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1 **Classification: Biological Science – Agricultural Sciences**

2

3 # A novel antimicrobial alternative of microbiota metabolic product deoxycholic
4 # acid controls chicken necrotic enteritis

5

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16 **Keywords:** Microbiome, bile acids, intestinal inflammation, COX, coccidiosis
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22
Abstract

23 Necrotic enteritis (NE) caused by *Clostridium perfringens* infection has reemerged as a
24 prevalent poultry disease worldwide due to reduced usage of prophylactic antibiotics. The
25 lack of alternative antimicrobial strategies to control this disease is mainly due to limited
26 insight into NE pathogenesis, microbiome relationships, and host responses. Here we
27 reported that the metabolic byproduct of microbial metabolism of bile acids to deoxycholic
28 acid (DCA), at as low as 50 μM, inhibited 82.8% of *C. perfringens* growth in Tryptic Soy
29 Broth ($P < 0.05$). Sequential *Eimeria maxima* and *C. perfringens* challenge strongly
30 induced NE, severe intestinal inflammation, and body weight (BW) loss in broiler chickens.
31 These negative effects were diminished by 1.5 g/kg DCA diet. At the cellular level, DCA
32 alleviated NE-associated ileal epithelial death and lamina propria immune cell apoptosis.
33 Interestingly, DCA reduced *C. perfringens* invasion into villi without significantly altering
34 the bacterial luminal colonization. Molecular analysis showed that DCA reduced
35 inflammatory mediators of *Infγ*, *Litaf* (*Tnfα*), *Il1β*, and *Mmp9* mRNA accumulation in ileal
36 tissue. Mechanically, *C. perfringens* induced elevated expression of inflammatory
37 cytokines of *Infγ*, *Litaf*, and *Ptgs2* (COX-2 gene) in chicken splenocytes. Inhibiting the
38 COX signaling by aspirin attenuated INFγ- or TNFα-induced inflammatory response in
39 the splenocytes. Consistently, chickens fed 0.12 g/kg aspirin diet resisted against NE-
40 induced BW loss, ileal inflammation, and villus apoptosis. In conclusion, microbial
41 metabolic product DCA prevents NE-induced BW loss and ileal inflammation through
42 curbing inflammatory response. These novel findings could serve as a stepping-stone for
43 developing next generation antimicrobial alternatives against NE.

44

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45 ## Significance Statement

46

47 Widespread antimicrobial resistance has become a serious challenge to both agricultural
48 and healthcare industries. Withdrawing antimicrobials without effective alternatives
49 exacerbates poultry productivity loss at billions of dollars every year, caused by intestinal
50 diseases, such as coccidiosis- and *C. perfringens*-induced NE. This study used the
51 endogenous microbial metabolic secondary bile acid product DCA to control NE and to
52 improve chicken growth performance through modulating host response. These findings
53 have opened new avenues for developing next-generation antimicrobial free regiments.

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54 # Introduction

55

56 Antimicrobial resistance is one of the emerging challenges requiring immediate
57 and sustainable counter-actions from agriculture to healthcare (1). Increasing
58 antimicrobial resistance has caused the emergence of multiple drug-resistant microbes
59 or “superbugs”. Recently, a “superbug” of an *Escherichia coli* strain resistant to the last
60 resort antibiotic, Colistin, was reported in USA (2). Overuse of antimicrobial agents in
61 medical and agricultural practice is contributing to exacerbating the episodes of emerging
62 antimicrobial resistant microbes (1). Withdrawing antimicrobials in poultry production,
63 however, has caused new problems for the poultry industry by reducing production
64 efficiency and increasing diseases, such as *Eimeria maxima*- and *Clostridium*
65 *perfringens*-induced necrotic enteritis (NE) (3). Clinical signs of NE include watery to
66 bloody (dark) diarrhea, severe depression, decreased appetite, closed eyes, and ruffled
67 feathers. Dissection of dead or severely ill birds shows that the intestine is often distended
68 with gas, very friable, and contains a foul-smelling brown fluid, with clearly visible necrotic
69 lesions (4). Although progress has been made toward understanding risk factors
70 influencing the outcome of NE such as *C. perfringens* virulence, coccidiosis, and feed (5),
71 few effective non-antimicrobial strategies are available.

72 The human and animal intestine harbors up to trillions of microbes and this
73 intestinal microbiota regulates various host functions such as the intestinal barrier,
74 nutrition and immune homeostasis (6-8). The enteric microbiota regulates granulocytosis
75 and neonatal response to *Escherichia coli K1* and *Klebsiella pneumoniae* sepsis (9),
76 suggesting the key role of the microbiota in protecting the host against systemic infection.

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77 At the gut level, fecal transplantation was reported decades ago to prevent *Salmonella*
78 *infantis* chicken infection (10). More recently, microbiota transplantation has shown
79 tremendous success against recurrent *Clostridium difficile* infection (11) and *Clostridium*
80 *scindens* metabolizing secondary bile acids have been shown to inhibits *C. difficile*
81 infection (12). Bile acids synthesized in the liver are released in the intestine and
82 metabolized by gut microbiota into final forms of secondary bile acids (13). Bile acids,
83 particularly the secondary bile acid DCA, are associated with a variety of chronic
84 diseases, such as obesity, diabetes, and colorectal tumorigenesis (14, 15). Recently, we
85 found that mouse anaerobes and their metabolic product DCA prevented and treated
86 *Campylobacter jejuni*-induced colitis in germ-free mice through attenuating host
87 inflammatory signaling pathways (16).

88 Cyclooxygenases (COX)-catalyzed prostanoids regulate various activities
89 including cell proliferation, apoptosis and migration (17), gastrointestinal secretion (18),
90 body temperature (19), inflammation (20), and pain sensation (21). COX-1 and COX-3
91 (gene *Ptgs1*, alternative splicing) constitutively expressed are important for intestinal
92 integrity. Inducible COX-2 (*Ptgs2*) activity is associated with various inflammatory
93 diseases including inflammatory bowel disease (22) and radiation-induced small bowel
94 injury (23). COX-2 increases gut barrier permeability and bacterial translocation across
95 the intestinal barrier (24, 25). Paradoxically, COX-2 enhances inflammation resolution
96 through prostaglandin D_2 (26). Although non-selective COX inhibitor aspirin is used to
97 prevent various chronic diseases, it inflicts intestinal inflammation to the healthy intestine
98 (27).

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99 Currently, limited knowledge is available on the relationship between NE
100 pathogenesis, the microbiome, and host inflammatory response. Here, we hypothesize
101 that the microbiota metabolic product DCA attenuates NE. Our findings demonstrated that
102 DCA decreases NE-induced BW loss, intestinal inflammation, *C. perfringens* invasion,
103 and villus death. Blocking the inflammatory downstream target COX signaling pathways
104 by aspirin reduces NE-induced intestinal inflammation. These findings could serve as a
105 stepping-stone for the development of new antimicrobial free prevention and therapeutic
106 strategies against NE.

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107 ## Results

108

109 ### DCA prevents *C. perfringens in vitro* growth

110 We previously reported that the secondary bile acid DCA prevents and treats *C.*
111 *jejuni*-induced intestinal inflammation in germ-free mice (28). This secondary bile acid
112 also inhibits *C. difficile in vitro* growth (12). Since *C. difficile* and *C. perfringens* are in the
113 same genus, we reasoned that DCA would prevent *C. perfringens* growth. To test this
114 hypothesis, we implemented *in vitro* inhibition experiments, in which *C. perfringens* was
115 inoculated in Tryptic Soy Broth (TSB) with sodium thioglycollate under anaerobic
116 condition. The TSB was also added with various concentrations of bile acids, including
117 conjugated primary bile acid taurocholic acid (TCA), primary bile acid cholic acid (CA),
118 and secondary bile acid DCA. Notably, DCA inhibited *C. perfringens* growth at 0.01 (-
119 33.8%) and 0.05 mM (-82.8%, clear broth), respectively, compared to control, while TCA
120 (-16.4%) and CA (-8.2%) barely prevented the bacterial growth (cloudy broth) even at 0.2
121 mM (Figure 1A and B). We then examined if other secondary bile acids were also
122 bacteriostatic in TSB. Interestingly, *C. perfringens* growth was only mildly inhibited by
123 lithocholic acid (LCA; -22.6 and -23.8%) and ursodeoxycholic acid (UDCA; -10.0 and -
124 25.3%) at 0.2 and 1 mM, respectively (Figure 1C and D). These results suggest that the
125 secondary bile acid DCA effectively curbs *C. perfringens in vitro* growth.

126

127 ### DCA prevented NE-induced productivity loss

128 To further address whether DCA reduces coccidia *E. maxima*- and *C. perfringens*-
129 induced necrotic enteritis (NE) in birds, we fed day-old broiler chicks with 1.5 g/kg CA or

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130 DCA diets and growth performance of body weight (BW) gain were measured. To induce
131 NE, the birds were infected with 20,000 sporulated oocysts/bird *E. maxima* at 18 days of
132 age and then infected with 10^9 CFU/bird *C. perfringens* at 23 and 24 days of age. Notably,
133 DCA (solid black bar) but not CA (straight line bar) diet promoted bird daily BW gain during
134 0-18 days of age compared to birds fed control diets (open bar, Figure 2 A). Body weight
135 gain was impaired in birds infected with *E. maxima* (Em) at 18-23 days of age (coccidiosis
136 phase). Subsequent *C. perfringens* infection further drove NE control birds (dotted bar)
137 into BW loss at 23-26 days of age (NE phase). Remarkably, DCA prevented productivity
138 loss at coccidiosis and NE phases compared to the NE control birds. Interestingly, the
139 primary bile acid CA diet attenuated body weight loss at NE phase but failed at coccidiosis
140 phase compared to the NE control birds.
141
142 **DCA prevented NE-induced histopathology**
143 Coccidiosis and NE induce severe intestinal inflammation. To have a thorough
144 insight into DCA impact on NE pathogenesis, we collected intestinal tissue at upper ileum
145 as Swiss rolls, processed with H&E staining, and performed histopathology analysis.
146 Notably, *E. maxima* infection induced severe intestinal inflammation (ileitis) as seen by
147 immune cell infiltration into lamina propria, crypt hyperplasia, and mild villus height
148 shortening compared to uninfected birds (Figure 2B). Furthermore, NE control birds
149 suffered worse ileitis as seen by necrosis and fusion of villi and crypt, massive immune
150 cell infiltration, and severe villus shortening. Notably, DCA diet dramatically attenuated
151 NE-induced ileitis and histopathology score (Figure 2B and C), while CA reduced NE-
152 induced ileitis and histopathology score. These results indicate that DCA promotes growth

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153 performance and resists against coccidiosis- and NE-induced BW loss and severe ileitis
154 and histopathology.
155

156 **DCA attenuates NE-induced intestinal cell necrosis and apoptosis**

157 Healthy intestinal epithelial cells have polarity (29) and their nuclei are located
158 toward the basal membrane (30), while stressed dying (apoptosis or necrosis) cells lose
159 polarity (31) and their nuclei disperse from basal to apical membranes (32). We then
160 sought to examine whether cell death is relevant in DCA-attenuating NE-induced ileitis.
161 Since it is difficult to find reliable chicken antibodies to detect apoptosis or necrosis in
162 chicken histology slides, we first resorted to classical histological analysis under high
163 magnification. Consistently, the epithelial nuclei (dark blue) in heathy control bird villi were
164 distributed close to the basal membrane (at the right side of the yellow dash line, Figure
165 3A lower panel left). In contrast, the nuclei in inflamed villi epithelial cells of Em and NE
166 birds were scattered from basal to the apical membranes, indicating epithelial cell death
167 in villi of those birds. Notably, the DCA diet prevented epithelial cell nucleus translocation
168 to apical side, suggesting cell death reduction. To further characterize the villus cell death,
169 we used terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNNEL)
170 assay, which detects later stage of cell apoptosis. Consistent with histopathology results,
171 coccidiosis and NE induced massive scatter (Em birds) or concentrated (NE birds)
172 apoptosis cells (green dots) in villus lamina propria, however, cellular apoptosis was
173 attenuated in the DCA treatment. These results indicate that DCA resists against
174 coccidiosis- and NE-induced cell death in villus epithelial and lamina propria cells.
175

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176 **DCA reduces *C. perfringens* invasion and NE-induced inflammatory response**

177 Given DCA inhibited *C. perfringens* growth *in vitro*, it is logic to reason that DCA
178 might also reduce *C. perfringens* intestinal overgrowth in NE birds. To examine this
179 possibility, we collected ileal digesta extracted total DNA and measured *C. perfringens*
180 colonization level in the intestinal lumen using real-time PCR of *C. perfringens* 16S rDNA.
181 Consistently, coccidiosis and NE were associated with increased luminal *C. perfringens*
182 colonization compared to uninfected birds (Figure 4A). Surprisingly, ileal luminal *C.*
183 *perfringens* load in DCA birds was not significantly different from NE control birds, while
184 bird productivity and histopathology were strikingly distinct between the two groups of
185 birds (Figures 2A-C). We, therefore, reasoned that the pathogen invasion into tissue was
186 the main driving factors of NE pathogenesis, but not the pathogen luminal colonization
187 level. Using a fluorescence *in situ* hybridization (FISH) technique, we found that while *C.*
188 *perfringens* was present deeply in the inflamed villus and crypt lamina propria of NE
189 control birds, the bacterium was barely detectable in the ileal tissue of DCA birds (Figure
190 4B). Because DCA reduced *C. perfringens* invasion and intestinal inflammation, we
191 evaluated the impact of DCA on various proinflammatory mediators in ileal tissue using
192 Real-Time PCR. Notably, *C. perfringens* strongly induced inflammatory *Infγ*, *Litaf* (*Tnfα*),
193 *Il1*β, and *Mmp9* mRNA accumulation in chicken ileal tissue, an effect attenuated by 51,
194 82, 63 and 93%, respectively, in DCA fed chickens (Figure 4C).

195

196 **COX inhibitor aspirin alleviates *C. perfringens*-induced inflammatory response in**
197 **splenocytes**

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198 Inflammatory events shape intestinal diseases and targeting the inflammatory
199 response attenuates disease progress such as inflammatory bowel disease (33) and
200 campylobacteriosis (34). To dissect how the host inflammatory response is involved in
201 NE-induced ileitis, we then used a primary chicken splenocyte cell culture system (35).
202 After isolation from 28-day old chickens, the splenocytes were infected with *C. perfringens*
203 (MOI 100) for 4 hours. Notably, *C. perfringens* increased inflammatory mediators of *Infγ*,
204 *Litaf*, *Il1*β, *Mmp9* and *Ptgs2* (protein COX-2) mRNA accumulation by 1.54, 1.69, 76.47,
205 1.72 and 8.65 folds, respectively, compared to uninfected splenocytes (Figure 5A). COX-
206 2 are important mediators in the inflammatory response (22). We then used COX inhibitor
207 aspirin towards *C. perfringens*-infected chicken splenocytes, but no inhibition of
208 inflammatory gene expression was observed (data not shown). We then used
209 inflammatory cytokines of recombinant murine INFγ and TNFα to challenge splenocytes
210 in the presence of aspirin. Remarkably, aspirin reduced INFγ-induced inflammatory gene
211 expression of *Infγ*, *Litaf*, *Il1*β, and *Mmp9* by 41, 27, 42, and 45%, respectively (Figure 5B).
212 Similarly, aspirin reduced TNFα-induced inflammatory gene expression of *Infγ*, *Litaf*, and
213 *Mmp9* by 49, 53, and 27%, respectively (Figure 5C). These data indicate that *C.*
214 *perfringens* induces inflammatory cytokines and COX-2 and inhibiting COX signaling by
215 aspirin reduces the cytokine-induced inflammatory response, suggesting that aspirin
216 poses protection potential against NE detrimental effects.

217

218 **Aspirin attenuates NE-induced productivity loss, histopathology and apoptosis**

219 To functionally assess the protective effect of aspirin against NE-induced ileitis,
220 broiler chickens fed with 0.12 g/kg aspirin diet (ASP) were infected with *E. maxima* and

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221 *C. perfringens* as describe before. Interestingly, ASP birds grew slower compared to
222 control diet birds during 0-18 days of age (Figure 6A). This is because aspirin inhibit all
223 COX isoforms and COX-1 and -3 are important for intestinal homeostats and growth.
224 Notably, ASP attenuated NE-induced BW loss by 60% in NE phase of 23-26 days of age,
225 while no difference between ASP and NE birds in coccidiosis phase of 18-23 days of age.
226 To further dissect the underlying cellular mechanism, we then used a histopathology
227 analysis. Consistently, ASP attenuated NE-induced intestinal inflammation and
228 histopathological score (Figures 6B and C). ASP also reduced NE-induced immune cell
229 apoptosis in villus lamina propria (Figure 6D). These data suggest that aspirin attenuates
230 NE-induced BW loss, intestinal inflammatory response, and villus cell death.
231
232

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233 # Discussion

234

235 Although NE has reemerged as a prevalent poultry disease worldwide in the antimicrobial
236 free era (3), the lack of comprehensive molecular mechanism insight into NE severely
237 hinders the development of antimicrobial alternatives to control this disease (36). Many
238 virulent factors of *Eimeria* and *C. perfringens* are identified but few findings are effective
239 to control NE in poultry production (5, 37), suggesting that we have overlooked important
240 players/factors in NE pathogenesis, such as microbiome and host response. Here we
241 reported that microbiota metabolic product DCA dramatically attenuates chicken NE by
242 reducing chicken inflammatory response. These new findings pave the path for exploring
243 novel antimicrobial alternatives to control NE.

244 It is a relatively new concept to manipulate microbiota and its metabolic products
245 against infectious diseases. Fecal transplantation was used in chickens decades ago to
246 prevent *S. infantis* infection (10). Microbiome plays an important role in susceptibility to
247 *C. difficile* infection (38). Anaerobe *C. scindens*-transformed secondary bile acids prevent
248 *C. difficile* germination and growth (12). Lithocholic acid and DCA but not primary bile
249 acid CA inhibit *C. difficile* vegetable growth and toxin production (12, 39). However,
250 whether secondary bile acids prevent or treat *C. difficile* infection in human or animal
251 models is still unknown. We recently found that orally gavaging DCA attenuates *C. jejuni*-
252 induced intestinal inflammation in germ-free mice (28). Based on the knowledge, we
253 reasoned that DCA may prevent *E. maxima*- and *C. perfringens*-induced chicken NE.
254 Indeed, dietary DCA but not CA prevents NE and its associated productivity loss. The
255 reduction of ileitis is coupled with reduced *C. perfringens* invasion and intestinal

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256 inflammation and cell death. Intriguingly, DCA failed to significantly reduce *C. perfringens*
257 ileal colonization, suggesting that the mechanism of DCA action is independent of
258 intestinal luminal colonization exclusion and is possible through modulating inflammation.
259 At the cellular level, the intestinal tract of NE-inflicted birds displays severe small
260 intestinal inflammation, showing massive immune cells infiltration into lamina propria,
261 villus breakdown, and crypt hyperplasia (40, 41). Intestinal inflammation is critical to clear
262 invaded microbes and to resolve inflammation, while overzealous inflammation causes
263 more bacterial invasion and further collateral damage and inflammation (42). Infectious
264 bacteria often hijack the inflammatory pathways to gain survival and invasion advantage.
265 For example, *Salmonella* Typhimurium induces extensive intestinal inflammation and
266 thrives on the inflammation (43). Consistent with this “over-inflammation” model, NE birds
267 with severe intestinal inflammation shows extensive BW loss, villus cell death, and *C.*
268 *perfringens* invasion. Conversely, DCA attenuating ileitis improves the growth
269 performance and dramatically reduces the NE pathology. Consequently, blocking
270 downstream inflammatory COX signaling by aspirin alleviates intestinal inflammation,
271 villus apoptosis and 60% NE-induced BW loss. These findings indicate that DCA inhibiting
272 inflammatory signaling pathways and targeting them could effectively prevent NE.
273 Altogether, our data reveal that the microbial metabolic product secondary bile acid
274 DCA attenuates NE, through blunting NE-induced host inflammatory response. These
275 findings highlight the importance of elucidating the molecular relationship between
276 infectious pathogen, microbiome, and host response. These discovers lay the first stone
277 on using microbiome and host inflammatory response to control NE and other intestinal
278 diseases.

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279

280 # Materials and Methods

281

282 ## Chicken experiment

283 All animal protocols were approved by the Institutional Animal Care and Use Committee
284 of the University of Arkansas at Fayetteville. Cohorts of 13 day-of-age broiler chicks
285 obtained from Cobb-Vantress (Siloam Springs, AR) were neck-tagged and randomly
286 placed in floor pens with a controlled age-appropriate environment. The birds were fed a
287 corn-soybean meal-based starter diet during 0-10 days of age and a grower diet during
288 11-26 days of age. The basal diet was formulated as described before (44). Treatment
289 diets were supplemented with 1.5 g/kg CA or DCA or 0.12 g/kg aspirin (all from Alfa
290 Aesar). Birds were infected with 20,000 sporulated oocytes/bird *E. maxima* at 18 days of
291 age and 10^9cfu/bird *C. perfringens* at 23 and 24 days of age. Chicken body weight and
292 feed intake were measured at d 0, 18, 23, and 26 days of age. Bird health status was
293 monitored daily after the pathogen infection. Birds were sacrificed at 26 days of age. Ileal
294 tissue and digesta samples were collected for RNA and DNA analysis. Ileal tissue was
295 also Swiss-rolled for histopathology analysis. Images were acquired using a Nikon TS2
296 fluorescent microscope. Ileal inflammation was scored by evaluating the degree of lamina
297 propria immune cell infiltration, villus shortening, edema, necrosis, crypt hyperplasia,
298 ulceration, and transmural inflammation using a score from 0 to 16.

299

300 ## *C. perfringens*-induced inflammatory response using primary splenocytes

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301 Splenocytes were isolated similarly to described previously (34). Briefly, chickens at 28
302 days of age were sacrificed and spleens were resected and homogenized using frosted
303 glass slides in RPMI 1640 medium supplemented with 2% fetal bovine serum, 2mM L-
304 glutamine, 50 µM 2-mercaptoethanol. After lysed the red blood cells, the collected cells
305 were plated at 2 x 10^6 cells/well in 6-well plates. The cells were pre-treated with 1.2 mM
306 aspirin for 45 min. Cells were then challenged with murine INFγ (1µg/ml, Pepro Tech),
307 TNFα (5 ng/ml, Pepro Tech), or *C. perfringens* (multiplicity of infection 100). The cells
308 were lysed in TRIzol (Invitrogen) at 2 or 4 hours after cytokines or *C. perfringens* treatment,
309 respectively.
310

311 **Real time RT-PCR**

312 Total RNA from ileal tissue or splenocytes was extracted using TRIzol as described
313 before (28, 45). cDNA was prepared using M-MLV (NE Biolab). mRNA levels of
314 proinflammatory genes were determined using SYBR Green PCR Master mix (Bio-Rad)
315 on a Bio-Rad 384-well Real-Time PCR System and normalized to *Gapdh.* Ileal digesta
316 DNA was extracted as described before (28) and the digesta bacteria were subject to
317 real-time PCR. The PCR reactions were performed according to the manufacturer's
318 recommendation. The following gene primers were used:
319 *Cp16S*_forward: 5'- CAACTTGGGTGCTGCATTCC-3'; *Cp16S* reverse: 5'-
320 GCCTCAGCGTCAGTTACAG-3'; *Mmp9*_forward: 5'-CCAAGATGTGCTCACCAAGA-3'
321 *Mmp9*_reverse: 5'-CCAATGCCCAACTTCTCAAT-3'; *Litaf* *(Tnfα)*_forward: 5'-
322 AGATGGGAAGGGAATGAACC; *Litaf* *(Tnfα)*_reverse: 5'-
323 GACGTGTCACGATCATCTGG-3'; *Il1β*_forward: 5'-GCATCAAGGGCTACAAGCTC-3';

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324 *Il1 β*_reverse: 5'-CAGGCGGTAGAAGATGAAGC-3'; *Infγ*_forward: 5'-
325 AGCCGCACATCAAACACATA -3'; *Infγ*_reverse: 5'-TCCTTTTGAAACTCGGAGGA-3';
326 *Ptgs2*_forward: 5'-ACCAGCATTTCAACCTTTGC-3'; *Ptgs2*_reverse: 5'-
327 CCAGGTTGCTGCTCTACTCC-3'; *Gapdh*_forward: 5'-GACGTGCAGCAGGAACACTA-
328 3'; *Gapdh*_reverse: 5'- CTTGGACTTTGCCAGAGAGG-3'.
329

330 **TUNNEL assay**

331 Cell apoptosis in intestinal tissue sections was visualized using TUNNEL assay. Briefly,
332 ileal tissue sections were deparaffinized with xylene bath for 3 times and rehydrated with
333 100%, 95%, and 70% ethanol. The tissue was then incubated with TUNNEL solution (5
334 µM Fluorescein-12-dUTP (Enzo Life Sciences), 10 µM dATP, 1 mM pH 7.6 Tris-HCl, 0.1
335 mM EDTA, 1U TdT enzyme (Promega) at 37° C for 90 min. The slides were counter-
336 stained with DAPI for nucleus visualization. The fluorescent green apoptosis cells were
337 evaluated and imaged using a Nikon TS2 fluorescent microscopy.
338

339 **Fluorescence in situ hybridization (FISH)**

340 *C. perfringens* at ileal tissue sections was visualized using FISH assay similarly as
341 previously described. Briefly, tissue sections were deparaffinized, hybridized with the
342 FISH probe, washed, stained with DAPI, and imaged using a Nikon TS2 fluorescent
343 Microscope system. The FISH probe sequence of Cp85aa18: 5'-
344 /Cy3/TGGTTGAATGATGATGCC-3' (46) was used to probe the presence of *C.*
345 *perfringens* similar to a previous report (45). Briefly, deparaffinized, formalin-fixed 5 µm
346 thick sections were incubated for 15 minutes in lysozyme (300,000 Units/ml lysozyme;

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347 Sigma-Aldrich) buffer (25 mM Tris pH 7.5, 10 mM EDTA, 585 mM sucrose, and 0.3 mg/ml
348 sodium taurocholate) at room temperature and hybridized overnight at 46 °C in
349 hybridization chambers with the oligonucleotide probe (final concentration of 5 ng/µl in a
350 solution of 30 percent formamide, 0.9 M sodium chloride, 20 mM Tris pH 7.5, and 0.01%
351 sodium dodecyl sulfate). Tissue sections were washed for 20 minutes at 48 °C in washing
352 buffer (0.9 M NaCl, 20 mM Tris pH 7.2, 0.1% SDS, 20% Formamide, and 10% Dextran
353 Sulfate) and once in distilled water for 10 seconds. The slide was stained with DAPI for 2
354 min and dried at RT, mounted with 50% glycerol. *C. perfringens* in intestinal tissue was
355 evaluated and imaged using a Nikon TS2 fluorescent microscopy.
356

357 **Bile acid C. perfringens inhibition assay**

358 *C. perfringens* in Tryptic Soy Broth (TSB) supplemented with 0.5% sodium thioglycollate,
359 with added TCA, CA or DCA (0, 0.01, 0.05, 0.1, or 0.2 mM, final concentration) or LCA or
360 UDCA (0, 0.2, or 1 mM, final concentration) was cultured overnight under anaerobic
361 conditions. The bacterial growth was monitored by (OD600nm) using a
362 spectrophotometer.

363 **Statistical Analysis**

364 Values are shown as mean ± standard error of the mean as indicated. Differences
365 between groups were analyzed using the nonparametric Mann–Whitney *U* test performed
366 using Prism 5.0 software. Experiments were considered statistically significant if *P* values
367 were <0.05.

368

369

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494 **Figure legends**

495

496 **Figure 1. DCA inhibits *C. perfringens in vitro* growth.** *C. perfringens* (10^3 CFU) were
497 inoculated into TSB supplemented with various concentrations of conjugated primary bile
498 acid TCA, primary bile acid CA, and secondary bile acid DCA. (A) Image of bile acids did
499 (clear broth) or did not (cloudy broth) inhibited *C. perfringens* growth. (B) OD600 reading
500 of the broth in A. (C) Image of bile acids did not inhibit *C. perfringens* growth. (B) OD600
501 reading of the broth in C. All graphs depict mean ± SEM. Results are representative of 3
502 independent experiments.

503

504 **Figure 2. DCA attenuates NE-induced productivity loss and histopathology.** Cohorts
505 of 13 broiler chickens were fed basal, 1.5 g/kg CA, or DCA diets. The birds were infected
506 with *E. maxima* and *C. perfringens* at 18 and 23 days of age, respectively. The birds were
507 sacrificed at 26 days of age. (A) Bird growth performance of daily body weight gain. (B)
508 H&E staining showing representative intestinal histology images. (C) Quantification of
509 histological intestinal damage score. Scale bar is 200 µm. All graphs depict mean ± SEM.
510 *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. Results are representative of 3 independent
511 experiments.

512

513 **Figure 3. DCA attenuates NE-induced intestinal cell death and apoptosis.** Cohorts
514 of 13 broiler chickens were fed different diets and infected as in Figure 2. (A)
515 Representative intestinal cell death (deviated nuclei) using H&E staining. (B)

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516 Representative villus cell apoptosis (green) using TUNEL assay. Scale bar is 20 μm (A)
517 and 10 μm (B). Results are representative of 3 independent experiments.
518
519 **Figure 4. DCA reduces *C. perfringens* invasion and inflammatory response.** Cohorts
520 of 13 broiler chickens were fed different diets and infected as in Figure 2. (A) Luminal *C.*
521 *perfringens* colonization level quantified by 16s RNA real-time PCR. (B) Presence of *C.*
522 *perfringens* (red dots) in ileal sections of NE birds, detected using fluorescence in situ
523 hybridization (FISH) assay. (C) Ileal *Infγ*, *Litaf* (*Tnfα*), *Il1β*, and *Mmp9* mRNA qPCR fold
524 change relative to uninfected birds and normalized to *Gapdh*. All graphs depict mean ±
525 SEM. NS, not significant; *, P<0.05. Results are representative of 3 independent
526 experiments.
527
528 **Figure 5. COX inhibitor aspirin alleviates *C. perfringens*-induced inflammatory**
529 **response in chicken splenocytes.** Splenocytes isolated from broiler chickens were
530 infected with *C. perfringens* (MOI 100) for 4 hr or stimulated with murine INFγ (1μg/ml) or
531 TNFα (5 ng/ml) for 2 hr in the presence of 1.2 mM aspirin. RNA was extracted, reverse-
532 transcribed, and quantified using a Bio-Rad 384 PCR platform. (A) *Infγ*, *Litaf*, *Il1β*, *Mmp9*,
533 and *Ptgs2* mRNA fold change normalized to *Gapdh*. (B) Gene expression fold change in
534 the presence of INFγ and aspirin. (C) Gene expression fold change in the presence of
535 TNFα and aspirin. All graphs depict mean ± SEM. *, P<0.05; ***, P<0.001. Results are
536 representative of 3 independent experiments.
537

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538 **Figure 6. Aspirin attenuates NE-induced productivity loss, histopathology, and**
539 **apoptosis.** Cohorts of 13 broiler chickens were fed basal and 0.12 g/kg aspirin diet. The
540 birds were infected and sampled as in figure 1. (A) Bird growth performance of daily body
541 weight gain. (B) H&E staining showing representative intestinal histology images. (C)
542 Quantification of histological intestinal damage score. (D) Representative cell apoptosis
543 (green) using TUNEL assay. Scale bar is 200 μm in B and 10 μm in C. All graphs depict
544 mean ± SEM. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. Results are representative of 2
545 independent experiments.

Figure 1. Deoxycholic acid (DCA) inhibits *C. perfringens* in vitro growth

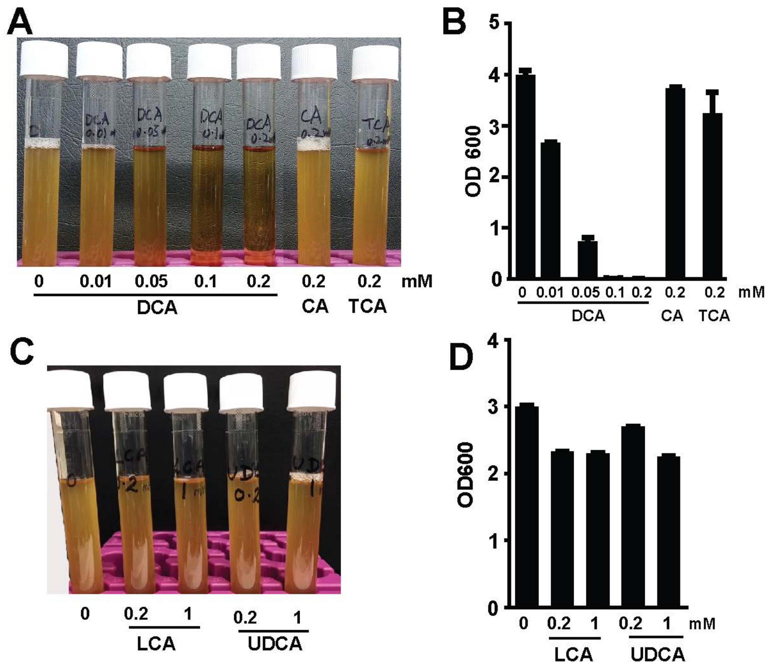
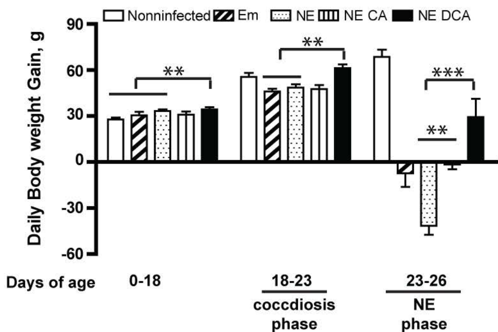


Figure 2. DCA attenuates NE-induced productivity loss and histopathology



B

Noninfected

NE

Em

NE + CA

NE + DCA

200 μM

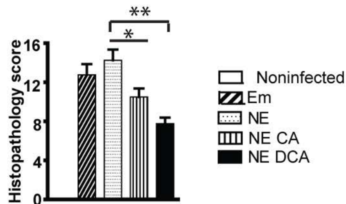


Figure 3. DCA attenuates NE-induced intestinal cell necrosis and appoptosis

A

Noninfected
Em
NE
NE + DCA
20 μM
20 μM
20 μM
20 μM

B

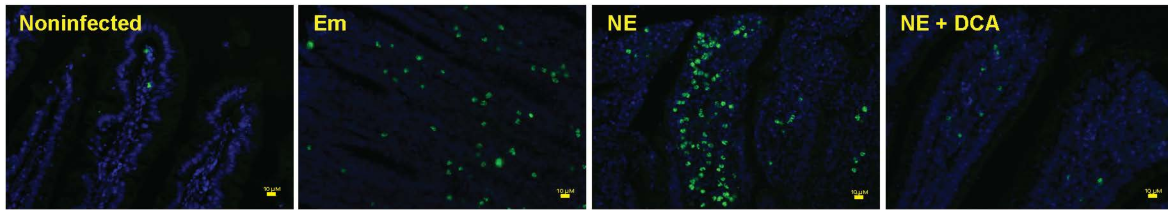


Figure 4. DCA reduces *C. perfringens* invasion and inflammatory response

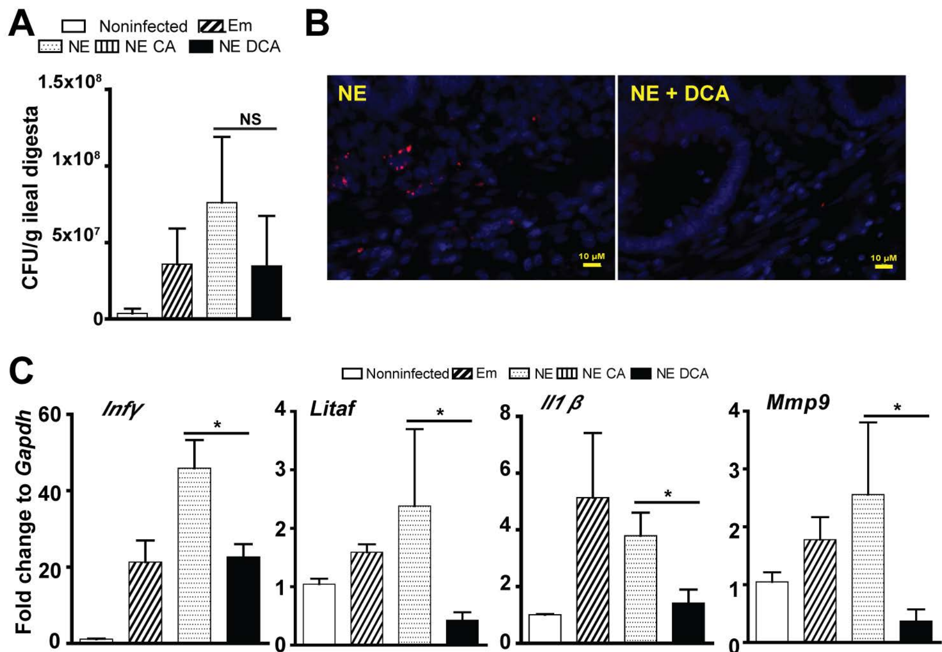


Figure 5. COX inhibitor Aspirin alleviates *C. perfringens*-induced inflammatory response in splenocytes

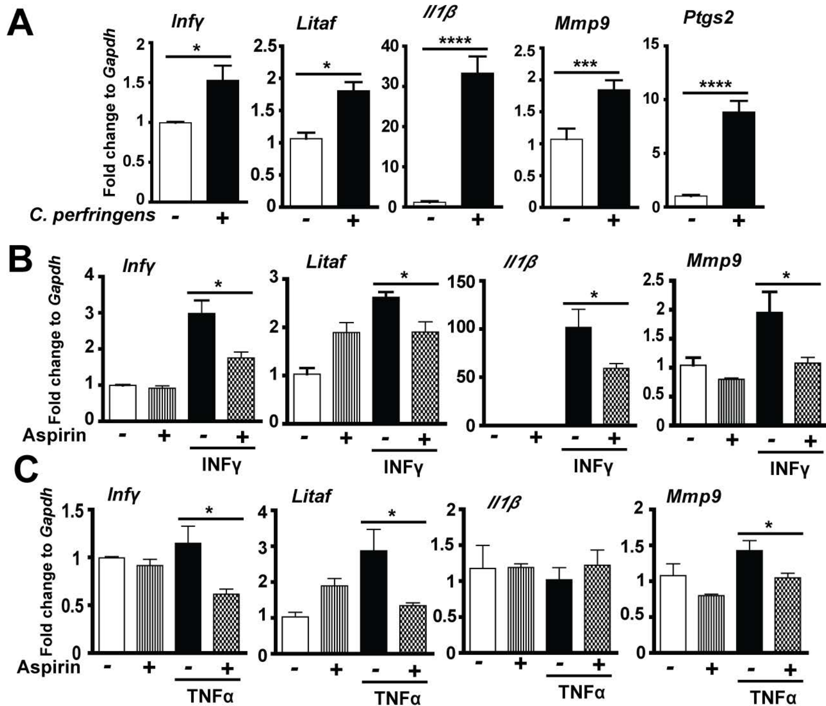


Figure 6. Aspirin atteuates NE-induced productivity loss, histopathology and apoptosis