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Research Note: *Clostridium perfringens* NetB and CnaA neutralizing nanobodies in feed reduce the incidence of poultry necrotic enteritis

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ABSTRACT Necrotic enteritis is a devastating disease to poultry caused by the bacterium *Clostridium perfringens*. As a novel approach to combating poultry necrotic enteritis, we identified and characterized several hundred single domain antibody fragments (or nanobodies) capable of binding either the NetB toxin or the collagen-binding adhesin (CnaA) of *C. perfringens*. Many of the nanobodies could neutralize the in vitro functions of NetB or CnaA with inhibitory concentrations in the nanomolar range. The nanobodies were also screened for proteolytic stability in an extract derived from gastrointestinal tract fluids of chickens. A collection of 6 nanobodies (4 targeting NetB and 2 targeting CnaA) with high neutralizing activity and high gastrointestinal tract extract stability were expressed and secreted by *Pichia pastoris* or *Bacillus subtilis*. Chickens were given a feed with 1 of the 2 nanobody-containing groups: 1) nanobody-containing *P. pastoris* supernatants that were semi-purified, lyophilized, and enterically coated, or 2) *B. subtilis* spores from strains containing the nanobody genes. Compared to untreated chickens (23.75% mortality), mortality of chickens receiving feed modified with the *P. pastoris* and *B. subtilis* products decreased to 11.25 and 7.5%, respectively. These results offer a new opportunity to improve the control of poultry necrotic enteritis by incorporating highly specific nanobodies or bacteria expressing these nanobodies directly into chicken feed.

Key words: *Clostridium perfringens*, necrotic enteritis, nanobody, feed

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INTRODUCTION

Poultry necrotic enteritis (**NE**) is a devastating gastrointestinal disease in commercial poultry production. The disease is estimated to cause a yearly global loss to producers of $6 billion USD and takes both a clinical form characterized by depression, diarrhea, anorexia, lesions in the small intestine, and increased mortality, and a subclinical form characterized by damage to the mucosa of the small intestine and poor performance, but without increased mortality (Emami and Dalloul, 2021). The disease often requires a predisposing factor, such as *Eimeria* infection or dietary changes, that disrupts the physical properties of the gastrointestinal tract and allows for the proliferation of *Clostridium perfringens* strains that possess the NetB toxin (Moore, 2016).

Many products to reduce NE are available or in development (Abd El-Hack et al., 2022). These include vaccines, products to improve overall gut health (such as prebiotics, probiotics, essential oils, and phytochemicals), products that improve feed quality, products to control coccidiosis, and products to reduce immunosuppressive diseases. However, despite these options, NE continues to cause tremendous losses to poultry producers and requires the development of new interventions. In the current study a new technology targeting 2 of the virulence factors of *C. perfringens* was utilized. Camelids, such as llamas, produce an unusual type of antibody consisting of only 2 heavy chains and the antigen-binding V_HH domain of these antibodies can be recombinantly expressed as single-domain antibodies or nanobodies (Muyldermans, 2013). These nanobodies are the smallest antibody fragment that retains the entire antigen binding site of the original antibody from which they are derived, and they are being developed for a wide range of applications. In this study, neutralizing nanobodies to 2 *C. perfringens* virulence factors that play key roles in necrotic enteritis: the pore-forming toxin NetB and the collagen-binding adhesin CnaA

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(Keyburn et al., 2008; Wade et al., 2016; Lacey et al., 2018) were developed.

MATERIALS AND METHODS

Antigen and Nanobody Expression and Purification

NetB (GenBank Accession #ABW71134.1, amino acid residues S31-L322) was expressed as an N-terminal fusion with His_6-thioredoxin (**TRX**), cleavable with Tobacco Etch Virus protease (**TEVp)** in *Escherichia coli* Rosetta-gami 2 grown overnight in autoinducing media, 100 μg/mL ampicillin at 30°C. The CnaA collagen binding domain (GenBank Accession #ALJ54440.1, amino acid residues G35-K336) was expressed as an N-terminal fusion with His_6-maltose binding protein (**MBP**), cleavable with TEVp in *E. coli* BL21 grown overnight in LB broth, 1 mM IPTG, 25 μg/mL kanamycin at 30°C. After cell lysis via sonication, the soluble fraction was passed over a HisTrap HP column (Cytiva) and the bound protein was eluted with an imidazole gradient (0−300 mM). After TEVp cleavage, the proteins were passed over a second HisTrap HP column and were collected in the flowthrough. The proteins were further purified with ion exchange and size exclusion chromatography using HiTrapQ HP (Cytiva) and Superdex 200 Increase 10/300 GL (Cytiva) columns, respectively, concentrated to ∼10 mg/mL in a final buffer of 20 mM HEPES, 150 mM NaCl, pH 7.4, aliquoted, flash frozen, and stored at −80°C. Uncleaved CnaA was also produced for use in the functional assay described below.

Nanobodies were expressed and purified using the same process described for NetB above up to the second HisTrap HP column. They were further purified using cation (HiTrap SP HP, Cytiva) or anion (HiTrapQ SP, Cytiva) exchange chromatography depending on the pI, concentrated to ∼10 mg/mL in a final buffer of 20 mM HEPES, 150 mM NaCl, pH 7.4, aliquoted, flash frozen, and stored at −80°C.

Llama Immunization and Nanobody Discovery

Typical llama immunization and phage-based nanobody discovery protocols were used throughout these studies (Baral et al., 2013). Briefly, llamas were injected intramuscularly with 100 μg of each antigen on days 1, 21, 42, and 63. On days 28, 49, and 70, lymphocytes were collected, and RNA was isolated from the lymphocytes. RNA was converted to cDNA, and nanobody genes were PCR-amplified and used to create M13 phage libraries, with each phage particle in the library carrying a single nanobody DNA sequence internally and expressing the nanobody fused to the phage gIII tip protein. Phage libraries were enriched for antigen-specific binders through 3 rounds of biopanning. Finally, binding of individual monoclonal phages was quantified using an anti-M13 antibody in an ELISA assay. Nanobody genes from phage with 4-fold greater binding on antigen-coated wells compared to PBS-coated wells were sequenced and those with unique complementarity determining regions were selected for in vitro characterization.

NetB Cytotoxicity Assay

Leghorn male hepatoma (**LMH**) cells (ATCC CRL-2117) were adhered to the surface of tissue-culture treated and gelatin-coated 96-well microtitre plates at 64,000 cells/well in Waymouth's media + 10% FBS overnight at 37°C and 5% CO_2. 50 nM NetB was preincubated with 10 to 40,000 nM nanobodies or buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) for 15 min at 37°C and 5% CO_2. Eleven μL of NetB/nanobody mixtures was applied to LMH cells in 100 μL of media and incubated for 5 h at 37°C and 5% CO_2. Cytotoxicity induced by NetB was measured using the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Mississauga, ON) following the manufacturer's instructions. NetB cytotoxicity in the presence of a nanobody was determined relative to NetB cytotoxicity in the presence of buffer only.

CnaA Collagen Binding Assay

Collagen type IV (Sigma -Aldrich, C7521) was dissolved in PBS at 20 μg/mL and coated on to wells of 96-well microtitre plates overnight at 4°C. The plates were washed with 200 μl of PBS and then blocked with 200 μL of 5% skim milk in PBS for 2 h at 37°C. MBP-CnaA (100 nM) was preincubated with nanobodies (12.5 −1600 nM) or buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) for 30 min at 37°C. The plates were washed 3x with 200 μL of PBS, and 100 μl of MBP-CnaA/nanobody mixtures were added to wells and incubated for 2 h at 37°C. The plates were washed 3x with 200 μL of PBS and incubated with 100 μL of 0.125 μg/mL of anti-His antibody-HRP conjugate for 1 h at room temperature. The plates were washed 3 x with 200 μL of PBS and 100 μL of TMB substrate was added and developed for 30 min. 50 μL of 1 M HCl was added to each well to stop the reaction and A_{450} was measured. CnaA binding to collagen in the presence of a nanobody was determined relative to CnaA binding to collagen in the presence of buffer only.

Gastrointestinal Tract Extract Stability Assay

A chicken jejunal extract was produced as follows: The contents of the jejunum were collected in a 50 mL conical tube, an equal volume of water was added, and the mixture was homogenized with a dounce homogenizer. The homogenized mixture was centrifuged for 10 min at 3,700 RCF and the supernatant was collected. The supernatant was centrifuged for 10 min at 23,000 RCF and the supernatant was collected, aliquoted, flash frozen, and stored at −20°C.

Reactions were set up in volumes of 8 μL on ice. Each tube consists of 2.4 μL chicken jejunal extract, 5 ug nanobody in 0.8 μL PBS, and 4.8 μL of 150 mM NaCl. Control reactions without jejunal extract were also set up containing 5 μg nanobody in 3.2 μL PBS and 4.8 μL of 150 mM NaC1. The tubes were incubated on ice for 5 min, followed by 42°C for up to 24 h. After incubation, 8 μL of preheated 2X SDS sample buffer was added to stop the reaction and nanobody stability was assessed using SDS-PAGE.

Production of P. pastoris *Supernatants Containing Nanobodies*

Individual nanobody genes were codon optimized for *Pichia pastoris* and nanobody proteins were secreted by *P. pastoris* strain BG11 with C-terminal 6X-histidine tags under the control of the methanol-inducible AOX promoter. Transformed cells were plated on yeast extract peptone dextrose (**YPD**) agar with 500 μg/mL zeocin and incubated for 2 d at 30°C. Twenty colonies for each nanobody were screened for nanobody secretion. The *P. pastoris* clone with the highest expression was chosen for fed batch fermentation. After fermentation, yeast biomass was removed using centrifugation and nanobodies were enriched from supernatants using 3 rounds of ultrafiltration with a 2 kDa molecular weight cut off. Supernatants enriched for nanobodies that did not pass through the filter were frozen and lyophilized.

Production of B. subtilis *Spores Expressing Nanobodies*

Individual nanobody genes were codon optimized for *Bacillus subtilis* and nanobody proteins were secreted by *B. subtilis* strain PY79 with C-terminal 6X-histidine tags under the control of the constitutive P_{veg} promoter (Lam et al., 1998). Based on small-scale expression tests, the clone with highest expression was selected for spore production. Cells were grown in 1 L of Difco sporulation medium (**DSM**). The DSM was produced in house with the following ingredients: 5 g/L peptone (Bioshop, PEP403), 3 g/L beef extract (Bioshop, BEF222), 1 g/L KCl (Bio Basic Canada Inc., 7447-40-7), 5 g/L D-glucose (Bioshop, GLU501), 1 mM $MgSO_4$ (Sigma, 7487-88-9), 10 μM $MnCl_2$ (Sigma, 13446-34-9), 0.5 mM $CaCl_2$ (Bioshop, CCL555), and 1 mM $FeSO_4$ (Sigma, 7782-63-0). After 72 h of growth spores were harvested by centrifugation. Pellets were air dried and ground into a powder. Spores/g were quantified by colony counting after heating samples (80°C for 20 min) to inactivate vegetative cells.

Experimental Necrotic Enteritis Infection of Chickens

Five hundred and sixty Ross 708 male broiler chicks (day-of-hatch; day 0) were assigned to 7 treatment groups with eight cages per treatment and ten chicks per cage (Aviagen Group, Huntsville, AL). Treatment groups were assigned to cages using a randomized complete block design (Cochran and Cox, 1992). The study began when chicks were placed in experimental cages. Only healthy birds were selected. Chicks were raised in Petersime-style battery cages (stocking density of 0.63 square feet per bird) in a solid-sided barn and maintained under ambient humidity. Feed and water were available ad libitum throughout the trial. Each cage contained 1 trough feeder and 1 trough drinker (10 bird to feeder/ drinker ratio, 24-inch x 3.5-inch trough). All birds were weighed by cage on days 0, 14, 22, and 28. The necrotic enteritis challenge model consisted of approximately 2,500 *Eimeria maxima* strain MX8 oocysts (kindly provided by Dr. Lorraine Fuller, University of Georgia) per bird gavaged on day 14 as the predisposing factor and *C. perfringens* strain CP4 (Thompson et al., 2006) gavaged on days 19 and 20 in 1.0 mL at concentrations of 3.0×10^8 and 7.0×10^8 CFU/mL, respectively.

B. subtilis spores were fed to chicks beginning at day 0 at a dose of 6×10^9 CFU/kg feed. The fed spores were an equal mixture of 6 *B. subtilis* clones, each expressing a different nanobody. Enterically coated yeast supernatants containing nanobodies were fed to chicks from day 18 to day 28 of the experiment at a dose of 5.3 g/kg feed. One control group received bacitracin methylene disalicylate (**BMD**) at a dose of 55 mg/kg feed an industry standard antibiotic treatment, from day 18 to day 28 (Zoetis Inc., Parsippany, NJ). The basal feed is shown in Table 1 and all treatments were applied to this basal feed as liquid applications. *B. subtilis* spores were added throughout the entire experiment since *B. subtilis* can colonize the GI tract of chickens (La Ragione and Woodward, 2003), so spores added prior to *C. perfringens* challenge may be beneficial. Conversely, nanobodies added directly to feed are expected to traverse through the GI tract, so there is limited value feeding them to chickens significantly in advance of the *C. perfringens* challenge.

NE mortality in birds that died during the trial was assessed by gross lesions during necropsy. NE mortality values reported are derived from a percent mortality for each cage containing ten chicks. NE lesion scores were determined on day 22 for 1 chicken per replicate to monitor progression of the disease using a scoring system of 0 = normal, 1 = mild, 2 = moderate, and 3 = severe (Hofacre et al., 1998). The trial was terminated on day 28. The necrotic enteritis challenge was conducted as per SPRG's institutional animal care and use committee approved protocol #NBNE2072022-97.

The statistical model based on our experimental design is $Y_{ij} = \mu + B_i + T_j + BT_{ij} + \varepsilon_{ij,}$ where Y represents the response parameter, B_i represents the block effect, T_j represents the treatment effect, BT_{ij} represents the interaction between the block and treatment effects, and ε_{ij} represents the experimental error. Statistical analysis was conducted with the STATISTIX for Windows software (Stata version 15.1, StataCorp LLC, College Station, TX). Groups were compared by ANOVA

Table 1. Basal experimental diet formulation and nutrient specifications.

Ingredient	Percent
Corn	56.659
Soybean Meal	35.231
Vegetable Oil	3.620
Dicalcium Phosphate	1.513
Calcium Carbonate	0.974
Filler[1]	0.547
DL - Methionine	0.333
Salt (NaCl)	0.330
L - Lysine	0.263
Sodium Bicarbonate	0.149
L - Threonine	0.113
Trace Mineral Premix[2]	0.100
Choline Chloride (60%)	0.068
Vitamin Premix[3]	0.050
L - Valine	0.027
Vitamin E Supplement	0.013
Phytase (5000 ftu/g)[4]	0.010
Calculated Nutrient Analysis:	
Dry Matter (%)	88.36
Crude Protein (%)	21.81
Calcium (%)	0.96
Available Phosphorus (%)	0.48
Metabolizable Energy (kcal/kg)	3055
Digestible Lysine (%)	1.2

[1]Products were added at the expense of sand (filler).
[2]Trace Mineral premix per pound of finished feed (0.100%): Manganese (Mn), 120.0 ppm; Zinc (Zn), 110.0 ppm; Calcium (Ca), 55 ppm, Iron (Fe), 30.0 ppm; Copper (Cu), 16.0 ppm; Iodine, 1.5 ppm; Selenium, 0.3 ppm. (Southeastern Minerals, Bainbridge, Georgia).
[3]Vitamin premix per pound of finished feed (0.05%): Vitamin A, 3750 IU; Vitamin D3, 2000 IU; Vitamin E, 16 IU; vitamin B_{12} (cobalamin), 10 μg; Biotin, 0.08 mg; Menadione, 1.25 mg; Thiamine, 1.0 mg; Riboflavin, 3.5 mg; d-Pantothenic Acid, 6.0 mg; Vitamin B6, 1.5 mg; Niacin, 27.5 mg; Folic Acid, 0.5 mg. (DSM Nutritional Products, Parsippany, NJ).
[4]Quantum Blue 5G (AB Vista, Marlborough, UK).

with a comparison of means using the Tukey-Kramer test and were considered statistically different at $P < 0.05$.

RESULTS AND DISCUSSION

Selection of Lead Nanobodies

A total of 6 llamas were inoculated with NetB and CnaA, resulting in the discovery of 192 and 217 unique nanobodies to the 2 antigens, respectively. More than 90% of these nanobodies were successfully purified from *E. coli* expression and screened in antigen-specific in vitro functional assays and stability assays.

All α-NetB nanobodies were screened for their ability to reduce the cytoxicity of NetB to tissue cultured LMH cells. Nearly 90% of the NetB-binding nanobodies tested were able to block NetB cytotoxicity with IC_{50} values below 1 μM. Figure 1A shows the results for 4 nanobodies (NBX-NB1, NBX-NB2, NBX-NB3, and NBX-NB4) that could reduce NetB cytotoxicity by >90% and had average IC_{50} values below 100 nM. The mean (and standard error of the mean) from 4 independent experiments for these nanobodies were: NBX-NB1, 33 ± 5.7 nM; NBX-NB2, 71 ± 10 nM; NBX-NB3, 8.4 ± 2.3 nM; NBX-NB4, 4.6 ± 0.3 nM. Neither the buffer in which the nanobodies are dissolved, nor a nanobody directed at another target reduced NetB toxicity.

All α-CnaA nanobodies were screened for their ability to reduce the binding of CnaA to collagen. Unlike the NetB-binding nanobodies, nearly all of which blocked NetB cytotoxicity, only about half of the CnaA nanobodies were able to block CnaA binding to collagen by at least 50% at the concentrations tested. Figure 1B shows the results for 2 nanobodies (NBX-CA1 and NBX-CA2) that could reduce binding of CnaA by >50% and had average IC_{50} values below 200 nM. The mean (and standard error of the mean) from 4 independent experiments for the 2 nanobodies were: NBX-CA1, 184 ± 20 nM; NBX-CA2, 169 ± 25 nM. Neither the buffer in which the nanobodies are dissolved, nor a nanobody directed at another target reduced CnaA binding to collagen.

All nanobodies were screened for proteolytic stability in a chicken jejunal extract. Figure 1C shows the results for the 6 nanobodies described above. All 6 were visible on SDS-PAGE for at least 1 h in the presence of the extract. Of the more than 400 α-NetB and α-CnaA nanobodies that were discovered and screened in this assay; the 6 nanobodies shown here are in the top 12.5% of the most stable proteolytically stable nanobodies in chicken gastrointestinal tract extracts.

Many other nanobodies had either high efficacy in the in vitro functional assays or high proteolytic stability. The 6 nanobodies described above had the best combination of in vitro function and stability, and thus were selected for use in in vivo studies as a collection.

Production of Materials for In Vivo Trial

The quality and quantity of nanobodies in the lyophilized yeast supernatant were determined before in vivo studies. To do this, a small amount of each powder was resuspended in buffer and the soluble fraction was used for characterization. Nanobody activity were maintained in the in vitro function assays described above for all 6 lead nanobodies (data not shown). Nanobody concentrations in the powders were estimated by comparing dissolved material to a standard curve of purified protein using SDS-PAGE analysis.

We sought to provide enough of each nanobody to the chickens such that each nanobody would reach an average concentration greater than the IC_{50} values determined above. Since the concentration of NetB used in the in vitro assay (5 nM) is similar to an estimated amount (Lee et al., 2021) in the gut digesta (2.2 nM), the in vitro IC_{50} provide a good starting point for in vivo studies using NetB-binding nanobodies. We are not aware of any such estimate of the in vivo CnaA concentration and the concentration used in our in vitro collagen binding assay (100 nM) may not be well-aligned with the in vivo concentration. After mixing materials for the 6 lead nanobodies, the total nanobody content in the lyophilized yeast supernatant was estimated to be ~11% by weight. The proportion of the 6 nanobodies

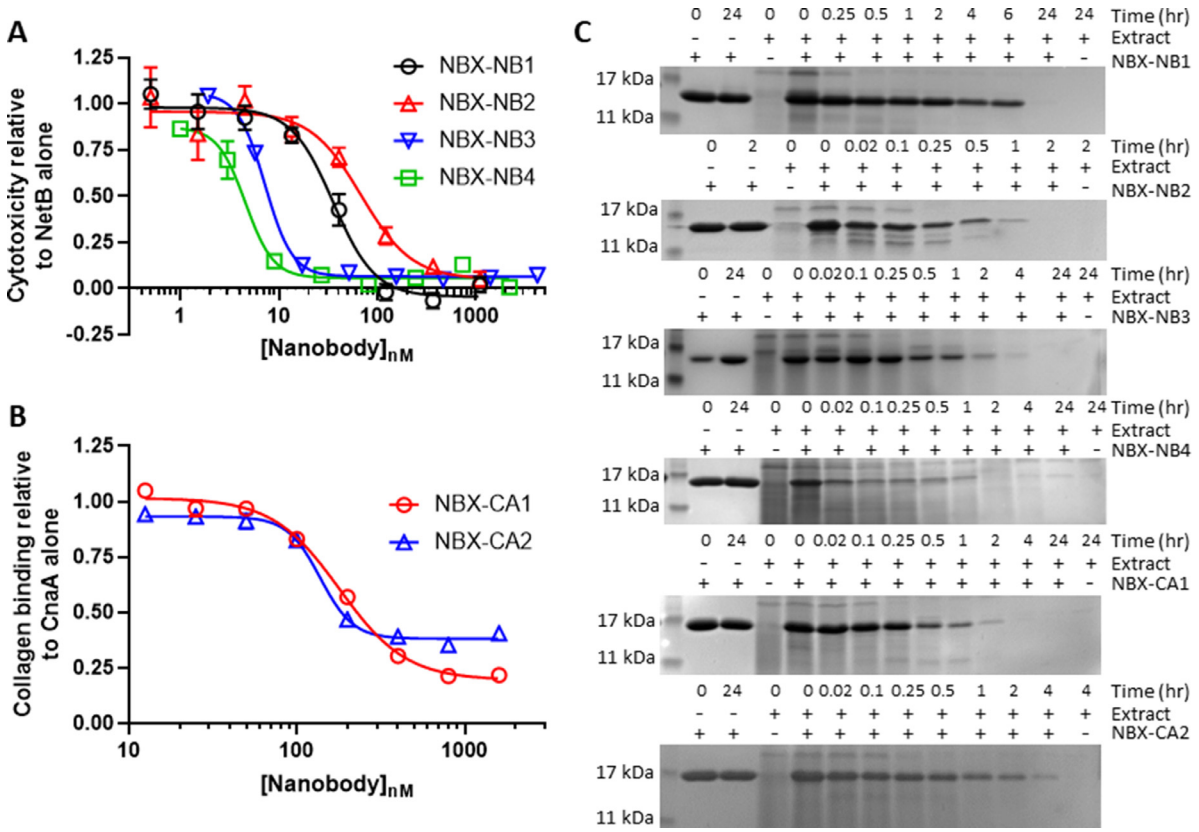

Figure 1. In vitro characterization of lead nanobodies. (A) Four α-NetB nanobodies (NBX-NB1, NBX-NB2, NBX-NB3, and NBX-NB4) block the cytotoxicity of NetB to tissue cultured LMH cells in a dose-dependent fashion. Data shown is from a representative experiment for each nanobody. Data points are the mean and standard error of the mean from triplicate wells. (B) Two α-CnaA nanobodies (NBX-CA1 and NBX-CA2) reduce the binding of CnaA to collagen in a dose-dependent fashion. Data shown is from a representative experiment for each nanobody. Data points are the mean and standard error of the mean from triplicate wells. (C) All 6 nanobodies survive for an hour or more in the presence of a chicken jejunal extract. For each nanobody, the SDS-PAGE gel shows the following from left to right: 11 and 17 kDa molecular weight markers; the nanobody, incubated at 42°C in the absence of extract at the start and endpoints of the experiment to show that any observed degradation is extract dependent; the extract without nanobody at the start of the experiment to aid in the identification of the nanobody on the gel; a time course of the nanobody incubated at 42°C in the presence of the extract; and the extract without nanobody at the end of the experiment to aid in the identification of the nanobody on the gel.

within the total nanobody content were: NBX-NB1 (0.7%), NBX-NB2 (7.4%), NBX-NB3 (1.1%), NBX-NB4 (2.3%), NBX-CA1 (19.3%), and NBX-CA2 (69%). The mixed lyophilized yeast supernatants were enterically coated using a lipid-based matrix (Jefo Nutrition) with a final inclusion rate of 18%. A similar, enterically coated mixture of nanobodies that target antigens from an unrelated bacterium, do not block the activity of NetB or CnaA, and are not expected to reduce necrotic enteritis was also produced with a final inclusion rate of 12%.

In Vivo Efficacy Trial

Clinical results of the in vivo efficacy trial are shown in Table 2. Unchallenged chicks (Group 1) showed $0\%^{b}$ necrotic enteritis mortality, while challenged/untreated chicks (Group 2) had $23.75\%^{a}$ mortality. Both the group receiving the yeast extract with active nanobodies (Group 4, $11.25\%^{a,b}$ mortality) and the group receiving *B. subtilis* secreting active nanobodies (Group 6, $7.5\%^{a,b}$ mortality) reduced mortality to levels equal to or lower than the BMD (antibiotic) control (Group 3, $11.25\%^{a,b}$ mortality). There were also decreases in mortality in groups receiving off-target nanobodies (Group 5, $17.5\%^{a,b}$ mortality) or *B. subtilis* without nanobodies (Group 7, $15\%^{a,b}$ mortality); however, the reductions were not as great as those groups that received active nanobodies targeting NetB and CnaA, indicating that the nanobodies play an active role in disease reduction. It is possible that the materials in the encapsulant and the *B. subtilis* bacterial cells themselves also contribute some overall benefit to gut health and account for a portion of the improvement seen with the nanobody-containing groups. NE lesion scores were determined for 1 chicken per replicate on day 22 to monitor disease progression in each group (Table 2). NE mortality was not a component of the scoring system, nor were any of the chickens that succumbed to NE included in the lesion scoring data. The lesion scoring data confirmed that NE lesions were detected in all the groups that were challenged and not in the unchallenged groups.

Performance parameters were also measured throughout the 28-day challenge and the results are shown in Table 3. Once adjusted for mortality, unchallenged chickens (Group 1) had a feed conversion ration (FCR) of 1.437^{b}, while the FCR of the challenged/untreated

Table 2. NE clinical data from battery cage efficacy trial.

Group #	NE challenge[1]	Experimental treatment	NE percent mortality[2]	NE lesion scores[2,3]
1	No	None	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
2	Yes	None	$23.75^{a} \pm 19.23$	$0.63^{a,b} \pm 0.74$
3	Yes	Bacitracin methylene disalicylate	$11.25^{a,b} \pm 11.26$	$0.63^{a,b} \pm 0.52$
4	Yes	Active nanobodies	$11.25^{a,b} \pm 17.27$	$1.12^{a} \pm 0.35$
5	Yes	Off-target nanobodies	$17.5^{a,b} \pm 16.69$	$0.75^{a,b} \pm 0.46$
6	Yes	*B. subtilis* secreting active nanobodies	$7.5^{a,b} \pm 7.07$	$0.50^{a,b} \pm 0.76$
7	Yes	*B. subtilis*	$15^{a,b} \pm 10.69$	$0.38^{a,b} \pm 0.52$
		ANOVA F Test Treatment P values	0.0299	0.0078

[1]Challenged chickens received *E. maxima* oocysts on day 14 and *C. perfringens* strain on days 19 and 20.
[2]Data shown are the means ± standard deviations. Means within a column lacking a common superscript differ ($P < 0.05$).
[3]NE lesions scores (0−3) were determined for 1 chicken per replicate on day 22.

Table 3. Performance data from battery cage efficacy trial.[1,2]

Group #	Feed intake (kg/cage)	Weight gain (kg)	Nonadjusted feed conversion ratio	Adjusted feed conversion ratio
1	$15.50^{a} \pm 1.25$	$1.12^{a} \pm 0.09$	$1.437^{b} \pm 0.066$	$1.437^{b} \pm 0.066$
2	$13.11^{a,b} \pm 1.53$	$1.01^{a} \pm 0.10$	$1.972^{a} \pm 0.408$	$1.607^{a} \pm 0.125$
3	$15.13^{a} \pm 2.37$	$1.09^{a} \pm 0.08$	$1.691^{a,b} \pm 0.076$	$1.565^{a,b} \pm 0.099$
4	$13.44^{a,b} \pm 1.83$	$0.99^{a} \pm 0.14$	$1.698^{a,b} \pm 0.313$	$1.540^{a,b} \pm 0.079$
5	$12.31^{b} \pm 1.49$	$0.96^{a} \pm 0.14$	$1.806^{a,b} \pm 0.377$	$1.567^{a,b} \pm 0.106$
6	$14.48^{a,b} \pm 1.21$	$1.09^{a} \pm 0.10$	$1.564^{b} \pm 0.101$	$1.485^{a,b} \pm 0.044$
7	$13.89^{a,b} \pm 2.07$	$1.04^{a} \pm 0.17$	$1.757^{a,b} \pm 0.207$	$1.585^{a} \pm 0.084$
ANOVA F Test Treatment *P* values	0.0062	0.0798	0.0058	0.0052

[1]Data shown are the means ± standard deviations. Means within a column lacking a common superscript differ ($P < 0.05$).
[2]Day 0 to Day 28 Performance Results

chickens (Group 2) rose to 1.607^{a}. The best FCR improvements compared to Group 2 were for the chickens fed the yeast extract with active nanobodies (Group 4, FCR of $1.54^{a,b}$) or *B. subtilis* secreting active nanobodies (Group 6, FCR of $1.485^{a,b}$) Both groups 4 and 6 outperform the FCR seen in the BMD control (Group 3, FCR of $1.565^{a,b}$). Like the observation above for necrotic enteritis mortality, the control treatments of off-target nanobodies (Group 5, FCR of $1.567^{a,b}$) and *B. subtilis* without nanobodies (Group 7, FCR of 1.585^{a}) also have some benefits to performance parameters compared to challenged/untreated chickens and may contribute to some of the benefit seen in the nanobody-containing groups. However, the data indicate that the active nanobodies make the most substantial improvements to feed conversion ratios.

To the best of our knowledge, this is the first study to indicate that nanobodies can reduce poultry necrotic enteritis when they are applied in poultry feed and when they are applied in a recombinantly expressed and semi-purified form. In contrast, a previous demonstration of the efficacy of nanobodies for poultry necrotic enteritis was restricted to a bacterial delivery system through in ovo and water administration (Gangaiah et al., 2022). Although different application methods may be useful under certain conditions, the ability to incorporate the material directly into poultry feed provides a very straightforward route of administration to farmers.

Despite several commercially available products for necrotic enteritis reduction, this disease remains a significant challenge to poultry producers and continues to lead to billions of dollars in losses around the world every year. The data presented here offer a new strategy for a highly specific and targeted approach that can be directly incorporated into poultry feed as a prophylactic against necrotic enteritis. The possibility also exists to utilize these molecules therapeutically as an alternative to or in conjunction with an antibiotic.

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DISCLOSURES

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