Factors that Modify the Microbiome in Perinatal and Neonatal Periods

Josef Neu, M.D.

University of Florida

Agenda

- **The Past**
  - Human Microbiome Project and Intestinal Microbe Host Interactions
- **Recent Studies**
  - Preterm Birth.
  - Mode of Delivery Effects
  - Environmental Effects:
    - Antibiotics
  - Microbes in Human Milk
- **The Future**
    - Multi-omics
    - Host Microbial Interactions

Environmental sample

Dilute

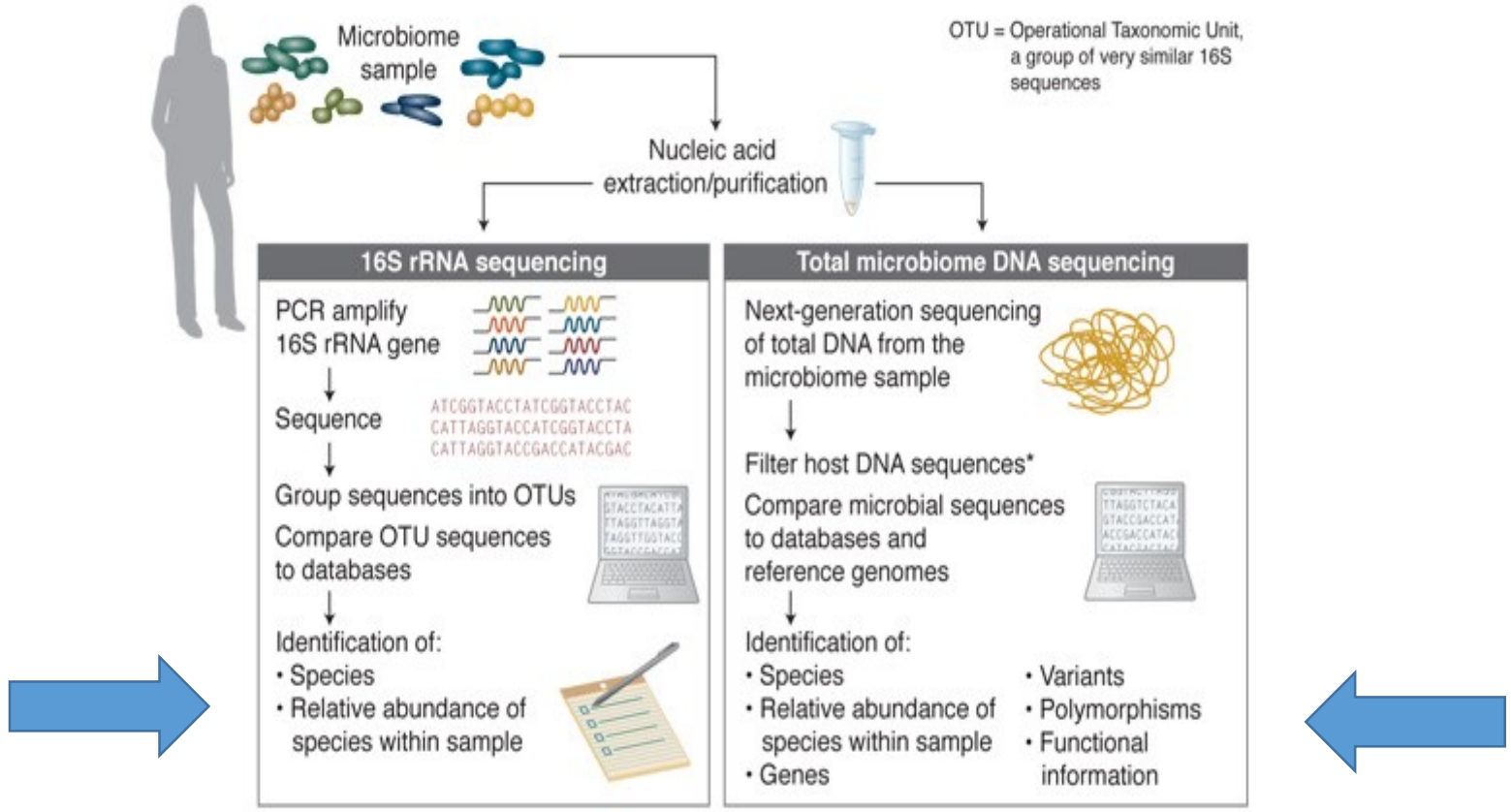
Microscope

Petri dish

"THE GREAT PLATE COUNT ANOMALY"

~100 times > cells than colonies, 99% unculturable

Major Sequencing Technologies

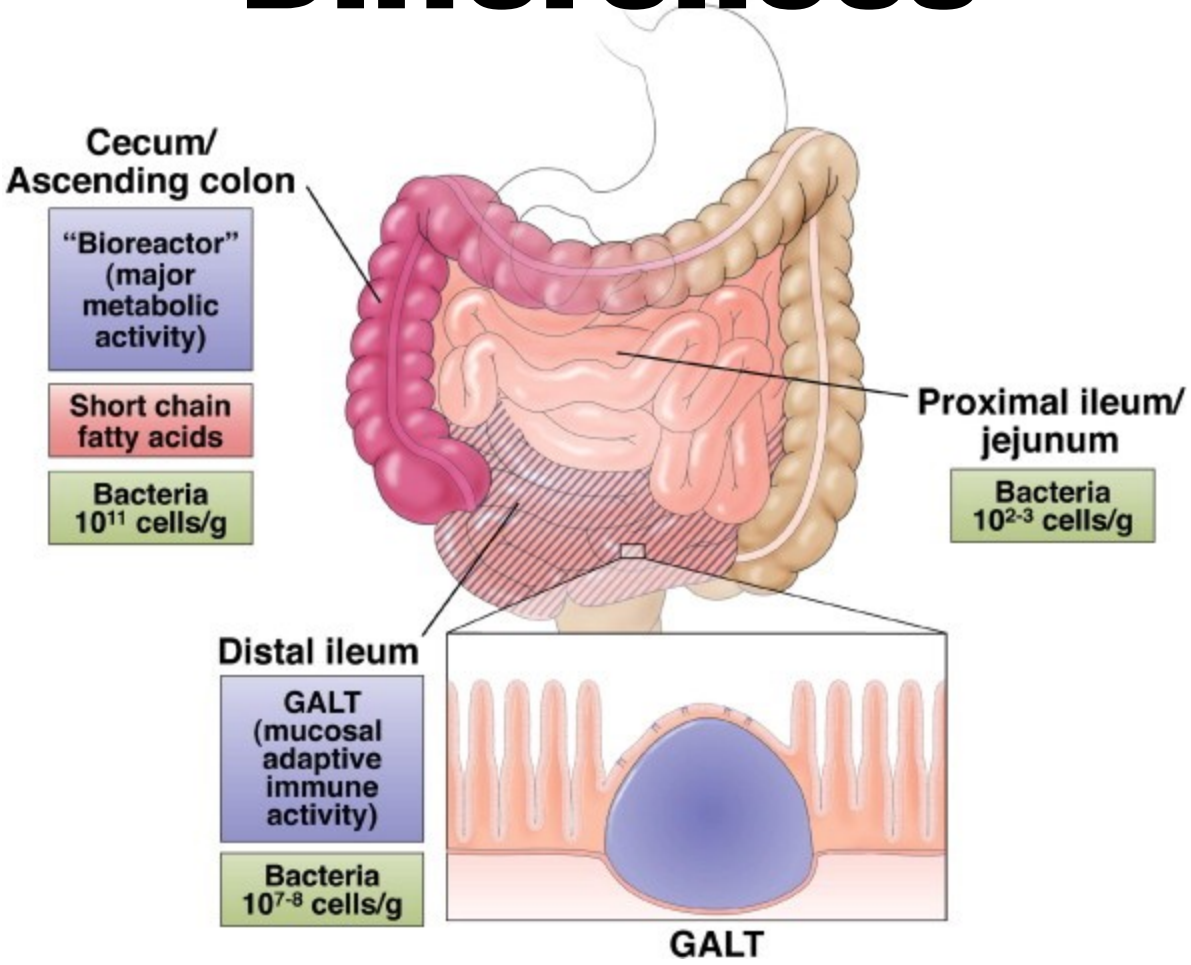


Adapted from : https://www.neb.com/tools-and-resources/feature-articles/addressing-challenges-in-microbiome-dna-analysis

Holobiont

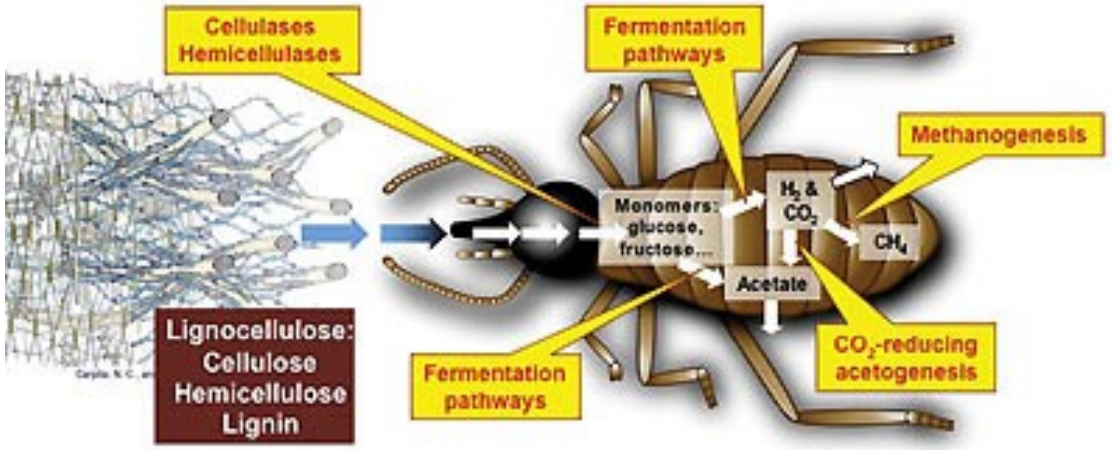
A “super or meta -organism” consisting of an assemblage of different species that form ecological units. It adjusts and transforms itself according to environmental changes causing evolution of the entire entity.

The Gut: Regional Microbial Differences



Neish, A. Gastroenterology vol. 136,No. 1, 2009

Mechanisms: Metabolomic (Bioreactor) Role



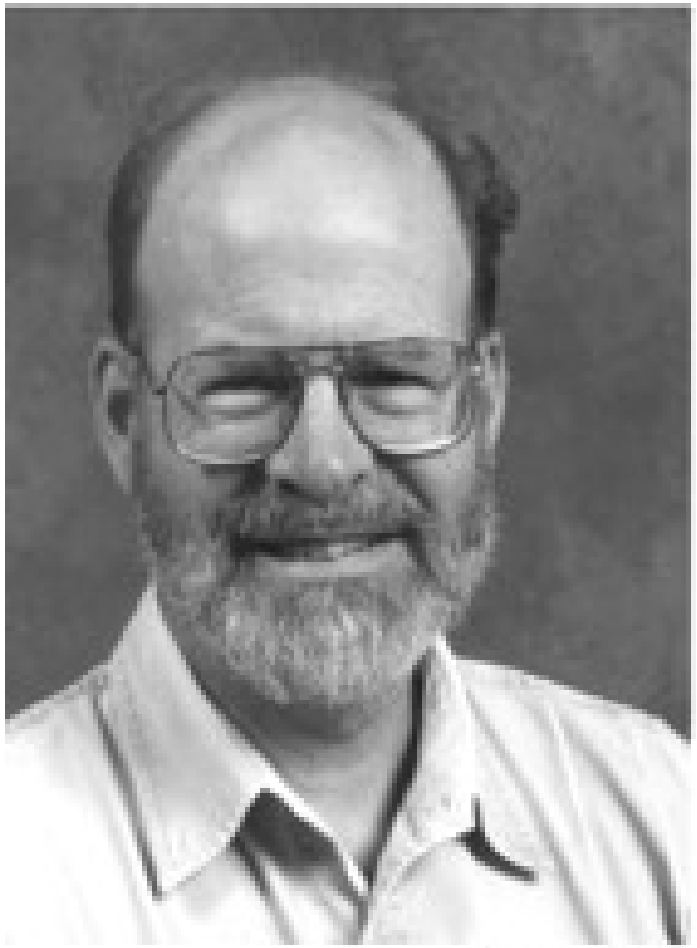
Gut bacteria and obesity
"Holy shit!"
Nov 12th 2009
From *The Economist* print edition
A new way of finding out how diet affects gut microbes

Roy C. Nutr. Clin Practice 21;351:2006
Turnbaugh, PJ Cell Host Microbe Volume 3, Issue 4, 17 April 2008, Pages 213-223

“TOLL” Proteins-1985

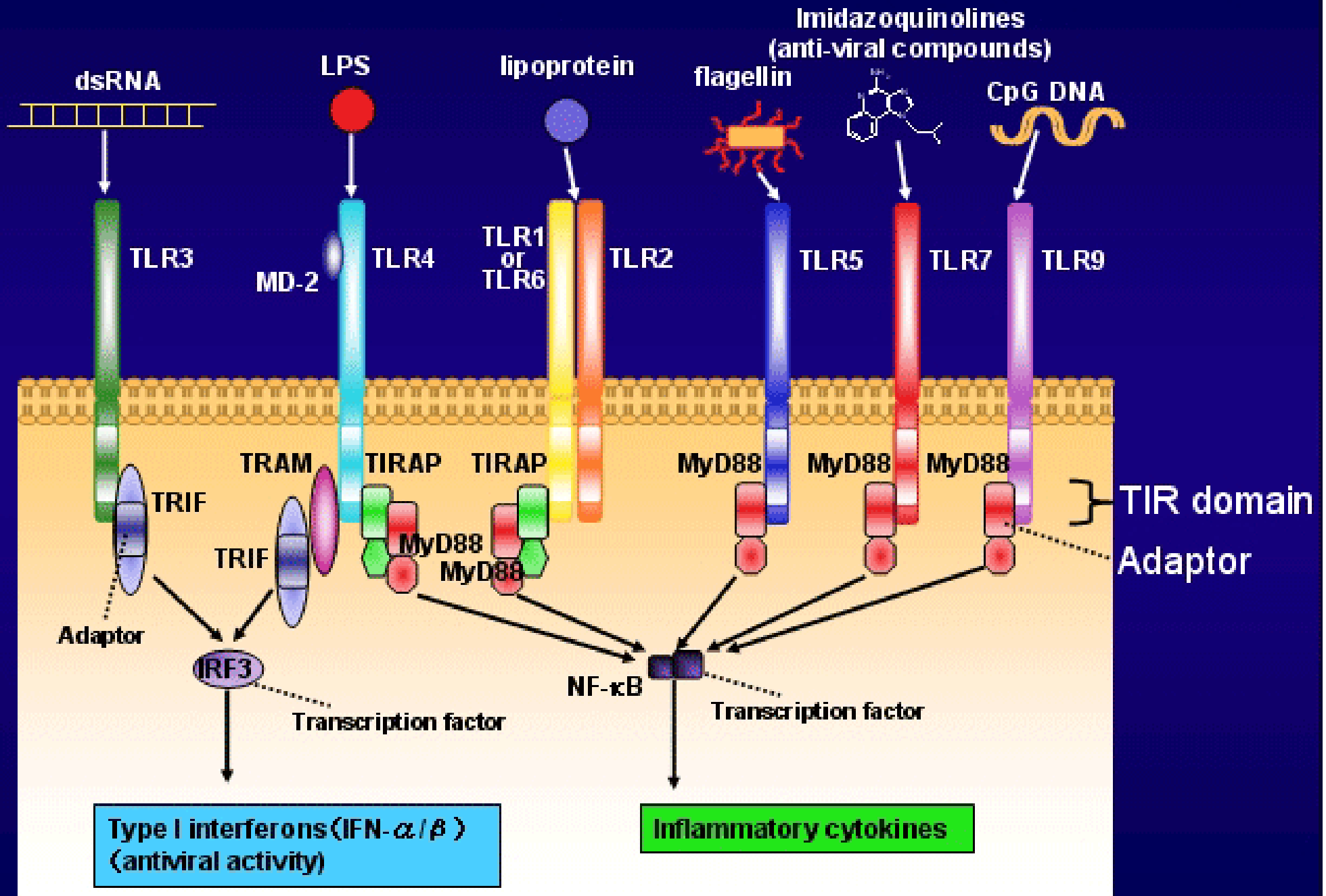
Christiane Nüsslein-Volhard

“Toll Receptors”-1996



"Father of Innate Immunity," Charles Janeway

Toll-like receptors: ligands and signaling pathways



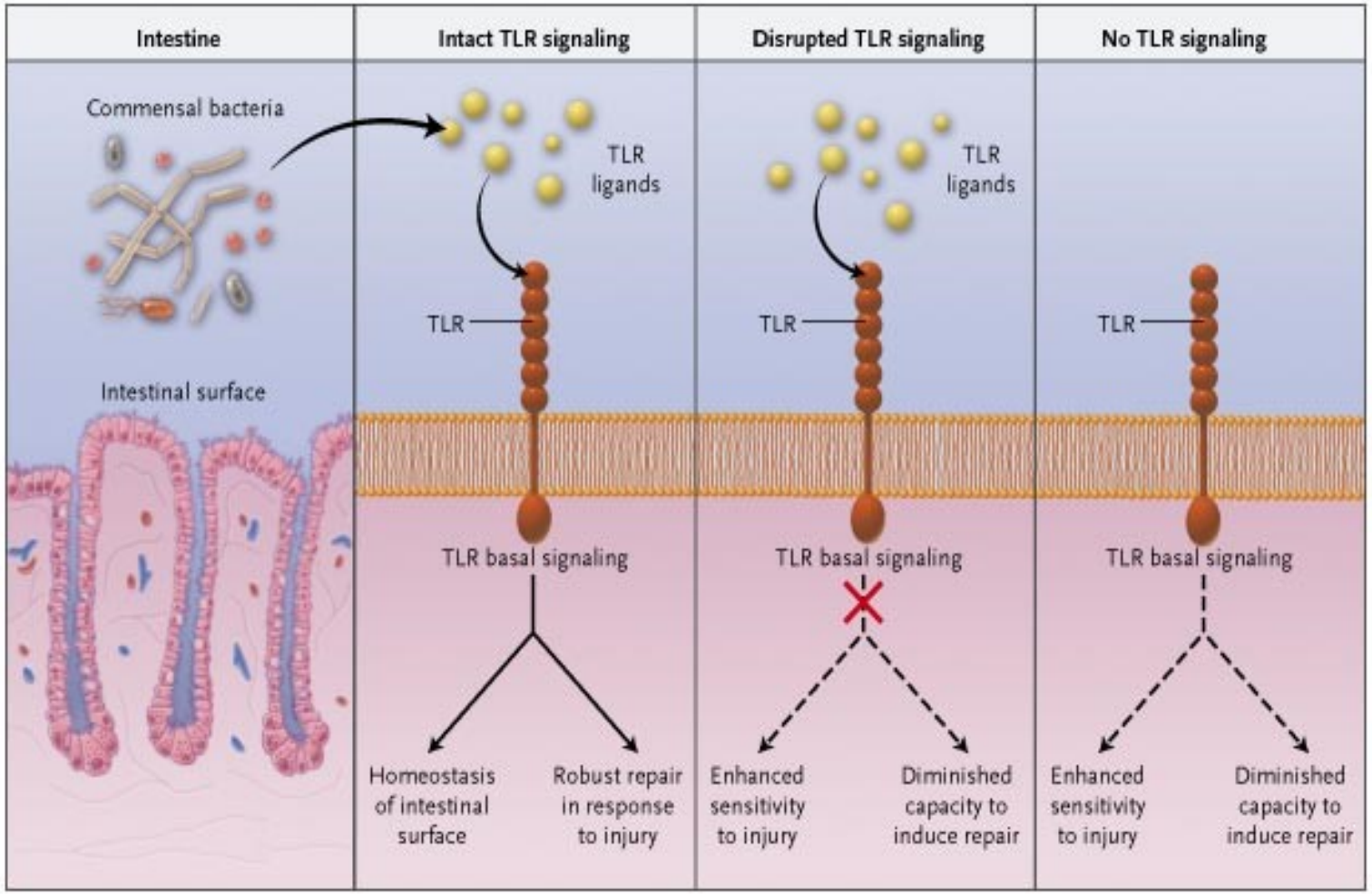
Cell, Vol. 118, 229–241, July 23, 2004, Copyright ©2004 by Cell Press

Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis

Seth Rakoff-Nahoum,[1] Justin Paglino,[2]
Fatima Eslami-Varzaneh,[3] Stephen Edberg,[2]
and Ruslan Medzhitov[1,*]
[1]Howard Hughes Medical Institute
and Section of Immunobiology
[2]Department of Laboratory Medicine
[3]Department of Pathology
Yale University School of Medicine
New Haven, Connecticut 06510

by commensal bacteria (Farrell and LaMont, 2002; Sartor, 2000). The nature and regulation of the host-commensal interactions in the gut is thus an area of intense scientific and clinical interest.

TLRs comprise a family of pattern-recognition receptors that detect conserved molecular products of microorganisms, such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), recognized by TLR4 and TLR2, respectively (Takeda et al., 2003). TLRs function as sen-

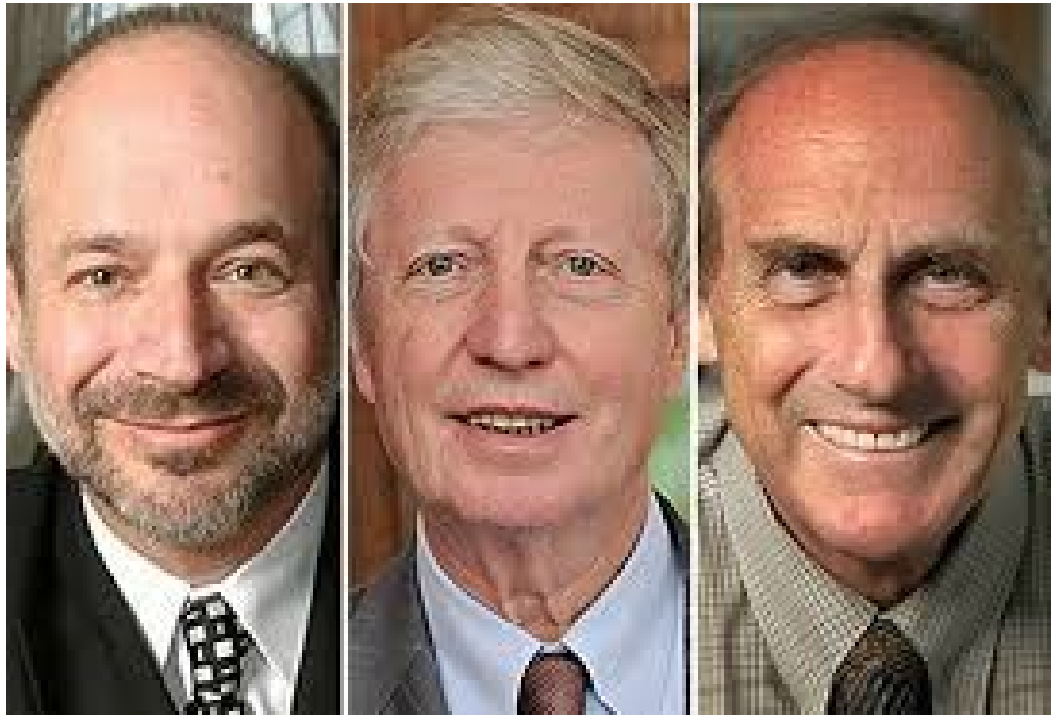


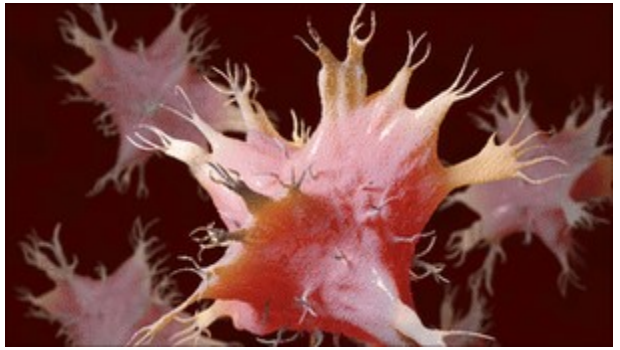
From Madara J. Building an intestine – architectural contributions of commensal bacteria. *New Engl. J. Med*. 2004; 351: 1685-86.

Lesson

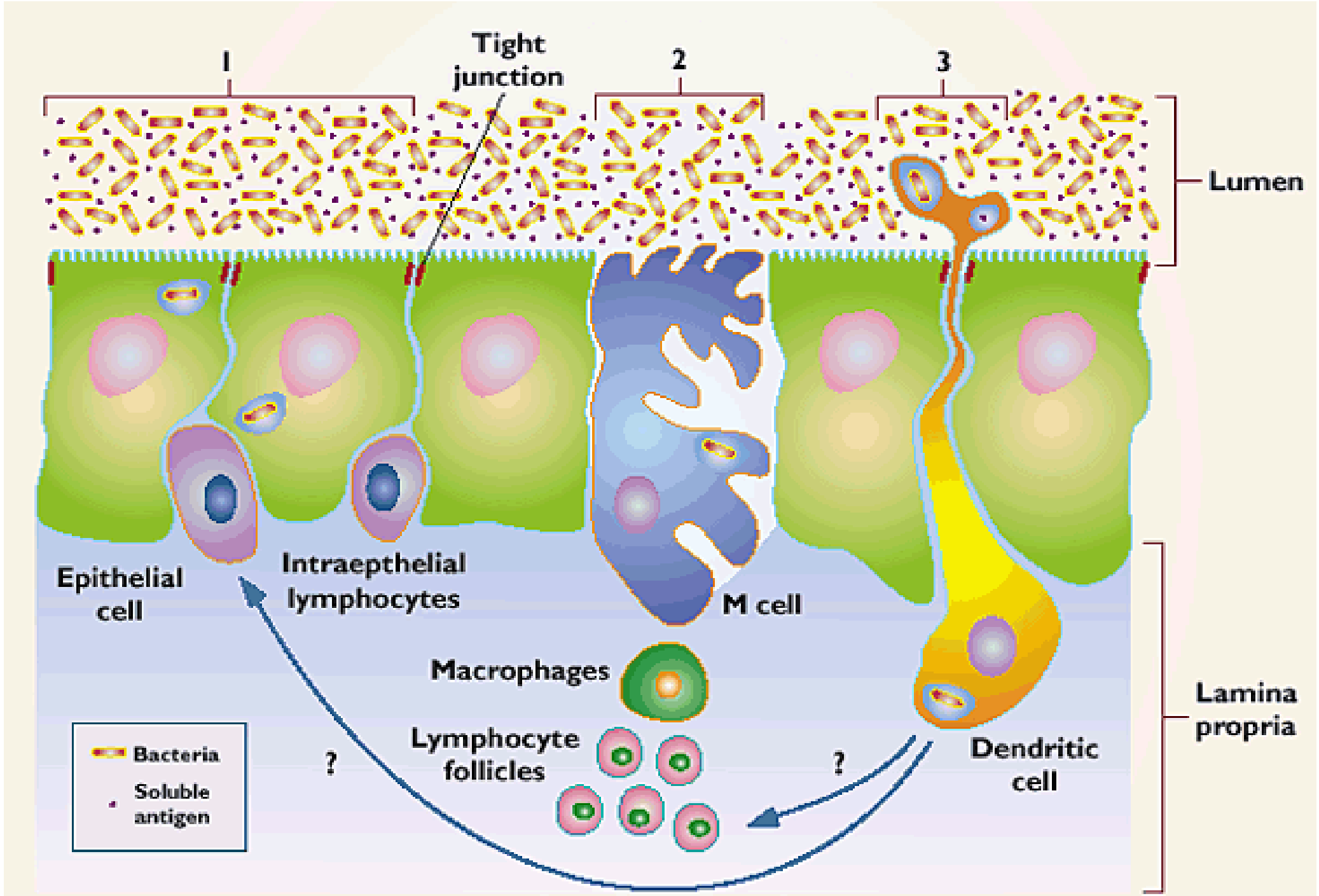
Low grade stimulation (“tickling”) of toll receptors can prevent high grade inflammation and intestinal damage and promotes intestinal homeostasis.

The Dendritic Cell and its Role in Adaptive Immunity





Bruce Beutler,
Jules Hoffman and
Ralph Steinman


1
Tight junction
2
3
Lumen
Epithelial cell
Intraepthelial lymphocytes
M cell
Macrophages
Lymphocyte follicles
Dendritic cell
?
?
Lamina propria
Bacteria
Soluble antigen

Questions

Amniotic fluid is sterile?

- True
- False

Placenta is sterile?

- True
- False

Meconium is sterile?

- True
- False

Human Milk is sterile?

- True
- False

Most infants first come into contact with microbes during birth — or so researchers have assumed.

Baby's first bacteria

THE WOMB WAS THOUGHT TO BE STERILE. SOME SCIENTISTS ARGUE IT'S WHERE THE MICROBIOME BEGINS.

By Cassandra Willyard

Soon after conception, a human embryo begins to assemble a remarkable organ crucial to its survival. The placenta is both a lifeline and a guardian: it shuttles

nearly 200 placentas collected from women giving birth at a hospital in St Louis, Missouri. When the researchers examined the samples under a microscope, they found bac-

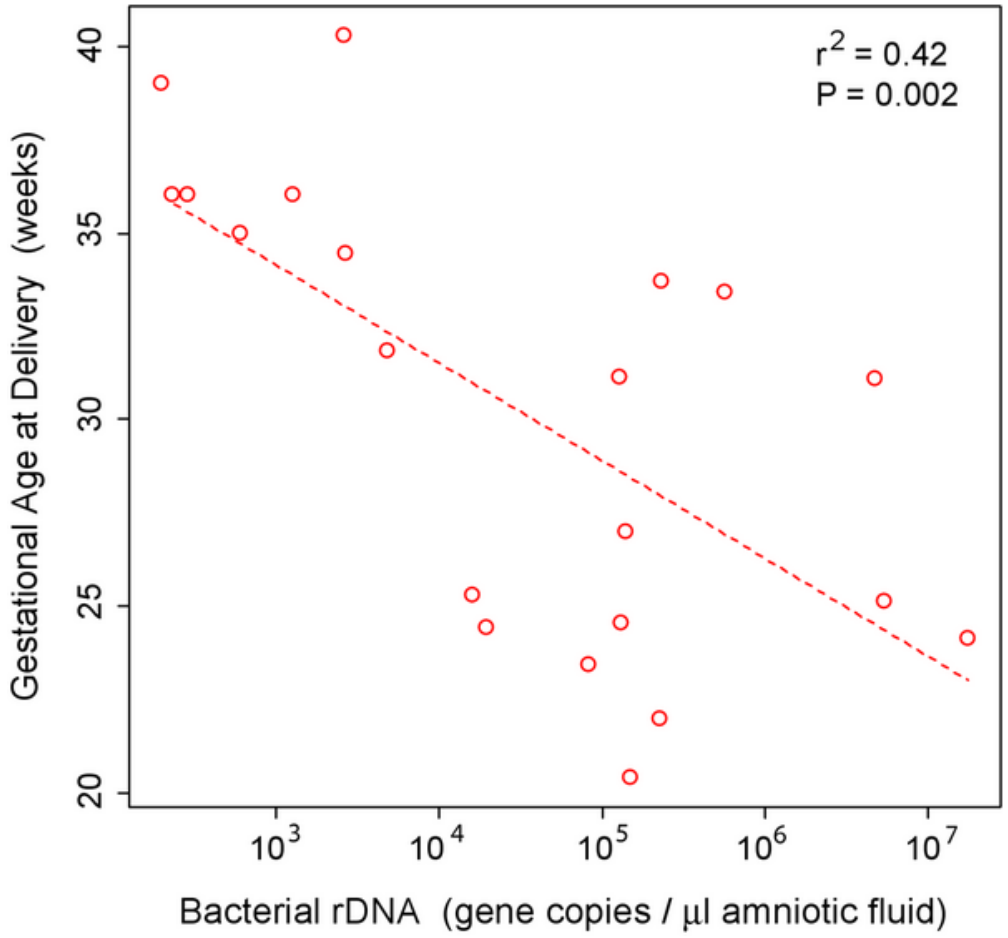
DO/REUTERS

A "Scientific Knife Fight"

Is it all "Fake News"?

Willyard. C. Nature: Jan 18, 2028

Is there a Fetal Microbiome? Might this Relate to Prematurity



DiGulio DB PLoS ONE. 2008 Aug 26;3(8):e3056.

RESEARCH ARTICLE

MICROBIOME

The Placenta Harbors a Unique Microbiome

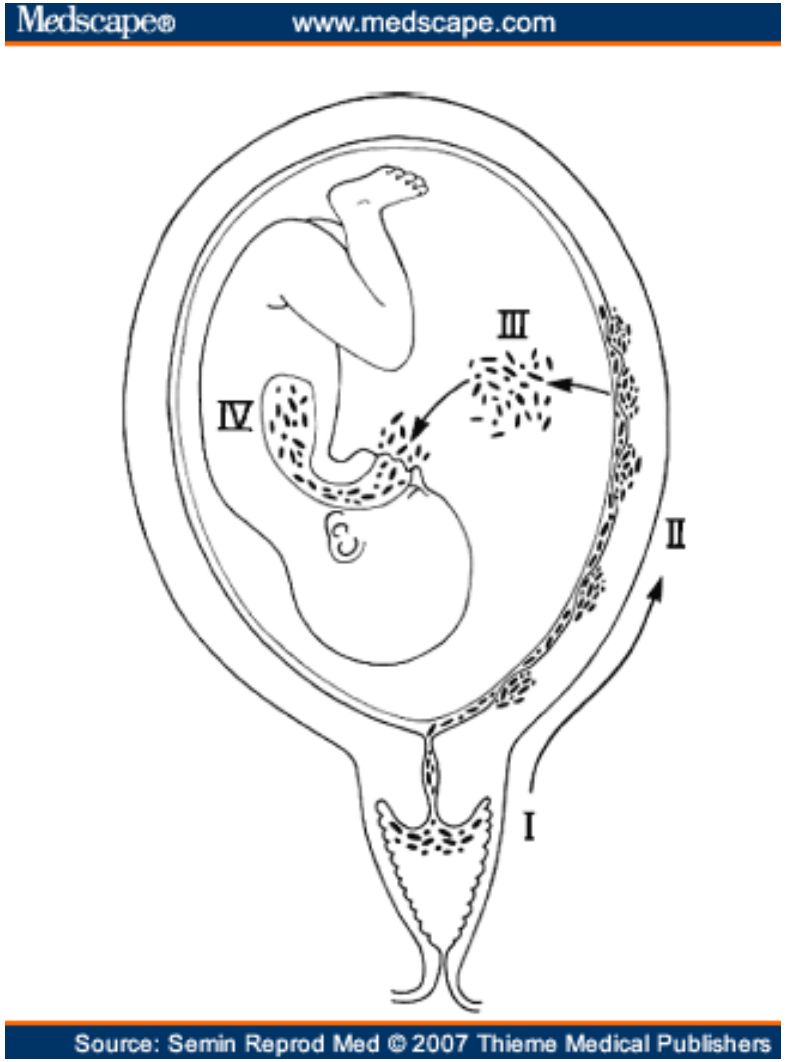
Kjersti Aagaard,[1,2,3]* Jun Ma,[1,2] Kathleen M. Antony,[1] Radhika Ganu,[1] Joseph Petrosino,[4] James Versalovic[5]

Humans and their microbiomes have coevolved as a physiologic community composed of distinct body site niches with metabolic and antigenic diversity. The placental microbiome has not been robustly interrogated, despite recent demonstrations of intracellular bacteria with diverse metabolic and immune regulatory functions. A population-based cohort of placental specimens collected under sterile conditions from 320 subjects with extensive clinical data was established for comparative 16*S* ribosomal DNA–based and whole-genome shotgun (WGS) metagenomic studies. Identified taxa and their gene carriage patterns were compared to other human body site niches, including the oral, skin, airway (nasal), vaginal, and gut microbiomes from nonpregnant controls.

INTRODUCTION

Aagaard, et.al. Science Translational Medicine, 21 May 2014

How Do the microbes get into the fetus? What are the implications?

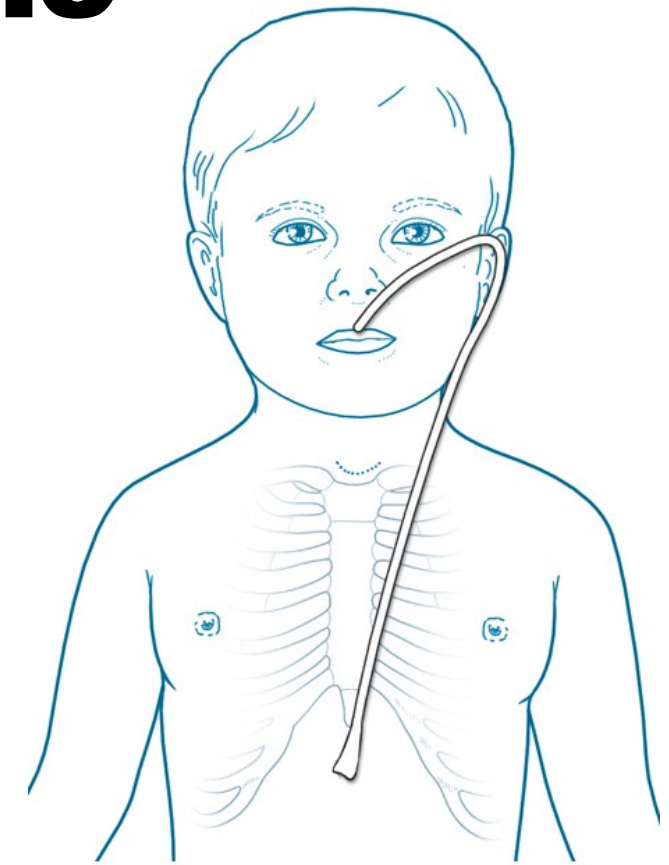


Over 50% of the microbes in meconium are also found in Amniotic Fluid suggesting fetal swallowing of colonized amniotic fluid.

Ardissone, et al. PLOS One 2014

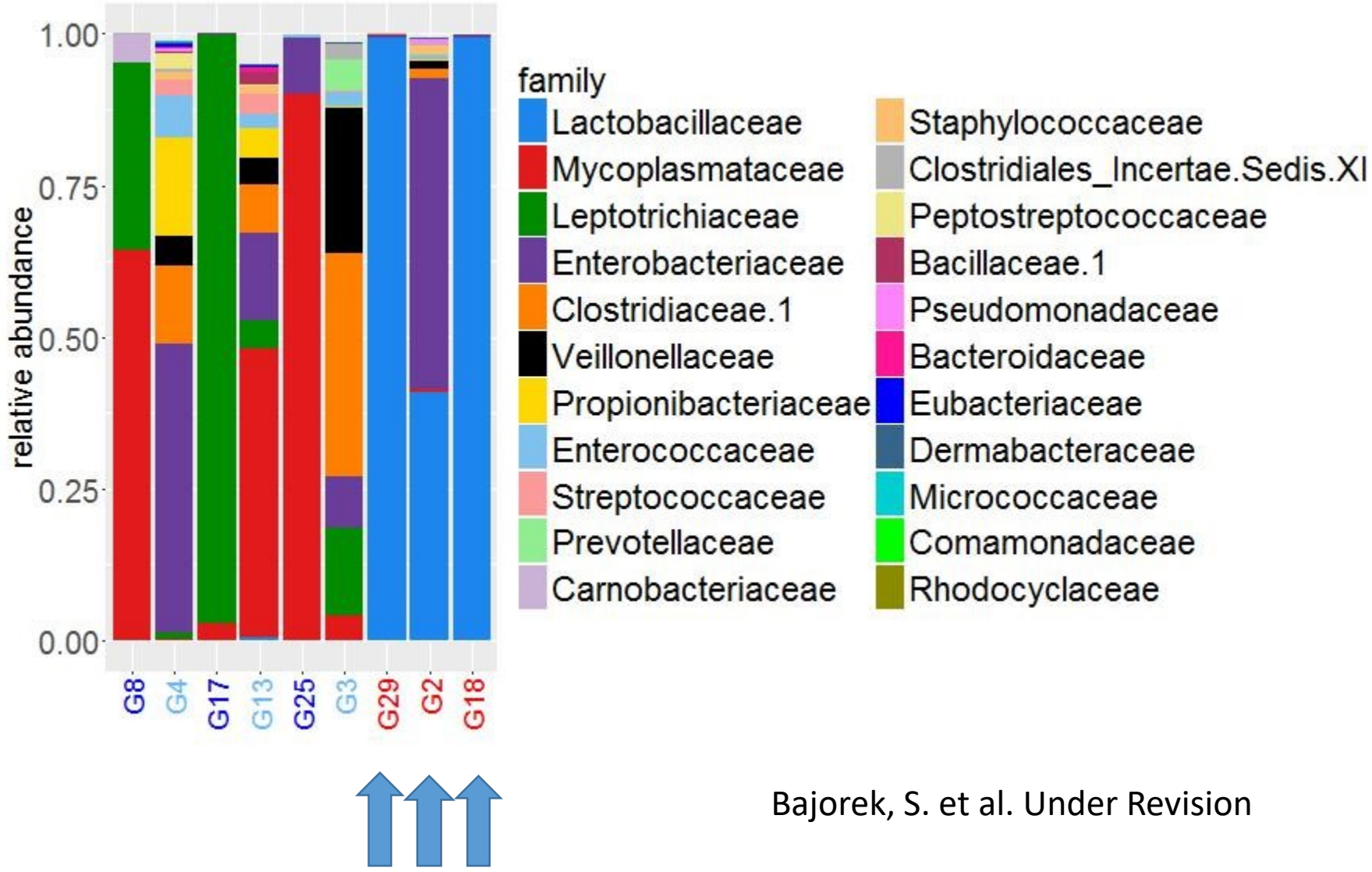
First Gastric Aspirate Microbiome

- Do first gastric aspirates as a reflection of amniotic fluid differ in term versus preterm infants?
- How do these compare to microbes present in other niches such as vagina or GI tract?
- Could these microbes relate to inflammation of the GI tract and preterm birth?



Source: Goodman DM, Green TP, Unti SM, Powell EC: *Current Procedures: Pediatrics:* www.accesspediatrics.com

Gastric Aspirates: Term versus Preterm



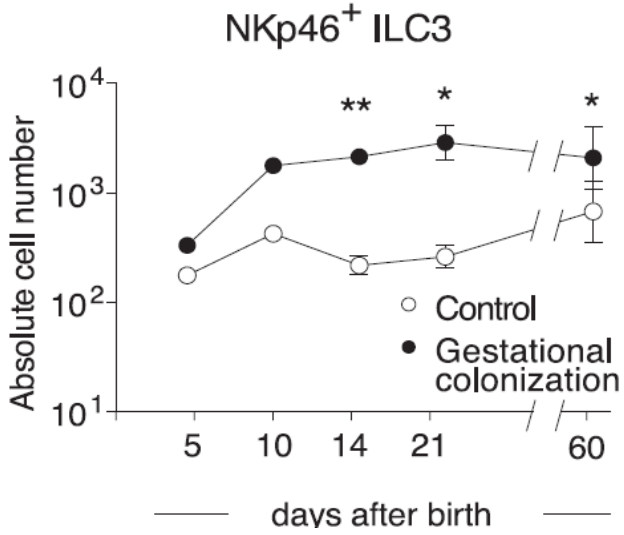
Bajorek, S. et al. Under Revision

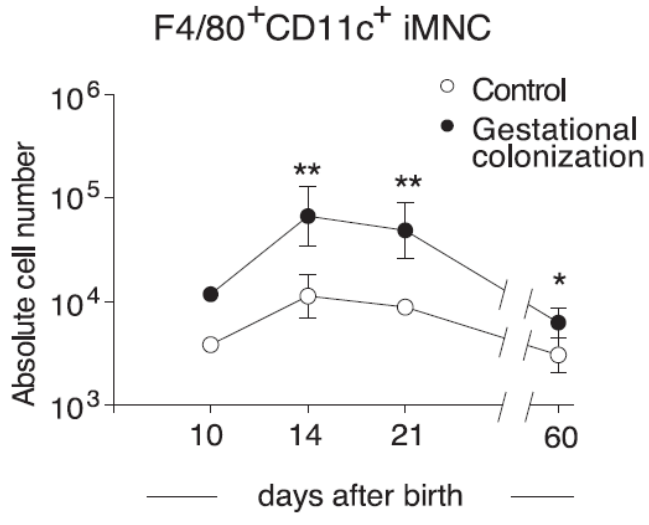
Sterile Womb Hypotheses: the Controversy

- Are the microbes found in the womb simply a “kitome”? U. Penn Group found from 6 placentas that using strict controls from the extraction kits that the microbes present in their studies were derived from the kits(Lauder, et al. Microbiome, 2016).
- Sheep fetuses have a placental and intestinal microbiome (where microbes can be cultured) and the placental appears to act as a conduit during times of stress that allow microbes to reach the brain (Zarate, M.A. Sci Rep. 2017 Jul 25;7(1)).
- “If humans do not have an in-utero microbiome, we would be the only species that doesn’t” (Susan Lynch, UCSF).
- Roberto Romero form NIH and other colleagues are planning to answer this question more definitively.

Mouse Study: Maternal Intestinal Microbiota Drives Early Postnatal Innate Immune Development

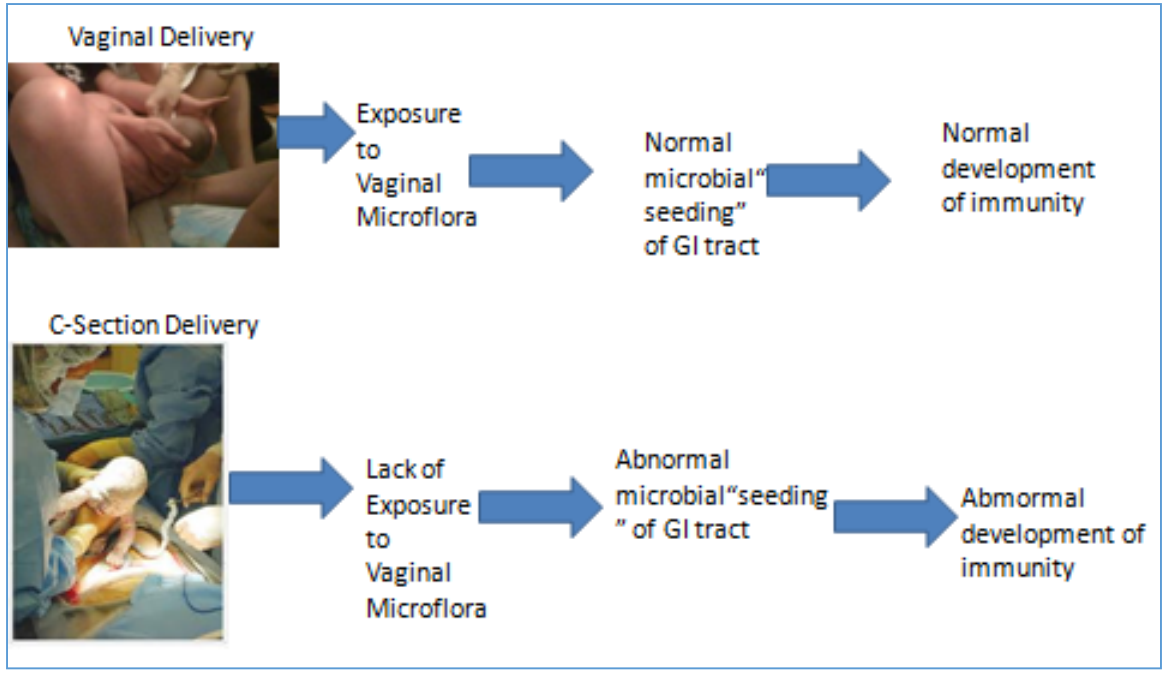
- Pregnant germ free mice colonized with E. coli HA 107 (strain does not persist in the intestine).
- Compared inoculated to non-inoculated offspring intestinal innate immune systems.





Gomez de Aguero, et al. Science. March 18,2016

Epidemiology Studies of C-section vs. Vaginal Delivery

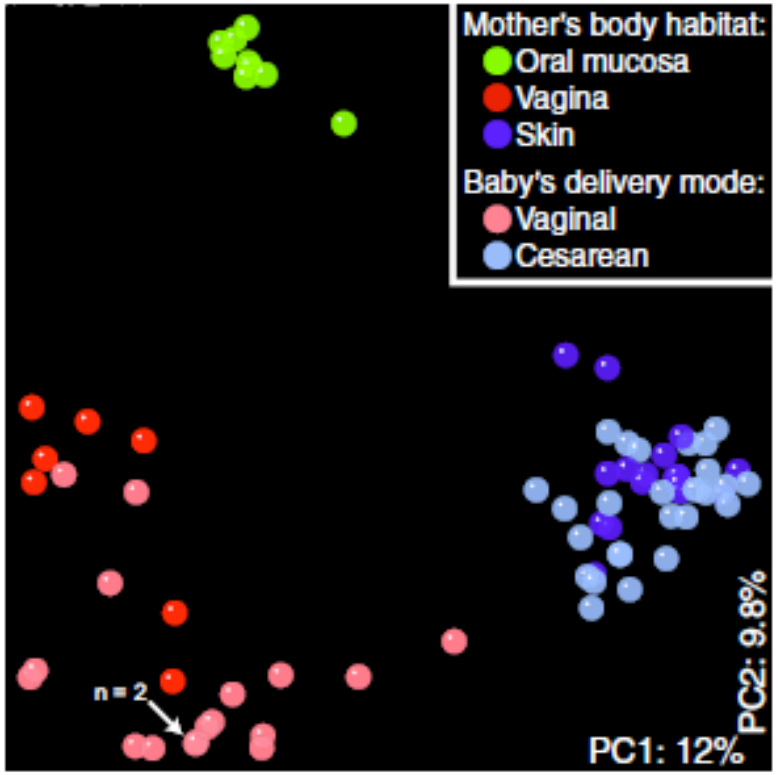


Higher odds of:

- **Allergic Rhinitis**
- **Asthma**
- **Celiac Disease**
- **Type 1 Diabetes**
- **Gastroenteritis**
- **Allergies**
- **Obesity**

*Neu J, Rushing, J.
Clinics in Perinatology 2011

Vaginal vs. C-section Delivery: Non-Culture Technique



Meconium obtained <24 hours after Delivery.

Vaginally delivered infants acquired bacterial communities resembling their own mother's vaginal microbiota and C-section infants harbored bacterial communities similar to those found on the skin surface. (Dominguez-Bellow, MG. Proc Natl Acad Sci U S A. 2010 Jun 29;107(26):11971-5)

nature medicine

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NATURE MEDICINE | BRIEF COMMUNICATION

Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer

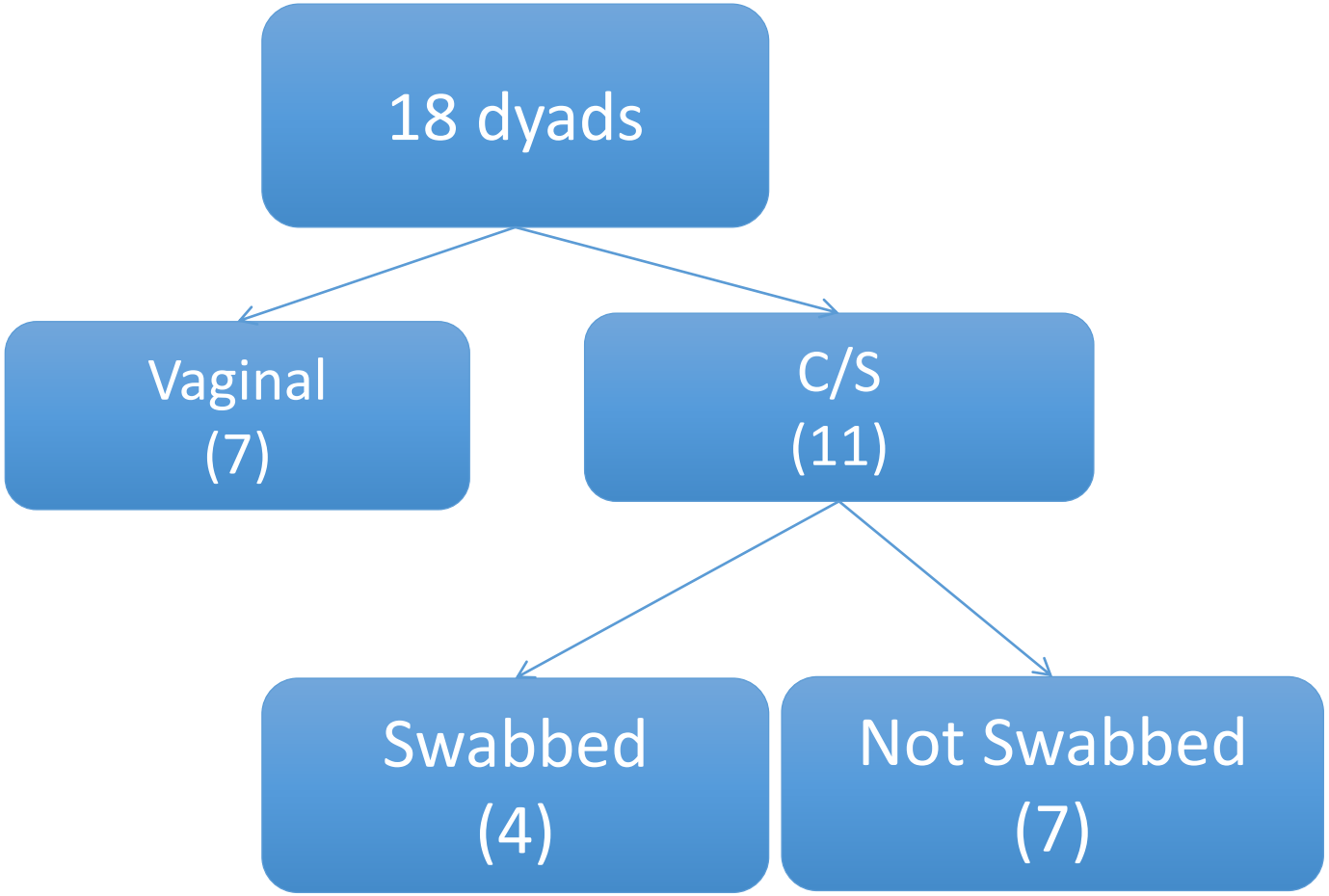
Maria G Dominguez-Bello, Kassandra M De Jesus-Laboy, Nan Shen, Laura M Cox, Amnon Amir, Antonio Gonzalez, Nicholas A Bokulich, Se Jin Song, Marina Hoashi, Juana I Rivera-Vinas, Keimari Mendez, Rob Knight & Jose C Clemente

Affiliations | **Contributions** | **Corresponding authors**

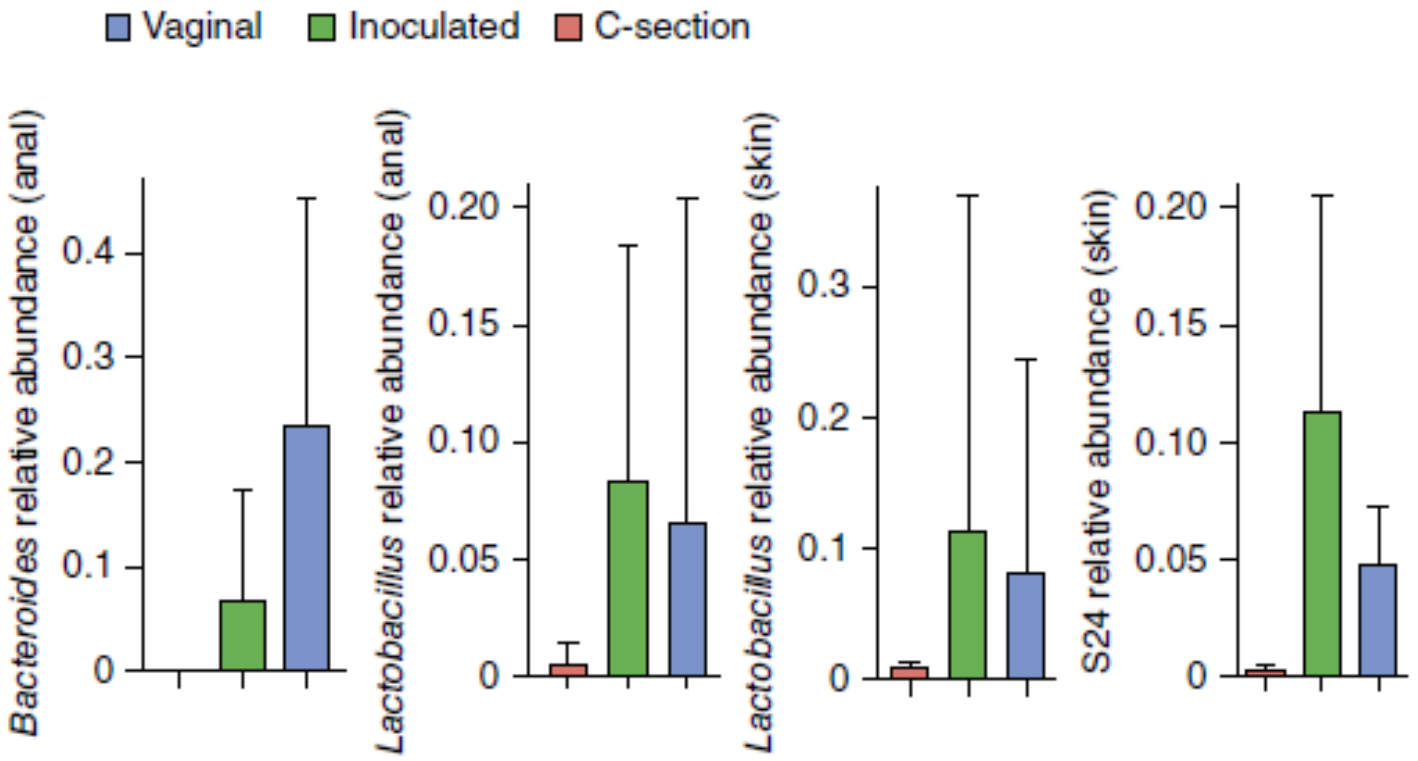
Nature Medicine (2016) | doi:10.1038/nm.4039

Received 03 July 2015 | Accepted 22 December 2015 | Published online 01 February 2016

Groups



Restitution of Vaginal Microbes



Dominguez-Bello, Nature Medicine, Feb. 2016

Practice Advisory: American College of Ob/Gyn Nov. 8, 2016

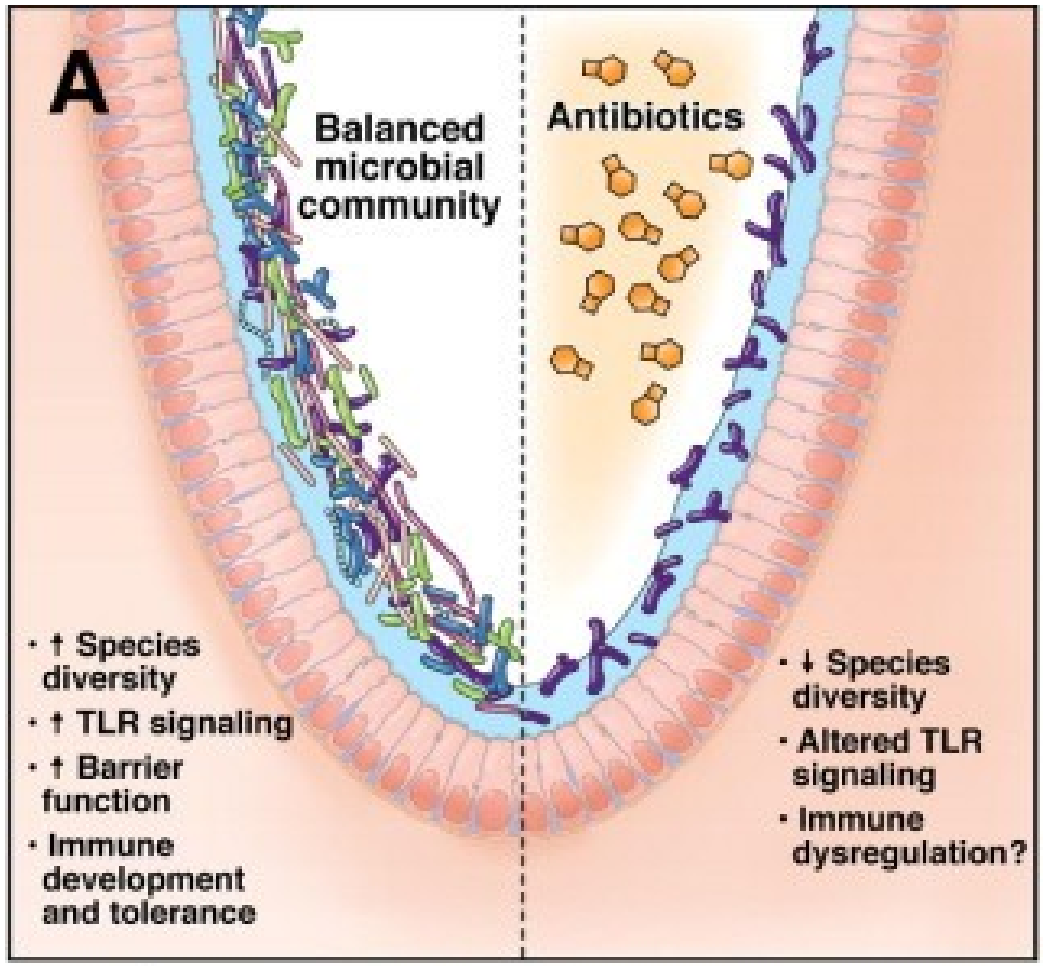
"With the increasing attention given to this concept in public media, providers are being faced with incorporating vaginal seeding into clinical practices. At this time, based on the lack of data about the safety and harms, and the potential for harm from transfer of pathogens associated with adverse neonatal outcomes, we recommend against the practice of vaginal seeding until better data are available establishing the safety and benefits of this practice."

Antibiotic Use, Dysbiosis and Adverse Outcomes

- Intrapartum antibiotic prophylaxis (IAP) is provided in 30% of Pregnancies.
- Majority of preterms less than 33 weeks gestation are treated with ampicillin and gentamicin for at least 48 hours.
- Average length of treatment of “standard of care” practice in preterm neonates is between 5 to 7 days.
- How do these practices affect the developing microbiome, microbial metabolites, resistance factors and neonatal disease.

Antibiotic Induced Dysbiosis

Early Antibiotic Use: What are we really doing?



Preidis and Versalovic, Gastroenterology 2009;136:2015-2031

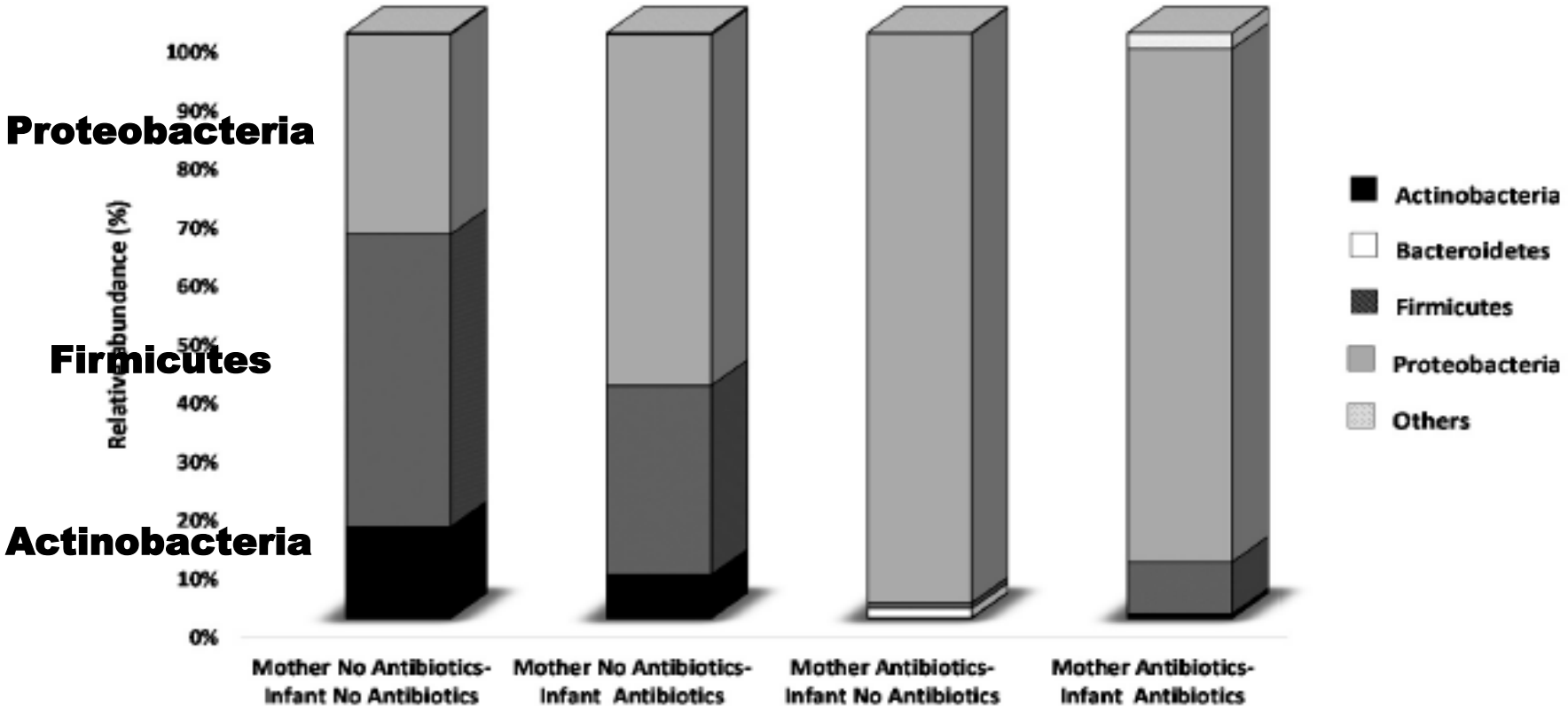
Variation in Duration of Initial Exposure

- Retrospective review of 50,261 neonates across 127 NICUs from California showed a forty-fold variation in antibiotic prescribing practice with similar burden of infection and mortality.
- What does this mean?
- “Forty-fold variation in NICU antibiotic prescribing practice across 127 NICUs with similar burdens of proven infection, NEC, surgical volume, and mortality indicates that a considerable portion of antibiotic use lacks clear warrant; in some NICUs, antibiotics are overused.”

Schulman, et al . Pediatrics 2015;135:826-33

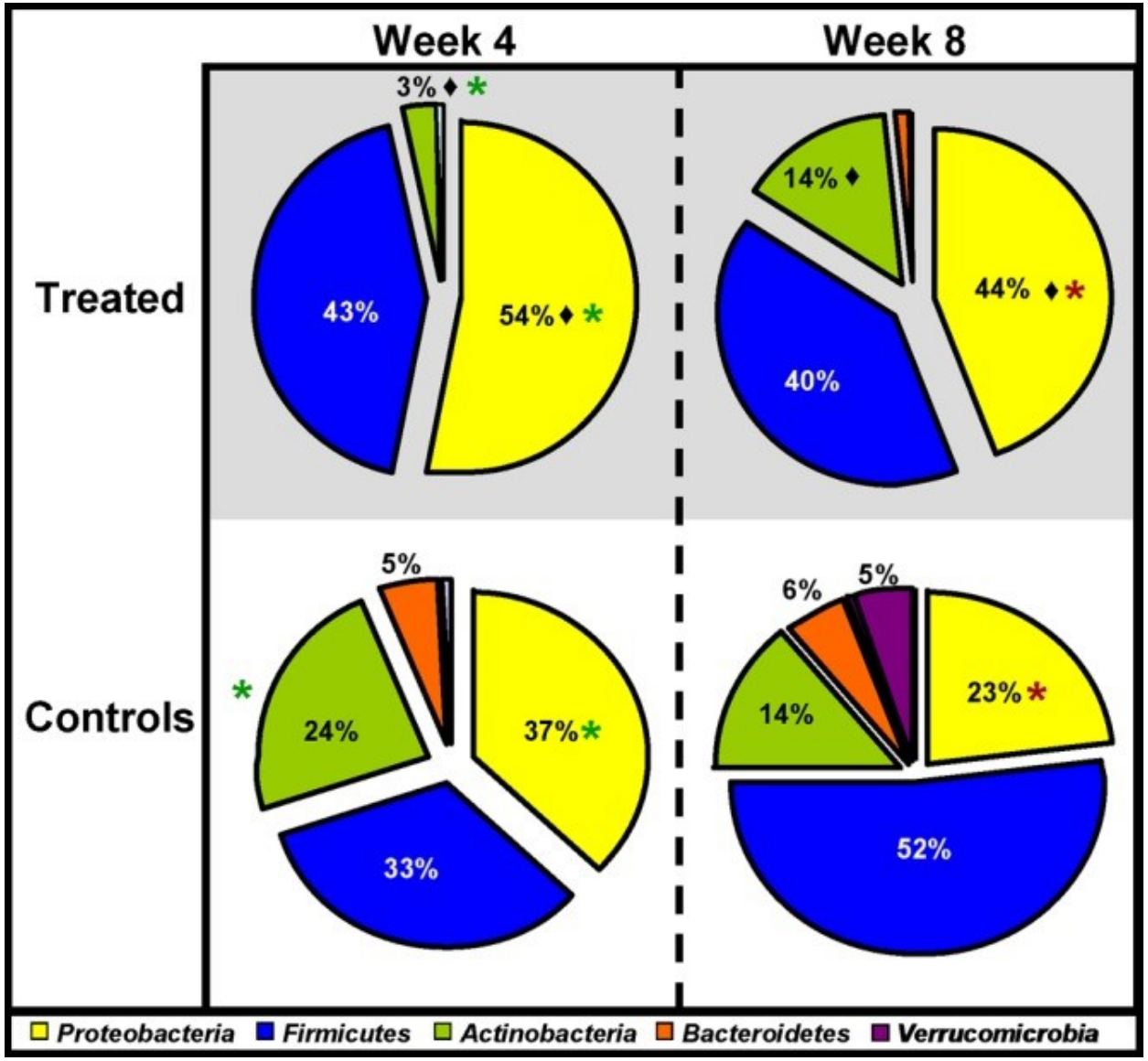
“ So I give a couple days antibiotics to my preterm patients, what does that matter if I change the microbes in the GI tract since I could potentially be saving the baby’s life by treating unrecognized early onset sepsis”---Anonymous Neonatologist.

Do Antenatal and/or Postnatal Antibiotics Affect Neonatal Microbiota? Phylum Level Distributions at 30 days depending on Antibiotic Use



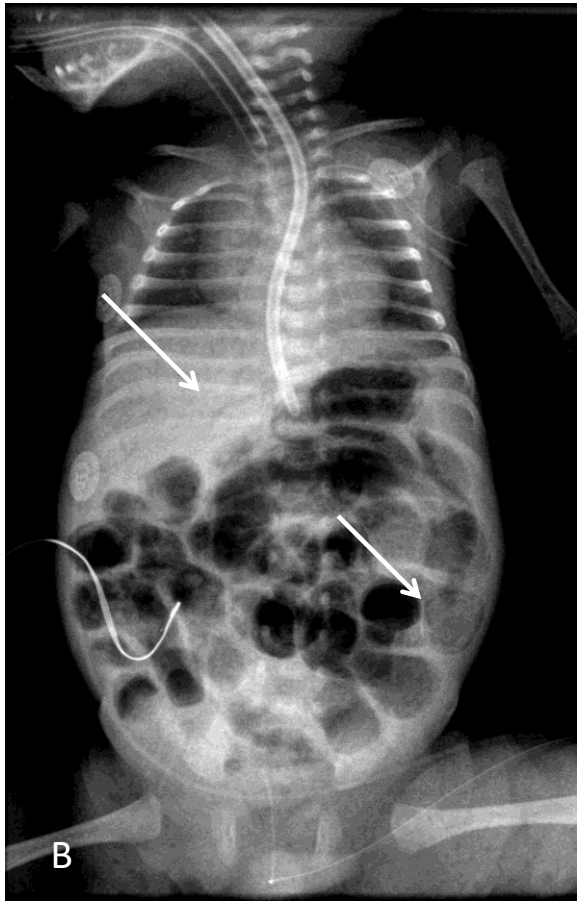
Arboleya, S., et al. Int. J. Mol. Scie. 2016, 17, 649

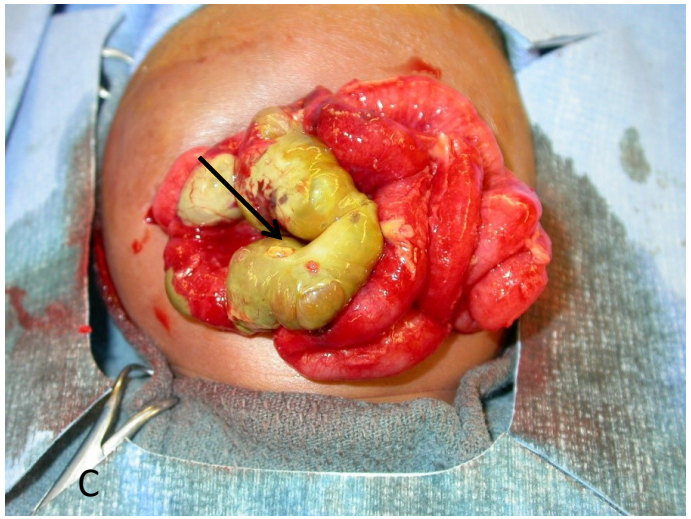
Effects of Ampicillin and Gentamicin within 48 hours after birth



Fouhy, R. et al. Antimicobial Agents
and Chemotherapy. Nov. 2012,
vol 56., Number 11.

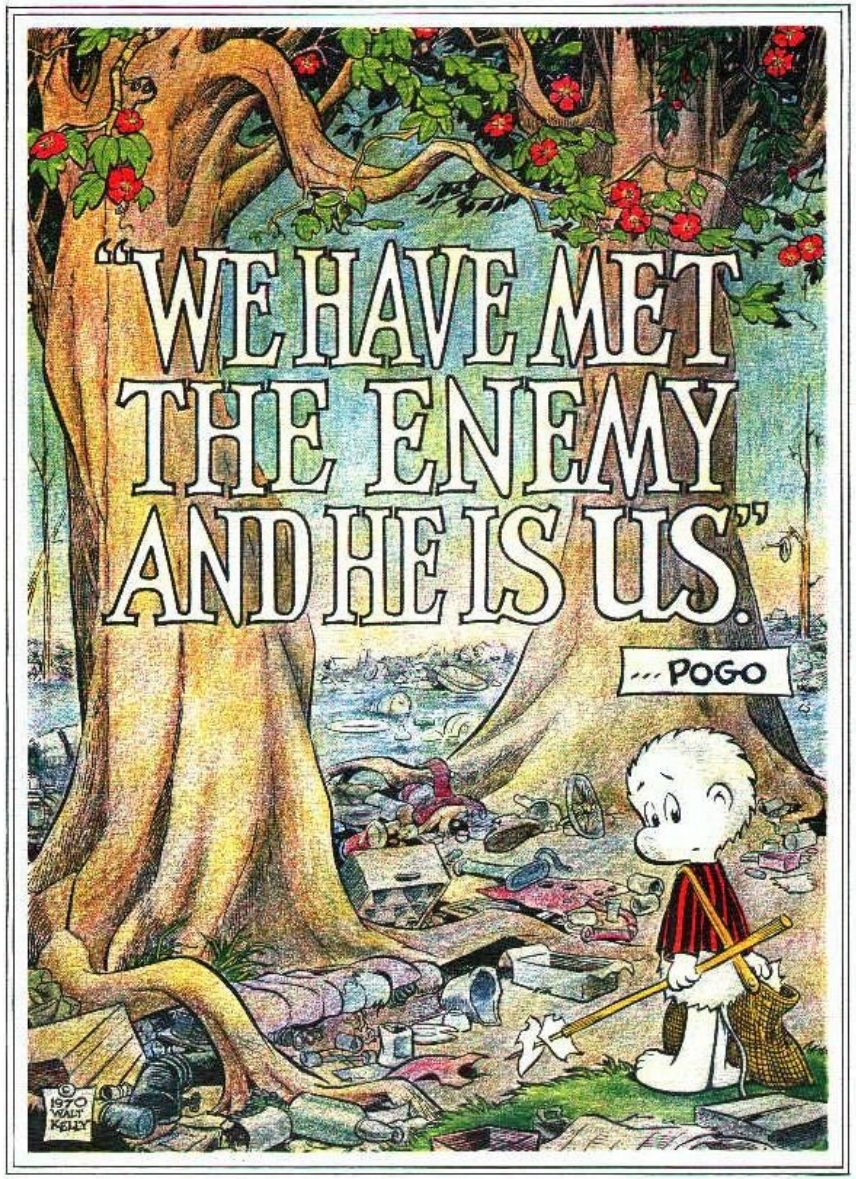
“Classic” NEC





Neu, J. and Walker , W. A. New England Journal of Medicine, Jan. 2011

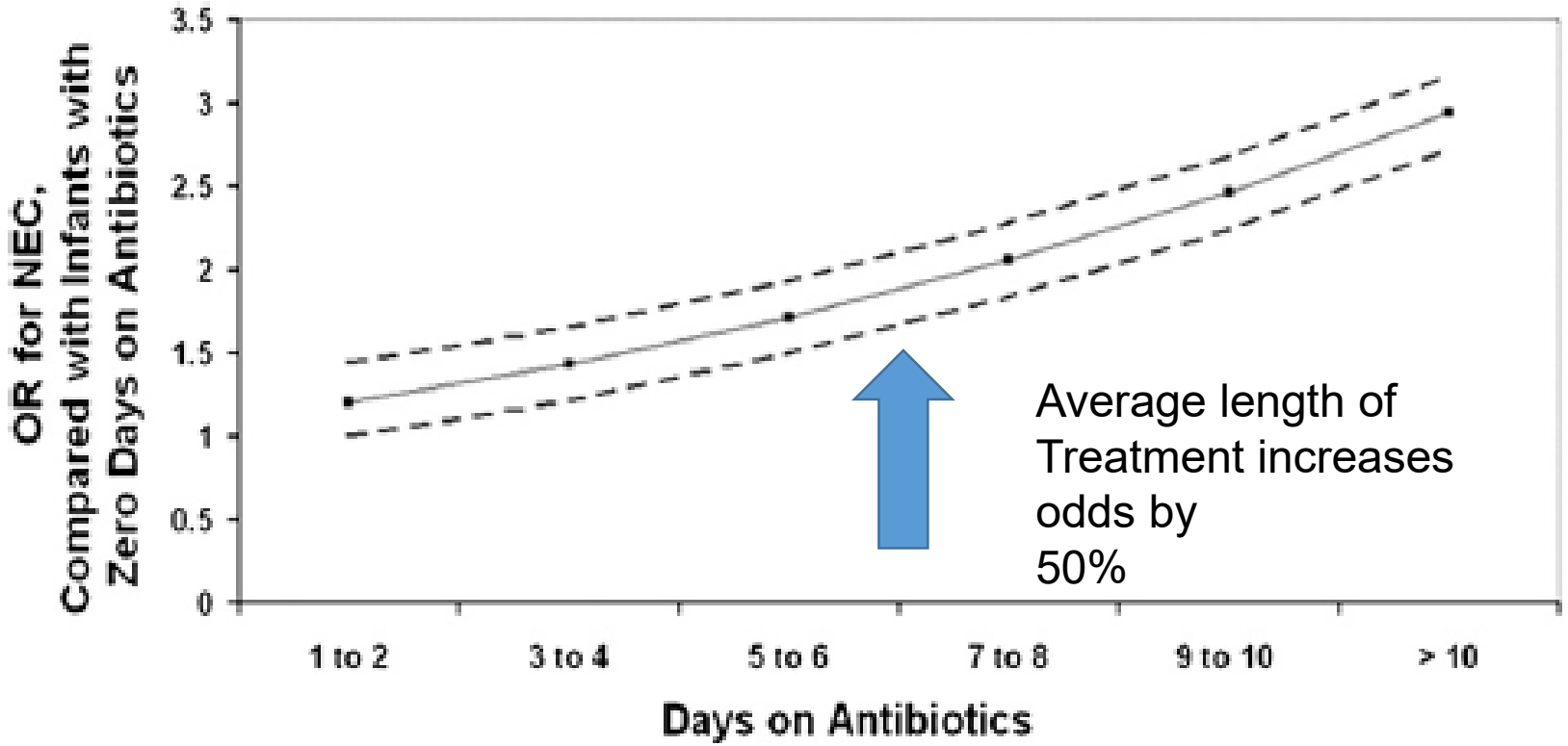
Do Common Neonatal Practices Cause NEC?

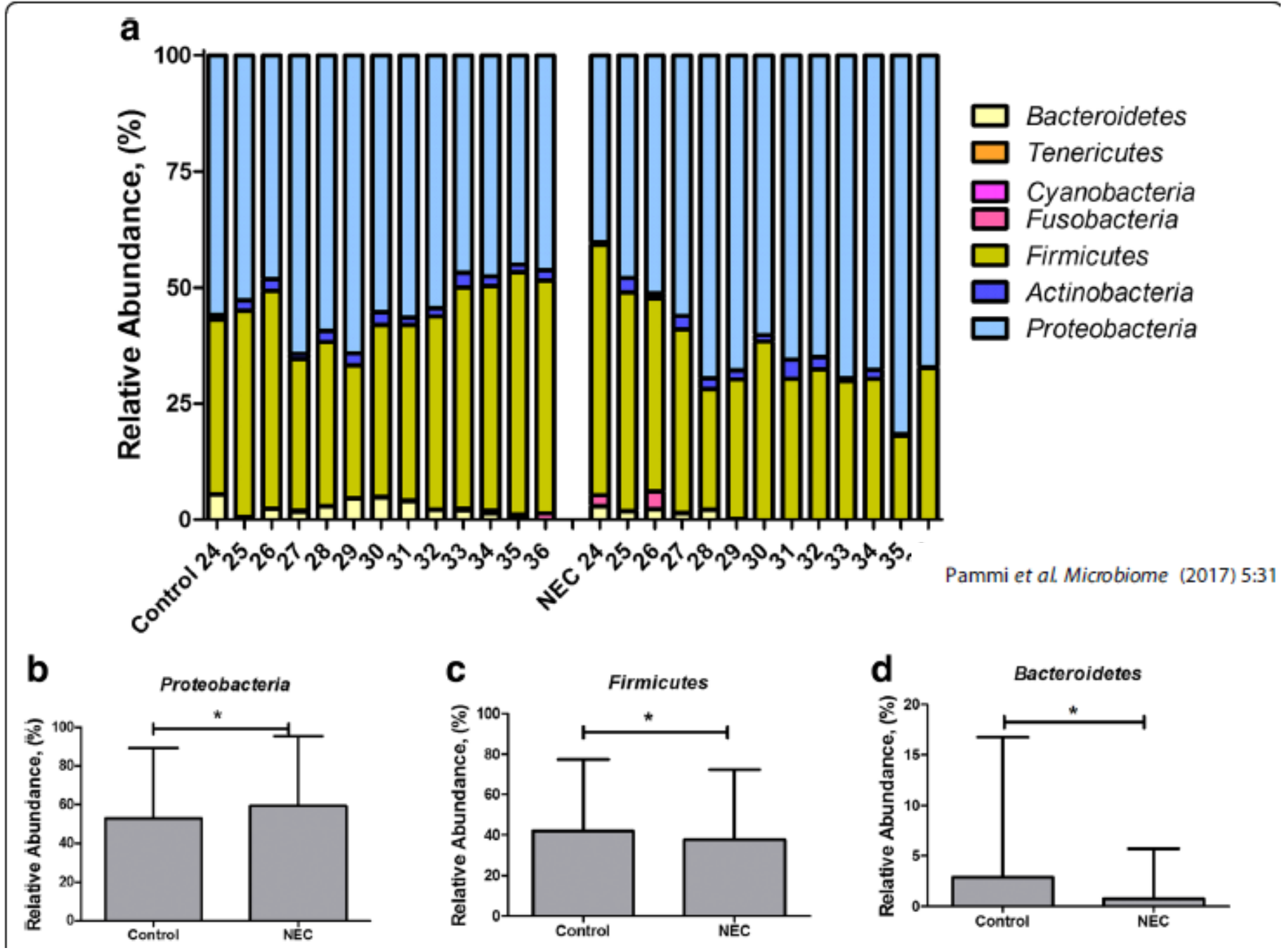


Causes of Inappropriate Colonization "DYSBIOSIS"

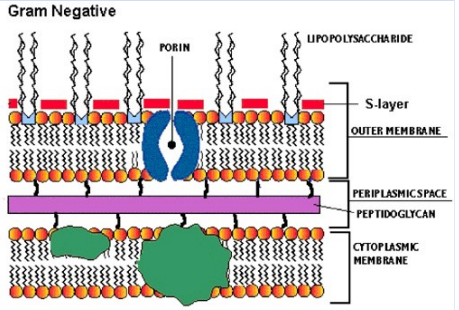
Odds Ratio of NEC with Increased Days on Antibiotics

Alexander, V.N. J. Pediatrics, Sept. 2011

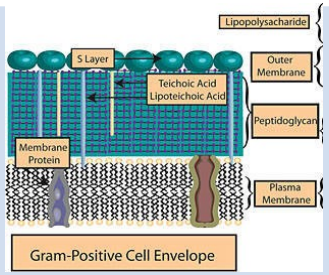



a
Relative Abundance, (%)
100
75
50
25
0
Control 24 25 26 27 28 29 30 31 32 33 34 35 36
NEC 24 25 26 27 28 29 30 31 32 33 34 35
Bacteroidetes
Tenericutes
Cyanobacteria
Fusobacteria
Firmicutes
Actinobacteria
Proteobacteria
Pammi et al. Microbiome (2017) 5:31
b
Proteobacteria
Relative Abundance, (%)
Control
NEC
c
Firmicutes
Relative Abundance, (%)
Control
NEC
d
Bacteroidetes
Relative Abundance, (%)
Control
NEC

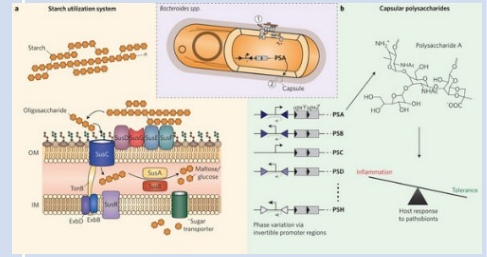
Comparison of three major phyla: Proteobacteria, Firmicutes and Bacteroidetes

Phylum	Gram Staining	Functional Relationship	Comment
Proteobacteria	Gram negative	High Lipopolysaccharide (LPS) content in cell wall. Abundance of Proteobacteria increased prior to exacerbations of inflammatory bowel diesease. Strong stimulator of TLR4. E. Coli, Klebsiella and Pseudomonas are representatives.	

Comparison of three major phyla: Proteobacteria, Firmicutes and Bacteroidetes

Phylum	Gram Staining	Functional Relationship	Comment
Firmicutes	Gram positive	Lactobacilli are a common class of the Firmicutes phylum. Have high lipoteichoic acid in the cell wall, but low LPS. Have excellent capacity for energy harvest. Produce butyrate in high quantities. Butyrate is a major fuel for colonocytes and important for maintenance of tight junctions.	

Comparison of three major phyla: Proteobacteria, Firmicutes and Bacteroidetes

Phylum	Gram Staining	Functional Relationship	Comment
Bacteroidetes	Gram negative, anaerobic, rod shaped bacteria.	Involved in fermentation of carbohydrates (propionate and acetate producers), utilization of nitrogenous substances, and biotransformation of bile acids. Bacteroides fragilis is a representative. The immunomodulatory molecule, polysaccharide A (PSA), of *B. fragilis* mediates the conversion of $CD4^+$ T cells into $Foxp3^+$ Treg cells that produce IL-10 during commensal colonization. PSA is not only able to prevent, but also cure experimental colitis in animals. Propionic acid is also a strong inducer of the Foxp3+ R regulatory pathway.	

Breast milk microbes Over time

Hunt, et al. PlosOne 2011

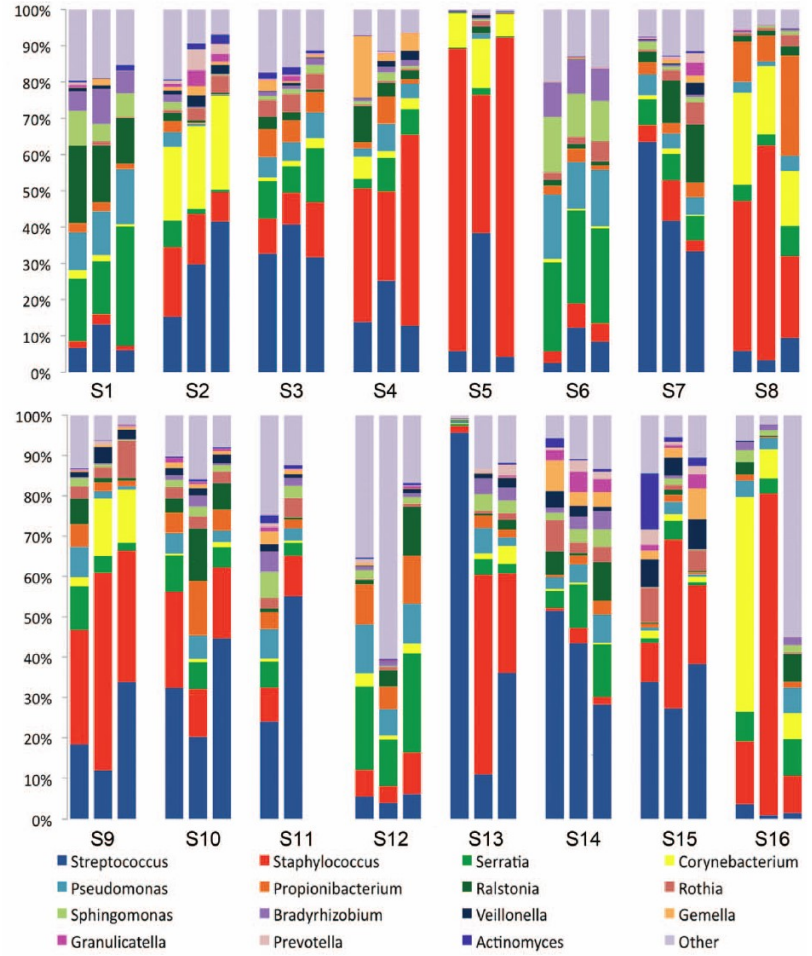


Figure 1. The community composition of the 15 most abundant bacterial genera in each of 3 milk samples from 16 subjects was diverse. The communities observed were found to be reasonably complex, and while consistent in composition over time for some subjects, a great deal of variation was observed over time in the samples of others.
doi:10.1371/journal.pone.0021313.g001

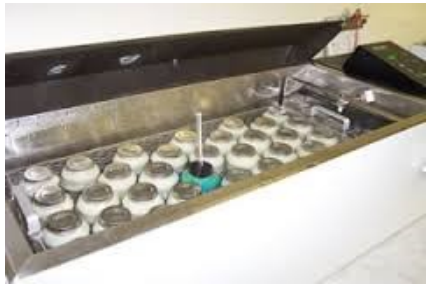
American Academy of Pediatrics Guidelines 2012

"The potent benefits of human milk are such that all preterm infants should receive human milk). Mother's own milk, fresh or frozen, should be the primary diet, and it should be fortified appropriately for the infant born weighing less than 1.5 kg. If mother's own milk is unavailable despite significant lactation support, pasteurized donor milk should be used.

Pediatrics
March 2012, VOLUME 129 / ISSUE 3

Donor Human Milk after Holder Pasteurization

Total Lipid	NA or ↓ 3.5-5.5%
Free fatty acids	↑ 83%
Lactose and oligosaccharides	NA
Total protein	NA or 4% reduction
Alkaline phosphatase (ALP)	↓ 99%
Bile salt-stimulated lipase (BSSL)	Abolished
Lactoferrin (LF)	NA or ↓44-91%
LF-iron-binding capacity	↓ 71%
IgA	NA or ↓ 20-60%
IgM	Abolished
IgG	↓ 34%
Lactoperoxidase (LP)	↓ 82%
LP activity	↓ 88%
Lysozyme	↓ 24-60%
Lysozyme activity	NA or ↓ 65-85%
TGF-β	NA
IGF-I	↓ 39%
IGF-II	↓ 10%
IGFBPs	↓ 7-19%
EGF	NA



Bacterial Load over Lactational Stages: FusA Gene PCR

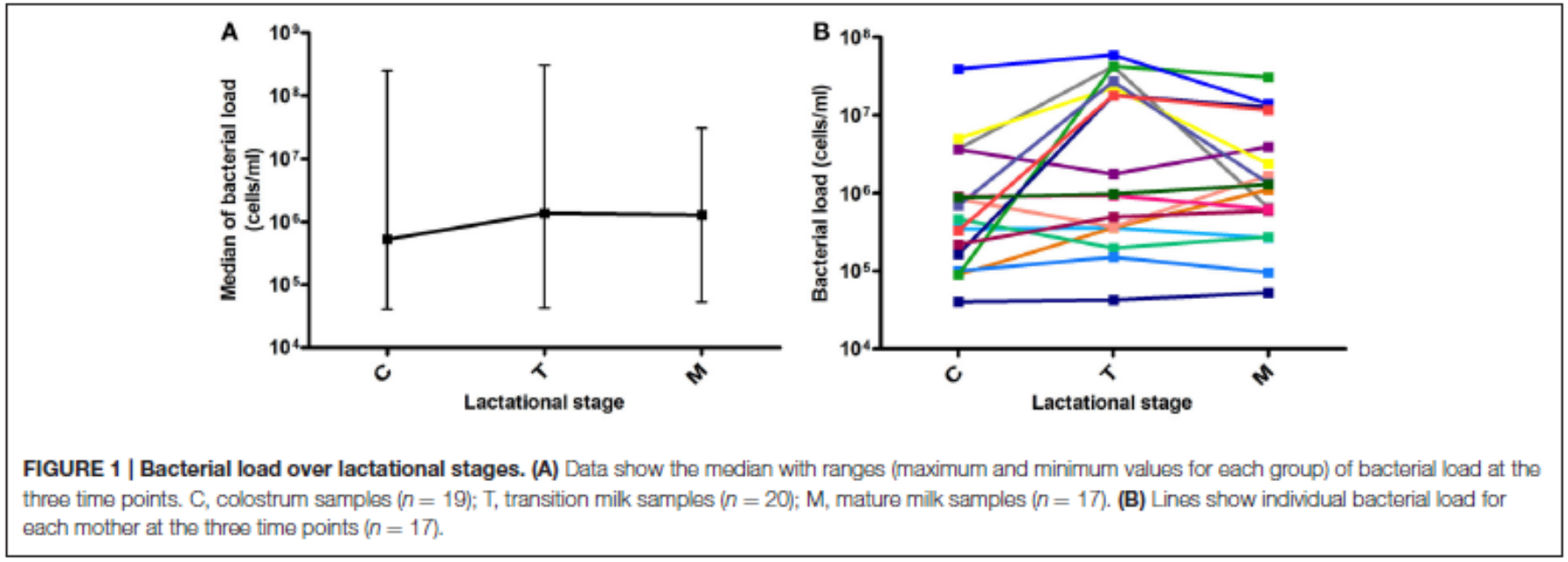


FIGURE 1 | Bacterial load over lactational stages. (A) Data show the median with ranges (maximum and minimum values for each group) of bacterial load at the three time points. C, colostrum samples ($n = 19$); T, transition milk samples ($n = 20$); M, mature milk samples ($n = 17$). **(B)** Lines show individual bacterial load for each mother at the three time points ($n = 17$).

Boix-Amaros, A. Frontiers in Microbiology. 20 April, 2016

Microbial Dose from Human Milk

- Assume intake of 800 ml/day
- Assume $10^{5\text{-}6}$ bacterial cells/ml
- This will provide 10 $^{7\text{-}8}$ bacterial cells (personalized?) daily, close to the dose in most probiotic studies.

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ORIGINAL RESEARCH
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Personalization of the Microbiota of Donor Human Milk with Mother's Own Milk

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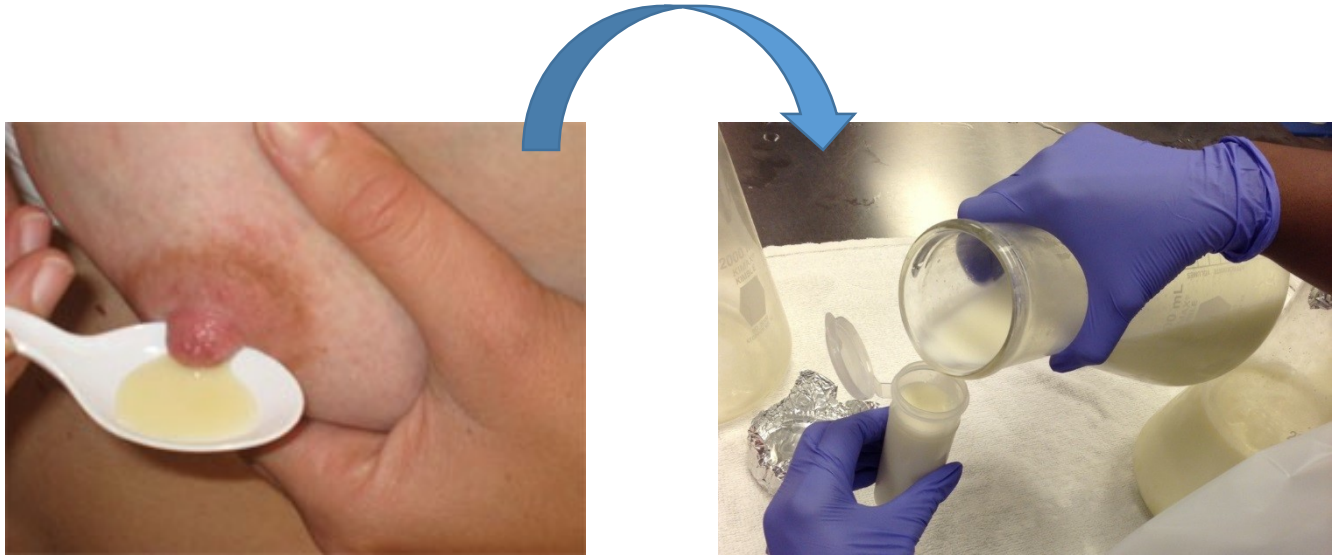
The American Academy of Pediatrics recommends that extremely preterm infants receive mother's own milk (MOM) when available or pasteurized donor breast milk (DBM) when MOM is unavailable. The goal of this study was to determine whether DBM could be inoculated with MOM from mothers of preterm infants to restore the live microbiota (RM). Culture dependent and culture independent methods were used to analyze the fluctuations in the overall population and microbiome, respectively, of DBM, MOM, and RM samples over time. Using MOM at time 0 (T0) as the target for the restoration

Frontiers in Microbiology, August 2017

Re-Faunation Experiments

- Donor Milk Refaunation: Adding mother's own milk to pasteurized donor breast milk (DBM) to add back potentially beneficial microbes: 10-30% dilution will expand a large part of microbiome after 4-8 hours.



Take Home Messages

- Studies of the Microbiome are opening new avenues in our understanding of Health and Disease.
- The Fetal Maternal Unit most likely is not “sterile” and prenatal exposure to microbes may be very important in subsequent development.
- Early life pre, peri and postnatal exposures such as antibiotic use and diet are particularly important in that they can result in life-long changes in health and disease.
- Future studies will utilize multi-omic approaches to better delineate how microbes interact with the environment and the host in the cause and/or prevention of disease.