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1 ***Salmonella*-vectored vaccine delivering three *Clostridium perfringens* antigens**
2 **protects poultry against necrotic enteritis**.
3
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23 **Abstract**

24 Necrotic enteritis is an economically important poultry disease caused by the bacterium
25 *Clostridium perfringens*. There are currently no necrotic enteritis vaccines available for
26 use in broiler birds, the most important target population. *Salmonella*-vectored vaccines
27 represent a convenient and effective option for controlling this disease. We used a
28 single attenuated *Salmonella* vaccine strain, engineered to lyse within the host, to
29 deliver up to three *C. perfringens* antigens. Two of the antigens were toxoids, based on
30 *C. perfringens* α-toxin and NetB toxin. The third antigen was fructose-1,6-bisphosphate
31 aldolase (Fba), an metabolic enzyme with an unknown role in virulence. Oral
32 immunization with a single *Salmonella* vaccine strain producing either Fba, α-toxoid and
33 NetB toxoid, or all three antigens, was immunogenic, inducing serum, cellular and
34 mucosal responses against *Salmonella* and the vectored *C. perfringens* antigens. All
35 three vaccine strains were protective against virulent *C. perfringens* challenge. The
36 strains delivering Fba only or all three antigens provided the best protection. We also
37 demonstrate that both toxins and Fba are present on the *C. perfringens* cell surface.
38 The presence of Fba on the cell surface suggests that Fba may function as an adhesin.
39

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40

41 **Introduction**

42

43 Keeping our food supply safe is one of many challenges facing the agriculture
44 industry. When rearing poultry, maintenance of a healthy flock requires a multi-faceted
45 strategy that includes good husbandry, strong biosecurity practices, proper feed
46 formulation, vaccination, and veterinary care. One element of poultry rearing has been
47 the inclusion of sub-clinical amounts of antibiotics in the feed to promote growth.
48 Recent concerns regarding the impact of this practice on increasing antibiotic resistance
49 in human pathogens has led to stricter regulations governing antibiotic use in food
50 animals and voluntary elimination of antibiotics by poultry producers and food providers.
51 While limiting the use of antibiotics on the farm may have long-range health benefits for
52 the human population, it leads to additional challenges for the poultry industry. Based
53 on results in other countries, it is well known that the incidence of necrotic enteritis (NE)
54 caused by *Clostridium perfringens* type A strains increases when antibiotics are
55 removed from the feed [1].
56 NE is an enteric disease causing chronic mucosal damage to the intestines with
57 a range of symptoms including general poor health, reduced appetite, reduced weight
58 gain, poor digestion and cholangiohepatitis. More severe symptoms, such as sudden
59 death, can also occur in afflicted flocks. There are often no overt symptoms of
60 pathology. Subacute infections are the most common, resulting in economic losses,
61 due to the reduced weight of the birds, and carcass condemnation, due to liver lesions,
62 after slaughter [2]. There are many predisposing factors, which include feed

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63 composition, stress, coccidiosis, and immunosuppression due to infection with certain
64 viruses [3]. This disease is estimated to cause annual global losses of up to $6 million
65 dollars to poultry producers.

66 Vaccination is one practical alternative to antibiotics. In a previous report, we
67 used a novel *Salmonella* Typhimurium vaccine vector to deliver two relevant clostridial
68 toxoid antigens, PlcC, a nontoxic carboxyterminal fragment of α-toxin, and a GST-NetB
69 fusion protein [4]. NetB is a pore-forming toxin that plays a central role in NE [5].
70 Although the role of α-toxin in pathogenesis is not clear, anti-α-toxin antibodies are
71 protective [6], possibly due to their ability to inhibit *C. perfringens* growth [7]. The *S.*
72 Typhimurium vaccine strain we used was engineered to display a near wild-type
73 phenotype at the time of immunization. After several rounds of replication in host
74 tissues, the strain lyses, releasing the *C. perfringens* antigens. This type of attenuated
75 strain is called a lysis strain [8]. Immunization with the lysis strain delivering PlcC and
76 GST-NetB was previously shown to elicit protective immunity against *C. perfringens*
77 challenge [4].

78 Fructose-1,6-bisphosphate aldolase (Fba) was previously identified as an
79 immunogenic protein in the supernatant of a virulent NE strain [9]. Chickens injected
80 with recombinant Fba were partially protected against NE after challenge with *C.*
81 *perfringens* [10]. In this work, we provide evidence that Fba is present on the surface of
82 *C. perfringens* and evaluate the efficacy of Fba delivered by a *Salmonella* lysis strain
83 with or without co-delivery of PlcC and NetB. We demonstrate that all three antigens
84 can be effectively delivered by a single *Salmonella* lysis strain, eliciting strong mucosal
85 and cellular responses. Inclusion of Fba enhanced protection against NE.

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86

87

88 **Results**

89 **Characterization of *Salmonella* vaccine strains.** All *S*. Typhimurium vaccine strains
90 required arabinose for growth (data not shown) and had similar growth characteristics in
91 LB broth (0.1% arabinose, 0.1% mannose) (**Fig. S1**). Production of PlcC, GST-NetB
92 and Fba by the various *Salmonella* vaccine strains were assessed by western blot. The
93 strain carrying pYA5130 (*plcC fba* GST-*netB*) produced Fba at levels similar to the
94 strain producing Fba alone (**Fig. 1**) and produced GST-NetB at levels similar to the
95 strain carrying pYA5112 (*plcC* GST-*netB*). The strain carrying pYA5112 (*plcC* GST-
96 *netB*)produced about 6-fold more PlcC than the strain carrying pYA5130 (*plcC fba* GST-
97 *netB*) (**Fig. 1**). This is not surprising, as the *plcC* leader sequence in pYA5112 was
98 modified to increase PlcC synthesis [4], while the *plcC* gene in pYA5130 does not carry
99 that modification.

100

101 **Figure 1**. Antigen production by vaccine strains as determined by western blot.
102 Vaccine strains were grown in LB as described in Materials and Methods. Antigen
103 production was induced by the addition of IPTG four hours prior to harvest. Membranes
104 were probed with the indicated anti-sera. Predicted mass of antigens are: PlcC, 18kDa;
105 GST-NetB, 59 kDa; Fba, 30 kDa. Lane 1, *Salmonella* vector control; Lane 2, *Salmonella*
106 strain carrying pYA5130 (*plcC, fba, netB*); Lane 3, *Salmonella* strain carrying pYA5112
107 (*plcC*, *netB*); Lane 4, *Salmonella* strain carrying pKR023 (*fba*).

108

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109 **Serum antibody responses**. In experiment 1, the triple antigen vaccine elicited a
110 significant increase in anti-Fba serum IgY titers when compared to the double-antigen
111 vaccine or vector control ($P < 0.0001$) (**Fig. 2A**). Modest increases in anti-PlcC and
112 anti-NetB responses were observed, although only the anti-NetB responses elicited by
113 the triple antigen vaccine achieved significance ($P < 0.02$). In experiments 2 and 3,
114 birds received 10-fold higher vaccine doses. Results from experiments 2 and 3 were
115 similar and the data were combined for analysis. In this case, the anti-Fba serum IgY
116 titers were 14 – 21-fold greater than controls, which was significant (**Fig. 2B**; $P < 0.02$).
117 We observed similar, significant increases in anti-NetB titers in birds immunized with
118 *Salmonella* vaccines producing NetB ($P < 0.05$). Despite the difference in PlcC
119 production by strains carrying pYA5112 and pYA5130 (Fig. 1), the serum responses
120 elicited by the two strains were comparable in both experiments (Fig. 2). However,
121 though we observed increased anti-PlcC titers in birds immunized with strains producing
122 PlcC, the increases were not statistically significant. This result is consistent with
123 results from our previous study in which we observed poor seroconversion to PlcC
124 when delivered by the same double antigen strain [4]. The anti-*Salmonella* LPS titers
125 in immunized birds were 8 – 32-fold higher than controls ($P < 0.0001$) (**Fig. 2B**).
126
127 **Figure 2**. Serum antibodies against *C. perfringens* and *Salmonella* antigens in
128 vaccinated and non-vaccinated birds as determined by ELISA. (A) Experiment 1; (B)
129 Experiments 2/3. Differences in responses compared to non-vaccinates and vector-
130 only controls are indicated *, $P < 0.0001$; **, $P < 0.02$. LPS responses were compared
131 to non-vaccinated controls.

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132

133 **Mucosal IgA, IgM and IgY responses**. We examined intestinal mucosal responses in
134 experiments 2 and 3 and have combined the data. The vaccine elicited strong,
135 significant mucosal responses to all antigens, including PlcC, although statistically
136 significant responses against every antigen were not observed for all isotypes (**Fig. 3**).
137 The mucosal IgA responses against PlcC were not significant for either strain delivering
138 PlcC, while mucosal IgM responses elicited by both strains were significant. The anti-
139 PlcC mucosal IgY response was only significant for the strain delivering two antigens.

140

141 **Figure 3.** Mucosal antibody responses to the indicated *C. perfringens* proteins and
142 *Salmonella* LPS as determined by ELISA. (A) Experiment 1; (B) Experiments
143 2/3. Differences in responses compared to non-vaccinates and vector-only controls are
144 indicated by *, $P < 0.0001$; **, $P < 0.02$. LPS responses were compared to non-
145 vaccinated controls.

146

147 Birds immunized with the strain delivering only Fba achieved significant anti-Fba
148 mucosal responses across all isotypes ($P < 0.02$). The anti-Fba IgM and IgA responses
149 were significant in birds immunized with the triple antigen strain. The anti-Fba IgY
150 responses, while elevated, were not significant ($P = 0.20$). All immunized birds
151 produced elevated anti-NetB responses, although the anti-NetB IgA responses in birds
152 that received the triple antigen strain did not achieve significance ($P = 0.08$)
153 **Cellular responses**. Immunization with the vaccine producing all three antigens elicited
154 strong anti-Fba and anti-PlcC cellular responses in blood lymphocytes and splenocytes

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155 (**Fig. 4**). Significant anti-NetB responses were observed in the splenocytes of birds
156 receiving the triple antigen strain ($P < 0.05$), but not in lymphocytes. Birds immunized
157 with the strain producing two antigens, PlcC and NetB, produced significant anti-NetB
158 responses in splenocytes ($P < 0.005$) and significant anti-PlcC responses in
159 lymphocytes ($P < 0.05$). Birds immunized with the strain producing Fba alone exhibited
160 significant cellular responses in splenocytes ($P < 0.0001$), while the response in
161 lymphocytes was not significant.
162
163 **Figure 4**. Cellular responses by splenocytes and lymphocytes from vaccinated and
164 non-vaccinated chickens. Significant differences compared to controls are indicated.
165 *, $P < 0.0001$; $^{\#}$, $P < 0.005$, $^{\&}$, $P < 0.05$.
166
167 **Protection studies**. Protection against challenge was assessed by scoring intestinal
168 lesions using a 6 point scoring system [11]. In experiment 1, birds immunized with
169 either the double antigen strain or the triple antigen strain had significantly lower lesion
170 scores than birds in the control groups (**Table 1**). There was no statistical difference
171 between the average lesion scores for the triple antigen and double antigen strain
172 groups ($P = 0.25$) in this experiment.
173
174 **Table 1**. Intestinal lesion scores from Experiment 1

Group	**Number of birds with the indicated score**							**Av.**
	0	**1**	**2**	**3**	**4**	**5**	**6**	**score**
Salmonella only (n = 8)	0	2	2	2	2	0	0	2.5

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plcC, netB (n = 9)	3	1	5	0	1	0	0	1.5*
plcC, fba, netB (n = 10)	4	4	2	0	0	0	0	0.8**
NV[#], challenged (n = 10)	0	0	3	4	3	0	0	3.0
NV, non-challenged (n = 5)	1	4	0	0	0	0	0	0.8

175

176 *P = 0.0055 vs non-vac

177 **P = 0.0001 vs non-vac

178 [#]NV – non-vaccinated

179

180

181 In experiment 3, we included a strain producing only Fba in addition to the strains
182 evaluated in experiment 1. Chickens immunized with any strain carrying *C. perfringens*
183 antigens had significantly lower lesion scores than non-vaccinated or *Salmonella* only
184 controls (**Table 2**), indicating protection against *C. perfringens* challenge. As in the
185 previous experiment, there were no statistical differences between the double antigen
186 and triple antigen groups (P = 0.19). However, there was a significant difference in
187 lesion scores between the Fba-only group and the double antigen group (P = 0.01),
188 indicating that Fba is contributing to protection against *C. perfringens* challenge.

189

190 **Table 2**. Intestinal lesion scores from Experiment 3

Group	**Number of birds with the indicated score**							**Av.**
	0	**1**	**2**	**3**	**4**	**5**	**6**	**score**
Salmonella only (n = 15)	0	1	3	7	2	2	0	3.1

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plcC, netB (n = 14)	2	4	5	2	0	0	1	1.9*
plcC, fba, netB (n = 17)	5	6	4	2	0	0	0	0.9**
fba (n = 16)	8	5	3	0	0	0	0	0.7**&
NV, challenged (n = 9)	0	0	1	2	4	0	2	4.0
NV, non-challenged (n = 6)	3	3	0	0	0	0	0	0.5

191

192 *P = 0.0012 vs non-vac, P = 0.0056 vs *Salmonella* only group

193 **P < 0.0001 vs non-vac or *Salmonella* only groups

194 [&]P = 0.01 vs *plcC*, *netB* group

195 There were no significant differences between any vaccinated group and the non-

196 challenged controls.

197

198 **Plc, NetB and Fba are displayed on the surface of *C. perfringens*.** To

199 evaluate the potential of rabbit antisera raised against rPlcC, rNetB and rFba to bind

200 directly to *C. perfringens*, we used an immunofluorescence assay. Previous work

201 showed that anti-PlcC antibodies bind to the surface of *C. perfringens* [7]. We

202 confirmed this observation. Rabbit antibodies raised against rPlcC bound to challenge

203 strain CP4 and another NE strain, JGS4143 (**Fig. 5**). Binding was dependent on the

204 presence of α-toxin, as no fluorescence was observed when we probed JGS5388, a

205 Δ*plc* derivative of JGS4143. In addition, when purified rPlcC was mixed with the

206 antisera prior to incubation with CP4, immunofluorescence was drastically reduced,

207 indicating that fluorescence was mediated by anti-α-toxin-specific antibodies present in

208 the serum. When we probed wild-type *C. perfringens* strains CP4 and JGS4143 with

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209 anti-NetB antisera, we observed immunofluorescence. We also observed
210 immunofluorescence using Δ*plc* strain JGS5388. However, no immunofluorescence
211 was observed using the naturally occurring NetB^{-} strain JGS4043 or when we mixed
212 purified rNetB with the anti-NetB antisera prior to incubation with CP4 (**Fig. 5,** right
213 column).
214
215 **Figure 5**. Indirect immunofluorescence detection of proteins on the surface of *C.*
216 *perfringens*. *C. perfringens* strains were incubated with polyclonal sera from rabbits
217 hyperimmunized with the indicated *C. perfringens* proteins. In the far right panel, sera
218 were mixed with 1 μg of the indicated proteins for 30 min prior to incubation with the *C.*
219 *perfringens* strains.
220
221 Interestingly, Fba appears to be present on the cell surface as well, since we
222 observed immunofluorescence of all strains when probed with anti-Fba antisera.
223 Fluorescence was lost when the sera was pre-incubated with purified rFba (**Fig. 5**, right
224 column). Incubation of *C. perfringens* with pre-immune sera or the secondary antibody
225 alone did not result in immunofluorescence (**Fig. S2**). As an additional control, we
226 incubated purified rFba with the anti-NetB antisera and rNetB with the anti-Fba antisera
227 prior to probing *C. perfringens* CP4. These incubations did not diminish
228 immunofluorescence (**Fig. S2**), indicating that protein-specific titration of each antibody
229 was required to prevent its binding to *C. perfringens*. This result supports our
230 interpretation that NetB and Fba are displayed on the surface of *C. perfringens*.

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231 **NetB and Fba facilitate adherence to eukaryotic cells**. The presence of Fba on the
232 *C. perfringens* cell surface raises the possibility that it plays a role in attachment to the
233 host epithelium. To investigate the possible role of Fba in adherence, we examined the
234 effect of pre-incubating Caco-2 cells with Fba prior to performing an attachment assay.
235 As controls, we included wells in which either NetB or the *Streptococcus pneumoniae*
236 protein PspA was substituted for Fba. Prior addition of Fba to the wells resulted in a
237 significant reduction in adherence ($P < 0.037$) (**Fig. 6**). We also found that NetB
238 inhibited adherence as well ($P < 0.002$). The irrelevant *S. pneumoniae* protein PspA
239 had no effect. These results suggest a possible role for Fba and NetB in adherence to
240 intestinal epithelial cells.
241
242 **Figure 6**. Adherence of *C. perfringens* CP4 to Caco-2 cells in the presence or absence
243 of purified rFba, rNetB or rPspA. Attachment results significantly different from the no
244 protein controls are indicated.
245
246 **Discussion**
247 Fba is an enzyme of the Embden-Meyerhof-Parnas glycolytic pathway that
248 converts D-fructose-1,6-bisphosphate into D-glyceraldehyde-3-phosphate and
249 dihydroxyacetone phosphate [12]. In *C. perfringens*, it also plays a role in the
250 catabolism of *myo*-inositol [13] and is negatively regulated by the VirR/VirS two
251 component system that also regulates production of a number of virulence factors
252 including α-toxin [14]. An enzyme important in central metabolism may seem to be an
253 unusual choice for inclusion in a vaccine. However, in several organisms, Fba is

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254 recognized as a "moonlighting protein", one that can perform two or more autonomous
255 functions [12]. Fba has been shown to serve as an adhesin in a number of dissimilar
256 bacterial pathogens, including *S. pneumoniae* [15], *Mycobacterium tuberculosis* [16]
257 and *Neisseria meningitidis* [17]. Our immunofluorescence data shows that a protein
258 cross-reactive with anti-Fba antisera is present on the surface of *C. perfringens* (**Fig. 5**),
259 a result consistent with a possible role for Fba as an adhesin in this organism. Our
260 tissue culture results support this as well, as adherence to Caco-2 cells is inhibited in
261 the presence of rFba (**Fig. 6**). However, the importance of this finding will need to be
262 confirmed using a chicken epithelial cell line. Similarly, NetB appears to aid in
263 adherence to epithelial cells. This is not surprising, since NetB is known to be toxic to
264 both chicken and human cells. NetB may bind to host cells via interactions with
265 cholesterol [18].

266 As a vaccine, immunization with the appropriate homologous Fba is partially
267 protective against infection with *S. pneumoniae* [19], *S. pyogenes* [20] and the fish
268 pathogen, *Edwardsiella tarda* [21]. In *C. perfringens*, Fba was previously identified as
269 one of several secreted proteins recognized by sera taken from chickens with acquired
270 immunity to necrotic enteritis [9]. In an initial assessment of its vaccine potential,
271 chickens given a single intramuscular injection with recombinant Fba were well
272 protected against a mild *C. perfringens* challenge [10]. Protection against a more
273 severe challenge required multiple injections. Fba delivered by a non-lysis attenuated
274 *S*. Typhimurium vaccine was also found to elicit partially protective immunity [22],
275 although protection was not as effective as when it was delivered by intramuscular
276 injection. In the current study, Fba delivered by an attenuated lysis *S*. Typhimurium

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277 strain elicited the strongest protection, significantly better than the vaccine delivering the
278 two toxoid antigens (**Table 2**). It is possible that, despite the careful strain design to
279 prevent loss of immunogenicity due to antigen load, there is some stress on the vaccine
280 strain delivering the two toxoid antigens, leading to lower immune responses against
281 each antigen. However, in the triple antigen strain, this deficiency appears to be offset
282 by the combined protective efficacy provided by each antigen. Although immunization
283 with the strain delivering Fba alone yielded the lowest lesion scores, it is likely that the
284 triple antigen strain will provide the broadest protection. This question will be examined
285 in future experiments.

286 It is of interest that, in this study, we achieved anti-Fba serum IgY titers similar to
287 the serum titers observed when Fba was delivered by a non-lysis strain [22].
288 Conversely, delivery by lysis strain elicited much greater mucosal responses, indicating
289 that mucosal immunity is more important than humoral immunity for protection against
290 challenge.

291 Previous work demonstrated that anti-PlcC antibodies bound to the surface of *C.*
292 *perfringens*, indicating the presence of α-toxin [7]. We extended those observations to
293 confirm that antibody binding did not occur in a Δ*plc* mutant and that binding is reduced
294 by incubation of antisera with purified PlcC (**Fig. 5**). Our data also indicate that NetB
295 toxin is present on the surface of *C. perfringens*. The presence of all three antigens on
296 the cell surface suggests that the anti-*C. perfringens* mucosal antibodies elicited by our
297 vaccine may bind directly to *C. perfringens*, facilitating opsonization and/or inhibiting
298 toxin secretion. If, as our data suggest, Fba serves as an adhesin for *C. perfringens*,
299 anti-Fba antibodies bound to *C. perfringens* cells may serve to prevent close contact of

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300 the bacterium with the host epithelium, an important step in pathogenesis [23, 24]. In
301 addition, anti-PlcC antibodies were previously shown to inhibit *C. perfringens* growth
302 directly, suggesting another mechanism for the action of these antibodies.
303 This work highlights the potential for a *Salmonella*-vectored vaccine to control
304 NE. Our findings show that the *Salmonella* lysis vector strain χ11802 was capable of
305 delivering up to three antigens simultaneously, generating humoral, cellular and
306 mucosal responses against all antigens. In particular, this strain is able to generate
307 strong mucosal responses as shown here and in a previous study [4]. Our results also
308 support the idea that intestinal mucosal responses are an effective deterrent against
309 lesion formation caused by *C. perfringens*.
310
311 **Materials and Methods**
312 **Animal care.** All animal experiments were conducted in compliance with the Arizona
313 State University Institutional Animal Care and Use Committee and the Animal Welfare
314 Act under protocol 16-1480R.
315 **Bacterial strains, plasmids and growth conditions**. Strains and plasmids used in
316 this study are listed in **Table 3**. All vaccine strains were routinely cultured at 37°C with
317 aeration. *Salmonella* were grown in Luria Broth (LB) (Bacto tryptone, 10 g/liter; Bacto
318 yeast extract, 5 g/liter; NaCl, 10 g/liter) with aeration. When required, media were
319 supplemented with ampicillin (Amp; 100 µg/mL), 2,6-diaminopimelic acid (DAP; 50
320 µg/mL), L-arabinose (Ara; 0.1% v/v) or mannose (Man; 0.1% v/v). Media were solidified
321 with 1.5% (wt/vol) agar as needed. Vaccine strains were supplemented with 0.1% Ara

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322 in plates. *C. perfringens* was cultured anaerobically on blood agar plates, in cooked
323 meat medium (CMM; Difco) or in fluid thioglycollate medium (FTG; Difco) for challenge.
324
325 **Table 3. Strains and plasmids used in this study**.

Strain or plasmid	Genotype/characteristics	Source or reference
Salmonella		
χ11802	ΔP_{murA25}::TT *araC* P_{BAD} *murA* Δ*asdA27*::TT *araC* P_{BAD} *c2* Δ(*wza-wcaM*)-*8* Δ*pmi-2426* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*recF126*	[4]
C. perfringens		
CP4	Wild-type	[25]
JGS4143	Wild-type	[26]
JGS5388	JGS4143 Δ*plc*	G. Songer
JGS4043	$NetB^-$	[27]
E. coli		
MGN055	φ80d *lacZ*ΔM15 *deoR Δ(lacZYA-argF)U169 supE44 gyrA96 recA1 relA1 endA1* Δ*asdA4* Δ*zhf-2*::Tn*10 hsdR17*	[28]
BL21(DE3)	F^- *ompT gal dcm lon hsdSB*(*rB*$^-$ *mB*$^-$) λ(DE3 [*lacI lacUV5*-T7*p07 ind1 sam7 nin5*])	Promega, Madison, WI

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M15 (pREP4)	□*lacZM15 thi mtl* Kan^R	Qiagen
Plasmids		
pQE30	Ap^r	Qiagen
pKR023	*fba* in pYA3681	This study
pKR032	*netB* in pQE30	This study
pYA3493	Non-lysis *asdA* plasmid vector	[29]
pYA3681	Lysis *asdA, murA*, plasmid vector	[8]
pYA4756	Codon-optimized *fba* in pUC57	Genscript
pYA5023	*plcC*, *fba*, GST-*netB* in pYA3493	This study
pYA5107	His-tagged *fba* in pET30a	This study
pYA5112	*plcC*, GST-*netB* in pYA3681	[4]
pYA5130	*plcC*, *fba*, GST-*netB* in pYA3681	This study

Plasmid constructions. The *fba* gene, codon-optimized for expression in *Salmonella*, was synthesized by Genscript (Piscataway, NJ, USA). To construct plasmid pKR023, the *fba* gene was amplified from plasmid pYA4756 using primers Fba-KpnI-F: (TTGGGGTACCTCATGGCACTGGTTAACGCAAAAG) and Fba-SacII: ATTACCGCGGCTATTAAGCTCTGTTTACTGA. Primers 3681-KpnI (TGGGGTACCAGATGGCACTGGTTAACGCAAAAG) and 3681-SacII (ATTACCGCGGCTATTAAGCTCTGTTTACTGA) were used to introduce KpnI and SacII sites into pYA3681. The *fba* gene was cloned into pYA3681, electroporated into

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337 *Escherichia coli* strain MGN055 and then subsequently moved into *S*. Typhimurium
338 strain χ11802. To construct plasmid pYA5130, primers 5023-KpnI-F
339 (CCATGGGGTACCAGATGAGTATTCAACATTTCCGT) and 5023-SacII
340 (ATTACCGCGGTTACAGATAATATTCGATTTTAATT) were used to amplify the *plcC-*
341 *fba-Gst-netB* gene cassette from pYA5023. The purified PCR product was digested
342 with KpnI and SacII. Primers 3681-KpnI and 3681-SacII were used to amplify the
343 plasmid sequences from pYA3681. The purified PCR product was digested with KpnI
344 and SacII. The two fragments were then ligated and electroporated into MGN055. A
345 plasmid of the expected size and DNA sequence was designated pYA5130.
346 **Immunofluorescence assay**. To determine if antibodies against NetB, PlcC, and Fba
347 bound to the bacterial cell surface, we performed an indirect immunofluorescence test.
348 Several fresh colonies of *C. perfringens* from a Trypticase Soy Agar with 5% Sheep
349 Blood plate (BD) were used to inoculate Brain Heart Infusion (BHI) Broth (BD). Cultures
350 were grown for 24 h and harvested by centrifugation at 4000 X *g* for 15 min. Pelleted
351 cells were resuspended with 10% formalin in phosphate buffered saline (PBS) and fixed
352 by overnight incubation at 4°C with slow rotation. Fixed cells were pelleted,
353 resuspended, and washed twice in PBS. 100µL of fixed cells were pelleted and blocked
354 with 2% BSA for 1 h at room temperature with slow agitation. Cells were pelleted and
355 resuspended in 200 µL of control sera, anti-sera, anti-sera + antigen or control protein,
356 or PBS (no sera control) diluted 1:50 in PBS and incubated overnight at 4°C with slow
357 rotation. Cells were washed with PBS-0.1% Tween 20 and goat anti-chicken IgG
358 antibody conjugated with fluorescein isothiocyanate (SouthernBiotech, Birmingham, AL)
359 diluted 1:500 in PBS was added. The resulting cell suspension was incubated for 3 h at

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360 room temperature with gentle agitation. Cells were washed 3 times with PBS-0.1%
361 Tween 20, resuspended in PBS and mounted for observation under a Leica TCS SP5
362 confocal microscope.
363 **Synthesis of recombinant antigens in χ11802.** To evaluate synthesis of PlcC, Fba
364 and GST-NetB, overnight static cultures of *S.* Typhimurium χ11802 carrying plasmids
365 pYA5112 (PlcC, GST-NetB), pYA5130 (PlcC, Fba, GST-NetB), pKR029 (Fba) or
366 pYA3681 (empty vector) were inoculated into LB supplemented with 0.1% arabinose
367 and 0.2% mannose and grown to an OD_{600} of 0.6. Then, 1 mM isopropyl-β-D-
368 thiogalactopyranoside (IPTG) was added to each culture and induced for an additional 4
369 h. The final cultures were adjusted to the same density using OD_{600} values. Equal
370 volumes of the adjusted samples were centrifuged at 16,000 × *g* for 5 min and the pellet
371 was resuspended with 100 µL sodium dodecyl sulfate (SDS)-loading buffer (100 mM
372 Tris–HCl, pH6.8; 4% SDS; 0.2% bromophenol blue; 20% glycerol; 200 mM β-
373 mercaptoethanol). The whole-cell lysates were subjected to SDS-polyacrylamide gel
374 electrophoresis (SDS-PAGE) and western blot as described using polyclonal rabbit sera
375 raised against the indicated antigens [4].
376 **Immunization of chickens.** Groups of one-day-old Cornish × Rock broiler chickens
377 were purchased from the Murray McMurray Hatchery (Webster City, IA). Chickens were
378 divided into separate pens, with 16-20 chicks per group. On the day of arrival, three
379 chicks were euthanized. Spleen and ceca were collected to confirm their *Salmonella*-
380 free status. The shipping boxes were also swabbed. Tissues were homogenized in
381 PBS and plated onto Brilliant Green plates for bacterial enumeration. Tissue samples
382 and swabs were also enriched in Rappaport-Vassiliadis medium for 48 h at 37°C and

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383 subsequently plated onto Brilliant Green. No *Salmonella* were detected in the birds nor
384 in the shipping boxes. The following day (day 0, the birds were 4 days of age), all
385 chicks were orally inoculated with approximately 1 x 10^8 CFU (experiment 1) or 1×10^9
386 CFU (experiments 2 and 3) of various *S*. Typhimurium vaccine strains, including the
387 vector-only control strain χ11802(pYA3681), in 100 µL of PBS. Additional groups of
388 control birds were not vaccinated. Thirty minutes later, the chicks were provided with
389 feed and water. The same dose of the same strain was given as a boost immunization
390 14 days later.

391 We performed three independent experiments. Intestinal mucosa samples and
392 cellular response data were not collected in experiment 1 and the strain carrying
393 pKR023 was not tested. In experiment 2, all vaccine strains were used and immune
394 response data were collected, but no challenge was performed. In experiment 3, all
395 strains were used and serum, mucosal and cellular response data were collected.

396 **Sample collection for analysis of immune responses.** To evaluate mucosal immune
397 responses (experiments 2 and 3), intestinal samples were collected as described
398 previously [30] with some modifications. Three birds from each group were necropsied
399 at 19 (experiment 2) or 21 (experiment 3) days after the 1st immunization (6 days after
400 the boost). The intestines were opened aseptically and the surface contents were
401 removed gently using a clean paper wiper. Then, approximately 3 g of intestinal
402 scrapings were collected using glass slides and resuspended in 30 mL of ice-cold PBS
403 containing Pierce™ Protease Inhibitor Mini Tablets. After shaking for 1 min, the
404 supernatants were collected by centrifugation at 4000 × *g* for 20 min at 4°C to evaluate
405 intestinal IgA, IgY and IgM antibody production. Blood was collected from wing veins of

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406 all remaining birds in each group at 21 days after the 1st immunization to assess the IgY
407 antibody responses to *C. perfringens* and *Salmonella* antigens in serum.
408 **Determination of antibody response by enzyme-linked immunosorbent assay**
409 **(ELISA).** ELISAs were performed in triplicate as described [31] to determine the IgY
410 responses against his-tagged PlcC, his-tagged Fba, his-tagged NetB and *S.*
411 Typhimurium lipopolysaccharide (LPS) in chicken sera and IgA, IgY and IgM responses
412 in intestinal washes. Biotinylated anti-chicken IgA (Alpha Diagnostic Intl. Inc), IgY
413 (Southern Biotechnology) or IgM (Bioss) antibodies diluted 1:10,000 were used to
414 detect the various antibody isotypes.
415 **Cellular proliferation assay**. A proliferation assay was performed to evaluate cell-
416 mediated immunity. Twenty-one days post primary immunization, blood and spleens
417 were harvested. Lymphocytes in the blood were harvested using the gentle swirl
418 technique [32] and plated in quadruplicate, in a 96-well plate at 10^5 cells/well in RPMI-
419 1640 without phenol red. Spleens were placed through a 70 µm cell strainer to obtain
420 single cell suspensions. Red blood cells were lysed with Red Blood Cell Lysis solution
421 (eBioscience). Splenocytes were then washed, suspended in RPMI and plated at 10^6
422 cells/well. Each set of cells was incubated at 37°C, 5% CO_2 for 72 h with or without 4
423 µg/ml of either His-Fba, *S*. Typhimurium LPS, His-NetB, His-PlcC, or 1 µg/ml PMA. Cell
424 proliferation was measured using the Vybrant®MTT Cell Proliferation Assay Kit
425 (Molecular Probes). Mean absorbance value of antigen stimulated wells divided by
426 mean absorbance of non-stimulated control wells was used to calculate stimulation
427 index.

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428 **Challenge with *C. perfringens*.** Chickens were fed an antibiotic-free starter feed
429 containing 21% protein for 20 days, at which time the feed was switched to a high
430 protein (28% protein), wheat-based feed containing 36% fish meal and zinc at 400 ppm
431 (customized by Reedy Fork Farm, NC) to predispose the birds to necrotic enteritis [11,
432 33]. Birds were challenged with virulent *C. perfringens* strain CP4 [25] in feed from day
433 28 to day 32 as described [11] with some modifications. Feed was withdrawn on day 27
434 for 15 h before challenge. On day 28, chickens were orally gavaged with 0.5 mL of an
435 overnight culture of *C. perfringens* CP4 grown in CMM medium. Immediately after
436 gavage, infected feed was provided thereafter for 5 consecutive days. To prepare
437 infected feed, *C. perfringens* was grown in CMM medium for 24 h at 37°C, which then
438 was inoculated into FTG medium at a ratio of 0.3% (v/v) and incubated at 37°C for 15 h
439 (morning challenge) or 23 h (evening challenge). The *C. perfringens* culture was mixed
440 with feed at a ratio of 1:1 (v/w). Infected feed was prepared freshly twice daily. All birds
441 were euthanized and necropsied the day following the final challenge (day 33).
442 **Lesion scoring.** Protection against *C. perfringens* challenge was assessed on the
443 basis of gross intestinal lesion scores at necropsy. On day 33, chickens were
444 euthanized with CO_2 and their small intestines (defined here as the section between the
445 gizzard and Meckel's diverticulum) were examined for visible gross lesions. Intestinal
446 lesions were scored as follows: 0 = no gross lesions; 1 = thin or friable wall or diffuse
447 superficial but removable fibrin; 2 = focal necrosis or ulceration, or non-removable fibrin
448 deposit, 1 to 5 foci; 3 = focal necrosis or ulceration, or non-removable fibrin deposit, 6 to
449 15 foci; 4 = focal necrosis or ulceration, or non-removable fibrin deposit, 16 or more foci;
450 5 = patches of necrosis 2 to 3 cm long; 6 = diffuse necrosis typical of field cases [11].

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451 **Attachment assay.** The human colon carcinoma cell line Caco-2 (ATCC® #HTB-37)
452 were obtained from the American Type Culture Collection (Manassas, VA) and cultured
453 in Dulbecco's' modified Eagle's medium (DMEM) with 4.5 g/L glucose (Corning,
454 Manassas, VA) containing 4mM L-glutamine, 1% sodium pyruvate, 1% non-essential
455 amino acids (NEEA), 100 U/ml penicillin, 100 µg/ml streptomycin, 20% heat inactivated
456 fetal calf serum. Caco-2 cells were seeded at 5 X 10^5 cells/mL in each well of a 24-well
457 tissue culture plate. Cells were allowed to grow to a confluent monolayer for 48 hours.
458 One hour prior to infection with CP4, media was replaced with antibiotic free DMEM.
459 Then, 1 µg/ml of purified protein (his-FBA, his-NetB, or his-PspA) or PBS was added to
460 the Caco-2 monolayer and incubated for 15 min at 37°C, 5% CO_2. The bacterial
461 inoculum was prepared as follows. CP4 cultures were grown anaerobically for 24 h in
462 BHI broth inoculated from a fresh blood agar plate. Cultures were pelleted and
463 resuspended in PBS to a target concentration of 5 X 10^5 CFU/ 20 µL. Caco-2
464 monolayers were infected with an MOI of 1:1 and then centrifuged for 3 min at 240 x *g*
465 and incubated for 1 hour at 37°C, 5% CO_2. To detach the Caco-2 monolayers, 200
466 µL/well of 0.25% Trypsin-EDTA was added to each well. Once the monolayer cells
467 detached, 800 µL/well of PBS was added. The trypsinized samples were serially diluted
468 and plated onto blood agar plates for *C. perfringens* enumeration. All counts were
469 normalized to the inoculum concentration and presented as percentage of the no
470 protein control.
471 **Statistical analysis.** All statistics were carried out using GraphPad Prism 6.0 (Graph-
472 Pad Software, San Diego, CA). Antibody titers and adherence data were analyzed
473 using two-way or one-way ANOVA followed by Tukey's posttest, respectively. Lesion

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474 scores were analyzed using a two-tailed Mann-Whitney test. The values were
475 expressed as means ± SEM, and differences were considered significant at $P < 0.05$.
476

477 **Acknowledgements**

478 The authors wish to thank Jacquelyn Kilbourne, Larisa Gilley, Nyja Brown, Donovan
479 Leigh, Penelope Roach and Melody Yeh for their expert technical assistance. We thank
480 Dustin McAndrew, Randall Dalbey and the DACT staff at ASU for expert care and
481 husbandry of our research animals.

482 **Competing interests**. KR is an inventor on US patent 8,465,755 and US patent
483 9,040,059. All other authors declare no competing interests.

484 **Funding**. This project was supported by Agriculture and Food Research Initiative
485 Competitive Grant no. 2016-67016-24947 from the United States Department of
486 Agriculture, National Institute of Food and Agriculture and startup funds from Arizona
487 State University to KR. The funders had no role in study design, data collection and
488 analysis, decision to publish, or preparation of the manuscript

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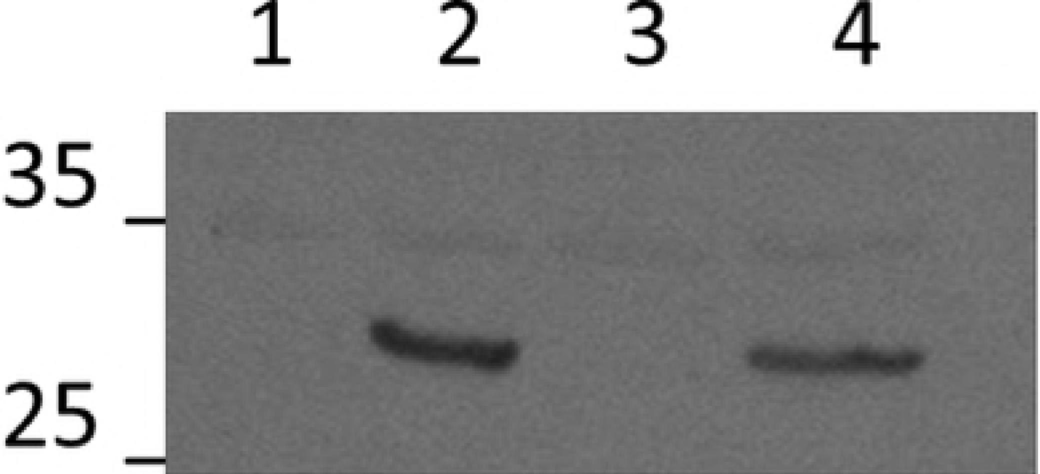
630 Supporting information captions

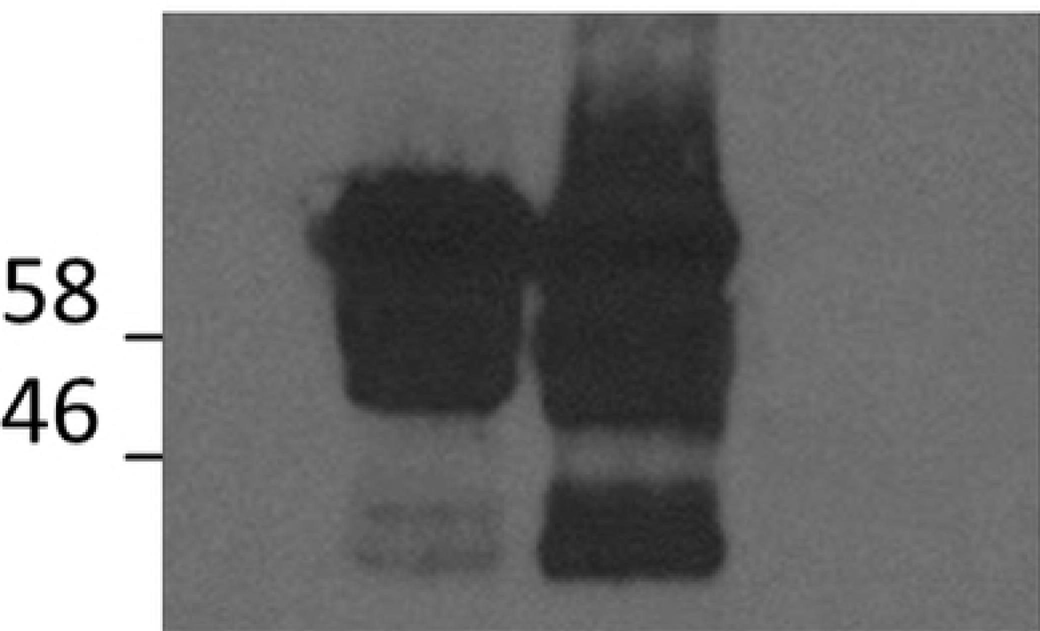
631 **Figure S1**. Growth curves of the χ11802 vaccine strains used in this study. Strains
632 were grown with aeration in LB supplemented with 0.1% arabinose and 0.1% mannose.
633 A. Optical density measurements; B. Colony forming units obtained by plating onto LB +
634 arabinose at the indicated times.
635
636 **Figure S2**. Immunofluorescence of *C. perfringens* strain CP4. Cells were incubated with
637 pre-immune sera (Pre-Bleed), secondary antibody only (2º α) or the indicated antisera
638 with or without prior incubation with the indicated recombinant proteins as outlined in the
639 Materials and Methods section.
640
641
642



Relative intensities

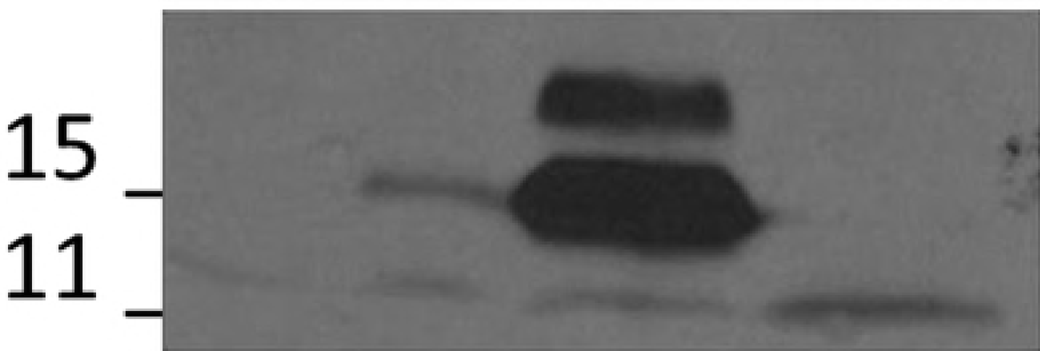
α FBA

Lane 2 – 1.0
Lane 4 – 0.8



α NetB

Lane 2 – 1.0
Lane 3 – 1.0



α Plc

Lane 2 – 1.0
Lane 3 – 6.0

A.

Log$_2$ Titer Serum IgY

2^{11}

2^{10}

2^{9}

2^{8}

2^{7}

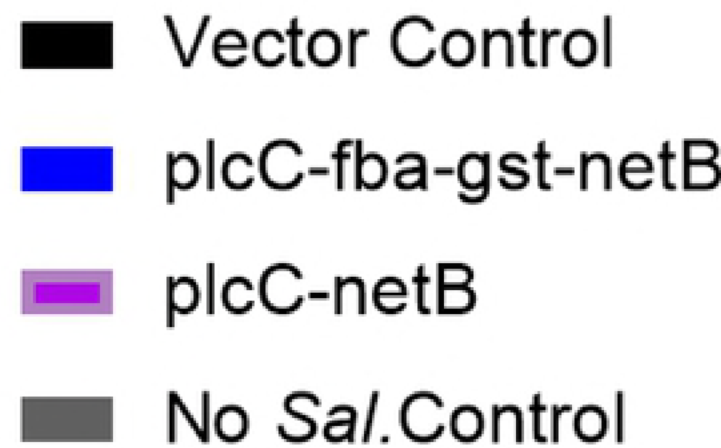
*

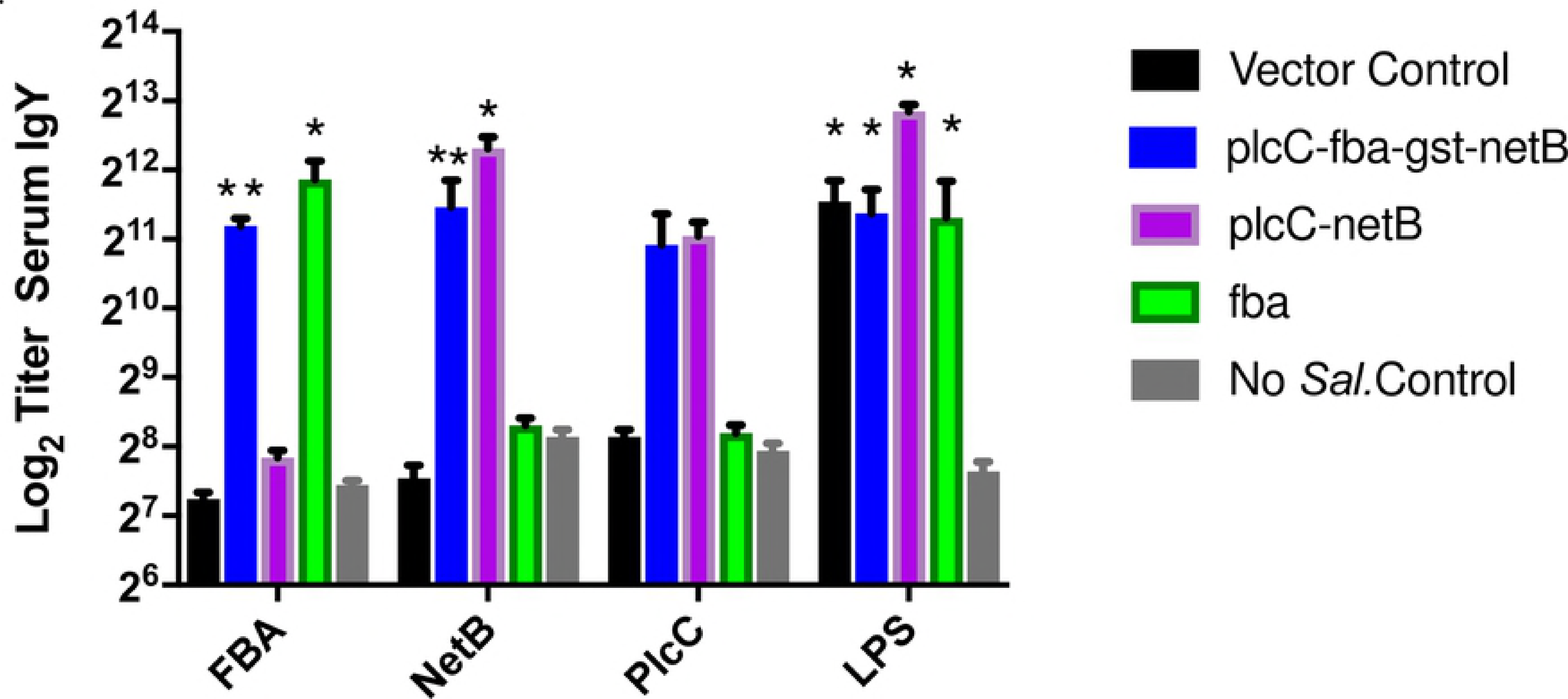
* *

FBA

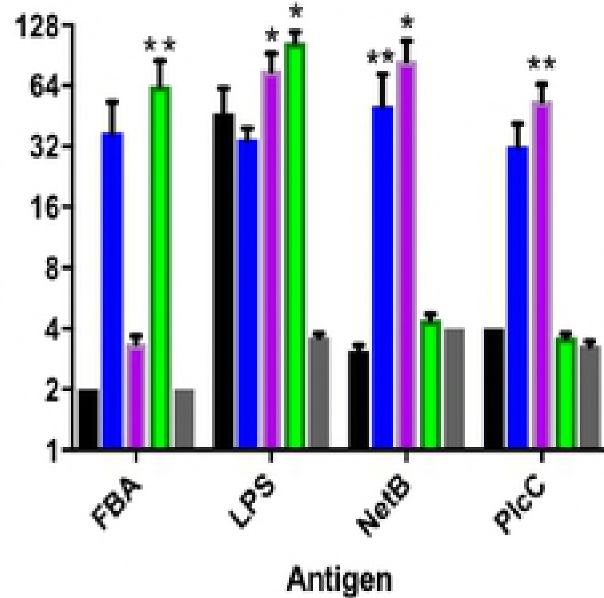
NetB

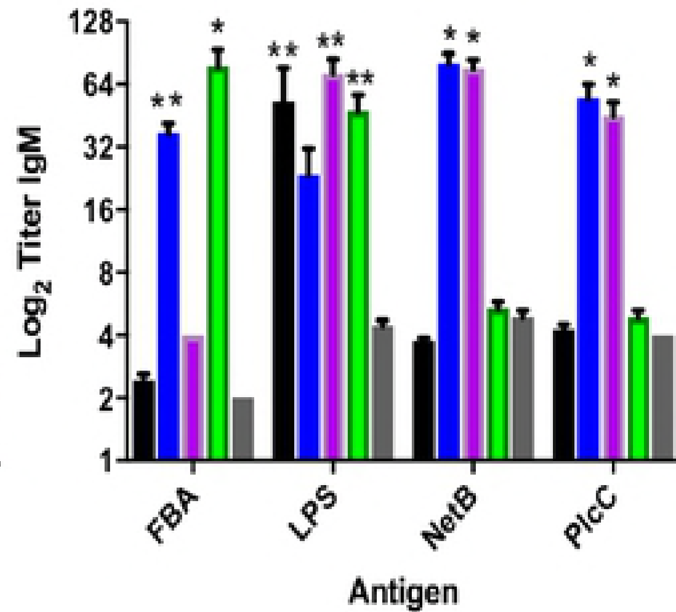
PlcC





B.
Log2 Titer Serum IgY
2^14
2^13
2^12
2^11
2^10
2^9
2^8
2^7
2^6
FBA
NetB
PlcC
LPS
Vector Control
plcC-fba-gst-netB
plcC-netB
fba
No Sal.Control





Intestinal IgA

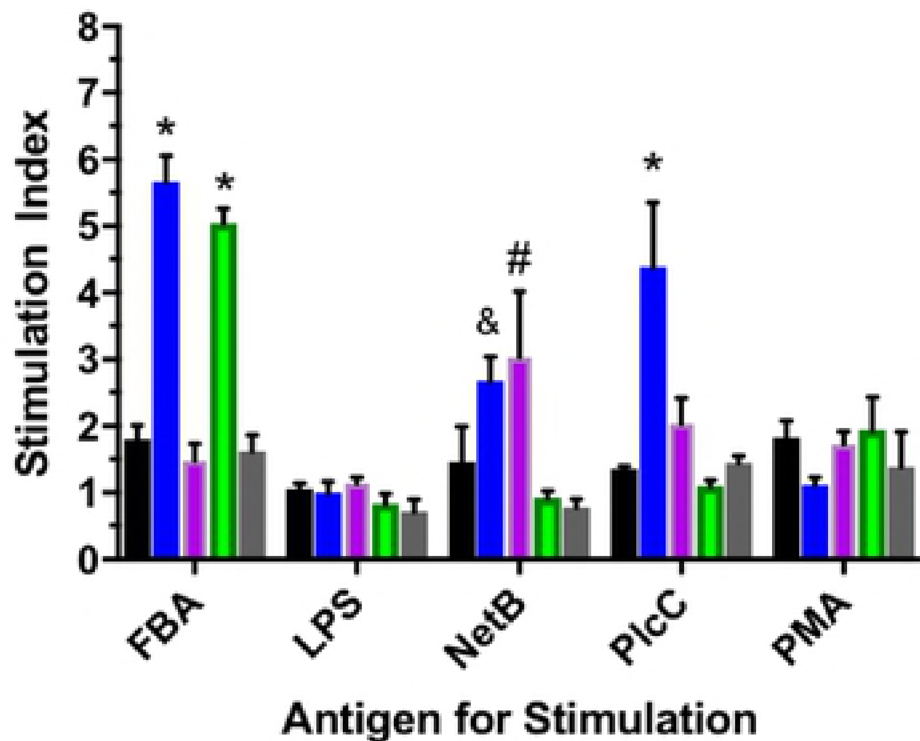
256
128
64
32
16
8
4
2
1

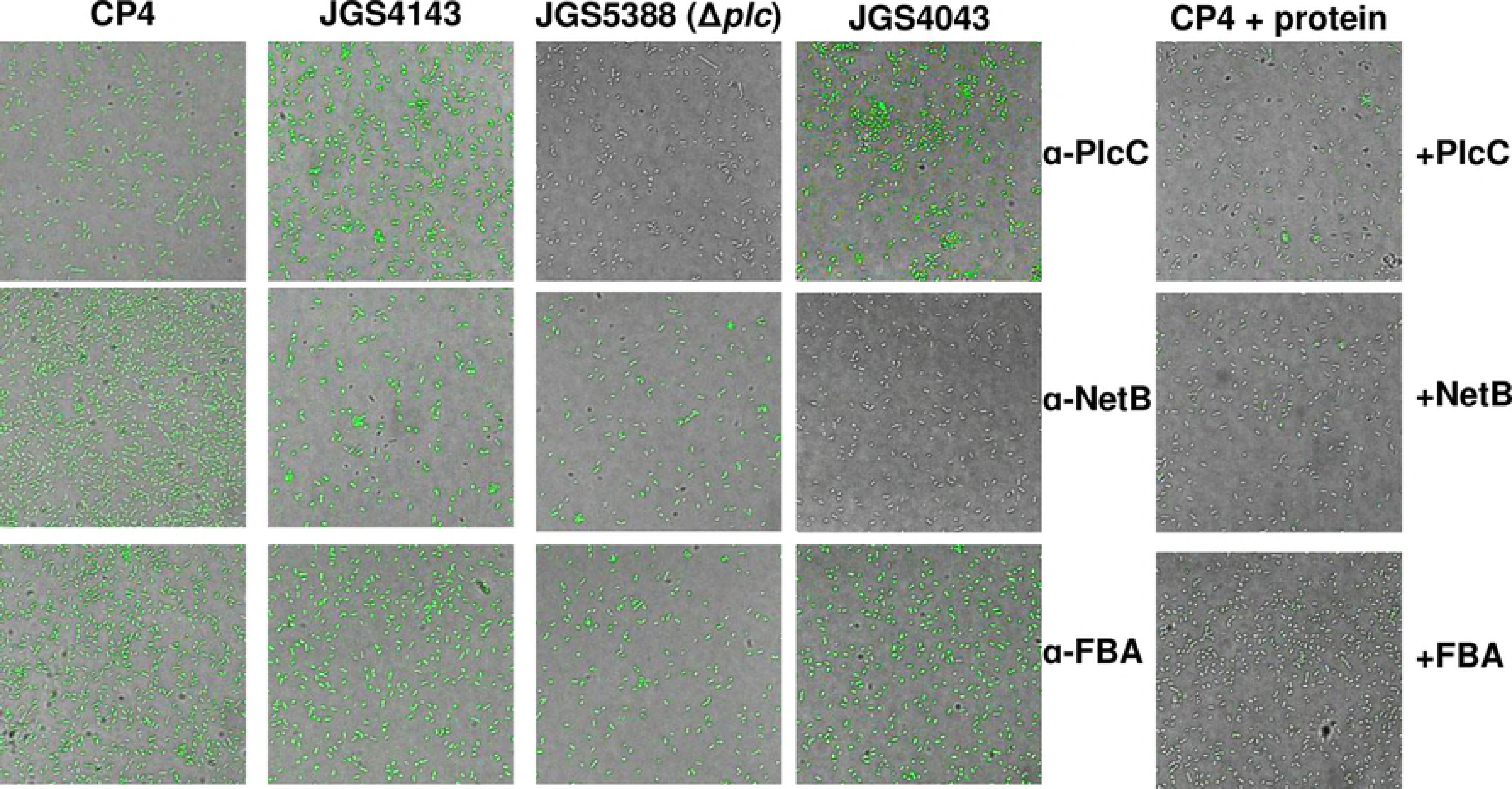
Log2 Titer IgA

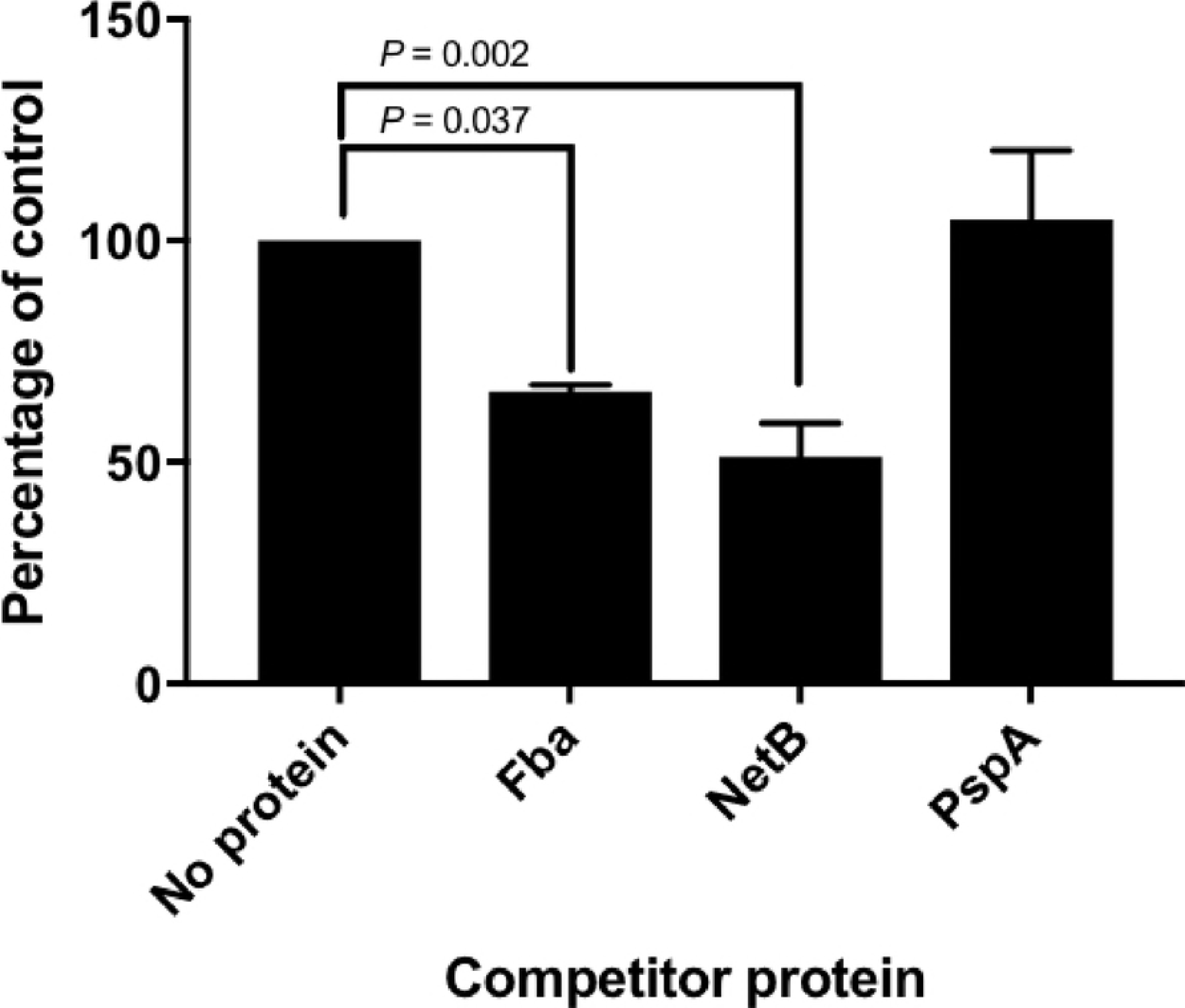
FBA
LPS
NetB
PlcC

Antigen

Vector Control
plcC-fba-gst-netB
plcC-netB
fba
No *Sal.*Control


Splenocytes
Stimulation Index
0
1
2
3
4
5
6
7
8
*
*
&
#
*
FBA
LPS
NetB
PlcC
PMA
Antigen for Stimulation
Lymphocytes
Stimulation Index
0
10
20
25
30
35
40
45
50
*
*
&
FBA
LPS
NetB
PlcC
PMA
Antigen for Stimulation
Vector Control
plcC-fba-netB
plcC-netB
fba
None


CP4
JGS4143
JGS5388 (Δplc)
JGS4043
CP4 + protein
α-PlcC
+PlcC
α-NetB
+NetB
α-FBA
+FBA
0
µm
100


150
100
50
0
Percentage of control
P = 0.002
P = 0.037
No protein
Fba
NetB
PspA
Competitor protein