Evaluation of a toxoid fusion protein vaccine produced in plants to protect poultry against necrotic enteritis

Joseph G Hunter [1,2] , **Shyra Wilde** [1] , **Amanda M Tafoya** [1] , **Jamie Horsman** [1] , **Miranda Yousif** [1] , **Andrew G Diamos** [1,2] , **Kenneth L Roland** [1] , **Hugh S Mason** Corresp. [1,2]

[1] Center for Immunotherapy, Vaccines and Virotherapy, The Biodesign Institute, Arizona State University, Tempe, AZ, United States

[2] School of Life Sciences, Arizona State University, Tempe, AZ, United States

Corresponding Author: Hugh S Mason
Email address: hugh.mason@asu.edu

Background. Necrotic enteritis (NE) is caused by type A strains of the bacterium *Clostridium perfringens*. Total global economic losses to the poultry industry due to NE is estimated to be over 2 billion dollars annually. Traditionally, NE has been effectively controlled by inclusion of antibiotics in the diet of poultry. However, recent concerns regarding the impact of this practice on increasing antibiotic resistance in human pathogens have led us to consider alternative approaches, such as vaccination, for controlling this disease. NE strains of *C. perfringens* produce two major toxins, a-toxin and NetB. Immune responses against either toxin can provide partial protection against NE.

Methods. We have developed a fusion protein combining a non-toxic carboxyl-terminal domain of a-toxin (PlcC) and an attenuated, mutant form of NetB (NetB-W262A) for use as a vaccine antigen to immunize poultry against NE. We utilized a DNA sequence that was codon-optimized for *Nicotiana benthamiana* to enable high levels of expression. The 6-His tagged PlcC-NetB fusion protein was synthesized in *N. benthamiana* using a geminiviral replicon transient expression system, purified by metal affinity chromatography, and used to immunize broiler birds.

Results. Immunized birds produced a strong serum IgY response against both the plant produced PlcC-NetB protein and against bacterially produced His-PlcC and His-NetB. Immunized birds were significantly protected against a subsequent in-feed challenge with virulent *C. perfringens* when treated with the fusion protein. These results indicate that a plant-produced PlcC-NetB toxoid is a promising vaccine candidate for controlling NE in poultry.

1 **Evaluation of a toxoid fusion protein vaccine**

2 **produced in plants to protect poultry against necrotic**

3 **enteritis**

4

5 Joseph G. L. Hunter[1,2A], Shyra Wilde[1AB], Amanda M. Tafoya[1], Jamie Horsman[A], Miranda

6 Yousif[1], Andrew G. Diamos[1,2], Kenneth L. Roland[1] and Hugh S. Mason[1,2] *

7

8 [1]Center for Immunotherapy, Vaccines and Virotherapy, The Biodesign Institute, Arizona State

9 University, Tempe, Arizona, USA

10 and

11 [2]School of Life Sciences, Arizona State University, Tempe, Arizona, USA

12 [A]These authors contributed equally to this work

13

14 [B]Current address: Department of Microbiology and Molecular Genetics, Emory University

15

16 *Corresponding Author

17

18 Email addresses:

19 Joseph Hunter - jghunte1@asu.edu

20 Shyra Wilde - shyra.lynn6@gmail.com

21 Amanda M. Tafoya - Amanda.Gonzales@asu.edu

22 Jamie Horsman - jhorsma1@asu.edu

23   Miranda Yousif - mlyousif@asu.edu

24   Andrew Diamos - adiamos@asu.edu

25   Kenneth L. Roland – Kenneth.roland@asu.edu

26   Hugh Mason - Hugh.Mason@asu.edu

Abstract

Background. Necrotic enteritis (NE) is caused by type A strains of the bacterium *Clostridium perfringens*. Total global economic losses to the poultry industry due to NE is estimated to be over 2 billion dollars annually. Traditionally, NE has been effectively controlled by inclusion of antibiotics in the diet of poultry. However, recent concerns regarding the impact of this practice on increasing antibiotic resistance in human pathogens have led us to consider alternative approaches, such as vaccination, for controlling this disease. NE strains of *C. perfringens* produce two major toxins, α-toxin and NetB. Immune responses against either toxin can provide partial protection against NE.

Methods. We have developed a fusion protein combining a non-toxic carboxyl-terminal domain of α-toxin (PlcC) and an attenuated, mutant form of NetB (NetB-W262A) for use as a vaccine antigen to immunize poultry against NE. We utilized a DNA sequence that was codon-optimized for *Nicotiana benthamiana* to enable high levels of expression. The 6-His tagged PlcC-NetB fusion protein was synthesized in *N. benthamiana* using a geminiviral replicon transient expression system, purified by metal affinity chromatography, and used to immunize broiler birds.

Results. Immunized birds produced a strong serum IgY response against both the plant produced PlcC-NetB protein and against bacterially produced His-PlcC and His-NetB. Immunized birds were significantly protected against a subsequent in-feed challenge with virulent *C. perfringens* when treated with the fusion protein. These results indicate that a plant-produced PlcC-NetB toxoid is a promising vaccine candidate for controlling NE in poultry.

49 **Key words**

50 Necrotic enteritis vaccine, poultry vaccine, *Clostridium perfringens*, plant-based antigen

51 production

52

53 **INTRODUCTION**

54   *Clostridium perfringens* (C. *perfringens*), a Gram-positive anaerobic spore-forming

55 bacterium, is the causative agent of necrotic enteritis (NE) in poultry and has become an

56 economically significant issue for the broiler industry [1]. The pathogenesis of NE is complex

57 and results from the concerted actions of a number of *Clostridial* genes combined with reduced

58 gut health generated by environmental factors, feed, mycotoxins or the presence of other

59 pathogens [2, 3]. NE typically occurs in broiler chicks at 2 to 6 weeks of age, but may occur in

60 older birds as well [4, 5]. The onset of NE at around 2 weeks is believed to be due to waning

61 levels of anti-Clostridium maternal antibodies [5]. The acute form of the disease leads to

62 increased mortality in broiler flocks, which can account for high losses of up to 1% per day,

63 reaching mortality rates up to 10-40% [6]. In the subclinical form, damage to the intestinal

64 mucosa caused by *C. perfringens* leads to poor productivity (reduced growth, reduced feed

65 efficiency) without mortality. The estimated cost of subclinical NE is around $0.05 per chicken

66 with a total global loss of nearly $2 billion per year [7, 8].

67   *C. perfringens* is ubiquitous; readily found in soil, dust, feces, feed, used

68 poultry litter and is normally present in the intestines of healthy chickens. The

69 enterotoxaemia that results in clinical disease most often occurs either following an alteration in

70 the intestinal microflora or from a condition that results in damage to the intestinal mucosa (e.g.

71 coccidiosis). High dietary levels of animal by-products such as fishmeal, or certain grains with

72   high levels of indigestible fiber including wheat, barley, oats, and rye predispose birds to NE.

73   Dietary fat source can also influence the *C. perfringens* population [9]. Current thinking is that

74   predisposing factors such as a high protein diet (e.g. fishmeal) and/or *Eimeria* infection result in

75   alterations of the chicken gut microbiota that allow incoming or resident *C. perfringens* strains to

76   become established [10].

77   Historically, *C. perfringens* outbreaks in the broiler industry were avoided using growth-

78   promoting antimicrobials in the diet. However, concerns regarding antibiotic resistance led to

79   restrictions on the use of antibiotics [11]. When antibiotics were banned in European and

80   Scandinavian countries, it led to increases in *C. perfringens* infections, including NE [12]. Thus,

81   other methods for controlling *C. perfringens* infections are needed.

82   Toxins have traditionally been targeted as antigens of interest for controlling clostridial

83   infections. The *C. perfringens* α-toxin, encoded by the *plc* gene, is the major virulence

84   determinant for gas gangrene [13]. Antibodies to *C. perfringens* α-toxin prevent gas gangrene in

85   mice [14]. The protein is divided into two domains; the amino-terminal domain and carboxy-

86   terminal domain [15]. The amino-terminal domain encodes the catalytic site responsible for

87   phospholipase activity [15-17], while the carboxy-terminal domain is involved in interactions

88   with phospholipids, targeting the enzyme to host cell membranes [18]. The α-toxin carboxy-

89   terminal fragment (aa 247 – 370) is non-toxic [19] and immunization with this fragment confers

90   protection against α-toxin and *C. perfringens* in a gas gangrene mouse model [14]. In chickens,

91   immune responses against the C-terminal domain (PlcC) can provide protection against intestinal

92   lesions following challenge with *C. perfringens* [14, 20-22].

93   For many years, the chromosomally-encoded α-toxin was considered to be the major

94   toxin associated with NE [13, 14, 23, 24]. The C-terminal domain of the α-toxin (PlcC) has been

95   studied extensively as a vaccine against *C. perfringens* infection, delivered as a purified protein

96   [25-27] or by live attenuated bacteria [22, 28]. Currently the only commercially available

97   vaccine for necrotic enteritis is composed of an α-toxoid derived from a *C. perfringens* type A

98   strain [29].

99   More recent studies have identified a β-like toxin, designated NetB toxin, linked to

100   necrotic enteritis. It was originally identified in a *C. perfringens* type A strain isolated from an

101   Australian outbreak of NE [30]. Keyburn et al. showed that *C. perfringens* supernatants contain a

102   factor that is toxic for chicken leghorn male hepatoma cells [30].   The factor was present in

103   supernatant from a *plc* knockout mutant, but not a *netB* knockout mutant of *C. perfringens*,

104   implicating NetB as the toxin. Further, the *C. perfringens netB* mutant was attenuated for

105   virulence in chickens, whereas a *plc* mutant of the same parent strain retained full virulence [31].

106   Screens for *netB* in *C. perfringens* field isolates indicate that the presence of the *netB* gene is

107   highly correlated with necrotic enteritis causing strains [30, 32, 33]. Thus, NetB has emerged as

108   a critical virulence factor. NetB functions by binding to cholesterol in membranes, forming

109   heptameric pores [34, 35]. There are a number of single amino acid substitutions in the rim loop

110   region of the protein, believed to interact with the host membrane, that significantly reduce its

111   toxicity. These include Y191A, R200A, W257A and W262A [34] and S254L, R230Q and

112   W287R [35].

113   NetB is also a protective antigen, providing significant protection against *C. perfringens*

114   challenge, particularly in combination with other immunogenic components [36-38]. In addition,

115   immunization with non-toxic rim mutants, W262A [36] and S254L [38], have been shown to

116   generate protective immune responses in chickens. A study that examined serum antibody levels

117   against *C. perfringens* α-toxin and NetB toxin in commercial birds from field outbreaks of NE

118   showed that the levels of serum antibodies against both α-toxin and NetB toxin were

119   significantly higher in apparently healthy chickens compared to birds with clinical signs of NE,

120   suggesting that these antitoxin antibodies may play a role in protection [39]. These results

121   indicate a correlation between the presence of antitoxin antibodies in the serum and protective

122   immunity against NE.

123   In a previous study, we used a live attenuated *Salmonella* vaccine to deliver PlcC, NetB,

124   or both to chickens. We found that birds immunized against both toxins developed fewer severe

125   lesions after *C. perfringens* challenge than birds immunized with either antigen alone [20]. Based

126   on this finding, we have been developing approaches to combine the immunogenic components

127   of each toxoid into a single protein. In this study, we describe a novel *plcC-netB* fusion gene

128   modified to encode the rim mutation W262A and codon-optimized for production in *Nicotiana*

129   *benthamiana*. The gene was introduced into *N. benthamiana* via *Agrobacterium tumefaciens*-

130   mediated transformation of a geminiviral replicon transient expression system. The resulting

131   fusion protein was purified and used to inject broiler chicks. Results indicate that the protein was

132   highly immunogenic and elicited partial protection against challenge with a virulent strain of *C.*

133   *perfringens*.

134

135   **MATERIALS AND METHODS**

136   2.1 Expression vector construction

137   A PlcC-NetB fusion protein gene was designed using codons preferred for N.

138   benthamiana [40]. Amino acid sequences were based on GenBank accessions *A*AP15462.1 for

139   PlcC and ABW71134.1 for NetB. For targeting of nascent protein to the endoplasmic reticulum

140   (ER), the barley alpha amylase signal peptide, followed by a 6His tag, was added to the N-

141   terminus of PlcC. A linker peptide GGSGGSGGPSGGSGG was inserted between the PlcC and

142   NetB sequences (Fig. 1). The fusion protein nucleotide sequences are in GenBank accession

143   numbers MH883808 and MH883809. The gene was synthesized commercially with flanking

144   XbaI and SacI sites. The 1402 bp XbaI-SacI fragment was ligated with geminiviral vector

145   pBYR2eK2M [41] to make pBYR2eK2M-6HplcCnetB. Removal of the 417 bp BamHI fragment

146   containing the PlcC and linker sequences yielded the ER-targeted 6His-NetB construct

147   pBYR2eK2M-6HnetB. To make the ER-targeted 6His-PlcC vector, we PCR end-tailored the

148   PlcC sequence to introduce a stop codon and SacI site at its 3' end, using the primer PlcC-Sac-R

149   (Table S1), inserting the resulting product to yield pBYR2eK2M-6HplcC. To produce the

150   cytosol-targeted proteins, the signal peptide was removed by introducing an XbaI site and start

151   codon 5' of the 6His sequence by PCR with primer 6H-Xba-F and using the ER-targeting

152   constructs above as templates. The resulting XbaI-SacI fragments were ligated with plasmid

153   pBYR2e3K2Mc-GFP [42, 43] digested XbaI-SacI, thus yielding the vectors pBYR2e3K2Mc-

154   c6HplcCnetB, pBYR2e3K2Mc-c6HplcC, and pBYR2e3K2Mc-c6HnetB.

155

156   2.2 Agroinfiltration of plants for protein production

157   Expression vectors were inserted into *Agrobacterium tumefaciens* EHA105 via

158   electroporation. PCR and restriction digestion of the purified plasmids were used to confirm the

159   resulting strains. Agrobacterium strains were grown overnight at 30°C in YENB (7.5 g Yeast

160   Extract and 8 g Nutrient Broth for 1 liter) + 50 mg/L kanamycin and 2.5 mg/L rifampicin.

161   Bacteria were pelleted for 10 minutes at 5,000g, then resuspended in infiltration buffer [10 mM

162   2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5 and 10 mM $MgSO_4$) with a final OD600 =

163   0.2. Suspensions were infiltrated into *N. benthamiana* leaves at 5- to 6-weeks of age using a

164 syringe without needle via a small puncture [43]. Infiltrated leaves were harvested at 5 days post

165 infiltration (DPI).

166

167 2.3 Protein extraction, acid precipitation clarification, and filtration

168   Total protein was extracted from agroinfiltrated leaf samples using a 1:3 (*w:v*) ratio of

169 extraction buffer (50 mM sodium phosphate, 200 mM sodium chloride, pH 8.1) at 4˚C. Leaf

170 samples were homogenized for 5-10 min until an even consistency was obtained using a two

171 speed Conair™ Waring™ Laboratory Blender (Fisher Scientific, Waltham, MA). Homogenized

172 leaf tissue was stirred at high speed for 10 minutes at 4°C to improve protein solubility.

173   Acid precipitation was performed on crude extracts after this 10-minute stirring period.

174 Note that no filtration, sedimentation, or centrifugation was performed before acid precipitation.

175 1 M phosphoric acid was added to the crude extract as it spun until a pH of 4.4-4.6 was reached,

176 as measured by a VWR Sb70 Symphony Ph Meter (VWR, Radnor, PA), Extract was stirred for

177 10 min at 4°C. 2 M Tris base was then added to the extract until pH reached 7.5-7.7, and the

178 mixture was stirred for another 10 min at 4°C before being filtered through Miracloth and

179 centrifuged at 17,000*g*, 4°C, for 30 min. The extract was then subjected to a second filtration

180 through Miracloth resulting in a clarified extract, which was passed through a 0.45-micron filter

181 for sterilization before metal affinity chromatography.

182   Three mL of Talon metal affinity resin (Clontech/Takara Bio, Mountain View, CA) was

183 spun down at 10,000*g*, 4°C, for 5 min and the supernatant removed from the bed. The resulting

184 1.5 mL resin bed was washed using 15 mL of extraction buffer (referred to below as wash

185 buffer) and once again spun down at 10,000*g*, 4°C, for 5 min. Supernatant was discarded and the

186 resin resuspended in 15 mL wash buffer before being added to a 20 mL column. A final wash of

187   20 mL was performed before the filtered extract was passed through the column. The resin was

188   washed with 60 mL of wash buffer, and elution of bound proteins achieved with PBS + 150 mM

189   imidazole, pH 7.4, collected in five 1 mL samples. Elution samples were dialyzed overnight in 1

190   liter of sterile PBS using either a 3 mL or 10 mL 3,500 MWCO Slide-A-Lyzer G2 dialysis

191   cassette (Thermo Scientific, Waltham, MA), following manufacturer's instructions. Elution

192   concentrations were determined both before and after dialysis by an A_{280} absorption using PBS

193   and PBS + 150 mM imidazole, respectively, as blank solutions. Protein concentrations were

194   confirmed by Coomassie staining of SDS-PAGE (below) analyzed with ImageJ [44].

195

196   2.4 SDS-PAGE and Western blot

197   Crude extracts, clarified extracts, and eluted samples were mixed with SDS sample buffer

198   (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02 % bromophenol blue) under reducing

199   conditions (0.5 M DTT) and samples boiled for 10 min and separated on 4-15% polyacrylamide

200   gels (Bio-Rad, Hercules, CA). Gels were stained with Coomassie stain (Bio-Rad) or electroblot

201   transferred to a PVDF membrane. PVDF membranes were blocked with 5% PBSTM (PBS with

202   0.05% Tween-20 and 5% nonfat dry milk) overnight at 4°C. Membranes were then washed with

203   PBST before incubation with a 1:10,000 dilution of rabbit anti-PlcC or rabbit anti-NetB

204   antibodies [20] in 1% PBSTM for 1 hour at 37°C. After washing a second time the membranes

205   were incubated with a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase conjugate in

206   1% PBSTM for 1 hour at 37°C. Bound antibody was detected with ECL reagent (Amersham

207   Biosciences, San Francisco USA). Stripping of blots for reprobing was performed using a

208   modified Abcam protocol. PVDF membrane was washed for 10 min in mild stripping buffer (for

209   1 liter: 15 g glycine, 1 g SDS, 10 mL Tween20, pH 2.2) per Abcam's recipe. PVDF membrane

210   was then rinsed twice in 20 mL of PBS for 10 minutes followed by two 5-min rinse steps in 20

211   mL PBST. 5% PBSTM was used to block the membrane before reprobing.

212

213   2.6 Production of recombinant proteins in *E. coli* for immune assays

214   His-tagged recombinant PlcC (rPlcC) and NetB (rNetB) were cloned by PCR using primer

215   sets Plc_pqe_f1/ Plc_pqe_r2 and netB_pqe_f3/ netB_pqe_r4, respectively (Table S1), using

216   pYA5130 as the template. The resulting PCR fragments were digested with BamHI and SalI and

217   ligated to BamHI/SalI-digested plasmid pQE30. Plasmids carrying the desired inserts were

218   moved into *E. coli* strain M15(pREP4) (Qiagen). Protein synthesis was induced by the addition

219   of IPTG according to the manufacturer's instructions. rPlcC and rNetB were purified by passage

220   over a TALON affinity column (Clontech Laboratories, Inc, Mountain View, CA) following the

221   manufacturer's recommendations. The GST-NetB protein shown in Figures 2 and 3 was purified

222   as previously described [20].

223

224   2.7 Chicken experiments

225   All animal experiments were conducted in compliance with the Arizona State University

226   Institutional Animal Care and Use Committee (Protocol 16-1480R) and the Animal Welfare Act,

227   in accordance with the National Institutes of Health guide for the care and use of laboratory

228   animals (NIH Publication No. 8023, revised 1978).

229   Groups of one-day-old Cornish × Rock broiler chickens were purchased from the Murray

230   McMurray Hatchery (Webster City, IA) and randomly sorted and placed in pens with pine

231   shavings on the floor. Food and water was supplied *ad libitum*. For experiments 1 and 2, groups

232   of birds were immunized subcutaneously with 50 µg of fusion protein (Exp. 1) or 100 µg of

233   fusion protein (Exp. 2) with 50 µg of Quil-A adjuvant. Birds were given three immunizations at

234   weekly intervals beginning at 8 days of age, with the final immunization at 22 days of age. Mock

235   vaccinated controls were injected with adjuvant only. At 27 days of age, blood was taken for

236   serum analysis. Three days later, when the birds were 30 days of age, birds were given an in-feed

237   challenge with virulent *C. perfringens* for 5 days. The birds were scored for intestinal lesions the

238   next day, at 35 days of age. For experiment 3, birds were injected with 50 µg of PlcC-NetB at 7

239   days of age and again at 12 days of age. Blood and tissues were taken for immunological

240   analysis ten days after the boost. Birds were challenged two weeks after the final boost, when the

241   birds were 26 days of age.

242

243   2.8 ELISA

244   ELISAs were performed in triplicate as described [25] to determine the IgY responses against his-

245   tagged PlcC, his-tagged Fba, his-tagged NetB and S. Typhimurium lipopolysaccharide (LPS) in

246   chicken sera and IgA, IgY and IgM responses in intestinal washes. Biotinylated anti-chicken

247   IgA (Alpha Diagnostic Intl. Inc), IgY (Southern Biotechnology) or IgM (Bioss) antibodies

248   diluted 1:10,000 were used to detect the various antibody isotypes.

249

250   2.9 Cellular proliferation assay

251   A proliferation assay was performed to evaluate cell-mediated immunity. Lymphocytes

252   in the blood were harvested using the gentle swirl technique [1] and plated in quadruplicate, in a

253   96-well plate at 10^5 cells/well in RPMI-1640 without phenol red. Spleens were placed through a

254   70 µm cell strainer to obtain single cell suspensions. Red blood cells were lysed with Red Blood

255   Cell Lysis solution (eBioscience). Splenocytes were then washed, suspended in RPMI and

256   plated at 10^6 cells/well. Each set of cells was incubated at 37°C, 5% CO_2 for 72 h with or without

257   4 μg/ml of either His-Fba, His-NetB, His-PlcC, or 1 μg/ml PMA. Cell proliferation was

258   measured using the Vybrant®MTT Cell Proliferation Assay Kit (Molecular Probes). Mean

259   absorbance value of antigen stimulated wells divided by mean absorbance of non-stimulated

260   control wells was used to calculate stimulation index.

261

262   2.10 Challenge with C. perfringens

263   Chickens were fed an antibiotic-free starter feed containing 21% protein for 20 days, at

264   which time the feed was switched to a high protein (28% protein), wheat-based feed containing

265   36% fish meal and zinc at 400 ppm (customized by Reedy Fork Farm, NC) to predispose the

266   birds to necrotic enteritis [45, 46]. Birds were challenged with virulent *C. perfringens* strain CP4

267   [47] in feed for 5 days as described [46] with some modifications. Feed was withdrawn on the

268   day prior to challenge for 15 h. The following day, chickens were inoculated with 1.0 mL of an

269   overnight culture of *C. perfringens* CP4 grown in cooked meat medium (CMM; Difco) by oral

270   gavage. Immediately after gavage, infected feed was provided thereafter for 5 consecutive days.

271   To prepare infected feed, *C. perfringens* was grown in CMM medium for 24 h at 37°C, which

272   then was inoculated into fluid thioglycollate medium (FTG; Difco) at a ratio of 0.3% (v/v) and

273   incubated at 37°C for 15 h (morning challenge) or 23 h (evening challenge). The *C. perfringens*

274   culture was mixed with feed at a ratio of 1:1 (v/w). Infected feed was prepared freshly twice

275   daily. All birds were euthanized and necropsied the day following the final challenge.

276

277   2.11 Lesion scoring

278   Protection against *C. perfringens* challenge was assessed on the basis of gross intestinal

279   lesion scores at necropsy. On day 33, chickens were euthanized with CO_2 and their small

280   intestines (defined here as the section between the gizzard and Meckel's diverticulum) were

281   examined for visible gross lesions. Intestinal lesions were scored as follows: 0 = no gross

282   lesions; 1 = thin or friable wall or diffuse superficial but removable fibrin; 2 = focal necrosis or

283   ulceration, or non-removable fibrin deposit, 1 to 5 foci; 3 = focal necrosis or ulceration, or non-

284   removable fibrin deposit, 6 to 15 foci; 4 = focal necrosis or ulceration, or non-removable fibrin

285   deposit, 16 or more foci; 5 = patches of necrosis 2 to 3 cm long; 6 = diffuse necrosis typical of

286   field cases [46]. Birds that died during challenge were assigned a score of 6.

287

288   2.12 Statistical analysis

289   All statistics were carried out using GraphPad Prism 6.0 (Graph-Pad Software, San

290   Diego, CA). Antibody titers and lesion scores were analyzed using one-way ANOVA followed

291   by Tukey's posttest. The values were expressed as means ± SEM, and differences were

292   considered significant at *P* < 0.05.

293

RESULTS

295   3.1 Construction of a PlcC-NetB fusion protein.

296   We designed a fusion protein comprising an N-terminal 6His tag, PlcC, linker

297   GGSGGSGGPSGGSGG, and NetB (**Fig. 1**). The long, flexible linker was used to facilitate

298   correct folding of the PlcC and NetB domains, and to permit unencumbered access of B-cell

299   receptors to the important immunogenic regions. PlcC contains the C-terminal receptor-binding

300   domain of the alpha toxin [18]. The NetB sequence contains a mutation W262A in the rim

301   domain [34], which substantially attentuates the toxicity of the protein in cell culture assays. We

302   noted that PlcC and NetB contain a total of 5 eukaryotic Asn-linked glycosylation sites (N-X-

303   S/T), which could result in the addition of glycans to the Asn residue when the protein is targeted

304   to the ER in plant cells. We constructed expression vectors to target proteins either to the ER

305   (pBYR2eK2M-6HplcCnetB, -6HplcC, and -6HnetB) or to the cytosol (pBYR2e3K2Mc-

306   c6HplcCnetB -c6HplcC, and -c6HnetB). We expected the cytosol targeted proteins to be

307   unglycosylated, while the ER-targeted proteins may be glycosylated at any combination of or all

308   of the 5 consensus N-X-S/T sites.

309

310   3.2 Production of PlcC-NetB and PlcC-NetB-NG fusion proteins in plants.

311   Using the methods outlined herein two different fusion proteins were produced. One,

312   targeted to the endoplasmic reticulum (ER), was glycosylated (PlcC-NetB). The other, targeted

313   to the cytoplasm, was non-glycosylated (PlcC-NetB-NG). Both fusion proteins were probed with

314   rabbit anti-PlcC and rabbit anti-NetB antibodies which yielded the same results on a western blot

315   (**Fig. 2**). PlcC-NetB also has incrementally increased band sizes above the initial band which

316   aligns with the singular PlcC-NetB-NG band at approximately 49 kDa, the expected relative size

317   of the fusion proteins (**Fig 2**). Both NetB and PlcC expressed individually were also probed and

318   appear at the expected sizes of 34 kDa and 15 kDa, respectively. While ER and cytoplasmic

319   targeted NetB and PlcC were produced only representative results are shown in Figure 2.

320   The process of purification for the PlcC-NetB protein is outlined in Figure 3. A

321   Coomassie-stained gel (**Fig. 3A**) shows a wild-type, uninfiltrated (WT) control followed by

322   crude extract which had already been acid precipitated. Note that the crude extract has highly

323   reduced plant protein contaminants, due to the acid precipitation that caused contaminating

324   proteins to become insoluble while the fusion proteins remain soluble. The flow-through fraction

325   contained bands of a similar profile to the crude extract on the Coomassie, while both washes

326   showed no detectable protein. Three elution fractions contained bands at approximately 49 kDa.

327   Anti-NetB antibodies (**Fig 3B**) reacted primarily with the standard and elution 2, while elutions 1

328   and 3 had visible but less prominent bands. When probed with anti-PlcC antibodies (**Fig 3C**),

329   similar bands were present in every lane except the blank and NetB standard lanes. Elution

330   fraction bands were as strong as those seen in the crude extract lane. Flow-through and wash

331   fractions had lighter but detectable bands when probed with anti-PlcC. Bands that reacted with

332   anti-PlcC antibodies were also seen at approximately 49 kDa.

333

334   3.3 Serum antibody responses to fusion protein antigens.

335   We evaluated the serum antibody responses in birds immunized with PlcC-NetB and in

336   control birds against the glycosylated fusion protein and against non-glycosylated recombinant

337   rPlcC and rNetB produced in bacteria. In experiments 1 and 2, the anti-PlcC-NetB titers were

338   significantly higher than controls (**Fig. 4**). The titers against rPlcC and rNetB were also

339   significantly elevated over controls. In experiment 3, where birds were immunized twice, only

340   the titers against the fusion protein were significantly higher than controls, although the serum

341   titers against rNetB were elevated.

342

343   3.4 Cellular responses against PlcC-NetB in Experiment 3.

344   Immunization with the fusion protein led to variable cellular responses. The rPlcC-NetB

345   fusion protein elicited weak, but significant, cellular responses against rNetB and rPlcC-NetB in

346   splenocytes (**Fig. 5**). No antigen-specific stimulation was detected in lymphocytes (data not

347   shown).

348

349   3.5 Protection against C. perfringens challenge.

350   Protection against challenge was assessed by scoring intestinal lesions using a 6 point

351   scoring system [46]. Immunization with the rPlcC-NetB fusion protein resulted in a significant

352   reduction in lesion scores in all three experiments (**Fig. 5**). Note that in Experiment 3, birds were

353   challenged a week earlier than in experiments 1 and 2. The younger age of the birds may have

354   contributed to the higher lesion scores for some birds. Two of the non-immunized birds died

355   during challenge. This was the only experiment in which any birds died following challenge.

356

DISCUSSION

358   There is a growing need for methods to control NE that do not rely on antibiotics.

359   Vaccination is a proven approach for controlling many poultry diseases and immunization with

360   toxoid antigens is known to provide some protection against necrotic enteritis [37, 48].

361   Immunizing with both α-toxoid and NetB toxoid provides better protection than when either

362   antigen is used alone [49]. Based on these observations, we designed a novel fusion protein

363   combining toxoids of both toxins. The use of a plant expression system to produce this antigen

364   has many advantages, including high yields, ease of stockpiling, rapid and easy scaling, quick

365   response to emerging pathogens, and lack of any animal components in the final vaccine product.

366   The production of a PlcC-NetB fusion was readily accomplished in *N. benthamiana* using

367   a geminiviral replicon transient expression system. A previously described *Salmonella*

368   expression system was used to attempt production a fusion protein between PlcC and GST-NetB.

369   However, production levels were low and the protein was rapidly degraded within the

370   *Salmonella* cells [20]. In contrast, the plant expression system was shown to produce the PlcC-

371   NetB fusion protein at a high capacity without degradation. High levels of protein production

372   were due to our use of the geminiviral replicon transient expression system which has been

373   recently optimized and has undergone improvement since the production of the PlcC-NetB

374   fusion protein [41, 42, 50]. Acid precipitation (a process by which the PI of proteins, and their

375   repulsive electromagnetic forces, equal zero and results in protein aggregation) proved to be an

376   effective clarification step, over other purification methods, as both the PlcC-NetB and PlcC-

377   NetB-NG fusion proteins proved to be acid stable [51]. This allowed for easy purification using

378   metal affinity chromatography because many of the plant protein contaminants were removed

379   from solution while the fusion proteins remained soluble. Metal affinity chromatography resulted

380   in substantial purification although contaminants could be seen upon concentration of elutions

381   (data not shown). However, contaminants were deemed not to be an issue for vaccination as they

382   were in such low quantities.

383   Since a plant expression system was used, native plant glycosylation of the fusion protein

384   PlcC-NetB occurred. An illustration of this can be seen in both Figure 2 and Figure 3 as there are

385   progressively higher bands above the primary fusion protein band at 49 kDa only in the ER

386   targeted variant. Glycosylation was a concern as an immune response could potentially be

387   directed towards the glycans rather than the fusion protein itself [52, 53]. This is the reason the

388   PlcC-NetB-NG fusion was pursued. PlcC-NetB-NG proved to be troublesome as it precipitated

389   after dialysis, a phenomenon that has been observed for rubisco, the most abundant plant protein

390   contaminant. Insolubility of PlcC-NetB-NG may also be caused by stripping of metal ions from

391   both PlcC and NetB that are normally present. According to the crystal structure of PlcC it likely

392   binds calcium ions, however, due to difficulty in forming a crystal structure calcium was

393   substituted for cadmium (15). The crystal structure of native NetB toxin has been shown to bind

394   magnesium (35). These metal ions could be removed during the final dialysis step, which might

395   affect protein structure and solubility. This may indicate that the glycoforms play a role in

396   protein stability and solubility as PlcC-NetB did not have solubility issues under the same buffer,

397   concentration, and purification conditions. Results indicate that, even with plant native

398   glycosylation, the PlcC-NetB fusion promoted an anti-toxoid response. We speculate that the

399   glycoforms could have possibly had an adjuvant effect as antibody titers for the fusion protein

400   were greater than *E. coli* produced PlcC and NetB delivered simultaneously [54]. This may also

401   be due to the fusion of both proteins as well. In any case, production of the PlcC-NetB fusion

402   protein was found to be efficient on a small scale which, due to the nature of plant expression

403   systems, is readily scalable. Moreover, the fusion protein vaccine was found to be effective when

404   injected into broiler birds as is discussed below.

405   In Experiments 1 and 2, three weekly injections with the fusion protein generated strong

406   serum IgY responses against both toxin components (**Fig. 4**). Vaccinated birds had geometric

407   mean titers of around 2,000 against recombinant NetB and PlcC proteins purified from *E. coli*

408   (**Fig. 4**). Titers against the PlcC-NetB fusion protein produced in plants were around 8,000 in

409   immunized birds. The difference in titers between single proteins and the fusion protein is

410   speculated to be due to enhanced immune response to the fused protein, since PlcC and NetB are

411   in close proximity during infection, or responses against the carbohydrate moieties on the

412   glycosylated protein leading to an enhanced immune response.

413   In Experiment 3 birds were immunized at 7 and 12 days of age which lead to a reduced

414   serum response. The chicken adaptive immune system does not fully mature until approximately

415   two weeks post-hatch, although humoral responses can be detected after immunization with a

416   protein antigen at 12 days of age [55]. In this regard, an adjuvanted NetB toxoid was shown to be

417   ineffective when injected into day-of-hatch chicks [56]. Thus, it is possible that immunizing the

418   birds at 7 days of age did not serve as a priming dose, such that the immunization at 12 days of

419   age was effectively the prime. Previous work showed that immunization at 12 days of age, but

420   not 7 days of age, can be boosted by injection at 29 days of age [55]. Although we did not

421   perform a vaccine boost, we infer that the birds were adequately primed since, despite the low

422   serum antibody responses, they were protected against challenge (**Fig. 5C**).

423

424   CONCLUSIONS

425   We have demonstrated that this novel toxoid antigen produced in plants is immunogenic and

426   protective in chickens. However, we acknowledge that a more practical immunization approach

427   is needed in order to have a positive impact on the broiler industry. We envision several options.

428   Immunization of parent stock is likely to induce maternal antibodies to protect chicks during the

429   first few weeks of life. This approach has been demonstrated to be effective with an α-toxoid

430   [29]. Alternatively, protein antigens can be successfully delivered via in ovo injection with an

431   adequate adjuvant. Delivery of the fusion protein via cornmeal could also be a cheap and

432   effective mass delivery method for vaccine produced in corn seeds. The effectiveness of these

433   approaches will be assessed in future studies.

434

435

ACKNOWLEDGEMENTS

436

446

FIGURE LEGENDS

447

448 **Figure 1**. Schematic of the his tagged PlcC-NetB fusion protein. Potential Asn-glycosylation

449 sites are indicated by a short line (-). The site of the W262A mutation is indicated.

450

451 **Figure 2.** Western blot comparing different fusion constructs. (**A**) Blot probed with rabbit anti-

452 NetB. (**B**) Blot used in (A) after stripping, probed with rabbit anti-PlcC. 6H-PlcC-NetB, ER

453 targeted fusion protein expressed in *N. benthamiana* leaf; PlcC-NetB-NG, cytosolically targeted

454 fusion protein expressed in *N. benthamiana* leaf; NetB-NG, cytosolically targeted NetB

455 expressed in *N. benthamiana* leaf; GST-NetB, bacterially expressed standard protein; PlcC-NG,

456 cytosolically targeted PlcC expressed in *N. benthamiana* leaf.

457

458   **Figure 3.** Purification of glycosylated PlcC-NetB fusion. (**A**) Coomassie-stained gel showing all

459   purification steps. (**B**) Western blot of all purification steps probed with rabbit anti-NetB. (**C**)

460   Western blot of all purification steps probed with rabbit anti-PlcC. Note: NetB standard is a

461   GST-NetB fusion with a mass of 59.9 kDa [20].

462

463   **Figure 4**. IgY serum titers from immunized and non-immunized birds, assayed against NetB,

464   PlcC, or NetB-PlcC fusion. All *P* values are compared to adjuvant only controls. Fusion refers to

465   his-tagged, glycosylated PlcC-NetB. (**A**) Experiment 1, * $P \le 0.009$ (**B**) Experiment 2, ** $P \le$

466   0.0025. (**C**) Experiment 3, [&] P < 0.0002.

467

468   **Figure 5**. Splenocyte responses to PlcC-NetB, PlcC and NetB after immunization with PlcC-

469   NetB in Figure 4, Experiment 3.

470

471   **Figure 6**. Intestinal lesions scores in (**A**) Experiment 1, (**B**) Experiment 2 and (**C**) Experiment 3.

472   Each dot represents the score of an individual bird.   Differences between immunized groups and

473   controls are indicated. * $P < 0.001$; ** $P = 0.004$; [&]$P = 0.0006$; [†]$P < 0.0001$.

474

475

476   **Table S1**. Primers used in this study*

Primer	DNA sequence
PlcC-Sac-R	CCC<u>GAGCTC</u>CTATTTGATGTTGTAAGTGGAGTTTCC
6H-Xba-F	GACT<u>CTAGA</u>ACAATGGCTCACCATCACCATCATCAC
Plc_pqe_f1	ACAT<u>GGATCC</u>GACCCGTCCGTGGGCAACAAC
Plc_pqe_r2	ACAT<u>GTCGAC</u>GTTTCCTAAGCTTCTATTTG
netB_pqe_f3	G<u>GGATCC</u>AGCGAACTGAACGAC
netB_Pqe_r4	<u>GTCGAC</u>GCTTTTACAGATAATATTC

477   *Underlined sequences indicate engineered restriction sites used for cloning
478

REFERENCES

1. Timbermont, L., et al., Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathol, 2011. **40**(4): p. 341-7.

2. Prescott, J.F., et al., The pathogenesis of necrotic enteritis in chickens: what we know and what we need to know: a review. Avian Pathol, 2016. **45**(3): p. 288-94.

3. Shivaramaiah, S., et al., The role of an early *Salmonella* Typhimurium infection as a predisposing factor for necrotic enteritis in a laboratory challenge model. Avian Dis, 2011. **55**(2): p. 319-23.

4. Cooper, K.K. and J.G. Songer, Virulence of *Clostridium perfringens* in an experimental model of poultry necrotic enteritis. Vet Microbiol, 2010. **142**(3-4): p. 323-8.

5. Cooper, K.K., J.G. Songer, and F.A. Uzal, Diagnosing clostridial enteric disease in poultry. J Vet Diagn Invest, 2013. **25**(3): p. 314-27.

6. McDevitt, R.M., et al., Necrotic enteritis; a continuing challenge for the poultry industry. World Poultry Sci J, 2006. **62**(2): p. 221-247.

7. McReynolds, J.L., et al., Evaluation of immunosuppressants and dietary mechanisms in an experimental disease model for necrotic enteritis. Poult Sci, 2004. **83**(12): p. 1948-52.

8. Van der Sluis, W., Clostridial enteritis is an often underestimated problem. World Poult., 2000. **16**: p. 42-43.

9. Knarreborg, A., et al., Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. Appl Environ Microbiol, 2002. **68**(12): p. 5918-24.

501   10.   Stanley, D., et al., Differential responses of cecal microbiota to fishmeal, *Eimeria* and

502   *Clostridium perfringens* in a necrotic enteritis challenge model in chickens. PLoS One,

503   2014. **9**(8): p. e104739.

504   11.   Butaye, P., L.A. Devriese, and F. Haesebrouck, Antimicrobial growth promoters used in

505   animal feed: Effects of less well known antibiotics on gram-positive bacteria. Clin

506   Microbiol Rev, 2003. **16**(2): p. 175-188.

507   12.   Van Immerseel, F., et al., *Clostridium perfringens* in poultry: an emerging threat for

508   animal and public health. Avian Pathol, 2004. **33**(6): p. 537-49.

509   13.   Titball, R.V., C.E. Naylor, and A.K. Basak, The *Clostridium perfringens* alpha-toxin.

510   Anaerobe, 1999. **5**(2): p. 51-64.

511   14.   Williamson, E.D. and R.W. Titball, A genetically engineered vaccine against the alpha-

512   toxin of *Clostridium perfringens* protects mice against experimental gas gangrene.

513   Vaccine, 1993. **11**(12): p. 1253-8.

514   15.   Naylor, C.E., et al., Structure of the key toxin in gas gangrene. Nat Struct Biol, 1998.

515   **5**(8): p. 738-46.

516   16.   Guillouard, I., T. Garnier, and S.T. Cole, Use of site-directed mutagenesis to probe

517   structure-function relationships of alpha-toxin from *Clostridium perfringens*. Infect

518   Immun, 1996. **64**(7): p. 2440-4.

519   17.   Titball, R.W., et al., Hemolytic and sphingomyelinase activities of Clostridium

520   perfringens alpha-toxin are dependent on a domain homologous to that of an enzyme

521   from the human arachidonic acid pathway. Infect Immun, 1991. **59**(5): p. 1872-4.

522   18.   Guillouard, I., et al., The carboxy-terminal C2-like domain of the alpha-toxin from

523   *Clostridium perfringens* mediates calcium-dependent membrane recognition. Mol

524   Microbiol, 1997. **26**(5): p. 867-76.

525   19.   Titball, R.W., A.M. Fearn, and E.D. Williamson, Biochemical and immunological

526   properties of the C-terminal domain of the alpha-toxin of *Clostridium perfringens*. FEMS

527   Microbiol Lett, 1993. **110**(1): p. 45-50.

528   20.   Jiang, Y., et al., Protection against necrotic enteritis in broiler chickens by regulated

529   delayed lysis *Salmonella* vaccines. Avian Dis, 2015. **59**(4): p. 475-85.

530   21.   Kulkarni, R.R., et al., A live oral recombinant *Salmonella enterica* serovar Typhimurium

531   vaccine expressing *Clostridium perfringens* antigens confers protection against necrotic

532   enteritis in broiler chickens. Clin Vaccine Immunol, 2010. **17**(2): p. 205-14.

533   22.   Zekarias, B., H. Mo, and R. Curtiss, III, Recombinant attenuated *Salmonella enterica*

534   serovar Typhimurium expressing the carboxy-terminal domain of alpha toxin from

535   *Clostridium perfringens* induces protective responses against necrotic enteritis in

536   chickens. Clin Vaccine Immunol, 2008. **15**(5): p. 805-16.

537   23.   Al-Sheikhly, F. and R.B. Truscott, The interaction of *Clostridium perfringens* and its

538   toxins in the production of necrotic enteritis of chickens. Avian Dis, 1977. **21**(2): p. 256-

539   63.

540   24.   Fukata, T., et al., Influence of *Clostridium perfringens* and its toxin in germ-free

541   chickens. Res Vet Sci, 1988. **44**(1): p. 68-70.

542   25.   Williamson, E.D. and R.W. Titball, A genetically-engineered vaccine against the alpha-

543   toxin of *Clostridium perfringens* protects mice against experimental gas gangrene.

544   Vaccine, 1993. **11**(12): p. 1253-1258.

545 26. Stevens, D.L., et al., Immunization with the C-Domain of alpha -toxin prevents lethal

546   infection, localizes tissue injury, and promotes host response to challenge with

547   *Clostridium perfringens*. J Infect Dis, 2004. **190**(4): p. 767-73.

548 27. Nagahama, M., et al., A recombinant carboxy-terminal domain of alpha-toxin protects

549   mice against Clostridium perfringens. Microbiol Immunol, 2013. **57**(5): p. 340-5.

550 28. Hoang, T.H., et al., Recombinant *Bacillus subtilis* expressing the *Clostridium perfringens*

551   alpha toxoid is a candidate orally delivered vaccine against necrotic enteritis. Infect

552   Immun, 2008. **76**(11): p. 5257-65.

553 29. Crouch, C.F., et al., Safety and efficacy of a maternal vaccine for the passive protection

554   of broiler chicks against necrotic enteritis. Avian Pathol, 2010. **39**(6): p. 489-97.

555 30. Keyburn, A.L., et al., NetB, a new toxin that is associated with avian necrotic enteritis

556   caused by *Clostridium perfringens*. PLoS Pathog, 2008. **4**(2): p. e26.

557 31. Keyburn, A.L., et al., Alpha-toxin of *Clostridium perfringens* is not an essential virulence

558   factor in necrotic enteritis in chickens. Infect Immun, 2006. **74**(11): p. 6496-500.

559 32. Keyburn, A.L., et al., Association between avian necrotic enteritis and *Clostridium*

560   *perfringens* strains expressing NetB toxin. Vet Res, 2010. **41**(2): p. 21.

561 33. Tolooe, A., et al., Prevalence of *netB* Gene among *Clostridium perfringens* isolates

562   obtained from healthy and diseased chickens. Journal of Animal and Veterinary

563   Advances, 2011. **10**(1): p. 106-110.

564 34. Savva, C.G., et al., Molecular architecture and functional analysis of NetB, a pore-

565   forming toxin from *Clostridium perfringens*. J Biol Chem, 2013. **288**(5): p. 3512-22.

566 35. Yan, X.X., et al., Structural and functional analysis of the pore-forming toxin NetB from

567   *Clostridium perfringens*. MBio, 2013. **4**(1): p. e00019-13.

568 36.   da Costa, S.P.F., et al., Protection against avian necrotic enteritis after immunisation with

569   NetB genetic or formaldehyde toxoids. Vaccine, 2013. **31**(37): p. 4003-4008.

570 37.   Keyburn, A.L., et al., Vaccination with recombinant NetB toxin partially protects broiler

571   chickens from necrotic enteritis. Vet Res, 2013. **44**(1): p. 54.

572 38.   Keyburn, A.L., et al., Maternal immunization with vaccines containing recombinant NetB

573   toxin partially protects progeny chickens from necrotic enteritis. Vet Res, 2013. **44**(1): p.

574   108.

575 39.   Lee, K.W., et al., *Clostridium perfringens* alpha-toxin and NetB toxin antibodies and

576   their possible role in protection against necrotic enteritis and gangrenous dermatitis in

577   broiler chickens. Avian Dis, 2012. **56**(1): p. 230-3.

578 40.   Geyer, B.C., et al., Transgenic plants as a source for the bioscavenging enzyme, human

579   butyrylcholinesterase. Plant Biotechnol J, 2010. **8**(8): p. 873-86.

580 41.   Diamos, A.G., S.H. Rosenthal, and H.S. Mason, 5' and 3' Untranslated Regions Strongly

581   Enhance Performance of Geminiviral Replicons in *Nicotiana benthamiana* Leaves. Front

582   Plant Sci, 2016. **7**: p. 200.

583 42.   Diamos, A.G. and H.S. Mason, Chimeric 3' flanking regions strongly enhance gene

584   expression in plants. Plant Biotechnol J, 2018.

585 43.   Huang, Z. and H.S. Mason, Conformational analysis of hepatitis B surface antigen

586   fusions in an Agrobacterium-mediated transient expression system. Plant Biotechnol J,

587   2004. **2**(3): p. 241-9.

588 44.   Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, NIH Image to ImageJ: 25 years of

589   image analysis. Nat Methods, 2012. **9**(7): p. 671-5.

590   45.   Coursodon, C.F., et al., TpeL-producing strains of *Clostridium perfringens* type A are

591   highly virulent for broiler chicks. Anaerobe, 2012. **18**(1): p. 117-21.

592   46.   Shojadoost, B., A.R. Vince, and J.F. Prescott, The successful experimental induction of

593   necrotic enteritis in chickens by *Clostridium perfringens*: a critical review. Vet Res,

594   2012. **43**(1): p. 74.

595   47.   Thompson, D.R., et al., Live attenuated vaccine-based control of necrotic enteritis of

596   broiler chickens. Vet Microbiol, 2006. **113**(1-2): p. 25-34.

597   48.   Cooper, K.K., H.T. Trinh, and J.G. Songer, Immunization with recombinant alpha toxin

598   partially protects broiler chicks against experimental challenge with *Clostridium*

599   *perfringens*. Vet Microbiol, 2009. **133**(1-2): p. 92-7.

600   49.   Fernandes da Costa, S.P., et al., Variable protection against experimental broiler necrotic

601   enteritis after immunization with the C-terminal fragment of *Clostridium perfringens*

602   alpha-toxin and a non-toxic NetB variant. Avian Pathol, 2016. **45**(3): p. 381-8.

603   50.   Rosenthal, S.H., A.G. Diamos, and H.S. Mason, An intronless form of the tobacco

604   extensin gene terminator strongly enhances transient gene expression in plant leaves.

605   Plant Mol Biol, 2018. **96**(4-5): p. 429-443.

606   51.   Wilken, L.R. and Z.L. Nikolov, Recovery and purification of plant-made recombinant

607   proteins. Biotechnol Adv, 2012. **30**(2): p. 419-33.

608   52.   Maverakis, E., et al., Glycans in the immune system and the altered glycan theory of

609   autoimmunity: a critical review. J Autoimmun, 2015. **57**: p. 1-13.

610   53.   Schahs, M., et al., Production of a monoclonal antibody in plants with a humanized N-

611   glycosylation pattern. Plant Biotechnol J, 2007. **5**(5): p. 657-63.

612   54.   Bosch, D. and A. Schots, Plant glycans: friend or foe in vaccine development? Expert

613   Rev Vaccines, 2010. **9**(8): p. 835-42.

614   55.   Mast, J. and B.M. Goddeeris, Development of immunocompetence of broiler chickens.

615   Vet Immunol Immunopathol, 1999. **70**(3-4): p. 245-56.

616   56.   Mot, D., et al., Day-of-hatch vaccination is not protective against necrotic enteritis in

617   broiler chickens. Avian Pathol, 2013. **42**(2): p. 179-84.

618

Figure 1

Schematic of the his tagged PlcC-NetB fusion protein

Potential Asn-glycosylation sites are indicated by a short line (-). The site of the W262A mutation is indicated.
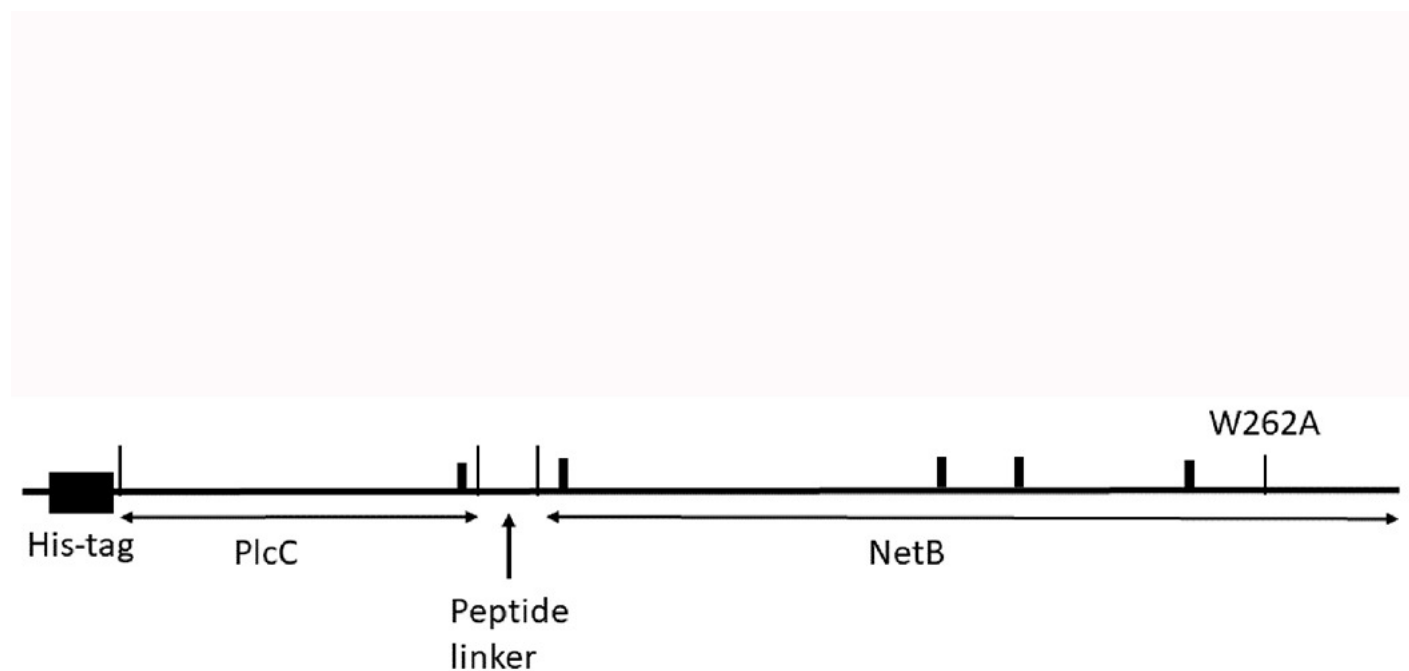
Figure 1. Schematic of the His-tagged PlcC-NetB Fusion Protein. Potential Asn-glycosylation sites are indicated by a short line (▌). The site of the W262A mutation is indicated.
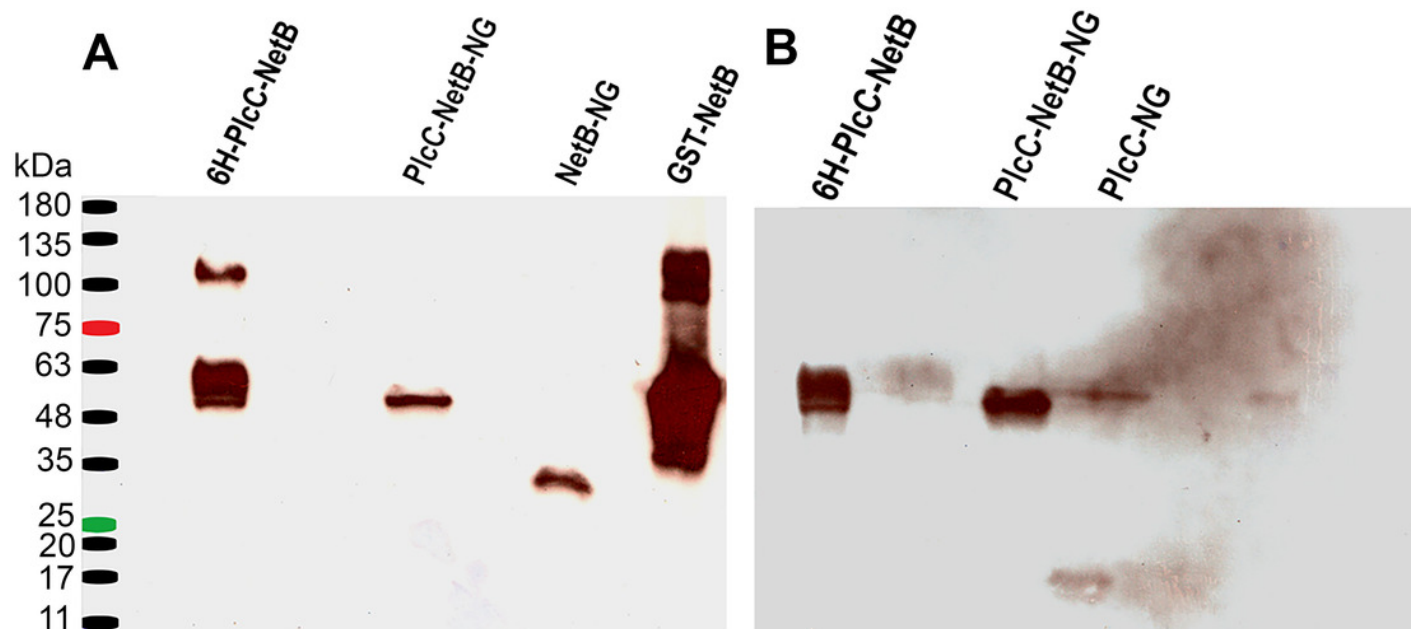
Figure 2

Western blot comparing different fusion constructs

(**A**) Blot probed with rabbit anti-NetB. (**B**) Blot used in (A) after stripping, probed with rabbit anti-PlcC. 6H-PlcC-NetB, ER targeted fusion protein expressed in *N. benthamiana*leaf; PlcC-NetB-NG, cytosolically targeted fusion protein expressed in *N. benthamiana*leaf; NetB-NG, cytosolically targeted NetB expressed in *N. benthamiana*leaf; GST-NetB, bacterially expressed standard protein; PlcC-NG, cytosolically targeted PlcC expressed in *N. benthamiana*leaf.

Figure 2. Western blot comparing different fusion constructs. (**A**) Blot probed with rabbit anti-NetB. (**B**) Blot used in (A) after stripping, probed with rabbit anti-PlcC. 6H-PlcC-NetB, ER targeted fusion protein expressed in N. benthamiana leaf; PlcC-NetB-NG, cytosolically targeted fusion protein expressed in N. benthamiana leaf; NetB-NG, cytosolically targeted NetB expressed in N. benthamiana leaf; GST-NetB, bacterially expressed standard protein; PlcC-NG, cytosolically targeted PlcC expressed in N. benthamiana leaf.

Figure 3

Purification of glycosylated PlcC-NetB fusion

(**A**) Coomassie-stained gel showing all purification steps. (**B**) Western blot of all purification steps probed with rabbit anti-NetB. (**C**) Western blot of all purification steps probed with rabbit anti-PlcC. Note: NetB standard is a GST-NetB fusion with a mass of 59.9 kDa [20].
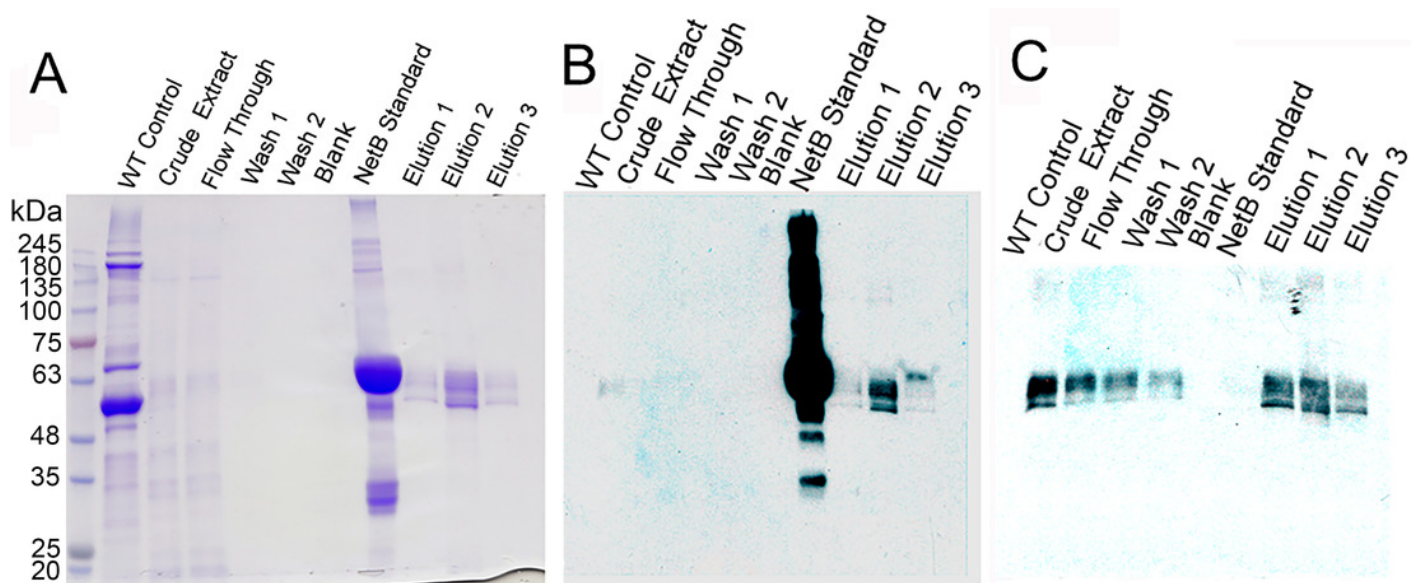

Figure 3. Purification of Glycosylated 6H-PlcC-NetB fusion. (**A**) Coomassie stain of all purification steps. (**B**) Western blot of all purification steps, rabbit anti-NetB probe. (**C**) Western blot of all purification steps, rabbit anti-PlcC probe. Note: NetB standard is a GST-NetB fusion.
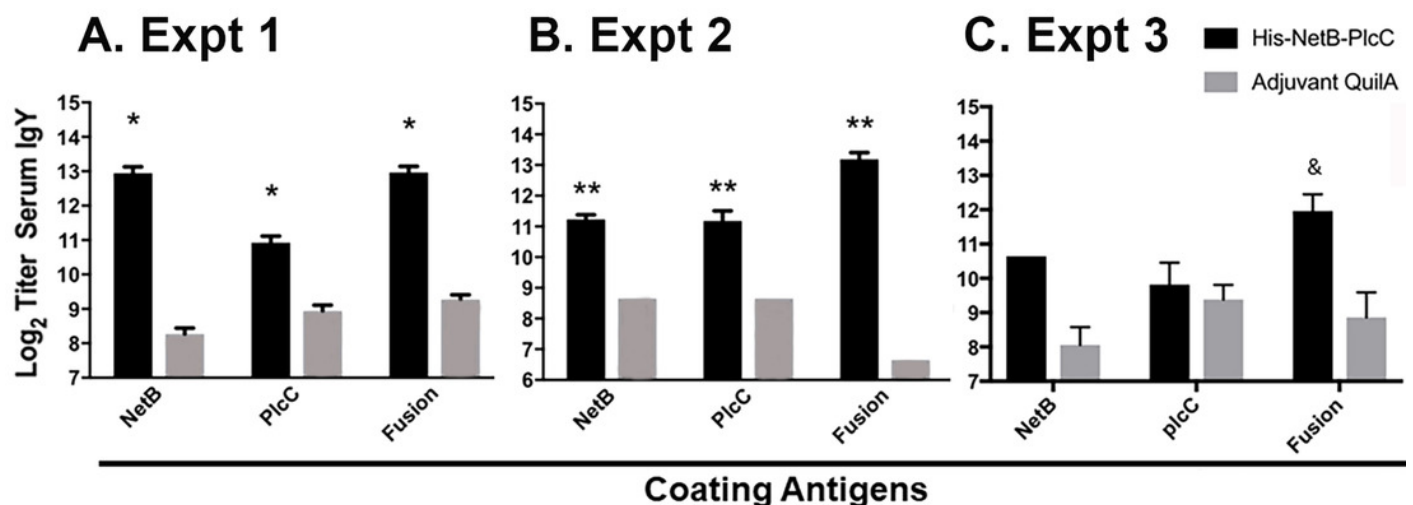
Figure 4

IgY serum titers from immunized and non-immunized birds, assayed against NetB, PlcC, or NetB-PlcC fusion

All P values are compared to adjuvant only controls. Fusion refers to his-tagged, glycosylated PlcC-NetB. (**A**) Experiment 1, * $P \le$ 0.009 (**B**) Experiment 2, ** $P \le$ 0.0025. (**C**) Experiment 3, [&]$P < 0.0002$.

Figure 4. IgY serum titers from immunized and non-immunized birds, assayed against NetB, PlcC, or NetB-PlcC fusion. All P values are compared to adjuvant only controls. Fusion refers to his-tagged, glycosylated PlcC-NetB. (**A**) Experiment 1, * P ≤ 0.009 (**B**) Experiment 2, ** P ≤ 0.0025. (**C**) Experiment 3, & P < 0.0002.

Figure 5

Splenocyte responses

Splenocyte responses to PlcC-NetB, PlcC and NetB after immunization with PlcC-NetB in Figure 4, Experiment 3.
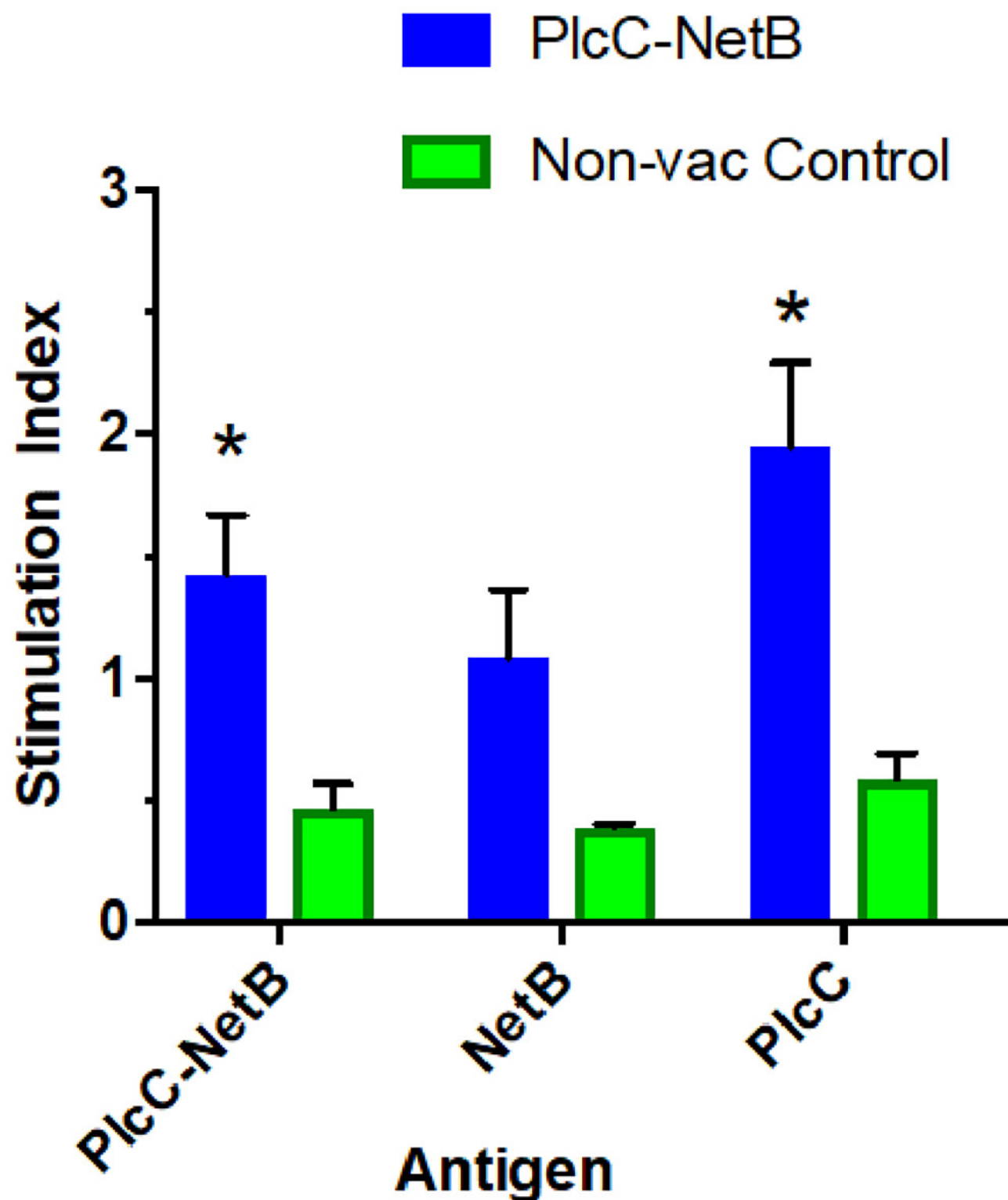
Figure 5. Splenocyte responses to PlcC-NetB, PlcC and NetB after immunization with PlcC-NetB in Figure 4, Experiment 3.

Figure 6

Intestinal lesions scores

Intestinal lesions scores in (**A**) Experiment 1, (**B**) Experiment 2 and (**C**) Experiment 3. Each dot represents the score of an individual bird. Differences between immunized groups and controls are indicated. * $P < 0.001$; ** $P = 0.004$; $^{\&}P = 0.0006$; $\dagger P < 0.0001$
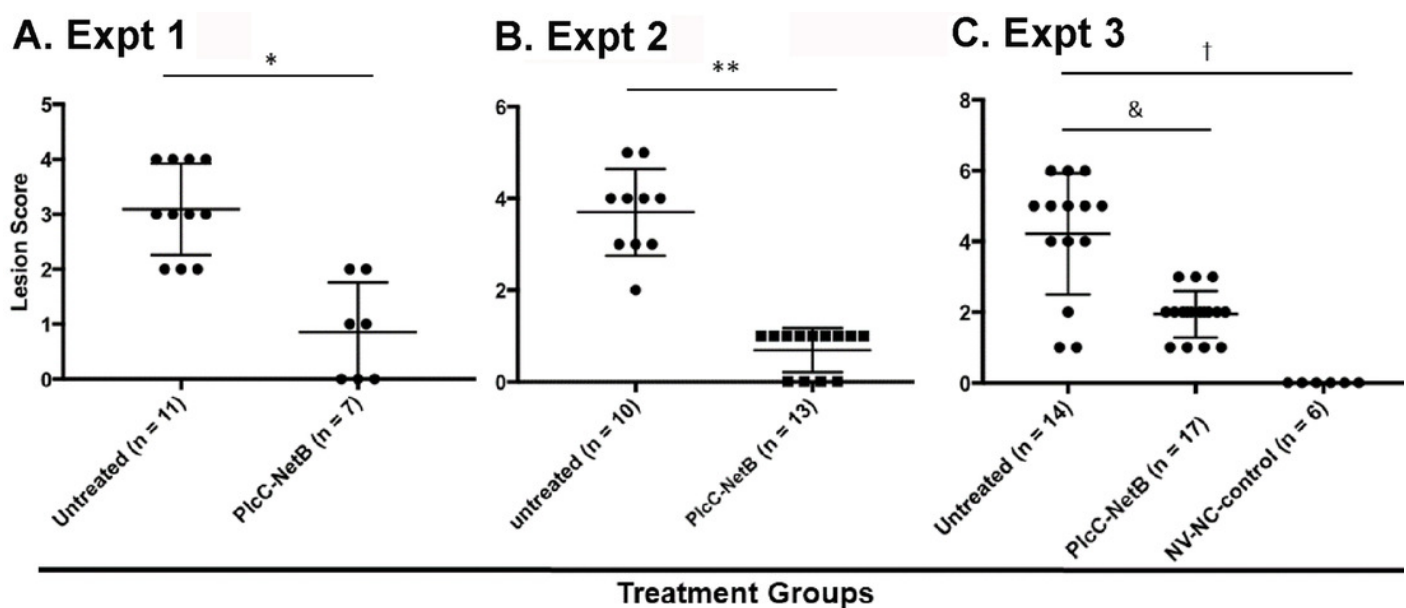

Figure 6. Intestinal lesions scores in Experiment 1 (**A**) Experiment 2 (**B**) and Experiment 3 (**C**). Each dot represents the score of an individual bird. Differences between immunized groups and controls are indicated. * $P < 0.001$; ** $P = 0.004$; $^{\&}P = 0.0006$; $\dagger P < 0.0001$