A New Model for Controlling Septicemia in Broiler Chickens Using Host Defense Peptides

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Table of Contents

List of Tables ii

List of Figures ii

A. Overview 1

B. Research Activities 2

1. Objective One: Screening of Host Defense Peptides for Efficacy 2

a. Use of *in vivo* screening 2
b. Bacterial killing assay 2

2. Objective Two: Establish that the HDPs ameliorate clinical signs of septicemia in chickens 3

a. Effect of Avian Cathelicidin 3 and Avian β Defensin 2 on septicemia caused by *E. coli* 3
b. Effect of a cocktail of six HDP on septicemia caused by *E. coli* 4
c. Effect of a cocktail of six HDP supplemented with Gamma-aminobutyric acid on septicemia caused by *E. coli* 6
d. Effect of a cocktail of six HDP given by in ovo injection on septicemia caused by *E. coli* 9

C. Conclusions 9

D. Communication of Research Results 10

E. Financial Report 10

List of Tables

Page

Table 1. Effect of Host Defense Peptides on Growth of *E. coli in vitro* 2

List of Figures

Page

Figure 1. Effect of Avian Cathelicidin 3 and Avian β Defensin 2 on septicemia (Trial A) 4

Figure 2. Effect of a cocktail of Host Defense Peptides on septicemia when birds are challenged 24 hours after treatment (Trial B) 5

Figure 3. Effect of a cocktail of Host Defense Peptides on Septicemia when birds are challenged 72 hours after treatment (Trial C) 5

Figure 4. The Effect of GABA and HDP on septicemia (Trial D) 6

Figure 5. The Effect of GABA and HDP on septicemia when challenged 24 hours after treatment (Trial E) 7

Figure 6 A. The Effect of GABA and HDP on septicemia (Trial F) with a high dose challenge of *E. coli* 8

Figure 6 B. The Effect of GABA and HDP on septicemia (Trial F) with a low dose challenge of *E. coli* 8

Figure 7. The effect of HDP delivered in ovo on septicemia produced by E. coli 9

A. Overview

Colisepticemia is a common infectious disease found in poultry which represents a substantial economic burden to the Saskatchewan and Canadian poultry industries. Septicemia is defined as acute invasion of the systemic circulation by pathogenic bacteria accompanied by signs of sepsis or septic shock. Septicemia caused by *E. coli* is a significant problem in young birds, especially under 2 weeks of age. *E. coli* infections have a significant economic impact due to the initial mortality. In addition, infected birds that survive often have significantly lower body weights compared with uninfected birds.

Host Defense Peptides (HDPs) are natural compounds with inherent antimicrobial activity which can also stimulate the host innate immune system, promote wound-healing, and are efficacious as vaccine adjuvants. Studies have begun to elucidate the distinct functions of HDPs (such as their ability to stimulate innate immunity) in mammalian systems which are separate from their antimicrobial activity. Although HDPs have been used in laboratory animals as inducers of innate immunity and as vaccine adjuvants, their activity in chickens has not been well-established. The objective of this study is to determine if HDPs can ameliorate septicemia in a young chick model.

We have carried out *in vitro* studies to determine the killing activity of a group of Host Defense Peptides (HDPs) against *E. coli*. We observed that a cocktail of 6 HDPs was most effective in killing *E. coli in vitro.* This cocktail included Bovine Myeloid Antimicrobial peptide Indolicidin, Protegrin-1, Porcine β defensin 1, Avian β defensin 2, and Avian Cathelicidin 3.

We have tested the effect of HDPs *in vivo* on septicemia caused by *E. coli.* We observed that a combination of two avian HDPs (Avian β defensin 2 and Avian Cathelicidin 3) were not effective in the prevention of septicemia and in high dose, appeared to increase the severity of the disease. Based on this observation we reduced the amount of each HDP used in subsequent experiments. We have also observed that the cocktail of 6 HDPs were not effective in the prevention of septicemia but were not toxic.

We have tested the combined effect of HDPs and gamma-aminobutyric acid (GABA) on septicemia. We observed that GABA alone is not effective in the prevention of septicemia. It also appears that the combination of HDPs and GABA delivered by subcutaneous injection or by in ovo injection, were not effective in the reduction of septicemia caused by *E. coli* in young birds.

B. Research Activities

1. Objective One: Screening of Host Defense Peptides for Efficacy

a. Use of *in vivo* screening

An *in vivo* screening screening method has been developed at VIDO using mice. Briefly mice are given an intraperitoneal injection of the host defense peptide and challenged 18 hours later with a intraperitoneal injection of the test bacteria. The peritoneal cavity is flushed with buffer 24 hours after the challenge and the bacteria cultured to quantify the number of bacteria surviving. We attempted to reproduce this model in two- day- old chicks. However we were unsuccessful as the level of bacteria recovered from the peritoneum varied widely in the untreated control birds. This variation was so great that we were unable to see an effect of the host defense peptides used. We therefore had to use a more conventional *in vitro* screening method.

c. Bacterial killing assay

In this assay we incubated E. coli with the individual host defense peptide or a mixture of peptides for 24 hours at 37^{o}C. The mixture was then plated on MacConkey agar and incubated for a further 24 hours. Decreased growth of the bacteria indicated that the HDP was active.

Table 1. Effect of Host Defense Peptides on Growth of *E. coli in vitro*

	Growth of Bacteria After Exposure to Host Defense Peptides								
Group	HDP tested	Concentration of HDP (micrograms per mL)							
		100	50	25	12.5	10	1.0	0.1	0
1	All HDP*	NG**	NG	NG	NG	NG	NG	NG	G
2	HDP minus Avian β defensin 2 and Avian Cathelicidin 3	NG	NG	NG	NG	G	G	G	G
3	HDP minus Bovine Myeloid Antimicrobial peptide and Protegrin-1	NG	G	G	G	G	G	G	G
4	HDP minus Indolicidin and Porcine β defensin 1	NG	NG	NG	NG	NG	NG	G	G
5	HDP minus Bovine Myeloid Antimicrobial peptide	NG	NG	NG	G	G	G	G	G
6	HDP minus Protegrin-1	NG	NG	G	G	G	G	G	G
7	Bovine Myeloid Antimicrobial Peptide alone	NG	NG	G	G	G	G	G	G
8	Protegrin-1 alone	G	G	G	G	G	G	G	G

*the cocktail of HDP included Bovine Myeloid Antimicrobial peptide, Indolicidin , Protegrin-1, Porcine β defensin 1, Avian β defensin 2, Avian Cathelicidin 3

**G: Growth NG: no growth

The cocktail of the 6 HDP inhibited the growth of the bacteria at the lowest concentration used.

It was interesting to note that protegrin-1 alone did not inhibit growth of the bacteria (Group 8) but appeared to contribute to the activity of the cocktail of 6 peptides (Group 1 compared to Group 6). It also appeared that the removal of Avian Cathelicidin 3 and Avian β Defensin 2 from the cocktail of peptides had a detrimental effect (Group 2 compared to Group 1) on the ability to kill bacteria.

2. Objective Two: Establish that the HDPs ameliorate clinical signs of septicemia in chickens

The *E. coli* infection model has been has been well-established in-house and will be an excellent means to evaluate efficacy of HDPs against septicemia. The HDP were given by subcutaneous injection into the right side of the abdomen of the bird. A field isolate of *E. coli* serogroup O2 (a virulent strain used extensively in-house for avian studies) was used as the challenge strain. The *E .coli* challenge is given as a subcutaneous injection into left side of the abdomen. Chicks were tagged to allow for identification were randomly allocated into animal isolation rooms.

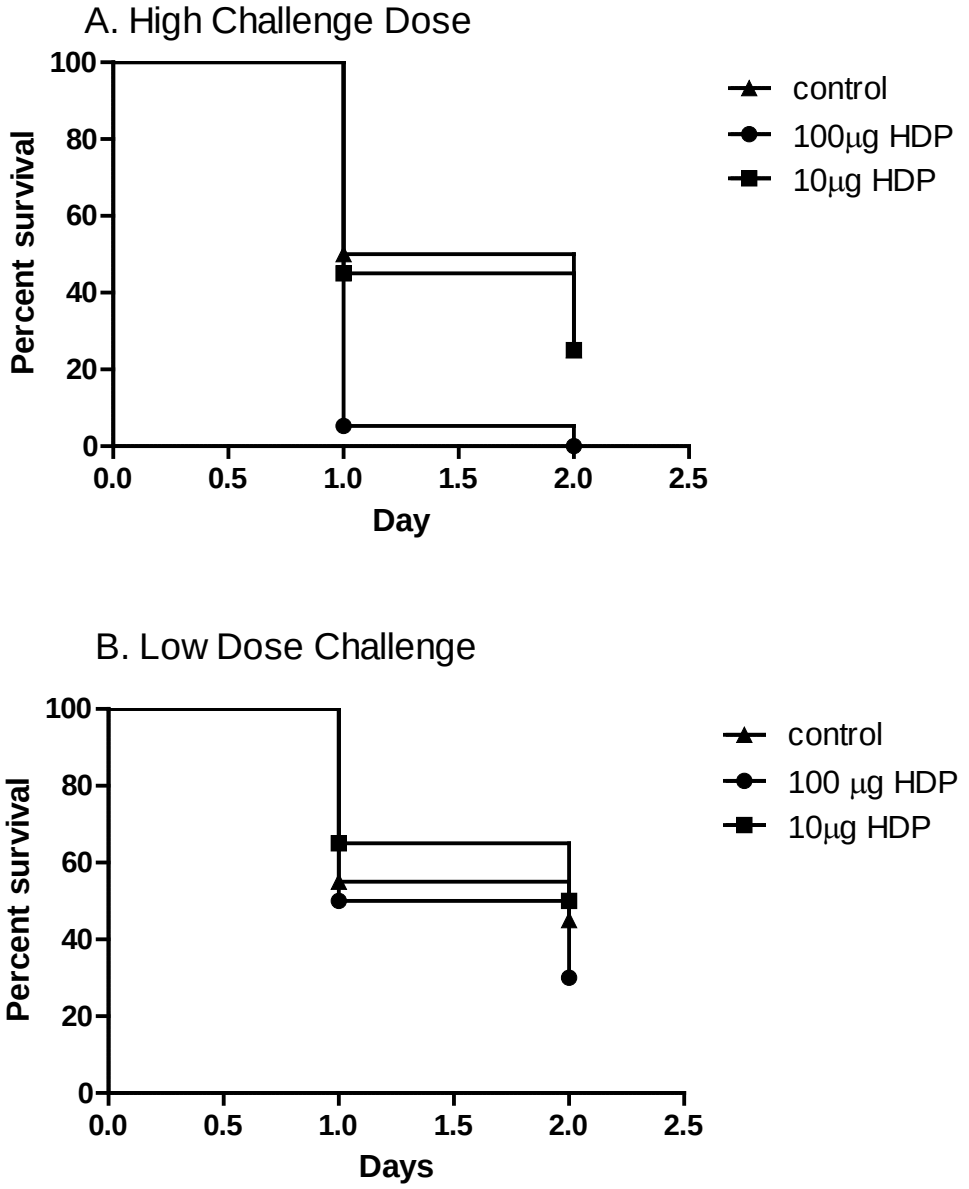
a. Effect of Avian Cathelicidin 3 and Avian β Defensin 2 on septicemia caused by *E. coli*

Preliminary research has shown that Avian Cathelicidin 3 and Avian β Defensin 2 have the ability to reduce clinical signs of a localized *E. coli* infection (cellulitis).

In this experiment (Trial A) we tested the effect of a combined administration of these two HDPs to determine if they were effective in ameliorating a systemic *E. coli* infection. Birds were treated with either 100µg or 10 of µg of each peptide at day of age. After 24 hours the birds were challenged with either 5 $X10^5$ (A) or $5X10^4$ (B) colony forming units of bacteria .

In this experiment the bacterial challenge was very severe resulting in 70 to 100% mortality in two days. However in both challenge doses there was more mortality in the groups of birds that received the 100 µg of HDP than the control group. We concluded that a dose 100 µg of HDP was detrimental. The 10µg dose appeared to have no effect.

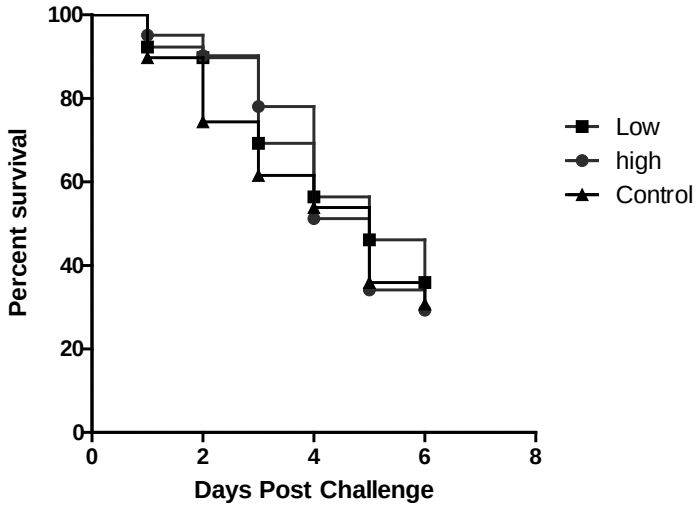
Figure 1. Effect of Avian Cathelicidin 3 and Avian β Defensin 2 on septicemia (Trial A)



b. Effect of a cocktail of six HDP on septicemia caused by *E. coli*

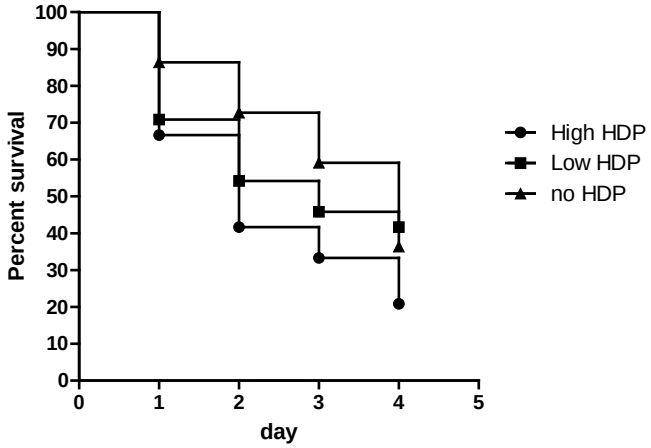
Based on our *in vitro* studies we decided to test a cocktail of six HDPs including Bovine Myeloid Antimicrobial peptide, Indolicidin , Protegrin-1 , Porcine β defensin 1, Avian β defensin 2 and Avian Cathelicidin 3 (Trial B). In this case the dose used was 10μg (high) or 1μg (low) of each peptide and the peptides were given at one day of age. The birds were challenged on day two (one day after the peptides were given) with 1.5 $X10^4$ cfu of bacteria. No difference was observed between the treatment and control groups.

Figure 2. Effect of a cocktail of Host Defense Peptides on septicemia when birds are challenged 24 hours after treatment (Trial B)



Since no effect of the HDPs was observed, the previous experiment was repeated but the challenge was not given until 72 hours after treatment with the HDPs to determine if this was a more effective interval between treatment and challenge (Trial C, Figure 3). Once again the HDPs appeared not to be effective in the reduction in the disease observed.

Figure 3. Effect of a cocktail of Host Defense Peptides on septicemia when birds are challenged 72 hours after treatment (Trial C)

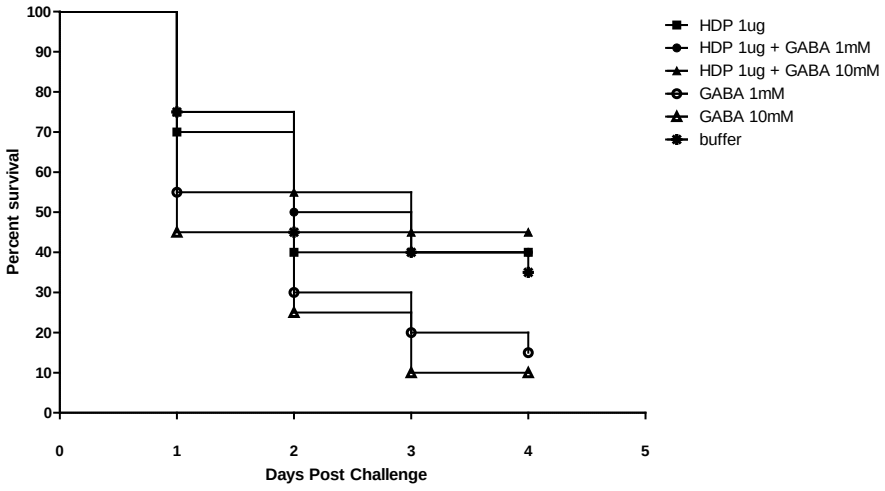


c. Effect of a cocktail of six HDP supplemented with Gamma-aminobutyric acid on septicemia caused by *E. coli*

Gamma-aminobutyric acid (GABA) is a non-protein amino acid. It is well known for its role as an inhibitory neurotransmitter of developing and operating nervous systems. It has also been shown to be involved in the healing process of cutaneous wounds and in the suppression of inflammation. Preliminary data produced by other researchers at VIDO suggested that GABA ,when combined with HDPs , might also be effective in prevention of septicemia in 4 week old birds. Based on this information we decided to test its activity in young chicks.

In our initial experiment we used either 1 or 10 mmol of GABA, alone or combined with 1µg concentrations of the six HDPs (Trial D). In this experiment the GABA and HDP were combined before injection. The birds received the treatment at one day of age and were challenged 72 hours later. It was clear that GABA alone, at these levels, had a negative effect on the birds as more animals in these groups died than in the negative control. The groups of birds that received both GABA and HDPs appeared to be the same as the groups that received either HDPs or buffer. It appears that the HDPs may have inactivated the GABA (Figure 4)

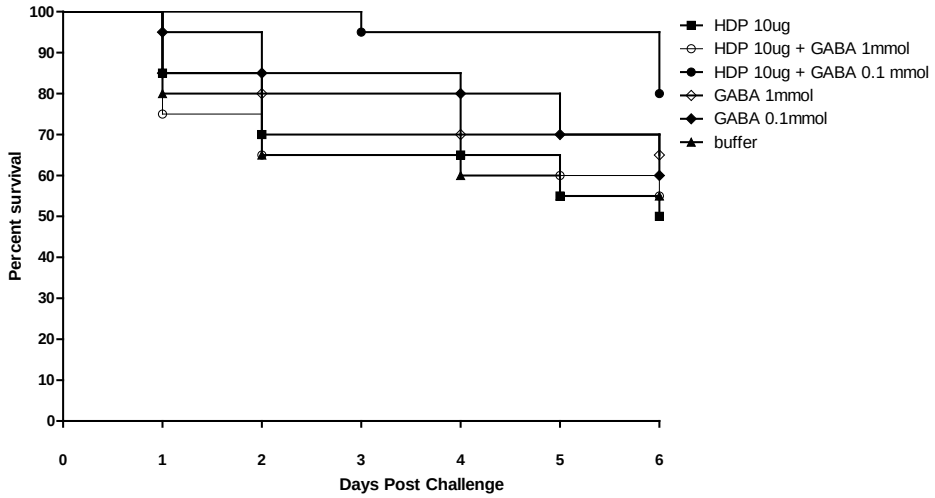
Figure 4. The Effect of GABA and HDP on septicemia (Trial D)



Based on the results of the first animal experiment with GABA we modified the experimental design for the subsequent animal trial. We reduced the dosage of GABA and used to 1 or 0.1 mmol. In this trial we did not mix the GAGA and the HDPs but gave them in two separate injections. We also increased the dosage of HDPs ten fold. Finally the animals were challenged 24 hours after treatment. Birds were challenged with either 1 $X10^5$ of bacteria (Trial E).

In contrast to the previous experiment GABA at a concentration of 1mmol did not appear to be toxic (Figure 5). The group treated with a cocktail of HDP and 0.1mmol had less mortality than any of the other groups. In this experiment the mortality observed, even in the negative control group, was lower than in other experiments.

Figure 5. The Effect of GABA and HDP on septicemia when challenged 24 hours after treatment (TrialE)



As the treatment with HDP 10µg with GABA 0.1 mmol appeared to be the most promising treatment (although not statistically different than the other groups) (Figure 5) we carried out another animal trial (Trial F) to determine if this trend was reproducible. . In this experiment the birds were housed in two rooms and were challenged with different concentrations of *E. coli*. No significant difference was observed between the treatment groups and the untreated control group at either dose of the challenge.

Figure 6 A. The Effect of GABA and HDP on septicemia (Trial F) with a high dose challenge of *E. coli*

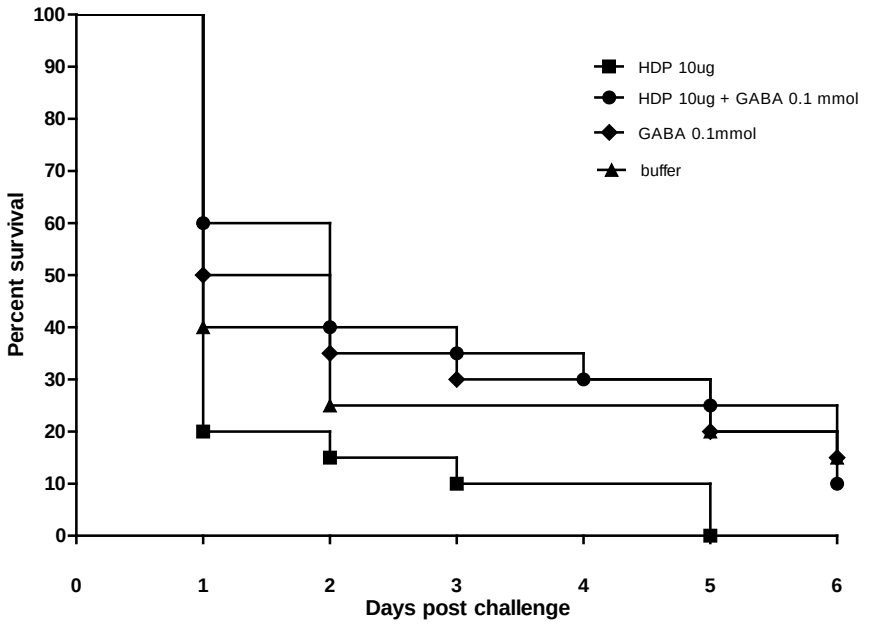
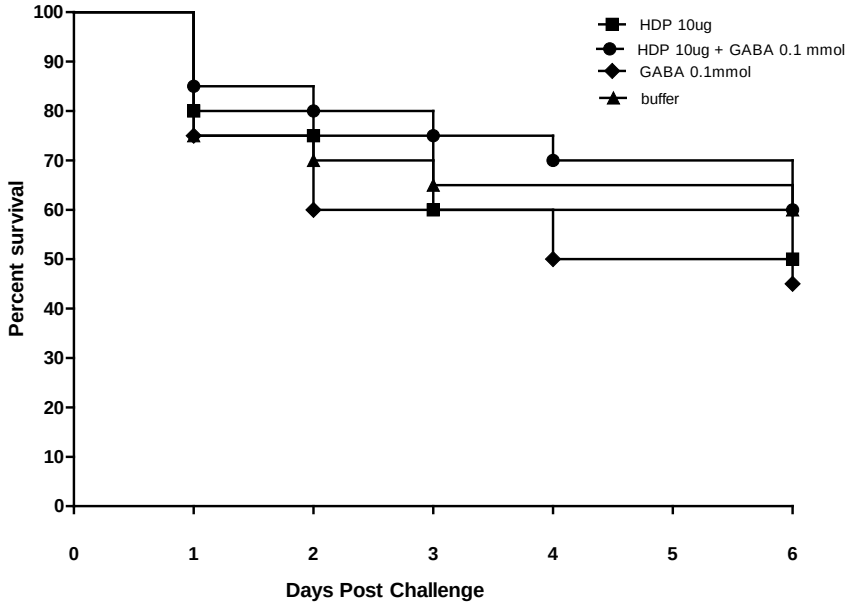


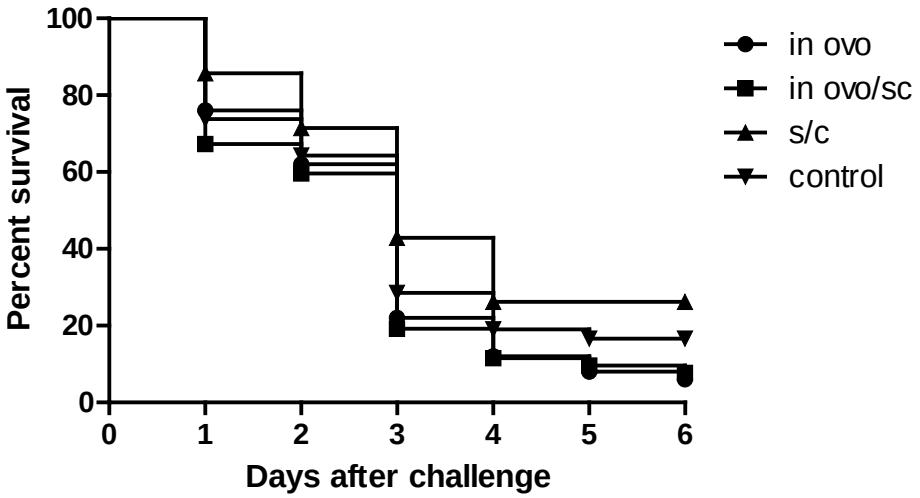
Figure 6 B. The Effect of GABA and HDP on septicemia (Trial F) with a low dose challenge of *E. coli*



d. Effect of a cocktail of six HDP given by in ovo injection on septicemia caused by *E. coli*

The final animal trial (Trial G) was carried out to determine if giving HDP by the in ovo route of delivery would be effective. Delivery of vaccines and other immune stimulants via in ovo injection on Day 18 of incubation is widely used throughout the poultry industry. The cocktail of HDP previously used were given in ovo at a concentration of 10 µg per peptide. For comparison birds were treated by s/c injection at day of age with the same cocktail of HDP or were treated both by in ovo vaccination and S/c injection. No difference was observed in the survival of any of the treated groups compared to the untreated controls suggesting that the treatment with HDP by in ovo injection alone or in combination with s/c was not effective in preventing septicemia caused by *E. coli* (Figure 7).

Figure 7. The effect of HDP delivered in ovo on septicemia produced by *E. coli*



C. Conclusions

We have shown that in vitro treatment with a cocktail of six HDPs (Bovine Myeloid Antimicrobial peptide, Indolicidin, Protegrin-1, Porcine β defensin 1, Avian β defensin 2, and Avian Cathelicidin 3) for 24 hours was effective in killing *E. coli*.

Treatment of day old chicks with Avian Cathelicidin 3 and Avian β defensin 2 by subcutaneous injection was not effective in the reduction of septicemia caused by *E. coli.* The cocktail of 6 HDPs, which had been shown to be antimicrobial, were not effective in the reduction of septicemia when they were delivered by subcutaneous injection or by in ovo

injection. In addition the supplementation of the cocktail of HDPs with GABA did not increase the efficacy of the HDP.

D. Communication of Research Results

We have presented two oral presentations about this work and have had a research paper accepted for publication.

Brenda Allan, Rachelle M. Buchanan, Shirley Hauta, Jan van den Hurk, and Heather L. Wilson. 2012. Innate Immune Cocktail Partially Protects Broilers Against Cellulitis and Septicemia. Accepted for publication in Avian Diseases

Brenda Allan. Immunotherapy to Enhance Innate Immunity in Young Chicks. Invited Seminar Sept 2012 Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton AB.

B. Allan and Heather Wilson. An Alternative to Antibiotics to Treat Cellulitis and Septicemia. 60th Western Poultry Disease Conference. Sacramento, California March 21-23, 2011.

E. Financial Report

Has been submitted under separate cover.