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Hanin Sarhan, Bridge Fellow at Indiana University

Group picture from 2022 CKS at ACS HQ in Washington, DC

ACS Scholar Adunoluwa Obisesan
BS, Massachusetts Institute of Technology, June 2021 (Chemical-biological Engineering, Computer Science & Molecular Biology)

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- ACS Webinars on Diversity
- ACS Volunteer and ACS Meetings Code of Conduct
- ACS Publications DEIR Hub
- NEW! Download DEIR Educational Resources
- C&EN Trailblazers
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Say hello in the questions window!
A synthetic microbiota designed through meta-analysis provides insight to community function in *Clostridioides difficile* resistance
Challenges in translating microbiome science

1. What defines a healthy microbial community?
2. Does a singular healthy microbiota exist?
3. What are the mechanisms that drive health?
4. How can we design functional microbial communities?

Proposed solution: meta-analysis

Microbiome meta-analysis

• >10 years of high throughput microbiome data in public repositories
• Not all of it is useful, but it allows for studying the human microbiome across populations and disease states
• MAGs have become an incredibly powerful tool for microbiome research
• Is there more we can learn from this data in aggregate?
**Clostridioides difficile**

- Opportunistic pathogen causing spectrum of disease
- Normally suppressed by healthy gut microbiome and triggered by antibiotics
- Treatment frequently followed by recurrent infection
- ~½ million annual infections in US and on the rise costing billions
- Fecal transplant has proven effective but has limitations

---

**Fecal transplant alternatives**

- Fecal transplants are highly efficacious but:
  - May carry MDR pathogens
  - May have undesirable off-target effects
  - Rely on human donors -> intrinsically irreproducible composition
  - Can we rationally design a synthetic fecal microbiome transplant (sFMT) alternative?
    - But what organism(s) should we put in it?
I. Design of Synthetic Communities

C. difficile meta-analysis

**Goal:** Identify the organisms most robustly anti-correlated with *C. difficile* colonization
Altered community composition with *C. difficile*

- Schubert 2014 (n=278)
- PRJNA379979 (n=124)
- Seekatz 2016 (n=98)
- Ling 2014 (n=80)
- Schneider 2017 (n=73)
- Zuo 2018 (n=50)
- Song 2013 (n=36)
- Weingarden 2015 (n=24)
- Seekatz 2018 (n=21)
- PRJNA259188 (n=14)
- Rojo 2015 (n=13)

Combined

<table>
<thead>
<tr>
<th>C. difficile</th>
<th>Shannon's log2(fold-change)</th>
<th>Faith's PD</th>
<th>Richness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased Cd.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Decreased Cd.</td>
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<tr>
<td>P &lt; 0.05</td>
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</tr>
<tr>
<td>P &gt; 0.05</td>
<td>-</td>
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</tr>
</tbody>
</table>

Random Forest model training

- Accurate predictions of *C. difficile* colonization in external validation studies (AUROC=0.81±0.2)
- ~200 features (organisms) with predictive ability
Predictive taxa

- Predictive taxa are enriched for negative predictors
- Predictive taxa cover a broad phylogenetic range
- *Clostridium scindens*, a known inhibitor of *C. difficile* is not predictive of *C. difficile* colonization *in vivo*

Identifying taxa for synthetic community

- Features anti-correlated with *C. difficile* are correlated with each other:
  - Evidence that they will form a stable community?
- We constructed:
  - sFMT1: 37 pure culture strains anti correlated with *C. difficile*
  - sFMT1+Cs: sFMT1 with *C. scindens*
  - ProCd: 25 pure culture strains positively associated with *C. difficile*
II. Characterizing Community Assembly and Function

Characterization in serial culture

- sFMT forms a stable community in vitro
**Composition *in vivo***

- sFMT colonization kinetics mimic a human fecal transplant

**In vivo vs in vitro**

- There are “waves” of succession during colonization similar to humans
- *In vivo* community composition and temporal dynamics are distinct from *in vitro*
• Metagenomic methods needed to differentiate strains for higher sensitivity and specificity
• Developed StrainR2: normalization based on effective unique genome size
• FPKM (Fragments per kilobase per million reads mapped)
• FUKM (Fragments per unique thousand hashed k-mers per million reads mapped)

Intraspecies competition

• What are the determinants of competitive exclusion in vivo?
sFMT metabolism *in vivo*

- SCFAs are derived from bacterial metabolism of non-digestible carbohydrates among other sources
- sFMT1 replicates metabolism of human-derived fecal transplant (hFMT)

sFMT bile acid transformation

- sFMT1 replicates many biotransformations observed in a complex human sample and addition of *C. scindens* leads to 7α-dehydroxylation
An unexpected observation

- 3-oxoLCA is a potent anti-inflammatory molecule acting on Th17 cells
- 3-oxoLCA is also an inhibitor of *C. difficile*
- How could 3-oxoLCA be produced in the absence of *C. scindens*

III. Measuring resistance to *C. difficile* infection
**C. difficile** exclusion *in vitro*

- **sFMT1 and hFMT** reduce **C. difficile** abundance by orders of magnitude
- **ProCD** (organisms positively correlated with **C. difficile**) has no significant effect

---

**Gnotobiotic infection model**

- Colonization reduces disease severity and virulence factor expression
IV. Determining sFMT Mechanism(s) of Action

Stickland fermentation

• Proline fermentation is an important pathway for *C. difficile in vivo*, could sFMT1 members be competing for proline?
Designing $\Delta$Stickland functional knockout

- Stickland fermenting strains predicted on basis of possessing proline reductase homologs (Nstrains=8)
- Verified *in vitro* using NMR

Testing $\Delta$Stickland functional knockout

- Compared 3 groups:
  - original sFMT1 (N=37 strains)
  - sStickland1 (N=8 strains predicted to reduce proline)
  - sFMT1$\Delta$Stickland1 (N=29 strains [37-8])
- NMR confirms functional knockout *in vivo*
**ΔStickland loses colonization resistance**

But can we refine further?
Reducing sStickland1 complexity

- Validated Stickland fermentation within sStickland1 members in vitro
- 2 strains of *Dorea longicatena* and *Peptostreptococcus anaerobius* demonstrate most convincing activity
- Contrasted:
  - Germ-free
  - sFMT1 (37 strains)
  - sStickland2 = JEB00029 + JEB000254
  - sFMT1ΔStickland2 = sFMT1 - sStickland 2
Translational targets

- Predictive power not driven by differential abundance, but differential presence
- These two species are found in ~20% of individuals without *C. difficile* while largely absent in carriers
- Could these be key predictors of susceptibility and/or potential therapeutic targets?

Conclusions

- Meta-analysis allowed the design of a functional synthetic community
- *C. scindens* may be dispensable for *C. difficile* resistance in a complex community, but strains which conduct Stickland fermentation of proline are necessary and sufficient
- Limitation of proline availability may be key to microbial suppression of *C. difficile*
Conclusions

- Synthetic microbiomes are tractable tools for mechanistic study coupling big data with experimental opportunities

- Synthetic fecal transplants and derivatives thereof (sFMT) may have potential for clinical translation

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Microbiome Mechanics: Building a Healthier Gut

Peptidoglycan’s Role in Gut Homeostasis

The Gut Microbiome

- The human digestive tract is populated with bacteria (~95% of the human microbiome is located here)

- Essentially a microbial organ within a host organism

- Commensal relationship
The Gut Microbiome

- Gut homeostasis affects our day-to-day functioning
- Two way relationship in terms of exchange of signaling molecules
- We only know of a few biologically active molecules being produced by gut microbiota

A major molecule that is now entering this small list is MDP, which happens to be a fragment of bacterial cell walls

Bacterial cell wall: Peptidoglycan

- Peptidoglycan (also known as sacculi) is a single LARGE molecule that surrounds the entire bacterial cell
- All bacteria are protected by this ‘jacket’ like structure
- Peptidoglycan is uniquely bacteria in nature (humans do not have any molecules similar to it)

**MDP Released by Gut Microbiota**

- Fragments (or ‘bricks’) from the cell wall are released by muramidases (e.g., lysozyme)

**Sensing of NOD2**

- MDP is a fingerprint of bacterial presence
- It gets detected by NOD2 inside mammalian cells
- This process was thought to be defensive in nature
  - signifying an infection
  - but this concept may not capture all that MDP does....
Alternative role for NOD2?

Enterococcus peptidoglycan remodeling promotes checkpoint inhibitor cancer immunotherapy

Potentiation of Immunity

- Could NOD2 activation from microbiome peptidoglycan lead to better immunological state?
- Can this improved state potentiate checkpoint cancer immunotherapy?
Potentiation of Immunity

- Gut bacterial enzyme was responsible for depolymerizing peptidoglycan into NOD2 agonists

- In mice devoid of gut bacteria, the supplementation of MDP was sufficient to replicate the anti-cancer phenotype
  - Has implications for drug design and better cancer immunotherapies
Alternative role for NOD2?

Regulation of appetite

• Could NOD2 operate in the brain?
  • If so, what physiology could it control?
• NOD2 expression in neurons could impact feeding and temperature in female mice

• Supplementation of MDP (peptidoglycan fragment) can modulate neuronal activity
Alternative role for NOD2?

Peptidoglycan Can Promote Growth

- What is the impact of directly feeding peptidoglycan?
  - Peptidoglycan can be readily isolated from bacteria, including those that harbor our guts
• Probiotic with *Lactobacillus plantarum* improves intestinal NOD2 stimulation and linear growth
Open Questions - NOD2 in Host Health

- Can we visualize peptidoglycan of gut bacteria in live animals?

- Can we isolate peptidoglycan from stool samples to analyze its composition and NOD2 activation level?

Goal #1 – Live Animal Imaging

We metabolically tagged the peptidoglycan of gut bacteria in live mice with near IR fluorophores.
Goal # 2 – Non-invasive Sacculi Isolation

We set out to isolate peptidoglycan from fecal samples to readily interrogate NOD2 signaling

• But how?
  
  • Fecal samples are very complex and it is not trivial to isolate bacteria/sacculi
  
  • We took advantage of a special property of sacculi: its resistance to SDS/heat/DNAase/RNAase/protease

Isolation of Sacculi

- Sacculi imaging
- Peptidoglycan analysis
- Binding profile
- NOD2 activation
The Gut Microbiome

• Peptidoglycan from gut bacteria operates as a biologically active mediator of host health via NOD2 sensing.
Molecular interactions in the human microbiome

The American Obesity Epidemic

Obesity is defined as a BMI over 30
Map: Elijah Wolfson for TIME • Source: N Engl J Med 2019;381:2440-50 • Created with Datawrapper
Obesity Increases Propensity of All-Cause Mortality

Moderate Flavonoid Consumption is Negatively Associated With Mortality
Flavonoids are a large family of plant secondary metabolites

- **Flavones**
  - Apigenin

- **Flavonols**
  - Quercetin

- **Flavanones**
  - Naringenin

- **Isoflavones**
  - Daidzein

- **Anthocyanidins**
  - Cyanidin

Gut bacterial flavonoid catabolism & cardiometabolic disease

- Fruits & veggies
- Flavonoids
- Poor absorption
- Monophenolic acids
- PPARγ↑
- Who? How?
- Cardiometabolic diseases
**Hypothesis:** Monophenolic acids stemming from microbial flavonoid catabolism are responsible for the anti-obesogenic effect of flavonoid consumption.

Berry extracts attenuate HFD-induced obesity

Osborn et al. (2022) PNAS
Diet Informs Gut Microbial Composition

![Graph showing the effects of different diets on gut microbial composition. The graph includes NMDS1 and NMDS2 axes, with points representing different diet groups. The x-axis shows NMDS1 values ranging from -0.4 to 1.0, and the y-axis shows NMDS2 values ranging from -0.4 to 0.2. The points are color-coded to represent HFD, HFD + Aronia, HFD + Black Currant, and HFD + Black Elderberry. The graph highlights significant differences between groups.]

Beta-Dispersion

- HFD vs HFD + Aronia *
- HFD vs HFD + Black Currant **
- HFD vs HFD + Black Elderberry **
- HFD + Aronia vs HFD + Black Currant *
- HFD + Aronia vs HFD + Black Elderberry *

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1

Osborn et al. (2022) PNAS

Berry Diets Promote Microbial Diversity

![Graph showing the relative percentage of different genera in the gut microbiome across different diet groups. The genera are color-coded to represent HFD Control, HFD + Black Currant, HFD + Black Elderberry, and HFD + Aronia. The Shannon diversity index is also shown, with bars indicating the diversity for each group.]

Osborn et al. (2022) PNAS
Targeted Mass Spec on Microbial Portal Blood Flavonoid Catabolites

4-Hydroxyphenylacetic acid is correlated with improved metabolic parameters

Hoyles et al. (2018) Nat Med
A Single Monophenolic Acid (4-HPAA) Reprograms Global Fat Storage

Non-Alcoholic Fatty Liver Disease (NAFLD)

NAFLD is often a consequence of obesity and is a risk factor for cardiometabolic disease
The Non-Alcoholic Fatty Liver Disease (NAFLD) Spectrum

RISK FACTORS INCLUDE:
- OBESITY
- DIABETES
- ARTERIAL HYPERTENSION
- HYPERLIPEMIA
- INSULIN RESISTANCE
- GENETIC FACTORS

PRESENCE OF HEPATIC STEATOsis WITH NO EVIDENCE OF HEPATOCyTAL INJURY

PRESENCE OF HEPATIC STEATOsis PLUS INFLAMMATION AND SCARRING.

Liver transplant / Death

BY 2025, NASH IS PROJECTED TO OVERTAKE HEPATITIS C AS THE LEADING CAUSE OF LIVER TRANSPLANTS IN THE U.S.

4-HPAA Reverses High Fat Diet-Induced Steatosis

Osborn et al. (2022) PNAS
AMPK Regulates Liver Lipid Metabolism

4-HPAA Induced Hepatic Activation the AMPK Pathway

Osborn et al. (2022) PNAS
4-HPAA Downregulates Hepatic Fatty Acid Synthesis

4-HPAA Directly Activates AMPK in Primary Hepatocytes in a Dose Dependent Manner

BJ Massey

Osborn et al. (2022) PNAS

Osborn et al. (2022) PNAS
Identification of the initiating step in flavonol catabolism

Yang et al. (2021) Nat Commun

? = flavone reductase
CHI = chalcone isomerase
EnoR = enoate reductase
PHY = phloretin hydrolase

Homologs of F. plautii Catabolic Genes are Rare in Human Fecal Microbiomes

Naseer Sangwan

Osborn et al. (2022) PNAS
Conclusions

• Supplementing a HFD with flavonoid-rich elderberry extract significantly attenuated HFD-induced obesity. 4-HPAA was enriched in the portal plasma of these mice.

• Continuous subcutaneous delivery of 4-HPAA was sufficient to reverse HFD-induced hepatic steatosis.

• This anti-steatotic effect is associated with the activation of AMP-activated protein kinase α (AMPKα).

• In a large survey of healthy human gut metagenomes, about two percent contained homologues of all four characterized bacterial genes required to catabolize flavonols into 4-HPAA.

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