to combat developing antibiotic-resistant bacteria [8]. Moreover, the last new class of antibiotics, oxazolidinones, was discovered with linezolid in 1987 and subsequently approved by the United States Food and Drug Administration (FDA) in 2000 [12, 13]. Consequently, there is growing interest in alternative antimicrobial strategies, such as antimicrobial peptides (AMPs), to address this therapeutic gap.

AMPs are peptide-based biomolecules composed of 5 to 100 amino acids and are typically cationic [14]. In nature, they are produced by the innate immune system and act as the first line of defense against infections in a wide variety of organisms, including humans, animals, and plants [12, 14]. Most conventional antibiotics have specific cellular targets, including cellular machinery involved in DNA and protein synthesis and components of bacterial cell walls [14]. In contrast, AMPs primarily cause rapid cell death by binding to anionic bacterial cell membranes and disrupting their integrity, leading to cell lysis [12, 14]. AMPs typically do not target eukaryotic cells, as their membranes usually have low anionic charge [14]. However, at higher concentrations, some AMPs may exhibit cytotoxic effects on eukaryotic cells [14]. Therefore, rigorous safety evaluation of AMPs is essential before regulatory approval, including in vitro assays, such as hemolysis and cytotoxicity tests, as well as in vivo experiments.

Many AMPs exert less selective pressure on bacteria than conventional antibiotics due to their nonspecific targets and diverse mechanisms of action, thus potentially reducing the development of antimicrobial resistance [8, 15–17]. In addition to their direct antimicrobial activity, some AMPs demonstrate immunomodulatory properties, recruiting immune cells or regulating cytokine levels to enhance the host response to infection [8, 18]. Cytokines are important signaling molecules that facilitate the growth, differentiation, and activation of immune cells [19]. In the present study, the effect of AMPs on the expression of the following cytokines was investigated: Interleukin (IL)-1β, IL-8, IL-6, IL-10, and interferon gamma (IFN-γ), as well as the transcription factor interferon regulator factor 4 (IRF-4). IL-1β, IL-8, and IL-6 are associated with activation of inflammation in chickens [19, 20]. Furthermore, IL-8 recruits heterophils to the infection site [20]. Conversely, IL-10 plays a role in tissue repair, angiogenesis, and attenuation of the inflammatory response and is an inducible feedback regulator of the immune response in chickens [19, 21, 22]. The roles of IRF-4 and IFN-γ are not well-characterized in chickens; however, there is evidence that IRF-4 plays a critical role in B cell development and maturation, similar to its mammalian counterpart [23], while IFN-γ generally functions to bridge the adaptive and innate immune systems [24, 25].

Previously, we screened 875 AMP candidates, identified using our artificial intelligence (AI) based prediction tools: AMPlify [26], rAMPage [27], and AMPd-Up [28], and used in vitro assays to characterize their antibacterial, hemolytic, and cytotoxic properties. While these computational tools have significantly accelerated the discovery of AMPs, a critical bottleneck remains in demonstrating whether AI-identified AMPs maintain their efficacy and safety when transitioned from in vitro assays to the complex, large-scale environment of commercial poultry production. In this study, we selected three lead AMPs (named TeBi1, TeRu4, and PeNi4) for further testing, due to their high antimicrobial activity and low toxicity. The three AMPs were tested in vivo as a prophylactic treatment prior to APEC inoculation (challenge trials).

Immunomodulatory effects of these AMPs were tested in tissue harvested from animals involved in the challenge trials and in HD11 cells, a chicken macrophage-like cell line. Lastly, the AMPs were tested for their safety and efficacy against ECM in a commercial production setting (pen trials). Overall, TeRu4 demonstrated activity in HD11 cell culture, while both TeBi1 and TeRu4 showed efficacy in challenge trials and maintained bird safety in commercial production settings, supporting their potential for future translational studies.

Materials and methods

In silico peptide selection

Candidate AMP sequences were predicted using three machine-learning-based software programs developed by our team: AMPlify, rAMPage, and AMPd-Up [26–28]. Candidate AMPs were evaluated for potential cytotoxicity using tAMPer [29], a machine-learning-based toxicity prediction tool, and those predicted to be cytotoxic were excluded from further evaluation.

Peptide synthesis

All AMPs for this study were synthesized by GenScript (Piscataway, NJ, USA) via solid-phase synthesis. The identities of the AMPs were confirmed by electrospray ionizing mass spectrometry (ESI–MS) while the minimum AMP purity specification was set at ≥ 85% for all peptides, as determined by the vendor using analytical HPLC. For in vitro assays and APEC challenge trials, AMPs were synthesized in 80 µg aliquots, while AMPs selected for pen trials were synthesized in 125, 250, or 500 mg aliquots. All aliquots were stored at −20 °C.

Bacterial species and growth

APEC strain EC317 [30] (clinical turkey isolate, obtained from VIDO-InterVac, Saskatchewan, CA) was used as the bacterial target for AMPs. Glycerol stocks were stored at −80 °C. The primary streaks were done by plating the stocks on 5% Columbia blood agar (Thermo Fisher Scientific, Waltham, MA, USA) and incubating for 20–24 h at 37 °C under aerobic conditions, as described by Richter et al. [8]. The secondary streaks were done by transferring 2–3 colonies from the primary streak on a new 5% Columbia blood agar plate. Colonies from the secondary streak were used for downstream analysis. Bacterial growth medium was prepared by dissolving 10.5 g Mueller Hinton Broth (Sigma-Aldrich, St. Louis. MO, USA) powder in 500 mL distilled water and autoclaving for sterility.

In vitro lead peptide selection

Minimum inhibitory concentrations (MICs) of candidate AMPs were determined in vitro against EC317, using antimicrobial susceptibility tests (ASTs) as previously described [8]. For the selection process, candidate AMPs with MICs ≤ 32 µg/mL against EC317 were designated as hit AMPs and selected for further in vitro testing. Following the protocol by Richter et al. [8], hemolytic activities of hit AMPs were evaluated in porcine red blood cells (RBCs). Porcine RBCs were used due to limited availability of high-quality fresh chicken RBCs during the study period. AMPs with low hemolytic activity ($HC_{50} \geq 128$ µg/mL, where HC_{50} is the AMP concentration that lyses 50% RBCs) were subsequently tested for their potential cytotoxicity to human kidney HEK293 cells according to the protocol described [8]. AMPs with $CC_{50} \geq 128$ µg/mL (CC_{50}, the AMP concentration that reduces cell viability to 50%) were designated as lead AMPs (Table 1). Additional in vitro assays were conducted on a panel of *E. coli* isolates and SL-29 chicken embryonic fibroblast cell line to evaluate the antimicrobial activity and cytotoxicity of selected peptides (Table S2).

Care and use of animals

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

Table 1 Physicochemical properties and in vitro activities of three lead AMPs TeRu4, TeBi1, and PeNi4

AMP	Sequence length	Net charge (+)	Amino acid sequence	EC317 MIC, µg/mL	RBC HC_{50}, µg/mL	HEK293 CC_{50}, µg/mL
TeRu4	30	8	SWLSKSVKKLVNKKNYTRLEKLAKKKLFNE	1.3	> 128	> 128
TeBi1	23	6	KIKIPWGKVKDFLVGGMKAVGKK	1.75	> 128	128
PeNi4	15	3	GLLGKVLGVGKKVLC	32	> 128	> 128

Listed for each AMP are their sequence length, net charge, and amino acid sequence. Each AMP was synthesized as the C-terminal carboxylic acid. In vitro activities include the antimicrobial activity against avian pathogenic *E. coli* (APEC) EC317 (minimum inhibitory concentration, MIC), the hemolytic activity against porcine red blood cells (RBCs) (hemolytic concentration 50%, HC_{50}), and cytotoxicity towards HEK293 cells (cytotoxic concentration 50%, CC_{50})

APEC challenge trials

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

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

The birds were given feed (Masterfeeds, Humboldt, SK, Canada) and water ad libitum. On d 4 after hatch, approximately half of the birds (between 8–17) were euthanized. The remaining birds were euthanized at 7 d after hatch. Birds were monitored two to four times per day for symptoms, and birds reaching a humane intervention point were euthanized. Only the birds euthanized on schedule on d 4 and 7 were included in the analyses. Bird survival was also tracked.

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

Immunomodulatory activity assays with HD11 cells

The effects of AMPs on in vitro cytokine production were assessed using an HD11 chicken semi-adherent macrophage-like cell line (obtained from VIDO-InterVac). HD11 cells were cultured in RPMI medium with 1X Glutamax and 10% Fetal Bovine Serum (Thermo Fisher Scientific) at 41 °C (normal chicken body temperature) and 5% CO_2 and were routinely passaged every 2 d.

Cell plating

HD11 cells were plated for experimentation between passages three to twelve. Suspended cells were collected with the growth medium, and adherent cells were obtained after washing with PBS and trypsinization with 0.25% Trypsin–EDTA in PBS (both from Thermo Fisher Scientific). The cells were centrifuged at 200 × *g* for 7 min and the pellet was resuspended in fresh medium. Trypan Blue (Thermo Fisher Scientific) was added to a sample of cells and live cells were counted on a Hausser Scientific Bright-Line Phase Hemacytometer (Fisher Scientific, Hampton, NH, USA). HD11 cells were plated at 2.5×10^4 cells per well on Nunclon Delta 96-well plates (Thermo Fisher Scientific) and incubated overnight.

HD11 experiment overview

In all experiments, HD11 growth medium was used to resuspend and dilute AMPs and other reagents, and all incubations were carried out at 41 °C and 5% CO_2. All experiments included a medium-only control and a lipopolysaccharide (LPS)-only control (final concentration of 25 ng/mL). In all experiments other than the AMP-only Screen, 8 μg/mL polymyxin B sulfate (PB) was used as a positive control as it is known to attenuate the LPS-triggered inflammatory response [31, 32]. Prior to treatment, the supernatant was removed from the wells, therefore only the adherent cell monolayer was used during the experiments.

Four experimental approaches were devised to investigate the immunomodulatory activity of AMPs. First, HD11 cells were treated solely with AMPs (AMP-Only Screen). Second, cells were treated with a range of AMP concentrations followed by treatment with LPS from *E. coli* O111:B4 (Sigma-Aldrich), to test if the AMPs could modulate the immune response triggered by LPS (AMP Preincubation Screen). If an AMP showed immunomodulatory activity in the AMP Preincubation Screen, it was then tested in the AMP Preincubation with Wash Step and the AMP and LPS Coaddition experiments.

AMP-only screen

HD11 cells were treated with 100 μL 16 μg/mL TeRu4, TeBi1, or PeNi4, and incubated for 6 h.

AMP preincubation screen

HD11 cells were treated with 100 μL of 1, 2, 4, 8, or 16 μg/mL TeRu4, TeBi1, or PeNi4, and incubated for 3 h. After the incubation, 100 μL LPS (50 ng/mL) was administered to the cells, resulting in a final concentration of 25 ng/mL LPS. The cells were incubated for an additional 3 h.

AMP preincubation with wash step

The cells were treated identically to the AMP Preincubation Screen but with the addition of a wash step prior to the addition of LPS. Briefly, after the first 3 h incubation, the medium (containing the AMP or PB) was removed from the wells and the cell monolayer was washed three

times using fresh medium. 100 μL LPS (25 ng/mL) was administered, and the cells were incubated for an additional 3 h.

AMP and LPS coaddition

AMP was resuspended and diluted to prepare a 2× concentration curve of 32, 16, 8, 4, and 2 μg/mL. PB and LPS were prepared at 2× concentrations of 16 μg/mL and 50 ng/mL, respectively. At regular time intervals, 2× AMP or 2× PB was added in equal volume to 2× LPS to yield a 1× mixture of AMP or PB, and LPS. Mixtures were vortexed for 1 min and incubated for 30 min prior to being administered to cells at regular time intervals in the order in which they were made. The cells were incubated for 3 h.

Cell harvest and RNA isolation

Supernatant was removed from HD11 cells after the final incubation. Buffer RLT from the miRNeasy Micro Kit (Qiagen, Mississauga, ON, Canada) with 2 mol/L DL-dithiothreitol (Sigma-Aldrich) was added to lyse adherent cells at a volume of 130 μL per well, with each technical replicate comprising two wells in the 96-well plate. HD11 cells in lysis buffer were disrupted by pipetting up and down, vortexed, and stored at −80 °C until RNA isolation. In total, three technical replicates per treatment were collected for RNA isolation and qPCR analysis.

Commercial pen trials

Each pen trial examined the efficacy against ECM and the safety of a single AMP. Cobb 500 or Ross 308AP broiler chicken breeds were used in pen trials (Table S1). All breeds were sourced from a commercial hatchery (Rossdown Farms & Natural Foods, Abbotsford, BC, Canada), and the specific breed used in each trial is summarized in Table S1. Of the seven pen trials, five were full-duration (35 d) pen trials and two were "Hatch Only" pen trials. The "Hatch Only" pen trials were conducted specifically to record hatchability and early-stage viability outcomes for AMP-dosage pairs where these parameters were not captured during the corresponding full-duration pen trial. For all pen trials, birds were sourced from older breeder flocks (a different flock for each trial).

AMPs were solubilized in 400 mL sterile diluent (SD) bags, obtained from the hatchery, to reach a concentration of 0.4 or 0.2 mg/mL, except in the 20 μg TeRu4 trial where PBS was used instead. An AMP-free SD bag was used for control. The AMP and control solutions were then taken to the hatchery, for in ovo injections on d 18 post-fertilization, where 50 μL AMP or SD solution was injected into the air sac of 1,680 to 2,352 eggs using the Embrex Inovoject injection machine (Zoetis Inc., Parsippany, NJ, USA). After the injections, eggs were incubated for 3 d. On the hatching day (d 0), pips, culls, late-stage embryo mortalities (deaths occurring between d 15 and 21 of incubation), post-hatch mortalities, and nonviable eggs/embryos (total of pips, culls, late-stage embryo mortalities, and post-hatch mortalities) were counted and recorded. For "Hatch Only" pen trials, the experiment concluded at this stage.

For the full-duration pen trials, the birds were then transferred to SJ Ritchie Research Farms (Abbotsford, BC, Canada) where they were cloacally sexed by professionals and then separated by sex and treatment. A total of 24 mini pens, within six blocks, were set up. The birds were weighed individually, then 50 birds were placed into each mini pen according to their sex and treatment, with each block containing each of the four experimental groups in a random order. Birds were provided ad libitum access to water and an all-vegetable, raised without antibiotic (RWA) feed (Trouw Nutrition, Chilliwack, BC, Canada). A step-down lighting program was implemented: from d 0 to d 21, birds experienced 6 h darkness per 24 h cycle; from d 21 to 28, 4 h darkness; and from d 28 to the end of the trial, 1 h darkness per day. Number of birds in each mini-pen was reduced to 12 on d 10 to prevent overcrowding of the mini-pens. Birds were checked three times per day throughout the trial and were weighed individually on d 10, 21, and 35. Any mortality during the pen trial was noted.

qPCR analyses

Multiplex qPCR assays were designed and separately validated for three chicken tissues: air sac, cecal tonsil, and spleen; and for the HD11 macrophage-like cell line. Primers and probes were designed for the normalizer genes ribosomal protein L8 (*rpl8*) and ribosomal protein S10 (*rps10*); the cytokine genes *IL-1β, IL-6, IL-8, IL-10,* and *IFN-γ*; and the gene for the transcription factor *IRF-4*. The primer and probe sequences are reported in Table S3.

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

The concentration of RNA from tissue samples was measured by NanoDrop (NanoDrop 1000 V3.8.1; Thermo Fisher Scientific) and diluted to a concentration

of approximately 100 ng/μL before complementary DNA (cDNA) synthesis, while RNA from HD11 cells was used neat due to its low concentration. All RNA samples were converted to cDNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), per the manufacturer's instructions.

cDNA from HD11 cells and chicken tissues was diluted 1/20 after synthesis and analysed by their respective qPCR assays with reaction conditions outlined in Table S4. All samples and controls were run in technical quadruplicates, and all plates included a non-template (negative) control and inter-plate standard as a positive control. Standard deviations between the Cycle Threshold (Ct) values of technical replicates were computed. If any deviation was greater than 0.5 cycles, the outlier value in that set of replicates was removed. Normalizer stability was verified by BestKeeper [33]. Mean values between technical replicates were taken into the next stage of analysis. Data was normalised against the geometric mean of *rpl8* and *rps10*, and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method, as previously described [34]. Final relative fold changes for each sample and cytokine were determined by comparing to the relevant control: median of the PBS-treated birds from d 4 (for challenge trials), median of the PBS-treated female birds (for pen trials), or the mean of medium-treated HD11 cells (for cell culture experiments).

Statistical analyses

Statistical analyses of the qPCR results were conducted in R version 3.6.3 [35]. The data were tested for normality by the Shapiro–Wilk and Levene's tests. They were then subjected to pairwise analyses by the Kruskal–Wallis and Wilcoxon rank-sum tests for non-parametric data with a significance threshold of $\alpha = 0.1$. For the challenge and pen trials, this significance cut off was selected due to the high variability of in vivo data. For the HD11 experiments, this threshold was appropriate as the smallest *P* achievable using a rank-sum test with $n = 3$ was approximately 0.08.

The rest of the challenge trial data was analyzed in R version 4.4.0 and 4.5.1 [35]. Hatchability rates of treated eggs compared to control eggs, as well as rates of bacterial detection in the birds' air sacs and pericardia, were evaluated using the Fischer's exact test. The presence or absence of bacteria detected in the birds' air sacs and pericardia was also compared to AMP dose using a Cochrane-Armitage trend analysis on both d 4 and 7. Cumulative weight gain of birds euthanized on d 4 and 7 was evaluated using a Wilcoxon rank-sum test, and survival was graphed and evaluated using the log-rank test and the "survminer" package [36]. Other challenge trial graphs and HD11 graphs were constructed using the packages "tidyverse", "readr", "janitor", "superb", "ggsignif", "rstatix", "DescTools", and "ggpubr" [37–45].

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

Statistical analyses of hatchability, survival, mean weights, feed conversion ratio, and flock uniformity in pen trials were conducted using R (version 4.1.3) and the packages: "survival", "survminer", "openxlsx", "readxl", "tidyverse", "zoo", "car", "ggplot2", "ggsignif", and "MHTdiscrete" [36, 40, 44, 46–52]. For pen trials, statistical comparisons between treatment and control groups used Wilcoxon rank-sum tests (hatchability and production parameters) and log-rank tests (survival), with production and survival analyzed separately by sex. *P* were corrected for multiple comparisons using the Šidák method.

Results

In vitro peptide selection

The 875 candidate AMPs identified by rAMPage [27], AMPlify [26], and AMPd-Up [28] were previously tested for antimicrobial activity and cellular toxicity in vitro [8]. Of these, 331 with MICs $\leq$ 32 μg/mL against EC317 were designated as hit AMPs. Among hit AMPs, 275 exhibited minimal hemolytic activity ($HC_{50} \geq 128$ μg/mL) against porcine RBCs. Of these, 62 also demonstrated minimal cytotoxicity ($CC_{50} \geq 128$ μg/mL) in HEK293 cells. These were classified as lead AMPs. Three representative lead AMPs (TeRu4, TeBi1, and PeNi4) were selected for further experiments. TeRu4 and TeBi1 demonstrated lower mean MIC values than PeNi4. The physicochemical properties of these AMPs and their in vitro activities are summarized in Table 1. These AMPs were evaluated in vitro using HD11 cells and in vivo using APEC challenge trials and pen trials.

qPCR primer design and validation

The sequences of the qPCR primers and probes, reaction conditions, and assay efficiencies are reported in Tables S2–S4. Primer efficiencies ranged from 93%–108% in the air sac, 92%–107% in the cecal tonsil, 87%–107% in the spleen, and 84%–101% in HD11 cells.

APEC challenge trials

To assess the prophylactic efficacy of TeBi1, TeRu4, and PeNi4 in chickens, fertilized eggs were injected in ovo at d 18 of incubation with either 1, 5, 10, or 20 μg AMP, or a PBS control. One day after hatch, the birds were

challenged with 5×10^4 CFU APEC strain EC317 by yolk sac injection.

Egg hatchability was between 90%–98% in all treatment groups, with no significant differences between treated birds and control birds (Table S6). Notably, when birds were treated with 20 µg AMP (Fig. S1), mortality occurred earlier in treated birds than controls within the first few days of post-infection, though cumulative survival by d 7 did not differ significantly between groups. Because of this high mortality rate, subsequent challenge trials tested the AMPs on a dose curve at 1, 5, or 10 µg. In the latter trials, there were no significant differences in survival probability with any dose of AMP (≤10 µg) compared to control (Fig. S2).

Among APEC-challenged birds, those treated with 1 or 5 µg of TeBi1 were approximately 25% heavier than control birds ($P=0.03$ and $P=0.07$, respectively, Fig. 1a), and birds treated with 10 µg TeBi1 were approximately 50% heavier than control birds ($P=2\times10^{-4}$) on d 7 post-hatch. This weight preservation likely reflects reduced disease burden rather than direct growth promotion, as no weight differences were observed in unchallenged pen trial birds. There were no significant differences in weight gain in birds treated with TeRu4 or PeNi4 (Fig. S3).

On d 7, birds treated with 10 µg TeBi1 had significantly lower rates of infection in both the air sac ($P=0.01$) (Fig. 1b) and pericardium ($P=0.03$) (Fig. 1c). Increased peptide dose was also generally correlated with decreased numbers of birds with detectable bacteria in either the air sac or the pericardium ($P=0.02$ and $P=0.008$, respectively, also on d 7). There were no significant differences in bacterial detection rates in the air sacs and pericardia of birds treated with TeRu4 or PeNi4 (Fig. S4), but for TeRu4-treated birds on d 4, there was a slight but similar trend of decreased numbers of infected birds with increasing AMP dose (air sac: $P=0.009$, pericardium: $P=0.1$), and for PeNi4, on d 4, in the air sac only, there was a slight trend of increasing numbers of infected birds with increased AMP dose ($P=0.1$).

Increased bacterial load in the air sac was correlated with increased levels of transcript abundance of certain cytokines in the air sac (Fig. 2), confirming that the APEC challenge model induces inflammatory responses. Across all three challenge trials, birds with light, moderate or heavy levels of active APEC infection in their air sacs had significantly higher relative abundance of *IL-1β* transcripts ($P=2\times10^{-4}$, 1×10^{-6}, and 7×10^{-13}, respectively) than birds with no bacterial growth in their air sacs (Fig. 2a). They are also positively correlated by Spearman's rho, with $\rho=0.58$ and $P=1\times10^{-19}$. The same trend, though weaker, is observed with *IL-8*. While $\rho=0.32$ ($P=3\times10^{-6}$) overall, birds with light, moderate, or heavy levels of APEC infection also had significantly higher relative abundance of *IL-8* transcripts ($P=0.03$, 3×10^{-6}, and 5×10^{-6}, respectively) than birds with no bacterial growth in their air sacs (Fig. 2b).

In the cecal tonsil (Fig. S5), qPCR results show minimal changes due to treatment with PeNi4, with only a 2-fold increase in the inflammatory cytokine *IL-8* in the 10 µg group on d 4 ($P=0.1$); and with TeRu4, a 2-fold

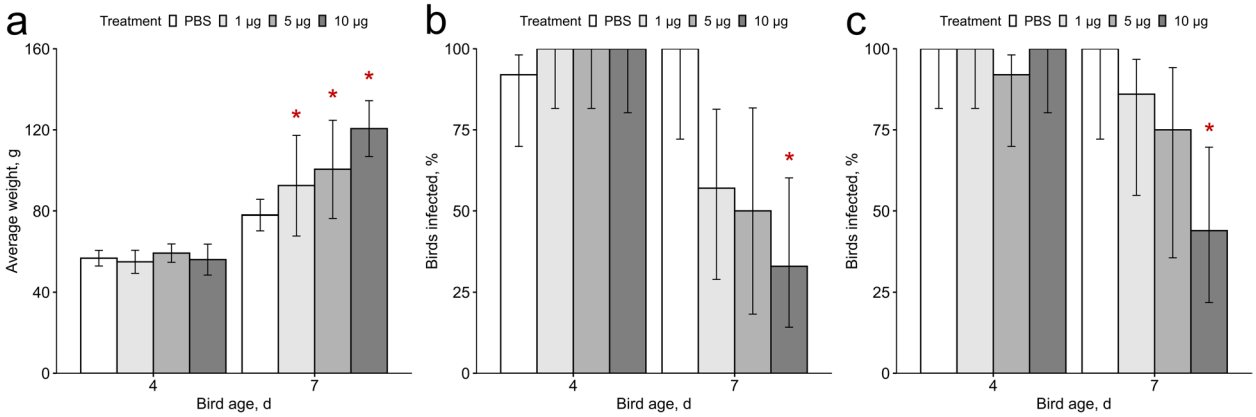


Fig. 1 Weight gain and bacterial detection rates in air sacs and pericardia in TeBi1 administered birds. **a** Weight gain of TeBi1 treated birds between d 4 and 7. Birds, euthanized on schedule, were weighed individually, and values shown are median ± median absolute deviation. On d 7, birds treated with 1 or 5 µg TeBi1 were approximately 25% heavier than control birds, and birds treated with 10 µg TeBi1 were approximately 50% heavier than control birds. Significance ($P\leq0.05$) relative to the phosphate buffered saline (PBS) control is indicated by an asterisk. **b** and **c** Percent of TeBi1 treated birds infected by avian pathogenic *E. coli* (APEC) on d 4 and 7 post-hatch in the **b**) air sacs and **c**) pericardia. Birds were euthanized as scheduled and aseptic swabs were taken from the tissues and streaked onto MacConkey agar. The percentage of birds in each treatment group that resulted in bacterial growth is shown, with the error bars denoting a 90% Wilson confidence interval. Each treatment group was compared to the PBS control by Fisher's Exact Test. Significance ($P\leq0.05$) relative to the PBS control is indicated by an asterisk. On d 7, there were significantly fewer birds with detectable bacteria in the 10 µg TeBi1 treatment group than the control group, in both tissues

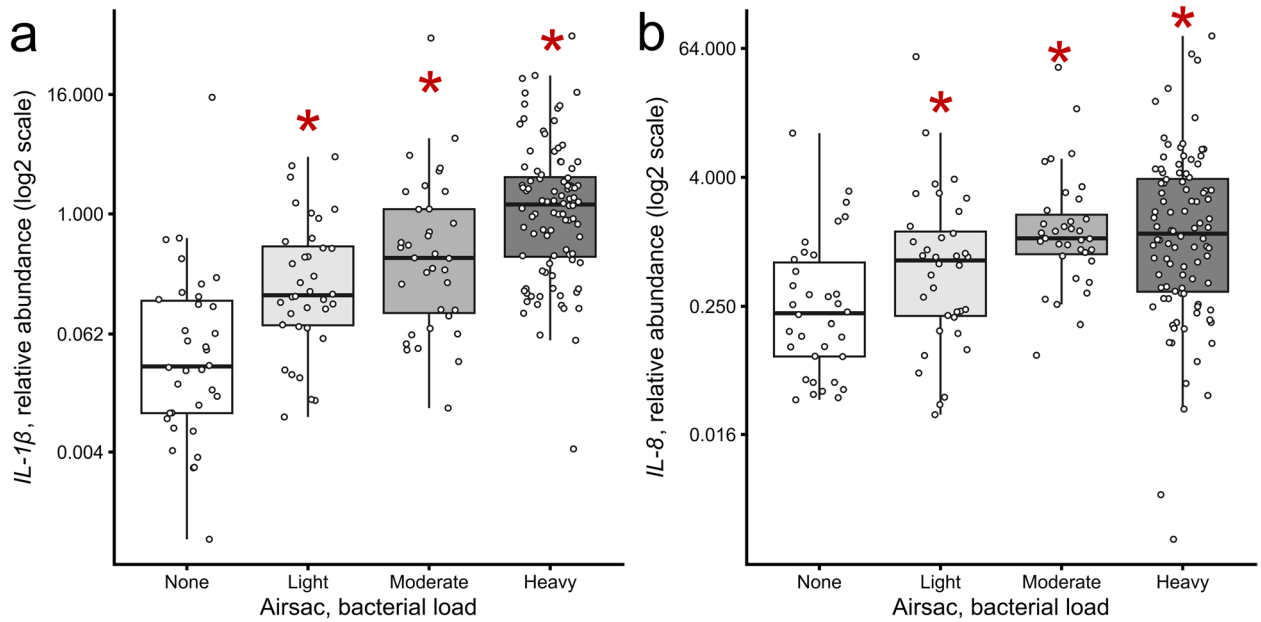


Fig. 2 Relative transcript abundance of **a**) *IL-1β* and **b**) *IL-8* in air sacs versus bacterial load. These birds were treated with ≤ 10 µg TeRu4, TeBi1, or PeNi4. Each data point represents the air sac of a single bird. The light, moderate, and heavy infection groups were compared to the non-infected group (none) using the Wilcoxon rank-sum test. Significance ($P \leq 0.05$) relative to non-infected group is indicated by an asterisk

decrease in *IL-8* in the 1 µg group on d 4 ($P=0.1$). In contrast, there are numerous changes due to treatment with TeBi1, including a 2.4-fold increase in *IL-8* transcripts in the 5 µg group on d 4 ($P=0.1$), and a 5-fold decrease in *IL-8* transcripts in the 10 µg group on d 7 ($P=0.06$). *IFN-γ* expression increased 1.4- and 2.5-fold ($P=0.03$) in the 5 and 10 µg groups, respectively, on d 4, but decreased 1.4- and 1.1-fold ($P=0.03$ and $P=0.09$, respectively) in the 1 and 10 µg groups, respectively, on d 7. With respect to *IL-1β,* on d 7, the 5 µg TeBi1 group showed a 5-fold decrease ($P=0.07$). For the anti-inflammatory transcript *IL-10,* there is a 2.5-fold decrease ($P=0.07$) with 5 µg TeBi1 on d 7. Finally, TeBi1 treatment was also associated with changes in *IRF-4* transcripts: the 10 µg group was associated with a 1.7-fold increase on d 4 ($P=0.04$), and the 5 µg group was associated with a 1.5-fold increase on d 7 ($P=0.1$).

In the spleen (Fig. S6), fewer responses are observed. For TeRu4, there is a 2-fold decrease in *IL-8* transcripts on d 7 ($P=0.1$) after treatment with 1 µg. For PeNi4, there is a 5-fold increase in *IL-10* transcripts on d 7 ($P=0.09$) after treatment with 5 µg. For TeBi1, while there are no differences in the inflammatory cytokines *IFN-γ*, *IL-1β*, or *IL-8*, there is a 3-fold increase in *IL-10* transcripts on d 4 ($P=0.03$) after treatment with 10 µg. There are also multiple increases in *IRF-4* transcripts: on d 4, a 2-fold increase after treatment with 1 µg ($P=0.1$), a 3-fold increase after treatment with 5 µg ($P=0.1$); and on d 7, a 1.3-fold decrease after treatment with 1 µg ($P=0.1$).

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

HD11 experiments

In addition to challenge trials, the ability of TeRu4, TeBi1, and PeNi4 to influence the cellular immune response was tested in an HD11 cell model system. In all HD11 experiments ($n=21$), the LPS induction control resulted in a wide-ranging, but consistent increase in relative fold change compared to the medium control for *IL-1β* (14–677), *IL-8* (8–249), and *IL-6* (55–3,284) (Fig. 4 and Figs. S7 and S8). Except for the AMP-only experiment, all stated fold-changes are relative to the LPS induction control. The induction of cytokine transcripts by LPS was significantly attenuated by PB in all but a single cytokine measurement from one experiment ($n=15$), with fold change decreases compared to LPS alone ranging from 6–166 for *IL-1β*, 2–34 for *IL-8*, and 11–335 for *IL-6* (Fig. 4 and Fig. S8). The preincubation, preincubation with wash step, and coaddition experiments were performed in biological triplicate, and the AMP-only experiments in biological duplicate. While only one representative biological replicate is shown per experiment,

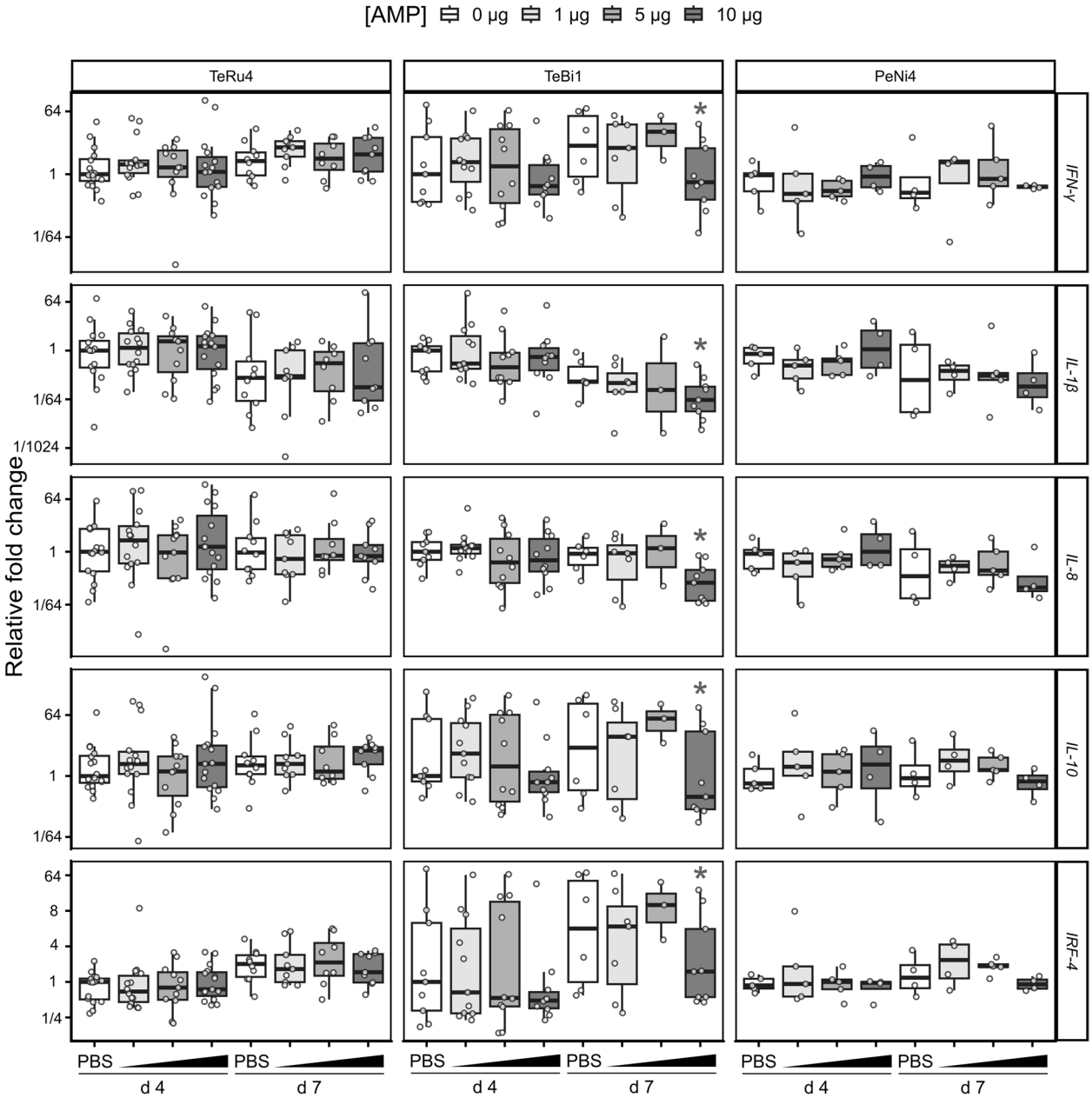


Fig. 3 Relative fold change of cytokine transcripts in the air sac of APEC challenged birds. Birds were treated before hatch with 1, 5, or 10 µg AMP and euthanized on d 4 or 7 after hatch, and all significance comparisons are relative to the phosphate-buffered saline (PBS) on their respective days. Asterisks indicate significance at $P \leq 0.1$. *IFN*, interferon; *IL*, interleukin; *IRF*, interferon regulatory factor

these selected replicates capture the overall trends in fold changes observed across replicates (unless otherwise stated). The fold change values stated are that of the representative replicate.

AMP-only screen of TeRu4, TeBi1, and PeNi4

To assess the ability of AMPs alone to induce cytokine production in HD11 cells, cells were incubated with 16 µg/mL of each AMP or 8 µg/mL PB, and cytokine levels were compared to a medium-only control. Treatment with TeRu4 resulted in a 2-fold increase in *IL-1β* (in one of two biological replicates) and 3-fold increase in *IL-8*, while TeBi1 lead to a 4-fold decrease in *IL-6* (Fig. S7). Treatment with PeNi4 did not significantly alter the abundance of any of the three cytokines, while TeBi1 had no significant effect on *IL-1β* and *IL-8*, and TeRu4 had

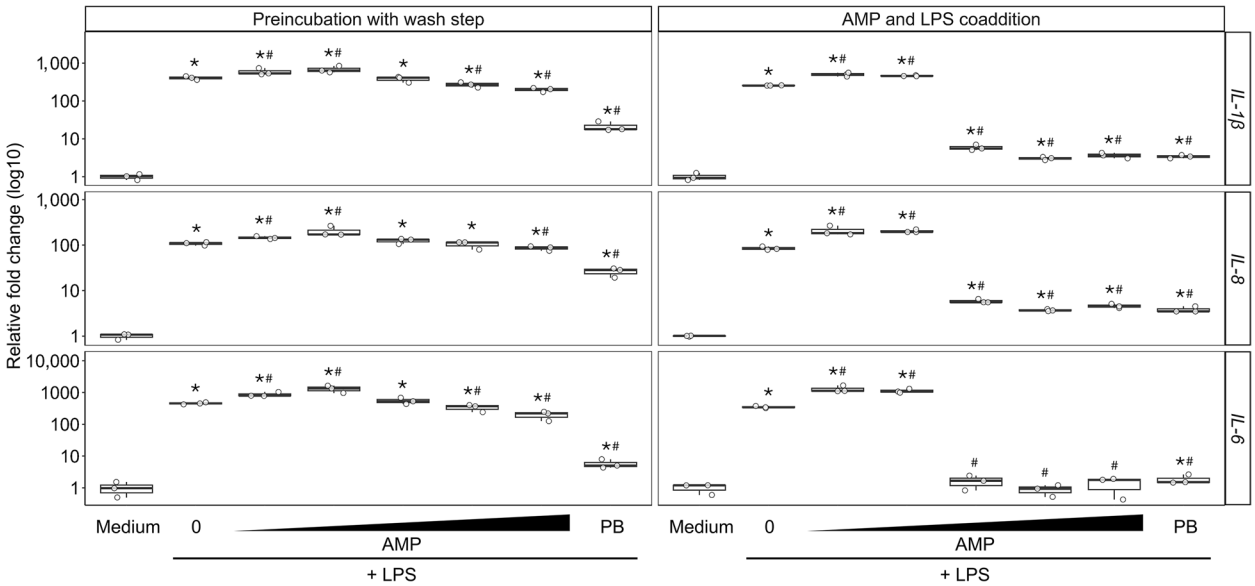


Fig. 4 Relative fold change of *IL-1β*, *IL-8*, and *IL-6* transcripts in HD11 cells treated with TeRu4. Results of the "preincubation with wash step" (left column) and "AMP and LPS coaddition" experiments (right column) are shown as determined by qPCR. In the preincubation with wash step experiment, cells were incubated for 3 h with medium, a range of 2-fold serially diluted antimicrobial peptide (AMP; 1 to 16 µg/mL TeRu4), or 8 µg/mL polymyxin B sulfate (PB), followed by three washes prior to the addition of 25 ng/mL LPS for an additional 3 h. In the coaddition experiment, cells were incubated for 3 h with medium, or with 25 ng/mL LPS mixed with a range of 2-fold serially diluted AMP (1 to 16 µg/mL TeRu4), or 8 µg/mL PB. A significant difference from the medium control is indicated by "*", and "#" indicates a significant difference from LPS treatment ($P < 0.1$, Mann-Whitney-U test). Open circles represent the fold change of one technical replicate relative to the medium control. The 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

no significant effect on *IL-6* (Fig. S7). Treatment with PB alone did not significantly or consistently change *IL-1β*, *IL-8*, and *IL-6* levels (Fig. S7).

Preincubation screen of TeRu4, TeBi1, and PeNi4

To examine if AMPs affect HD11 response to LPS, cells were preincubated with the designated AMP or PB, followed by treatment with LPS. Here and in subsequent experiments, medium only and LPS only served as negative and positive controls, respectively. A 2-fold decrease was observed for *IL-1β* and *IL-6* following preincubation with 8 µg/mL TeRu4 (Fig. S8). Preincubation with 16 µg/mL TeRu4 led to a significant reduction in *IL-1β*, *IL-8*, and *IL-6* levels by 13-fold, 4-fold, and 28-fold, respectively (Fig. S8). Similarly, treatment with 8 µg/mL PB significantly decreased *IL-1β*, *IL-8* (in 2 of 3 biological replicates), and *IL-6* abundance by 10-fold, 3-fold, and 20-fold, respectively (Fig. S8). While a significant 4-fold increase was observed for *IL-8* at 4 µg/mL TeRu4, this observation was only seen in two of three biological replicates (Fig. S8). Preincubation with TeBi1 and PeNi4 did not consistently induce a significant change in *IL-1β*, *IL-6*, and *IL-8* transcript levels across biological replicates (Fig. S8). The attenuation of LPS-induced responses of *IL-1β*, *IL-8*, and *IL-6* transcripts after TeRu4 preincubation prompted further investigation via the subsequent experiments to provide some insight into possible mechanisms of action.

Preincubation experiment with wash step

The effects on HD11 cells of transient exposure to TeRu4, followed by its removal and the addition of LPS, were examined. A significant decrease in *IL-1β*, *IL-8*, and *IL-6* transcripts remained after preincubation with 8 µg/mL PB (21-, 5-, and 80-fold respectively) and 16 µg/mL TeRu4 (2-, 1-, and 2-fold, respectively) (Fig. 4). Preincubation with 8 µg/mL TeRu4 induced a significant decrease in *IL-1β* levels by 2-fold and *IL-6* by 1-fold in two of three biological replicates (Fig. 4). Unlike in the previous preincubation experiment (without a wash step), treatment with 1 and 2 µg/mL TeRu4 followed by the wash step resulted in a 1- and 2-fold increase respectively in *IL-1β* and *IL-8* transcript levels (but not consistently in all biological replicates) (Fig. 4). For *IL-6* in this experiment, treatment with 1 and 2 µg/mL TeRu4 resulted in a 2- and 3-fold increase respectively across all three biological replicates (Fig. 4).

Coincubation of TeRu4 and LPS

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

Commercial pen trials

Pen trials were designed to test the efficacy against ECM and the safety of treating chickens with TeBi1, TeRu4, and PeNi4 in a commercial production setting. To account for differences in breed and trial design (e.g., "Hatch Only" vs. Full-duration pen trials), results for each trial were analyzed independently and are reported separately below. Late-stage viability (viable embryos from d 15 of incubation until placement in pens) was between 88% and 98% in all pen trials. No statistically significant differences were detected between the treatment and control groups (Fig. S9). There were no significant differences between the treatment and control groups for post-hatch mortality, culls, pips, and late embryo mortality.

For the first 10 d, there were no statistical differences between the survival probability of males and females (Fig. S10). Thus, the sexes were combined for subsequent analyses. No significant differences in survival probability were detected between treatment and control groups for any AMPs during the first 10 d of the pen trials, indicating that the tested AMPs had no measurable impact on ECM (Fig. S11). However, for the full duration of the pen trials (35 d), sex-specific differences in survival probabilities were observed for TeRu4 at 20 µg and TeBi1 at 10 µg (Fig. S12). For these two pen trials, the sexes were kept separate for analysis (Fig. 5 and Fig. S13). Notably, TeRu4 at 20 µg significantly increased the survival probability of female birds compared to the controls by approximately 4.9% by d 35 ($P=0.04$); this sex-specific effect was not observed in males and requires prospective validation. Moreover, TeBi1 at 20 µg significantly increased the survival probability of all birds compared to control by approximately 4.4% by d 35 ($P=0.02$); this benefit was not sex-specific unlike TeRu4. There was no other

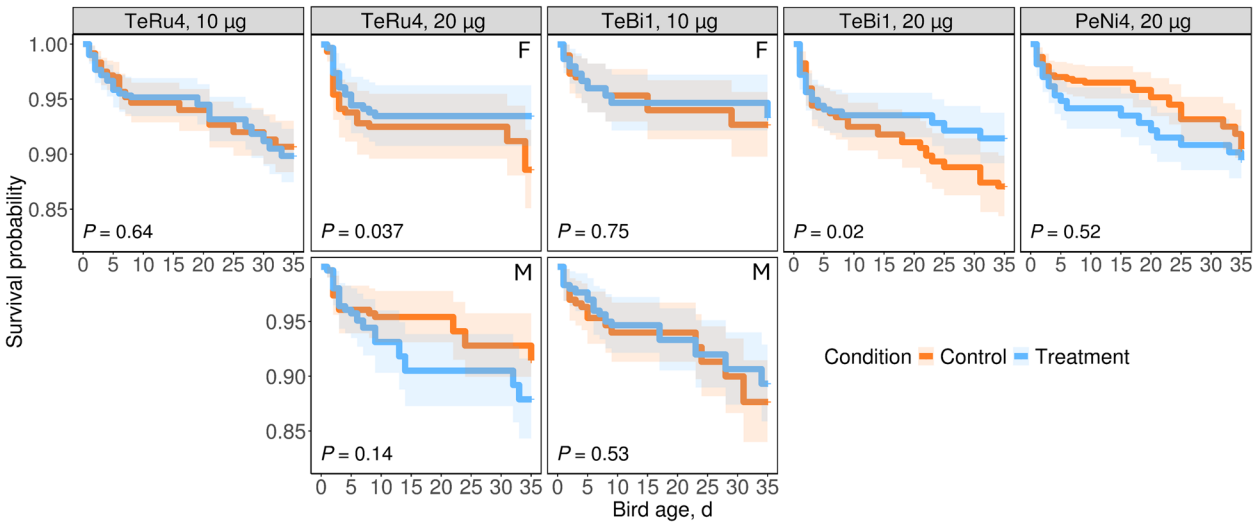


Fig. 5 Kaplan-Meier curves depicting the survival probabilities of groups during pen trials. Columns represent AMPs at specific dosages and each AMP-dosage combination corresponds to a separate pen trial. Sex-specific differences in survival probabilities were observed in the TeRu4 (20 µg) and TeBi1 (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

difference in survival probabilities between the treatment and control groups by d 35. In the 10 µg TeRu4 and TeBi1 trials, survival curves for both sexes closely overlapped (Fig. S13).

Significant differences in mean weight between treatment and control groups were observed only in the 20 µg TeBi1 trial (Fig. S14). Notably, on d 0, mean weights in the treatment groups were approximately 2.8% lower in males and 2.4% lower in females compared to controls ($P=0.02$ and 0.03). However, on d 10, no significant difference in mean weights were observed. Moreover, in the 10 µg TeRu4 trial, numerous birds of both sexes exhibited lower body weights on d 21 and 35, particularly in the treatment group, although this difference was not statistically significant. This observation may suggest subclinical effects of TeRu4 at lower doses, high biological variability among individual birds, or environmental effects, all of which warrant further investigation. By d 35, no significant differences in mean weight were detected (Fig. S14).

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

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

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

After treatment with 10 µg of TeRu4, female birds had a 1.8-fold increase ($P=0.1$) in *IFN-γ* transcripts relative

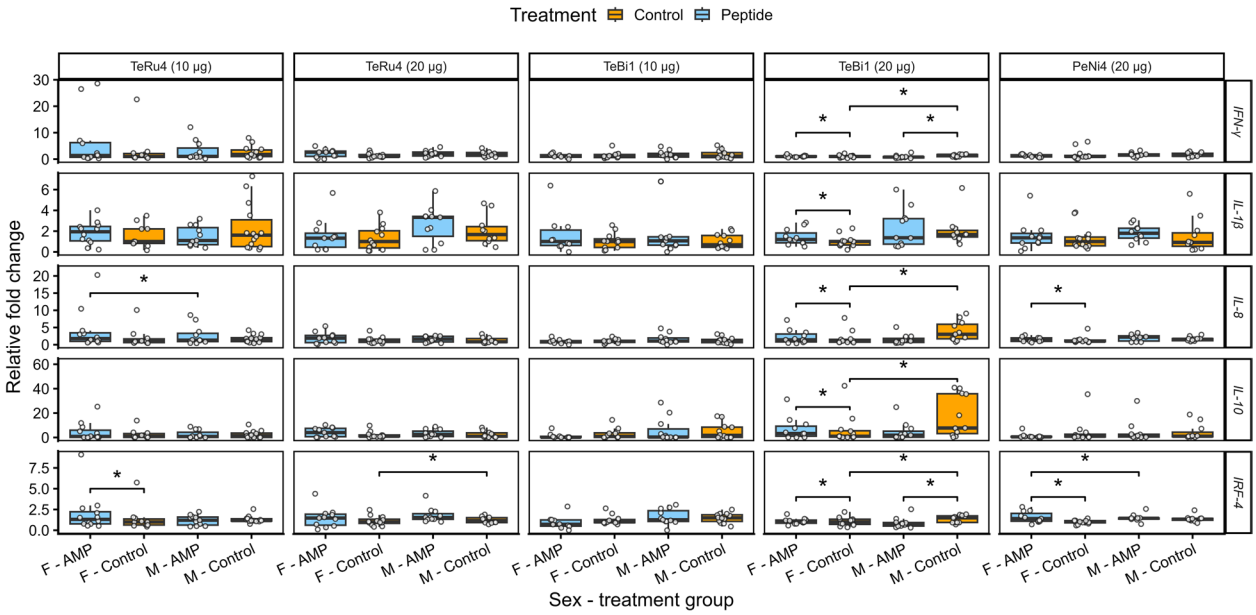


Fig. 6 Relative fold change of cytokine transcripts in the spleens of pen trial birds. These birds (no pathogen challenge) were treated with 10 or 20 µg TeRu4, TeBi1, or PeNi4. Birds were euthanized 10 d after hatch. Asterisks indicate significance at $P \leq 0.1$. F, Female birds; M, male birds. Additional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

to (female) control birds, and in that trial, male controls birds had a 1.2-fold increase ($P=0.1$) in *IRF-4* transcripts relative to female control birds. After treatment with 20 μg of TeRu4, male birds had a 1.3-fold increase ($P=0.08$) in *IRF-4* transcripts relative to control birds.

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

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

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

Discussion

The present study demonstrates the potential for machine-learning identified antimicrobial peptides to improve early health outcomes in broiler chickens while maintaining production performance under commercial conditions. Across complementary in vivo and in vitro assays, two of the three lead AMPs, TeBi1 and TeRu4, showed efficacy and immunomodulatory activity consistent with their intended roles as antibiotic alternatives for preventing APEC-associated diseases. Here, AI-assisted discovery is relevant for enabling large-scale candidate generation and triage that would be impractical by hand. However, extensive experimental validation in vitro, in vivo as well as in a commercial production environment remained essential, as in silico predictions cannot yet reliably predict real-world efficacy. All outcome data from the in vivo and in vitro assays were analyzed using nonparametric methods, Kruskal–Wallis for omnibus comparisons and Wilcoxon rank-sum for pairwise contrasts. Given the inherent variability of in vivo data and the exploratory nature of this study, a significance threshold of $\alpha=0.1$ was used, as specified in the methods.

First, in controlled challenge trials, pre-hatch administration of TeBi1 was associated with reduced post-hatch bacterial burden and improved early growth. At 10 μg per egg, TeBi1 significantly lowered culture detection of APEC in the air sac and pericardium by d 7 and yielded an approximate 50% increase in body weight relative to controls at the same time point. These findings support a direct antibacterial contribution in vivo and align with TeBi1's favorable in vitro profile [27]. Concordant decreases in pro-inflammatory cytokine transcripts in air sac tissue at d 7 (Fig. 3) further suggests that limiting bacterial load mitigates downstream inflammatory responses. In contrast, TeRu4 and PeNi4 did not reproducibly reduce organ-specific bacterial detection in the challenge setting at tested doses up to 10 μg, indicating peptide-specific differences in in vivo antibacterial activity despite their performance in in vitro experiments. Consistent with this, positive correlations between air sac bacterial loads and *IL-1β* ($\rho=0.58$) and *IL-8* ($\rho=0.32$) expression (Fig. 2) both support the validity of the infection model and indicate that AMP-mediated reductions in bacterial burden translate to dampened inflammatory signaling.

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

Third, pen trials provide critical evidence of field-relevant safety and performance. In ovo administration of TeBi1 or TeRu4 at 10 or 20 μg per egg did not adversely affect hatchability, FCR, or flock uniformity through d 35. Importantly, survival probabilities improved modestly but significantly in two settings: a 4.9% increase in females with 20 μg TeRu4 and a 4.4% increase in all birds with 20 μg TeBi1 by d 35. Although effect sizes were

small, they are operationally meaningful at commercial scale and were achieved without detectable adverse effects in growth or efficiency. These findings support a favorable benefit-risk profile for both peptides when delivered through standard in ovo automation.

Together, the in vivo and in vitro data outline complementary roles for these leads. TeBi1 appears to act primarily through direct antibacterial activity in the immediate post-hatch period, reducing tissue colonization and the associated inflammatory response. TeRu4 shows consistent anti-inflammatory activity in a macrophage model and over 35 d translates to a survival advantage in females at higher dose, suggesting that immunomodulation can contribute to resilience under farm conditions. These sex-specific responses may reflect differences in immune system maturation rates or hormone-immune interactions, as male broilers typically show different growth and immune development patterns than females [53, 54]. The patterns observed in both survival and cytokine transcript baselines warrant additional investigation into sex-dependent pharmacodynamics (PD), immune set-points, and potential interactions with breeder age and genetics.

While the results presented here are encouraging, this study also has several limitations. First, the challenge trials used a single APEC strain and a prophylactic in ovo regimen, limiting inference across heterologous strains and alternative administration strategies. Broader pathogen panels, additional exposure routes, and therapeutic or peri-hatch dosing schedules should be evaluated to define generalizability and optimal use cases. Because culture-based detection provides only semi-quantitative resolution, future challenge trials should incorporate CFU enumeration to more precisely characterize bacterial burden, as well as standardized lesion and pathology scoring. Second, critical gaps remain before regulatory submission, including pharmacokinetics (PK), peptide stability in the embryonic and early post-hatch milieu, and residue depletion profiles—all essential for establishing withdrawal periods and ensuring food safety. While our preliminary investigations demonstrated that TeRu4 and TeBi1 (2 mg/mL) maintain full antimicrobial activity in sterile distilled water when stored at 4 °C over 14 d (Fig. S18), more rigorous experiments are required to characterize their stability under physiological conditions. In addition, the absence of PK/PD and exposure–response data limits our ability to define a mechanistic basis for dose selection and to quantitatively interpret the safety window suggested by the early mortality observed at 20 μg. While mammalian cell surrogates provided a conservative initial screen corroborated by low toxicity in SL-29 chicken fibroblasts (Table S2), the early mortality at 20 μg/egg highlights the necessity of using such in vivo signals to refine the safety window beyond the limitations of in vitro models. Establishing peptide distribution, clearance, and exposure thresholds in ovo will be essential for refining dose ranges and confirming the biological rationale for future efficacy studies. Third, gene expression endpoints were measured at discrete time points and in a single cell line or selected tissues. High-resolution temporal profiling, including protein-level cytokine measurements and cellular phenotyping in multiple cell lines and tissues, would sharpen mechanistic inference, validate transcript-level patterns that served as early indicators, 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 could translate to substantial numbers at commercial scale, while early bacterial control reduces the need for subsequent therapeutic interventions. A formal cost–benefit analysis accounting for treatment costs, implementation expenses, and variable mortality baselines across production systems is needed to establish commercial viability. The complementary mechanisms of TeBi1 (direct antibacterial) and TeRu4 (immunomodulatory) suggest potential for combination strategies that leverage both activities, though this hypothesis requires experimental testing as peptide-peptide interactions and combined effects on immune signaling remain unpredictable. Future work should benchmark these AMPs against current antimicrobial interventions and animal husbandry standards to establish comparative efficacy and identify specific use cases where these AMPs provide advantages over existing approaches. These may include evaluation of alternative delivery formats, such as water supplementation during high-risk periods, to optimize protection throughout the production cycle.

Conclusions

In conclusion, the 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.

Abbreviations

AI	Artificial intelligence
AMP	Antimicrobial peptide
APEC	Avian pathogenic *Escherichia coli*
AST	Antimicrobial susceptibility test
CC_{50}	Concentration of cytotoxicity 50%
cDNA	Complementary DNA
Ct	Cycle threshold
CV	Coefficient of variation
ECM	Early chick mortality
FCR	Feed conversion ratio
FDA	United States Food and Drug Administration
HC_{50}	Concentration of hemolysis 50%
HD11	Chicken macrophage-like cell line
HEK293	Human embryonic kidney 293 cell line
IFN-γ	Interferon gamma
IL	Interleukin
IRF-4	Interferon regulator factor 4
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
PB	Polymyxin B sulfate
PBS	Phosphate-buffered saline
PD	Pharmacodynamics
PK	Pharmacokinetics
RBCs	Red blood cells
RWA	Raised without antibiotic
SD	Sterile diluent
qPCR	Quantitative polymerase chain reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40104-026-01417-8.

Additional file 1: Table S1. Antimicrobial peptide (AMP) and dosage tested on two chicken breeds. Table S2. Additional in vitro testing for select AMPs. Table S3. qPCR assay primer/probe sequences. Table S4. qPCR reaction conditions. Table S5. qPCR assay efficiencies by assay and tissue type. Table S6: Hatchability of eggs in APEC challenge trials. Fig. S1. Kaplan–Meier survival plots of chickens treated with 20 µg TeRu4, TeBi1, or PeNi4. Fig. S2. Kaplan–Meier survival plots of birds treated with either 1, 5, or 10 µg AMP. Fig. S3. Weight gain of chickens treated with TeRu4 and PeNi4. Fig. S4. APEC infection percentages in birds across multiple post-hatch time points and tissues. Fig. S5. Relative fold change of cytokine transcripts in the cecal tonsil of APEC challenged birds. Fig. S6. Relative fold change values of cytokine transcripts in the spleen of APEC challenged birds. Fig. S7. Relative fold change of *IL-1β*, *IL-8*, and *IL-6* transcripts lipopolysaccharide (LPS)-treated HD11 cells. Fig. S8. Relative fold change of *IL-1β*, *IL-8*, and *IL-6* transcripts AMP-preincubated HD11 cells. Fig. S9. Raw percentage values for hatchability parameters across various AMPs and dosages. Fig. S10. Kaplan–Meier curves survival plots of the bird sexes on d 10 of the pen trials. Fig. S11. Kaplan–Meier survival plots of control and treatment groups on d 10 of the pen trials. Fig. S12. Kaplan–Meier survival plots of the bird sexes on d 35 of the pen trials. Fig. S13. Kaplan–Meier survival plots of experimental groups on d 35 of the pen trials. Fig. S14. Mean weights of female and male birds during pen trials across different AMPs and dosages. Fig. S15. Feed conversion ratio (FCR) of male and female birds during pen trials. Fig. S16. Flock uniformity of female and male birds during pen trials across different AMPs and dosages. Fig. S17. Relative cytokine transcripts in the spleens of pen trial birds treated with 10 µg TeBi1. Fig. S18. Stability of TeRu4 and Tebi1 in sterile water over 14 d.

Additional file 2: Representative quality control data for AMPs.

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Authors' contributions

Conceptualization: EF, CCH, IB. Funding acquisition: EF, LMNH, FH, FB, CCH, and IB. Investigation: ED, TIP, SEM, AHD, HNKW, AY, UBB, MM, KW, MK, VB, VT, LR, WK, EB, WRC, VCT, CCH, IB. Methodology: SEM, LC, MEH, AY, WK, EB, WRC, VCT, EF, LMNH, FH, CCH, IB. Project administration: MK, FB. Supervision: CCH and IB. Visualization: ED, TIP, SEM, AHD, and HNKW. Writing – original draft: ED, TIP, SEM, AHD, and HNKW. Writing – review & editing: AY, UBB, MM, KW, MK, VB, VT, LR, WK, EB, WRC, VCT, RLW, EF, LMNH, FH, FB, CCH, IB.

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Data availability

All data generated or analyzed during the present study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

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

Consent for publication

Not applicable.

Competing interests

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

Author details

[1]BC Cancer, Vancouver, BC V5Z 1L3, Canada. [2]British Columbia Centre for Disease Control, Public Health Laboratory, Vancouver, BC V6Z R4R, Canada. [3]Genome Science and Technology Graduate Program, University of British Columbia, Vancouver, BC V6T 1Z4, Canada. [4]Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC V8P 5C2, Canada. [5]Canada's Michael Smith Genome Sciences Centre at BC Cancer, Vancouver,

BC V5Z 4S6, Canada. [6]Amphoraxe Life Sciences Inc., Vancouver, BC V6L 3C9, Canada. [7]SJ Ritchie Research Farms, Abbotsford, BC V4X 2N4, Canada. [8]Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan, Saskatoon, SK S7N 5E3, Canada. [9]Communicable Disease Prevention and Response, British Columbia Centre for Disease Control, Vancouver, BC V6Z R4R, Canada. [10]School of Population and Public Health, University of British Columbia, Vancouver, BC V6T 1Z3, Canada. [11]Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC V6T 1Z4, Canada. [12]Department of Chemistry, University of Victoria, Victoria, BC V8P 5C2, Canada. [13]Department of Medical Genetics, University of British Columbia, Vancouver, BC V6T 1Z3, Canada.

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References

1. Cuperus T, van Dijk A, Matthijs MGR, Veldhuizen EJA, Haagsman HP. Protective effect of in ovo treatment with the chicken cathelicidin analog D-CATH-2 against avian pathogenic *E. coli*. Sci Rep. 2016;6:26622. https://doi.org/10.1038/srep26622.
2. Allan B, Wheler C, Köster W, Sarfraz M, Potter A, Gerdts V, et al. In ovo administration of innate immune stimulants and protection from early chick mortalities due to yolk sac infection. Avian Dis. 2018;62(3):316–21. https://doi.org/10.1637/11840-041218-Reg.1.
3. Rezaee MS, Liebhart D, Hess C, Hess M, Paudel S. Bacterial infection in chicken embryos and consequences of yolk sac constitution for embryo survival. Vet Pathol. 2021;58(1):71–9. https://doi.org/10.1177/0300985820960127.
4. Nguyen TTT, Allan B, Wheler C, Köster W, Gerdts V, Dar A. Avian antimicrobial peptides: in vitro and in ovo characterization and protection from early chick mortality caused by yolk sac infection. Sci Rep. 2021;11:2132. https://doi.org/10.1038/s41598-021-81734-2.
5. Sarfraz M, Nguyen TTT, Wheler C, Köster W, Gerdts V, Dar A. Characterization of dosage levels for in ovo administration of innate immune stimulants for prevention of yolk sac infection in chicks. Vet Sci. 2022;9(5):203. https://doi.org/10.3390/vetsci9050203.
6. Hu J, Afayibo DJA, Zhang B, Zhu H, Yao L, Guo W, et al. Characteristics, pathogenic mechanism, zoonotic potential, drug resistance, and prevention of avian pathogenic *Escherichia coli* (APEC). Front Microbiol. 2022;13:1049391. https://doi.org/10.3389/fmicb.2022.1049391.
7. Bean-Hodgins L, Kiarie EG. Mandated restrictions on the use of medically important antibiotics in broiler chicken production in Canada: implications, emerging challenges, and opportunities for bolstering gastrointestinal function and health — a review. Can J Anim Sci. 2021;101(4):602–29. https://doi.org/10.1139/cjas-2021-0015.
8. Richter A, Sutherland D, Ebrahimikondori H, Babcock A, Louie N, Li C, et al. Associating biological activity and predicted structure of antimicrobial peptides from amphibians and insects. Antibiotics. 2022;11(12):1710. https://doi.org/10.3390/antibiotics11121710.
9. Wallinga D, Smit LAM, Davis MF, Casey JA, Nachman KE. A review of the effectiveness of current US policies on antimicrobial use in meat and poultry production. Curr Environ Health Rep. 2022;9(2):339–54. https://doi.org/10.1007/s40572-022-00351-x.
10. Schmerold I, Van Geijlswijk I, Gehring R. European regulations on the use of antibiotics in veterinary medicine. Eur J Pharm Sci. 2023;189:106473. https://doi.org/10.1016/j.ejps.2023.106473.
11. Destoumieux-Garzón D, Mavingui P, Boetsch G, Boissier J, Darriet F, Duboz P, et al. The one health concept: 10 years old and a long road ahead. Front Vet Sci. 2018. https://doi.org/10.3389/fvets.2018.00014.
12. Durand GA, Raoult D, Dubourg G. Antibiotic discovery: history, methods and perspectives. Int J Antimicrob Agents. 2019;53(4):371–82. https://doi.org/10.1016/j.ijantimicag.2018.11.010.
13. Leach KL, Brickner SJ, Noe MC, Miller PF. Linezolid, the first oxazolidinone antibacterial agent. Ann N Y Acad Sci. 2011;1222:49–54. https://doi.org/10.1111/j.1749-6632.2011.05962.x.
14. Bahar AA, Ren D. Antimicrobial peptides. Pharmaceuticals. 2013;6(12):1543–75. https://doi.org/10.3390/ph6121543.
15. Hancock REW, Sahl H-G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol. 2006;24(12):1551–7. https://doi.org/10.1038/nbt1267.
16. Fjell CD, Hiss JA, Hancock REW, Schneider G. Designing antimicrobial peptides: form follows function. Nat Rev Drug Discov. 2012;11(1):37–51. https://doi.org/10.1038/nrd3591.
17. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature. 2002;415(6870):389–95. https://doi.org/10.1038/415389a.
18. Rima M, Rima M, Fajloun Z, Sabatier J-M, Bechinger B, Naas T. Antimicrobial peptides: a potent alternative to antibiotics. Antibiotics. 2021;10(9):1095. https://doi.org/10.3390/antibiotics10091095.
19. Garcia P, Wang Y, Viallet J, Macek Jilkova Z. The chicken embryo model: a novel and relevant model for immune-based studies. Front Immunol. 2021;12:791081. https://doi.org/10.3389/fimmu.2021.791081.
20. Kogut MH. Dynamics of a protective avian inflammatory response: the role of an IL-8-like cytokine in the recruitment of heterophils to the site of organ invasion by *Salmonella enteritidis*. Comp Immunol Microbiol Infect Dis. 2002;25(3):159–72. https://doi.org/10.1016/S0147-9571(01)00035-2.
21. Rothwell L, Young JR, Zoorob R, Whittaker CA, Hesketh P, Archer A, et al. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. J Immunol. 2004;173(4):2675–82. https://doi.org/10.4049/jimmunol.173.4.2675.
22. Wu Z, Hu T, Rothwell L, Vervelde L, Kaiser P, Boulton K, et al. Analysis of the function of IL-10 in chickens using specific neutralising antibodies and a sensitive capture ELISA. Dev Comp Immunol. 2016;63:206–12. https://doi.org/10.1016/j.dci.2016.04.016.
23. Yu Y, Cheng L, Xu Z, Zhang Y, Ou C, Wang Q, et al. Tissue distribution and developmental changes of interferon regulatory factors in chickens and effects of infectious bursal disease virus infection. Microb Pathog. 2021;152:104601. https://doi.org/10.1016/j.micpath.2020.104601.
24. Santhakumar D, Rubbenstroth D, Martinez-Sobrido L, Munir M. Avian interferons and their antiviral effectors. Front Immunol. 2017. https://doi.org/10.3389/fimmu.2017.00049.
25. Yuk S-S, Lee D-H, Park J-K, Tseren-Ochir E-O, Kwon J-H, Noh J-Y, et al. Pre-immune state induced by chicken interferon gamma inhibits the replication of H1N1 human and H9N2 avian influenza viruses in chicken embryo fibroblasts. Virol J. 2016;13(1):71. https://doi.org/10.1186/s12985-016-0527-1.
26. Li C, Sutherland D, Hammond SA, Yang C, Taho F, Bergman L, et al. AMPlify: attentive deep learning model for discovery of novel antimicrobial peptides effective against WHO priority pathogens. BMC Genomics. 2022;23:77. https://doi.org/10.1186/s12864-022-08310-4.
27. Lin D, Sutherland D, Aninta SI, Louie N, Nip KM, Li C, et al. Mining amphibian and insect transcriptomes for antimicrobial peptide sequences with rAMPage. Antibiotics. 2022;11(7):952. https://doi.org/10.3390/antibiotics11070952.
28. Li C, Sutherland D, Richter A, Coombe L, Yanai A, Warren RL, et al. De novo synthetic antimicrobial peptide design with a recurrent neural network. Protein Sci. 2024;33(8):e5088. https://doi.org/10.1002/pro.5088.
29. Ebrahimikondori H, Sutherland D, Yanai A, Richter A, Salehi A, Li C, et al. Structure-aware deep learning model for peptide toxicity prediction. Protein Sci. 2024;33(7):e5076. https://doi.org/10.1002/pro.5076.
30. Allan BJ, van den Hurk JV, Potter AA. Characterization of *Escherichia coli* isolated from cases of avian colibacillosis. Can J Vet Res. 1993;57(3):146–51.
31. Yang S, Wang H, Zhao D, Zhang S, Hu C. Polymyxins: recent advances and challenges. Front Pharmacol. 2024;15:1424765. https://doi.org/10.3389/fphar.2024.1424765.
32. Schromm AB, Paulowski L, Kaconis Y, Kopp F, Koistinen M, Donoghue A, et al. Cathelicidin and PMB neutralize endotoxins by multifactorial mechanisms including LPS interaction and targeting of host cell membranes. Proc Natl Acad Sci USA. 2021;118(27):e2101721118. https://doi.org/10.1073/pnas.2101721118.
33. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – excel-based tool using pair-wise correlations. Biotechnol Lett. 2004;26(6):509–15. https://doi.org/10.1023/B:BILE.0000019559.84305.47.
34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001;25(4):402–8. https://doi.org/10.1006/meth.2001.1262.

35. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2024. Available from: https://www.R-project.org/.
36. Kassambara A, Kosinski M, Biecek P. survminer: drawing survival curves using "ggplot2." 2016;0.5.0. https://doi.org/10.32614/CRAN.package.survminer.
37. Signorell A. DescTools: tools for descriptive statistics. 2014;0.99.60. https://doi.org/10.32614/CRAN.package.DescTools.
38. Wickham H, Hester J, Bryan J. readr: read rectangular text data. 2015;2.1.5. https://doi.org/10.32614/CRAN.package.readr.
39. Wickham H, Henry L, Pedersen TL, Luciani TJ, Decorde M, Lise V. svglite: an "SVG" graphics device. 2015;2.2.1. https://doi.org/10.32614/CRAN.package.svglite.
40. Wickham H, Averick M, Bryan J, Chang W, McGowan L, François R, et al. Welcome to the tidyverse. J Open Source Softw. 2019;4(43):1686. https://doi.org/10.21105/joss.01686.
41. Kassambara A. ggpubr: "ggplot2" based publication ready plots. 2016;0.6.1. https://doi.org/10.32614/CRAN.package.ggpubr.
42. Kassambara A. rstatix: pipe-friendly framework for basic statistical tests. 2019;0.7.2. https://doi.org/10.32614/CRAN.package.rstatix.
43. Firke S. janitor: simple tools for examining and cleaning dirty data. 2016;2.2.1. https://doi.org/10.32614/CRAN.package.janitor.
44. Ahlmann-Eltze C, Patil I. ggsignif: significance brackets for "ggplot2." 2017;0.6.4. https://doi.org/10.32614/CRAN.package.ggsignif.
45. Cousineau D. superb: summary plots with adjusted error bars. 2021;0.95.19. https://doi.org/10.32614/CRAN.package.superb.
46. Therneau TM. survival: survival analysis. 2001;3.8–3. https://doi.org/10.32614/CRAN.package.survival.
47. Schauberger P, Walker A. openxlsx: read, write and edit xlsx files. 2014;4.2.8. https://doi.org/10.32614/CRAN.package.openxlsx.
48. Wickham H, Bryan J. readxl: read excel files. 2015;1.4.5. https://doi.org/10.32614/CRAN.package.readxl.
49. Zeileis A, Grothendieck G, Ryan JA. zoo: S3 infrastructure for regular and irregular time series (Z's ordered observations). 2004;1.8–14. https://doi.org/10.32614/CRAN.package.zoo.
50. Fox J, Weisberg S, Price B. car: companion to applied regression. 2001;3.1–3. https://doi.org/10.32614/CRAN.package.car.
51. Wickham H, Chang W, Henry L, Pedersen TL, Takahashi K, Wilke C, et al. ggplot2: create elegant data visualisations using the grammar of graphics. 2007;3.5.2. https://doi.org/10.32614/CRAN.package.ggplot2.
52. Yalin Zhu, Wenge Guo. MHTdiscrete: multiple hypotheses testing for discrete data. 2016;1.0.1. https://doi.org/10.32614/CRAN.package.MHTdiscrete.
53. Leitner G, Dan Heller E, Friedman A. Sex-related differences in immune response and survival rate of broiler chickens. Vet Immunol Immunopathol. 1989;21(3–4):249–60. https://doi.org/10.1016/0165-2427(89)90035-4.
54. Younis MEM, Jaber FA, Majrashi KA, Ghoneim HA, Shukry M, Shafi ME, et al. Impacts of synthetic androgen and estrogenic antagonist administration on growth performance, sex steroids hormones, and immune markers of male and female broilers. Poult Sci. 2023;102(1):102244. https://doi.org/10.1016/j.psj.2022.102244.