Analysis of antibiotic associated changes in turkey intestinal microbiota, and studies of *Campylobacter* growth

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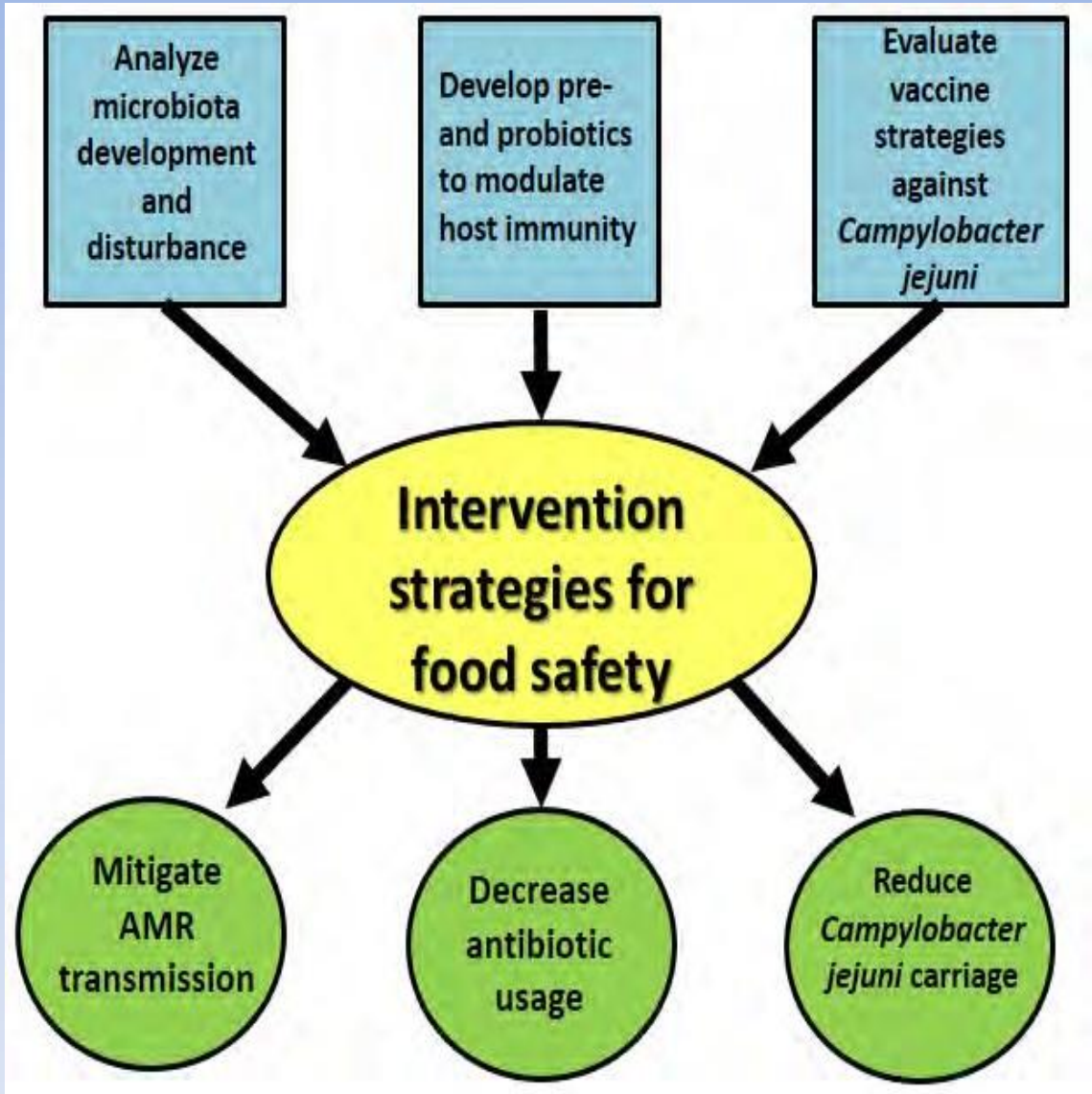
- Effects of antibiotics on composition of intestinal microbiota
- Transfer of antimicrobial resistance genes in the intestinal microbiota
- Test and develop pre-harvest methods to control *Campylobacter* spp. colonization in turkeys
- Common goal is to promote food safety



Outline

- Antibiotic effects on the turkey intestinal microbiota
  1. Bacitracin dosage drives structural and antibiotic resistant changes in the turkey cecal microbiome

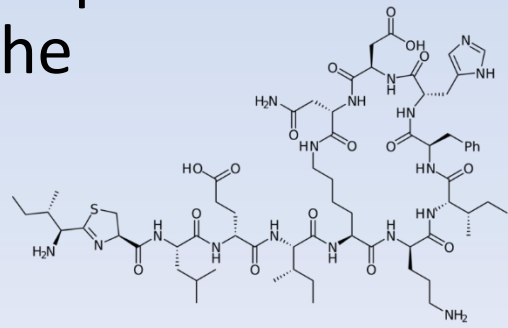
- *Campylobacter* interactions in the avian intestinal environment
  1. Detection of *Campylobacter* in the turkey gut
  2. Avian intestinal mucus modulates *Campylobacter jejuni* gene expression in a host-specific manner

Need to define their mode of action

- In 2017, FDA restricted the off-label and feed-efficiency usage of medically important antibiotics
  - Non-medically important antibiotics will continue to have therapeutic and sub-therapeutic uses

- Bacitracin is a bactericidal peptide that inhibits the cell wall biosynthesis (gram-positive)

- Bacitracin methylene disalicylate (BMD) is important to poultry production and isn't impacted by the new guidance



Objectives

- Characterize the effects of therapeutic and sub-therapeutic bacitracin methylene disalicylate on the turkey gut bacterial
- Membership
- Antibiotic resistance genes
- Function
- Host gene expression

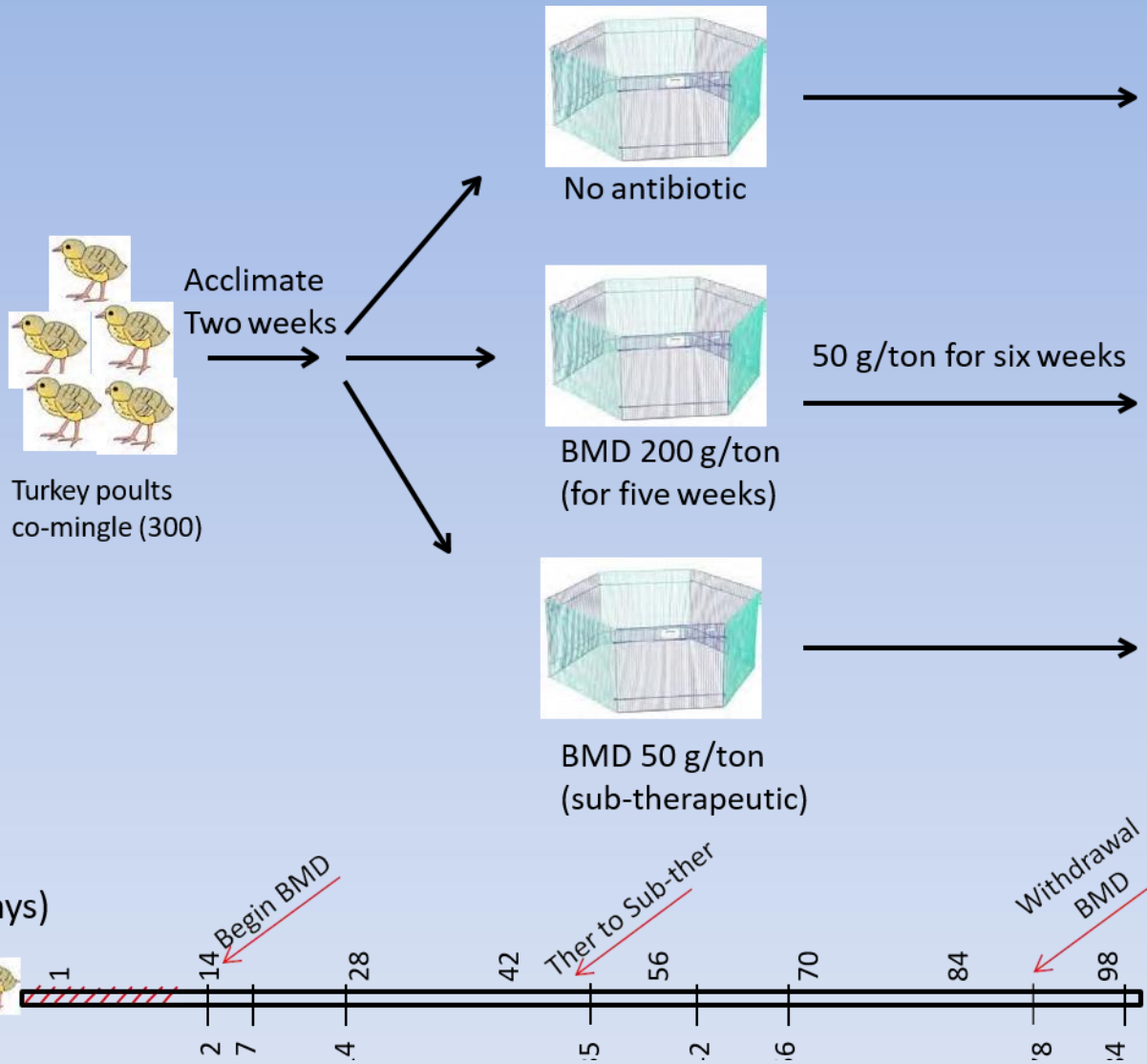
Poultry Information: Bacitracin Methylene Disalicylate—Type A Medicated Article (Antibacterial)

Active Drug Ingredient		Each pound contains feed grade bacitracin methylene disalicylate equivalent to 50 grams bacitracin (Master Standard).
Composition		A dried precipitated fermentation product obtained by culturing *B. licheniformis* Tracy on media adapted for microbiological production of bacitracin; calcium carbonate.
For Manufacturing Medicated Poultry and Livestock Feeds Only		
Mixing Directions		Prepare an intermediate premix containing 5 grams per pound by mixing 1.0 lb. of BMD 50 with 9.0 lbs. of soybean meal or ground corn. Then add 0.8 to 50 lbs. of intermediate premix per ton of finished feed.
Species	Grams Bacitracin Per Ton of Feed	**Indications for Use**
Broiler and Replacement Chickens	4 – 50	For increased rate of weight gain and improved feed efficiency.
	50	As an aid in prevention of necrotic enteritis caused or complicated by *Clostridium* spp. or other organisms susceptible to bacitracin methylene disalicylate.
	100 – 200	As an aid in control of necrotic enteritis caused or complicated by *Clostridium* spp. or other organisms susceptible to bacitracin methylene disalicylate.
		CAUTION: To control a necrotic enteritis outbreak, start medication at the first clinical signs of disease. The dosage range permitted provides for different levels based on severity of the infection. Consult a poultry diagnostic laboratory or pathologist to determine the diagnosis and advice regarding the optimal level of drug. Administer continuously for 5 to 7 days or as long as clinical signs persist, then reduce medication to prevention level (50 g/ton).
Laying Hens	10 – 25	For increased egg production and improved feed efficiency during the first seven months of production.
Growing Turkeys	4 – 50	For increased rate of weight gain and improved feed efficiency.
	200	As an aid in control of transmissible enteritis in growing turkeys complicated by organisms susceptible to bacitracin methylene disalicylate.
Pheasants	4 – 50	For increased rate of weight gain and improved feed efficiency.
Quail	5 – 20	For increased rate of weight gain and improved feed efficiency in quail not over 5 weeks-of-age.
	200	For prevention of ulcerative enteritis in growing quail due to *Clostridium colinum* susceptible to bacitracin methylene disalicylate. Feed continuously as the sole ration.

Note: Where minimum levels are shown, increase the antibiotic concentration within approved range when necessary to fit the feeding program, and to insure adequate levels of antibiotic in the complete ration.

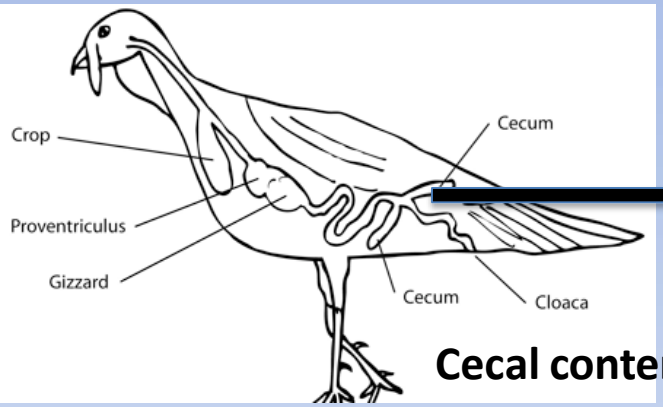
For Use in Animals Only.
For Use in Dry Feeds Only.
Not for Use in Liquid Type B Medicated Feeds.

NADA 46-592, Approved by FDA



Samples collected (Days relative to BMD start)

methods

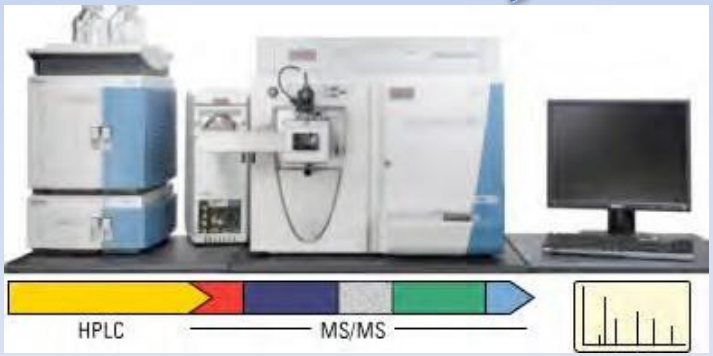


Cecal contents

DNA

HUMAnN2,
RGI, CARD
Comprehensive Antibiotic Resistance Database

16S rRNA gene seq.
(membership)



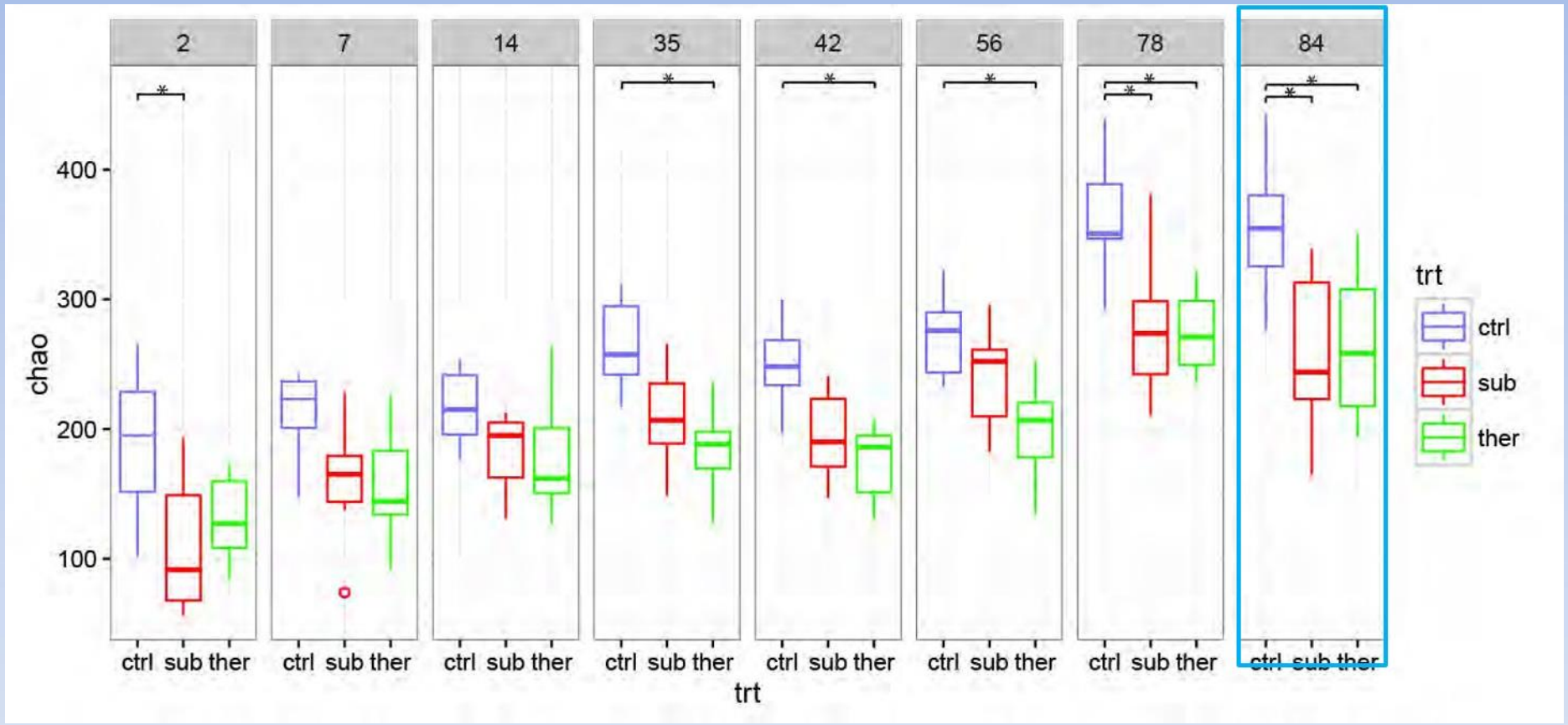
Global metabolomic analysis
(Metabolon Inc.)

16S rRNA gene sequence analysis to assess the microbiota

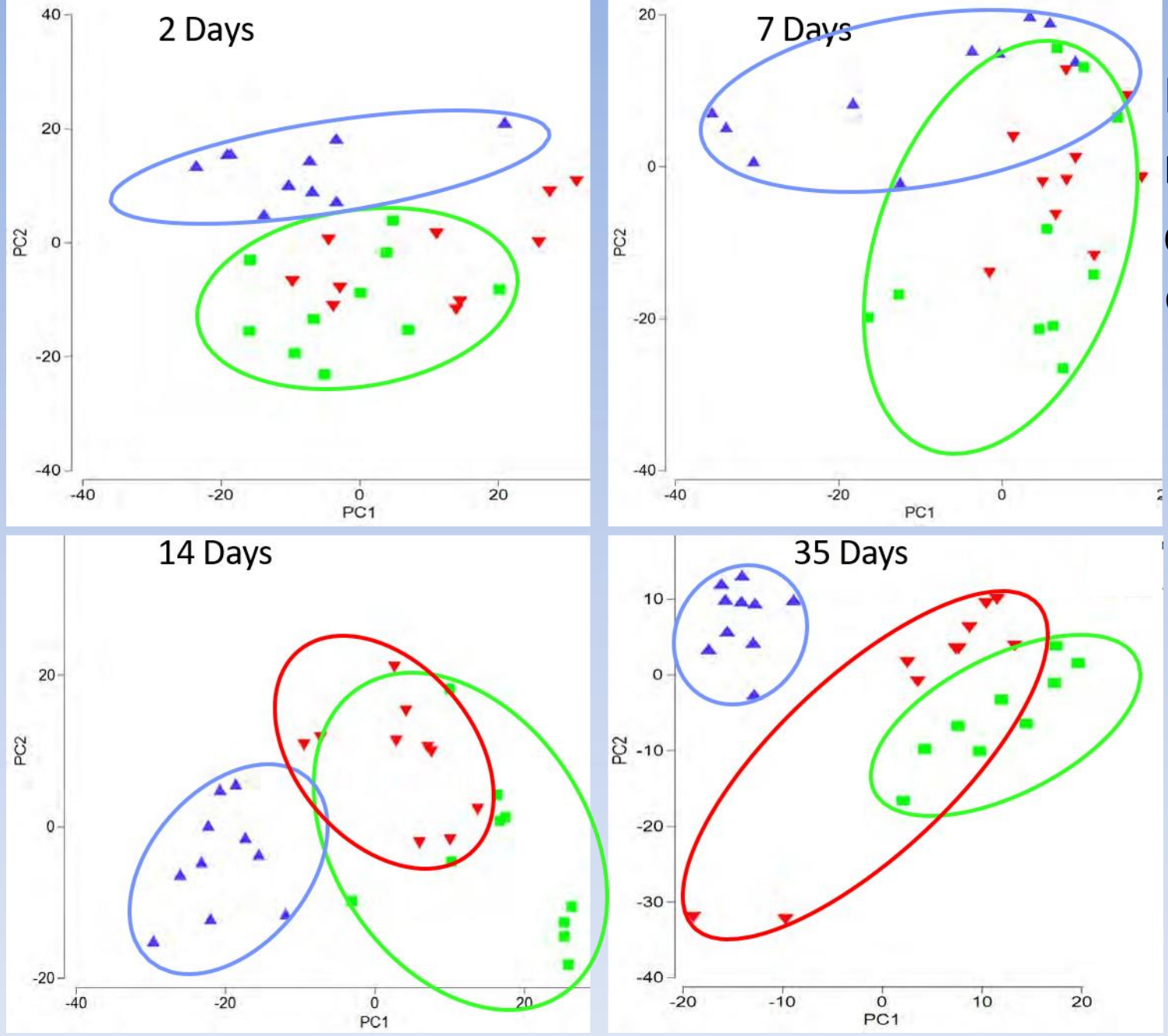
16S rRNA gene

Slide credit: Heather Allen

Reduced bacterial richness with antibiotic treatment



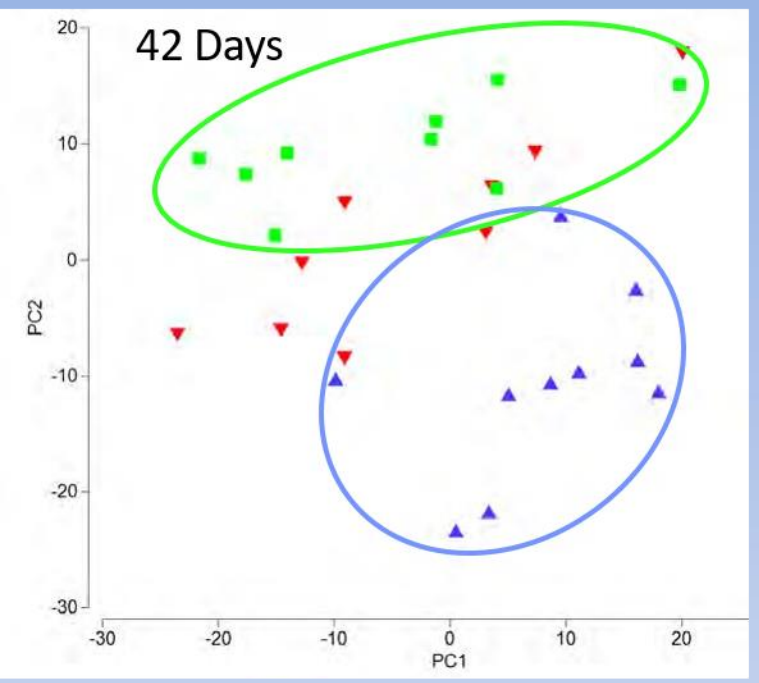
Credit: Tim Johnson

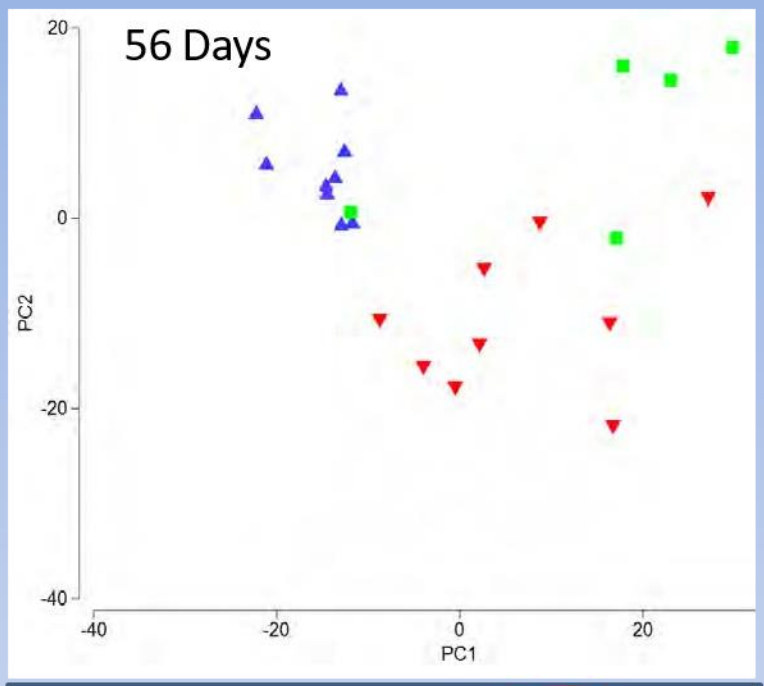


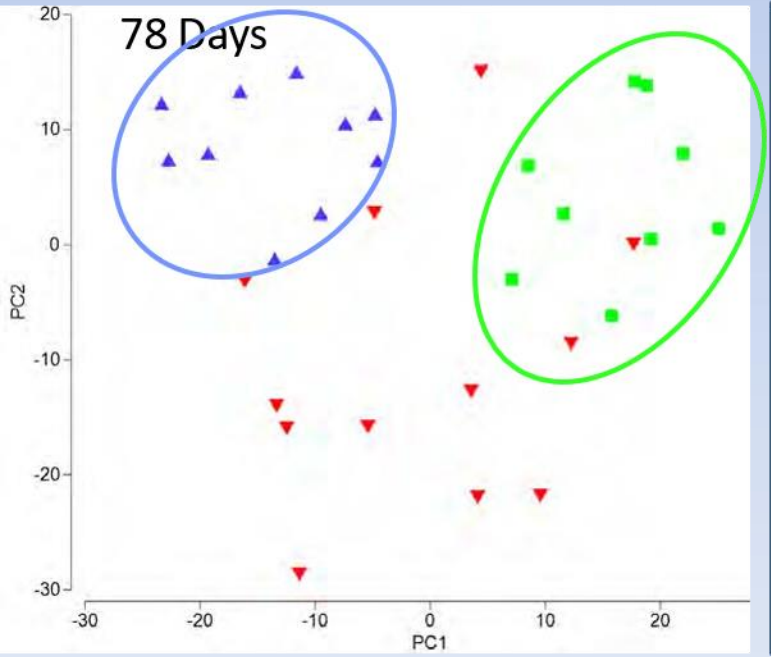
BMD drives microbiota changes

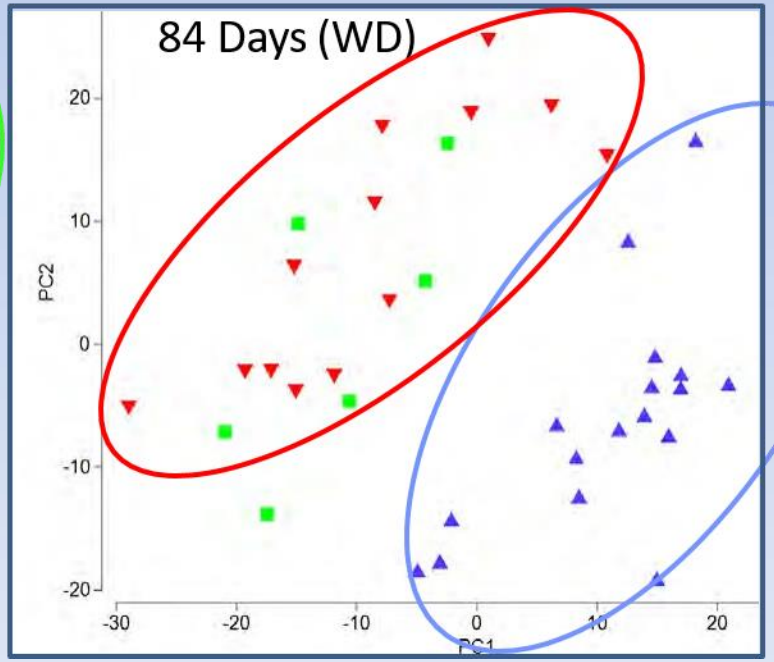
Circle=corr p<0.05

PCA analysis of OTU-based bacterial 16S rRNA gene sequence abundances









BMD drives microbiota changes

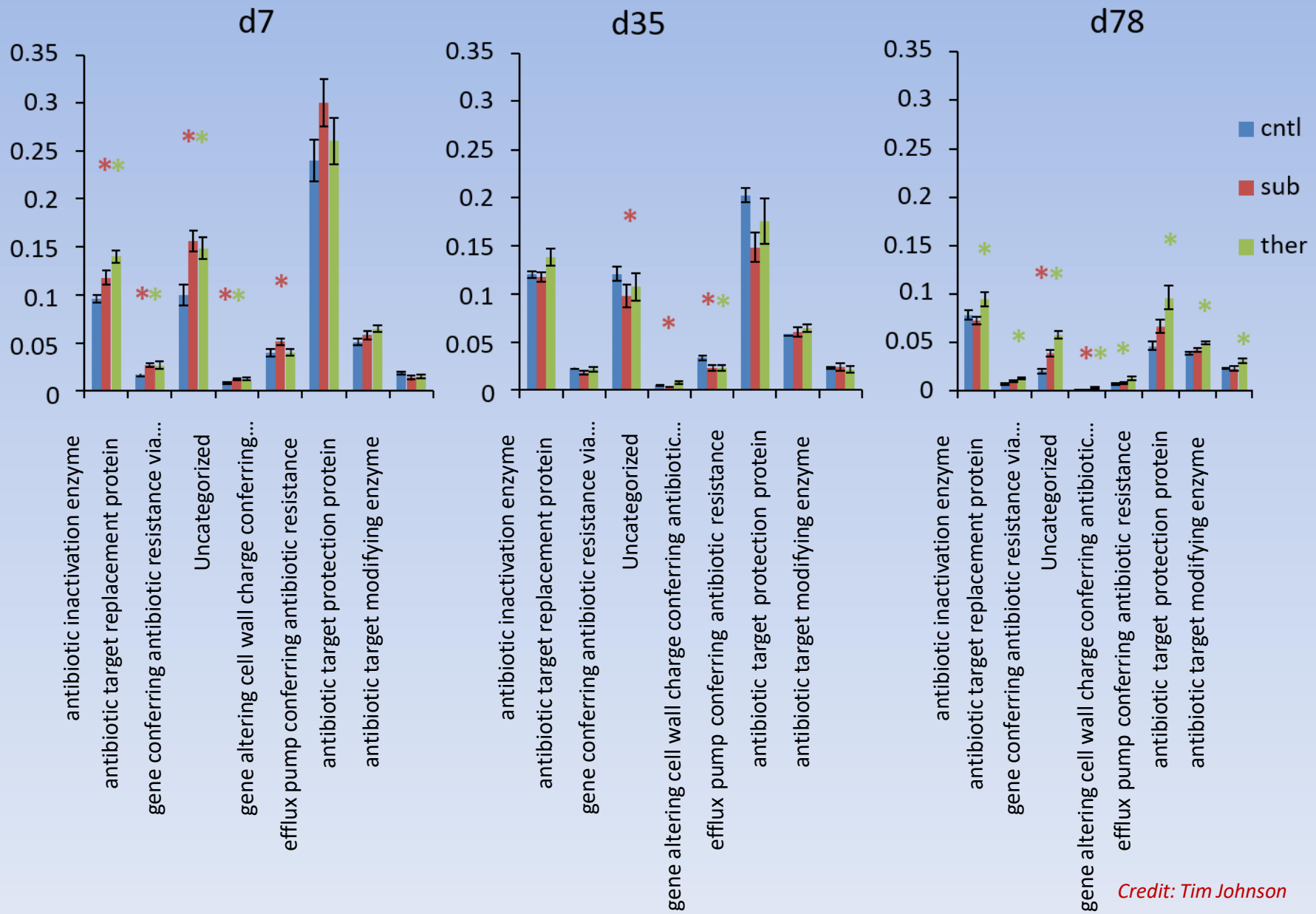
Circle=corr $p<0.05$

medication
- control
- sub
- ther*

* Ther. group was reduced to subtherapeutic

PCA analysis of OTU-based bacterial 16S rRNA gene sequence abundances

Increase of resistance genes in BMD metagenomes



Credit: Tim Johnson

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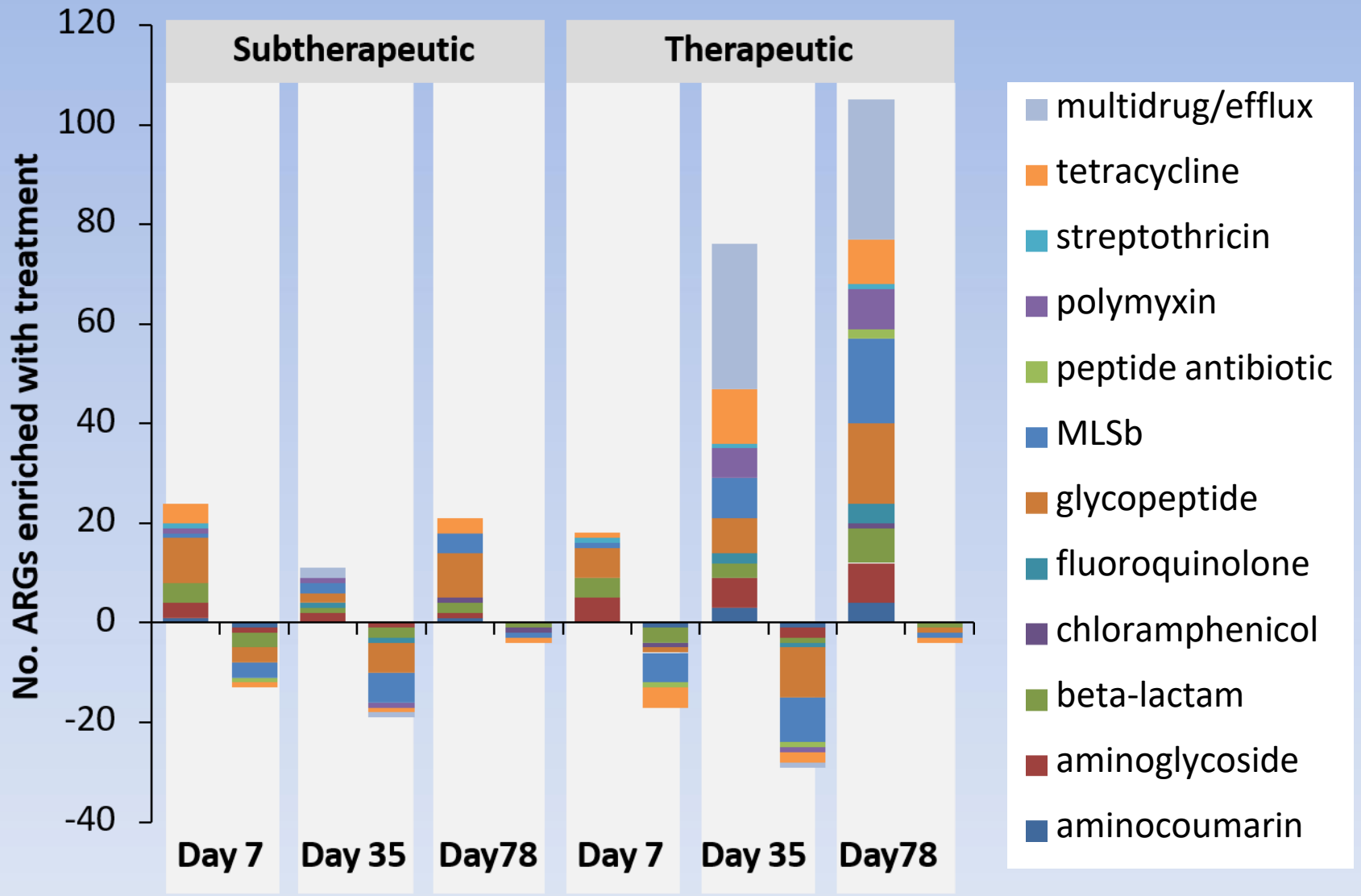
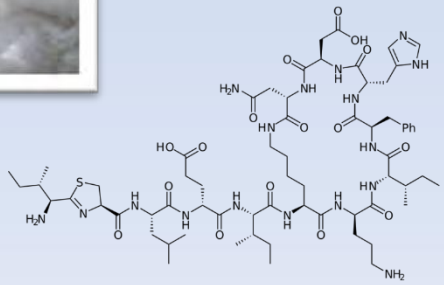


Credit: Tim Johnson

Summary

- In-feed BMD results in short- and long-term effects on the turkey intestinal microbiota
    - Reducing species richness
    - Increase in AMR genes in therapeutic BMD
- Relationship to animal health and food safety?



Campylobacter jejuni in the avian gut

- *C. jejuni* is considered an avian commensal, poultry are regarded as the main reservoir for human exposure (Epps et al. 2013)

- Understanding how *C. jejuni* interacts within the host-intestinal environment may provide insights into how it is is so apt at colonizing the avian gut

Evaluation of different *Campylobacter jejuni* isolates to colonize the intestinal tract of commercial turkey poults and selective media for enumeration

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(in press Poultry Science)

Day of hatch hybrid breed turkey poults (n=72) group housed

Poults are free of *Campylobacter* prior to colonization – enrichment of cecal contents prior to colonization (n=5)

D20 of age separated into 3 different rooms

D21 of age oral gavage 1 mL of:
a) Sterile Bolton's broth (mock-inoculated) n=15
b) NCTC 11168 ($5x10^8$ cfu) n = 24
c) NADC 20827 ($4x10^8$ cfu) n = 28

Euthanize poults at 3, 7 and 21 days after oral colonization

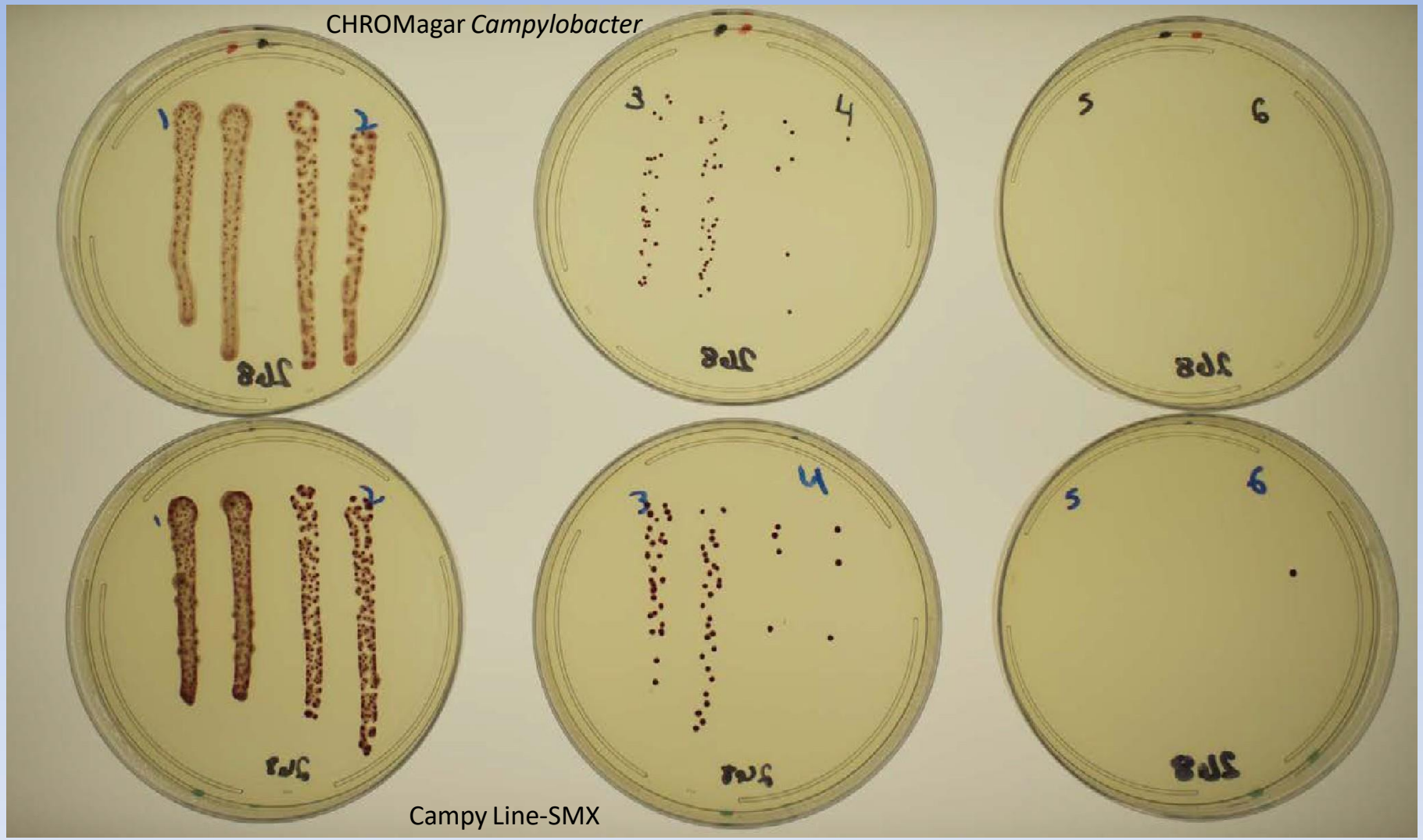
Intestinal contents harvested

Direct culture enumeration from ileal, cecal or colon contents:

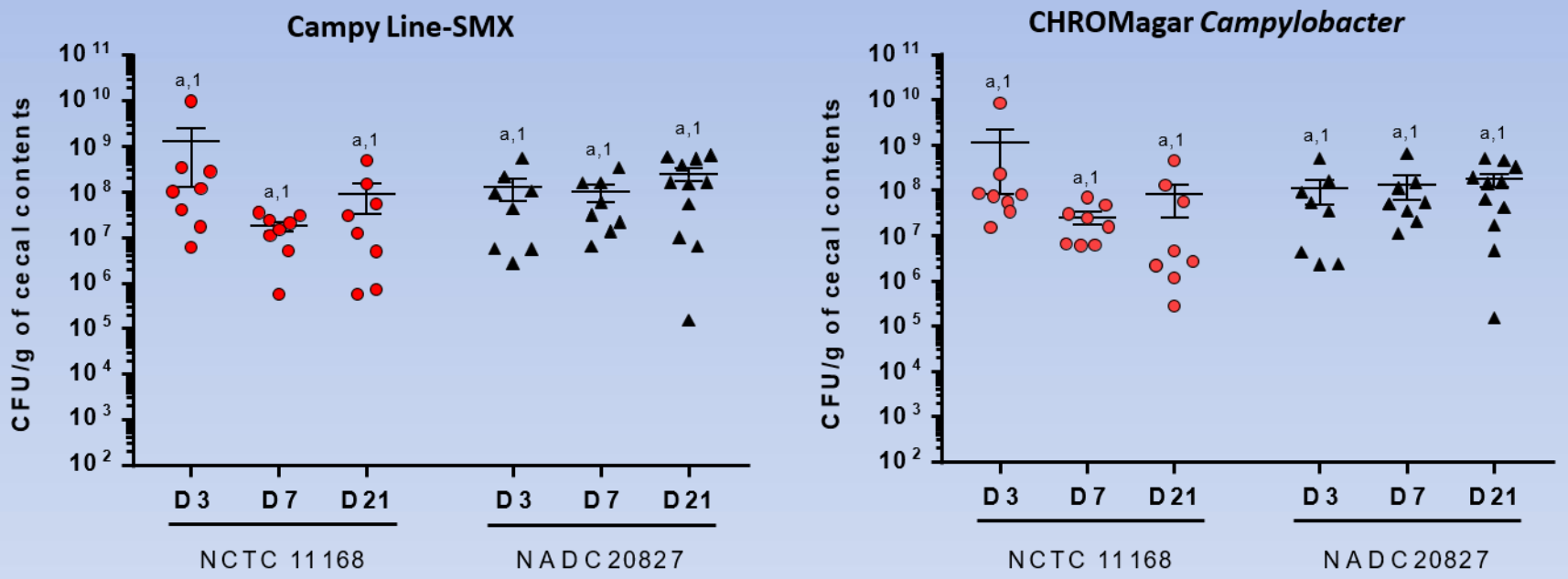
1g of contents 10-fold diluted and track plated on **Campy-Line agar supplemented with sulfamethoxazole** (SMX; 2.5 μg/mL), **Campy Cefex agar** or **CHROMagar *Campylobacter*** incubating at 42°C for up to 48h (microaeropillic)

Post culture validation of recovered colonies on each media using qPCR for *C. jejuni* HipO and *Campylobacter* 16S rRNA

CHROMagar and Campy Line-SMX agar recover *Campylobacter* cells with little background



Wild-type *C. jejuni* isolates NCTC 11168 and NADC 20827 persistently colonize the cecum of poults



✓NCTC 11168 and NADC 20827 persistently colonize the turkey cecum and colon

–Isolates failed to persistently colonize ileum

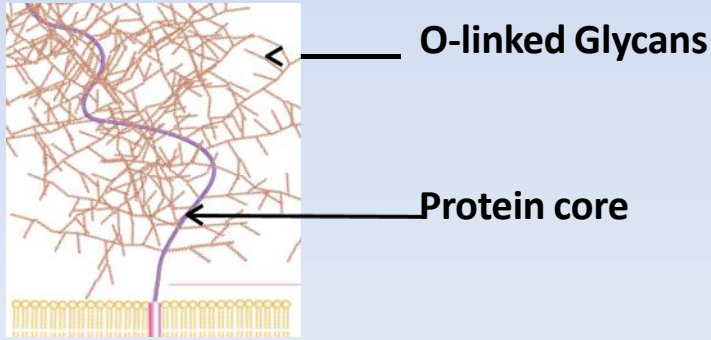
✓Campy Line-SMX and CHROMagar *Campylobacter* very useful to direct-plating enumerate *C. jejuni* from cecal and colon samples

–No significant difference in the number of each *C. jejuni* isolate

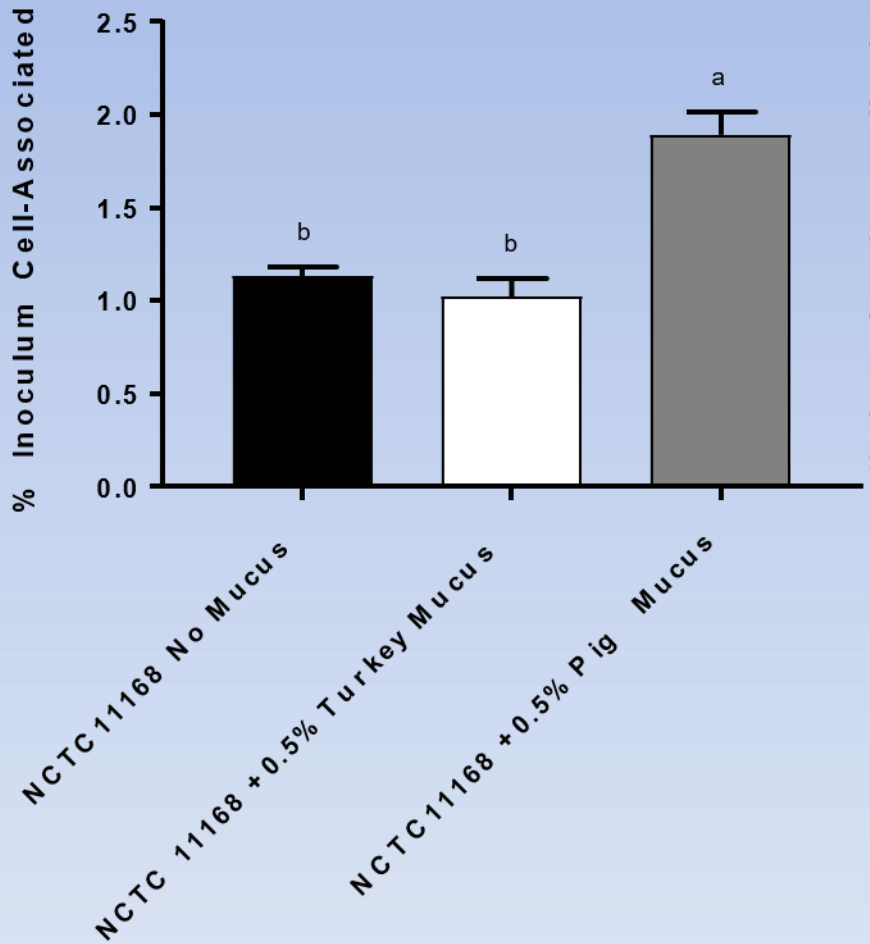
–Campy Cefex agar was not useful to enumerate C. jejuni from turkey intestinal samples

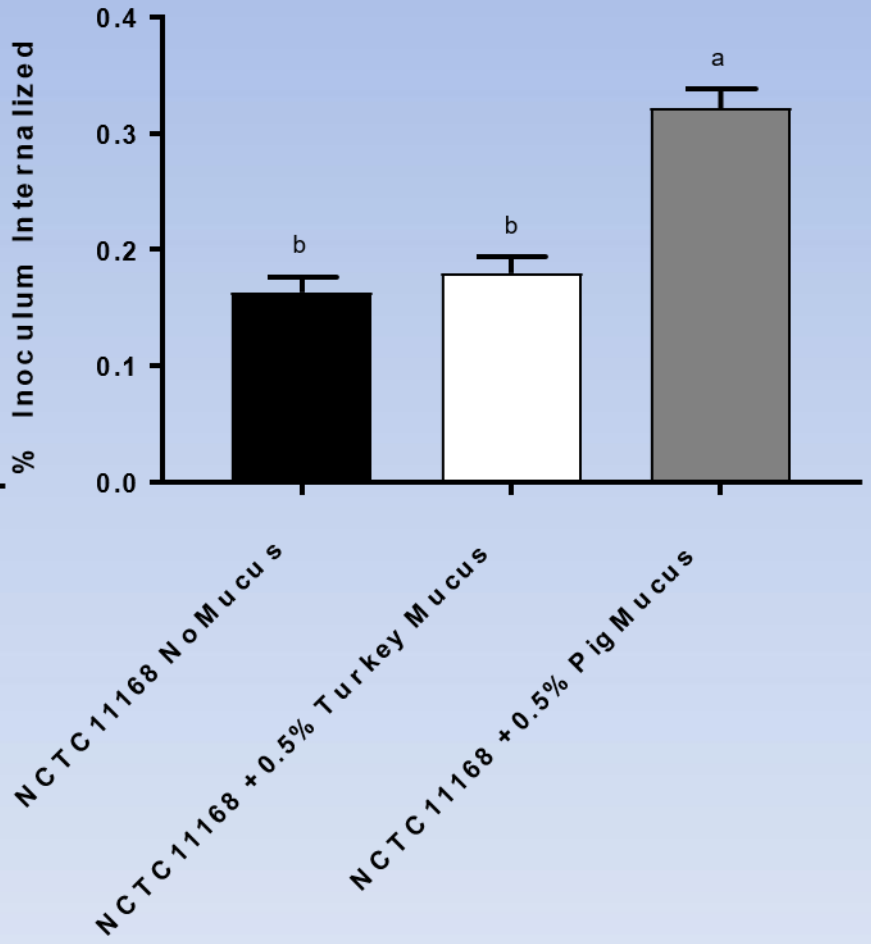
Campylobacter Mucus association

- Mucus colonization is essential for *C. jejuni* persistence in the avian gut
- Mucins modulate the pathogenicity of *C. jejuni* in a species-specific manner i.e. pathogenic in humans, commensal in chickens
  - *C. jejuni* preferentially binds mucin from the chicken gut over other species
- In this study, we characterized the transcriptome of *C. jejuni* NCTC11168 after exposure to purified mucus from avian (chicken and turkey) or mammalian (pig, sheep, and cow) hosts



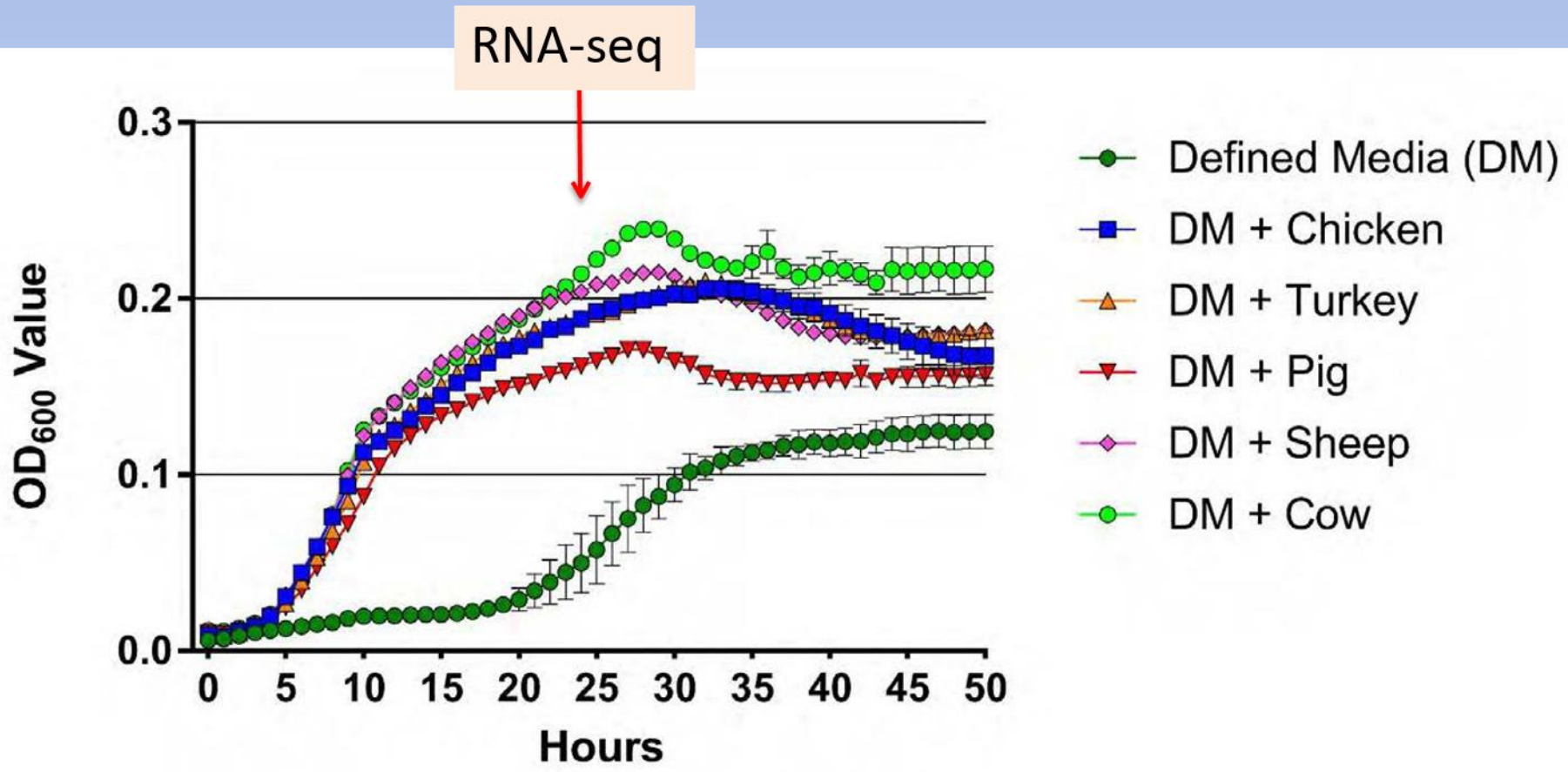
Pig mucus enhanced *C. jejuni* adherence and invasion *in vitro*





Credit: Matt Sylte

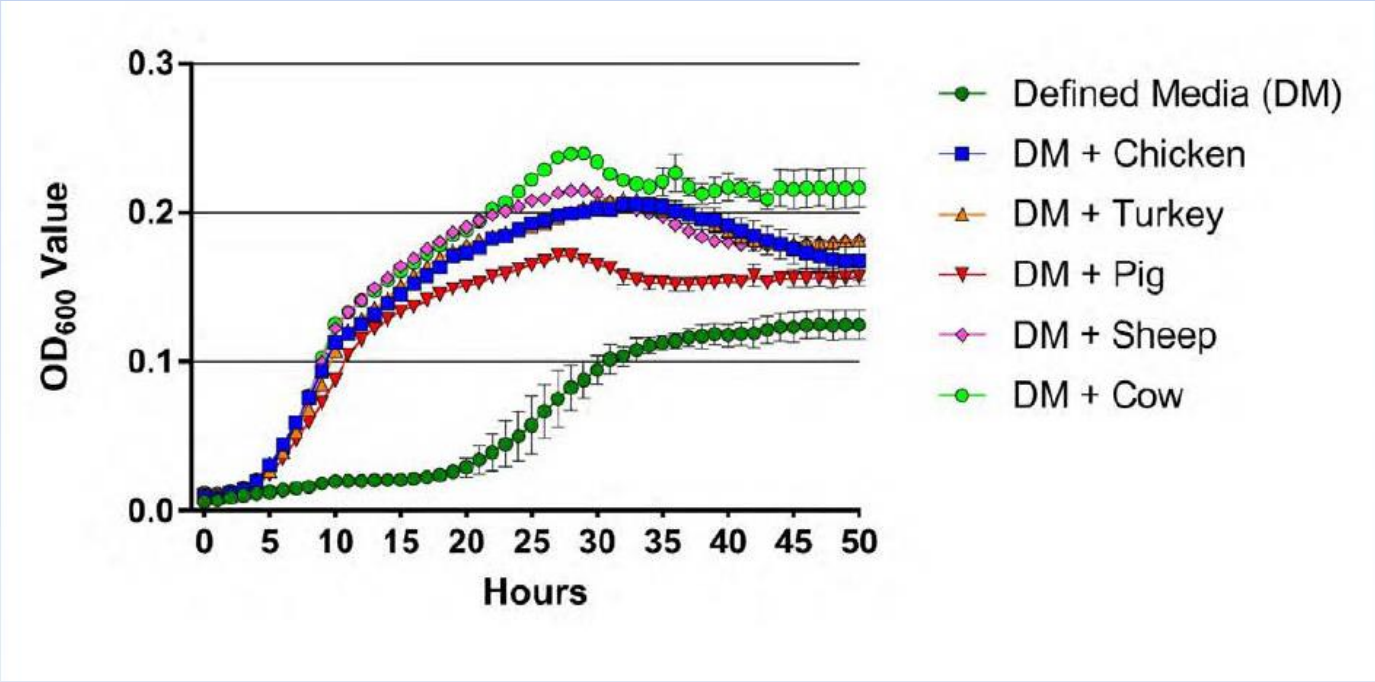
C. jejuni growth on mucus (0.5% w/v)



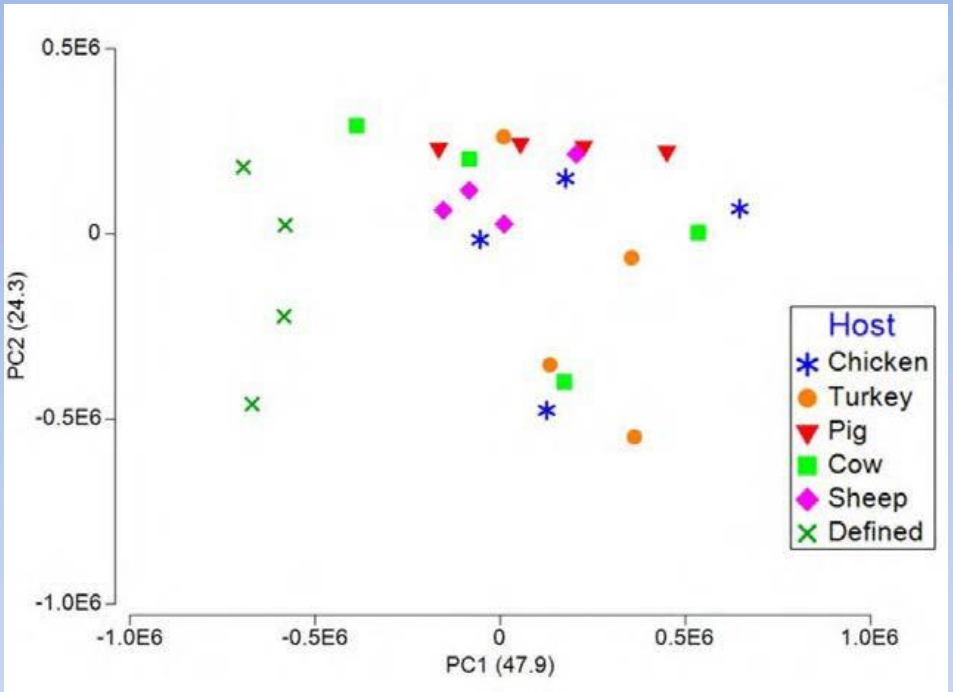
Defined media (MCLMAN), Alazzam, B. *et al*., 2010

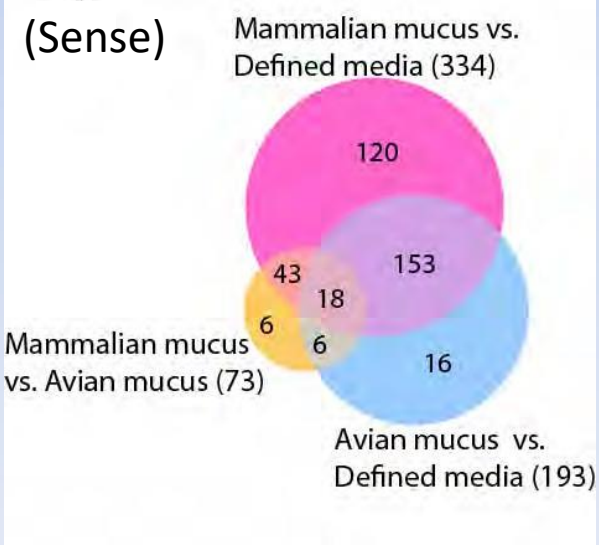
Many genes differentially expressed

- 33 genes were increased in *C. jejuni* grown in mucus, while 208 genes were increased in defined media (*FDR p*< 0.01, fold change >4)



Transcriptomes from *C. jejuni* grown in mucus differs from defined media





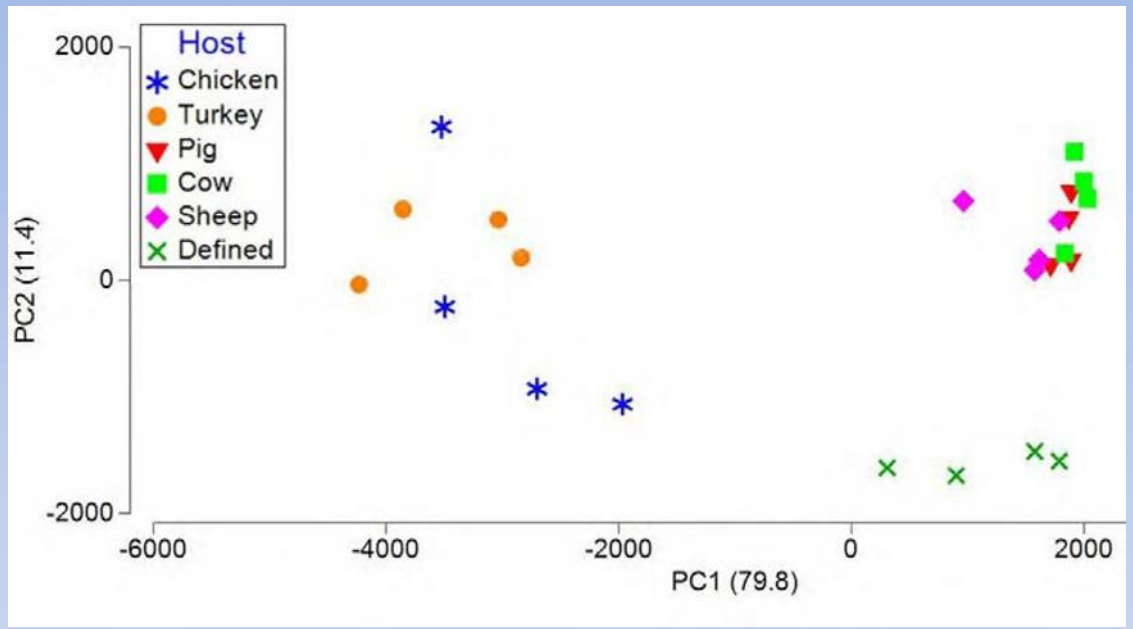
Genes increased in *C. jejuni* grown in avian mucus relative to mammalian mucus

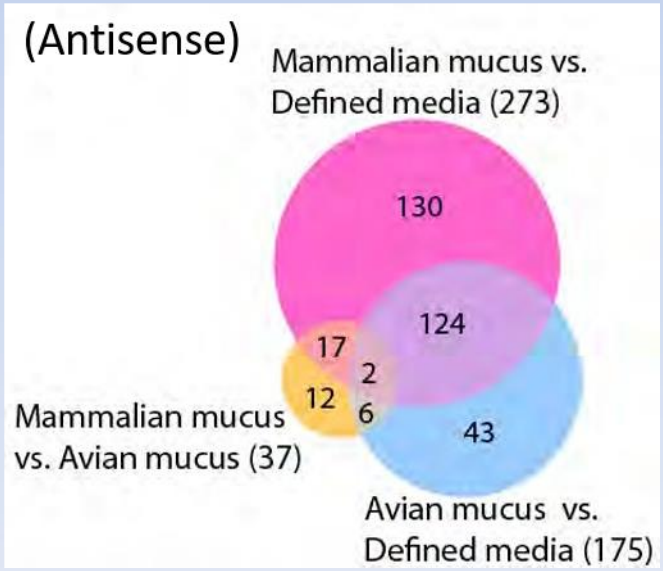
	Gene	Fold change	Description
iron uptake systems	**ceuB**	15	Iron compound ABC uptake transporter permease protein
	ceuC	19	enterochelin uptake permease
	ceuD	8	enterochelin uptake ATP-binding protein
	ceuE	5	Enterochelin uptake periplasmic binding protein
	cfbpA	19	Ferric iron ABC transporter, iron-binding protein
	cfbpB	11	Ferric iron ABC transporter, permease protein
	cfbpC	8	Putative iron-uptake ABC transport system ATP-binding
	cfrA	89	Ferric receptor CfrA
	chuA	87	Haemin uptake system outer membrane receptor
	chuB	84	Haemin uptake system permease protein
	chuC	159	Haemin uptake system ATP-binding protein
	chuD	93	Haemin uptake, periplasmic haemin-binding protein
	Cj0177	136	iron transport protein
	Cj0178	74	Putative outer membrane siderophore receptor
	Cj0241c	9	Hemerythrin domain protein
	Cj0818	51	lipoprotein
	Cj0819	68	Small hydrophobic protein
	Cj1383c	179	hypothetical protein, co expressed with haemin uptake
	Cj1384c	92	hypothetical protein, co expressed with haemin uptake
	Cj1397	20	Ferrous iron transport protein A, putative
	Cj1587c	7	ABC-type siderophore export system
	Cj1613c	17	Putative heme oxygenase
	Cj1658	14	High-affinity Fe2+/Pb2+ permease precursor
	Cj1660	14	Fe2+ ABC transporter, substrate binding protein
	Cj1661	15	Fe2+ ABC transporter, permease protein 1
	Cj1662	14	Fe2+ ABC transporter, permease protein 2
	Cj1663	11	Fe2+ ABC transporter, ATP-binding subunit
	exbB1	89	Ferric siderophore transport system, biopolymer transport
	exbB2	24	Ferric siderophore transport system, biopolymer transport
	exbD1	134	Ferric siderophore transport system, biopolymer transport
	exbD2	80	Biopolymer transport protein ExbD/TolR
	p19	17	Periplasmic protein, high-affinity Fe2+ transport
	tonB1	19	Ferric siderophore transport system, periplasmic binding
	tonB2	25	Ferric siderophore transport system, periplasmic binding
	tonB3	11	Ferric siderophore transport system, periplasmic binding
Oxidative Stress	**perR**	11	Peroxide stress regulator
	trxB	7	Thioredoxin reductase
	katA	117	Catalase
	Cj1386	44	Ankyrin-repeat containing protein
	Cj1308	5	Putative acyl carrier protein
	Cj0556	5	2-pyrone-4,6-dicarboxylic acid hydrolase
	Cj0379c	8	Probable sulfite oxidase
	bioC	4	O-methyltransferase
	ahpC	11	Alkyl hydroperoxide reductase subunit C
	acpP2	8	Putative acyl carrier protein
Membrane assoc.	**Cj1668c**	5	Putative periplasmic protein
	Cj1665	10	Possible lipoprotein thiredoxin
	Cj1664	13	Possible periplasmic thiredoxin
	Cj1621	5	Putative periplasmic protein
	Cj1406c	6	Putative periplasmic protein
	Cj1381	7	putative lipoprotein
	Cj1376	15	periplasmic protein
	Cj1372	5	Phospholipid ABC transporter protein
	Cj1207c	4	Putative lipoprotein thiredoxin
	Cj1021c	5	Putative periplasmic protein
	Cj0926	6	Membrane protein
	Cj0378c	9	Membrane protein
	Cj0176c	15	lipoprotein
Other functions	**cetB**	5	Signal transduction protein, energy taxis
	Cj0878	4	hypothetical protein
	Cj0422c	6	H-T-H containing protein
	Cj0444	13	pseudo gene
	Cj0459c	11	hypothetical protein
	Cj0717	8	Glutathione-dependent thiol reductase
	cgb	5	Bacterial haemoglobin, nitrosative stress
	Cj0880c	5	hypothetical protein
	Cj0963	5	DNA polymerase, bacteriophage-type
	Cj1208	5	5-formyltetrahydrofolate cyclo-ligase
	dba	14	disulfide bond formation protein
	dnaN	8	DNA polymerase III beta subunit

Genes increased in *C. jejuni* grown in mammalian mucus relative to avian mucus

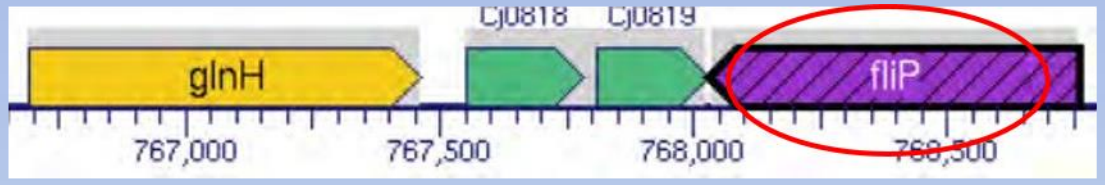
Gene	Fold change	Description
fdxA	-14	4Fe-4S ferredoxin, iron-sulfur binding
rrc	-14	Rubrerythrin, oxidative stress tolerance

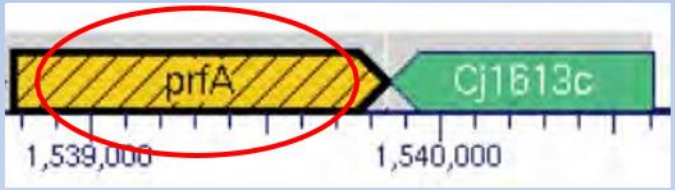
Antisense transcriptomes from mucus differs between hosts

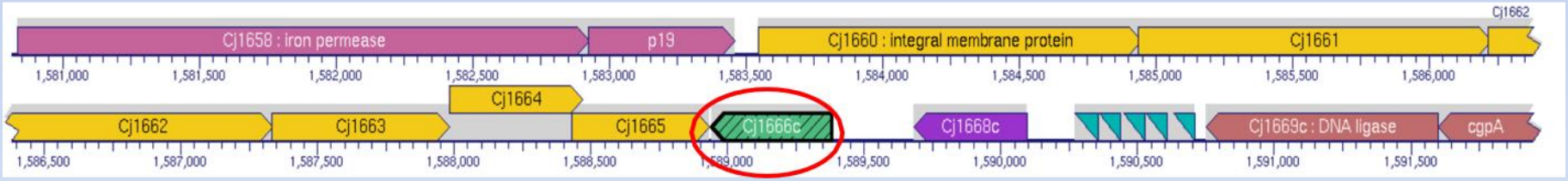


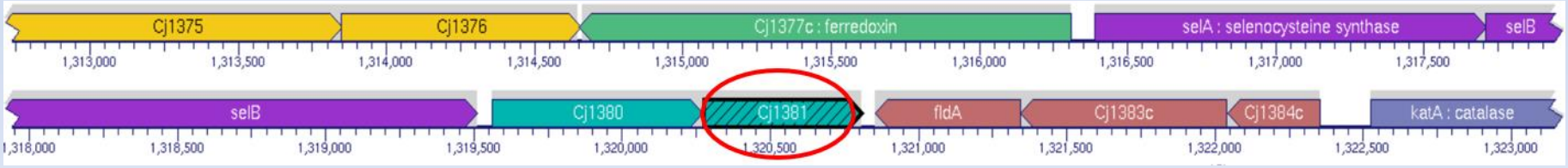


Gene neighborhoods of asRNAs include other upregulated genes









Summary

- Avian mucus modulates *C. jejuni* growth, adherence and invasion of host cells and gene expression, as well as upregulation of genes essential for colonization
  - Iron acquisition and oxidative stress genes

- Antisense RNAs were differentially expressed between avian and mammalian mucus, and associate with differentially expressed adjacent genes

- Understanding the environmental signals of mucus colonization may suggest interventions to reduce *C. jejuni* colonization

Future

- Ecology of AMR
  - Mobilization of AMR genes
  - Host range for some of the AMR-containing mobile genetic elements
- Succession of the microbiota in poultry
  - Are maternal or environmental inputs driving succession?
  - Can microbiota succession be modulated?
- Role of microbiota on *Campylobacter* colonization
  - Why are some flocks consistently positive or negative?
  - Does bacterial membership or functional capacity impact *Campylobacter* colonization?

• Acknowledgements

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- Adrienne Shircliff

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- Timothy Johnson

USDA Crop Production & Pest Control Research

- Guohong Cai

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