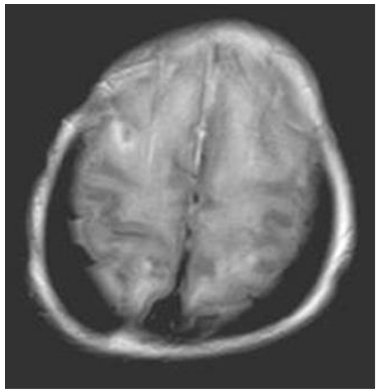
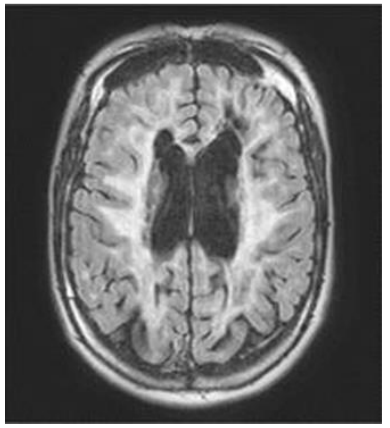
Early Life Physiological and Psychosocial Stress Imprints Gut Microbiome in Preterm Infants

To investigate the regulation of early life stress by the brain-gut-microbiota signaling mechanism and explore non-invasive microbial and immune-inflammatory predictors of neurodevelopment.

Background and Significance



- The U.S. ranks one of the highest in the world for the number of preterm births.
  < 37 weeks of gestation; 10% in 2014



- In NICU, infants are exposed to numerous early life stressors/pain during critical periods of neurodevelopment.
- 40% NICU survivors have at least 1 neurodevelopmental deficit.
- **Yet, mechanisms of early life experiences that alter infants' health outcomes remain largely unknown.**

The Brain-Gut-Microbiota Axis

- Components: Central nervous system (CNS), Hypothalamus-pituitary-adrenal (HPA) axis, Sympathetic-parasympathetic autonomic nervous system, Enteric nervous system, and Intestinal microbiota.

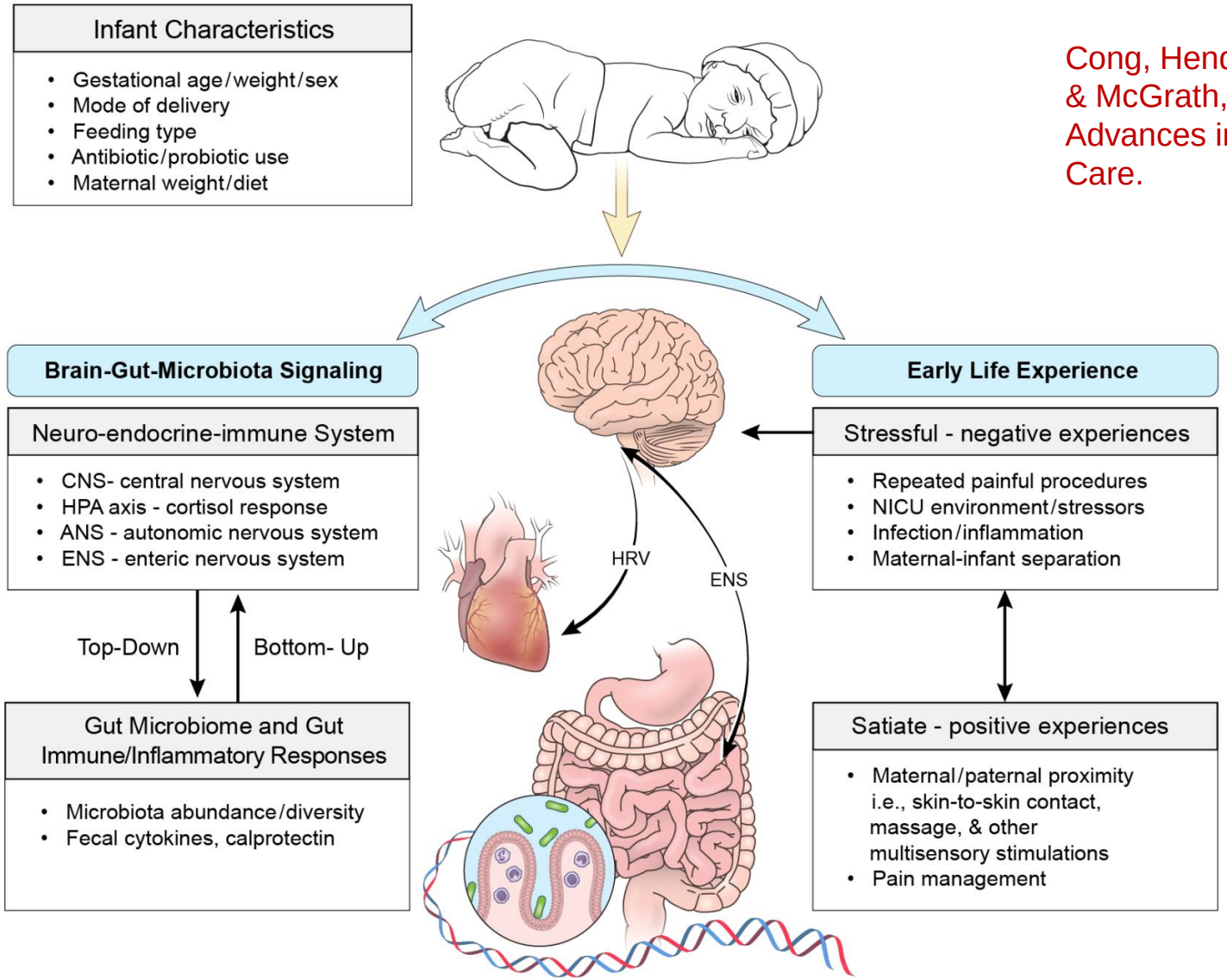
- Bidirectional communication network:

  Top-down: brain to influence the motor, sensory and secretory modalities of the GI tract

  Bottom-up: gut to affect brain function (hypothalamus and amygdala).

Video: https://www.youtube.com/watch?v=5DTrENdWvvM

Brain-Gut-Microbiota Signaling System



Cong, Henderson, Graf, & McGrath, (2015), Advances in Neonatal Care.

Gut Microbiome Patterns in Infants

- Colonization begins with facultative anaerobic organisms, followed by the development of obligate anaerobes, including *Bifidobacterium, Bacteroides,* and *Clostridium.*

- Full-term, breast-fed infant serves as the health standard or the “norm” for newborns.

- ***Factors:*** delivery mode, feeding, medication use, hospital environment, other early life experiences, and host genetics.

Gut Microbiome Patterns in Infants

- *Dysbiosis* of gut microbiota persists during infancy, especially in preterm infants, and then may reach a stable configuration at age 2 - 3 yrs.

- Preterm infants: demonstrate reduced levels of obligate anaerobes.

- Preterm infants: increased levels of facultative anaerobes, i.e., Enterobacteriaceae and Enterococcaceae

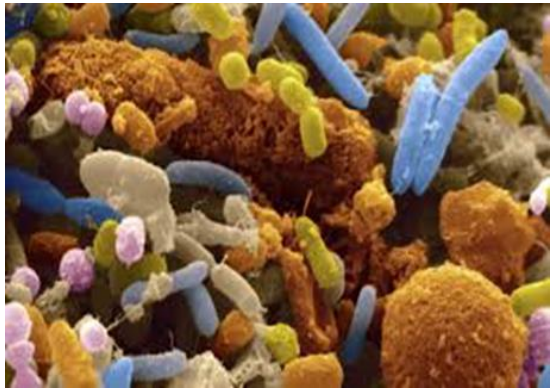
- https://www.youtube.com/watch?v=Pb272zsixSQ

Study Aims

Aim 1: Determine preterm infants' gut microbiome patterns over first 3-4 weeks

Aim 2: The linkage of gut microbiome patterns with early life stress/pain

Aim 3: The linkage of gut microbiome with neurodevelopmental outcomes.



Methods

- Design: Prospective longitudinal study.
- Setting: Level IV CCMC NICU at two sites, Hartford and Farmington, CT.
- Subjects: Stable preterm infants (26 – 32 weeks gestation), follow-up for 3-4 weeks.

Methods

- IRB approval and obtain consent from parents.
- Early life stress are measured daily.

NISS: Neonatal Infant Stressor Scale (Newnham, et. al, 2009)

- Feeding types (Mother's, Donor's, Formula)
- Neurodevelopmental outcomes, at 36-38 weeks CA.

NNNS: NICU Neurobehavioral Scale (Lester, et. al, 2004)

Methods

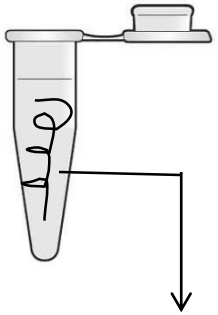
- Stool samples are collected daily starting 0 – 5 postnatal days for 3-4 weeks.

  Culture-independent DNA-based Genomic Technologies:

  Gut microbiota community profiles are determined by 16S rRNA sequencing and analysis

Investigating the Infant Microbiome

http://www.reuters.com

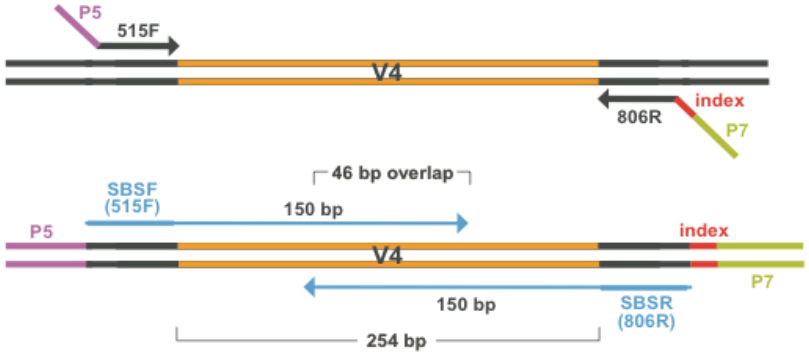


DNA Isolation from Microbial Community within Fecal Sample

PCR Amplification of the V4 Hypervariable Region of the 16S rRNA Gene

Illumina MiSeq Amplicon Sequencing

Illumina.com



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Quantitative Insights Into Microbial Ecology

qiime

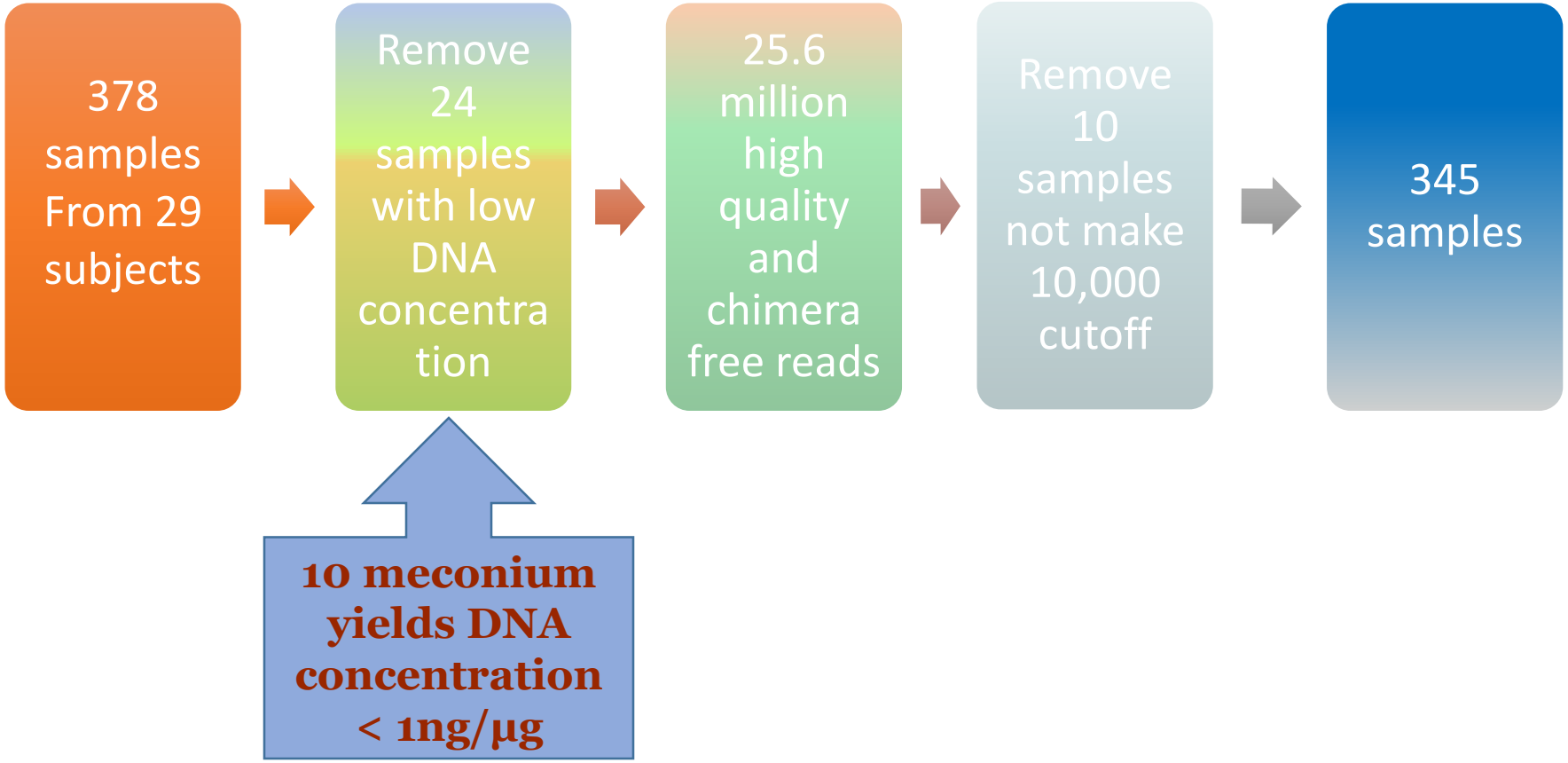
Microbial Composition Analyses

Results from First Cohort

Clinical characteristics of the initial 29 subjects:

		N (%)		Mean	SD
Gender	Male	14 (48 %)	Gestational Age (wks)	31.3	1.7
	Female	15 (52%)	Birth weight (g)	1460	445.3
Ethnicity	Hispanic	9 (31%)	SNAPEII	8.6	10.5
	Non-Hispanic	20 (69%)			
Delivery	Vaginal	12 (45%)			
	C-section	16 (55%)			
Race	White	22 (75 %)			
	African American	5 (17%)			
	Asian	1 (3%)			

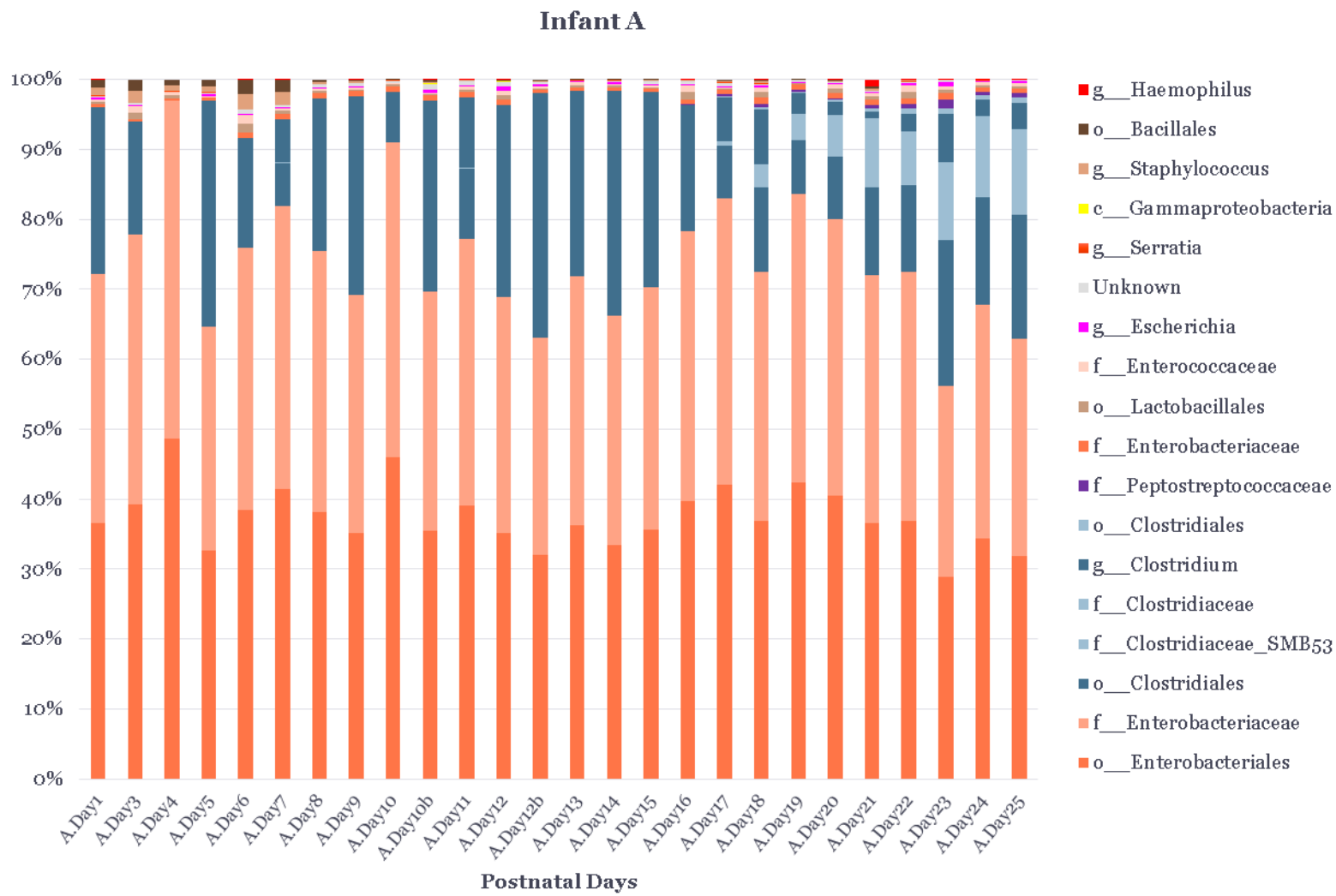
Workflow and Quality Control of Stool Samples

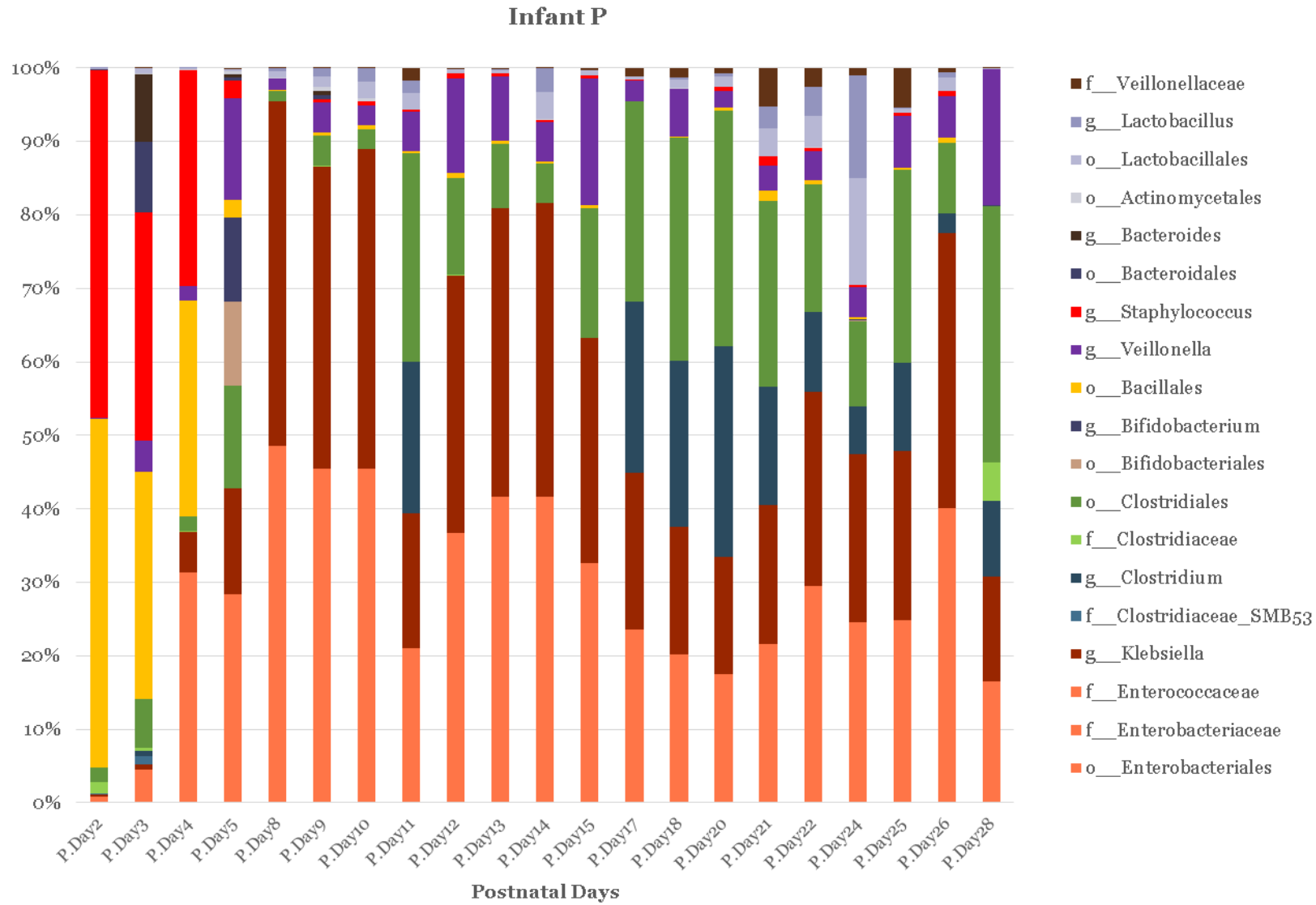


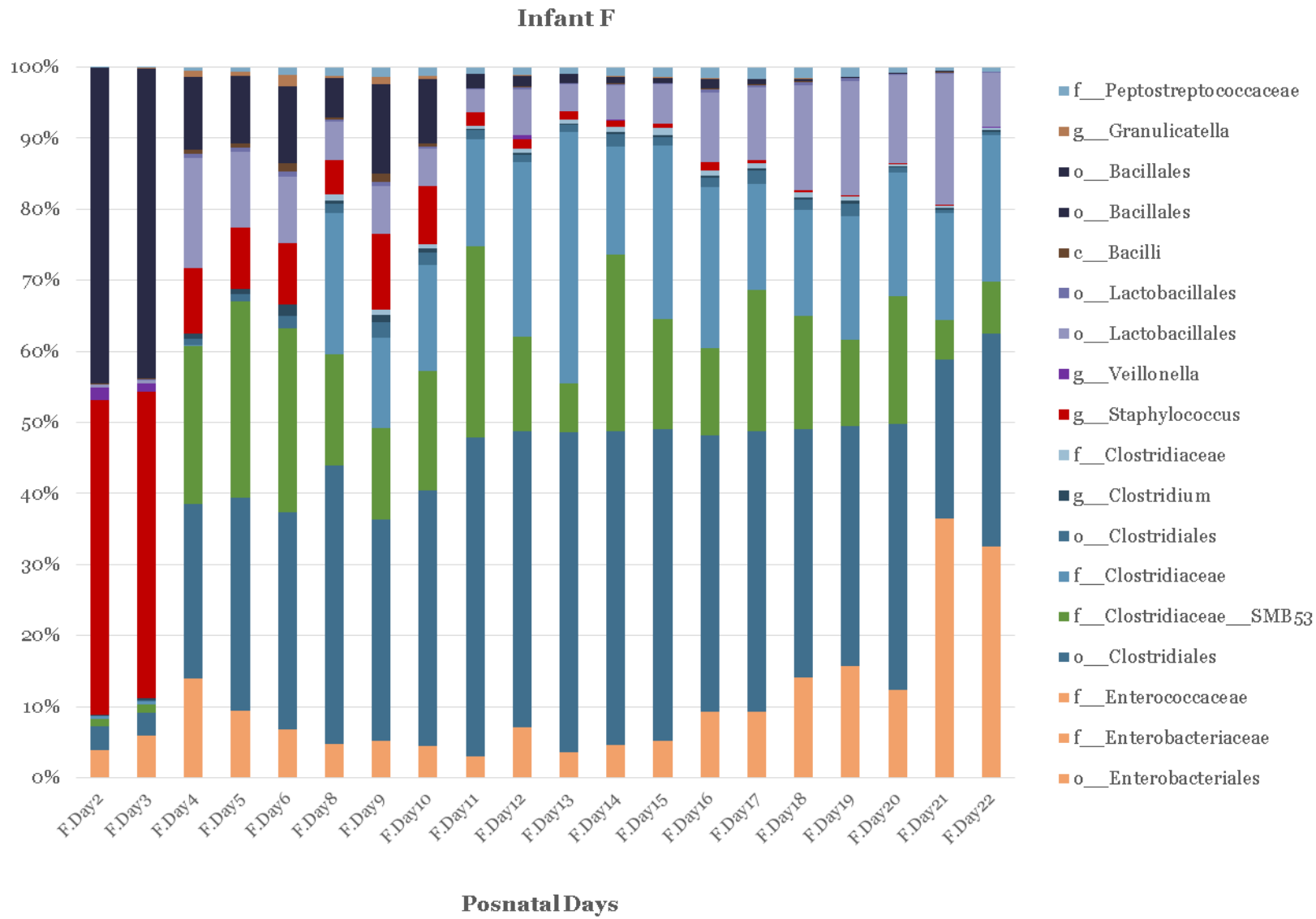
Result 1: Microbiome community composition in preterm infants

- The most abundant phyla:
  - Proteobacteria (54.3%)
  - Firmicutes (39.2%)
  - Bacteroidetes (3.9%)
  - Actinobacteria (2.4%).

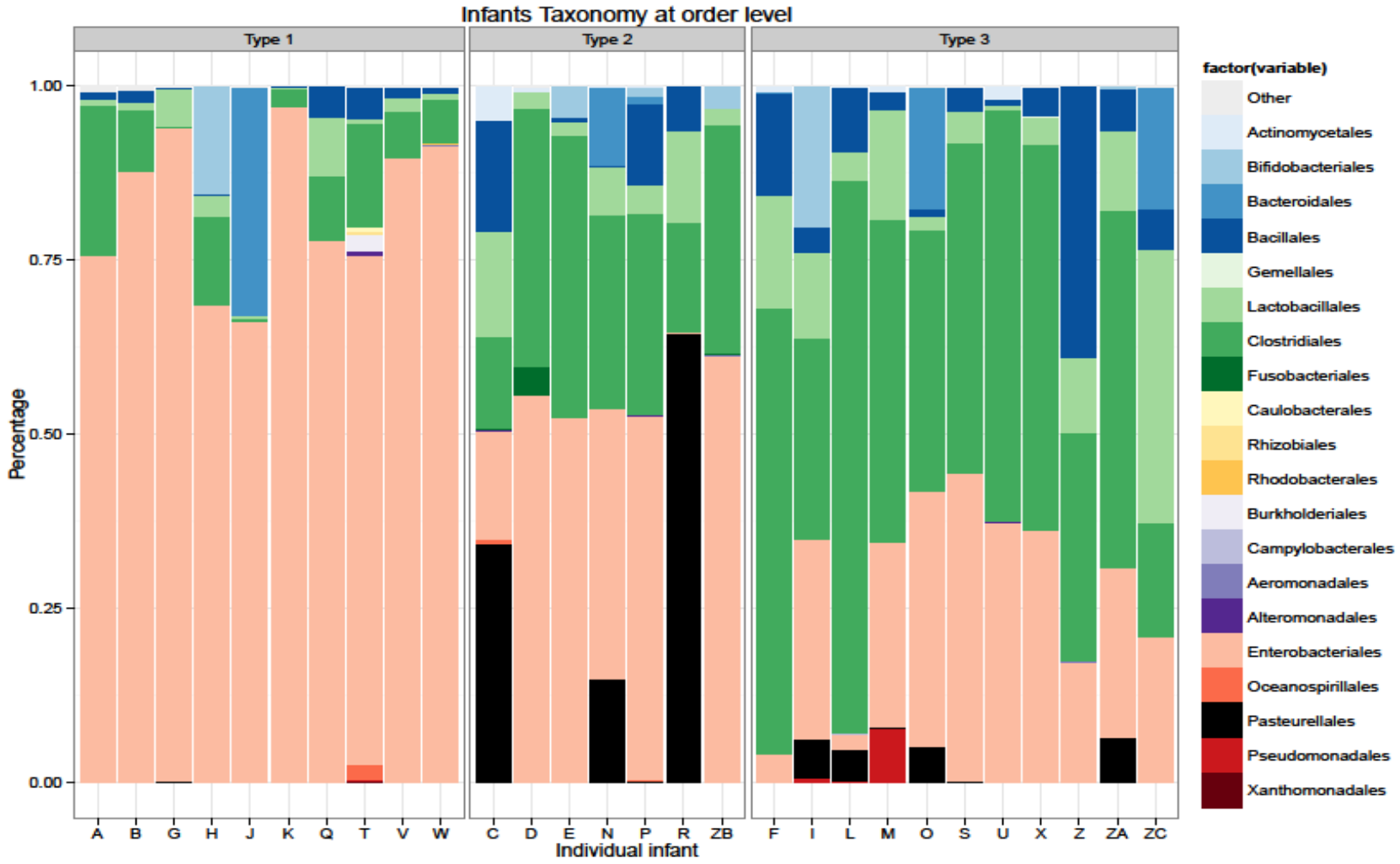
- What contributes to changes in the diversity of the microbiome (Linear mixed-effects models):
  - Time (postnatal days)
  - Gender
  - Feeding type (using mother's breastmilk or not)


Infant A
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
A.Day1
A.Day3
A.Day4
A.Day5
A.Day6
A.Day7
A.Day8
A.Day9
A.Day10
A.Day10b
A.Day11
A.Day12
A.Day12b
A.Day13
A.Day14
A.Day15
A.Day16
A.Day17
A.Day18
A.Day19
A.Day20
A.Day21
A.Day22
A.Day23
A.Day24
A.Day25
Postnatal Days
g__Haemophilus
o__Bacillales
g__Staphylococcus
c__Gammaproteobacteria
g__Serratia
Unknown
g__Escherichia
f__Enterococcaceae
o__Lactobacillales
f__Enterobacteriaceae
f__Peptostreptococcaceae
o__Clostridiales
g__Clostridium
f__Clostridiaceae
f__Clostridiaceae_SMB53
o__Clostridiales
f__Enterobacteriaceae
o__Enterobacteriales


Infant P
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
P.Day2
P.Day3
P.Day4
P.Day5
P.Day8
P.Day9
P.Day10
P.Day11
P.Day12
P.Day13
P.Day14
P.Day15
P.Day17
P.Day18
P.Day20
P.Day21
P.Day22
P.Day24
P.Day25
P.Day26
P.Day28
Postnatal Days
f__Veillonellaceae
g__Lactobacillus
o__Lactobacillales
o__Actinomycetales
g__Bacteroides
o__Bacteroidales
g__Staphylococcus
g__Veillonella
o__Bacillales
g__Bifidobacterium
o__Bifidobacteriales
o__Clostridiales
f__Clostridiaceae
g__Clostridium
f__Clostridiaceae_SMB53
g__Klebsiella
f__Enterococcaceae
f__Enterobacteriaceae
o__Enterobacteriales


Infant F
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
F.Day2
F.Day3
F.Day4
F.Day5
F.Day6
F.Day8
F.Day9
F.Day10
F.Day11
F.Day12
F.Day13
F.Day14
F.Day15
F.Day16
F.Day17
F.Day18
F.Day19
F.Day20
F.Day21
F.Day22
Posnatal Days
f__Peptostreptococcaceae
g__Granulicatella
o__Bacillales
o__Bacillales
c__Bacilli
o__Lactobacillales
o__Lactobacillales
g__Veillonella
g__Staphylococcus
f__Clostridiaceae
g__Clostridium
o__Clostridiales
f__Clostridiaceae
f__Clostridiaceae__SMB53
o__Clostridiales
f__Enterococcaceae
f__Enterobacteriaceae
o__Enterobacteriales

3 Microbial Patterns by Individual Infants Cong, Xu, Janton, Henderson, Matson, McGrath, Maas, Graf, (2016), PloS One



Result 2: Microbiome Patterns and Gender

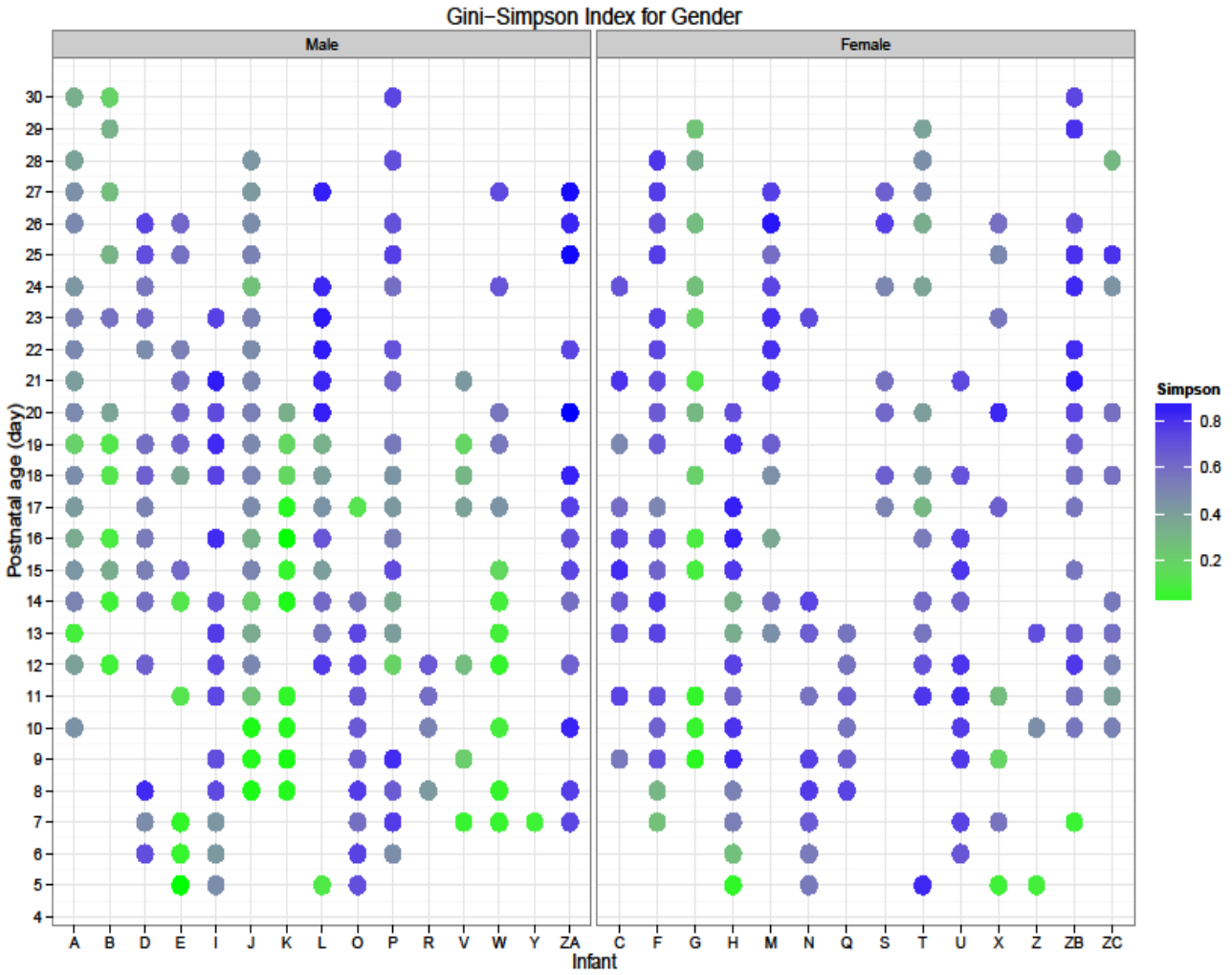
Cong, Xu, Janton, Henderson, Matson, McGrath, Maas, Graf, (2016), PloS One

α-diversity Index:

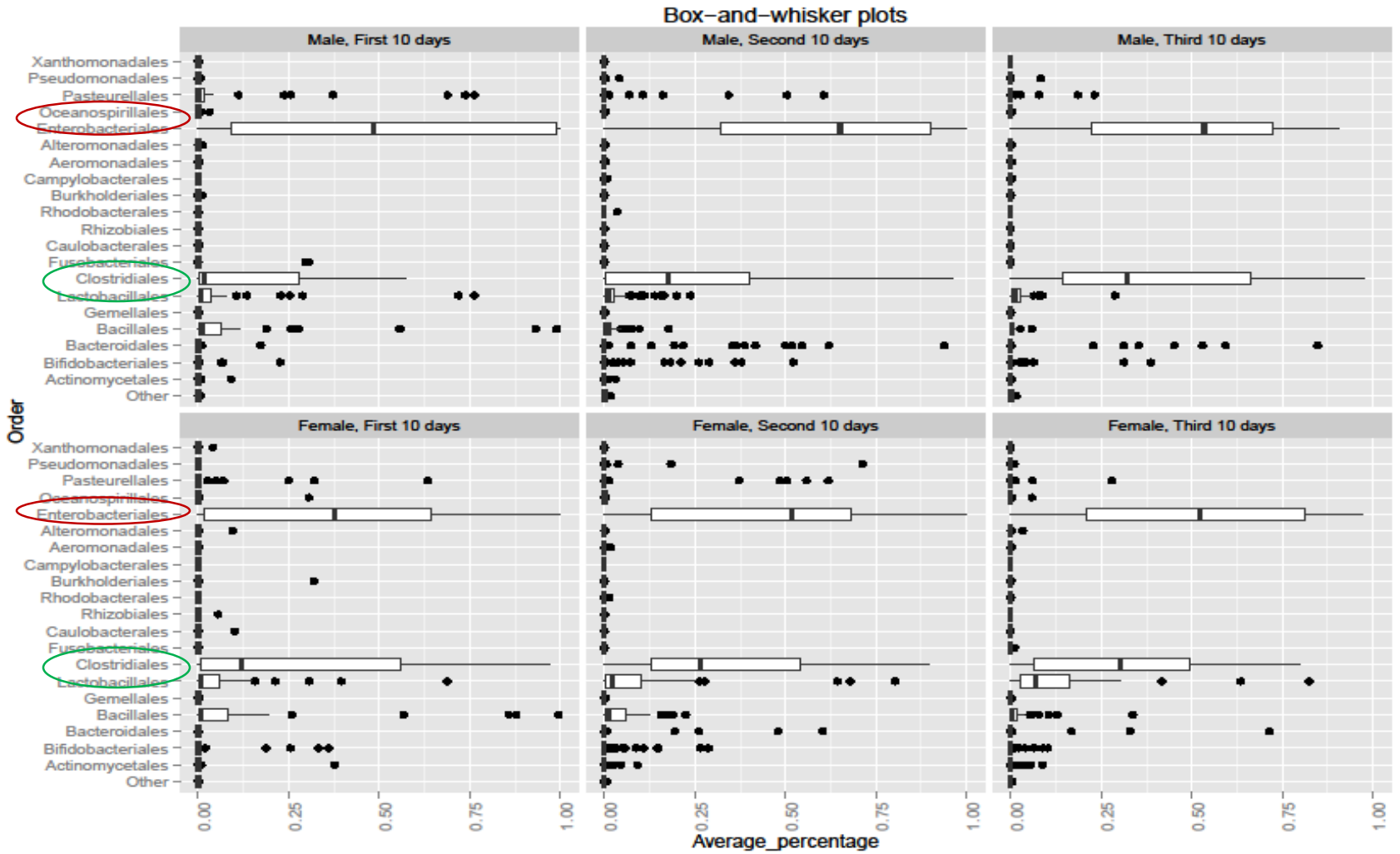
Left: males (0.48±0.26)

Right: females (0.58±0.22)

P < 0.05



Microbiome Communities and Gender (abundance) Cong, Xu, Janton, Henderson, Matson, McGrath, Maas, Graf (2016)



Result 3: Gut Microbiome and Feeding Types

Kingdom

Phylum

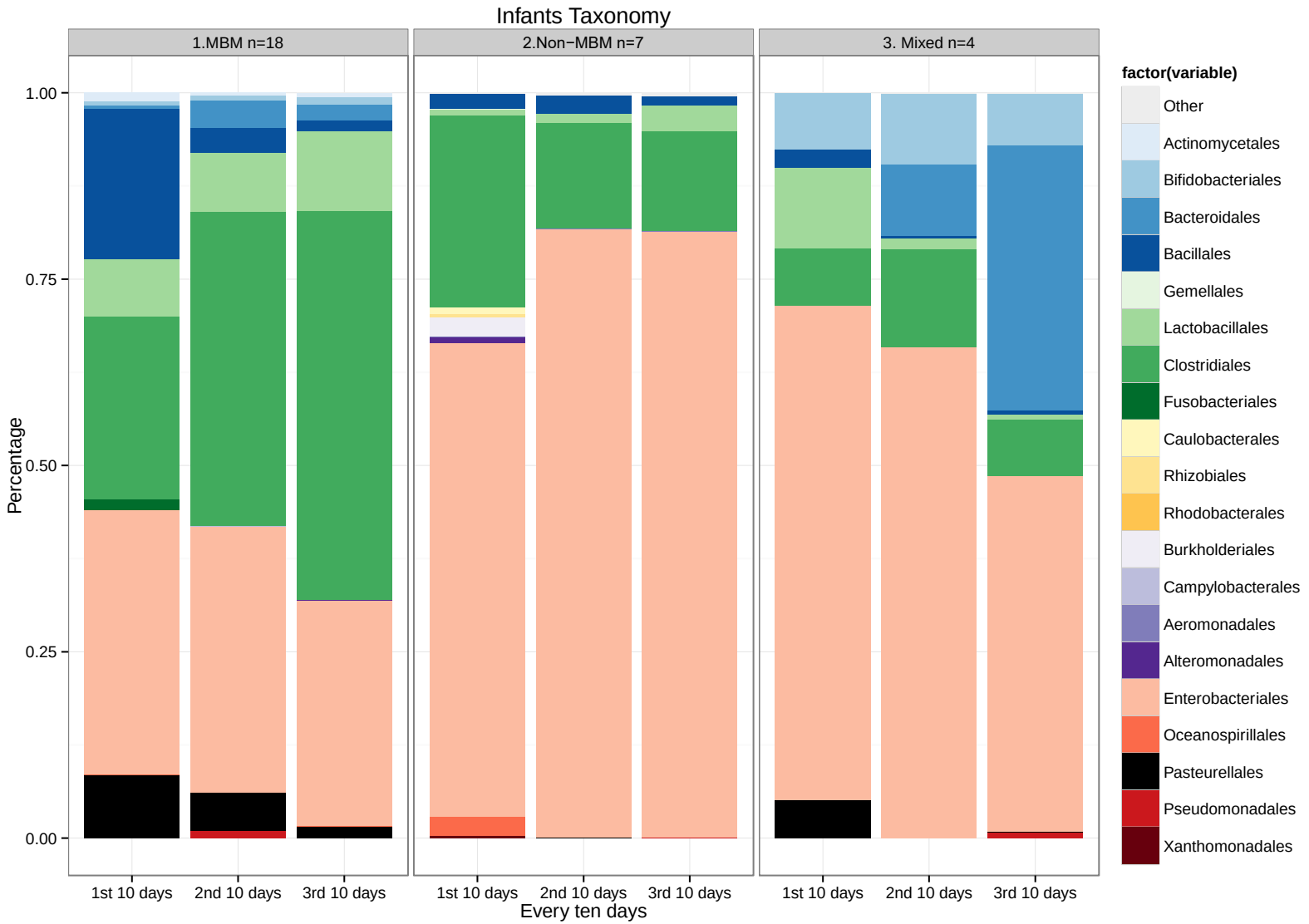
Class

Order

Family

Genus

Species

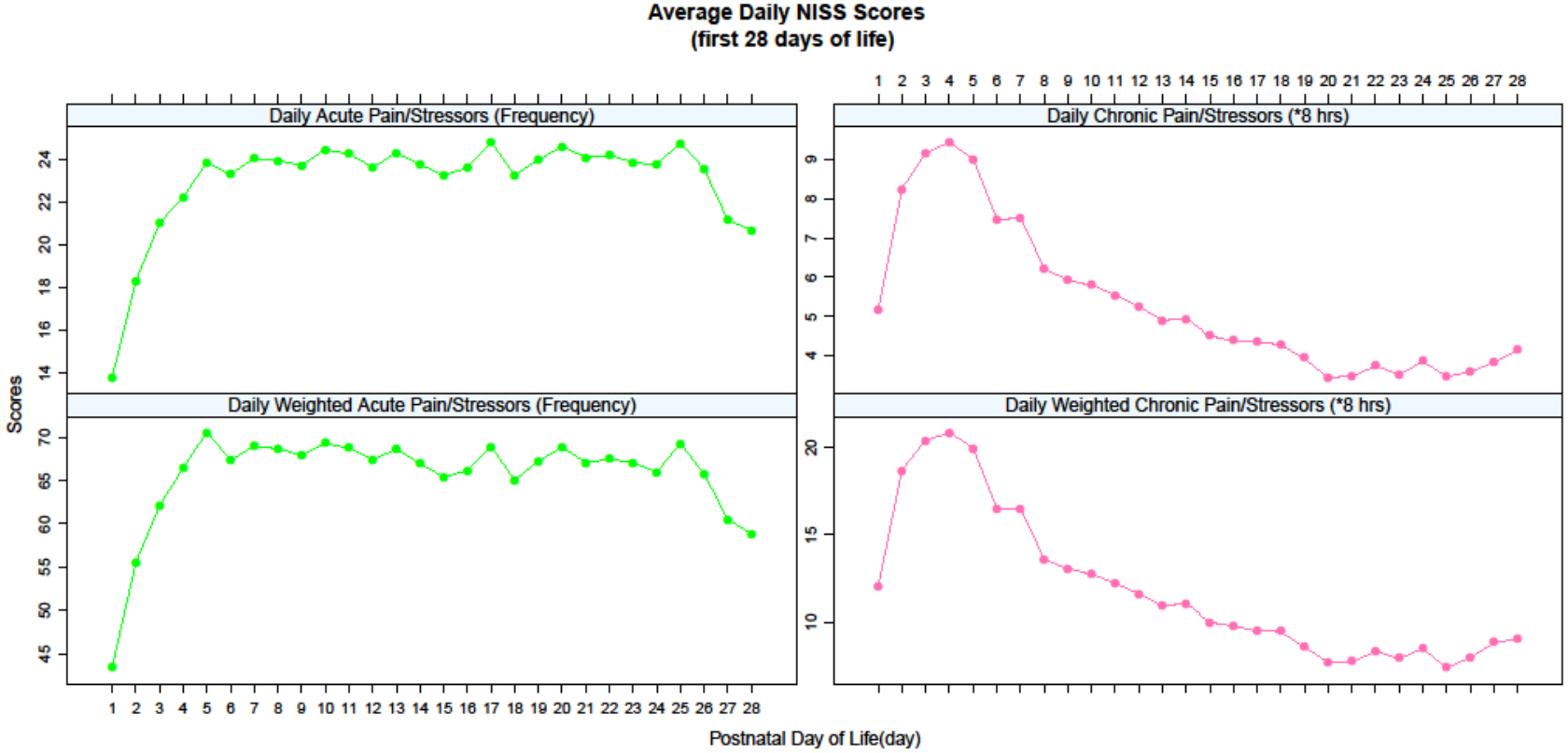


Results 4: Cumulative Pain/Stressors in the NICU

- Data from initial 50 subjects:
- **Acute pain/Stressor** daily: 23.4 ± 7.2

  diaper change, heel sticks, arterial blood draw
- **Chronic procedures** daily 5.1 ± 3.2 hours

  PICC in situ, NG tube in situ, CPAP, systemic infection



Developing a new scale measuring cumulative pain/stressors in the NICU

Accumulated Pain/Stressor Scale (APSS) in NICUs – based on Neonatal Infant Stressor Scale (NISS, Newnham, et al. (2009) in Australia)

- Focus group study
- National survey

- 68 procedures
- 9 categories: daily care, feeding, imaging, blood draw, peripheral venous access, central venous access, respiratory care, surgeries and major procedures, and infection

- Xu, Cong, Walsh, S. (in press). Development of Accumulated Pain/ Stressor Scale (APSS) in NICUs: A National Survey. Pain Management Nursing.

Results 5: Pain/Stressors, Gut Microbiome, and Neurobehavioral Outcomes

Cumulative Pain/Stress and Neurobehavioral Outcome

GLM Regression Analysis: NNNS scale

	NNNS- Stress/ Abstinence (p – value)	NNNS- Habituation (p-value)
Daily acute pain/stressors	0.028	0.016
Daily chronic pain/stressors	0.051	0.005
Gender	>0.05	0.022
Birth GA	>0.05	>0.05
Weight	>0.05	0.004
SNAPPE-II	>0.05	0.041
Direct BF	>0.05	0.002
Skin-to-Skin (KC)	>0.05	0.001

Generalized linear mixed models:

- NISS Acute and chronic pain/stressors: significantly associated with infant neurobehavioral outcomes (NNNS- Stress/Abstinence subscale scores), when controlling for birth GA, birth weight, delivery mode, severity of illness, and direct breastfeeding and kangaroo care contacts, $p<0.05$.

- NISS scores were negatively correlated to NNNS – Habituation subscale scores, $p<0.05$-0.01.

- **Indicating that infants who experienced more accumulative pain/stressor had worse stress responses at 36-38 weeks CA.**

- **Indicating infants who experienced less painful/stressful procedures had better habituation and regulation responses.**

Linkages of Pain/Stressors, Gut Microbiome, and Neurobehavioral Outcomes –

Indicator species of microbiota in different levels of pain/stressors experienced in the NICU

Phylum	Order / Genus	Indicator Value	Phylum	Order / Genus	Indicator Value
Infants Experienced Low level of *Acute* Pain/Stressors			**Infants Experienced Low level of *Chronic* Pain/Stressors**		
Actinobacteria	*Bifidobacteriales/Bifidobacterium*	0.65**	*Bacteroidetes*	*Bacteroidales/Bacteroides*	0.50*
Infants Experienced High levels of *Acute* Pain/Stressors			**Infants Experienced High level of *Chronic* Pain/Stressors**		
Firmicutes	*Lactobacillales/Enterococcus*	0.78**	*Firmicutes*	*Lactobacillales/Enterococcus*	0.85**
Firmicutes	*Lactobacillales/other*	0.72**	*Firmicutes*	*Lactobacillales/other*	0.80**
Firmicutes	*Lactobacillales/Granulicatella*	0.69**	*Firmicutes*	*Other/other*	0.77**
Proteobacteria	*Enterobacteriales/Pantoea*	0.52**	*Firmicutes*	*Lactobacillales/Granulicatella*	0.68**

Note: ** p < 0.01; * p < 0.05

Indicator species of gut microbiota with NNNS

Phylum	Order / Genus	Indicator Value
Infants with Less (better) NNNS-stress response		
Bacteroidetes	*Bacteroidales/Bacteroides*	0.55**
Infants with high (worse) NNNS-stress response		
Firmicutes	*Lactobacillales/other*	0.72**
Proteobacteria	*Enterobacteriales/Pantoea*	0.68**
Firmicutes	*Clostridiales/other*	0.56**
Proteobacteria	*Aeromonadales/other*	0.52**

Note: ** p < 0.01

Conclusions

- Over the first 30 days of early life, gut microbiome diversity begins low and increases daily after birth.
- Preterm infants' gut microbiome community is often dominated by Enterics.
- Preterm infants experience numeric acute and prolonged chronic painful/stressful procedures.
- Time (postnatal days of life), feeding, gender, and pain/stress experience affect the composition of the gut microbiome.
- Gut microbiome indicator species may be omic markers of pain/stressors for infant neurodevelopment.

Multidisciplinary Collaborations

Research Team:

- Xiaomei Cong, PhD, RN
- Joerg Graf, PhD
- Wendy A. Henderson, PhD, RN
- Jacqueline McGrath, PhD, RN
- Adam Matson, MD
- Naveed Hussain, MD
- Stephen Walsh, PhD
- Kendra Maas, PhD
- Wanli Xu, MS, RN, PhD student
- Dorothy Vittner, MS, RN, PhD student
- Ana Diallo, RN, PhD student
- Shari Galvin, BS, RN
- Megan Fitzsimons, BS, RN
- Laura Keating, BS, RN
- Angela Dejong, BS, RN

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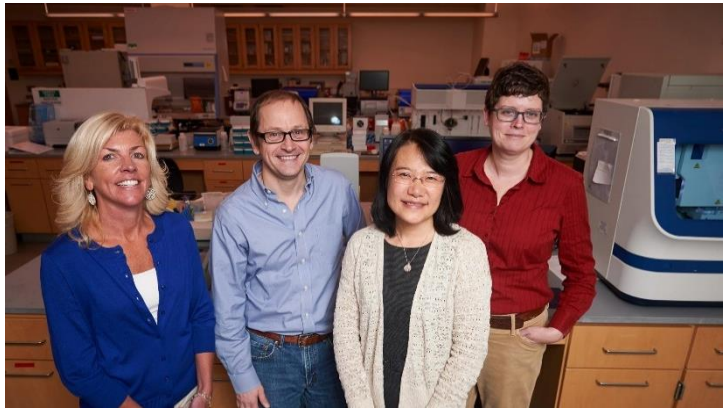
- UConn School of Nursing
- UConn Dept. of Molecular & Cell Biology
- Connecticut Children's Medical Center
- Stevenson Fund Support

CT Children's Medical Center NICU Staff Members and Families



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