CpG-ODN induced antimicrobial immunity in neonatal chicks involves a substantial shift in serum metabolic profiles

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Synthetic CpG-ODNs can promote antimicrobial immunity in neonatal chicks by enriching immune compartments and activating immune cells. Activated immune cells undergo profound metabolic changes to meet cellular biosynthesis and energy demands and facilitate the signaling processes. We hypothesize that CpG-ODNs induced immune activation can change the host’s metabolic demands in neonatal chicks. Here, we used NMR-based metabolomics to explore the potential of immuno-metabolic interactions in the orchestration of CpG-ODN-induced antimicrobial immunity. We administered CpG-ODNs to day-old broiler chicks via intrapulmonary (IPL) and intramuscular (IM) routes. A negative control group was administered IPL distilled water (DW). In each group (n = 60), chicks (n = 40) were challenged with a lethal dose of Escherichia coli, two days post-CpG-ODN administration. CpG-ODN administered chicks had significantly higher survival (P < 0.05), significantly lower cumulative clinical scores (P < 0.05), and lower bacterial loads (P < 0.05) compared to the DW control group. In parallel experiments, we compared NMR-based serum metabolomic profiles in neonatal chicks (n = 20/group, 24 h post-treatment) treated with IM versus IPL CpG-ODNs or distilled water (DW) control. Serum metabolomics revealed that IM administration of CpG-ODN resulted in a highly significant and consistent decrease in amino acids, purines, betaine, choline, acetate, and a slight decrease in glucose. IPL CpG-ODN treatment resulted in a similar decrease in purines and choline but less extensive decrease in amino acids, a stronger decrease in acetate, and a considerable increase in 2-hydroxybutyrate, 3-hydroxybutyrate, formic acid and a mild increase in TCA cycle intermediates (all P < 0.05 after FDR adjustment). These perturbations in pathways associated with energy production, amino acid metabolism and nucleotide synthesis, most probably reflect increased uptake of nutrients to the cells, to support cell proliferation triggered by the innate immune response. Our study revealed for the first time that CpG-ODNs change the metabolomic landscape to establish antimicrobial immunity in neonatal chicks. The metabolites highlighted in the present study can help future targeted studies to better understand immunometabolic interactions and pinpoint the key molecules or pathways contributing to immunity.

Upon microbial entry, pathogens are sensed by host's innate immune system through several pattern recognition receptors, predominantly toll-like receptors (TLRs)1–5. These receptors recognize pathogen-associated molecular

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patterns or molecules such as lipopeptides, lipoteichoic acid, flagellin, lipopolysaccharides, and unmethylated CpG motifs containing oligodeoxynucleotides (ODNs). This recognition leads to cell signaling cascades, which induce the secretion of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor as well as chemokines that attract phagocytic heterophils and macrophages to the site of infection. This cascade ultimately leads to the development of adaptive immunity against the invading pathogens. Chicken TLR21 and human TLR9 recognize CpG-ODNs containing GTCGTT motifs, and both have similar intracellular localization, signaling cascades, and cytokine induction patterns. CpG-ODNs have great potential as immunotherapeutic agents and vaccine adjuvants against infections and cancer. Several studies in humans, mice, cattle and sheep, fish, and chickens reported that CpG-ODNs initiate immune responses by activating immune cells and inducing cytokine secretion. Our laboratory reported for the first time that standalone CpG-ODN treatment can protect against bacterial infections in chickens. We showed that CpG-ODN administration protects chickens against Escherichia coli and Salmonella typhimurium infections. Other studies have also demonstrated the antimicrobial function of CpG-ODN against Salmonella enteritidis infection. We recently demonstrated that CpG-ODN treatment accelerates immune development by enriching immunological niches in chicks. Furthermore, our recent data established a cause-and-effect relationship by showing that the levels of CpG-ODN-induced immune enrichment strongly correlate with the levels of protection against E. coli infection. Regardless of recent advances, further investigations are needed to understand the mechanisms of CpG-ODN induced antimicrobial immunity better.

Several recent studies in humans and mice have suggested that energy metabolism significantly regulates immune cell fate and functions. Macrophages and dendritic cells (the sentinel cells) were shown to have increased glucose metabolism and increased expression of the glycolytic enzymes, glucose-6-phosphate dehydrogenase and hexokinase. Naïve resting T lymphocytes utilize oxidative phosphorylation for ATP generation, whereas activated T lymphocytes utilize aerobic glycolysis and glutaminolysis in the main and glutaminolysis in the main and glutaminolysis. It was recently reported that the activation of TLR4 by bacterial lipopolysaccharides in neutrophils increases glucose consumption. Cells stimulated via pattern recognition receptors (PRR) and pathogen-associated molecular pattern (PAMP) interactions undergo profound metabolic changes, which is important not only for the signaling processes but also for biosynthesis and energy production.

Metabolomics offers an excellent route for exploring the molecular connections between immunity and metabolism. In particular, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) can be applied to identify metabolites (the end products of biological responses) in biofluids to better understand disease-induced and immunity induced processes and responses. Despite the potential involvement of the metabolic changes in shaping the immune responses, to the best of our knowledge, no extensive metabolomic profiling for CpG-ODN-induced immunity has been carried out.

In the present study, we hypothesized that CpG-ODNs potentially regulate metabolic pathways that control the development of antimicrobial immunity. Antimicrobial immunity, as induced by intramuscular (IM) administration of CpG-ODN, has been the gold standard to assess challenges against several bacterial pathogens. We recently found that intrapulmonary (IPL) delivery of CpG-ODN also induces antimicrobial immunity in a dose-dependent manner. Therefore, in this study, we compared NMR-based serum metabolomic profiles in chickens treated with IM versus IPL CpG-ODNs or distilled water (DW) control.

Results

Immunoprotective efficacy of intramuscular and intrapulmonary delivery of CpG-ODN compared to distilled water control group against E. coli septicemia. Chicks that received CpG-ODN either through the IM or IPL routes or DW controls were challenged with lethal doses (1 × 10⁵ or 1 × 10⁶ CFU) of a pathogenic strain of E. coli. During the seven days of challenge experiments, chicks that received CpG-ODN were significantly protected compared to saline controls (Fig. 1). We found that CpG-ODN delivery through the IM route induced a higher survival against bacterial challenge compared to IPL CpG-ODN delivery. We also calculated the daily mean CCS for each chick through the seven-day observation period after E. coli challenge. The birds that received CpG-ODN IM or IPL had significantly lower CCS values (P < 0.001) compared to the DW controls (Fig. 2a); the lowest CCS values were in birds that received IM CpG-ODN. To measure the bacterial loads in different groups, we used a semi-quantitative estimate of E. coli isolation on Columbia sheep blood agar by the quadrant streaking method. In this method, clockwise streaking is done on the agar plate, and bacterial thinning occurs as streaking goes from quadrant 1 to quadrant 4. Therefore, the isolation of bacterial colonies in the higher quadrant suggests higher bacterial load in the sample. The number of birds with various bacterial loads is shown in Fig. 2b. These data clearly indicate CpG-ODN treatment led to a substantial reduction in bacterial loads. When birds in each group were divided into low and high bacterial load categories as mentioned in the materials and methods section, we found that the CpG-ODN administered birds had a statistically lower bacterial load in contrast to the DW controls: χ² = 9.911, P = 0.007 (Fig. 2c). Birds that succumbed to challenge or were euthanized had lesions such as pericarditis, airsacculitis or combination of airsacculitis together with pericarditis or pericarditis.

Effects of CpG-ODNs on serum metabolites. A total of 40 metabolites per experimental group were identified and quantified by NMR. These metabolites were further utilised to find differential biomarkers, by means of univariate and multivariate analysis. First, for quality control measures, unsupervised principle component analysis (PCA) was conducted on all samples together, including pooled samples (Fig. S1). Along with other supporting evidence, this work led to the removal of two outliers from the control group, but not from any experimental group. To further evaluate the group clustering owing to metabolic differences, discriminant analysis was applied per CpG-ODN delivery method vs. DW control. Specifically, a partial least squares discrimi-
ODN administration via intramuscular (IM)26–28,30 or intrapulmonary (IPL)29,34 routes can induce antimicrobial CpG-ODN induced antimicrobial immunity in chickens. We have extensively reported that standalone CpG-demands45. To the best of our knowledge, the present study is the first report on serum metabolic profiling of protein production, to support rapid B-cell and T-cell division and to fulfill increased energy and anabolic During an immune response, profound metabolic changes occur to facilitate cell signaling, to enhance cytokine Discussion

Figure 1. Survival percentages of the birds following lethal E. coli infection. Day-old neonatal chicks were treated with 50 µg of CpG-ODN intramuscularly (IM) or mucosal delivery via intrapulmonary (IPL) route [6 mg CpG-ODN aerosolized in a closed 0.036 m³ acrylic chamber containing 60 birds for 30 min] or treated with aerosolized distilled water (DW) as control. On the second-day post-treatment, the birds in each group (n = 40) were challenged with 1 × 10⁵ CFU or 1 × 10⁶ CFU of E. coli per bird, subcutaneously in the neck. The mortality was recorded until seven days post-challenge. Birds that received IM CpG-ODN (blue) and IPL CpG-ODN (red) treatments showed significantly better survival than the DW control (green) group (P < 0.05) over seven days post challenge.
information about the metabolome in various tissues in normal healthy chickens. However, no metabolomic data has been collected relating to CpG-ODN induced immune responses in chickens. Therefore, the present study was designed to investigate a potential regulation of metabolic pathways by CpG-ODNs to induce antimicrobial immunity. Here, we used NMR-based metabolomics to explore the potential of immuno-metabolic interactions in the orchestration of CpG-ODN-induced antimicrobial immunity. In doing so, we attempted to identify critical molecules or pathways associated with immunoprotective phenotypes based on their characteristic serum metabolite profiles. Our findings clearly suggest that immune-metabolic interactions are involved in CpG-ODN-mediated immunity. In particular, this study revealed a wide array of differential metabolic signatures in CpG-ODN treated chickens.

In the present study, chickens treated with intramuscular (IM) CpG-ODNs were better protected against bacterial infection than the intrapulmonary (IPL) group (Figs. 1, 2). This difference between the two CpG-ODN delivery methods corresponded well with the observed alteration in metabolic profile between the treatment groups, despite of natural variation in response between birds in the same group (Fig. 4). To better illustrate the metabolic alterations within their biochemical context, Fig. 5 presents a metabolic pathways map incorporating the t test results comparing each treatment to control (Tables 1, 2). It is well known that CpG-ODN causes the immune system's stimulation, resulting in pro-inflammatory cytokine secretion and activation of immune cells such as monocytes, macrophages, and lymphocytes. We recently reported an increased number of mononuclear...
cells infiltrating into the lungs of chickens within 24 h of CpG-ODN treatment. Given that immune cells must grow and divide rapidly upon activation, despite lacking nutrient stores, they require energy-rich resources, including sugars, amino acids, and fatty acids present in the extracellular environment. Therefore it is expected that the levels of these metabolites, during immune activation, would potentially decrease in the serum. In particular, immune activation requires amino acids for protein synthesis, nucleotides for DNA/RNA synthesis, sugars for energy production and fatty acids for lipid membrane production. As immune cell activation is certainly metabolically demanding, we assume that the metabolomics changes in serum that we observed in the present study could be the result of increased enrichment and activation of immunological niches, leading to enhanced uptake of metabolites by cells during immunological responses. Since blood bathes every organ and every tissue in the body, it essentially reflects the net metabolic changes resulting from the physiological and

Table 1. Selected Student's t-test results comparing IPL CpG-ODN to DW control. DW controls n = 18; IPL CpG n = 20. Correction for multiple comparisons was conducted using FDR (n = 40 metabolites). Between-group direction and fold-change of the mean raw metabolite concentrations are also presented. IPL CpG-ODN vs. DW control.

| Metabolite | IPL/DW | Fold change | t test P value | FDR—adjusted P value |
|------------|--------|-------------|----------------|---------------------|
| Choline    | Down   | 1.35        | 1.27E−05       | 5.08E−04            |
| Acetate    | Down   | 1.93        | 1.24E−04       | 0.002               |
| Hypoxanthine | Down   | 1.56        | 3.74E−04       | 0.005               |
| Proline    | Down   | 1.34        | 6.22E−04       | 0.006               |
| 2-Hydroxybutyrate | Up  | 1.60        | 7.85E−04       | 0.006               |
| 3-Hydroxybutyrate | Up  | 1.45        | 0.001           | 0.007               |
| Cytidine   | Down   | 1.56        | 0.001           | 0.007               |
| Aspartate  | Down   | 1.30        | 0.002           | 0.008               |
| Threonine  | Down   | 1.37        | 0.003           | 0.012               |
| Formate    | Up     | 1.49        | 0.008           | 0.030               |
| Fumarate   | Up     | 1.19        | 0.010           | 0.035               |
| Glycine    | Down   | 1.19        | 0.011           | 0.035               |
| Citrate    | Up     | 1.17        | 0.015           | 0.047               |
| Uridine    | Down   | 1.32        | 0.018           | 0.050               |
| Myo-inositol | Down  | 1.39        | 0.020           | 0.052               |
| Glutamine  | Down   | 1.13        | 0.037           | 0.092               |
| Isoleucine | Up     | 1.15        | 0.041           | 0.095               |
| Tryptophan | Down   | 1.14        | 0.043           | 0.095               |
metabolic needs or stresses in different tissues in the animal body. However, our serum metabolomics data do not distinguish between the possibilities of differential use or synthesis or uptake of metabolites in various tissues following the CpG-ODN administration. Additional studies and techniques will be required to investigate the complex issues of differentiating between the use, synthesis, and uptake of substances.

Several studies have reported that amino acids are critical in immune cell proliferation and function. A study in mice demonstrated that serine and glycine play an important role in T cell proliferation and function. In our study, we found a significant decrease in serum levels of essential and also non-essential amino acids. Altered levels of essential amino acids which are required for protein synthesis, included threonine (35% decrease in IPL treatment and 50% decrease in IM treatment), glycine (20–30% decrease in both treatments), and lysine (35% decrease, only in IM treatment). A recent study demonstrated that the metabolism of a single amino acid (proline) can have a profound effect on the immune responses to pathogens. This study reported that mitochondrial proline catabolism controls innate immunity in Caenorhabditis elegans by regulating reactive oxygen species (ROS) homeostasis. In both nematodes and birds, antimicrobial ROS produced by macrophages and neutrophils aids in bacterial killing. In our study, we found a substantial reduction in the serum proline levels that corresponded with increased antimicrobial immunity in both the IPL CpG-ODN and IM CpG-ODN groups. We hypothesize that the CpG-ODN induced increased leukocytes in various immunological niches, and enhanced activation of macrophages and heterophils, which probably led to greater utilization of serum proline. Further studies to test the role of proline utilization and CpG-ODN induced antimicrobial immunity would be of great interest.

Apart from their role as building blocks in protein synthesis and providing alternative substrates to the TCA cycle, amino acids are also precursors in nucleotide synthesis and play a vital role in cell proliferation. Specifically, glutamine is a key amino acid that is further metabolised into purines and downstream nucleotides to generate DNA and RNA. Increased uptake of glutamine and its precursors aspartate and also proline was observed after administration of CpG-ODN via IM and also IPL. Within the purine metabolism pathway, the three intermediates hypoxanthine, cytidine and uridine showed a uniform decrease across the CpG treatments.

In addition to the amino acid changes noted above, we also observed reduced acetate levels in serum upon the administration of CpG-ODN. The source of acetic acid might be either a catabolism product of ketone bodies, or metabolism of acetyl-CoA, an essential metabolite of carbohydrate and fatty acid metabolism. As mentioned earlier, cell proliferation also increases the demands for fatty acid synthesis, hence acetate acid might be utilised in this direction. On the other hand, fatty acid oxidation provides energy to the cellular activities.

| Metabolite      | IM/DW | Fold change | t test P value | FDR—adjusted P value |
|-----------------|-------|-------------|----------------|----------------------|
| Betaine         | Down  | 2.46        | 7.16E−08       | 2.86E−06             |
| Proline         | Down  | 1.51        | 8.45E−07       | 1.69E−05             |
| Serine          | Down  | 1.35        | 2.33E−05       | 3.11E−04             |
| Alanine         | Down  | 1.29        | 8.64E−05       | 8.64E−04             |
| Choline         | Down  | 1.41        | 1.26E−04       | 9.28E−04             |
| Glycine         | Down  | 1.31        | 1.86E−04       | 9.28E−04             |
| Threonine       | Down  | 1.49        | 1.73E−04       | 9.28E−04             |
| Tyrosine        | Down  | 1.30        | 1.48E−04       | 9.28E−04             |
| Cytidine        | Down  | 1.64        | 2.31E−04       | 0.001                |
| Glutamine       | Down  | 1.24        | 4.54E−04       | 0.002                |
| Hypoxanthine    | Down  | 1.51        | 9.11E−04       | 0.003                |
| Lysine          | Down  | 1.37        | 0.001          | 0.004                |
| D-Glucose       | Down  | 1.15        | 0.001          | 0.004                |
| Acetate         | Down  | 1.26        | 0.007          | 0.019                |
| Aspartate       | Down  | 1.22        | 0.008          | 0.020                |
| 2-Hydroxybutrate| Up    | 1.36        | 0.009          | 0.021                |
| Uridine         | Down  | 1.40        | 0.009          | 0.021                |
| Valine          | Down  | 1.16        | 0.017          | 0.037                |
| Carnitine       | Down  | 1.37        | 0.029          | 0.057                |
| Methionine      | Down  | 1.17        | 0.032          | 0.068                |
| Tryptophan      | Down  | 1.12        | 0.037          | 0.073                |
| Leucine         | Down  | 1.19        | 0.050          | 0.093                |
| Malonate        | Down  | 1.25        | 0.051          | 0.093                |
| Creatine        | Up    | 1.50        | 0.068          | 0.109                |
| Myo-inositol    | Down  | 1.29        | 0.066          | 0.109                |

**Table 2.** Selected Student’s t-test results comparing IM CpG-ODN to DW control. DW controls n = 18; IM CpG n = 20 (apart from carnitine, where two missing values were removed). Correction for multiple comparisons was conducted using FDR (n = 40 metabolites). Between-group direction and fold-change of the mean raw metabolite concentrations are also presented. IM CpG-ODN vs. DW control.
when in need through β-oxidation in mitochondria or peroxisomes. An increased fatty acid uptake in the liver results in ketogenesis, where fatty acids undergo incomplete oxidation. Lower serum acetate levels resulting from CpG-ODN administration might be indicating that immune cells must start dividing and that there is a need for extra energy. As a result, fatty acids started to undergo oxidation to provide that extra energy. Acetate also appears to play a signaling role in the immune system. A recent report has shown how acetate promotes T-cell effector functions through epigenetic modifications and that rapidly dividing cells (including both tumor and immune cells) can use acetate (instead of glucose) as an alternative fuel.

We also found that CpG-ODN administration by both IPL and IM resulted in increased levels of 2-OH butyrate, which can originate in several amino acids via 2-ketobutyrate. In humans 2-OH butyrate is related to lipid oxidation, oxidative stress and deficient energy metabolism. Interestingly, serum levels of 2-OH butyrate increased by 60% after the IPL treatment and only by 35% in the IM treatment. Further metabolic differences were observed between the two administration routes, with a slight decrease in glucose measured only in the IM group, while a 45% increase in the ketone body 3-OH butyrate was observed only in the IPL-CpG-ODN group, indicating ketogenesis was being used to provide for higher energy demands. In addition, an increase

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**Figure 4.** Combined box-and-whisker and dot plots showing the concentrations of selected metabolites in serum from neonatal chicks 24 h post-treatment. DW control, n = 18 (green); IM CpG-ODN, n = 20 (blue) and IPL CpG-ODN, n = 20 (red). Values are log-transformed concentrations in μM. One-way ANOVA results are detailed in supplementary Table S1.
in TCA cycle intermediates was recorded only after IPL administration, although the lactate-to-pyruvate ratio did not differ between the experimental groups. Altogether, these data suggest differences in the extent and rate of energy metabolism adaptations, potentially due to systemic vs. mucosal targeting by IM and IPL CpG-ODN administration methods, respectively.

Beyond its function as an energy source, a previous study in cattle reported that beta-hydroxybutyrate (3-OH butyrate) abrogates the antimicrobial function of neutrophils against E. coli. Several other studies showed that 3-hydroxybutyrate upregulates mRNA abundance of proinflammatory cytokines such as IL-1β in mouse macrophages, IL-8 in bovine liver, and several pro-inflammatory cytokines in calf hepatocytes. The cytokine mRNA data should be interpreted cautiously, as some cytokines such as IL-1β are highly regulated post-translationally. In our previous studies, we found a significant increase in the expression of several proinflammatory cytokines, including IL-1β and IL-6 following CpG-ODN administration. In the present study, we found an increased serum level of 3-hydroxybutyrate in the IPL CpG-ODN group, but how this would affect cytokines at the protein level needs further studies.

Our metabolomic analysis also highlighted two crucial metabolites, choline and betaine. Serum choline levels were reduced by 35–40% in the CpG-ODN treated birds while betaine levels were significantly lower (2.5-fold) only in the birds that received intramuscular CpG-ODN treatment. Choline is a precursor in the synthesis of acetylcholine, phosphocholine, and is an important intermediate in phospholipid metabolism. It is well documented that there is increased consumption of phosphocholine under severe oxidative and systemic inflammatory conditions. Choline is oxidized to betaine, and both are linked to the folate-dependent one-carbon cycle.
metabolism. In chickens, choline plays a significant role in improving the humoral and cellular immunity, and betaine has been shown to increase lymphocyte infiltration in infected mucosa. Betaine also enhances phagocytic and nitric oxide production by blood monocytes and heterophils. A recent study in cattle reported that T cell proliferation was linearly enhanced in vitro with increasing doses of choline. Reduced choline levels in our study could also be contributing to the low betaine levels, however betaine is also supplemented via the feed due to its nutritional importance.

As noted earlier, increased utilization of choline, betaine, and proline by immune cells can enhance the antimicrobial activity of various immune cells. Additionally, given that choline, betaine, and proline are effective osmolytes, their reduced levels in the serum would prevent bacterial proliferation due to a reduced accumulation of osmoprotectants (betaine, choline, and proline), which play an essential role in bacterial growth such as in the case of E. coli and Staphylococcus aureus. It has been reported that betaine provides the best osmotic protection to E. coli growth followed by choline (which is converted to betaine) and proline. Interestingly, in our study, we found that IM CpG-ODN administration dramatically reduced the serum levels of betaine followed by proline and choline, and provided better protection against E. coli compared to IPL-CpG-ODN, which only showed significantly reduced serum levels of choline and proline but not betaine. More significant reduction of bacterial growth supporting osmoprotectants in IM CpG-ODN vs. IPL CpG-ODN correlates well with the rank order of their protection data (IM-vs. IPL-CpG-ODN).

Overall, the metabolomic data from this study suggest that CpG-ODN-mediated antimicrobial immunity involves a number of significant metabolic changes in the host that enhance immunity and antagonize microbial proliferation in the host. CpG-ODN induced metabolomics data generated by the current study provides a unique and little-appreciated approach to identify regulatory molecules or pathways that give protective immunity to chickens against bacterial infections. The metabolites highlighted in the present study can help future targeted studies better understand antimicrobial metabolomic profiles and pinpoint the key molecules or pathways contributing to immunity.

Materials and methods

Housing and maintenance of experimental chickens. This work was carried out in compliance with the ARRIVE guidelines. The animal study was approved by the Animal Research Ethics Board, University of Saskatchewan (protocol number 20070008) and adhered to the guidelines of the Canadian Council on Animal Care. Euthanasia was performed by cervical dislocation following the AVMA guidelines for the euthanasia of animals. Day-old broiler chickens (Ross 308 strain) were obtained from a commercial hatchery in Saskatchewan. Groups of chicks were allocated randomly into an animal isolation room at the Animal Care Unit, Western College of Veterinary Medicine in Saskatoon, Saskatchewan, and chicks were maintained following the procedure as described earlier. Briefly, water and commercial broiler starter ration (23% crude protein, 1% calcium, 0.45% available phosphorous) were provided ad libitum. Air from each room was exhausted through a HEPA filter, and non-recirculated intake air was provided at a rate of 15–20 air changes/hr. Air pressure differentials and strict sanitation were maintained in this isolation facility. Broilers were raised at 32 °C for the first 7 days of life (average weight ~ 180 g); after that, the temperature was decreased by 0.5 °C per day until a room temperature of 27.5 °C was reached. Light (30 lx) was provided for 24 h/d during days 0 to 2 (post-hatch). Darkness was introduced at 3 d post-hatch with 1 h of dark added daily until 4 h of darkness was achieved.

CpG-ODN delivery. The CpG-ODN [TCGTCGTTTGTGTTTGTGTT(T705)] was free of endotoxin and produced with a phosphorothioate backbone (Operon Biotechnologies, Inc; Huntsville, AL, USA). Synthetic CpG-ODN was diluted in sterile DW and delivered by the IPL route. Briefly, the CpG-ODN solution was aerosolized as micro-droplets (particle size of 0.5–5 µm) using a Compressor Nebulizer (705–470) unit (AMG Medical Inc; Montreal, QC, Canada) in a closed 0.036 m³ acrylic chamber containing 60 birds for 30 min (6 mg CpG-ODN/chamber) that maintained atmospheric oxygen exchange. The control group of birds (n = 60) were aerosolized with DW for 30 min in the acrylic chamber using a similar compressor nebulizer. Another group of birds (n = 60) were administered with CpG-ODN (50 μg/100 μl/bird) by IM injection to the left thigh. The temperature was maintained at 28–30 °C in the acrylic chamber during the administration of CpG-ODN or DW.

E. coli culture and animal model. In order to confirm the immune protection induced by CpG-ODN delivery, a parallel E. coli challenge study was performed to the birds. A field isolate of E. coli from a turkey with septicaemia was used as the challenge strain according to our previously established animal model. The E. coli belonged to serogroup O2 was nonhemolytic, serum resistant, aerobactin producing and had K1 capsule with type I pilus. Aliquots of the bacterial isolate were stored at −80 °C in brain heart infusion broth (Difco, Detroit, Mich.) supplemented with 25% (wt/vol) glycerol (VWR Scientific Inc., Montreal, QC, Canada). In order to challenge the birds, bacteria were cultured on 5% Columbia sheep blood agar for 18–24 h at 37 °C. One colony of E. coli was added to 100 mL of Luria broth (Difco LB broth, Miller, Becton Dickinson and Company; Sparks, MD, USA) in a 250 mL Erlenmeyer flask. The culture was grown at 37 °C for 16–18 h, shaking at 150 rpm. This stationary phase culture contained approximately 1 × 10⁸ colony forming units (CFU) of bacteria per mL, which was then further diluted into saline to the concentration of bacteria required to challenge birds. The E. coli challenge dose was confirmed by plating serial dilutions of the diluted culture in duplicate on 5% Columbia sheep blood agar plates, incubating for 18 h at 37 °C, then counting the number of colonies.

The E. coli challenge study was performed according to the well-established animal model that we documented earlier. Briefly, on the second-day post-treatment, the birds in each group (n = 40) were challenged with 1 × 10⁶ CFU (n = 20) or 1 × 10⁷ CFU (n = 20) of bacteria per bird, subcutaneously in the neck. They were closely monitored three times a day for the most critical period of three days post-challenge and two times a
Metabolomics analysis of serum.  

Sample collection. Twenty chicks from each group were euthanized 24 h post-CpG-ODN treatment in the morning between 9 am to 10 a.m. Blood was immediately collected into serum tubes by severing the necks of the chicks with a sharp pair of scissors. After about 30 min of blood collection, the clotted blood samples were then centrifuged at 1000 g force for 15 min, and serum was separated into 1.5 mL microcentrifuge tubes. Serum samples were stored at −80 °C, transported on dry ice to The Metabolomics Innovation Centre (TMIC) facility at the University of Alberta in Edmonton, and stored at −80 °C until further analysis.

Sample preparation and NMR spectroscopy. Serum samples were thawed on ice and prepared in two batches according to a randomization template, with the addition of pooled samples for quality control. Plasma and serum samples contain a significant concentration of large molecular weight proteins and lipoproteins, which can seriously compromise the quality of 1H-NMR spectra though the generation of intense, broad lines that interfere with the identification and quantification of lower abundance metabolites. Deproteinization can eliminate these peaks. Deproteinization of the serum samples was done by centrifugation and ultrafiltration using 3-kDa cut-off centrifuge filter units (Microcon YM-3; Sigma-Aldrich, St. Louis, MO), following a previously reported deproteinization procedure. The deproteinized serum samples (280 μL) were then transferred to a 1.5 mL microcentrifuge tube followed by the addition of 70 μL standard NMR buffer solution (1 mM DSS (disodium-2, 2-dimethyl-2-silapentane-5-sulphonate), in 10% D2O). These samples (a total volume of 350 μL) were then transferred to a 3 mm NMR tube for spectral analysis. All 1H-NMR spectra were collected on a Bruker Avance III Ascend 700 MHz spectrometer with a 5 mm cryo-probe (Bruker Biospin, Rheinstetten, Germany). 1H-NMR spectra were acquired at 25 °C using the first transient of the noesy-presaturation pulse sequence, which was chosen for its high degree of quantitative accuracy. Spectra were collected with 128 transients using a 4 s acquisition time and a 1 s recycle delay.

NMR compound identification and quantification. Before spectral analysis, all free induction decays (FIDs) were zero-filled to 240 k data points, and a line broadening of 0.5 Hz was applied. The methyl singlet of the added DSS served as an internal standard for chemical shift referencing (set to 0.00 ppm) and for quantification. All 1H-NMR spectra were processed and imported into the Chenomx NMR Suite 8.1 software (Edmonton, Canada). The Chenomx NMR Suite software allows for a quantitative analysis of an NMR spectrum by manually fitting spectral signatures from an internal database to the spectrum. Specifically, the spectral fitting for metabolite was done using the standard Chenomx 700 MHz metabolite library. Most of the visible peaks are annotated with a compound name. Each spectrum was processed and analyzed by at least two experienced NMR spectroscopists to minimize compound misidentification and misquantification. Forty metabolites passed the NMR quality measures and underwent further statistical analysis.

Data processing and statistical analysis. The Metabo Analyst 4.0 software was used for statistical analysis of the metabolomics data. Data were log-transformed prior to univariate analysis, and also autoscaled prior to multivariate analysis. Principal Component Analysis (PCA) was performed for quality-control assessment. Partial Least Squares-Discriminant Analysis (PLS/DA) was used to classify samples and suggest potential biomarkers for treatment effect. Several univariate analysis tests were also employed. In particular, analysis of variance (ANOVA) was conducted to compare metabolite levels between all three groups, with Tukey’s HSD post-hoc analysis to indicate significant pairs. Student’s t test was used to compare between two experimental groups in terms of fold-change analysis and assessment of significance with regard to metabolite differences. In all tests, P values were further corrected for multiple comparisons by applying the Benjamini–Hochberg method of false discovery rate (FDR), and considering an FDR-adjusted P value of 0.1 as a threshold for inclusion in tables and figures. Pathway enrichment analysis was performed in the Metabo Analyst 4.0 software on log-transformed and auto scaled data, against the Gallus Gallus pathway database. For each two-group comparison, it consisted of an ANCOVA test with the use of a relative-betweenness centrality algorithm for pathway topology analysis.

The significance of the observed differences in chick survival and the cumulative clinical score (CCS) were analyzed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA) with a significance level of P < 0.05.
The survival data and bacterial scores of both $1 \times 10^5$ CFU and $1 \times 10^6$ CFU of *E. coli* challenge were combined for clarity of analysis and presentation. The level of significance with regard to differences among groups in survival patterns and median survival times were analyzed using the log-rank test and chi-square statistic. Clinical scores assigned at each time point were summed up to 7 days post-challenge to generate CCS and thereby daily mean CCS were calculated for each group. Two-way ANOVA was performed with Dunnett’s multiple comparison tests to compare the significant differences in mean CCS. For the statistical analysis on bacterial loads (quadrant streaking method), birds in each group were divided into two categories (low or high bacterial load). A chi-square test of independence was performed to examine the relationship between the CpG treatment method and the ability to recover viable bacteria based on this categorization. The results were interpreted with a statistical significance of *P* < 0.05.

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**Author contributions**
K.B.G., K.A.A. and S.G. conceived and designed experiments. K.B.G., N.K., K.A.A., S.G., D.S.W., R.M. and P.W. analyzed data. K.B. G., K.A.A., N.K. and S.G. wrote paper. K.B.G., K.A.A., S.P., B.L., L.E.A., R.K., T.G. and M.L. performed experiments. M.E., S.K.T. and D.S.W. provided materials and edited manuscript. All authors reviewed and approved the manuscript.

**Competing interests**
The authors declare no competing interests.

**Additional information**

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