Analysis of virulence and antibiotic resistance mechanisms of *Salmonella* and development of intervention strategies

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Focus of our research program

The effects of antibiotics on *Salmonella*

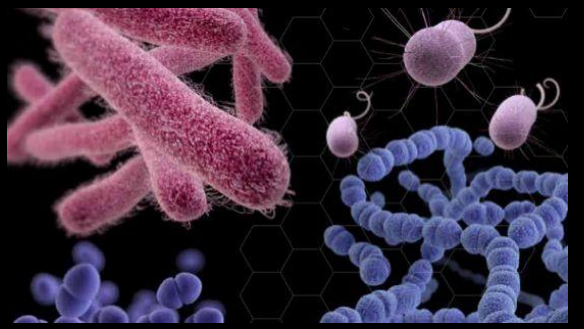
- Horizontal gene transfer by bacteriophage induction
- Activation of virulence mechanisms
- Enhanced resistance due to gene duplication and amplification

Alternatives to antibiotics and *Salmonella* interventions

- *Salmonella* DIVA vaccine
- Beneficial prebiotics & probiotics
- Feed additives

Antibiotics
The 20th century wonder drug

World Health Organization (WHO) considers bacterial resistance to antibiotics a serious and growing threat



World Health Organization
World Antibiotic Awareness Week
13-19 November 2017

Our time with
ANTIBIOTICS
is running out.

Antibiotics are in danger of losing their effectiveness due to misuse and overuse, and in many cases they aren't even needed.

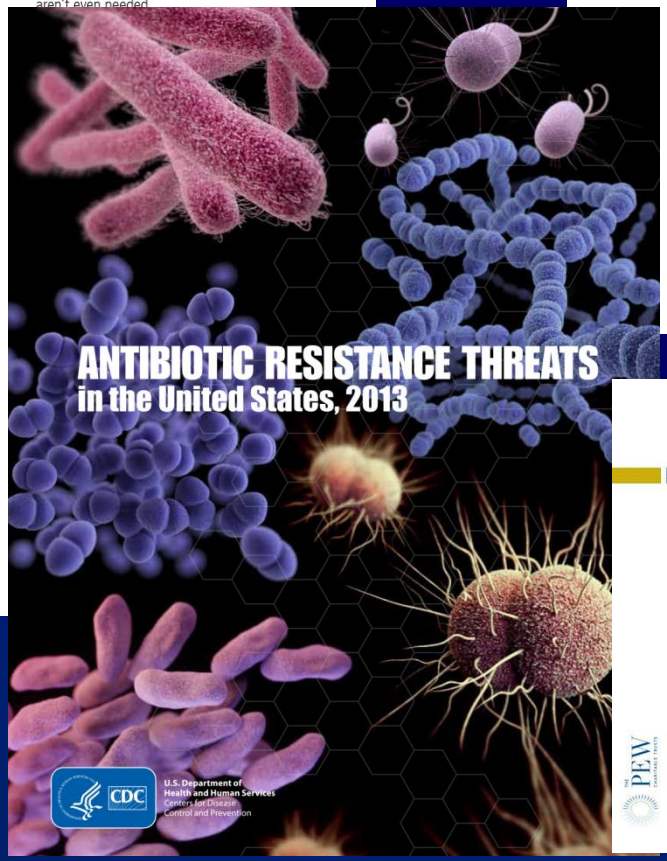
HANDLE ANTIBIOTICS WITH CARE

NATIONAL STRATEGY FOR COMBATING ANTIBIOTIC-RESISTANT BACTERIA

Vision: The United States will work domestically and internationally to prevent, detect, and control illness and death related to infections caused by antibiotic- resistant bacteria by implementing measures to mitigate the emergence and spread of antibiotic resistance and ensuring the continued availability of therapeutics for the treatment of bacterial infections.

September 2014

THE WHITE HOUSE
WASHINGTON



Regulatory—FDA

- Guidance #209, #213
  - Voluntary, no growth promoters after 2016
- Veterinary Feed Directive
  - Help move OTC to Rx

PEW

FDA

DRUG-RESISTANT NON-TYPHOIDAL SALMONELLA

THREAT LEVEL
SERIOUS

This bacteria is a serious concern and requires prompt and sustained action to ensure the problem does not grow.

100,000
DRUG-RESISTANT *SALMONELLA* INFECTIONS PER YEAR

1,200,000
SALMONELLA INFECTIONS PER YEAR

$365,000,000
IN MEDICAL COSTS PER YEAR

Non-typhoidal *Salmonella* (serotypes other than Typhi, Paratyphi A, Paratyphi B, and Paratyphi C) usually causes diarrhea (sometimes bloody), fever, and abdominal cramps. Some infections spread to the blood and can have life-threatening complications.

RESISTANCE OF CONCERN

Physicians rely on drugs, such as ceftriaxone and ciprofloxacin, for treating patients with complicated *Salmonella* infections. Resistant infections are more severe and have higher hospitalization rates. Non-typhoidal *Salmonella* is showing resistance to:

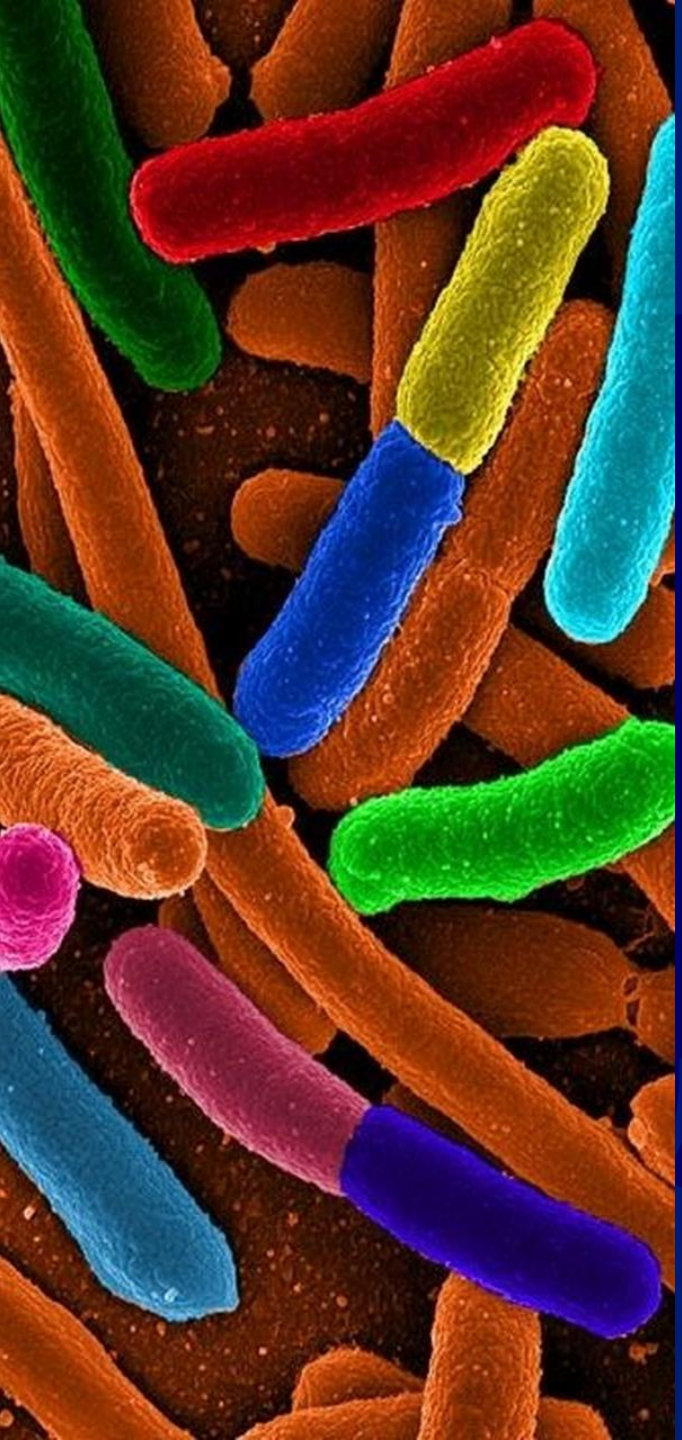
Annual estimations in the U.S.

1.2 million cases
23,000 hospitalizations
450 deaths
$365 million associated costs

	Percentage of all non-typhoidal *Salmonella**	Estimated number of illnesses per year	Estimated illnesses per 100,000 U.S. population	Estimated number of deaths per year
Ceftriaxone resistance	3%	36,000	12.0	13
Ciprofloxacin resistance or partial resistance	3%	33,000	10.9	12
Resistance to 5 or more antibiotic classes	5%	66,000	21.9	24
Any resistance pattern above	8%	100,000	34.1	38

*3-year average (2009–2011)
For more information about data methods and references, please see technical appendix.

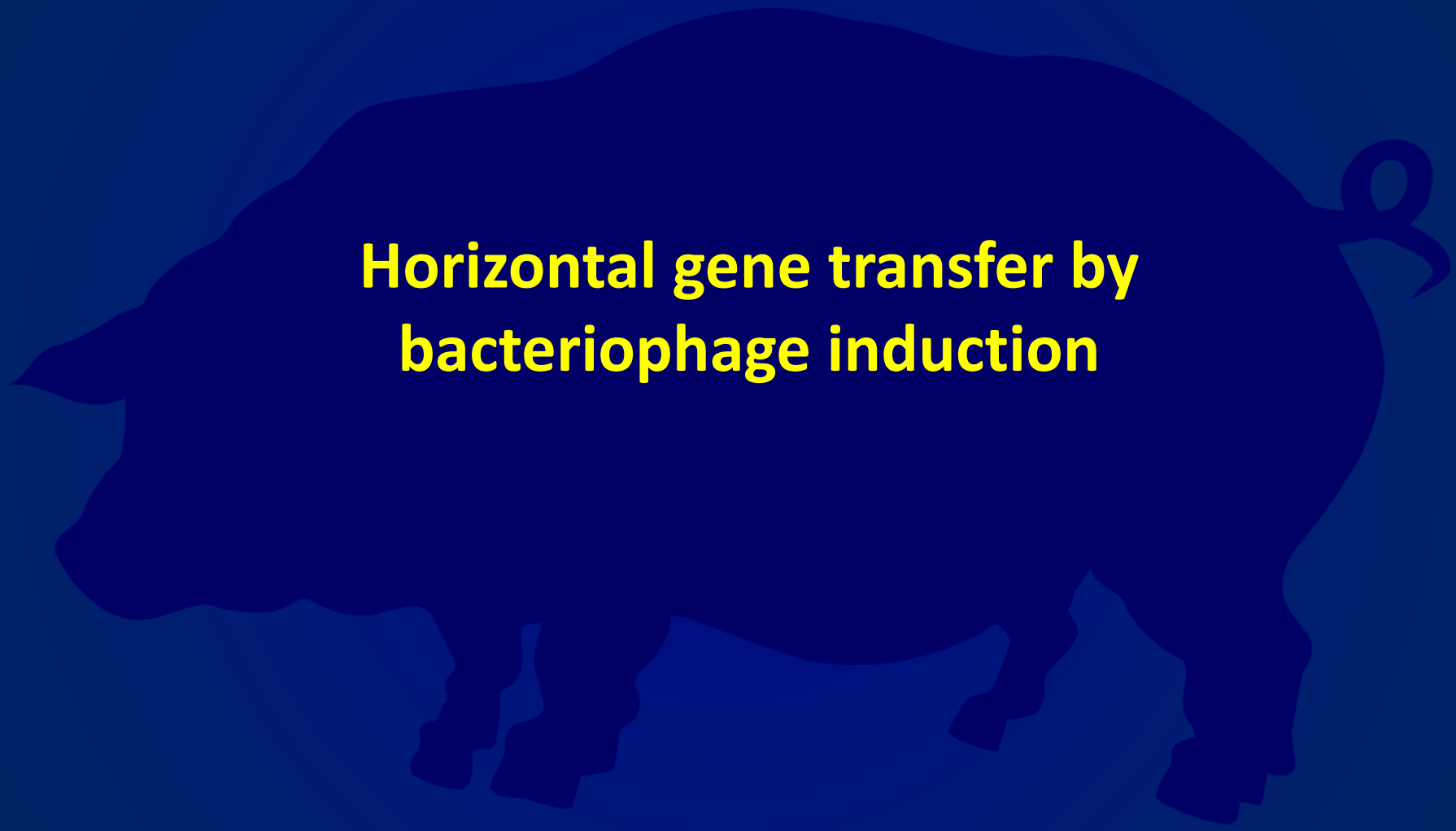
U.S. Department of Health and Human Services
Centers for Disease Control and Prevention



The effects of antibiotics on Salmonella

Horizontal gene transfer by bacteriophage induction

Activation of virulence mechanisms


Horizontal gene transfer by
bacteriophage induction

Carbadox in swine production

Carbadox is an antibiotic fed to 1/3 of nursery age pigs in the U.S. for the control of enteric diseases in swine at weaning, e.g., *Brachyspira hyodysenteriae*

Carbadox is mutagenic and has a 42 day withdrawal period prior to slaughter

It has no analog in humans; unclear if or how it will be regulated in the future

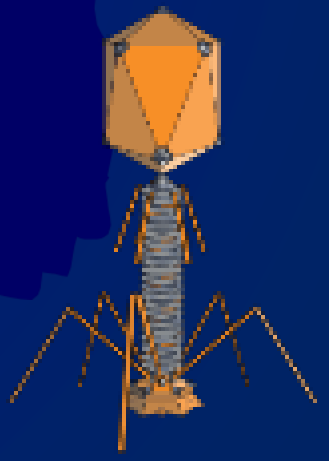
Carbadox activates bacteriophage-induced cell lysis and phage release in *Salmonella*

What is a bacteriophage (aka, prophage or phage)?

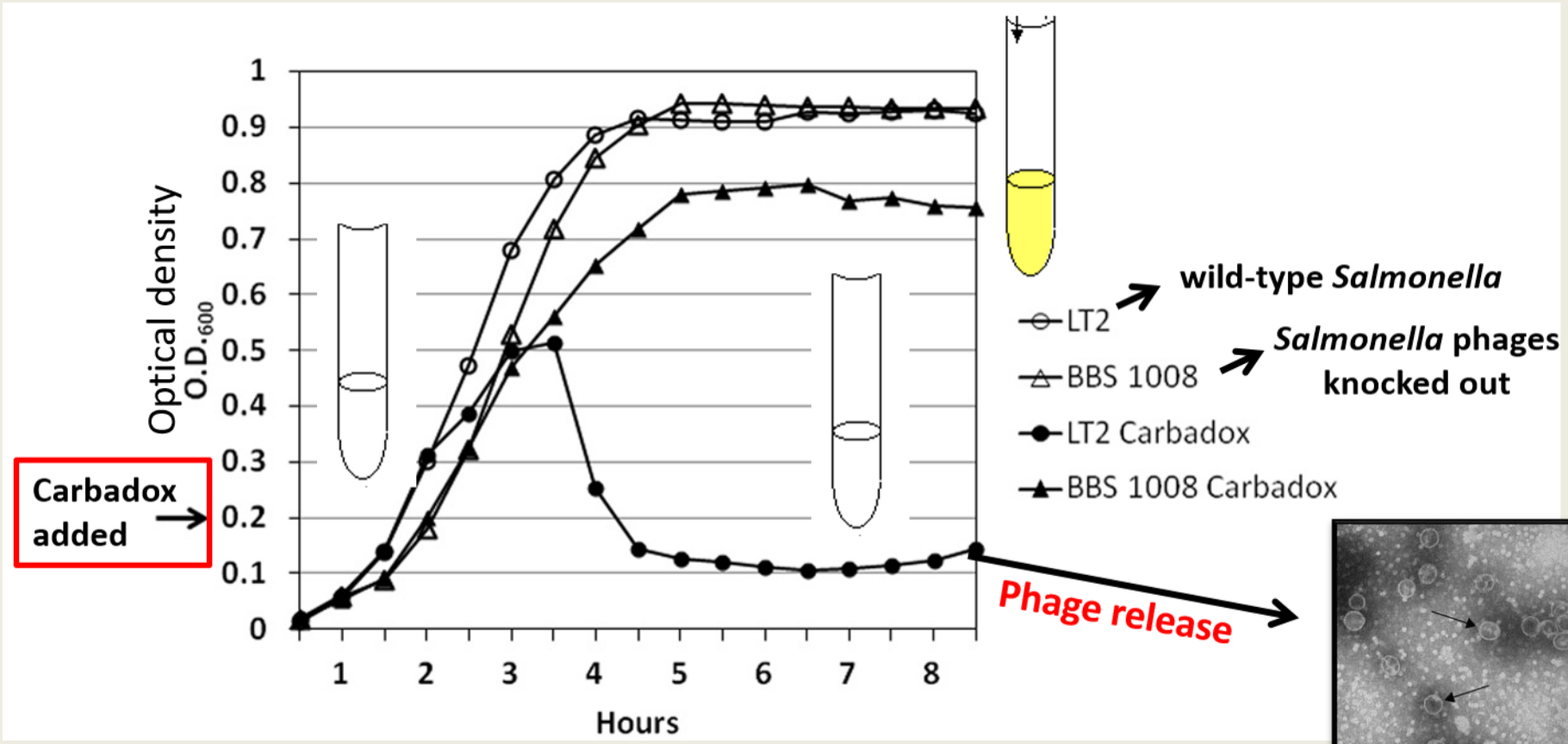
- Viruses that infect and replicate within bacteria; very common and very diverse

Many *Salmonella* contain multiple prophage

- Some are inducible (e.g. stress response)
- Some can package and transfer host DNA (generalized transduction)



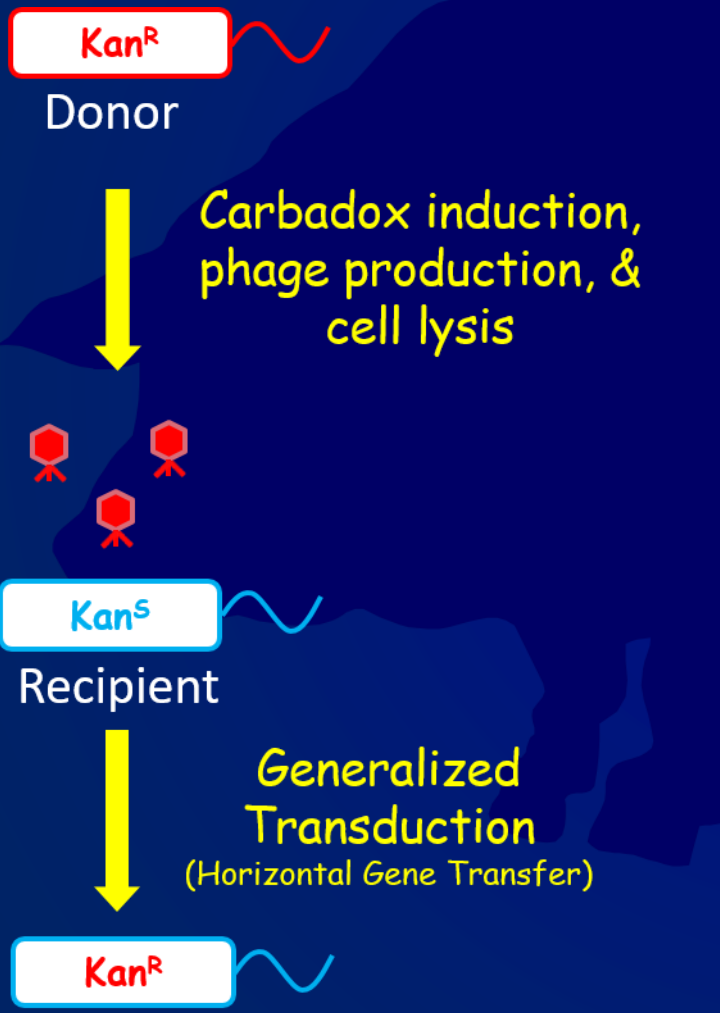
Carbadox activates bacteriophage-induced cell lysis and phage release in *Salmonella (2)*



2.5 µg/ml (ppm)

Bearson, Allen et al. *Frontiers in Microbiology* 5:52.

Carbadox induces phage-mediated, horizontal gene transfer of virulence genes and antibiotic resistance genes in multidrug-resistant (MDR) *S.* Typhimurium



Generalized Transduction

Salmonella Donor Strain	Not Induced	Carbadox Induced
LT2	0	0
UK1	0	0
SL1344	0	0
χ4232	0	0
DT104-NCTC13348	0	6 ± 1.7
DT104-530	0	291 ± 100.8
DT104b-5414	<1	117 ± 52.8
DT120-150	0	124 ± 19.3
DT120-305	<1	420 ± 94.8
DT120-613	<1	86 ± 16.7
DT193-1434	0	0
DT208-2348	0	0

Multidrug-resistant *S.* Typhimurium

Average frequency of generalized transduction per 0.5 ml of lysate from numerous *S.* Typhimurium donor strains into BBS 243.

Do other *Salmonella* serovars contain generalized transducing phage capable of horizontal gene transfer?

DNA sequence analysis suggests yes:

Arizonae*, Cholerae, Choleraesuis*, Dublin, Hadar, Heidelberg*, Houtenae, Johannesburg, Mississippi, Montevideo, Newport, Paratyphi A*, Pullorum*, Rubislaw, Schwarzengrund, Tennessee, Typhimurium*, Uganda, Wandsworth and Welteverden

Based on genome sequence scanning by Sherwood Casjens and/or genome alignments to Prophage 1 *int* in DT104 using BioCyc*

Can other antibiotics induce phage-mediated gene transfer from MDR *Salmonella*?

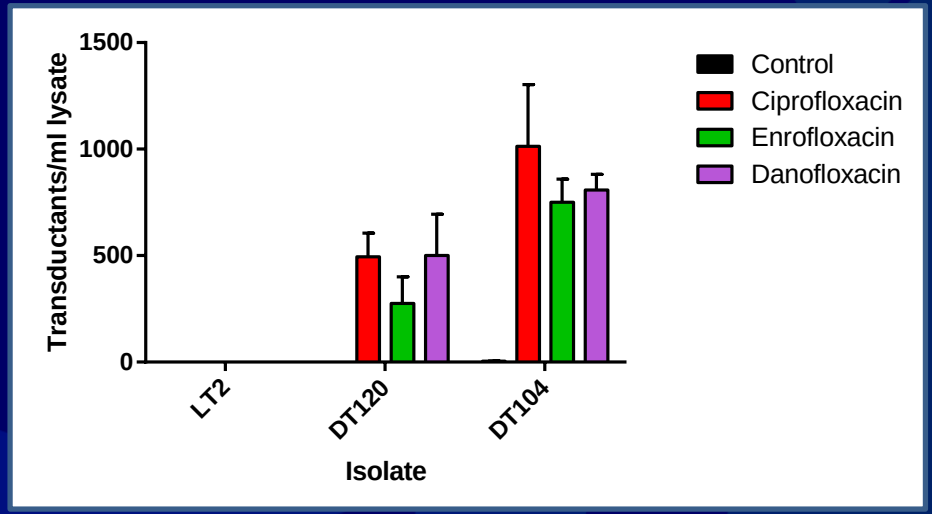
Fluoroquinolones - broad spectrum antibiotic

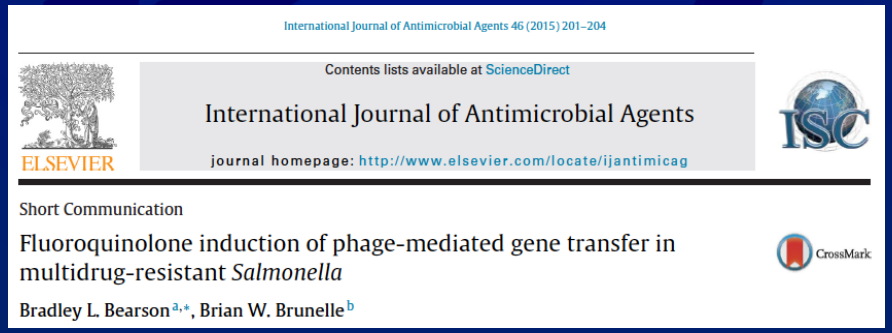
Human Medicine

- Ciprofloxacin

Veterinary respiratory diseases

- Enrofloxacin (Baytril®)
- Danofloxacin (Advocin™)

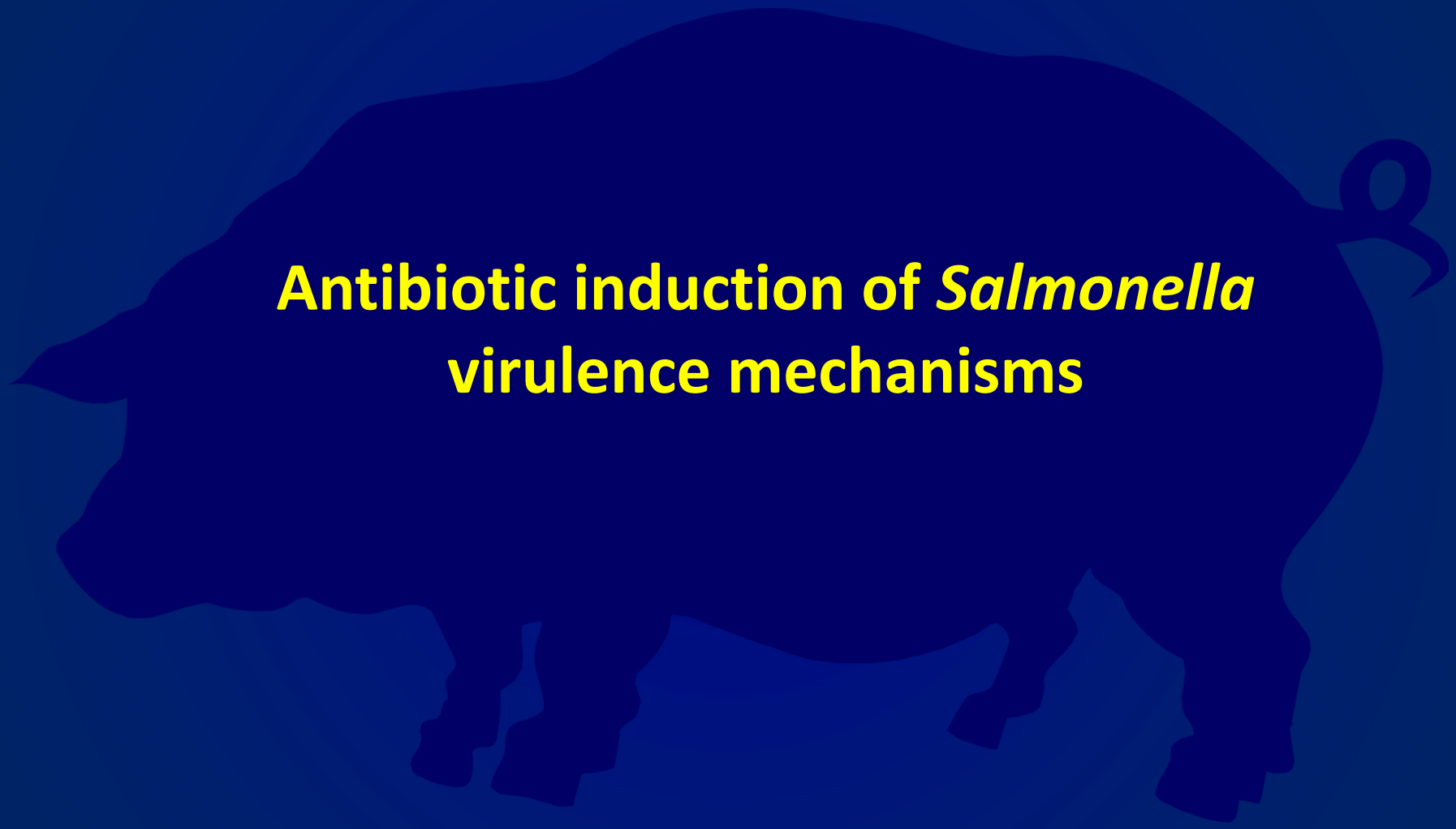



International Journal of Antimicrobial Agents 46 (2015) 201–204
Contents lists available at ScienceDirect
International Journal of Antimicrobial Agents
journal homepage: http://www.elsevier.com/locate/ijantimicag
ELSEVIER
ISC
Short Communication
Fluoroquinolone induction of phage-mediated gene transfer in multidrug-resistant *Salmonella*
CrossMark
Bradley L. Bearson[a,*], Brian W. Brunelle[b]

The Take-Home Message

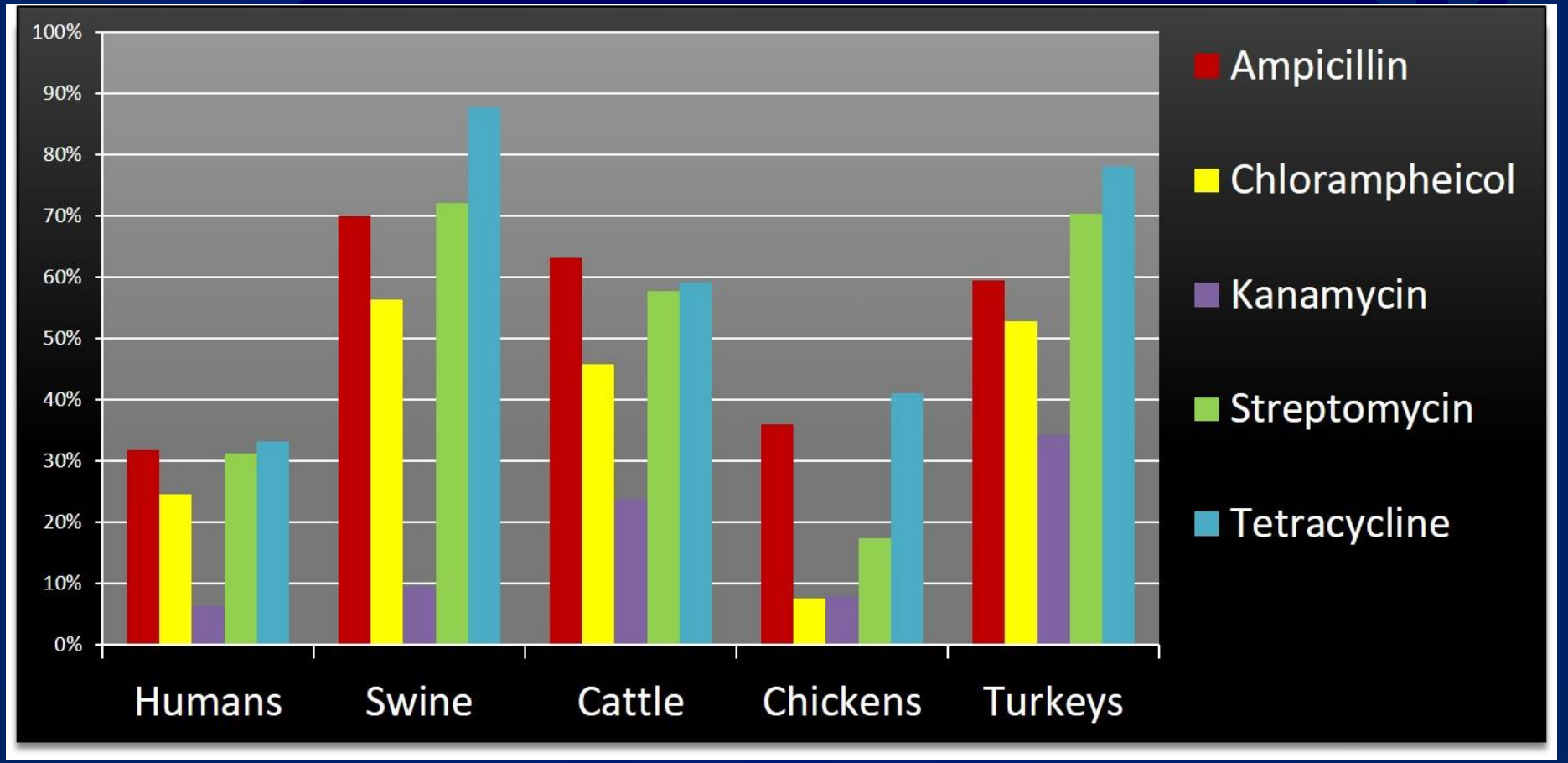
Certain *Salmonella* strains including MDR S. Typhimurium contain generalized transducing prophage capable of packaging and transducing *Salmonella* DNA

Transfer of antibiotic resistance, virulence, or other fitness genes

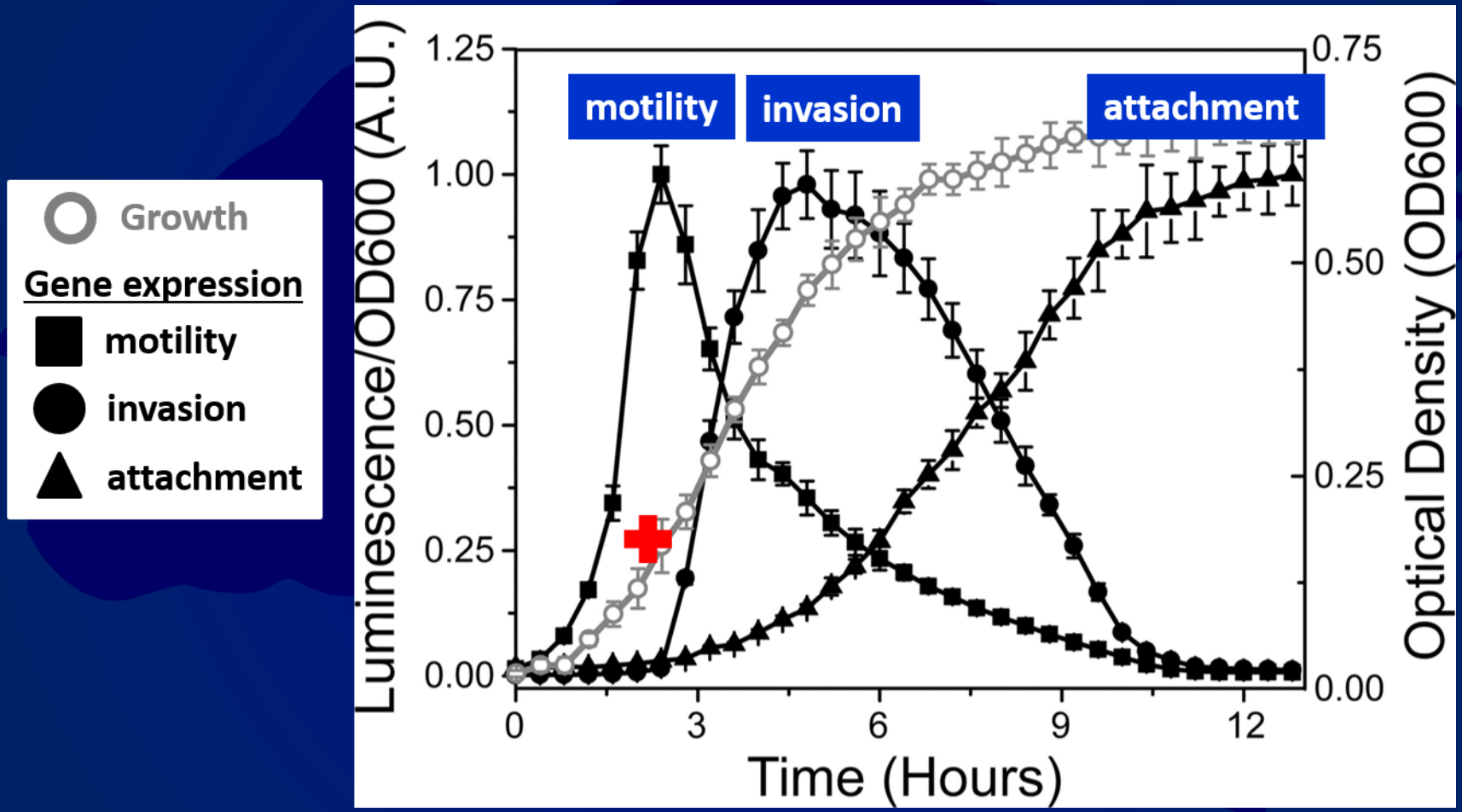


Antibiotic induction of *Salmonella* virulence mechanisms

National Antimicrobial Resistance Monitoring System (NARMS): 12-year average of *Salmonella* antibiotic resistance in humans and animal commodities



Virulence gene regulation *in vitro*



Saini et al. 2010. J. Bacteriol. 192(21):5767-5777

What are the exposure effects of tetracycline/chlortetracycline on MDR *Salmonella?*

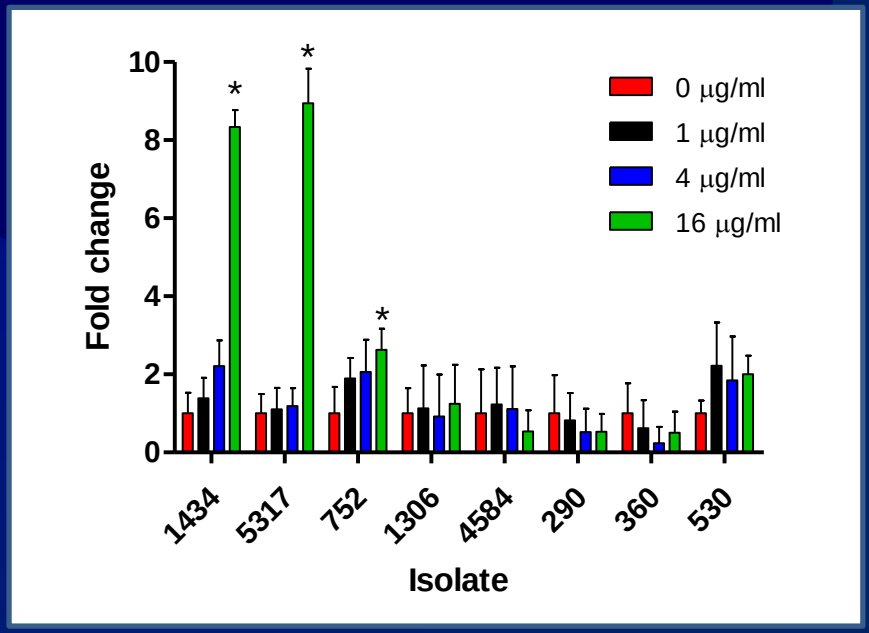
Invasion

In early log-phase growth when *Salmonella* is typically non-invasive, tetracycline induces invasion gene expression and accelerates cellular invasion in some MDR *Salmonella*.

Induction of invasion gene expression (37 genes)

	Tetracycline			
Gene	1434	5317	752	530
avrA	6.0	8.2	*1.2*	2.6
hilA	13.4	14.6	*1.4*	4.0
hilC	*-1.5*	*1.1*	-9.5	-2.5
hilD	6.1	5.6	*1.2*	3.4
iagB	20.2	17.0	3.3	6.4
invA	9.4	11.2	2.7	3.5
invB	8.1	5.9	*1.0*	3.2
invC	10.6	11.4	3.2	3.1
invE	26.4	16.0	*1.3*	1.7
invF	18.9	25.5	*1.6*	1.7
invG	14.5	12.3	*1.1*	*1.4*
invH	6.7	4.8	*-1.3*	2.6
invI	18.1	11.0	2.7	4.9
invJ	12.1	13.4	2.5	2.7
orgAa	10.4	10.2	3.5	4.6
orgAb	2.4	*1.9*	*1.5*	*1.4*
prgH	5.9	6.5	*1.5*	2.5
prgI	3.0	2.0	-2.2	*-1.2*
prgJ	2.8	2.7	-1.7	*-1.2*

Early log invasion of Hep-2 cells



Brunelle, Bearson et al. *BMC Microbiology* 13:202.

What are the effects of tetracycline exposure on MDR *Salmonella?*

Attachment

Fimbriae are appendages involved in attachment
13 fimbrial operons in *Salmonella* Typhimurium

Only 3 are considered to be inducible *in vitro*

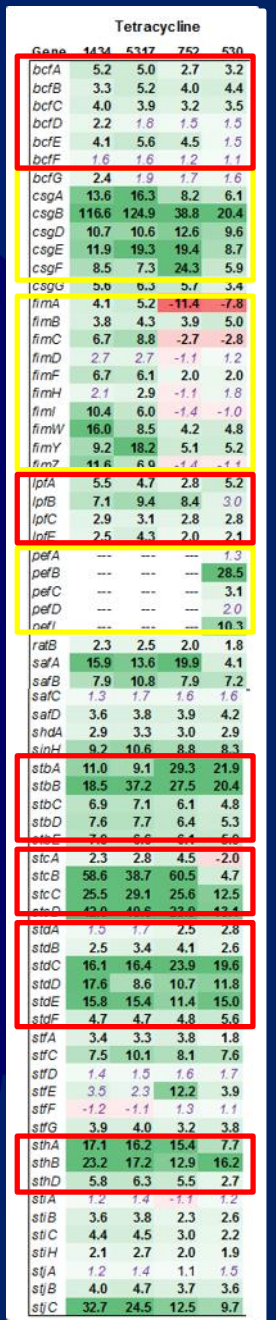
- *csg, fim,* and *pef*

6 are associated with persistence in mice

- *bcf, lpf, stb, stc, std,* and *sth*

Tetracycline induced 10 operons

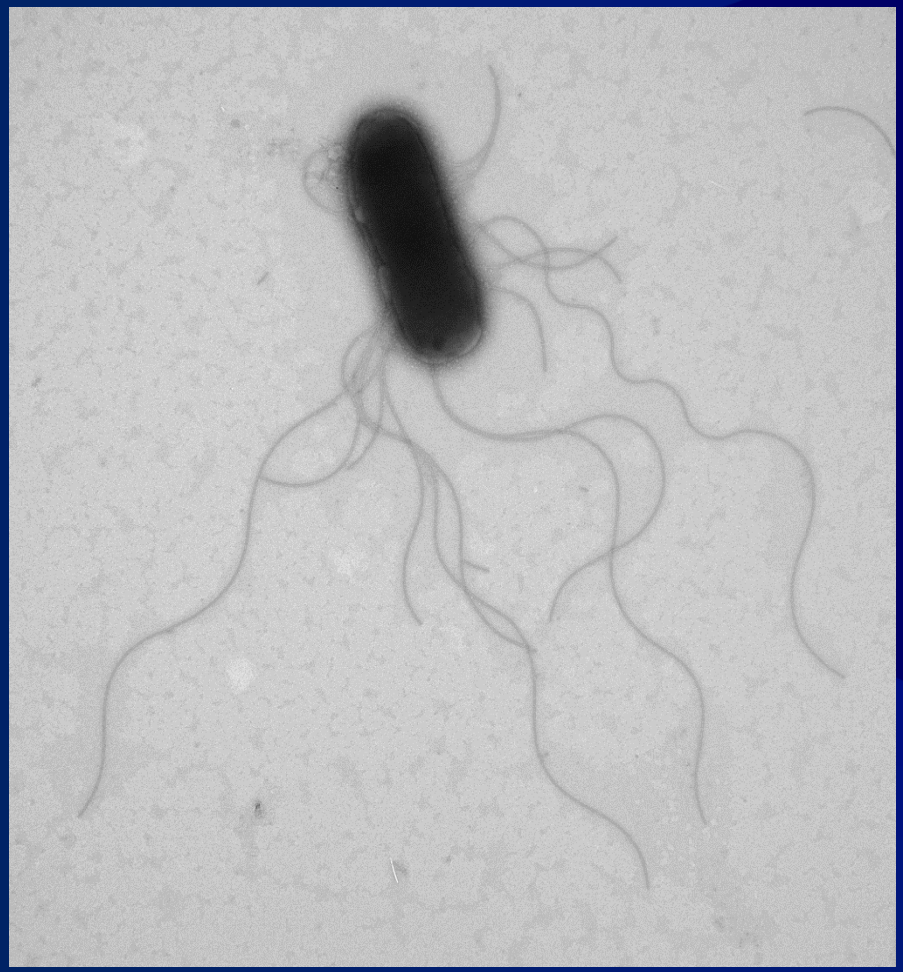
- *bcf, csg, fim, lpf, pef, saf, stb, stc, std,* and *sth*

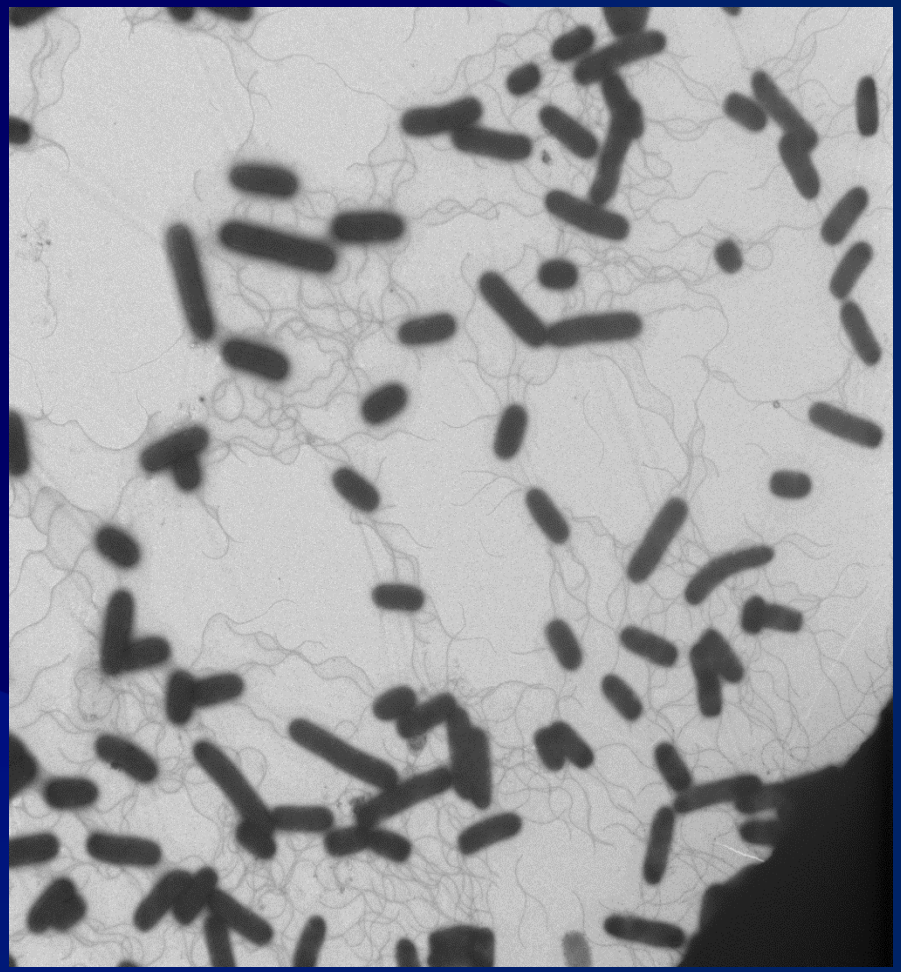


Tetracycline				
Gene	1434	5317	752	530
bcfA	5.2	5.0	2.7	3.2
bcfB	3.3	5.2	4.0	4.4
bcfC	4.0	3.9	3.2	3.5
bcfD	2.2	1.8	1.5	1.5
bcfE	4.1	5.6	4.5	1.5
bcfF	1.6	1.6	1.2	1.1
bcfG	2.4	1.9	1.7	1.6
csgA	13.6	16.3	8.2	6.1
csgB	116.6	124.9	38.8	20.4
csgD	10.7	10.6	12.6	9.6
csgE	11.9	19.3	19.4	8.7
csgF	8.5	7.3	24.3	5.9
csgG	5.6	6.3	5.7	3.4
fimA	4.1	5.2	-11.4	-7.8
fimB	3.8	4.3	3.9	5.0
fimC	6.7	8.8	-2.7	-2.8
fimD	2.7	2.7	-1.1	1.2
fimF	6.7	6.1	2.0	2.0
fimH	2.1	2.9	-1.1	1.8
fimI	10.4	6.0	-1.4	-1.0
fimW	16.0	8.5	4.2	4.8
fimY	9.2	18.2	5.1	5.2
fimZ	11.6	6.9	-1.4	-1.1
lpfA	5.5	4.7	2.8	5.2
lpfB	7.1	9.4	8.4	3.0
lpfC	2.9	3.1	2.8	2.8
lpfE	2.5	4.3	2.0	2.1
pefA	---	---	---	1.3
pefB	---	---	---	28.5
pefC	---	---	---	3.1
pefD	---	---	---	2.0
pefI	---	---	---	10.3
ratB	2.3	2.5	2.0	1.8
safA	15.9	13.6	19.9	4.1
safB	7.9	10.8	7.9	7.2
safC	1.3	1.7	1.6	1.6
safD	3.6	3.8	3.9	4.2
shdA	2.9	3.3	3.0	2.9
sinH	9.2	10.6	8.8	8.3
stbA	11.0	9.1	29.3	21.9
stbB	18.5	37.2	27.5	20.4
stbC	6.9	7.1	6.1	4.8
stbD	7.6	7.7	6.4	5.3
stbE	7.0	6.6	6.1	5.0
stcA	2.3	2.8	4.5	-2.0
stcB	58.6	38.7	60.5	4.7
stcC	25.5	29.1	25.6	12.5
stcD	13.0	10.6	23.0	13.1
stdA	1.5	1.7	2.5	2.8
stdB	2.5	3.4	4.1	2.6
stdC	16.1	16.4	23.9	19.6
stdD	17.6	8.6	10.7	11.8
stdE	15.8	15.4	11.4	15.0
stdF	4.7	4.7	4.8	5.6
stfA	3.4	3.3	3.8	1.8
stfC	7.5	10.1	8.1	7.6
stfD	1.4	1.5	1.6	1.7
stfE	3.5	2.3	12.2	3.9
stfF	-1.2	-1.1	1.3	1.1
stfG	3.9	4.0	3.2	3.8
sthA	17.1	16.2	15.4	7.7
sthB	23.2	17.2	12.9	16.2
sthD	5.8	6.3	5.5	2.7
stiA	1.2	1.4	-1.1	1.2
stiB	3.6	3.8	2.3	2.6
stiC	4.4	4.5	3.0	2.2
stiH	2.1	2.7	2.0	1.9
stjA	1.2	1.4	1.1	1.5
stjB	4.0	4.7	3.7	3.6
stjC	32.7	24.5	12.5	9.7

Brunelle, Bearson et al. *Frontiers in Microbiology* 5:801.

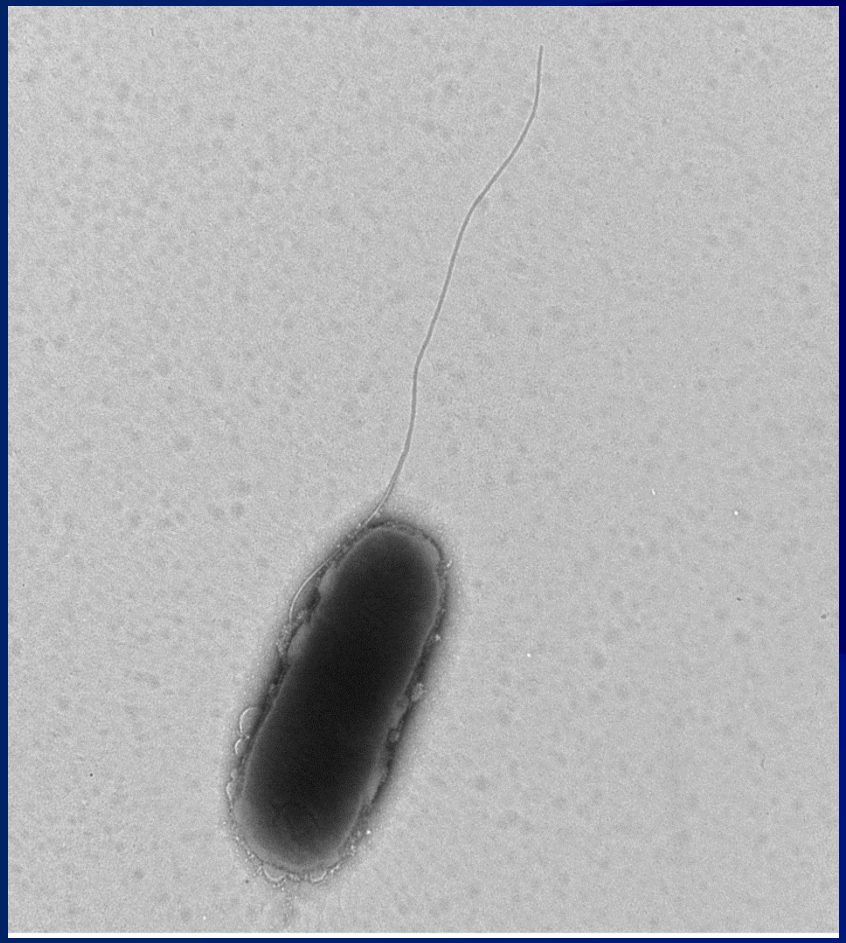
Electron Microscopy: no Tetracycline

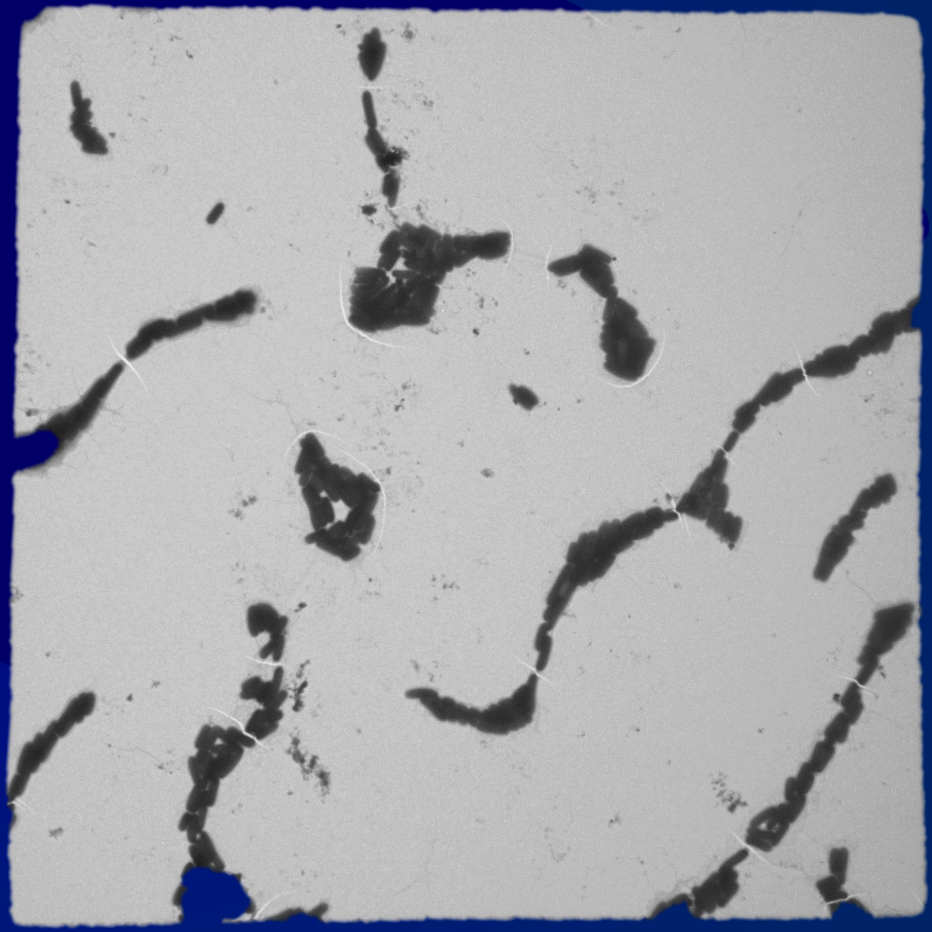




1434, no tetracycline

Electron Microscopy: with Tetracycline





1434 16 ug/ml tetracycline

The Take-Home Message

Effects of tetracycline/chlortetracycline exposure on MDR *Salmonella*:

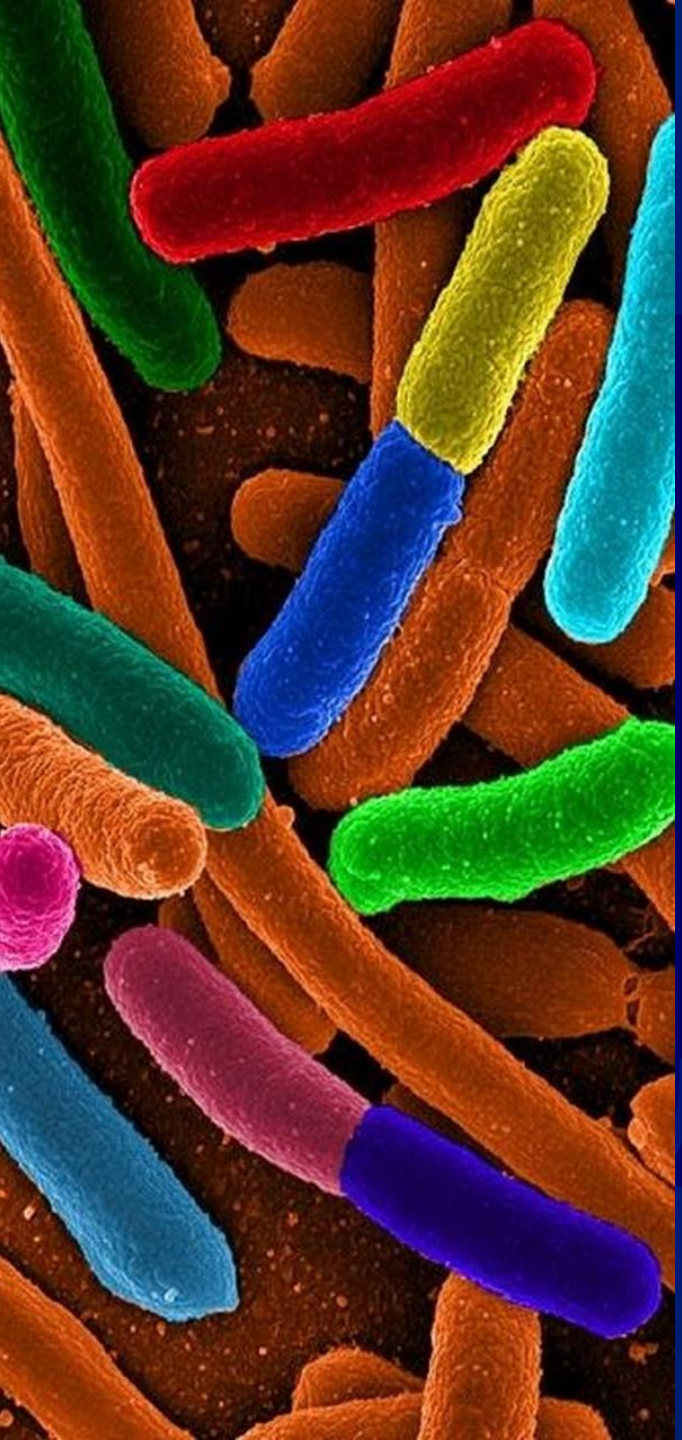
- Differentially regulated ~3,000 genes
- Accelerated progression of pathways that normally take 8+ hours, including induced invasion and attachment during early-log growth
- Activated ten fimbriae operons

Tetracycline-inducible invasion phenotype:

Provides MDR *Salmonella* with an additional competitive advantage that influences colonization potential and may be associated with increased morbidity among patients with MDR *Salmonella* infections

Our goal of investigating the effects of antibiotics on MDR *Salmonella* is to identify and characterize antibiotics that stimulate bacterial virulence mechanisms (as well as those that do not).

This information will assist veterinarians and food animal producers when determining the proper antibiotic therapy for infectious disease treatment that does not unintentionally complicate an illness and compromise animal management.



Alternatives to Antibiotics and *Salmonella* interventions

Salmonella DIVA vaccine

Beneficial prebiotics & probiotics

Feed additives

Identify on-farm intervention strategies to reduce the human foodborne pathogen *Salmonella* in food animals

Livestock and poultry colonized with *Salmonella* are often asymptomatic carriers of the human pathogen.

Salmonella-carrier animals can enter the food chain, contaminating slaughter plants and meat products.

These animals have the potential to shed the pathogen in their feces, resulting in unrecognized disease transmission/spread and contamination of the environment.


Salmonella DIVA vaccine

The *Salmonella* challenge

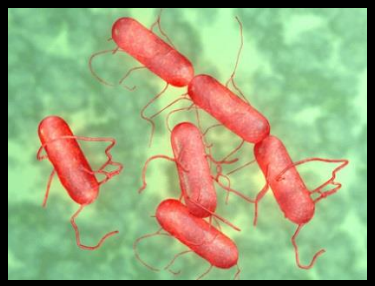
>2,500 serovars

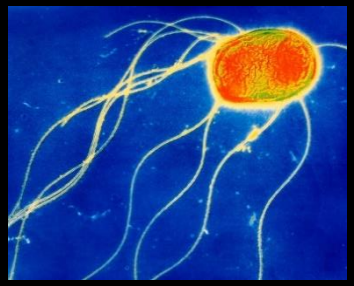
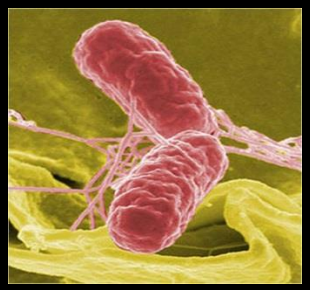
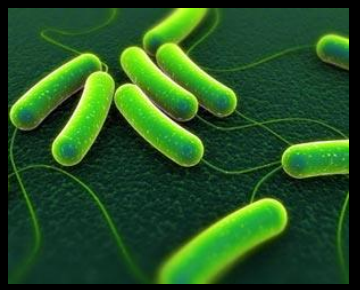
Ubiquitous in nature

Wide host range

Gastrointestinal to Systemic disease

Livestock are often asymptomatic carriers



Limitations of current *Salmonella* vaccines

Serovar-specific protection

Vaccination may interfere with *Salmonella* surveillance programs to identify *Salmonella*-positive herds or flocks

Our vaccine goal:

Attenuated *S.* Typhimurium strain

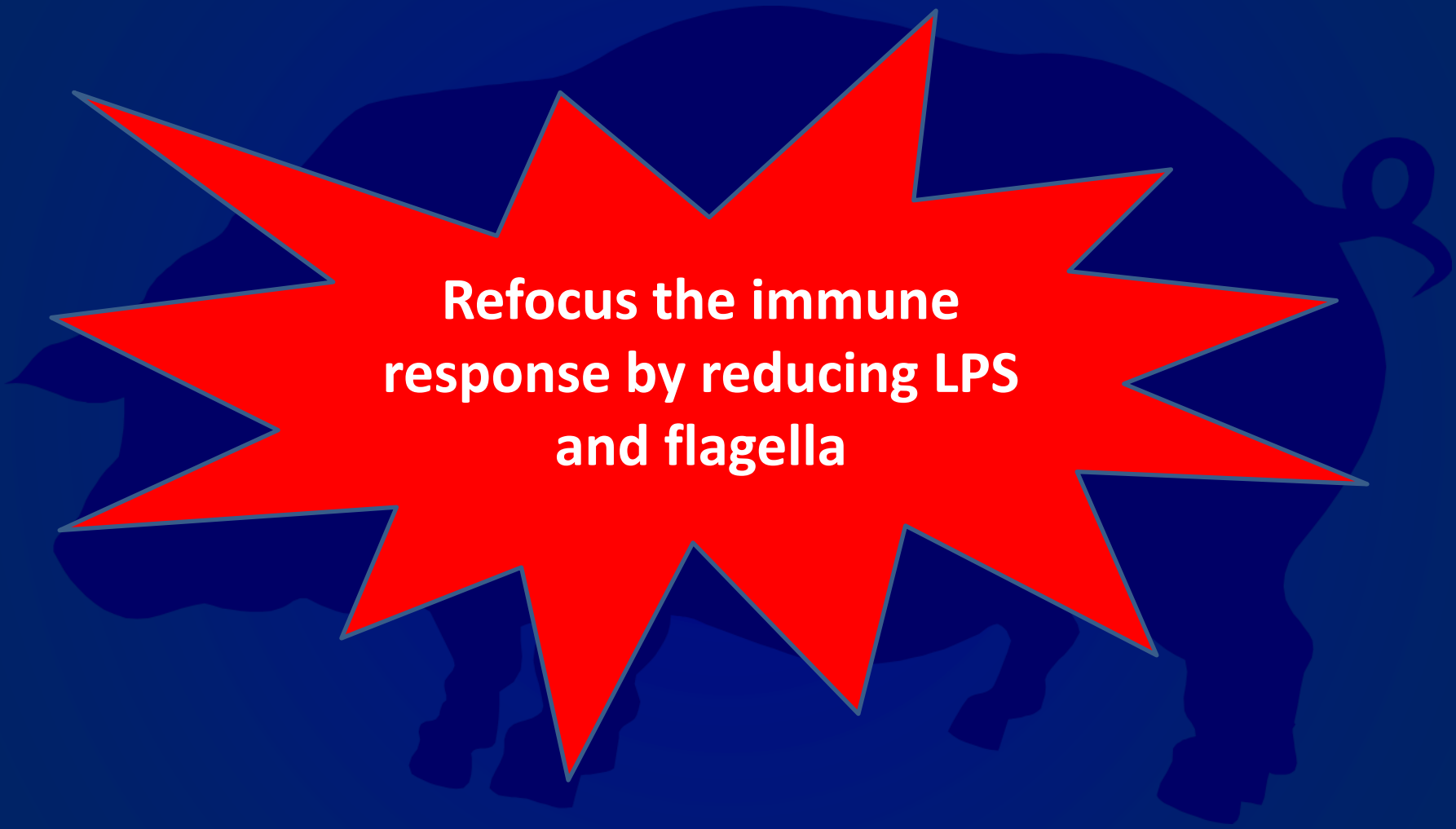
Reduction in disease and colonization

Cross-protection against multiple serovars

DIVA (Differentiation of Infected from Vaccinated Animals)

Reduce the variable, immunodominant *Salmonella* antigens

Increase conserved, *Salmonella* outer membrane proteins


Refocus the immune
response by reducing LPS
and flagella

Reducing serovar specific immunity: part 1

Decrease variable, immunodominant antigens

RfaH antiterminator

RfaH prevents premature transcription termination of long mRNA operons, including immunodominant:

- LPS (lipopolysaccharide)
- Flagellar/chemotaxis

Differentiate the >2,500 *Salmonella* serovars by their differences in LPS and flagella

Kauffman and White classification scheme

frontiers in VETERINARY SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 09 October 2014
doi: 10.3389/fvets.2014.00009

An *rfaH* mutant of *Salmonella enterica* serovar Typhimurium is attenuated in swine and reduces intestinal colonization, fecal shedding, and disease severity due to virulent *Salmonella* Typhimurium

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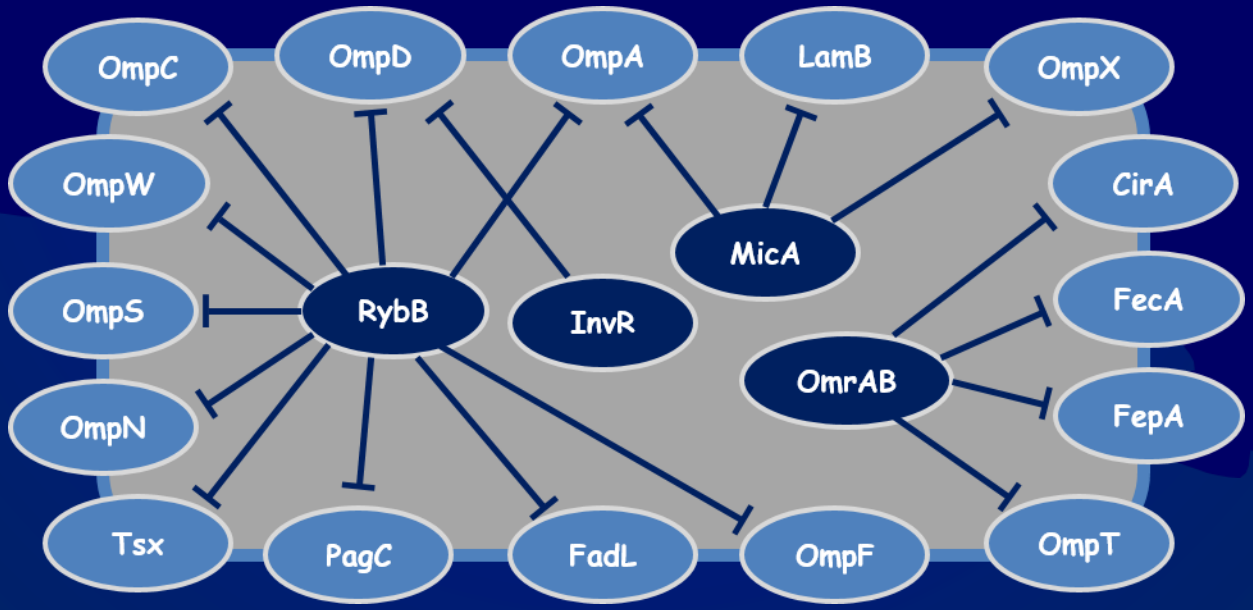
Reducing serovar specific immunity: part 2

Increase conserved outer membrane proteins

Small Regulatory RNA (sRNA)

- 50-250 nucleotides in length
- Generally untranslated RNA
- Encoded in intergenic regions
- Regulate their targets at post-transcriptional level



Adapted from Vogel. 2009. Mol. Microbiol. 71:1-11.

Salmonella Typhimurium BBS 866 live vaccine in swine

Attenuated in pigs

Differentiation of infected from vaccinated animals (DIVA)

Cross-protective against wild-type challenge

Choleraesuis (systemic disease) and Typhimurium (gastrointestinal disease)

- Reduced clinical disease (body temperature and diarrhea)
- Reduced fecal shedding (2-log reduction in ST)
- Reduced tissue colonization (1-2 log reduction in ST & SC)
- Reduced bacteremia (blood culture, spleen, liver in SC)
- Increased average daily gain (SC)

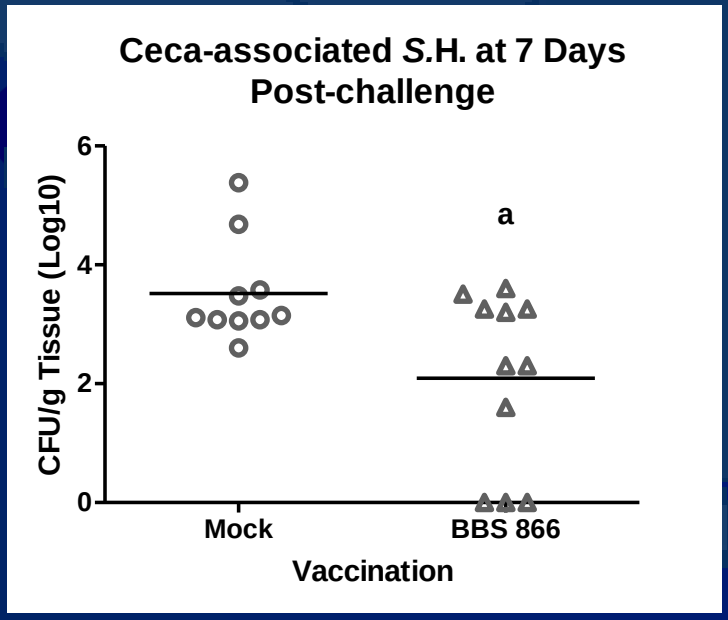
Vaccine. 2016. 34:1241-6.
Journal Medical Microbiology. 2017. 66:651-661.

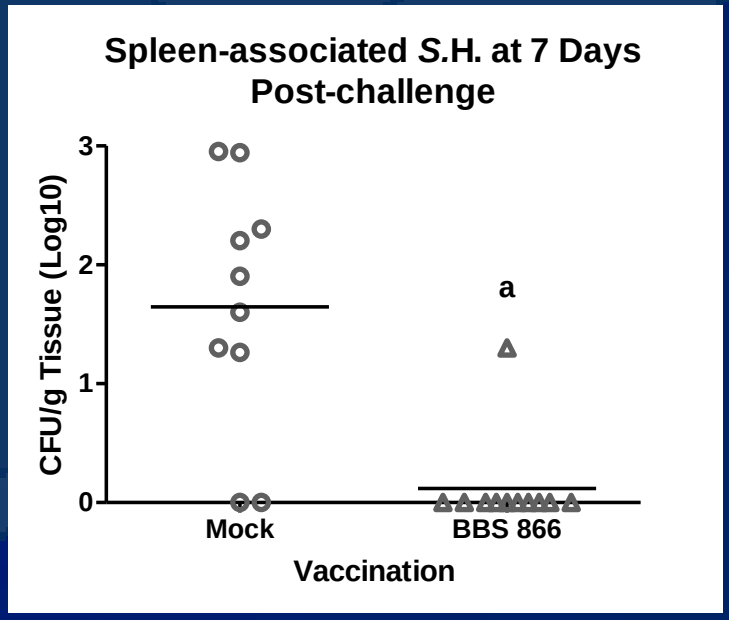
Vaccination significantly decreased *Salmonella* Heidelberg isolation from the spleen and ceca in turkeys

2 days old → Vaccinate (10^9)

2 weeks old → Booster vaccinate (10^9)

5 weeks old → MDR Heidelberg challenge (10^9)





The Take-Home Message (2)

Salmonella Typhimurium BBS 866 live vaccine

Attenuated in pigs & turkeys

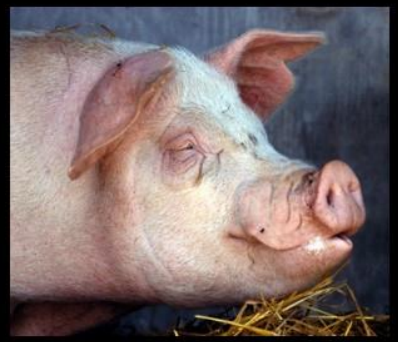
Differentiation of infected from vaccinated animals (DIVA)

Cross-protective against wild-type challenge Cholerasuis (pigs) and Heidelberg (turkeys)

Vaccine was exclusively licensed by Huvepharma in August 2017

U.S. patent was issued for the vaccine in January 2018

Analyzing the porcine intestinal microbiota in response to *Salmonella* in search for potential swine-specific probiotics



Low versus High *Salmonella* shedding in pigs

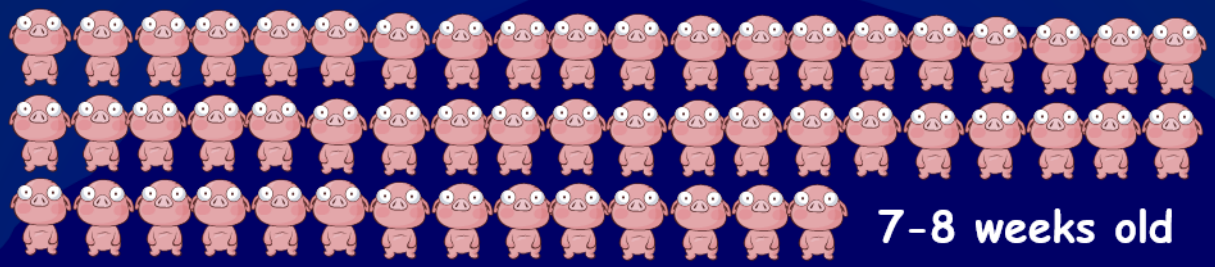
On the farm, as well as within experimental infections, the shedding of *Salmonella* from infected pigs varies from low-shedding to persistently high-shedding.

Are there differences between the microbiota composition in the gastrointestinal tract of "will be" Low-shedders and "will be" High-shedders prior to inoculation with *Salmonella* that could influence *Salmonella* colonization and persistence?

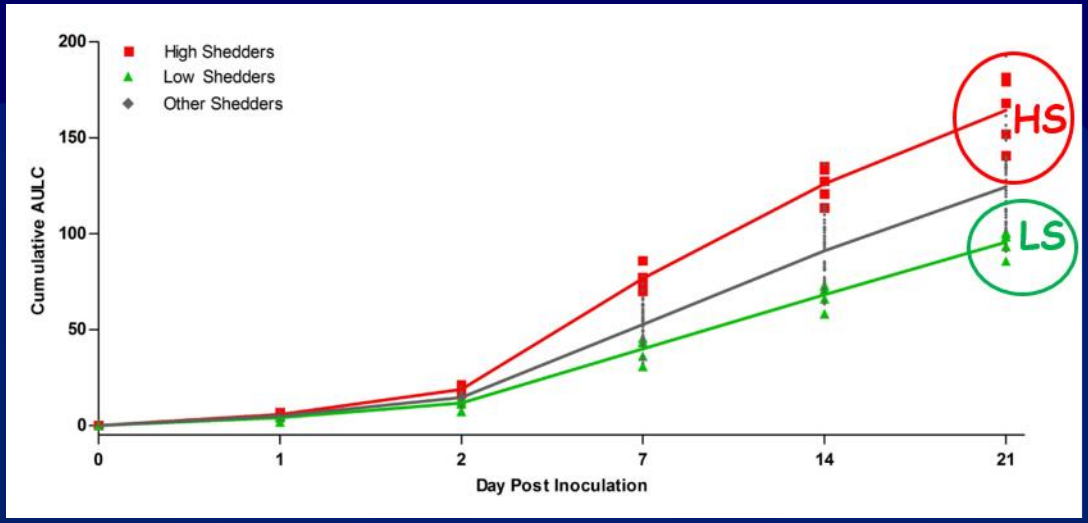
What microbiota alterations occur after introducing *Salmonella* into the GI tract and do the microbiota changes correlate with *Salmonella* shedding status?

Salmonella shedding in swine

Salmonella enterica serovar Typhimurium (1 billion)

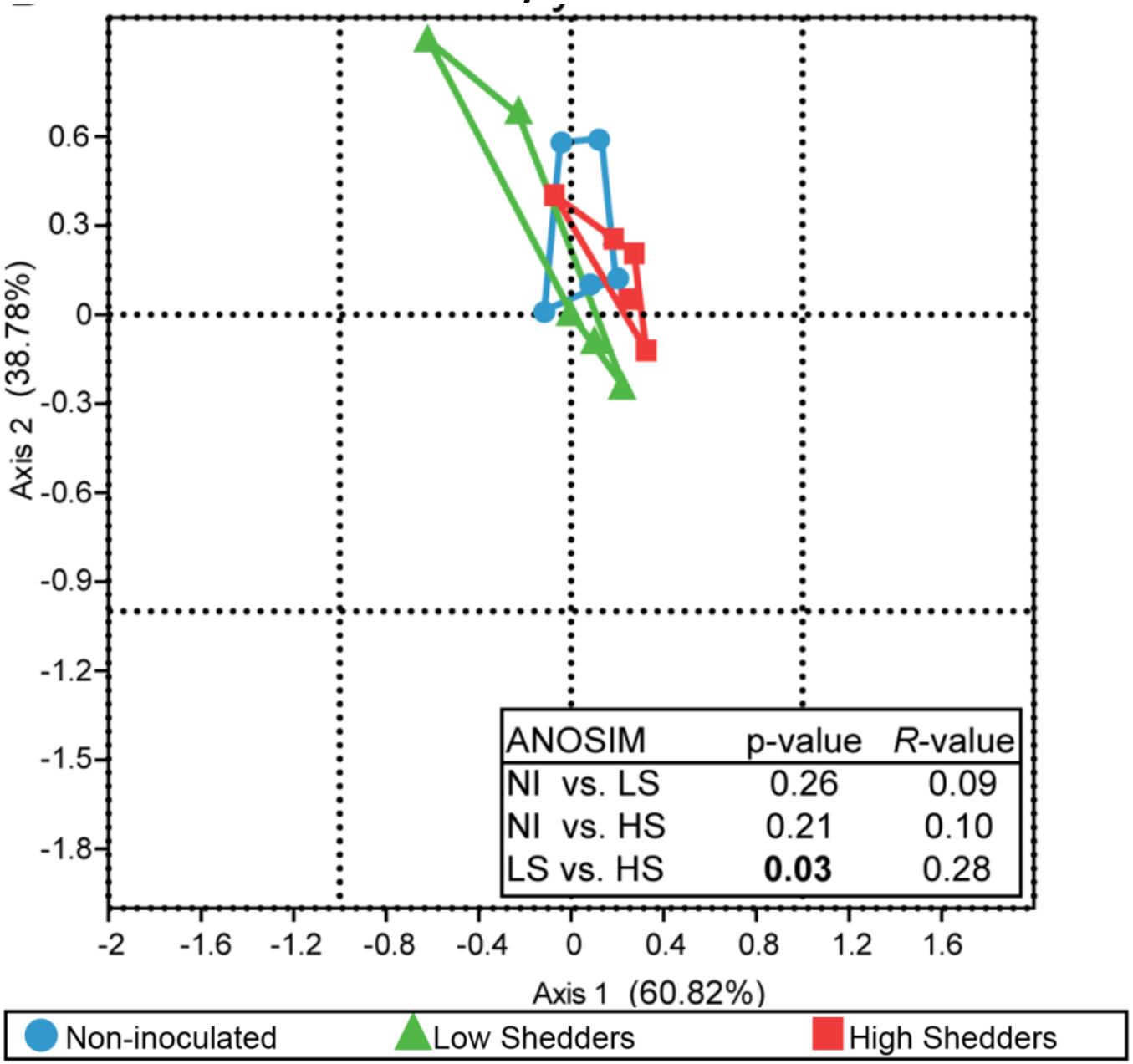


Litter-mate, mock-inoculated controls

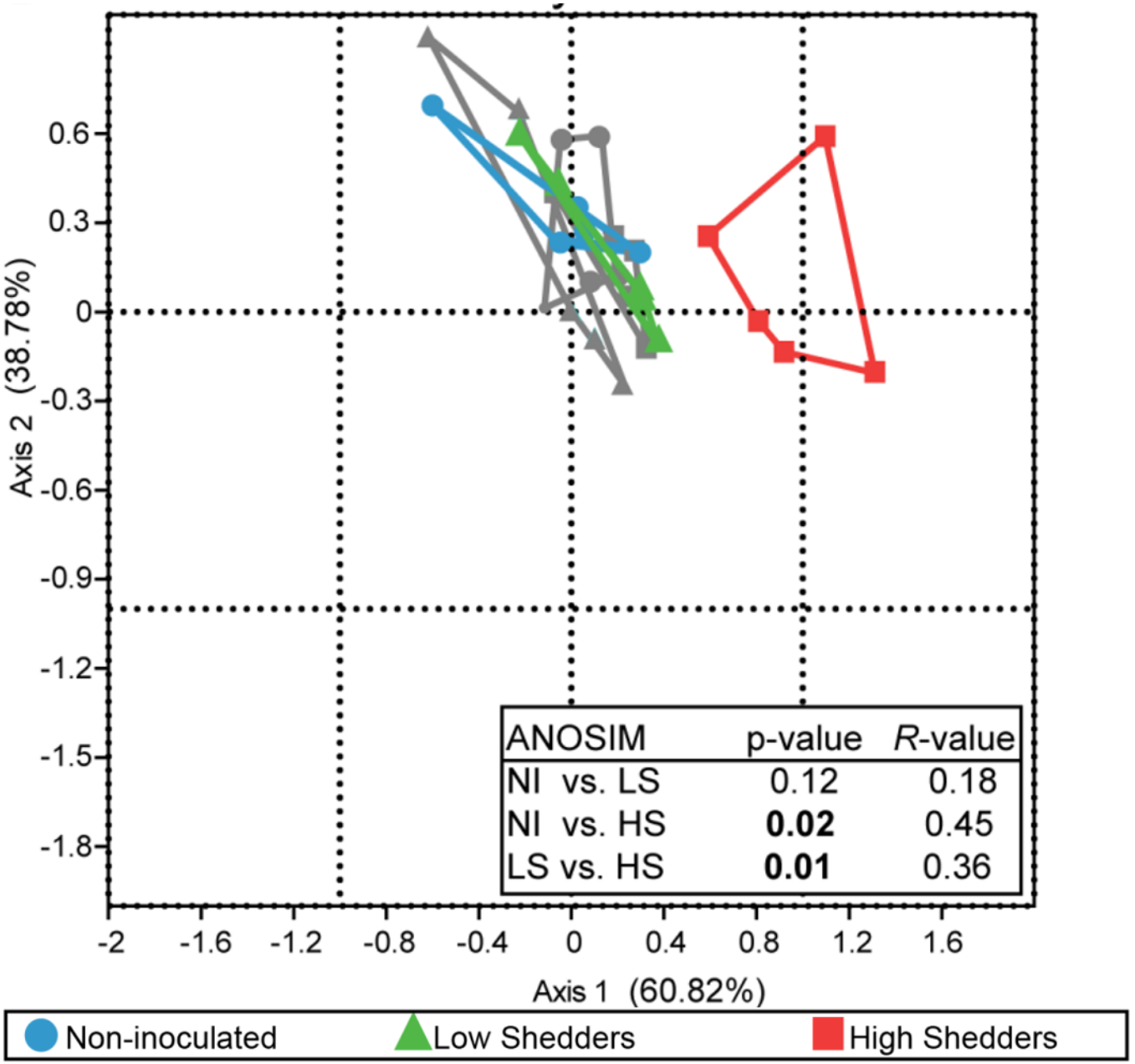


Bearson, Allen et al. *Infection, Genetics and Evolution* 16:330-40.

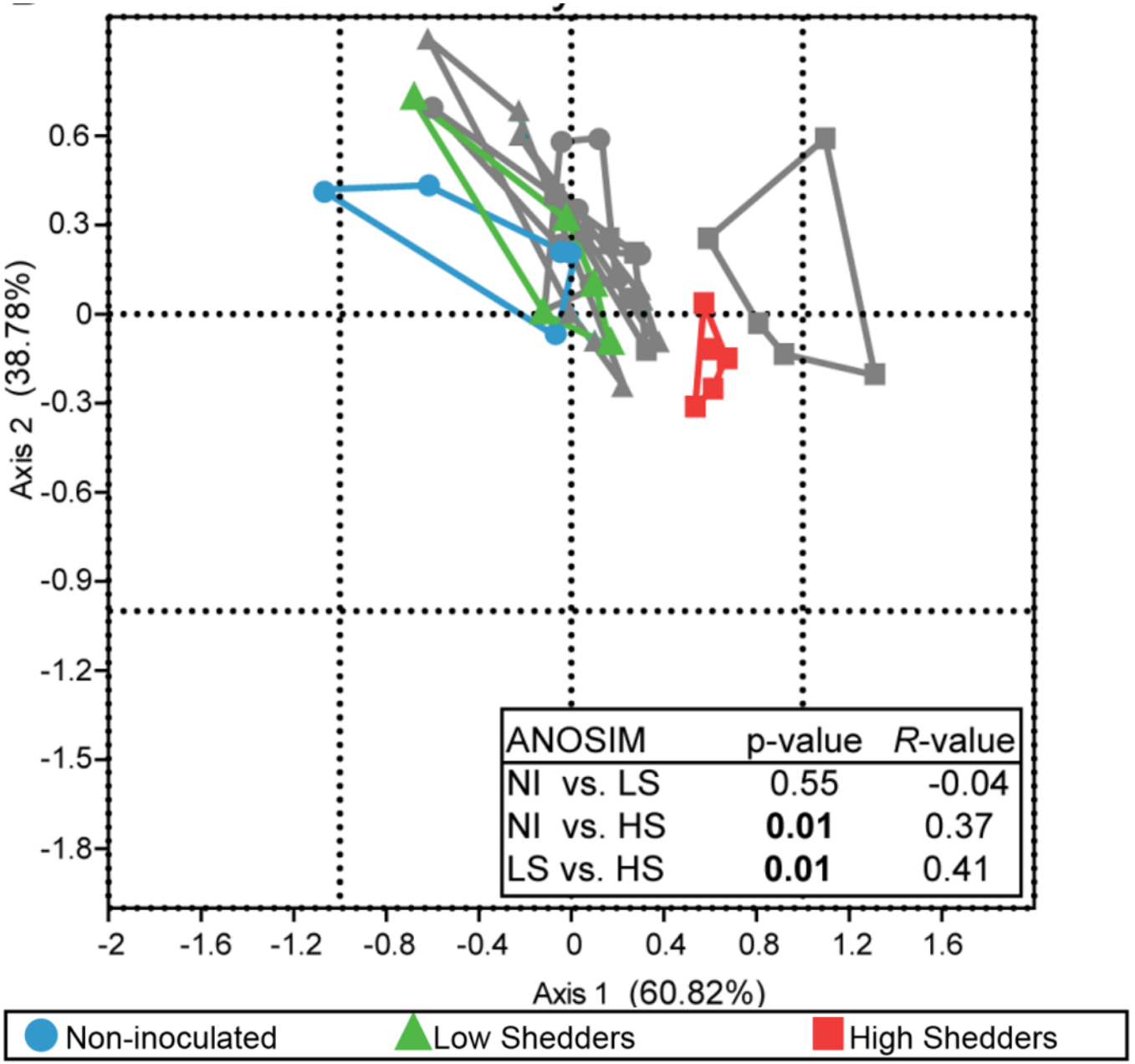
Day 0


0.6
0.3
0
-0.3
-0.6
-0.9
-1.2
-1.5
-1.8
Axis 2 (38.78%)
-2
-1.6
-1.2
-0.8
-0.4
0
0.4
0.8
1.2
1.6
Axis 1 (60.82%)
ANOSIM p-value R-value
NI vs. LS 0.26 0.09
NI vs. HS 0.21 0.10
LS vs. HS 0.03 0.28
Non-inoculated
Low Shedders
High Shedders

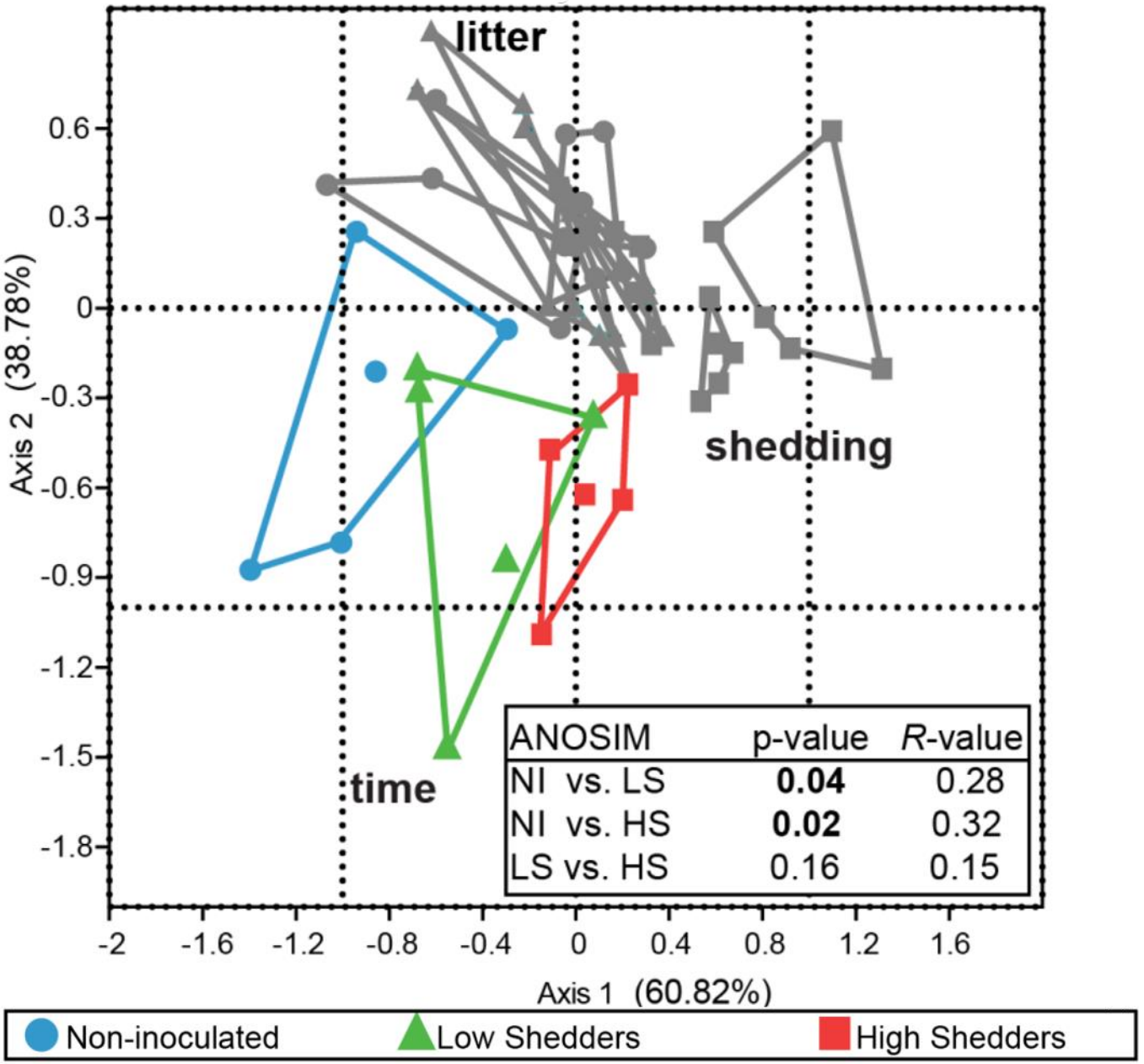
Day 2


0.6
0.3
0
-0.3
-0.6
-0.9
-1.2
-1.5
-1.8
Axis 2 (38.78%)
-2
-1.6
-1.2
-0.8
-0.4
0
0.4
0.8
1.2
1.6
Axis 1 (60.82%)
ANOSIM p-value R-value
NI vs. LS 0.12 0.18
NI vs. HS 0.02 0.45
LS vs. HS 0.01 0.36
Non-inoculated
Low Shedders
High Shedders

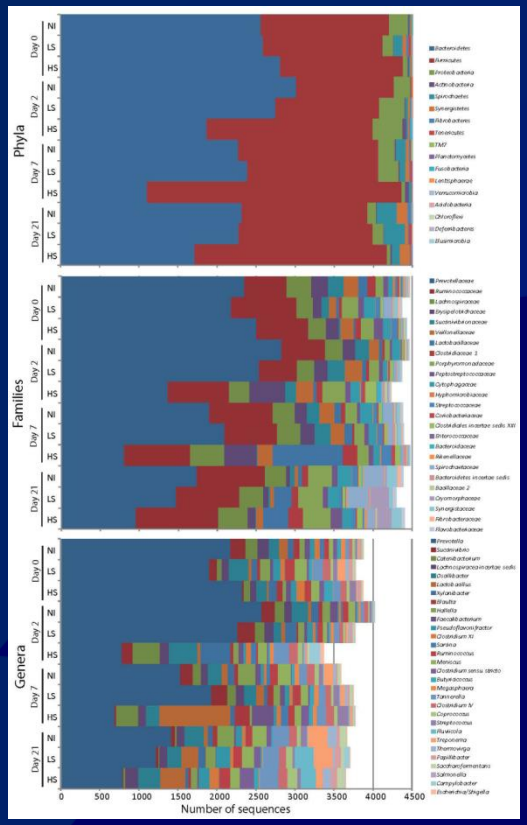
Day 7


0.6
0.3
0
-0.3
-0.6
-0.9
-1.2
-1.5
-1.8
Axis 2 (38.78%)
-2
-1.6
-1.2
-0.8
-0.4
0
0.4
0.8
1.2
1.6
Axis 1 (60.82%)
ANOSIM p-value R-value
NI vs. LS 0.55 -0.04
NI vs. HS 0.01 0.37
LS vs. HS 0.01 0.41
Non-inoculated
Low Shedders
High Shedders

Day 21



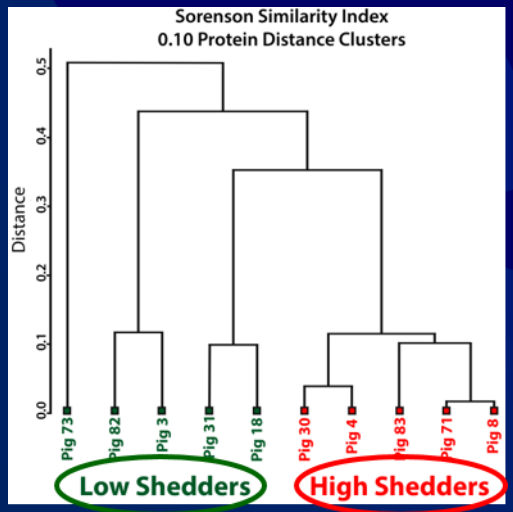
ANOSIM	p-value	*R*-value
NI vs. LS	**0.04**	0.28
NI vs. HS	**0.02**	0.32
LS vs. HS	0.16	0.15



Phylotype analysis

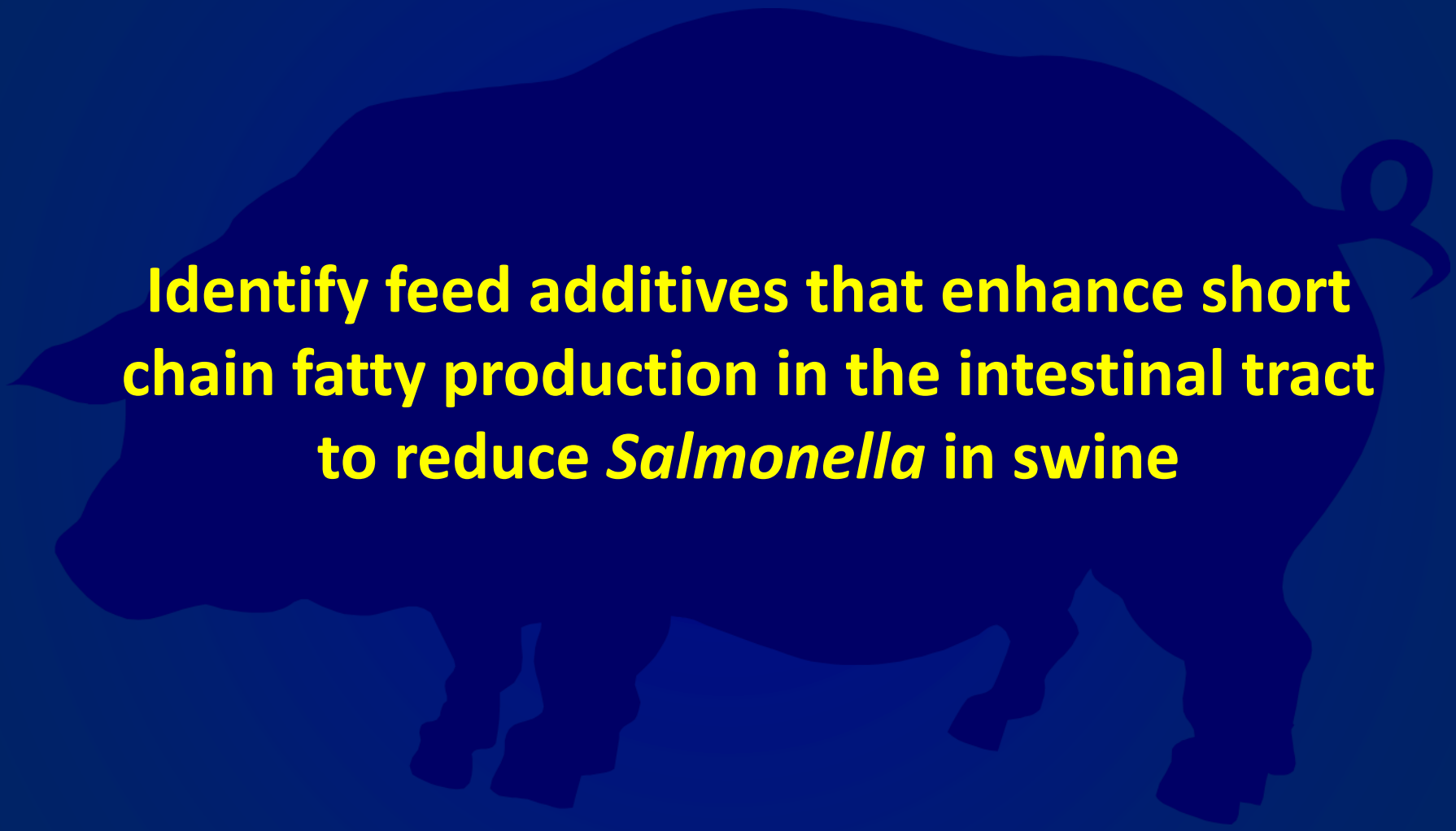
Prior to *Salmonella* challenge, the "will-be" low shedder pigs had a significantly greater relative abundance of *Ruminococcaceae*, members of which are:

- enriched in the mucosal environment
- have shown decreased abundance in other animals with gut disturbance
- produce short-chain fatty acids, including butyrate



Butyrate-producing bacteria

The diversity of butyrate-producing bacteria is different in swine that become high shedders versus low shedders.



Identify feed additives that enhance short chain fatty production in the intestinal tract to reduce *Salmonella* in swine

Potential mechanism for reducing *Salmonella* colonization or shedding

Encouraging short change fatty acid (SCFA) production, particularly butyrate, might reduce the niche for microbes that use respiration by limiting electron acceptor availability

Oxidation of butyrate in epithelial cells consumes most available oxygen (i.e. less available O_2)

SCFAs encourage robust mucus barrier

- More AMPs, more mucus, more IgA
- Limits the chance microbes will contact host tissues and elicit inflammatory response
- Less immune derived reactive oxygen species (ROS)

Feed trial with *Salmonella* challenge

6 diets

Control

Raw Potato Starch (5%)

Acid blend: 0.3% butyric acid-propionate-medium chain fatty acid mix

ZnO (2,000 mg/kg) + CuCl (200 mg/kg)

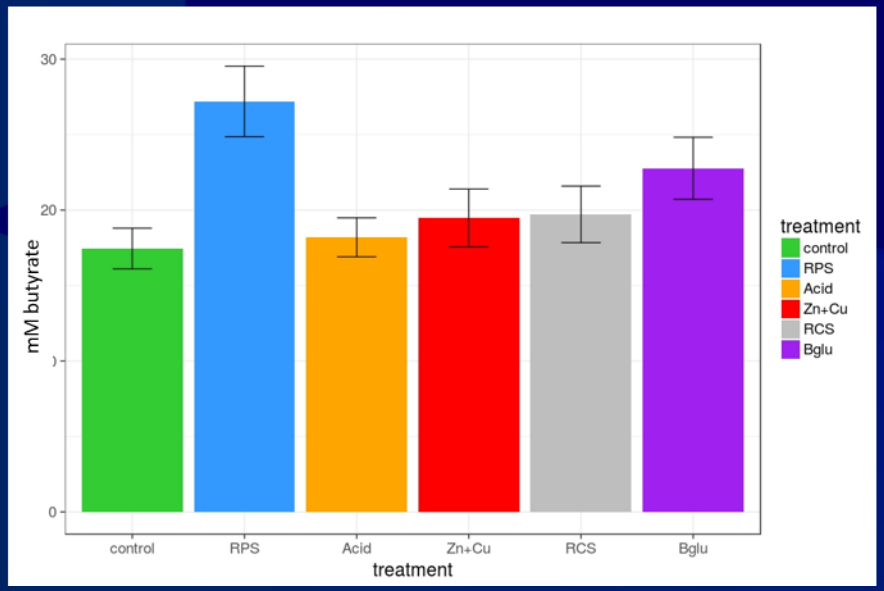
High amylose corn starch (5%)

Beta-glucan (0.05%, yeast based)

- **Ten pigs/treatment group (started feed trial at 3-weeks of age)**
- **After 4 weeks on the various diets, pigs were challenged with $1x10^8$ CFU *Salmonella enterica* serovar I 4,[5],12:i:- (feed additives continued)**
- **Fecal shedding was monitored for 21 days**
- **At necropsy, butyrate concentrations was measured in the cecal contents**

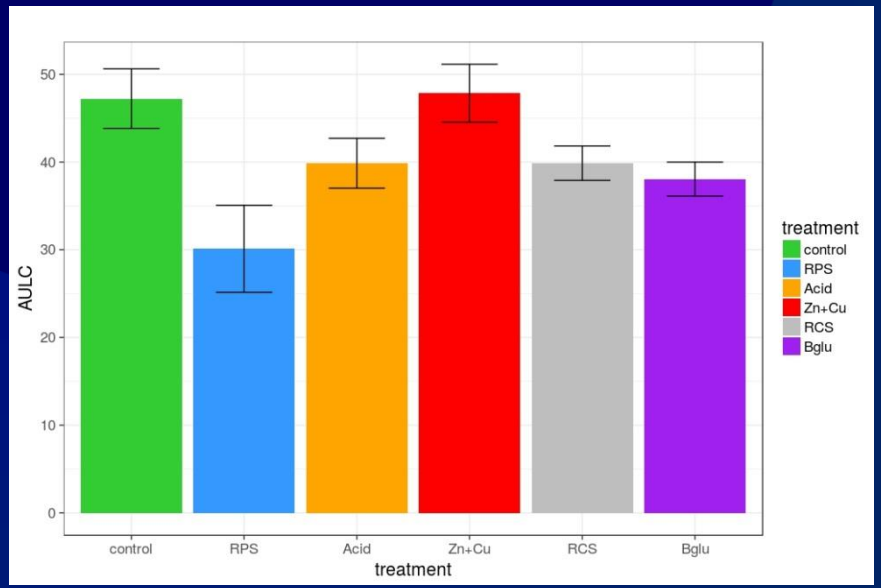
Feed trial with *Salmonella* challenge (2)

Butyrate production is increased in the cecal contents of *Salmonella* challenged pigs fed raw potato starch and β-glucan



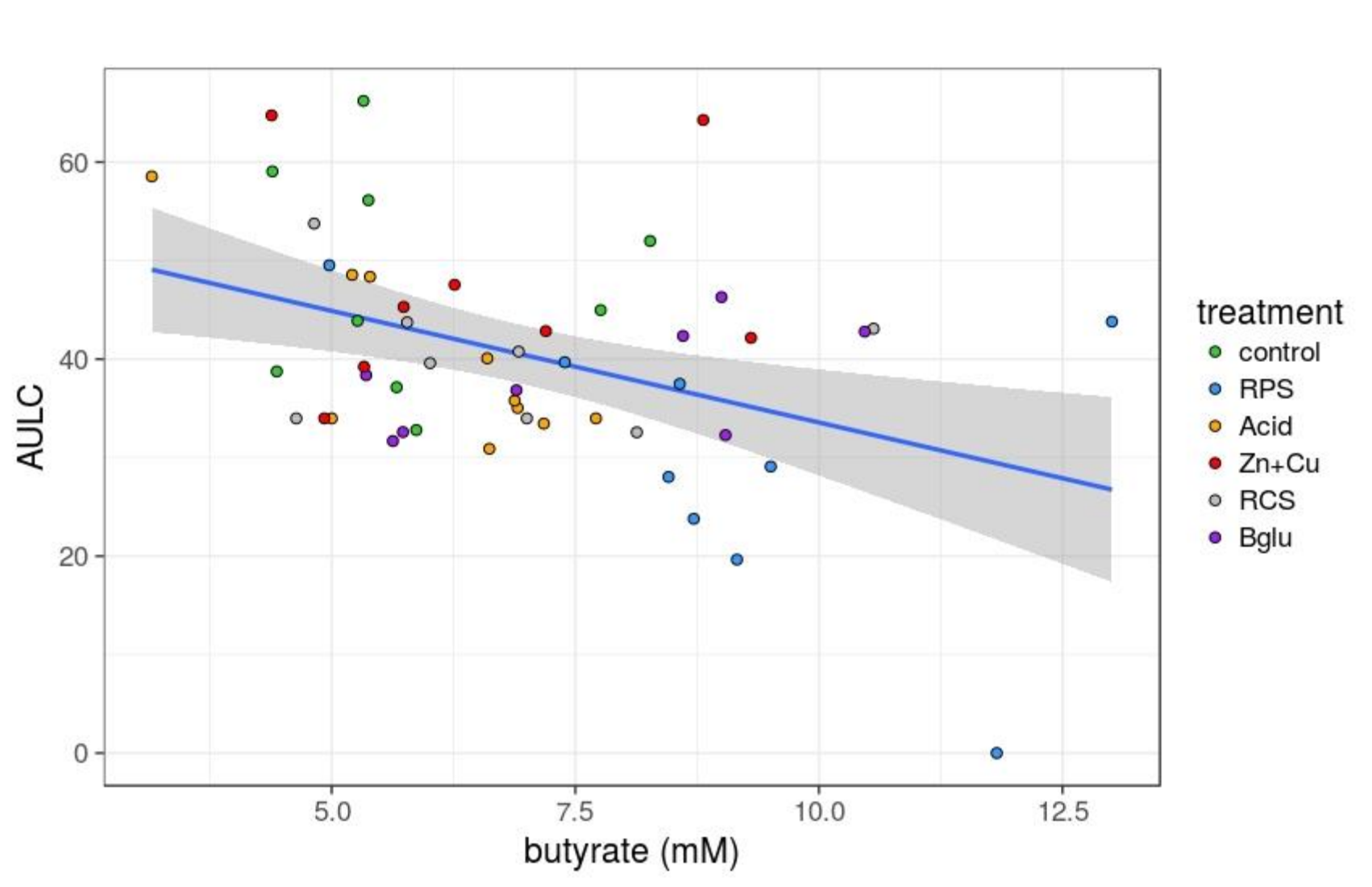
Wilcoxon: control vs RPS, p=0.004; control vs Bglu, p=0.036

Cumulative *Salmonella* fecal shedding over the 3-week study was significantly reduced in pigs fed raw potato starch and β-glucan



Wilcoxon: control vs RPS, p=0.013; control vs Bglu, p=0.043

Correlation between butyrate concentration in cecal contents and cumulative *Salmonella* fecal shedding



Spearman: -0.4, p=0.003

The Take-Home Message

The microbial composition of the intestinal tract can influence *Salmonella* shedding in pigs.

Feed components that promote SCFA-producing microbes (e.g. butyrate-producers) may benefit the host by enhancing swine gut health and reducing *Salmonella* colonization and shedding.

Probiotics + Prebiotic =

Thank you!

Heather Allen
David Alt
Darrell Bayles
Brad Bearson
Brian Brunelle
Brian Kerr
Jalusa Kich
Mike Kogut
Torey Looft
Thad Stanton
Julian Trachsel
Chris Tuggle