Alternatives to Antibiotics
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Challenges and Solutions in Animal Health and Production
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Host defense peptide with anti-microbial and immunomodulatory activities as antibiotic alternatives

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Topics to be discussed

- Antimicrobial and host defense peptides
- Chicken coccidiosis
- Chicken-derived synthetic peptide: cNK-2
- Nanoencapsulation of cNK-2

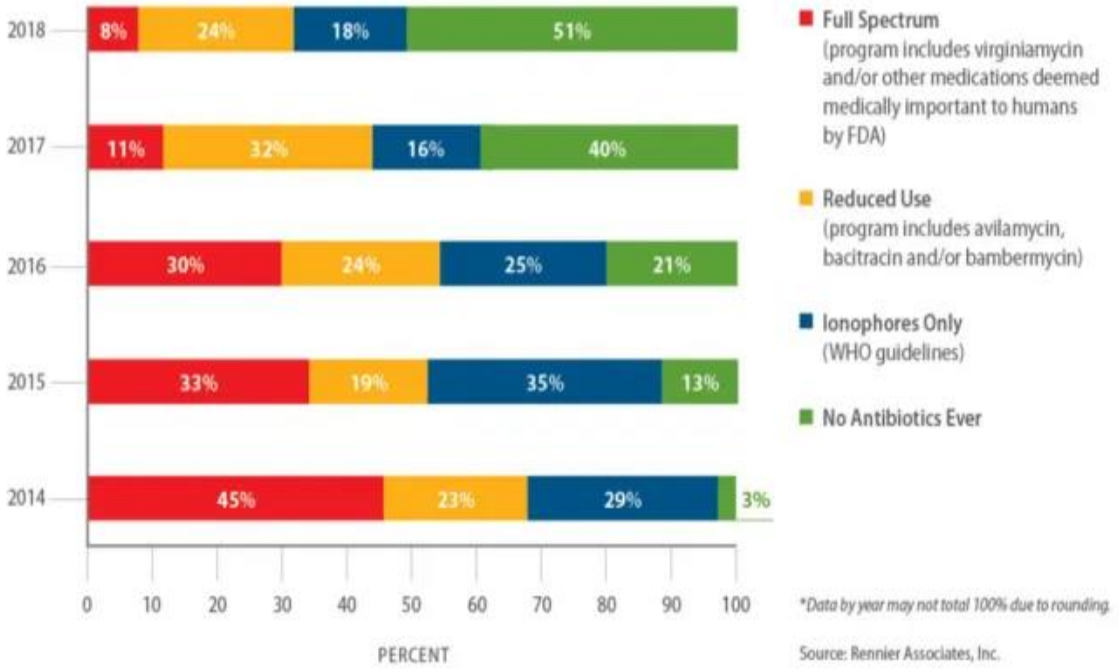
Issues in poultry production

- AGPs: Supplemented in the feed more than 60 years
- Issues on resistance to antibiotics

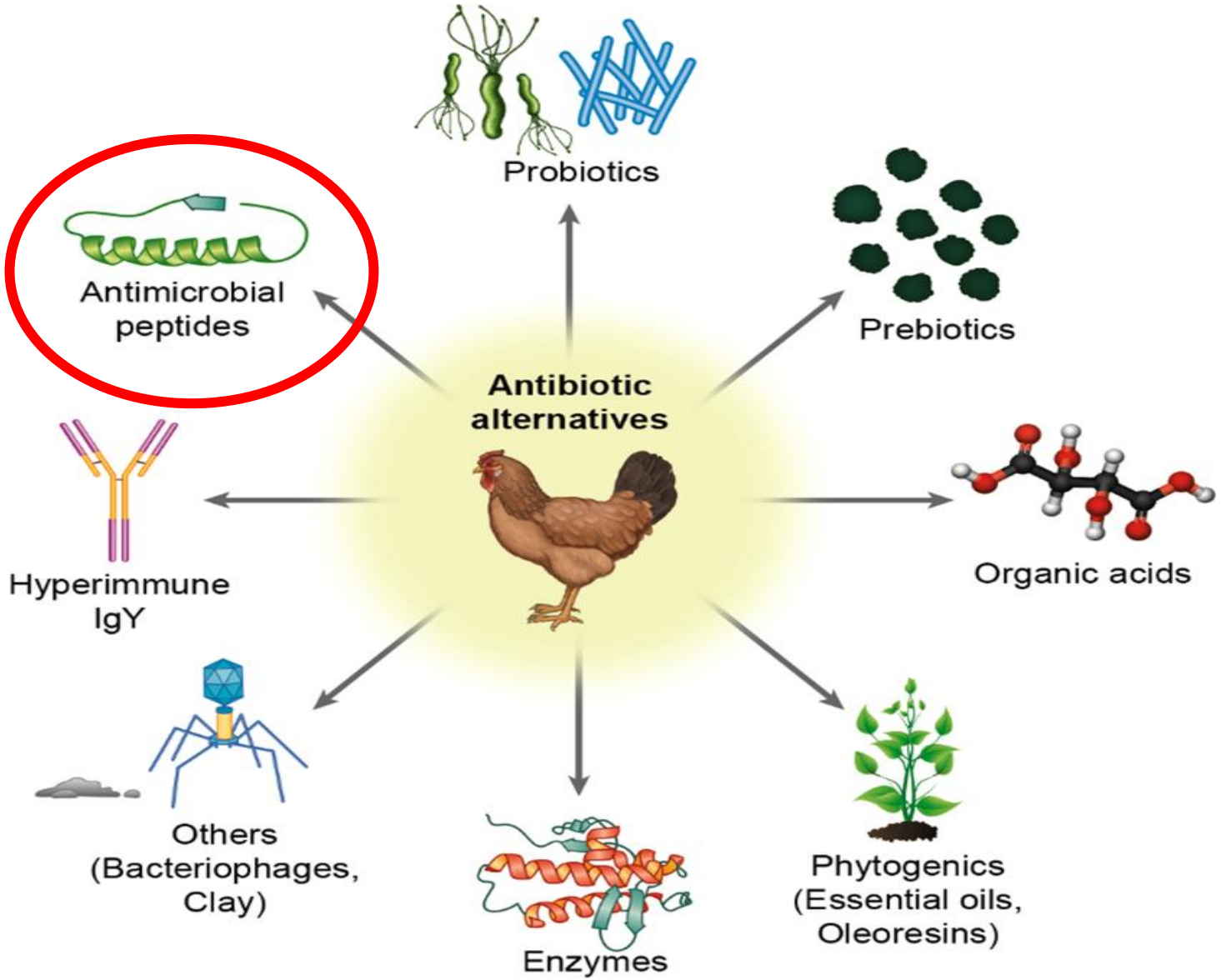
  and consumer's awareness
- Decreasing in use of AGPs
- Increasing poultry health problem and therapeutic

  antibiotics use
- Increasing demand for alternatives

extracted from The Poultry Site



Alternatives to antibiotics

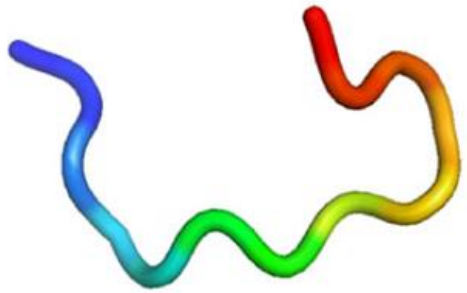


Gadde, Kim, Lillehoj et al. 2017. Animal Health Research Reviews

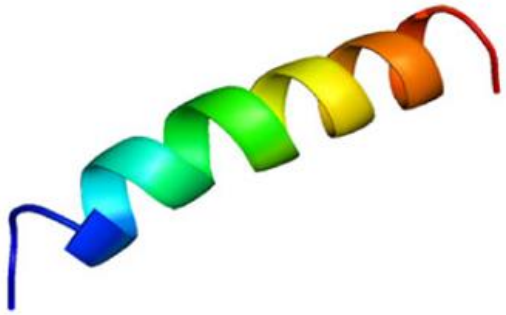
Antimicrobial peptides (AMPs) and host defense peptides (HDPs)

- Oligopeptides produced by all known species
- Part of innate immune response
- Broad spectrum antimicrobial activity
- More than 4,000 natural AMPs

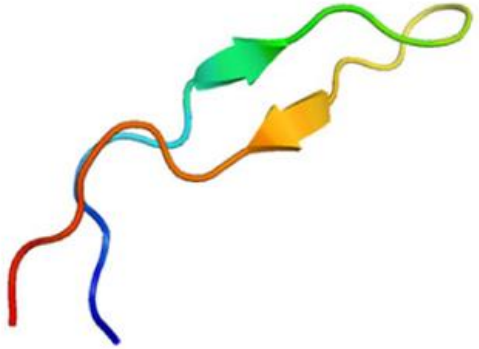
Structure type: Extended
Example: Indolicidin
Ref: Rozek *et al* 2003 **PDB:** 1QXQ



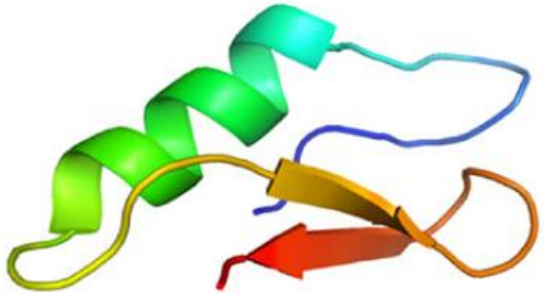
Structure type: α-helical
Example: Magainin 2
Ref: Geselle *et al* 1997 **PDB:** 2MAG



Structure type: β-sheet
Example: Lactoferricin
Ref: Hwang *et al* 1998 **PDB:** 1LFC

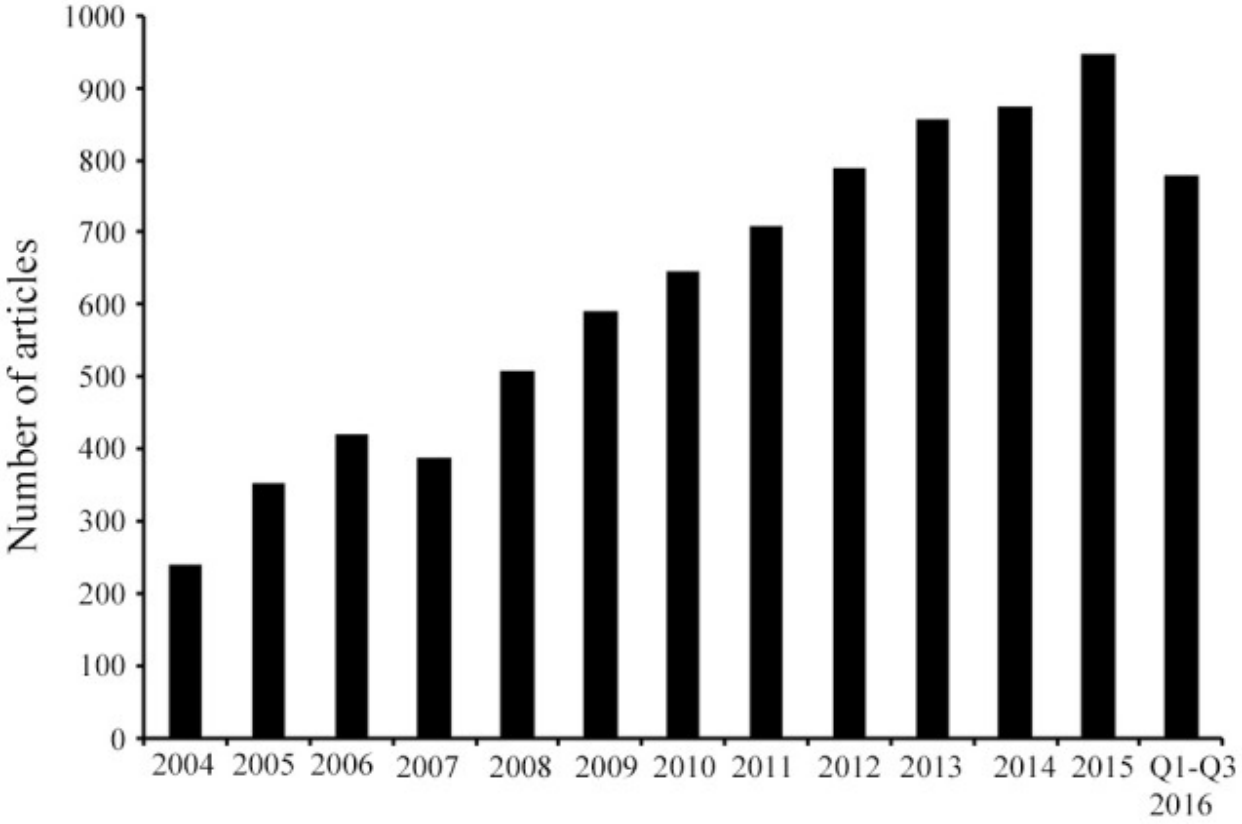


Structure type: Mixed
Example: Plectasin
Ref: Mygind *et al* 2005 **PDB:** 1ZFU



Extracted from AMR Centre

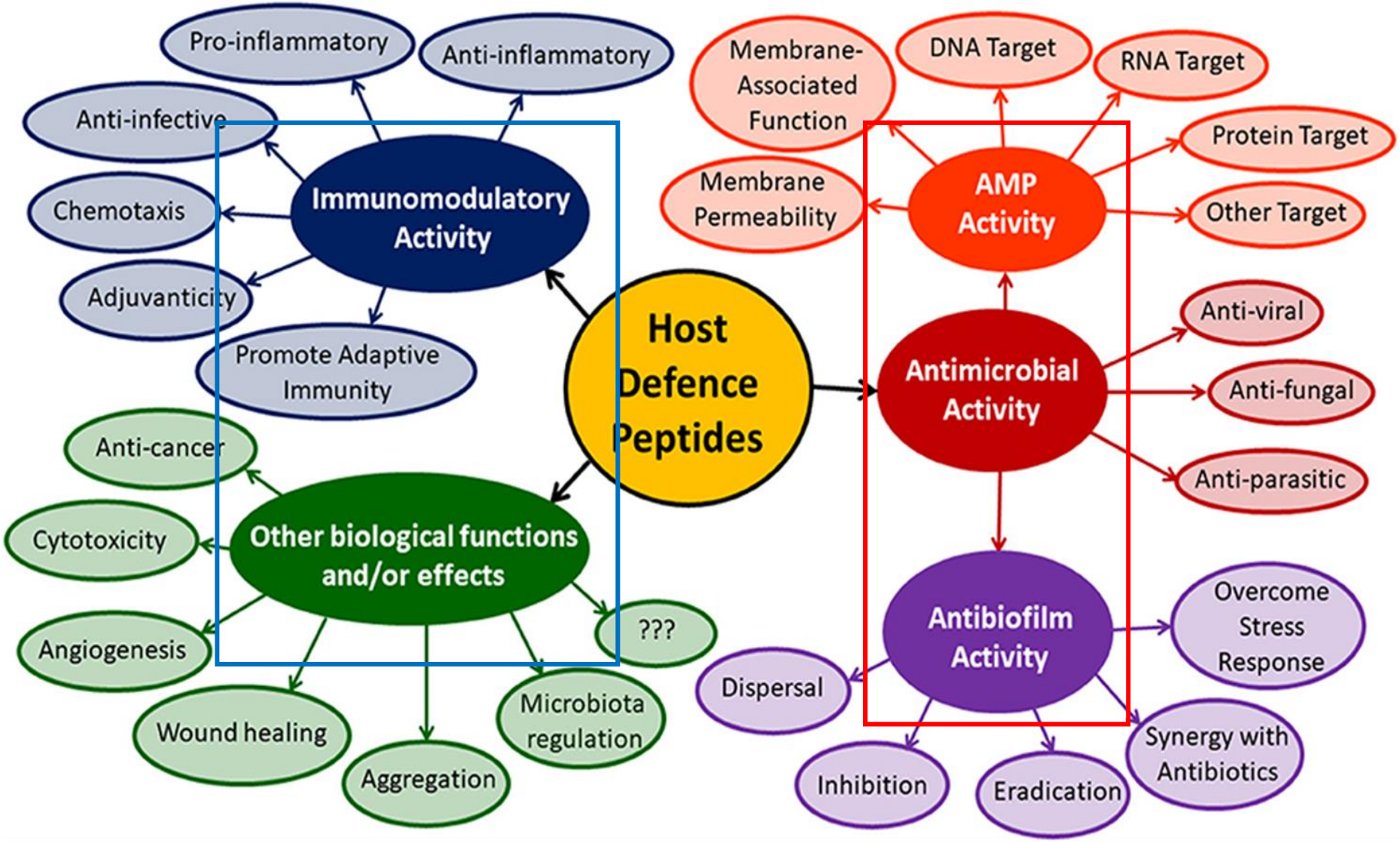
Increasing interest and limitations AMPs



Mahlapuu et al, 2016. Frontiers in Cellular and infection microbiology

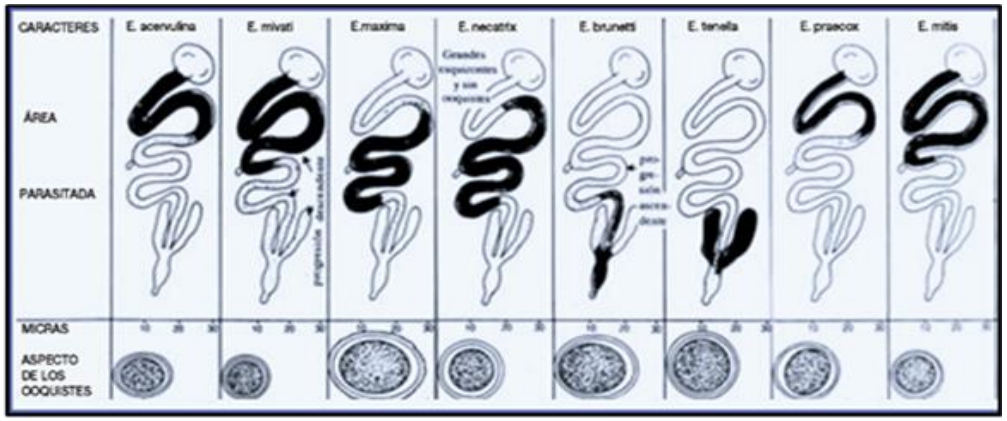
- High sensitivity of antimicrobial activities to environment
- Poor bioavailability
- High production cost
- Regulatory hurdles

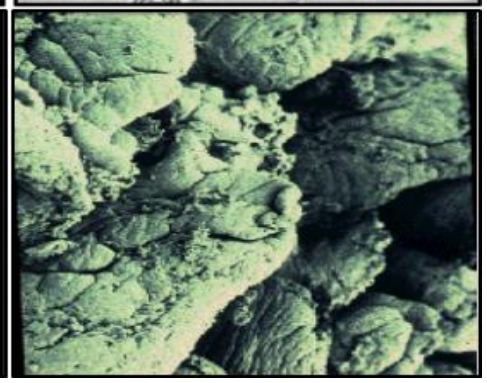
Functions of AMPs and HDPs

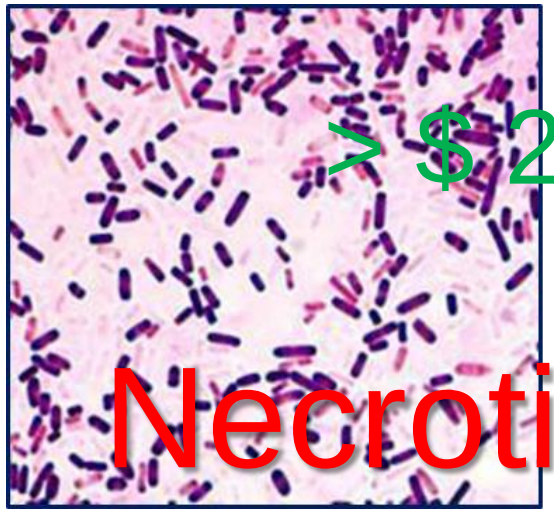


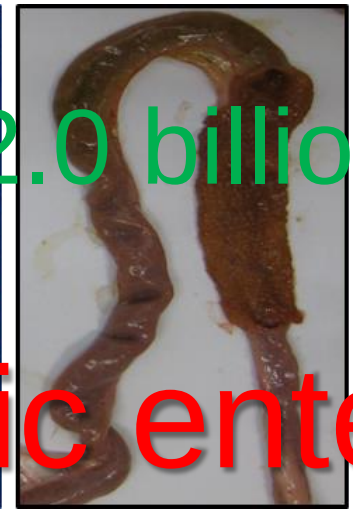
Haney et al., 2019. Frontiers in Chemistry

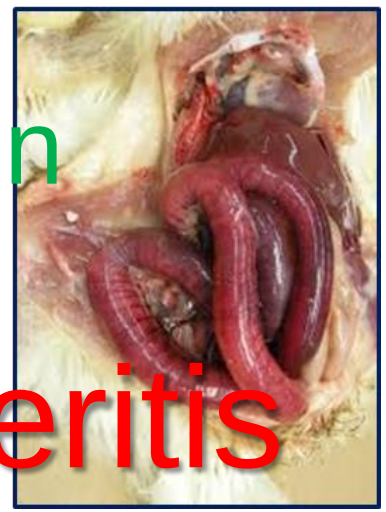
Chicken coccidiosis











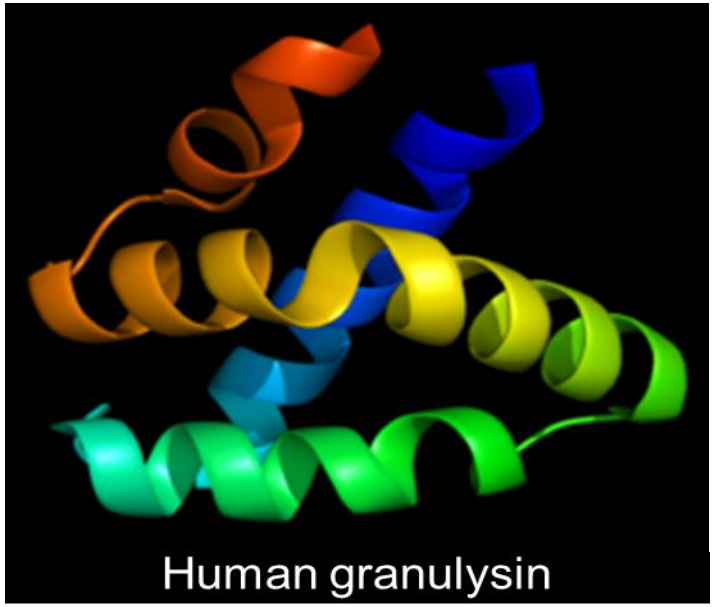
2019 Research Priorities of the American Association of Avian Pathologists

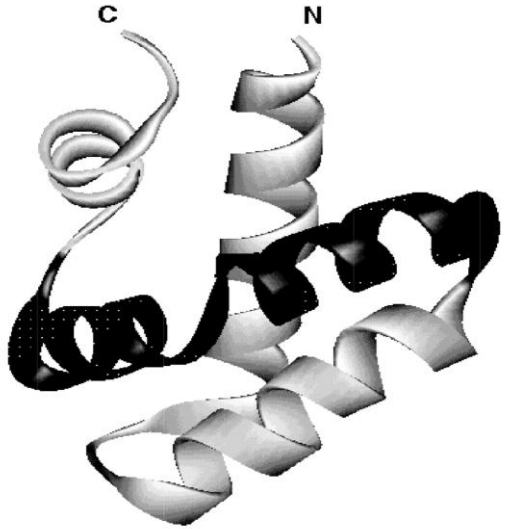
Natalie Armour[A], Mark Burleson[B], Eric Gingerich[C], Seiche Genger[D], Travis Schaal[E], John Glisson[F], Naola Ferguson-Noel[G] and John Smith[H]

Table 2. Broiler Research Priorities In Category 1: Health/Disease

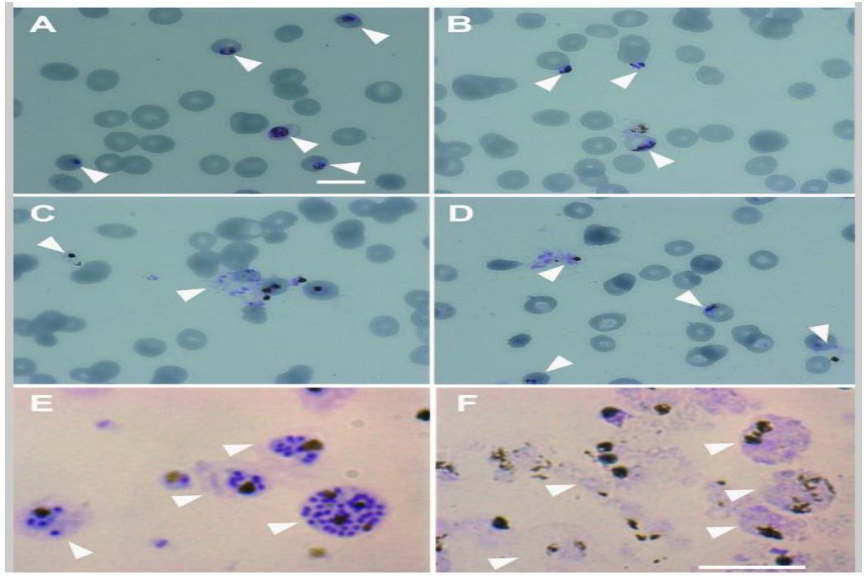
Rank	Score[a]	Subcategory	Research Needs Statement
1	4.1	Intestinal Health	Develop non-antibiotic strategies to optimize gut health, increase resistance to intestinal pathogens and improve feed conversion
1	4.1	Clostridial Diseases	Investigate risk factors contributing to the development of Necrotic Enteritis, including the role of feed ingredients
2	4.0	Coccidiosis	Determine the most effective non-ionophore rotation strategies for the control of coccidiosis and subsequent Necrotic Enteritis, for preserving the long-term efficacy of the drugs, and for ameliorating resistance.
3	3.9	Reovirus	Investigate the epidemiology of reoviruses, and the emergence of novel reovirus strains
3	3.9	Histomoniasis	Determine risk factors for the development of Histomoniasis. Determine whether early coccidiosis, breed/strain and sex impact the risk of developing Histomoniasis
3	3.9	Infectious Bronchitis	Determine the epidemiology, risk factors and effective control strategies for nephropathogenic Infectious Bronchitis
4	3.8	Intestinal Health	Conduct research to understand the intestinal microbiome and immunity, host-pathogen interactions and how various feed ingredients and additives modulate these functions to affect intestinal health

NK-lysin: homologue of human granulysin

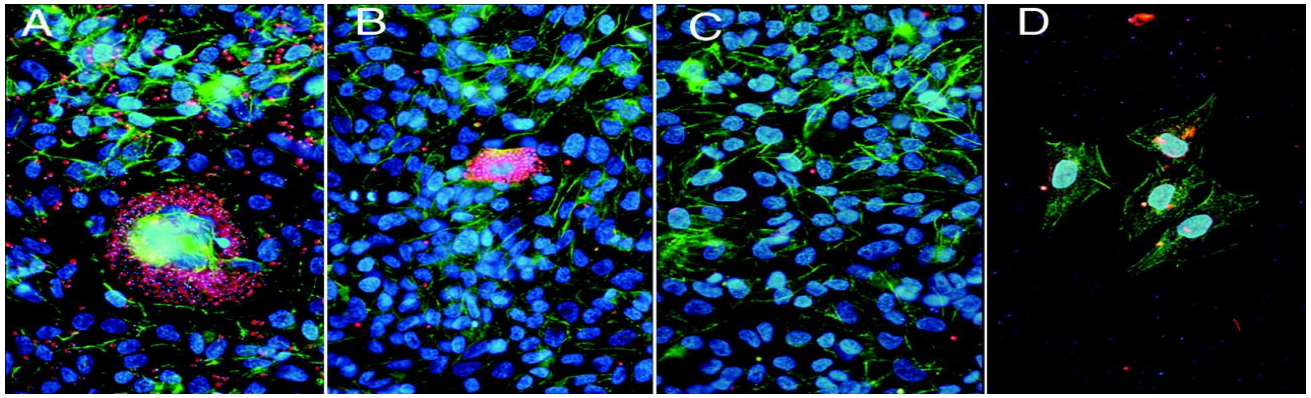




Porcine NK-lysin



Gelhaus et al., 2008. Antimicrobial Agents and Chemotherapy



Jacobs et al., 2003. Antimicrobial Agents and Chemotherapy

NK-2: Cationic core region of NK-lysin (27 a.a.)

pNK-2 effects on parasites

(Trypanosoma cruzi, Plasmodium falciparum)

NK-lysin homologues have paraticidal effect

History of NK-lysin in chicken coccidiosis

Clusters That Contain More Than 14 ESTs

Contig ID	Gene description	Organism	Accession No.	No. of ESTs
Contig171	NK-lysin	Equus caballus	CD728315	87
Contig1648	Apolipoprotein AIV	Gallus gallus	CD731936	69
Contig42	Fatty acid binding protein	Gallus gallus	CD735219	51
Contig1279	Immunoglobulin α heavy chain	Gallus gallus	CD735924	43
Contig1234	2',5'-oligoadenylate synthetase	Gallus gallus	CD730844	24
Contig944	ATP synthase β-subunit	Cyprinus carpio	CD732620	24
Contig971	Interferon regulatory factor 6	Ovis aries	CD732407	22
Contig1300	Jun-binding protein	Gallus gallus	CD739778	20
Contig1325	Acidic ribosomal phosphoprotein (P0)	Gallus gallus	CD737516	20
Contig1000	Angiotensin converting enzyme	Gallus gallus	CD731489	19
Contig608	α-tubulin	Gallus gallus	CD736033	19
Contig1524	34/67 kDa laminin receptor	Cricetulus griseus	CD737204	18
Contig1792	Actin related protein 2/3 complex, subunit 1B (ARPC1B)	Homo sapiens	CD737537	18
Contig733	GAPDH	Gallus gallus	CD735039	18
Contig992	Unknown	Unknown	CD729072	18
Contig528	Na^+-dependent nucleoside transporter	Oryctolagus cuniculus	CD737431	17
Contig352	Unknown	Unknown	CD733292	16
Contig1247	Ferritin heavy chain	Gallus gallus	CD740150	15

Min, Lillehoj et al., 2005. Molecular Biotechnology

Chicken NK-lysin is the most expressed in *Eimeria*-infected intestine

Chicken NK-lysin: cloning

```
NK-lysin, chicken       ---------------MAAALIVLLALGAAVQVAVTEPPRDDHRDLDAGSHWEQQWHLLQD  45
NK-lysin, porcine       -------------------------------PGLAFSGLTPEHSALARAHPCDGEQFCQN  29
NK-lysin, bovine        ------------------------------------------------------------
NK-lysin, equine        MKKMGCGGRLSSCPTMTSRALLLLASALLGTPGLTFSGLNPESYDLATAHLSDGEQFCQG  60
Granulysin, human       ---------------MATWALLLLAAMLLGNPGLVFSRLSPEYYDLARAHLRDEEKSCPC  45

                                      1  2                              3
NK-lysin, chicken       GSAAWDADEGDAMGPGKGIKCRFCVSLVKKVQKIVGDDPDEDAINNALNKVCSTGR-RQR  104
NK-lysin, porcine       LAPEDPQGDQLLQREELGLICESCRKIIQKLEDMVGPQPNEDTVTQAASRVCDKMK-ILR  88
NK-lysin, bovine        ---EDPQGDLLLQGEELSLRCGSCRRIIQHLMDKLGDQPDENTVIEEASKVCSKMR-LLK  56
NK-lysin, equine        LTQEDLQGDLLTERERQGIACWSCRKILQKLEDLVGEQPNEATINEAASRVCRNLG-LLR  119
Granulysin, human       LAQEGPQGDLLTKTQELGRDYRTCLTIVQKLKKMV-DKPTQRSVSNAATRVCRTGRSRWR  104
                              .:       .    *  ::::: . :  .* : :: :  .:** .     :

                          4
NK-lysin, chicken       SICK----------------------------------------QLLKKLRQQLSDALQNN  125
NK-lysin, porcine       GVCK----------------------------------------KIMRTFLRRISKDILTG  109
NK-lysin, bovine        GLCKSIMKKFLRTIAEDIVAGKTSQVICVDIKMCKSKPVGFIKKIMRTCLRLISRDILAG  116
NK-lysin, equine        GACK----------------------------------------KIMRTCLRLISRDILAG  140
Granulysin, human       DVCR----------------------------------------NFMRRYQSRVTQGLVAG  125
                        . *:                                        ::::    ::  :  .

                                5     6
NK-lysin, chicken       DDPRDVCTTLGLCKG-----  140
NK-lysin, porcine       KKPQAICVDIKICKEKTGLI  129
NK-lysin, bovine        KKPQEVCVDIKLCKHKAGLI  136
NK-lysin, equine        KKPQEVCVDIKLCKHKAGLI  160
Granulysin, human       ETAQQICEDLRLCIPSTGPL  145
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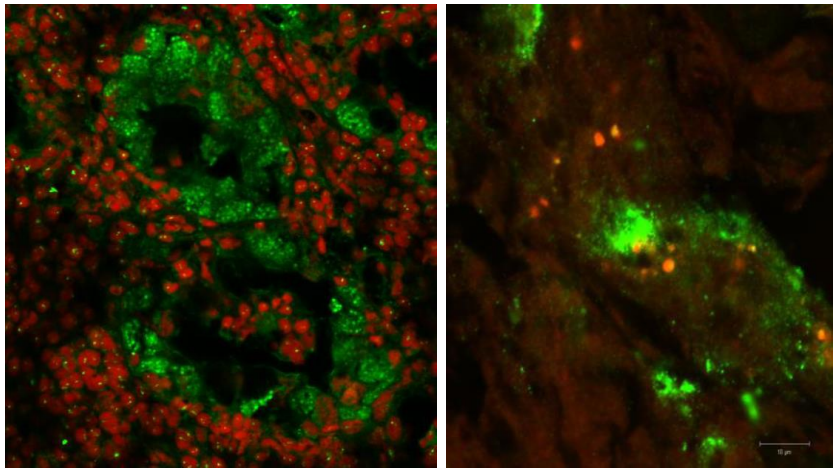
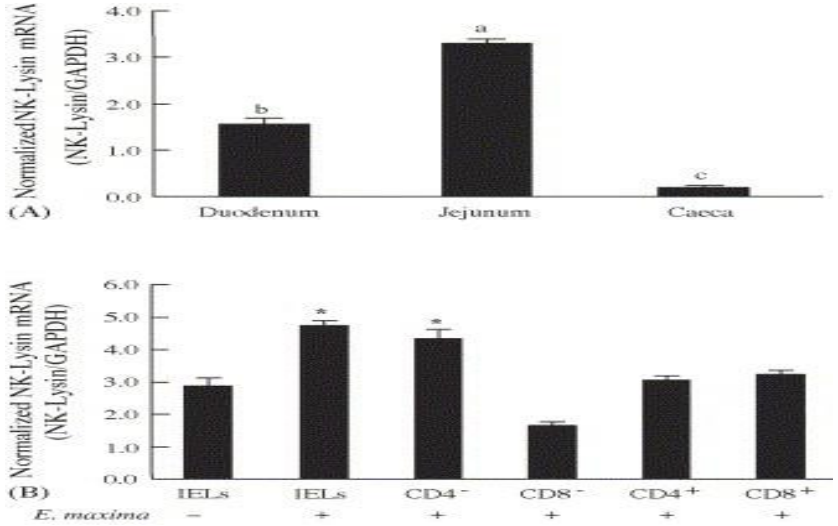
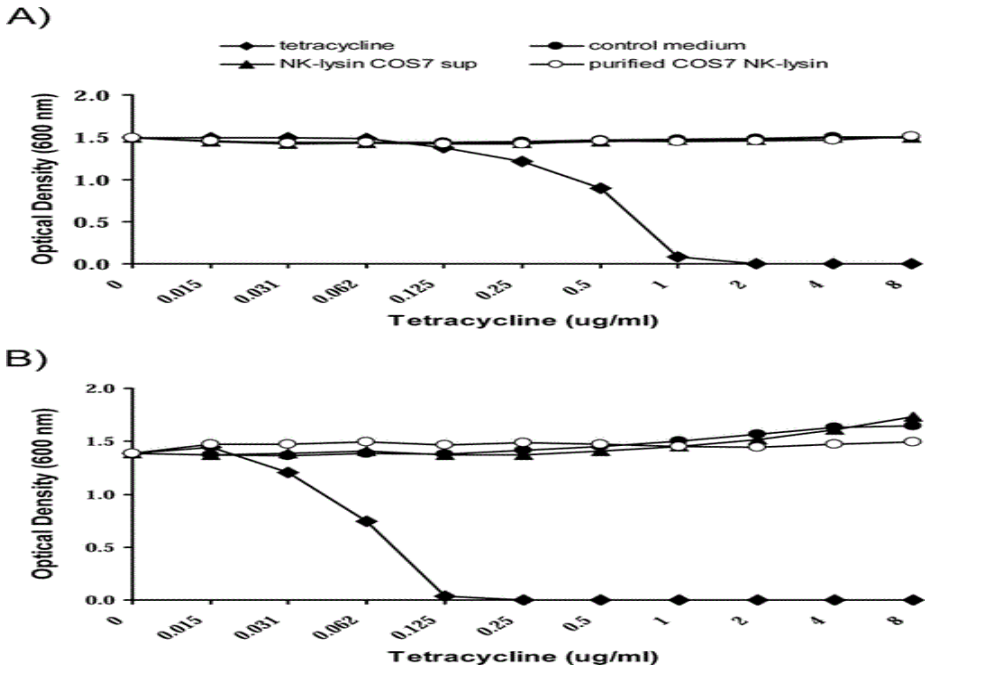




Hong and Lillehoj et al., 2006. Veterinary Immunology and Immunopathology

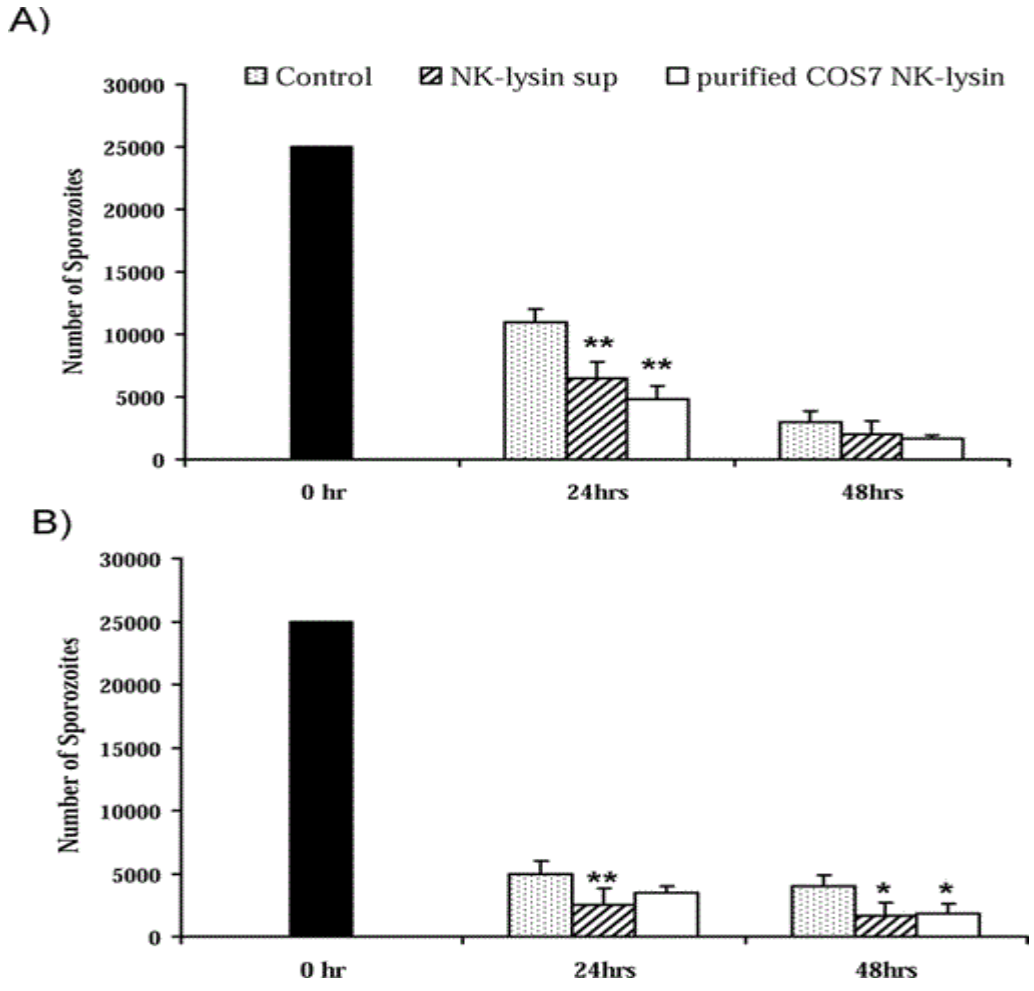
Chicken NK-lysin expressed in cytotoxic T cells and the intestine

Antimicrobial activity of cNK-lysin



Organism	Strain	5-µl Spot result	10-µl Spot result
Bacillus subtilis	STOP	neg[A]	neg
Salmonella Montevideo	S1	neg	neg
Salmonella Heidelberg	S2	neg	neg
Clostridium perfringens	A. 1113	neg	neg
Clostridium difficile	#3	neg	neg
Clostridium difficile	27.1	neg	neg
Campylobacter jejuni	C3 Dreeson	neg	neg
Campylobacter jejuni	C2 Dreeson	neg	neg
Enterobacter sakazaki	A. 51329	neg	neg
Vibrio harveyi	TAES2	neg	neg
Klebsiella pneumoniae	A. 13883	neg	neg
Escherichia coli O157:H7	265RC1	neg	neg
Enterobacter cloacae	A. 23355	neg	neg
Enterococcus faecium	50-52	neg	neg
Staphylococcus aureus	A. 25923	neg	neg
Proteus vulgaris	A. 13315	neg	neg

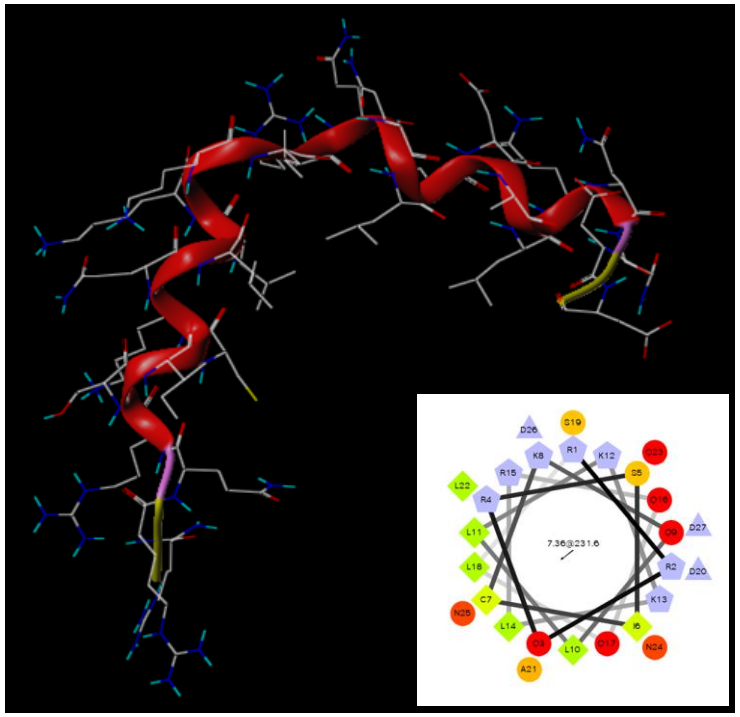
[A]neg = No visible zone of inhibition.

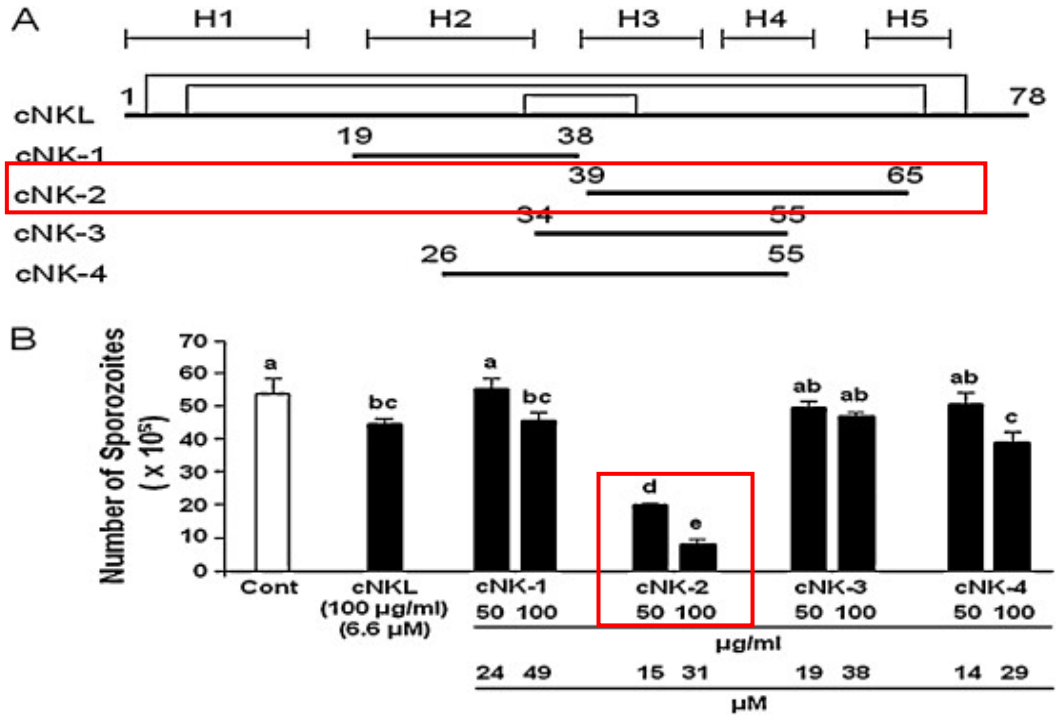


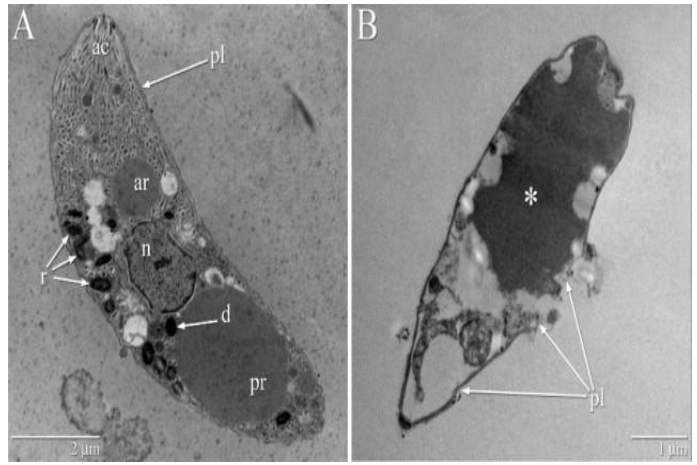
Hong and Lillehoj et al., 2008. Avian Diseases

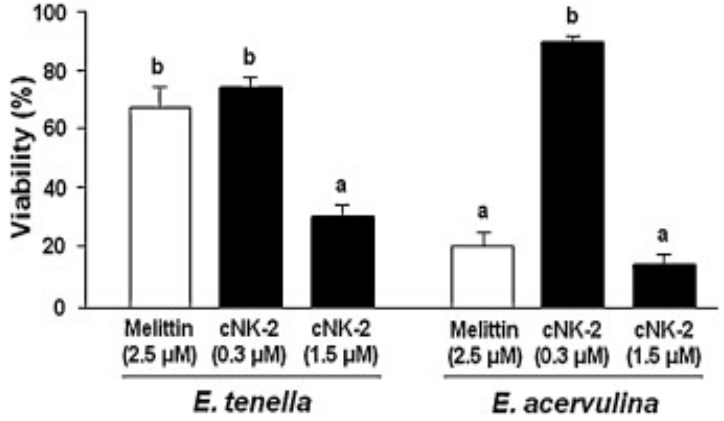
Chicken NK-lysin effects on *Eimeria* spp.

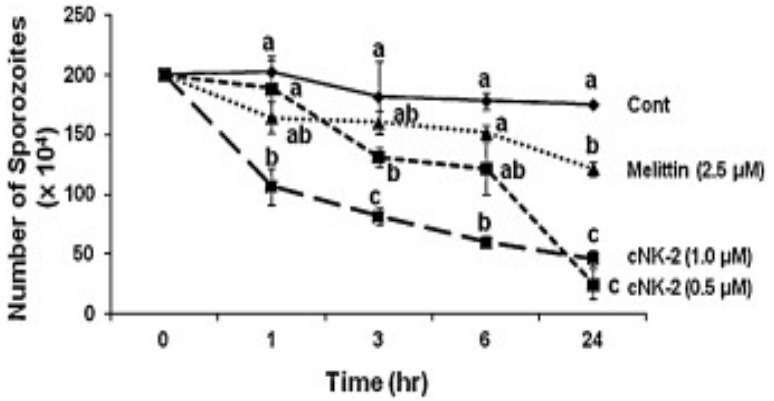
Chicken NK-lysin-derived peptides

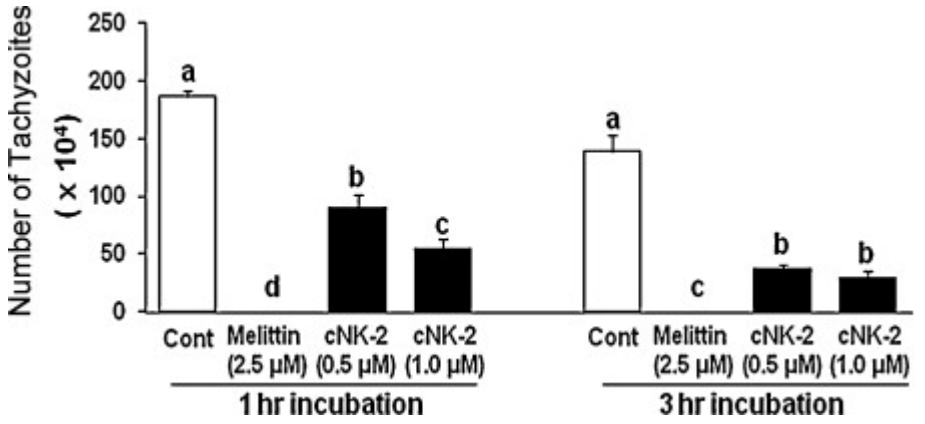






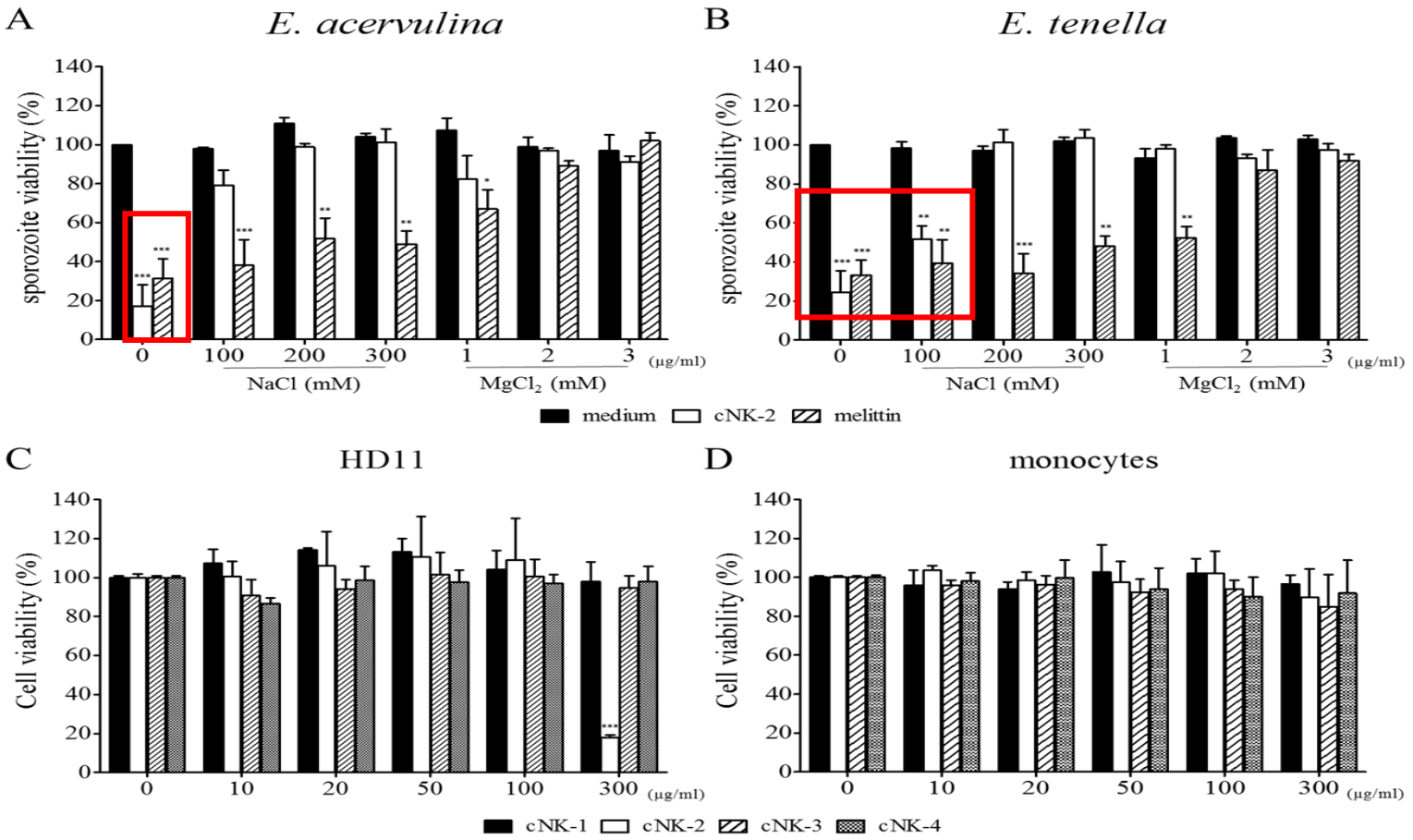






Lee and Lillehoj et al., 2013. Veterinary Parasitology

Chicken NK-2

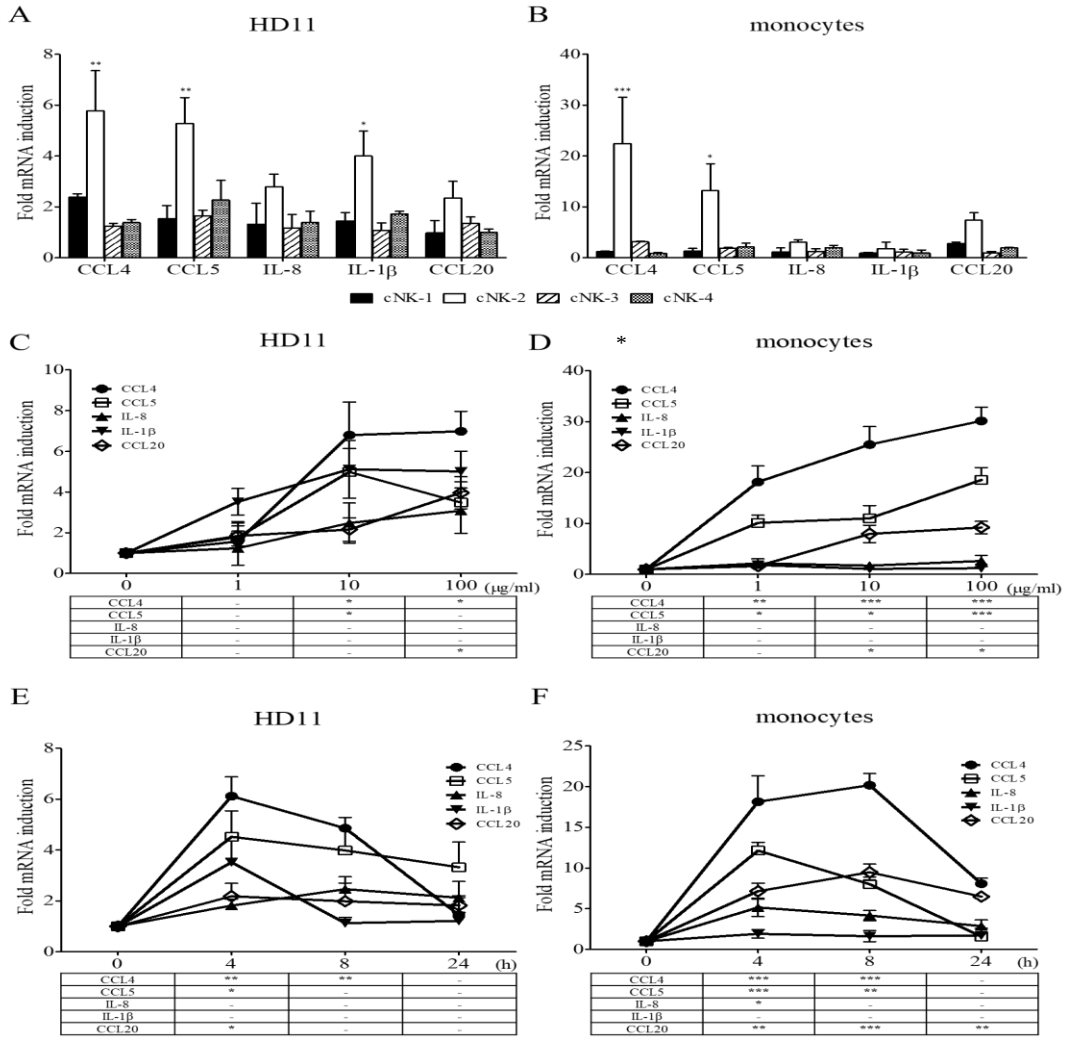


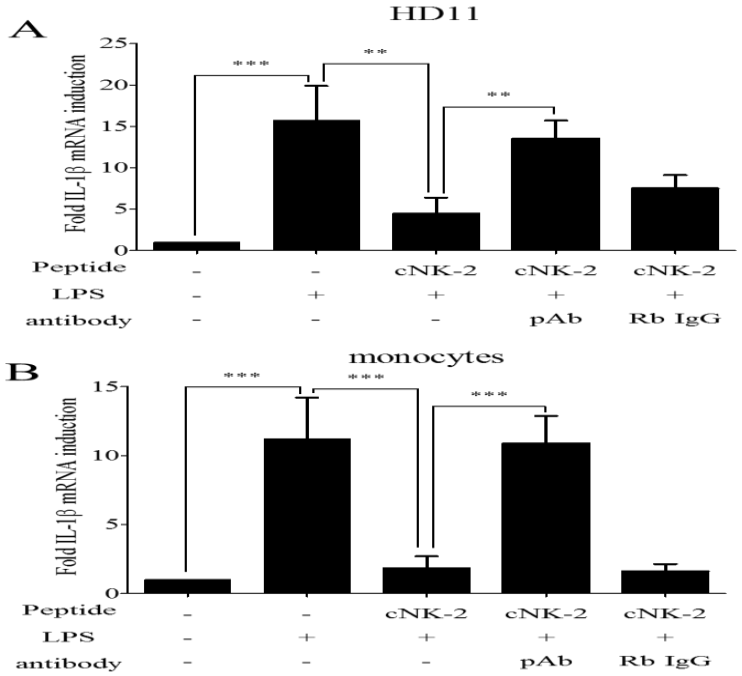
Kim, Lillehoj and Min, 2017. Scientific Report

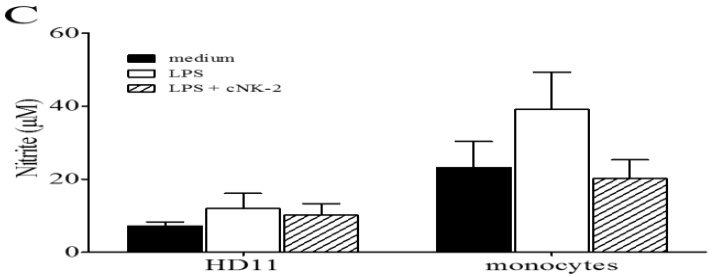
Somewhat attenuated antimicrobial activity in physiological salt condition

No cytotoxicity on chicken cells

Immunomodulation of cNK-2



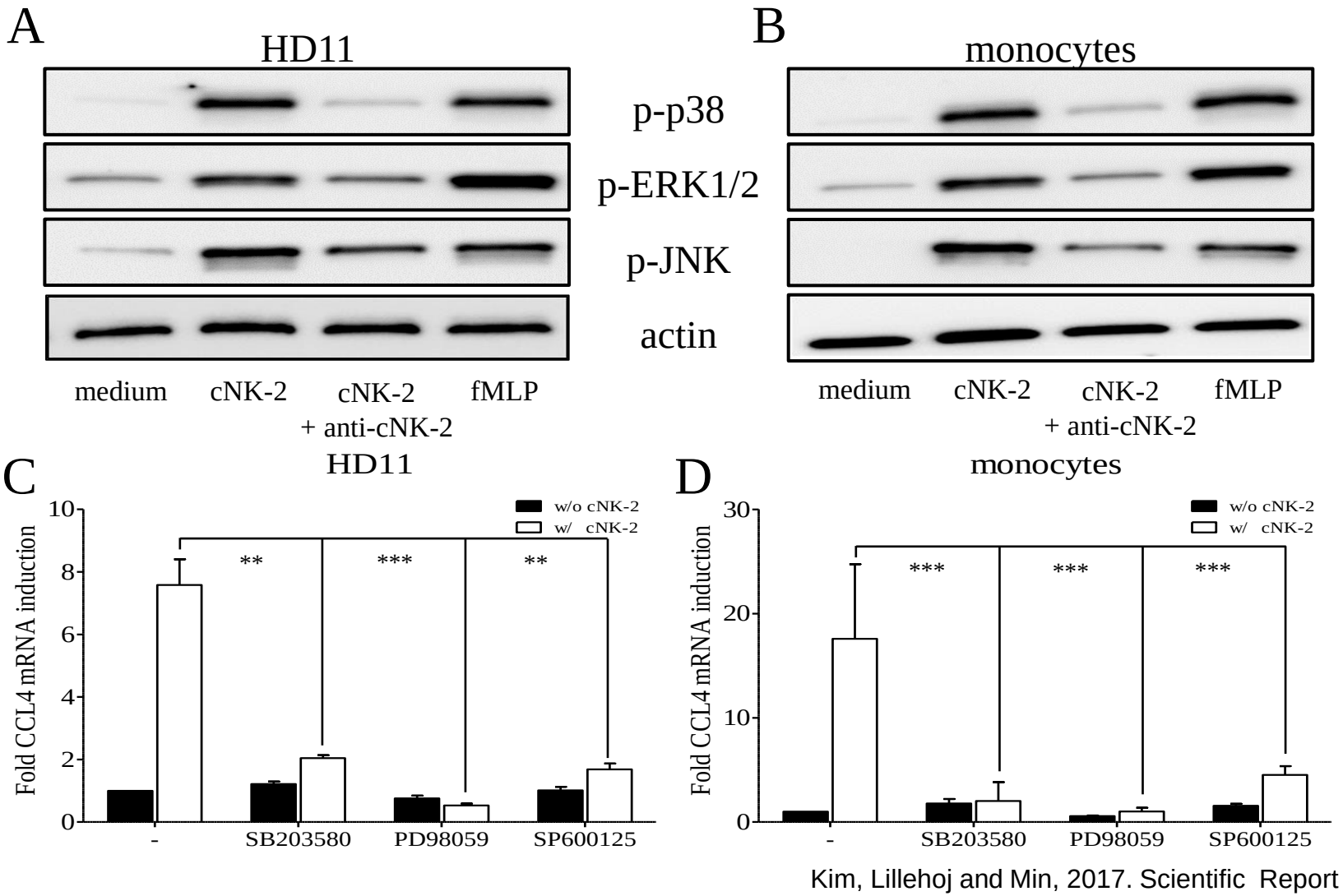




Kim, Lillehoj and Min, 2017. Scientific Report

Chicken NK-lysin modulates immune responses

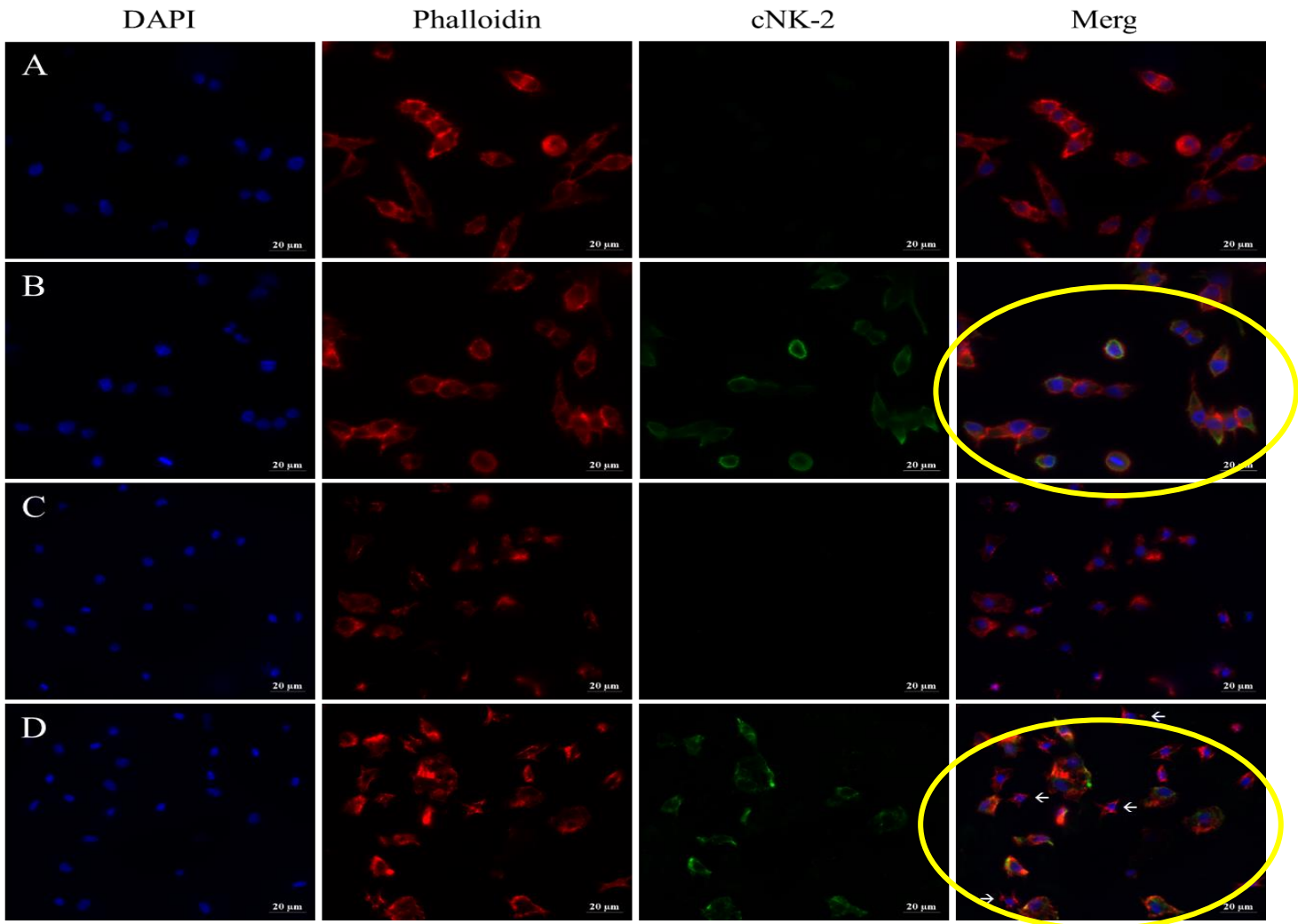
Immunomodulation of cNK-2



Kim, Lillehoj and Min, 2017. Scientific Report

Immunomodulation of cNK-2 is regulated through MAPK pathways

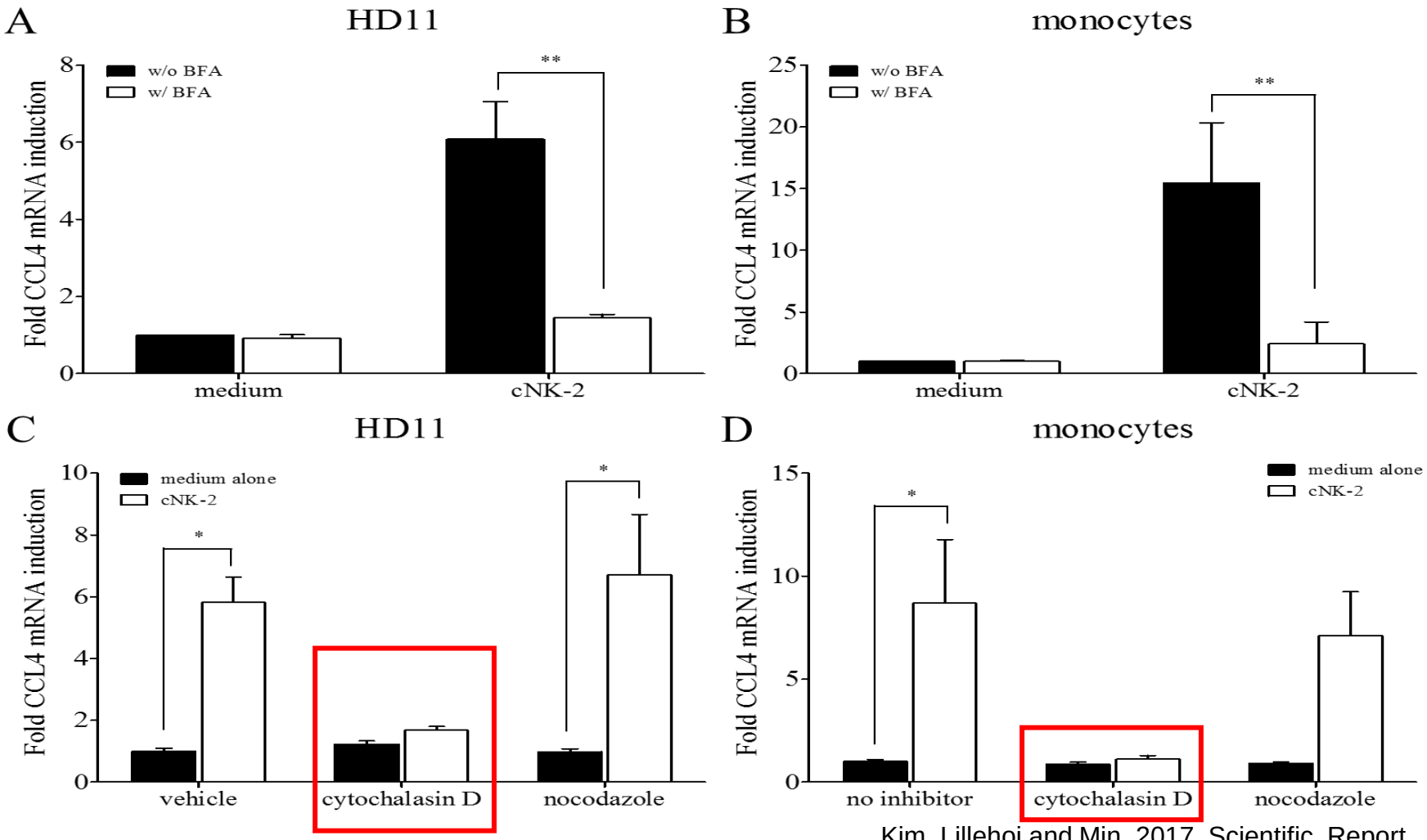
Internalization of cNK-2



Kim, Lillehoj and Min, 2017. Scientific Report

cNK-2 internalized into host cells

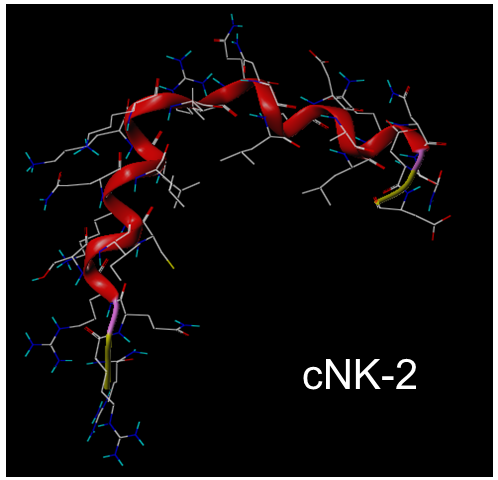
Immunomodulation of cNK-2



Kim, Lillehoj and Min, 2017. Scientific Report

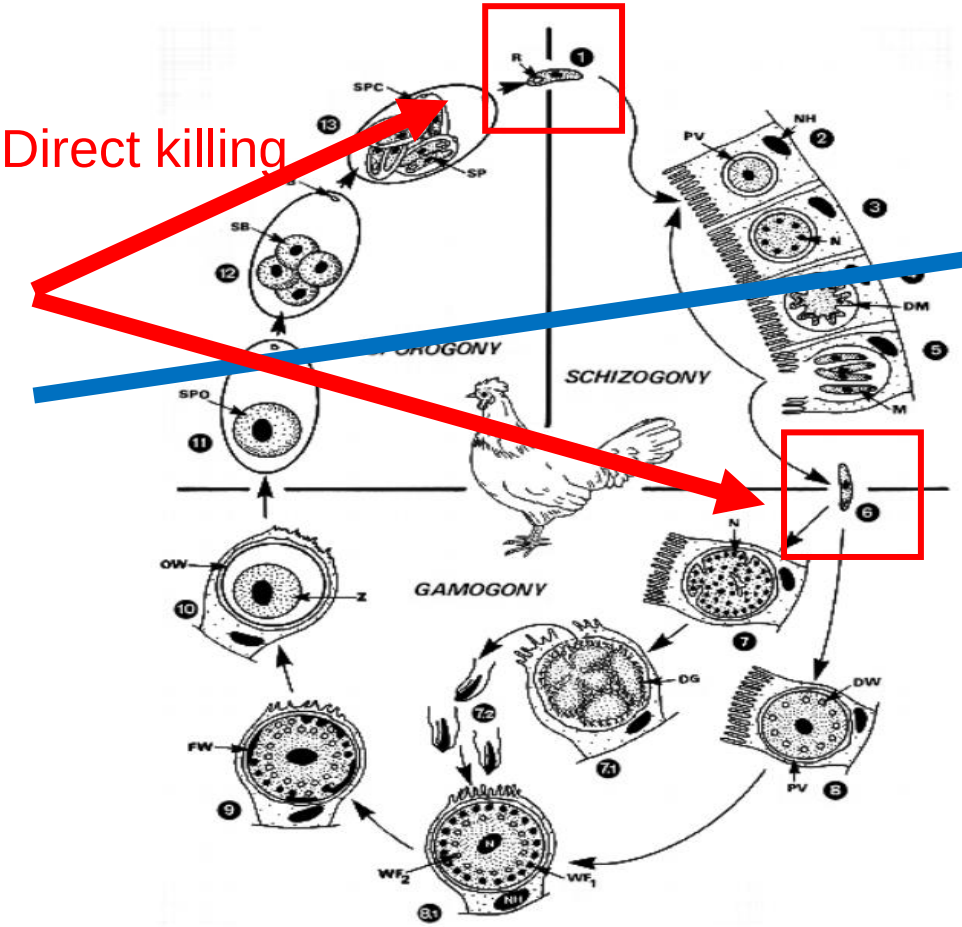
Endocytosis of cNK-2 is regulated by actin polymerization

Applications of chicken NK-lysin derived peptide

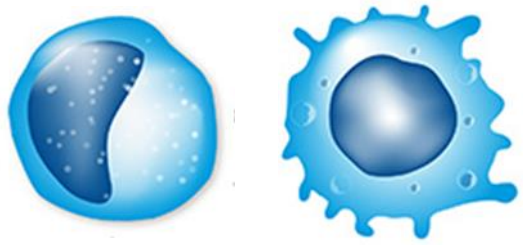


DELIVERY?

Direct killing



Toward pathogen

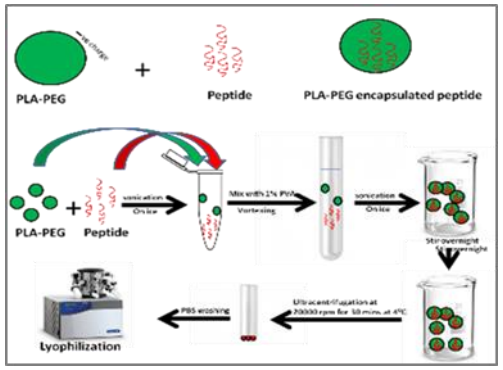


Immunomodulation

- Chemokine induction
- Anti-inflammation
- Signaling pathway activation

Toward host

Nanoencapsulation of cNK-2

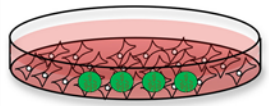


Encapsulated NPs

PLA-PEG

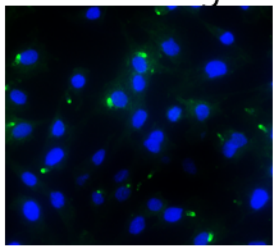
Characterization of NPs
- efficiency
- size
- ζ-potential
- release profile
- stability
- bioactivity
- toxicity

Treatment into chicken intestinal epithelial cells

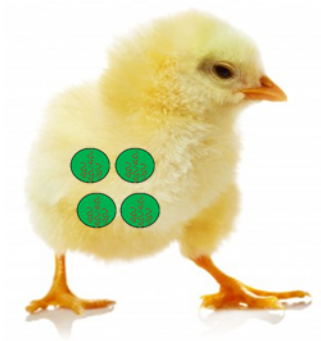


in-vitro *Eimeria* infection

Assessment
- antimicrobial activity against sporozoites
- sporozoite invasion
- NPs tracking



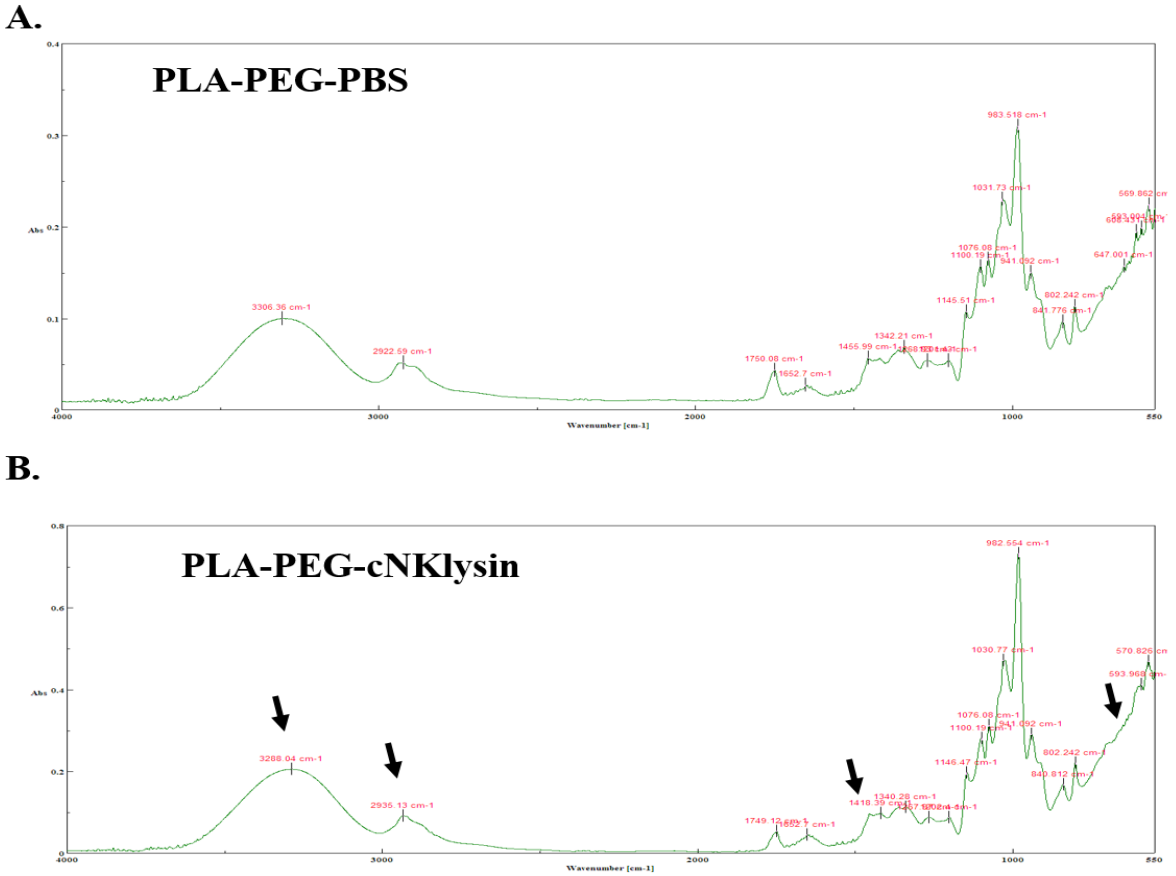
Oral delivery to chickens

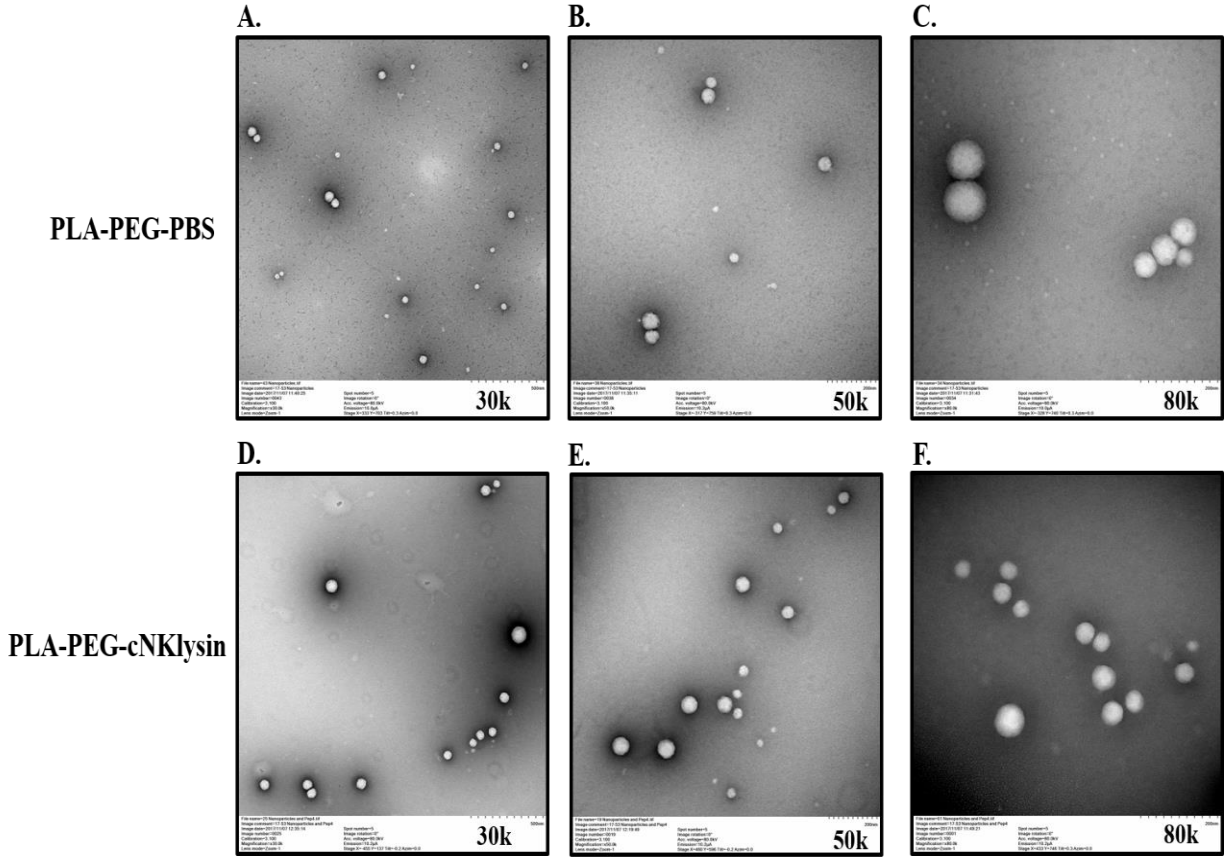


in-vivo *Eimeria* infection

Assessment
- body weight gain
- oocyst shedding
- intestinal lesion
- immune response
- NPs tracking

Nanoencapsulation of cNK-2



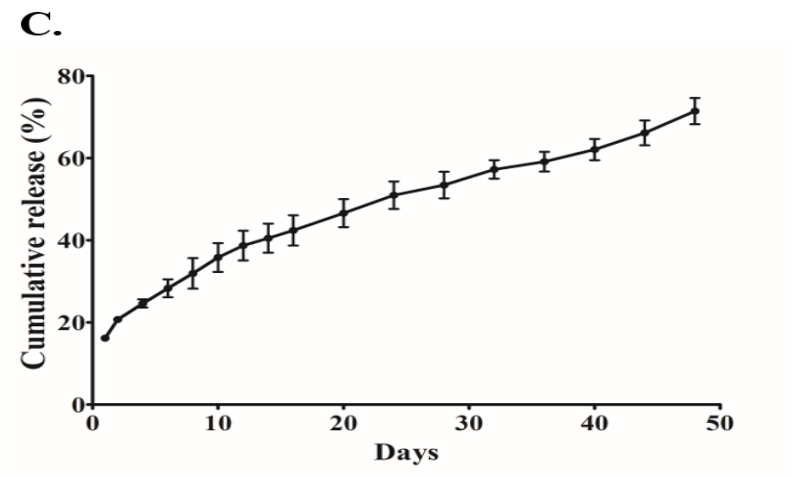


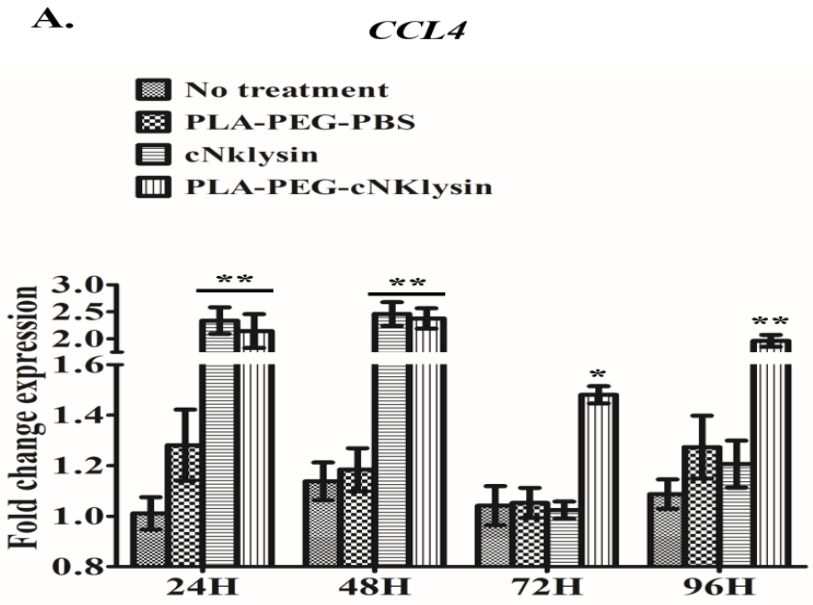
Chaudhari, Kim, and Lillehoj, unpublished data

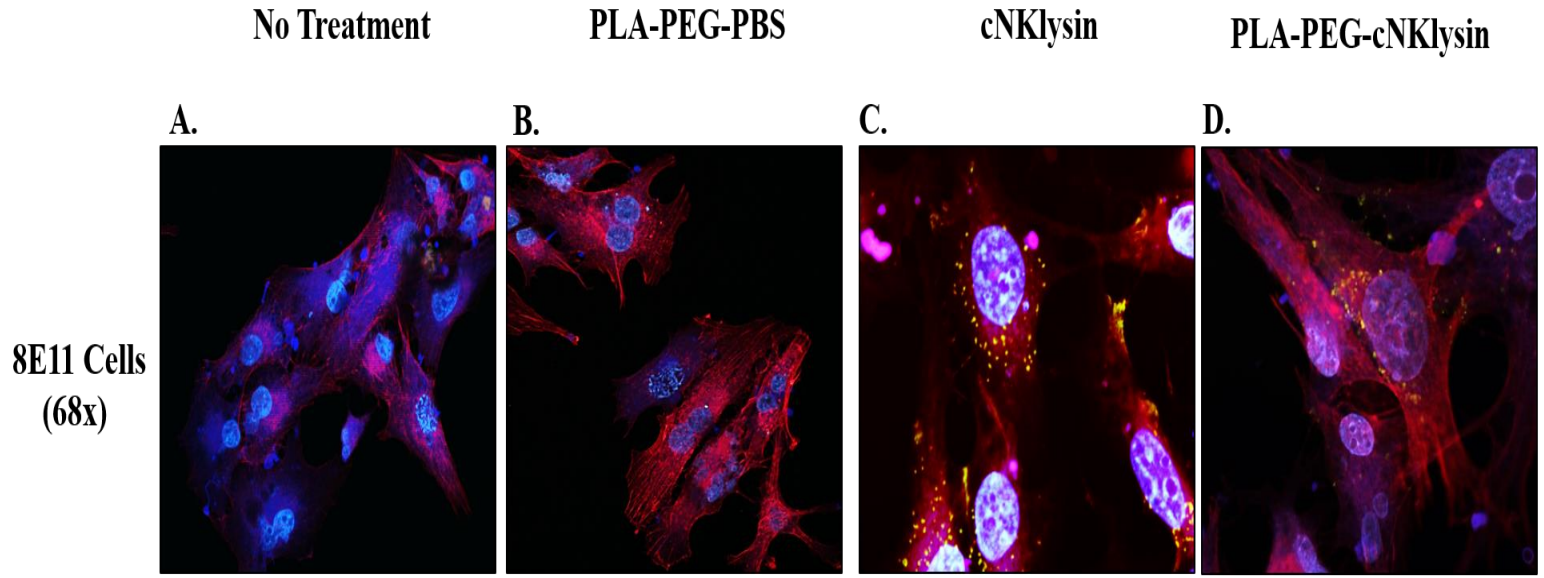
Shifted pattern in functional groups

Size ranged 85-120 nm and spherical in shape with smooth surface

Nanoencapsulation of cNK-2



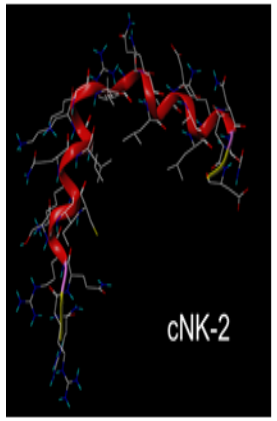




Chaudhari, Kim, and Lillehoj, unpublished data

Retaining immunomodulatory property and slow release

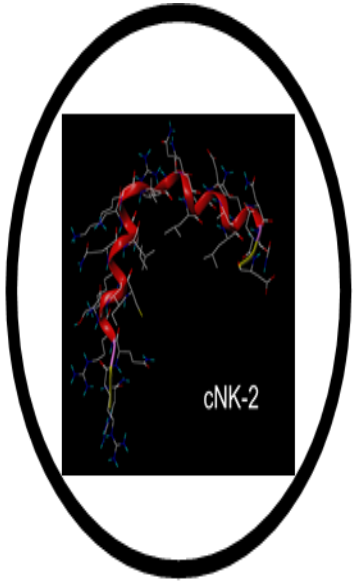
Nanoencapsulation of cNK-2



active cNK-2

- Antimicrobial
- Immunomdulatory
- Unstable in the body

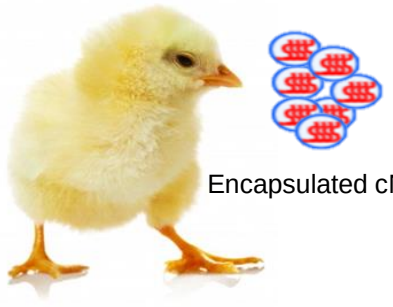
Encapsulation in nanoparticles



Encapsulated cNK-2

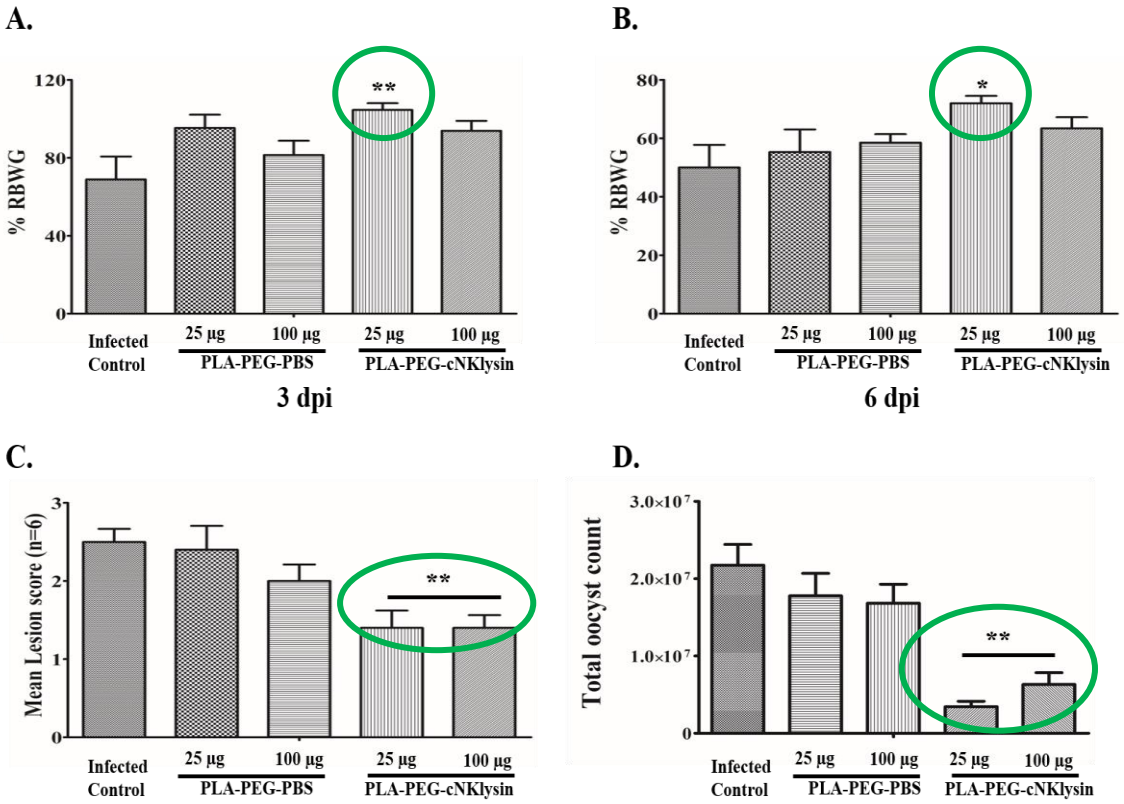
- Retaining functional properties
- Slow release
- Stability

Nanoencapsulation of cNK-2

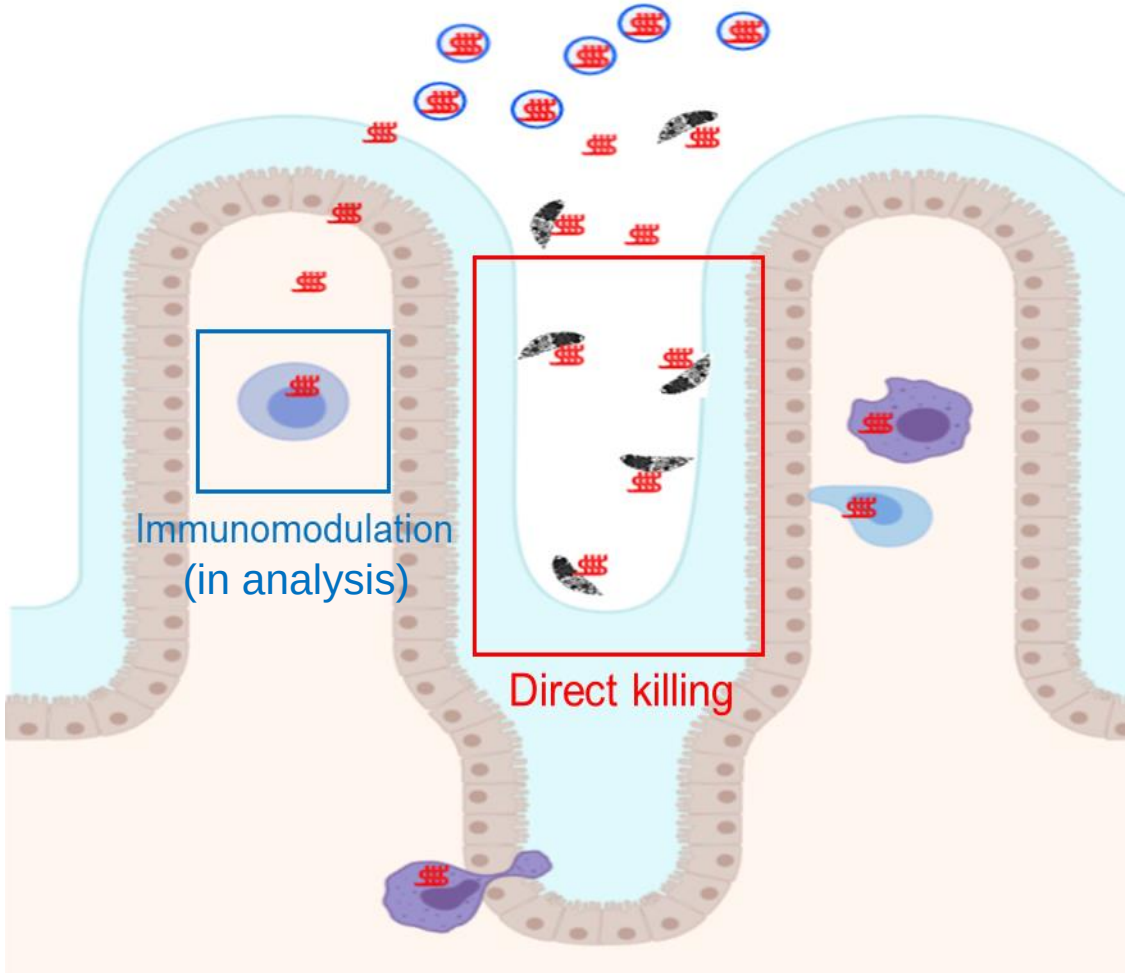


Encapsulated cNK-2

Eimeira spp.



Chaudhari, Kim, and Lillehoj, unpublished data



Summary

- Chicken NK-lysin has been identified as the most expressed gene in *Eimeria*-infected intestinal lymphocytes.
- Chicken NK-lysin derived peptide, cNK-2 has strong anti-coccidial effects as well as immunomodulation.
- Oral administration of nanoencapsulated cNK-2 increased growth performance and reduced intestinal lesion and oocyst shedding in coccidiosis.

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Thank you