Development of a subunit vaccine targeting *Clostridium perfringens* enzymes for the control of necrotic enteritis in broilers

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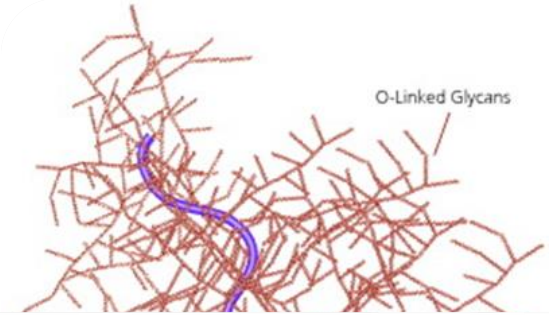
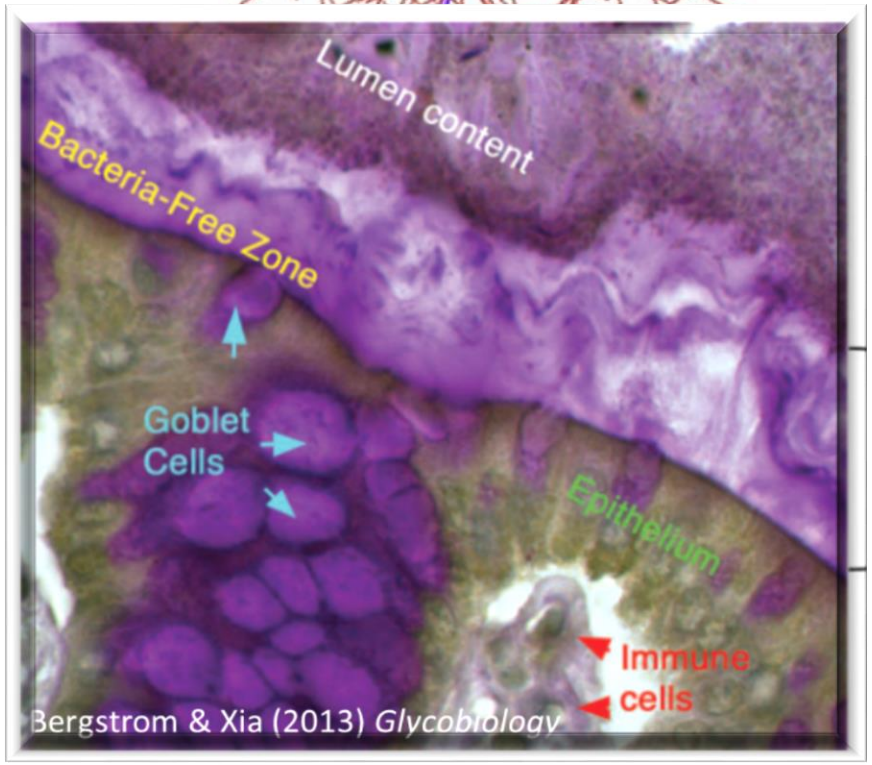
Mucins

- Majority are type *O*-glycans
  - Cross-linked connections that create high viscosity
- Protect epithelia
  - Physical barrier
  - Support innate & adaptive immunity
  - Pathogens must subvert to initiate infection
- Foraged by bacteria for nutrition

Reviewed by Bergstrom & Xia (2013) *Glycobiology*

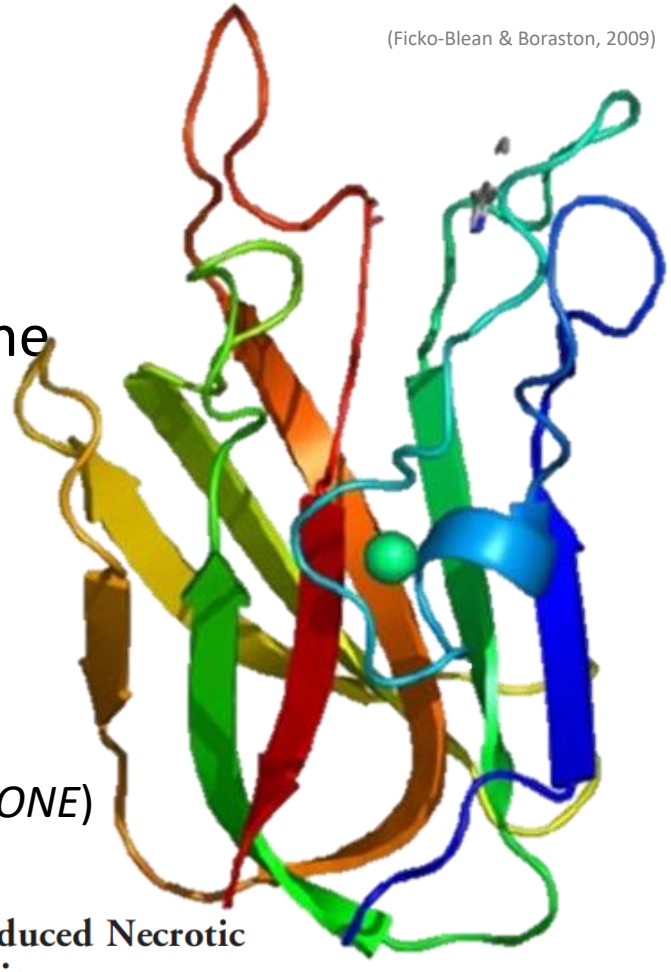




Antigenic Targets of *Clostridium perfringens*

- Glycoside hydrolases
  - Galactosidases and glucosaminidases
    - *O*-linked *N*-acetylgalactosamine and *N*-acetylglucosamine are major components of GIT mucin
  - Carbohydrate binding modules
    - Recognize CHO
    - Enzyme orientation and association
    - Abundant and highly conserved (Ficko-Blean et al. (2012) *PLoS ONE*)

(Ficko-Blean & Boraston, 2009)



AVIAN DISEASES 53:409-415, 2009

Immunization of Broiler Chickens Against *Clostridium perfringens*–Induced Necrotic Enteritis Using Purified Recombinant Immunogenic Proteins

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Antigen Selection

Identify conserved, immunoprotective mucolytic peptide sequences

- Generate hyper-immune sera

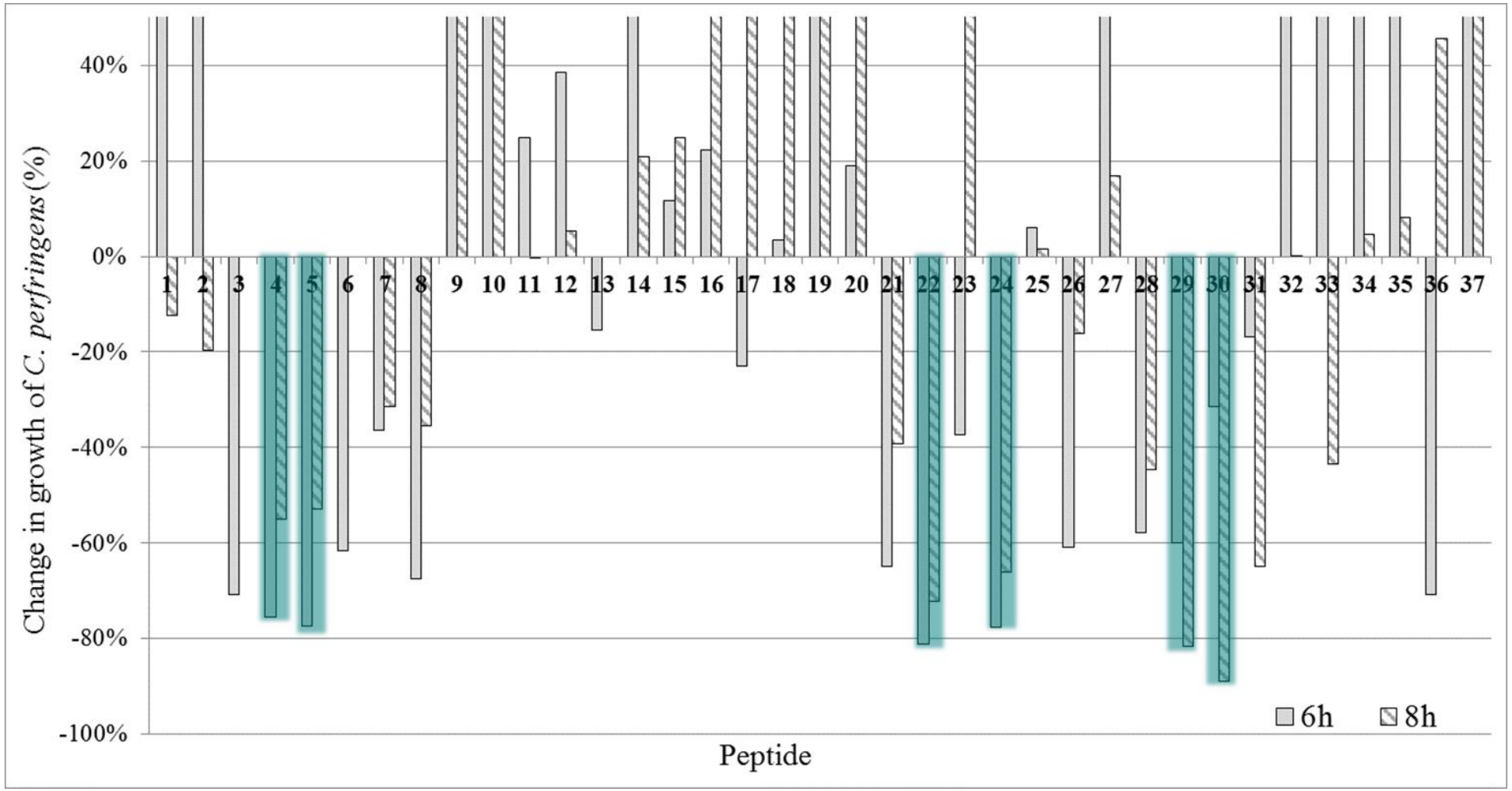
Evaluate hyper-immune sera growth inhibition of *C. perfringens* in mucin broth

Evaluate protective capabilities of selected peptides as vaccine components in broilers given an NE challenge

- Impact on body weight (BW) and lesion scores (LS)

Antigen Selection

Anti-mucinase antibody reduction of *Clostridium perfringens* growth in mucin broth

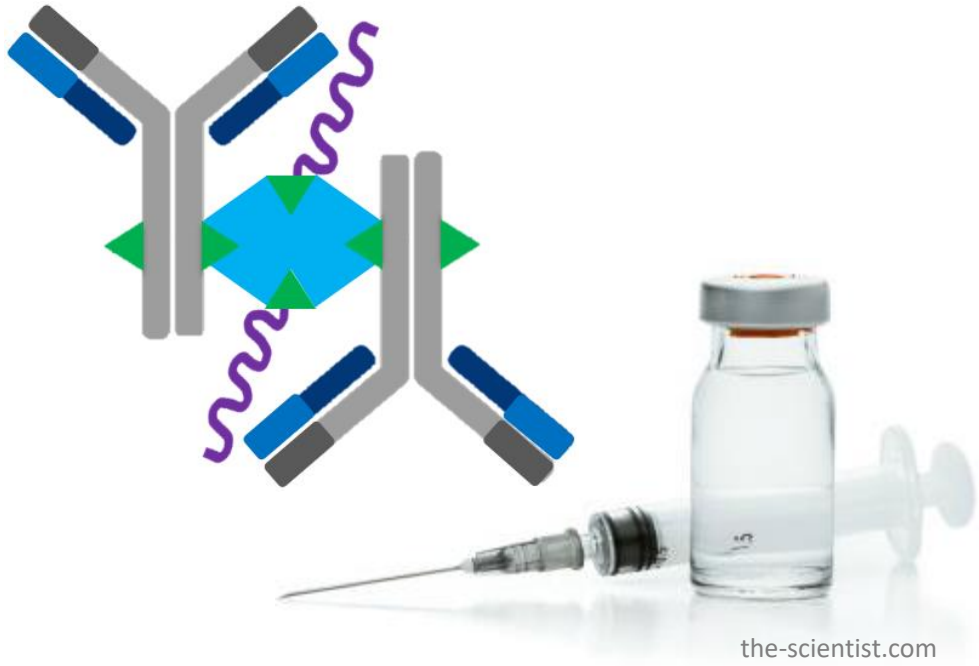


Proof of Concept Vaccination Experiment

Treatment Groups:

1. Non-vaccinated Non-challenged Control (**NVNC**)
2. Non-vaccinated Challenged Control (**NVCC**)
3. Peptide 4 (**VP4**)
4. Peptide 5 (**VP5**)
5. Peptide 22 (**VP22**)
6. Peptide 24 (**VP24**)
7. Peptide 30 (**VP30**)
8. Peptides 4, 5, 22, 24, 30 (**MC**)

$n_{2\text{-}7}$ = 20 birds
$n_{1\&8}$ = 10 birds



d1
Place all birds on Salinomycin (50ppm)

d2
10^4 CFU/bird SE G2-8
SC Vaccinate

d19
SC Vaccinate

d24
Remove Salinomycin G2-8

d27
Weigh all birds
Aminister EM G2-8

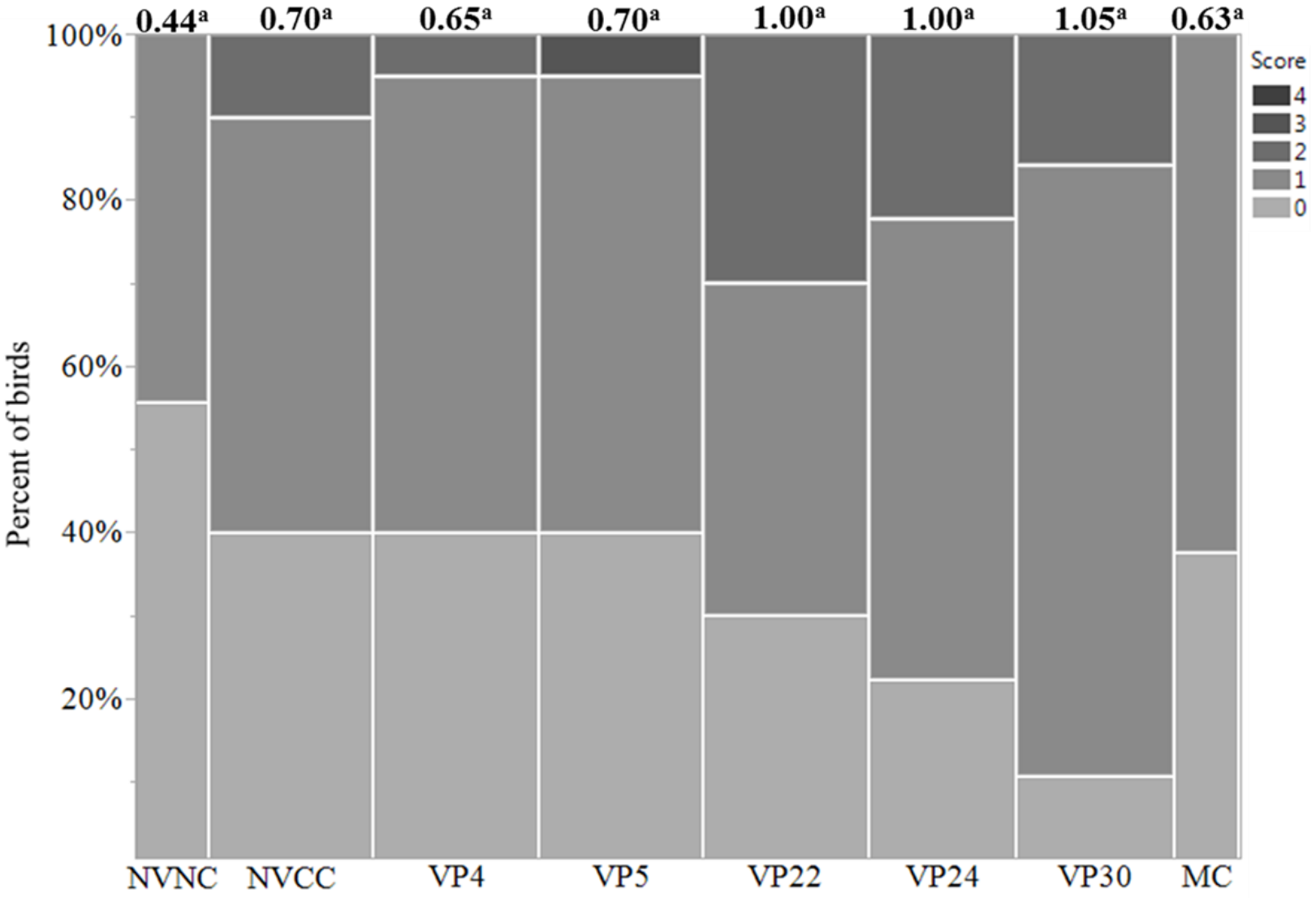
d32
Administer CP G2-8

d34
Weigh and lesion score

SE—*Salmonella* Enteritidis SC—Subcutaneous EM – *Eimeria maxima*

Proof of Concept Vaccination Experiment

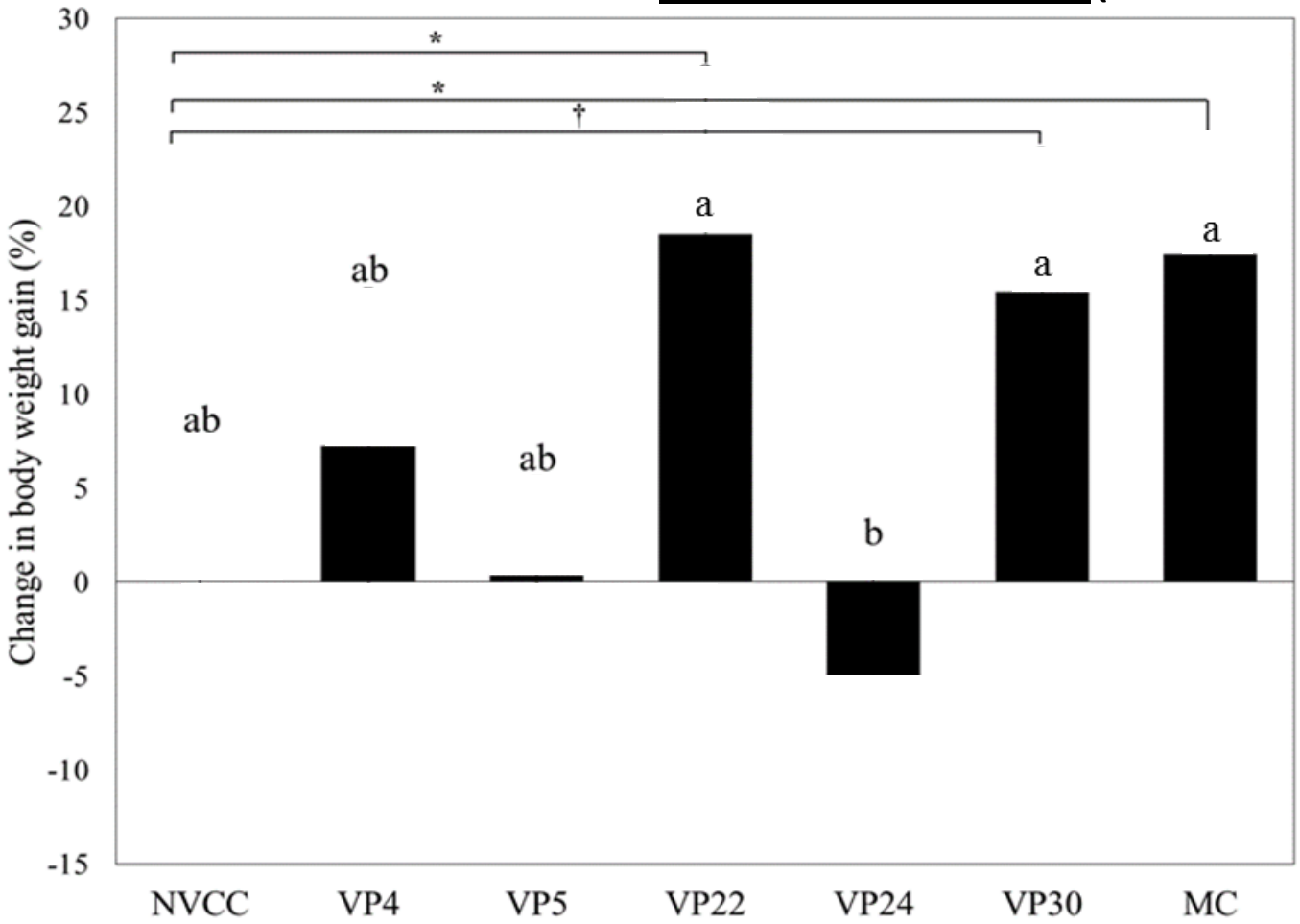
Distribution of necrotic enteritis lesion scores after vaccination with mucinase antigens



Proof of Concept Vaccination Experiment

Effect of Mucinase Antigen Vaccination on %Δ BWG Relative to NVCC (d27-d34)

Group	d27-34 BWG %
NVNC	52.18±1.80a
NVCC	33.24±2.51b



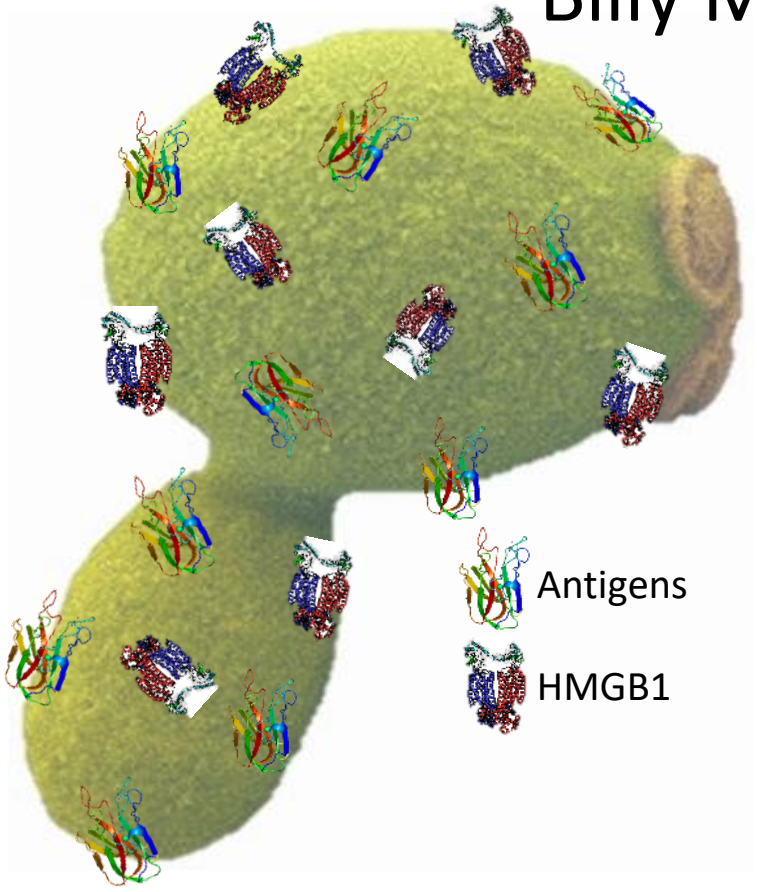
*significantly different from positive control ($P < 0.05$)

†significantly different from positive control ($P = 0.0661$)

Proof of Concept
Necrotic Enteritis Vaccine:
Anticoccidial Subunit Vaccine +
Alpha Toxin Antigens +
Mucinase Antigens

Subunit Vaccine

Poster #52
Billy M. Hargis



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Eimeria maxima vaccination via *Pichia pastoris* recombinant vector for coccidia protection in broiler chickens

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Abstract

Coccidiosis remains one of the most devastating protozoal diseases faced by the global poultry industry, and multiple drug options for control are classified as antibiotics, which limits treatment options. Moreover, coccidiosis vaccines are known to contribute markedly to bacterial enteropathies such as necrotic enteritis. Many strategies, including vaccination, are used to control this illness, yet outcomes are variable. Here we describe the application of a novel recombinant vaccine targeting *Eimeria* spp. in *Eimeria maxima* (EM) inoculated broilers. A new *Pichia pastoris* vaccine-vector expressing thrombospondin-related adhesive protein (TRAP) family, rhomboid protease (ROM5) and high mobility group box 1 (HMGB1) protein has been developed. Experiment 1 compared the previously-developed *Bacillus*-vectored TRAP-ROM5-HMGB1 and *Pichia*-vector efficacy against EM M6 inoculation. In experiment 2, timing and the delivery of the vaccines were compared. Results showed that there were no significant differences in body weight gain (BWG) or percent change in BWG (%ChangeBWG) relative to the positive control in experiment 1. In experiment 2, BWG was not significantly higher in chickens that were vaccinated via drinking water at day-of-hatch or at d5 then boosted with the same vaccine. No differences were observed for lesion scores (LS) in any of the experiments. However, quantification of oocysts per gram (OPG) of feces was markedly and significantly lower in some groups vaccinated with a form of the *Pichia*-vectored vaccine especially at the level of accumulative oocysts shedding or oocysts shedding per bird in experiment 2. Overall, this approach to vaccination, or augmentation of live oocyst-based vaccines, appears promising.

INTRODUCTION

- *Eimeria* infections are is an endemic and consistent issue facing the poultry industry, reducing performance and sometimes high morbidity/mortality.
- Current vaccination strategies involve inoculating a flock with virulent wild-type *Eimeria* and reliant on maintaining a house environment to allow multiple rounds of natural cycling.
- Difficulty in maintaining *Eimeria* cycling in the house results in poor vaccination coverage/protection in flocks.
- Maintained virulence of the vaccine strain occasionally induces coccidiosis disease in the flock itself.
- An alternative vaccine, that does not rely on *Eimeria* cycling would be very helpful.
- Two protective *Eimeria* epitopes (TRAP and ROM5) were identified and expressed on *Bacillus* or *Pichia*, along with HMGB1 immunostimulatory protein, to serve as vaccine vectors.

METHODS

Animal housing and procedures

Exp 1 & 2 used DOH Cobb-Vantress broiler chicks. Chicks were comingled in a single room on wood shavings in all experiments. Chicks were initially placed on a standard broiler diet with Clinacox (1lb/ton, 0.2%, Diclazuril; Huvepharma, USA), and anti-coccidial treatment was removed 4-6 days prior to *Eimeria maxima* (EM) inoculation. Feed and water were provided *ad libitum* for the duration of all experiments with the exception of a feed withdrawal period 6h prior to vaccination. Body weights were recorded at time of EM inoculation and either 5 days post inoculation 6dpi in order to calculate body weight gain (BWG) and percent change in body weight gain (%ChangeBWG) relative to the positive control. Additionally, fecal samples were randomly collected from comingled birds prior to EM inoculation to verify birds were uninfected prior to intentional exposure. A subset of birds from each treatment were moved to wire floor cages 4dpi for fecal collections and enumeration of oocyst shedding per gram of feces (OPG). After final weights were measured, remaining comingled birds were euthanized and lesion scores (LS) were recorded using a scale of 0-4 (Johnson and Reid, 1970). All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee at the University of Arkansas.

Eimeria maxima preparation

Purified cultures of EM M6 strain were used in these experiments. Oocysts were enumerated and resuspended in sterile 0.9% (w/v) normal saline and administered via oral gavage (OG) to all birds except those in the non-challenged control.

Vaccination procedure

The *Bacillus* and *Pichia* vectors used expressed antigenic *Eimeria* epitopes TRAP and ROM5, as well as immunostimulatory molecule HMGB1. Feed was removed 6h prior to oral gavage with respective vaccine treatments. All vaccines in were mixed directly into standard mannosylated chitosan adjuvant (MCA). The MCA stock solution (1.5% w/v) and *P. pastoris* suspension were combined at a 1:2 ratio for a final MCA concentration of 0.5%. For the drinking water (DW) groups, brooder rings were assembled to keep birds near the waterer in which the vaccine was supplied. After a 24h period, brooder rings were removed, and remaining water was measured

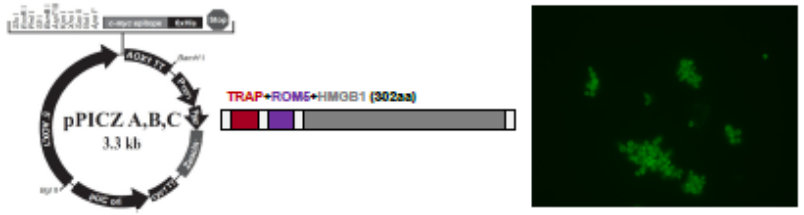

Figure 1. *Pichia* vaccine vector construct design and immunofluorescent staining depicting cell surface expression of inserted epitopes.

RESULTS

Table 1. Vaccination and challenge scheme for Exp 1 & 2.

Exp	Treatment ID	Vector	Dose (CFU)	Route	Adjuvant (1.5% w/v)	# of Oocysts/bird
1	Negative Control	-	-	-	-	1x10⁴ oocysts/bird
	Positive Control	-	-	-	-	
	Bacillus	MCA	$5x10^9$	OG (d1)	OG	
	Pichia	MCA	$2.5x10^9$	OG (d1)	OG	
	Bacillus	MCA	$1x10^9$	DW (d4)	DW	
	Pichia	MCA	$2.5x10^9$	DW (d4)	DW	
2	Negative Control	-	-	-	-	6x10⁴ oocysts/bird
	Positive Control	-	-	-	-	
	Pichia	MCA	$1X10^9$	DW (DOH)	DW	
	Pichia	MCA	$1X10^9$	OG (DOH)	OG	
	Pichia	MCA	$1X10^9$	OG (d5)	OG	
	Pichia	MCA	$1X10^9$	DW (d5)	DW	

All vaccine vectors contained: TRAP *Eimeria* protective T-cell epitope, ROM5 *Eimeria* epitope, and chicken HMGB1 immunostimulatory protein.
MCA (mannosylated chitosan adjuvant) used in all vaccine treatments.
Routes: OG = oral gavage, DW = drinking water

Table 2. Body weight gain after EM challenge for Exp 1 & 2.

Exp	Treatment ID	7d BWG (g)	7d %ChangeBWG
1	Negative Control	369.6 ± 9^a	100 ± 2.4^a
	Positive Control	308.2 ± 9.1^b	81.2 ± 2.5^b
	Bacillus	299.9 ± 7.2^b	80.8 ± 1.9^b
	Pichia	307.5 ± 7.5^b	83.2 ± 2^b
	Bacillus	288.2 ± 6.4^b	78 ± 1.7^b
	Pichia	288.6 ± 9.6^b	78 ± 2.6^b
2	Negative Control	502.4 ± 12.5^a	100 ± 2.5^a
	Positive Control	309.2 ± 14.2^b	61.6 ± 2.8^b
	Pichia	336.9 ± 12.7^b	67.1 ± 2.5^b
	Pichia	338.5 ± 15.6^b	67.4 ± 3.1^b
	Pichia	348.2 ± 12.6^b	69.4 ± 2.5^b
	Pichia	354.5 ± 10.5^b	70.6 ± 2.1^b

Table 3. Lesion scores for Exp 1 & 2.

Exp	Treatment ID	Lesion Scores (%)					Mean
		0	1	2	3	4	
1	Negative Control	86.2	13.8	0	0	0	0.1 ± 0.1^a
	Positive Control	0	0	0	36.8	63.2	3.6 ± 0.1^b
	Bacillus	0	0	10.5	57.9	31.6	3.2 ± 0.1^b
	Pichia	0	0	0	50.0	50.0	3.5 ± 0.1^b
	Bacillus	0	5.3	15.8	26.3	52.6	3.3 ± 0.1^{ab}
	Pichia	0	0	0	50.0	50.0	3.5 ± 0.1^b
2	Negative Control	5.3	10.5	5.3	0	0	1.2 ± 0.3^a
	Positive Control	0	0	5.0	40.0	55.0	3.5 ± 0.1^b
	Pichia	0	0	5.0	55.0	40.0	3.4 ± 0.1^b
	Pichia	0	0	0	55.6	44.4	3.4 ± 0.1^b
	Pichia	0	5.3	0	47.4	47.4	3.5 ± 0.1^b
	Pichia	0	0	0	50.0	50.0	3.4 ± 0.2^b

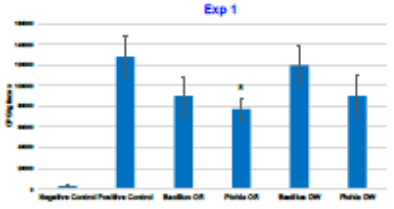

Figure 2. Oocyst shedding (days 6 & 7 combined) for Exp 1.

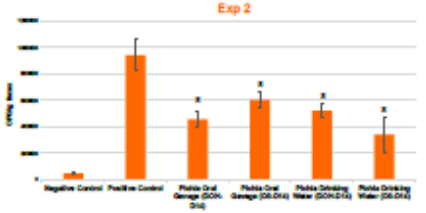

Figure 3. Oocyst shedding (days 6 & 7 combined) for Exp 2.

Alpha-toxin antigen

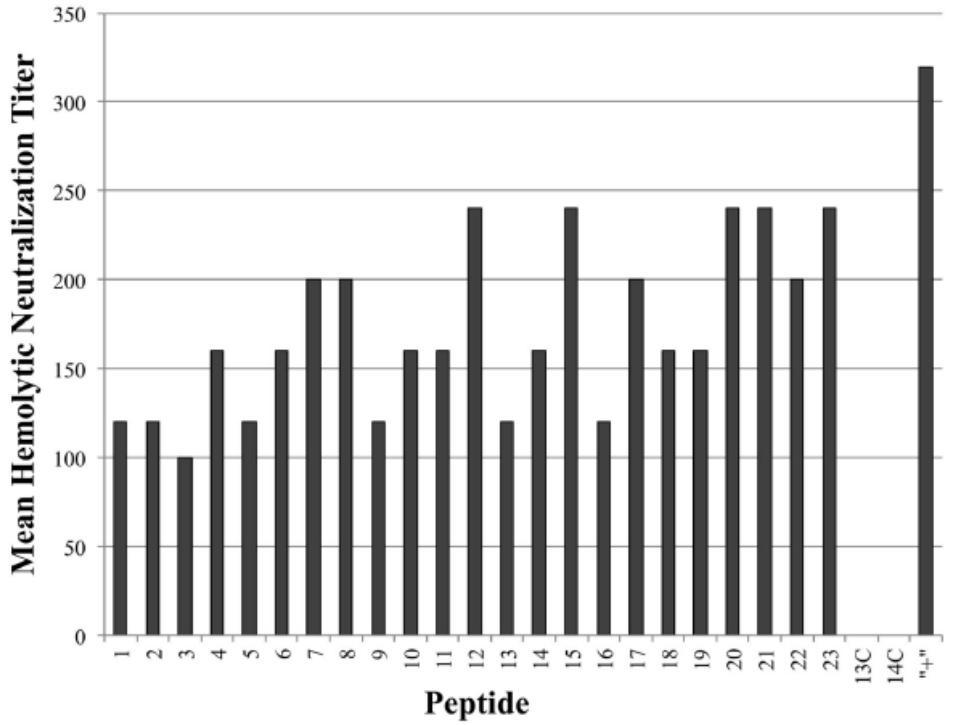

FIGURE 3 | Mean hemolytic neutralization titers. Hemolytic neutralization titers reported as the inverse of the highest serum dilution factor capable of completely neutralizing the hemolytic activity of *Clostridium perfringens* alpha-toxin. Hyperimmune serum against alpha-toxin obtained from USDA APHIS was used as positive control serum and the corresponding group was labeled as "+" on chart.

frontiers in Veterinary Science

ORIGINAL RESEARCH
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A Fast and Inexpensive Protocol for Empirical Verification of Neutralizing Epitopes in Microbial Toxins and Enzymes

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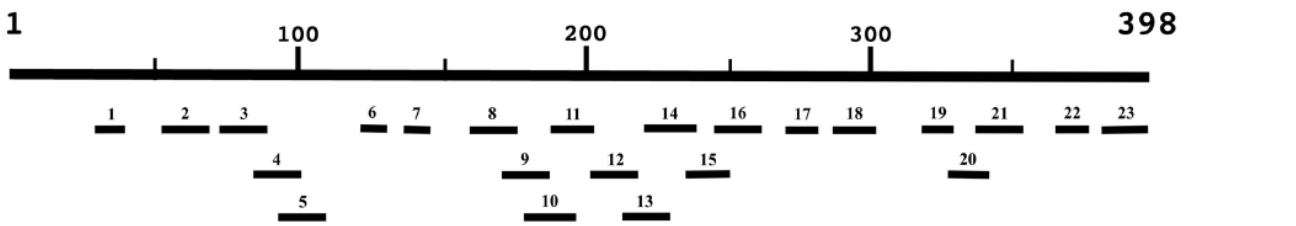
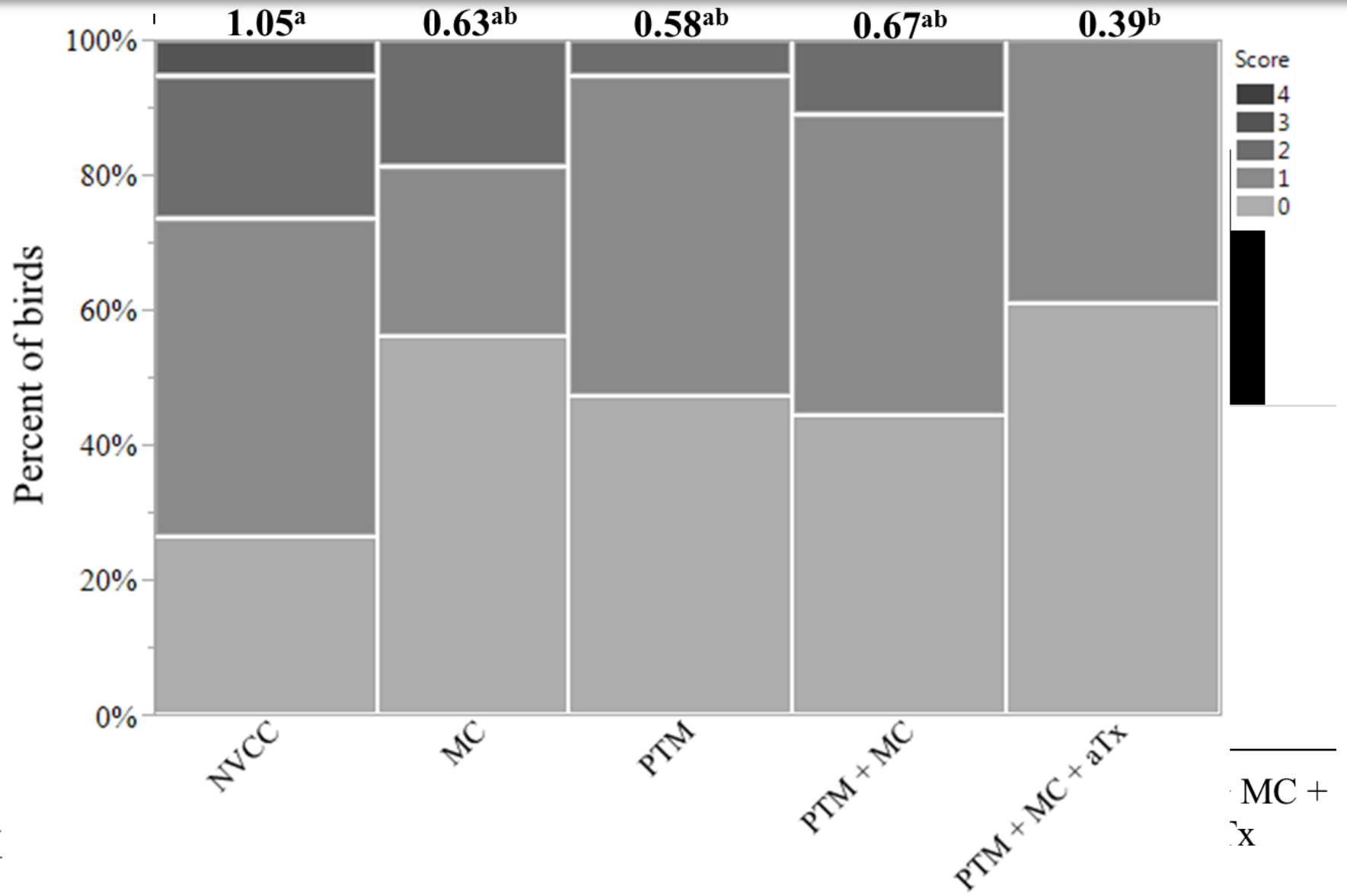


FIGURE 1 | Schematic of 23 peptides generated based on the *Clostridium perfringens* alpha-toxin amino acid sequence (CAA35186.1). Linear peptides were selected based on the ease of synthesis using Immune Epitope Database and Analysis Resource publically available B-cell epitope prediction algorithms (figure not to scale) (17).

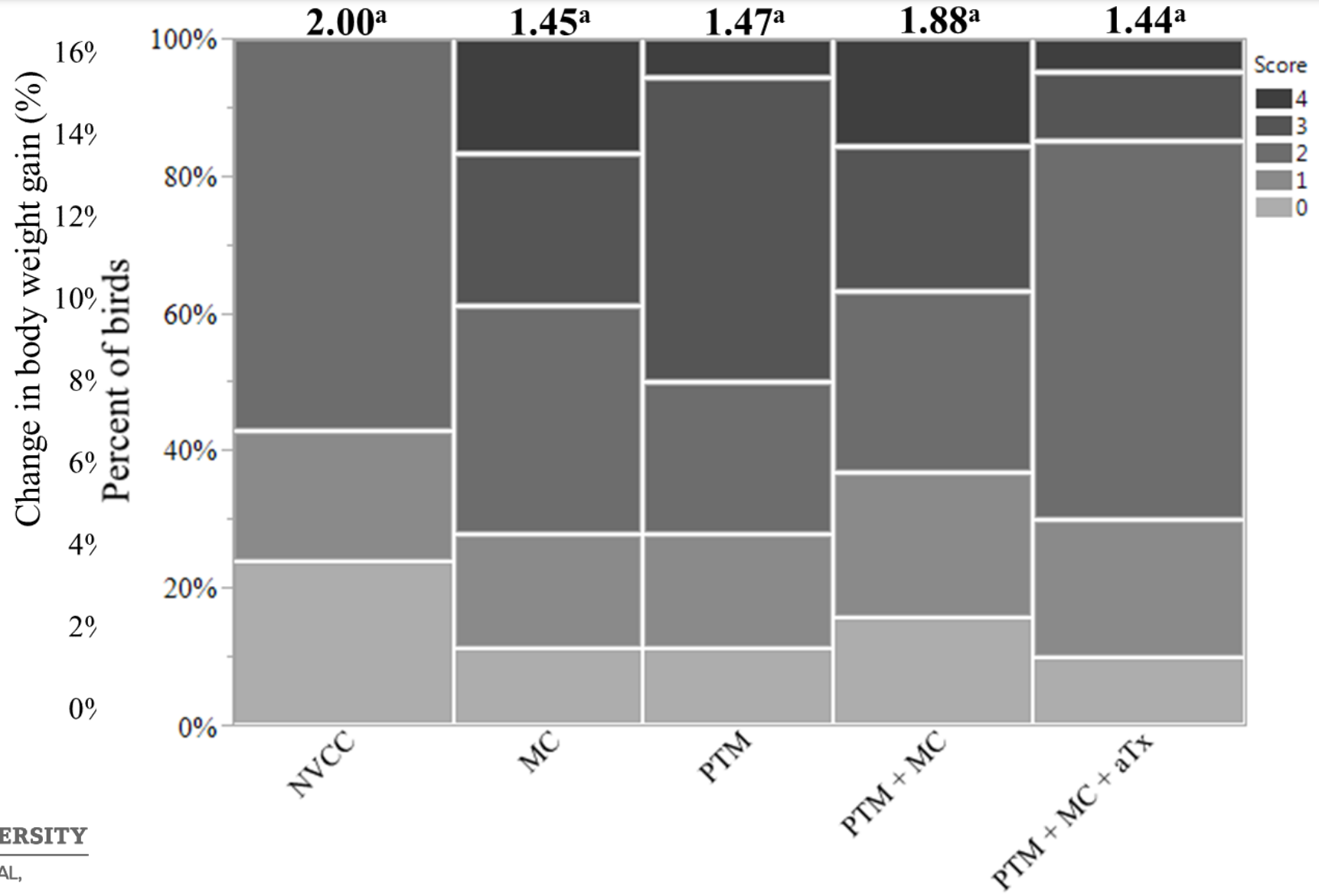
Proof of Concept NE Vaccine Experiments

Group name	Vaccination
NVCC	Non-vaccinated Challenge Control
MC	Mucinase antigens
PTM	*Pichia*-vectored *Eimeria* antigens
PTM + MC	*Eimeria* + mucinase antigens
PTM + MC + aTx	*Eimeria* + mucinase + alpha-toxin antigens

Proof of Concept: Experiment 2



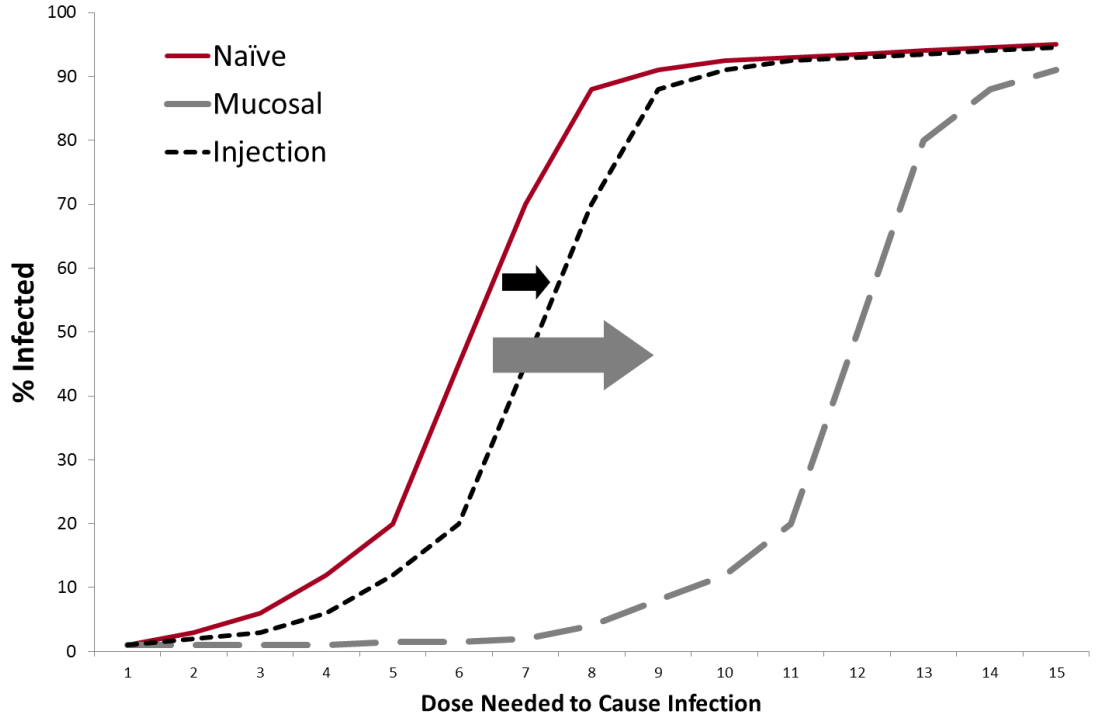
Proof of Concept: Experiment 3



Summary & Conclusions

- Antibodies against CBM of mucinases slowed *in vitro* growth of *Clostridium perfringens*
- Combination of mucinase + *Eimeria* + α toxin antigens provided protection against subclinical disease

- Next Step: Generate mucosal IgA with vaccination
  - Combined *Pichia* subunit vaccine



Thank You

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