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CENTER FOR BIOLOGICS EVALUATION AND RESEARCH  
CENTER FOR DRUG EVALUATION AND RESEARCH

FECAL MICROBIOTA FOR TRANSPLANTATION:  
SCIENTIFIC AND REGULATORY ISSUES CENTER FOR  
BIOLOGICS, EVALUATION AND RESEARCH (FDA) AND THE  
NATIONAL INSTITUTE FOR ALLERGY AND INFECTIOUS  
DISEASES (NIH)

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A G E N D A

**Welcome and Opening Remarks:**

KAREN MIDTHUN, MD  
Director, CBER/FDA

FRED CASSELS, PhD  
Branch Chief of Enteric and  
Hepatic Diseases, DMID/NIAID

**Session I: The Microbiome in Health and  
Disease Part I:**

**Moderator:**

MELODY MILLS, PhD  
NIAID/NIH

**Panelists:**

LITA PROCTOR, PhD  
National Human Genome Research  
Institute

PHILLIP TARR, MD  
Washington University, School of  
Medicine in St. Louis

YASMINE BELKAID, PhD  
National Institute of Allergy and  
Infectious Diseases

ERIC G. PAMER, MD  
Sloan-Kettering Institute

VINCENT B. YOUNG, MD, PhD  
University of Michigan

**Session II: The Microbiome in Health and  
Disease Part II**

**Moderator:**

DAVID RELMAN, MD

**Panelists:**

ROBERT BRITTON, PhD  
Michigan State University

LINDA S. MANSFIELD, MS, VMD, PhD  
Michigan State University

EMMA ALLEN-VERCOE, PhD  
University of Guelph

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P R O C E E D I N G S

(8:43 a.m.)

MS. MIDTHUN: Good morning, can you hear me? Okay, very good. Well, first off I'd like to welcome all of you. Thank you so much for coming today. I'm Karen Midthun, the Director of the Center for Biologics Evaluation and Research which is one of the Centers within the Food and Drug Administration. And the products that we're going to be discussing today fall within our purview.

And I just want to say that it really is a pleasure to be able to bring all of you together. We really see this workshop as an opportunity to foster information exchange regarding the regulatory and scientific issues associated with fecal microbiota for transplantation. Lately, as you know, there has been a lot of interest in this area and using fecal microbiota transplantation to treat recurrent *C. difficile* infection as evidenced by numerous reports in the popular press, published meta-

analyses of clinical use and a recently published open label clinical trial.

FDA began receiving inquiries about FMT about a year and a half ago and it raised a number of questions about FDA regulation including when an investigational new drug application is needed. We decided that we should hold a workshop on FMT that would bring together bench scientists, clinical researchers and those with regulatory expertise as well as other stakeholders to discuss various aspects of this quickly evolving field.

To this end, FDA, that is the Center for Biologics Evaluation and Research partnered with NIH, in particular NIAID in the Division of Microbiology and Infectious Diseases and it resulted in today's workshop. And I also very much want to thank those who were so integrally involved in developing the agenda for this workshop, engaging all those who we thought would be really important to be able to bring information to this workshop. I think we recognize that it was a lot of work

and we're very grateful to the collaboration with NIAID and also with others who have helped bring this together.

We're really fortunate in a sense that there is a rapid growth in the application that is that the rapid growth and in the interest in FMT coincides with a scientific revolution that is occurring in terms of understanding the importance of the human microbiome in shaping who we are and how our bodies function. This revolution has been fueled by really amazing technological developments in terms of our abilities to gather vast amounts of DNA sequence data on our microbial partners and by advances in our understanding of how our microbiota influence human health.

It's our hope that this growing scientific knowledge in combination with the application of rigorous clinical studies will usher in a new era in the treatment of human disease and the maintenance of human health. Again, I would like to express my appreciation for your participation in this workshop and

look forward to a very productive exchange of information and ideas.

And with that, I would like to turn the podium over to Dr. Fred Cassels. Thank you so much.

DR. FRED CASSELS: Thank you very much, Karen. My name is Fred Cassels. I'm the Chief of the Enteric and Hepatic Diseases Branch or EHDB. I'll be showing great restraint by not using humor related to feces.

I'm filling in for Dr. Carole Heilman; she's the Division Director Micro Infectious Diseases of DMID who very much wanted to attend but sends her regrets. DMID supports research to control and to prevent all human infectious disease agents except for HIV where we have a separate division of AIDS. Grants are the foundation of DMID. We also have product development and clinical efforts assisting academics and companies to develop products for the benefit of public health.

From our Division I would point out Dr. Richard Gorman who is in the back. He'll be moderating Session III. He's the Associate

Director for Clinical Research. From our branch, again dealing with Enteric and Hepatic Diseases Dr. Melody Mills in the front row here who will also be moderating the first session, has worked with Scott Stibitz and the FDA Steering Committee. Melody and Scott are the co-chairs. And her involvement is initially due to her grant portfolio which includes the human gut microbiome but also due to her organizational skills and her passion in this area.

I'd also like to point out Dr. Ryan Ranallo in the back. And due to his clostridium difficile portfolio is heavily involved in this area of research as well as product development and testing.

Kudos to the FDA for their outreach efforts to result in this type of meeting that includes such a strong scientific base, community component and as well as the regulatory aspects. This is an important topic and it's an important time to address these issues. The Steering Committee I'd also like to applaud for a very well thought out

and very comprehensive agenda. The broad range of speakers and stakeholders includes many aspects of the government, Human Genome Research Institute, the Intramural Program within NIAID bringing a research perspective, many from the Extramural NIAID and our group that brings that sort of programmatic perspective and notwithstanding the FDA with their public health and regulatory perspective.

Many academics are here, grantees and physicians. Some are treating patients with FMT. Others are conducting clinical trials. Another important aspect of the attendees is industry. They're examining FMT and leveraging all this microbiome data into potential products that hopefully will improve and save lives. So, we also have a healthy mix of PhDs, VMD and many MDs which is very appropriate for this meeting.

Much focus will be spent on the current practices although we will have a historical perspective really for the veterinary angle which is important to examine

as we look into the future. This field is at a critical stage. Many say fecal transplants are very safe and effective and the interest is heightened as Karen mentioned from New England Journal paper in January. Others are legitimately saying, not so fast. We must examine and assess the risk and benefit. These are legitimate complex issues.

Perhaps one solution down the road will be microbial replacement, some mixture of bacteria free of extraneous materials. But this is in the future. This very important meeting will examine in-depth all of these issues without blinking. Let's as a group help and work with the FDA in this incredibly promising area. Thank you.

Melody Mills will now moderate the first session entitled the Microbiome in Health and Disease.

MS. MILLS: Good morning, everyone. I just have a few logistical issues I need to make clear as we get started. Some housekeeping things as it were. So, first and foremost as you know there's signs saying

there's no food and drinks allowed in the auditorium. They're quite serious about this so please don't bring anything in. Please, if you've got something step outside and please take anything with you out of this auditorium so that we're allowed to use it again tomorrow as planned.

So, the other thing is to make sure you turn your phones off please. We have a transcriber so if you have any questions it's important to use a microphone. Please identify yourself, give your name and your affiliation and please don't be embarrassed if you forget and I remind you.

In terms of the speakers, we have a visual timer set up for you. So, there it's a red, yellow, green. When it turns yellow it means that you have five minutes left in your presentation. There is a pointer up there that you can use, a green pointer. You can also use to advance the slides. You can also use the up and down arrows on the computer.

In terms of the microphones at the podium, if you plan to stand still, please try

to place yourself in-between the two microphones so that the transcriber and everyone else can hear you. There's also a lavalier if you prefer to move around. And if anybody needs any help just look at me beseechingly and I'll run up there, okay?

So, let's go ahead and get started. The first talk will be the NIH Human Microbiome Project, the overview of goals and results. We're happy to welcome Lita Proctor from the National Human Genome Research Institute. She was the project science for the Human Microbiome Project and we have tried to ground this first day in the science of the Microbiome. So, let's get started. Thank you.

MS. PROCTOR: Let me get hooked up here. Podiums tend to overwhelm me because I'm so short. Let me just confirm I can advance, excellent. Okay.

My name is Lita Proctor. I'm a Program Director in the National Human Genome Institute. I have the privilege of overseeing the Human Microbiome Project which is actually

a total of eight years of activity funded by the Common Fund. We just finished phase one. And we're getting ready to launch a shorter three year phase two and after consulting with Melody I decided I was going to highlight just a couple of things from the Human Microbiome Project.

It was a massive activity. Many of the -- several of the PIs from the HMP are actually in the audience. So, I decided for the purposes of this workshop just to call out some of the activities. So, I want to describe the motivation for the Human Microbiome Project. Quite a bit of historical and scientific thinking went into developing it, what the objectives were and the structure of the HMP and in particular I want to call out a flagship activity called the Healthy Cohort Study which I think is particularly relevant to this group. And I want to call out some of the key data that came out of this Healthy Cohort Study.

Because I'm going to be talking about one particular paper I actually brought

some handouts and I'll point out which data I'm going to show and then you can decide if you want a handout or not. So, as you probably already know the idea that bacteria may play a role in human health has actually been around for a while. About the oldest citation I could find was from 1909. So, the concept that bacteria play a role in health has been around for a while. However, most of our attention, of course, has been on trying to treat infectious disease and through vaccinations and antibiotics and so on we've made tremendous progress of reducing many, many kinds of infectious disease.

At the same time, though, scientists have seen and epidemiologists have seen increases in autoimmune diseases such as MS or asthma or Type I Diabetes. So, the question becomes here we've won the battle effectively to remove really significant infectious disease but at the same time we saw this great emergence of autoimmune diseases in human populations. So, what was causing that? It wasn't a change in the genetics of the people.

This is only occurring over a few decades this rapid increase.

At the same time, particularly in my home institute, there is this major effort to sequent the human genome and towards the end of the Human Genome Project activity the microbiological community came to NHGRI and to NIH to say, hey, wait a minute. You don't have the whole story. We really need to be studying the comprehensive genomic inventory of the large portion of cellular life within the human body, the endogenous, the microbiota. That was before the term microbiome was coined.

So, that was the sort of second motivation behind the HMP. And so, in 2007, that's right, I think. Fall 2007 the NIH through the Common Fund which is an office in the Office of the Director which funds trans-NIH catalytic activities, funded this five year NIH program. It is quite a large undertaking. As you can see, I just simply wanted to show you all the different institutions. I think it was 80 plus

institutions and over 400 scientists that interacted across this five year program.

And what was the goal of the program? The goal of the program was to conduct a survey of the microbiome in humans, a kind of mapping activity of the human microbiota. And it was accomplished in a couple of different ways. One was through a kind of a taxonomic analysis of the microbial communities in different parts of the human body as well as an analysis of their collective genetic potential through, at that time, a fairly new procedure called metagenomics. And if there was any major driving question between HMP it was to ask the question is there such a thing as a core microbiome with a core in health or a core in disease.

Two cohort studies were undertaken to address those questions and to conduct the survey. One is the famously called Healthy Cohort Study in which each individual was clinically verified to be healthy across five body sites and in fact, I'm going to go into

some detail around how the Healthy Cohort Study was conducted so that you can understand the context of the data I'm going to show you. But there were 300 adults, sort of young to mid-age across five areas of the body and visited several times over about two years.

There's also a set of what we call demonstration projects which were actually projects to look at the association of the microbiome with various diseases. I won't go into any detail any more in this talk but just to show you that they tended to cluster around three areas of the body, skin, GI and urogenital. And it's everything from diseases to just conditions. So, you can see, of course, the lion's share were in the GI tract. That was a very extensive undertaking. If you have an interest in discussing it, we can talk about it offline.

So, I said we carried out a Healthy Cohort Study but in fact it's very hard to define healthy. So, as the way that the HMP defined healthy was to actually consult a wide variety of specialists in each part of the

body and talk to them about what would they consider a healthy or a normal condition of that body site. And so, a combination of inclusion and exclusion criteria were utilized to define healthy in this kind of super-healthy or carefully vetted cohort.

So, let me give you an example. Inclusion criteria, okay, the age range was limited to try to remove some variability. Women had to be willing to give a vaginal specimen and be on a regular menstrual cycle. BMI range was limited between 18 and 35 and so on. So, there were a number of inclusion criteria for enrollment.

But let me tell you about the exclusion criteria cause this is when it really starts to make you realize how carefully vetted this cohort was. Okay, there were five or six different kind of categories of exclusion criteria; number one in the oral. You know, there are all kind of things that you couldn't have any missing teeth. You actually couldn't have any kind of oral disease at all and if you did, and in fact, I

understand 80 percent of the initial recruits did have oral disease. They had to go and get it treated, be off antibiotics before they could re- enroll in the study.

Skin. There were all kinds of exclusion criteria around skin, acne that wasn't on the face. I'm not going to name them all. Here's an example, scalp dandruff that doesn't clear up. Think about it. Could you actually, I don't think I could, could you actually meet all these exclusion criteria?

Female specific exclusion criteria. They couldn't be pregnant or lactating. They couldn't have any kind of UTI or active STD in the last two months. They couldn't have undergone a hysterectomy. Their vaginal pH had to be greater than four point five and so on and so forth. So, all of these very precise body specific exclusion criteria for participation in this study.

Here's one about medications, okay? They could not have had influenza vaccine in the last month. They couldn't be using any kind of antibiotics, probiotics,

immunomodulators. They couldn't be using any other kind of immunosuppressive agents and they couldn't be using commercial probiotics, okay? Very careful criteria.

Other kinds of diseases and conditions that were part of the exclusion criteria included any kind of acute disease at the time of enrollment, any kind of history of cancer, any kind of unstable diet history; I'm not going to read them all. I just want to get the overall point that if you get your annual exam you don't nearly go to this depth in terms of vetting whether you're healthy or not. Your doctor says oh you're doing great. Your blood pressure's fine.

So, and there's some more criteria. So, I guess the point I wanted to make is that these people went through this extensive assessment prior to enrolling in the study and then within 30 days of going through all those studies they underwent a very extensive sampling regime and so on. And every time, if they were revisited, they had to go through the same survey to make sure they were, in

fact, free of disease and all those other criteria.

So, what did we analyze in the Healthy Cohort Study? Well, I mentioned that we wanted to know which organisms were present and how was that done? Through this phylogenetic analysis so using the 16S ribosomal RNA gene, which is a classic now, universal marker for doing phylogenies and because there was the opportunity to do so we also wanted to ask the question what's the functional potential, what's the genetic potential in this community? And that was done through metagenomics and comparing the data against metabolic pathway databases and so on.

So, there's actually quite a bit of information I can share but I'm going to just highlight a couple of things that I actually want to spend most of my time talking about another part of the data. What's interesting about this is the fact we didn't see any core per se, let me just go to the take home, we didn't see any core per se when it came to

gender, age, weight, ethnicity or race. In fact, the microbes when analyzed at all taxon levels really were distinct by body site. So, whether they were men or women or whatever the microbes know where they're living. They don't really care who they're living on. I think that's sort of a shorthand way to put it. Okay?

And another kind of take-home is remember these are healthy people. So, there was an opportunity to kind of predict what's the global pool of microbes that might be associated with healthy people and the HMP predicts that there will be upwards of 10,000 microbial species that are associated with healthy people. We don't all carry 10,000 species. We carry around 1,000 species per person but the global pool of potential microbes that can be associated with healthy people is about 10,000.

And what's also particularly interesting is that because we were able to do metagenomic analysis, or rather the investigators were able to do metagenomic

analysis, there may be upwards of eight million, eight million with an M, unique microbial genes that are associated with the microbial communities on our body sites. And if you contrast that against the human genome which is 20-25,000 genes, you can see that the microbial genetic signal is hundreds of times more than what we get from our own human genome.

Now, I'm going to close this talk and highlight one aspect because I think based on talking with Melody this is a really important part of it. Now, I've hopefully convinced you that this Healthy Cohort was very, very carefully vetted. And because we had 16S data and metagenomic data we were also able to ask some other questions. And one important question is simply are healthy adults carriers of potential pathogens? It doesn't mean they're acting as pathogens but can we see a genetic signal, a genomic signal for potential pathogens in these super- vetted healthy people?

And what we did or what the

investigators did is they used the NIAID PATRIC database which is the master database of all pathogens, not only priority one pathogens but all pathogens and they compared the HMP metagenomes and I think 16s but primarily metagenomes against the 300 plus bacterial genomes that are in PATRIC's Genome Finder. And this is where I come to this graphic. Because it's a very complicated graphic and I have cut it down for you, I've made paper copies for those of you who actually want to take a copy home. So, just come up here and grab a copy from the green folder.

This is work done by Curtis Huttenhower's lab as well as some of the other HMP investigators. So, this is a phylogenetic tree. So, there are four phyla. The Firmicutes, I don't know if I memorized these all correctly, the Firmicutes, the Actinobacteria, the Bacteroidetes and the Proteobacteria, four phyla. So, phyla for those of you who don't know is the most broad level of taxonomic classification. And what I

want to show you here is they were able to show not only what they call commensal microbes in this population but also potential pathogens.

So, let me quickly walk you through this graphic cause it's very complicated. These are the four phyla and the members in those four phyla. If there's a star it means it's a potential pathogen based on the PATRIC database. And in the outer circle you'll see here that they compared six different body sites; stool in blue, cheek plaque and tongue in the kind of orange to red zone, the nose microbiome in green, the vaginal microbiome in purple and skin in gray. So, what you're seeing is the prevalence of these microbes whether commensals or pathogens in these six body sites.

And secondly or thirdly rather, these bars on the outside show you the microbial abundance in the body site where they were most present. So, there's a lot of data.

Let me just tell you I've taken this

circle and cut it up into four pie pieces. And we're going to walk around the circle. So, starting on the -- oops, I've forgotten which side it started on. Starting on the northwest quadrant of this pie, here's one quarter of the pie. It's the Firmicute branch and I'm going to walk you through this again. In each graphic I have the potential pathogens that are the stars. These are the six body sites in the color coding. Here's that bar graph where it shows you the relative abundance or the abundance of that particular microbe and the intensity of the colors indicate their prevalence in each body site. So, those are the kind of legends for each of these pieces of the pie.

Okay, let me give you two examples. Here's lactobacillus, very, very prevalent in the vagina, no pathogens. So, that's sort of one clean example and that's flanked actually by two groups, the streptococcus and the staphylococcus which conclude, of course, not only commensal members but also potential pathogen members. And you can see that for

staph and strep that they tend to be most prevalent in the oral cavity. And there are three subsites in the oral cavity shown in these data.

And what I've done for you here is called out the species names of the Firmicutes that were found in this metagenomic analysis of HMP Healthy Cohort data. So, several different species of potential pathogens that can be found in healthy people in this staphylococcus and streptococcus data.

Okay, walking around now we're still going clockwise around the pie. We're looking now at the Actinobacteria and the Bacteroidetes phyla. And same kind of context, potential pathogens are starred. The six body sites, the bar length shows the microbial abundance and the intensity of colors show the species prevalence at each body site. What you can see right away is that both in the Actinobacteria phyla and the Bacteroidetes phyla there are a number of difference genera as well as species within each genera that appear to be present and

healthy people that in the PATRIC database are marked as potential pathogens.

Again, I'm not arguing, we're not arguing that these are pathogens but the genomic signal for them is present in healthy people. An example would be one that probably a lot of us know, *propionibacterium acnes* which is the causative agent of acne on skin is very abundant on healthy skin. It's just there. So, they're present a hundred percent of the time and yet they're not causing acne because these people have already been excluded for any acne.

What's another example? Well, that's probably enough for examples. Oh, here's another one, *corynebacterium*, many different species of *corynebacterium* are found in a wide variety of body sites. I think that we did notice though that the two major body sites that seemed to have the most numbers of pathogens are in the oral cavity and the oral microbiome and in the gut microbiome. So, stool and oral, if you look at that full pie chart, the full circle, you'll see that it's

primarily the oral cavity and the gut tract that seem to be the home of most of these potential pathogens.

Moving around the pie still, we're now in the southeast quadrant. We're looking primarily at the Proteobacteria phyla. There are several, four or five genera of potential pathogens that are found in healthy people primarily in the oral cavity. You can see that here because it's in the yellow, orange and burgundy bars. A lot of campylobacteria species, a lot of nesseria species and some even haemophilus influenza found in the oral cavity.

And then we're now finally back around to the last quadrant, the last pie chart and this is the example where we're back to the Firmicutes but very, very few potential pathogens in these genera. Only a few in the fusobacteria and the veillonella.

So, I hope this is useful. The idea is that these were carefully vetted healthy young adults that were free of any kind of disease and so on and yet they still seem to

carry microbial members in their microbiomes across their body sites that suggest they could be potential pathogens. So, I think that's something important to remember and secondly that this ring should show you that most of the ones that show up brightly are in the gut and in the oral cavity. So, it appears that the gut and oral cavity of the five major body regions that we examined appear to be the home of most of these potential pathogens.

And that based on comparing against the PATRIC database that the totally count was something like 80 plus potential pathogen sequences found in the HMP metagenomes. And of course, I've got to remind you we only looked at bacteria. So, we didn't do any kind of work on -- I didn't show any data from the viral work. So, remember that there are many kinds of not only bacteria face but also you carry otic viruses found in the healthy microbiome.

I'm closing with telling you if you want to learn more about what the HMP

accomplished, we just this past June published, June 2012 rather, just published two very high profile papers in Nature. They're all open access as well as a suite of companion papers that are also open in open access journals in the PLOS collection. And they're very easy to find. I've got the DOI numbers here.

Are you going to circulate the slides from this talk? Is that the plan? Post them for everything?

MS. MILLS: I'm not sure we've decided that yet.

MS. PROCTOR: Okay. I mean you're welcome to share mine. So the reference is for not only that very complex pie chart but also this are in my slides. And if you want to look at the data, the best place to go is our HMP data announcement and coordination center where they have an embarrassment of riches when it comes to tools, data, value added derived data sets and so on.

And with that, I believe I'm done. Thank you. I think I have time for questions

cause it's still green. I must have raced right through it. If you have a lot of questions, I'm going to direct you to the primary investigator on this chart. So, I'm sitting in the middle of the first row. So, if you're interested in getting a hard copy of this please just stop by.

MS. MILLS: So, Lita, I did have one question for you. So, it seems like there was an exceptionally extensive exclusion criteria but a relatively standard inclusion criteria. So, I think the point, one of the points you're making to distill it is that this was an atypically healthy group of people for their age in the general population?

MS. PROCTOR: Yes.

MS. MILLS: So, do you think that the screening modality really sort of set the highest bar for screening?

MS. PROCTOR: That was the intent. Again, this was meant to be a baseline study, you know, what can we actually call healthy? So, these specialists from all these fields were consulted to ask what would you consider

to be a healthy state for that particular body site. And then in aggregate all that advice came together to form that very extensive list of exclusion criteria.

So, I would have to say they were young and they were super healthy. So, yes, that's not necessarily the same thing as normal. Right? Right. Any other questions? Yes, oh you had one Scott.

DR. STIBITZ: So, you speak of the  
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MS. PROCTOR: You have to talk into a microphone. That's awkward. Talk in my microphone.

DR. STIBITZ: Oh, very good. So, you speak of the signature of particular pathogens. Was that defined as just say 16S ribosomal sequences for those taxonomic groups or were you looking for the presence of specific virulence factors or even assembling metagenomes?

MS. PROCTOR: So, as I understand the way the study was conducted, we weren't looking for any kind of virulence factors at

all. It was 16S and/or metagenomics, so just the genomics sequence. No genomes were assembled from the metagenome. I don't think that the sequence depth was sufficient to be able to assemble metagenomes. And I think maybe Dirk Jeevers, who's in the audience here somewhere -- have I got that right?

MR. JEEVERS: I can quickly add a lead to that. Basically, we made biomolecular markers for all organisms for which we have a genome and mapped the metagenomic (inaudible) onto those markers and so --

MS. PROCTOR: But there wasn't any opportunity to actually assemble genomes other than metagenomes?

MR. JEEVERS: No, the assembly typically, even if you go very deep it's a very challenging task to assemble metagenomics data so ranging between a couple of kbs to and that megabates but definitely not whole genomes.

MS. PROCTOR: Okay, thanks. There was a question down here.

MR. KUNDE: So, these are genetic

markers for the AT pathogenic bacteria?

MS. MILLS: Can you please identify yourself?

MR. KUNDE: Yes, I'm Sachin Kunde from Helene DuBois. But what does it mean if they are not able to -- are they virulent? Are we culturing them? I mean just having a presence of a gene does it mean anything?

MS. PROCTOR: It doesn't mean anything except that based on what we know the genomic signature are of these pathogens they are present in healthy people. And if we're talking about then transplanting material from healthy people to not so healthy people, I think we just want to -- I just want to put out there that we should be aware that the material could contain pathogens and that in a different environment, those microbes that are not pathogenic under one condition could in fact become pathogenic.

That's really my main point in showing the data from the Healthy Cohort Study. Phil?

MR. TARR: You described this cohort

as being young and healthy and those are phenotypes that are short-lived. Any opportunity to see what they're doing now?

MS. PROCTOR: You know, as I -- I think it's an excellent question. So, there's been a lot of interest in the community to revisit the HMP Healthy Cohort because we put so much time into it. But as I understand the way the clinical study was run and the way that the IRB was written, we cannot revisit them. We cannot revisit them. So, unfortunately there was no provision made upfront to allow for this particular cohort to be followed through time. I mean it would be the perfect little Framingham study of the microbiome and we don't have that.

In the back there?

SPEAKER: How much family history was vetted as part of screening? Because there's a lot of family history related issues and that you may think it is a normal host but the patient may be genetically predisposed to certain things.

MS. PROCTOR: First of all, when I

think of family history I was thinking more kind of phenotype or so on but in terms of genomics, no genomic sequences were done on these healthy cohorts. There were some genomic data that came out as a result of the metagenomic analysis but there was no deliberate genomic analysis done of the Healthy Cohort. So, I don't think we know that yet.

There may be an opportunity in the future to see some of those data as we try to figure out if we can actually analyze those human genome data because that wasn't part of the approval process for the original cohort study.

SPEAKER: The reason I ask this is because what we consider as normal may not be normal hosts, normal or supposedly healthy people may not truly be healthy. They may actually become diseased very soon.

MS. PROCTOR: Oh, I understand what you're saying. I mean they could be genetically predisposed towards one disease or another. But at time in their life when they

went through the Healthy Cohort Study, they were considered to be healthy no question about it.

I thought there was a hand way in the back somewhere.

MS. MILLS: I think we have time for one more questions.

MS. PROCTOR: Okay. Yes, ma'am.

MS. HAYS: Ann Hays, University of Virginia. I also had the same question in terms of family history because there's so many very healthy long-lived individuals who give a history of their parents also being similar. But the other thing is you mentioned that they had, that they were allowed to have had antibiotics and I was curious as to what the -- how long the interval was.

MS. PROCTOR: I don't remember and I don't know if anybody else in the audience who was part of it can remember. But the reason they were allowed to use antibiotics is -- was it six months Vince? Is because it turned out when they started the recruitment that 80 percent of the potential participants had oral

disease. Most of us have oral disease and they couldn't have oral disease if they were going to be part of the Healthy Cohort. So, they were sent off to their dentist and everything treated and if they used antibiotics, then they had to be off antibiotics for six months prior to re-enrolling. That was the motivation behind that.

MS. HAYS: But then the 20 percent of people who don't have oral disease and who don't need those antibiotics would be considered to have a more healthy microbiome?

MS. PROCTOR: No, they were part of the cohort. So, they weren't pulled out as a separate subset of the cohort. They were part -- they were reintroduced as part of the regular cohort. And I'm going to have to stop there but you know we can have a conversation afterwards. Thank you so much.

MS. MILLS: Okay, our next speaker is Dr. Phil Tarr from the Washington University School of Medicine in St. Louis. He'll be speaking on fecal transplants for

Clostridium difficile, microbial Messiah or medical misadventure. I think that's our favorite title of the session and I'd like to say that Dr. Tarr also was the principal investigator for one of the human demonstration projects in the HMP.

DR. TARR: Thank you very much, Melody. Thank you for inviting me to this session. This is a remarkable time in gastroenterology, microbiology and human biology and this is coming together much more rapidly than we ever thought it would even just a few years ago. And my title reflects the fantastic opportunities that are available to us as we discuss fecal or polymicrobial transplantation and the potential for harm which is something that we should always strive to avoid. I should disclose that last year I received an honorarium for a single lecture at a company one of whose products I will mention today.

So, the background that I think we can all agree on is the current data that's evolved in the past 24 months or so suggests

that fecal microbial transplants do have some benefits. Subjects reported were improved subjectively and most recently objectively in the New England Journal paper but the value is not yet well quantified. These are not massive studies and the databases that exist are still rather local and are not populated to such an extent that we can really calculate the benefit.

So far, this is an intervention that appears safe but again there's not been enough subjects to quantify the risk. We have the recent, relatively recent issue with Vioxx where it was given to a small number of people and low frequency but highly regrettable adverse events, myocardial events did not become apparent until after market dissemination. And practitioners, we all need some basis for efficacy and safety before we recommend or administer any intervention to one of our patients.

Additional background, in the past five years, none of us even those who were involved in the field would have guessed that

we would have found that these microbial communities are so vibrant and that they really drive good and bad phenotypes in animal experiments and now it appears in humans. At the very least they're associated with good and phenotypes. Our understanding of the biology underlying these phenotypes is embryonic. We are really still quite phenomenologic in what we're observing. And as we embark on any new intervention especially with a biologic that may be quite powerful and potentially risky and that can be administered with minimal infrastructure, we need to be cognizant of all the regulatory, ethical and liability issues and I'm certain that others will discuss that over the next couple of days.

So, first as practitioners we all say are we doing any harm before we do anything? And when we consider this globally and I'll discuss these various risks as sort of a survey of what could be out there and what could be in this biomass, we have to consider risk from donors, bacteria in that

stool that might cause disease, viruses that might cause disease in the recipient and if not disease, are we conveying antibiotic resistance genes when we transfer intestinal contents from one subject to another. And I will close with a few lessons from *C. difficile* microbiology.

So, at the very first, risk from the donor and 10, years ago there was a big surge in directed donations after much discussion with the FDA and Red Cross, family members and friends and neighbors were allowed to donate because of concerns about the safety of the blood supply. There's been fantastic advances in diagnostics of blood. So, blood is exceptionally safe and back then there were studies that suggested that donors who are known to families, designated donors, directed donors, had higher levels of virologic exclusion criteria than did random volunteers. And I have not followed this literature until in preparation for this talk and I see a paper came out late last year that suggests that this problem persists, that directed donors

have three to about eight times the frequency of being positive for HIV, Hepatitis B, HCV and HTLV.

So, knowing somebody seems to be the preferred way to select donors or living with somebody is being a preferred way to select donors in the studies that have been published may not be, in fact, safer from random donor pools, at least based on the blood donation literature. So, from this we can say don't make assumptions about your donors..

Now, there's risks from bacteria that we know about and this is an abstract from the van Nood paper from New England Journal. And all of these papers have methodologies very similar to this. Lita just showed that we have only the scantest idea of what is in the -- of the bacteria that are in the stool from our sequencing of healthy young people. And it is reasonable that the donors undergo a first pass microbiologic assessment but the stool evaluations in my opinion and the data that have been submitted to date in publications do not offer considerable

confidence that there are no pathogens in that transplanted substance.

So, all that's described is that they were cultured presumably for -- screened for enteropathogenic bacteria and for *C. difficile*. And the methodologies for this were not described. And looking forward I hope that we apply the most stringent ability to find pathogens especially at low densities as a standard.

Now, again, this is not just the group from the Netherlands. A group from Rhode Island talked about testing for bacterial culture and this is a group from Minnesota talking about donors underwent stool screening that included routine enteric pathogens. Again, no details and microbiologic testing protocols are quite variable and quite different in their sensitivity.

Furthermore, all of this standard microbiologic detection protocols that are out there now are based on diagnosing an infection at the height of an infection in a

well-handled specimen taken to a good competent laboratory. In other words, specimen has a high density of the enteric pathogen and this is 125 year old technology but interestingly it's set at just about the right sensitivity and specificity for diagnosing individuals with acute bacterial enteric infections. And people who have worked in microbiology laboratories will know this and for the rest of the audience I'd like to review.

When a specimen is handed in at the height of an illness for a stool culture, one takes the stool and splits it out into various analysis protocols. There may be some enrichment in a broth. It may be plated right on an agar plate; the agar plate will have varying indicator substances in it and inhibitory substances in it. And in the end the microbiologist examines about 100 colonies because it's plated for isolation. This an E. coli, O157-H7 that's not fermenting sorbitol unlike its mates which do. This is easily detected by a good microbiologist.

Now, this specimen was on a patient who was on day five of illness and you can see that this white colony which was the only one on the plate was about one percent of all the isolated colonies that the microbiologist could examine. Now, at the height of a bacterial enteric infection people are excreting about ten to the eighth, ten to the ninth viable pathogens per gram of stool. So, that means you could have ten to the sixth or ten to the seventh pathogens, Salmonella, Shigella. E. coli is rather unlikely to be carried by a healthy human and completely miss it on standard enteric microbiologic evaluations as appear to have been performed in the published microbiota transplants studies.

So, the way to move forward I would propose is to take lessons from the Food and Veterinary microbiology field where food borne pathogens are not highly abundant in food. They don't become highly abundant until they get into a human and start replicating in any given analyte. And generally this involves

looking for pathogens among many non-pathogens and we tag these usually with antibodies and beads that are really magnets to find these needles in the haystack. And then we concentrate it with a magnet pulling it off to the side, washing away the lookalikes and you can see that one out of a hundred then becomes one out of two in plating on an agar plate becomes very easy to distinguish.

So, looking forward, I hope that we can apply much more in-depth analyses to these specimens. And to look for pathogens that we know to look for. And to date we've been using in the published studies techniques that are slanted towards high abundance organisms at heights of infection.

Now, we also have data from a current study that sepsis comes from the gut in a susceptible host. This is 11 premature infants with late onset neonatal sepsis at St. Louis Children's Hospital collected over approximately a two year period. Seven of them had group B streptococci, *Serratia marcescens* or *E. coli* in their stool before

sepsis. Two of them died but we were able to find those organisms in their stool. Several percent of the same human subjects also had these organisms in their stool. These children were born with sterile stools. They did not have any gastrointestinal illnesses. They would have been, had they been older, likely to be called healthy donors. There was no reason to suspect by history or physical examination that anything was wrong with their gut.

And this demonstrates the organisms that be commensal in one host can be highly injurious to another. These were highly susceptible neonates presumably having acquired these infections from an adult or somebody else in that unit. So, again, what we know to look for, a stool culture isn't going to look for *Serratia marcescens* but put into the wrong host it could cause a disaster.

So, from bacteria we conclude to summarize the risk, standard culture techniques are really not excluding pathogens present in low density in asymptomatic donors.

And one person's commensal is another person's bloodstream isolate so you have to be very careful that the subject who is receiving this donation will not become a highly vulnerable host or is not a highly vulnerable host in the short order. Many of the studies published have excluded people who are highly likely to be injured by such an intervention and that was rightly so. But as we may get into expanded indications, this is going to become riskier and riskier potentially.

Now, viruses have not yet been scrutinized in the Human Microbiome Project to the extent that bacteria have. And in the studies that were reported for microbial transplantation the analyses largely limited to serology. And serology is quite good for evidence of a past or a recent past infection where there's viral invasion, your antibodies are long-lived or perhaps even for life after Hepatitis A or B or C infection but there was minimal viral analysis of stool.

There's not much viral analysis of stool that is FDA approved in the United

States. As far as I'm aware one can all a microlab and test for Rotavirus, Adenovirus 40 and 41, and if your lab is set up appropriately, norovirus diagnostics are becoming widely disseminated. So, they're out there now. These were not sought in the studies and we need to be careful about viral transmission.

A recent study completed by Dr. Lori Holtz and David Wang in collaboration with Dr. Carl Kirkwood in Melbourne looked at children with diarrhea. Two cohorts in the northern territory and Melbourne and some of these children had classic viral pathogens. But many as in many viral etiology studies had nothing identifiable and the viruses that she found in stool, that Lori found in stool, were based on viromic analyses, mass sequencing of the stool, not a specific kit analysis. Specimens were obtained, nucleic acids were extracted. This was not 16S based, this is metagenomic based. These people are mostly interested in viruses and throw out all the other sequences that they get.

And from the virus samples that they sought, 87 stools underwent mass sequencing. 70 percent of them, approximately, had viral sequences in them. About half of them had two or more viruses. 30 different viral families were detected and this shows the distribution. So, again, only about 30 percent of people had no viruses, these children had no viruses in them. Now, this is a slightly skewed sample in that these children had diarrhea but I think that it is highly likely that these viruses were there before the diarrhea as well as afterwards. And were not the cause of the diarrhea, they were samples of convenience and she is now trying to extend this to normal childhood cohorts without diarrhea to try to define at least the childhood human virome. But they're certainly going to be present.

Sequencing demonstrated rotavirus, noroviruses, adenoviruses and astroviruses many of which, some of which were detected by antigen testing, many of which were not. She found enteroviruses as we know do hang out in the gut even though they generally cause

non-gut disease. This is a nearly ubiquitous virus that everybody has. She found parainfluenza in the stool. It doesn't have to be a gut pathogen to be in your gut, plant viruses and novel viruses including a recently described polyomavirus. So, the virome is a completely unexplored territory and is not really being investigated for safety.

She expanded her work to look at by PCR at specimens, children from Seattle and the Gambia, very high frequency of adenovirus in their stool from the Gambia, very high frequency of enterovirus, though not from Seattle, at least not when the stools were collected at that time of year. So, again, the virome is out there and we're not addressing it. The donor virome clearly needs definition in general but certainly if we're starting to get into interventions using this donated substances and again what you find negative on an antigen test could -- you're only scratching the surface of what's in there.

So, antibiotic resistance. So, we

classically think of antibiotic resistance as a human who has gotten a lot of antibiotics variably, appropriately and that preexisting organisms in the gut are then selected because all the other ones are killed. And in a Darwinian battle and yet there may be many other ways to transmit antibiotic resistance genes without such a selection. And I'll elaborate on some work by Dr. Gautam Dantas and Aimee Moore.

So, certainly we know about selection and people who work in cancer settings see this day after day. Antibiotic use in North American children is diminishing. So, it's not just a situation where children are exposed to tremendous amounts of antibiotics and then resistant bacteria flourish. There may be this under the radar reservoir of resistance genes and these would probably be acquired non-selectively by subjects from parents, soil and animals. We do not live in a gnotobiotic environment.

So, the way we look for bacterial resistance classically is to take a

polymicrobial substance and plate it on an agar plate or put it in a broth with an antibiotic and if it can grow it can be selected. This is classic microbiology. But it turns out that when we apply this to a group of children nearly 500 children from Seattle, stools collected about a decade ago, and just took their stool, these children had never seen Fluoroquinolone. They actually had not seen a lot of antibiotics overall and no one close to them in their household had seen ciprofloxacin in the past month or taken it, at least not on the questionnaire. And just take their stool; three percent of them produced ciprofloxacin resistant E. coli.

Several produced *Stenotrophomonas maltophilia*. This is *Achromobacter xylosoxidans* which can be a pathogen in immune compromised individuals and *Enterobacter aerogenes*. So, these are organisms in stool that were antibiotic resistant to ciprofloxacin 10 years ago. We are repeating this study now in St. Louis and it looks like we're seeing a slightly higher percentage in

stool in children who've never had antibiotic selection. So, many of these studies seems to have used recent antibiotics as an exclusion criterion for a donor. That does mean that there's not a resistance in those bacteria that are being donated.

Additionally, we have been locked into this concept of it doesn't grow on a ciprofloxacin plate then there must not be anything resistant to ciprofloxacin in there or any other resistance factor. And in fact, that turns out not to be the case. So, classically what we have done is do direct selection from the stool. But in a series of elegant experiments from Gautam Dantas when he was in Boston and now repeated in St. Louis, if you take the stool and you extract DNA from it and put that DNA, no amplification, you put that DNA into an E. Coli that can grow on anything and then select for antibiotic resistance, you find genes in that stool that are dramatically different than -- you'll find resistances in that stool that would not be elicited by plating that stool on an agar

plate.

So, these are genes from bacteria that can't be grown on those agars with those antibiotics. They are largely from anaerobes and one needs to take the DNA out of that biomass and put that into an E. coli and then grow that E. coli and then sequence out those genes and the flanks of those genes to try to get some idea of the organisms in which they originated.

I will skip through some of this technology here and show that if you take stool, DNA directly from stool and put it into an E. Coli you will then select for organisms that actually have very little homology to what's known in pathogenic databases or just in the NCBI database in general. And they tend to be -- that DNA that confers antibiotic resistance tends to come from anaerobes.

If you grow up the bacteria aerobically and then take the DNA from it and sequence the DNA that confers resistance and look at those flanks you will find many organisms that you do recognize. So, what you

look for is highly tech -- what you find is highly technology dependent on what you're using on these stools.

Now, in children, these are 22 healthy children, Gautam Dantas and Aimee Moore are now examining, are now applying this technique and they are finding multiple classes of antibiotic resistance that are not apparent just by growing that stool on antimicrobial plates. These are single time specimens and you can see the children acquired these resistances quite early. These are resistances to tetracycline and ciprofloxacin, not ciprofloxacin in view of the way that the genetics are conveyed cause ciprofloxacin is a point mutation, but tetracycline and strianam and chloramphenicol and no child in North America is going to be exposed to those bacteria, to those antimicrobials in the first few months of life.

He is now looking at this longitudinally in 12 infants in Seattle. This is actually twin pairs and he's finding the

same thing in a prospective continuing cohort. Children come home from the nursery and soon thereafter acquire bacteria that have in their DNA a massive array of antimicrobial resistance factors. So, again, if you're transmitting this to another human they probably have their own repertoire of antimicrobial resistance factors in their own anaerobes but if they are all or about to become quite ill, this will not work to their advantage.

Now, there's a few final lessons from *C. difficile* that I'd like to discuss. All the tests for *C. difficile*, everyone tests for *C. difficile* before using this as a donor product and that's quite reasonable but the test details are not specified. But even if they were the tests that are out there are again geared towards moderate to high level *C. Difficile* in stool and we just do not know the lower limits of carriage of *C. difficile*. But it is probably higher than any test can state.

In other words, most of us in this room would probably test negative for *C.*

difficile using a variety of antigen tests or nucleic acid amplification tests that are now commercially available. And yet, a higher proportion of us probably do carry very low level C. difficile. Now, the standard way to look for C. difficile was first cytotoxin assay in stool. That may not be all that sensitive but it seemed to have been fairly specific and associated with disease. Growing the bug, it got its name difficile because it was difficult to grow back in the 1930s. So, that's cumbersome. The antigen tests have variable reliability. There's about 12 antigen tests that have been approved by the FDA.

One recently approved is from Cepheid and their limits of detection is reported in their 510K document to the FDA was about 41-255 colony forming units of C. Difficile per swab. And I made an estimate that perhaps there's a hundred milligrams on a swab. So, this test which seems to be fairly good and is being used increasingly frequently will detect about 400-2500 viable C. difficles per gram

of stool.

Recently, Carey-Ann Burnham's group, a diagnostic microbiologist, has been able to get down to 10 colony forming units per gram of stool using a combination of heat, cycloserine mannitol broth and blood agar plating to find hemolytic colonies and then testing them. So, the tests that we use are a couple of letters of magnitude higher than may be achieved by even more intense microbiologic analysis for C. diff before saying this donation does not have C. Diff in it.

So, again, I just want to get back to the patients. We all want to avert this terrible complication of C. difficile colitis. There's a spectrum of C. difficile injury to humans. This is the Bristol Stool Chart that our laboratory uses to try to gauge just how diarrheal the stools really are when they're submitted for C. difficile testing. And there's a spectrum where you have very mild cases that resolve spontaneously or with one a few days of metronidazole or vancomycin. Then there's horribly life- threatening C.

difficile.

My guess is the patients who have been published because of exclusion criteria, somebody expected to live for at least a year after the study started, were in this zone. And there's a terrible paradox here namely the closer you get to this area the more you may benefit from a fecal transplant but the riskier that fecal transplant will be because of the bioactivity in that substance. So, as we look forward we would need to carefully monitor what we're doing and have great databases..

Summarize, standard diagnostics for classic pathogens are far from adequate to assure that those pathogens are not present. That biomass harbors viruses, antibiotic resistance genes, potentially sleeper pathogens. Beware of unintended consequences. To paraphrase Donald Rumsfeld, we don't know namely how to measure the risk of that biomass. So, whether or not it has a specific pathogen we hope that stool will be beneficial but we also need to have some equipoise here

and recognize that we may be causing harm.

Looking forward, we really have got to improve our ability to know what's in there. Lita showed the first glimpse of that population systematically collected, thorough registries to track significant adverse events. Looking forward, the numbers and the literature do not yet give me enough confidence to recommend this as a safe procedure and informed consent in our patients has got to be critical no matter what strategy we take. Thank you.

MR. RAMESH: Sir, I mean I sincerely appreciate your conscience and --

MS. MILLS: Can you please identify yourself?

MR. RAMESH: (inaudible) Ramesh, (inaudible) for infectious disease. The concerns are great but the issue here is we are utterly exposed to a whole lot of biomass every day. Just like eating salads and I have little kids that wade in the kiddie pool. So, we are all getting fecal transplants every day. So, fear, I think we don't want to over

-- I mean it's important to state concerns but I think we can exceed the concerns by or we can cry too much fear in people. That's what I don't want.

DR. MCDONALD: Cliff McDonald, CDC. Thank you, Dr. Tarr for a great talk. We've had a chance to talk over the phone before. One question I would just challenge you on one point. There's a comment you said about that most of us are probably colonized with *C. difficile*. And I would -- this is something that certainly we agonize over a lot at CDC and this issue of where is *C. difficile* coming from. And I would say the epidemiology generally does not support that although there might be some increasing evidence that there's more of that.

There have been questions about whether *C. diff* might hang out in the appendix for example or something like this but in fact, there's evidence on the flip side that a rich microbiome might even hang out in the appendix, in the biofilms there. But one piece of evidence is just that you see higher

rates around other people with C. difficile. Now, certainly when you culture people in this room using standard methods you'll find three percent of healthy adults colonize but you're pointing out that maybe they're there in lower numbers.

But again, the epidemiology doesn't really support that cause we do see this herd rate where you really need the other people around you to get C. difficile generally. Now, that may just be more virulent strains or something like that but and generally it's either carriers that we can detect or people with symptomatic disease. So, I would just clarify that point.

DR. TARR: I think I will amend that to say a higher proportion might be colonized than we've detected to date.

MS. GARGES: I'm Sue Garges, NIAID. From the Australian study I was surprised not that two-thirds had viral genomes in their stool but that one-third didn't. So, do you think is that because they had diarrhea and things were, pardon the expression, flushed

out? Or is that really we don't have great methods yet for detecting those viral genomes there?

DR. TARR: Well, this was done by mass sequencing so if you had every nucleic acid, if you had a hundred percent certainty that you'd rarified every sequence that could be obtained I could share your surprise. But I think that right now we don't -- there's no 16S equivalent with which to construct rarification curves. It is probable that -- I can't completely answer but I can say that there was a gradient. Northern Territory children had more than Melbourne children. So, there may be limits to what actually somebody will have.

But until you get every nucleic acid in there sequenced and censused and accounted you can't really say that they were absent.

MR. RUBIN: Hi, I'm David Rubin from the University of Chicago. Thanks for a very stimulating discussion. My question is about the children with these mutations and resistant organisms. It begs the question

what the rest of the family or specifically the mother's organisms might like look. And I'm suspecting that you're already trying to look at that but can you tell us a little bit?

DR. TARR: We are looking at it and the sequences are not out yet. And we don't know yet.

MR. RUBIN: And the mom's history of antibiotic exposure et cetera?

DR. TARR: Minimal, minimal. People are of -- it's interesting. In North America, people are now shying away from antibiotics culturally.

MR. KUNDE: Sachin Kunde from Helene Dubois Children's Hospital. My question is in the same lines of colonization. Do we have data on what percentage of patients or subjects with formed stools have false positive C. diff PCR? Because when we perform fecal transplantation we are required to do this screening with C. diff and sometimes the stool may be formed from a donor and if it's positive, what does that mean?

DR. TARR: Interesting --

MR. KUNDE: What do we -- what data we have on that?

DR. TARR: I can answer with some of our laboratory data. So, we have another cohort in St. Louis, C. diff in children is incredibly common. Every one of your children will have acquired C. diff for several months, cleared it, acquired it again over the first two years of life based on our analysis of several dozen children already.

It's extremely common. They're a fully toxigenic C. diff. When we get a piece, we screen with PCR, every time we get a positive screen we can find that bug by culture. We can also find it in a subset of those where we cannot, where we do not get a positive with PCR. So, we generally believe that the PCR does reflect the viable C. Diff at much lower levels than we -- it can be a nuisance when somebody comes in with the mildest form of diarrhea. C. diff is sought, it's found and we're very ambivalent that it's the cause of the diarrhea.

So, I think it's not a bad test and

does represent viability. Linda?

MS. MANSFIELD: Linda Mansfield, Michigan State University. So, to follow up on that, does that mean that there might be C. diff spores in the household?

DR. TARR: We would imagine but we're limited only on the -- we're limited only to the stool, yeah. It certainly, the child was not born with C. diff in the stool. So, where it came from is open question.

DR. MCDONALD: Just a comment about both, maybe a comment, allow you to respond also from both yours and Dr. Proctor's talk. Is that it does seem like we're right now straddling paradigms. I mean both your talk and Dr. Proctor's talk have brought to light and reminded us, I think reminded most of us, we're already aware that stool has pathogens in it. That's one reason why we tried to keep stool away from each other but and that's a longstanding trait we have and reinforce with public health, all that.

But the flip side of this course is that it may be very, very important what ratio

it's there with other things. And that's -- and I think Dr. Proctor's talk was even on that. So, I think we should recognize, I think, in these two days that we are straddling paradigms. It's not -- I think the future may be that it's this whole idea of pathogen present is a pathway towards disease will be maybe someday replaced with pathogen without other things present is a pathway to disease.

DR. TARR: Right.

DR. MCDONALD: In C. diff I think we already have that a little bit. Here we have an iatrogenic disease that has been largely wrought through the eradication of some of those other factors. And so, as we go through this I think that let's keep that as an honest and open thing on the table.

DR. TARR: Right, and that's where good registries will be an incredible asset as we enter this brave new world. You're right. This is new biology, new regulation I imagine, new therapeutics that we don't have good frameworks for handling so we've got to be

very careful and disciplined moving forward.  
Okay, thank you.

MS. MILLS: Thanks everyone. Our next speaker is Dr. Yasmine Belkaid from the National Institute of Allergy and Infectious Diseases. She'll be speaking to the control of tissue immunity by commensals.

DR. BELKAID: So, hi. So, my talk is going to be a bit different, I guess, from the ambience of this meeting. It's very much on the basic side. And I thank very much the organizer for inviting me and present some our ideas and how some of them could maybe relate to some of the approaches that are taken by clinicians today. So, my talk is going to be very much about experimental approach to tests, the crosstalk between commensal and pathogens.

So, all of you that are in this room know very well that all different surface of the body will be invaded very soon after birth with a very diverse and abundant microbiota. But what is actually quite remarkable is the fact in the same tissues that are actually

home to these microbes are also the ones that actually are going to be the primary target of infection and definitely the way of entrance of pathogens into the body.

So, clearly the interaction between pathogen and commensal is quite complex. Of course, pathogen and commensal can be contextual. We have heard that before but at the same time these two forms of organisms can influence massively each other. And really relevant to the topic of fecal transplant of course, there is competition that can take place between commensals and pathogens. Of course, it has been also shown in various settings and actually acute infection can induce these biotas of the microbiota and emergence of more inflammatory strains such as *E. coli*, for example. And for the purpose of my talk what we're going to discuss is actually the fact that the microbiota can also influence immunity to pathogens.

So, a few years ago, we and others have actually shown that in the GI tract the presence of the resident microbiota is

extremely important for the induction of appropriate immunity against oral infection. And if you actually deplete these microbiota via antibiotic treatment or in germ-free settings the animal will no longer be able to develop productive immunity and T-cells and B-cell response against the invading microbe.

But, of course, most of the studies that have been done have been at the level of the GI tract but we have to remember then this commensal microbiota invade different kind of tissues and all of them will develop a very unique mode of interaction with these microbes that are residing. But actually how these microbes control local immunity have been poorly explored.

So, what we decided to explore in the laboratory a couple of years ago is really how all the tissues that are actually colonized by the microbiota can actually be controlled by these microbes and how actually they could influence immunity to pathogens. So, the tissue we decided to explore was actually very much a collaboration with Julie

Segre at the NIH that has pioneered the skin microbiota and really reveal then the skin is really a mosaic of different community of microbes that are very conserve in different sides of the body.

And this work was actually initiated by a graduate student Shruti Naik that decided to explore the possibility that this microbe can actually control local immunity. So, in the skin these microbes reside in very structures. They will be present in the sebaceous glands and hair follicles. The skin is, of course, the largest organ of the body. It's the most exposed surface. It is a physical barrier and of course it harbors very complex microbiota.

So, the first experiment that Shruti did was actually to look at what happened in the skin of an animal that is actually devoid of the microbiota. And as you can see here in the mice that is actually containing the microbiota you have actually a high frequency of gamma delta T cells and classical T cells able to produce IL-17, that is the name,

potent cytokine for the control of infection but can also have inflammatory potential.

If you look at the germ free mice, the mice that is devoid of this microbe you can see that IL-17 production is severely impaired in these mice and there is a reduction of the capacity of the classical T cells and gamma delta T cells to produce IL-17. It is actually quantified here where you can see there is a significant reduction of the frequency of T cells able to produce inflammatory cytokine at steady states when the mice have no skin commensal. This is also true for cells to be able to produce (inaudible) gamma.

What was very important in this study is actually the fact that this control was independent of the gut microbiota. Then clearly we actually revealed that actually in these settings the skin microbiota was able to control local immunity independently of the function of the gut microbiota. So, they actually reveal then tissues are likely to be controlled independently of each other by the

residing microbiota.

So, importantly Shruti decided to explore the possibility then this manipulation of the skin immunity may have functional consequences for the control of pathogens. And in this case, we utilize a skin commensal, a skin pathogen that is actually leishmania major, that is actually a protozoan parasite that is exclusively infecting the skin and remain very localized. This infection induces strong Th1 responses. It is self-sustained, self-controlled.

There will be the development of an acute lesion that is happily controlled after a few weeks. So, the first observation that Shruti made is the fact that you infect an animal that is devoid of the skin microbiota there is no longer inflammation. It is actually what you have in the mice that contain the skin microbiota they develop an ulcer, there is a necrotic lesion. But if you don't have any commensals, the mice are not capable to develop strong inflammatory responses and do not develop pathology. And

this is actually summarized here by the lesion size.

So, as in many other settings in this case the pathology is really due to an aberrant reactivity to the commensal. But this response of the commensal has also benefit because in absence of the commensal, what you have is an absence of productive immunity against the parasite. This is actually what you see here in the mice that has a skin commensal it can develop a different gamma response that control the infection. If you don't have the skin commensals, there is actually no development of productive immunity against the parasite.

So, the next step was of course to see this was linked to the skin commensal and for that we utilized a skin commensal of human at that beginning that is staph epidermidis that is, of course, present in the skin of most of us, this is the actual commensal. And you can see that she actually colonized a mice that has no commensals just with one species of skin commensal. This is sufficient to

restore, of course, a pathology but also the protective immunity against the parasite. It's actually seen tens of parasite number. The mice that don't have commensals have actually an enhanced level of parasite. If you restore the mice with just one skin commensal we can restore the capacity of this animal to develop protective immunity. And importantly similar results were obtained if utilized as skin commensal of rodents which is not something that is due to an aberrant host microbe interaction.

So, the mechanism by which these commensals are actually capable to promote immunity is, in fact, via the capacity to promote IL-1 production in the skin. So, this actually was shown using the following approach is if you take a germ free mice. Again, they have a little amount of IL-17 in the skin that, of course, is important for the control of infection. If you add staph ep you restore this IL-17. If you block IL-1 by using an antagonist an (inaudible), you can actually block the capacity of the commensals

to boost this IL-17 response that our study states is a very healthy response of the tissue.

It is also true in the context of inflammatory responses due to leishmania major. If you actually infect the germ free mice it won't develop a good immunity against the parasite, you add the skin commensal, you boost Th1 responses. If you block IL-1, you block the capacity of this mice to develop appropriate Th1 response against the parasite. So, this actually suggests that in the skin IL-1 pathway and amplification of this pathway is extremely important to promote the capacity of those skin commensal to boost local immunity.

So, relevant to the topic of this discussion we can also do that in an animal that already has a microbiota. In fact, we can manipulate the skin microbiota of an intact mice. And this is done using the following approach. If you had to actually take a mice that is raised in a facility that actually has indigenous microbiota and

topically apply staphylococcus epidermidis, there is actually no lesion, no injections. This is basically a transplant. You can actually follow what happened in the skin.

So, at steady state condition what you have is a certain fraction of cells able to produce IL-17. If you actually add back staphylococcus epidermidis at the surface of the skin of the mice without injecting it, it is actually sufficient to dramatically boost its capacity of T cells to produce IL-17 in the tissue. And you can see here that you have very high frequency of CT-4 T cells able to produce IL-

In response to this topical application. So, in the skin of a mice that has actually a microbiota, we can actually boost immunity by adding back topical commensal. Importantly, this also leads to reduction of population with regulatory property that will actually regulate immune responses.

So, this, of course, has actually relevance to psoriasis in which actually the

blockade of IL-17 has been proved to be effective to limit the inflammatory responses during those flares. Suggesting that actually maybe increase of the commensal population at a certain state can actually promote inflammatory responses that could in some case lead to inflammation. But we could also speculate and actually increasing those commensals may also have beneficial effect in certain circumstances by boosting the capacity of the host to control other infection.

And I think what is quite important is the fact then if you come back six months later on those mice it actually is still possible to grow the staphylococcus epidermidis from the skin and the IL-17 level is still sustained in complete absence of inflammation. So, this actually raised the possibility and actually we could potentially manipulate the skin microbiota and potentially transfer new skin microbiota on people that may have actually a tendency to harbor pathogenic microbes.

So, the mechanism, the way the skin

commensals work, actual what we are proposing is the fact and they actually manipulating the level of IL-1 in the skin. And they do that probably to the interaction of the hair follicle. They also block the capacity of keratinocyte to produce IL-1 receptor antagonist (inaudible) the results. This IL-1 can actually promote immunity by acting on innate cells. We haven't shown that but we actually are proposing that. But what we actually have found is the fact that this IL-1 is actually capable to act directly on the lymphocytes. And it does not do that by actually modifying the capacity of the cells to be primed or to migrate. But what this IL-1 produced in response to commensal colonization is to directly stimulate the capacity of T cells that reside in the skin to produce IL-17 and interferon-gamma. Both cytokine are going to be quite important for the control of infectious disease.

So, for the conclusion of this part the skin microbiota is actually quite important to controlling immunity. It does

that by controlling the level of activation of T cells but also the capacity to produce cytokine. These skin commensals are very important to promote local immunity to pathogen. We have shown that of course with leishmania major but we are now in the process of looking in the context of staphylococcus aureus which is, of course, a pathogen of very high relevance to human. The capacity of the skin commensals to promote immunity or cures via mechanism that actually distinct and specific from the -- distinct from the good microbiota are really suggesting that each tissue are going to be capable to be controlled by the microbiota in a unique manner and maybe learning how to do that in human maybe an important asset to manipulating the healthy states in different tissues.

So, what I would like to discuss with you for the next few minutes of this talk is actually the other way around. What is actually the consequences of infection for a relationship with the microbiota and what could be actually the long-term consequences

of infection or an establishment of a healthy relationship with these microbes.

So, in the GI tract and it's something that all of you are quite familiar with, we of course separate it from this microbe by various layers of structural and immunological barrier. And this constitutes together what has been defined as mucosal firewall that is of course the layer of epithelial cells, mucous responses, IGA, antimicrobial peptides. All these responses really synergize to really isolate us from these extremely abundant amount of microbes that of course contain a large amount of antigen. And really the idea is actually the want to develop this immunity against those antigens because of course a barren tract activity of the commensals would actually have potential consequences, negative consequences.

In addition of this very structural responses, what you have in the GI tract is various layers of regulation. And in particular you have a population of cells that is expressed regulatory T cells feature Foxp3

that is very important to limit human responses against the commensal. And actually beautiful work that was done by various authors including Workin Washu has revealed an actually certain microbes in the GI tract can promote the induction and the function of regulatory T cells population that in term limit immunity against commensal antigen.

And if you actually take some regulatory T cells that reside in the gut and if you actually look at the specificity of these T regs certain fraction of these cells actually specific for commensal antigens. So, this is a very important feature of the regulatory pathway of the GI tract.

But of course everything will be just fine if we were never infected. The GI tract is probably the most open access for pathogen and is an environment that is constantly rechallenged to exposure to various acute pathogens which means then this very nice relationship we have in the context of whole aesthetic study states is really often challenged by encounter with inflammatory

settings.

So, what is not really clearly understood is what happened in the context of this infection for this healthy relationship with the microbiota. So, a few years ago we decided to address the following question: can acute infection induce immune response against pathogen and what could be the consequences of this immunity for the immune system?

So, the model of infection decided to utilize for that is the model of *Toxoplasma gondii* infection, of course in humans is a very different pathogen. But in mice induce an acute GI infection that is actually rapidly controlled and lead to a chronic stage of infection. The acute stage of infection is characterized by massive tissue damage in the GI tract and a strong induction of a Th1 response that not only killed the parasite but also create tissue damage as often in those infection the pathology is due to aberrant response to the pathogen.

If you look at what happened in the GI tract of the mice that is acutely infected

you can see a massive infiltrate of CD-4 T cells. You can see *T. gondii* of course that is present in red but the regulatory T cells population that's usually is 10 percent of the CD-4 T cells present have vanished. And this actually has happened and been described now in other infection, actually acute infection disrupts the (inaudible) of regulatory T cells and these cells are no longer present in the GI tract.

An additional bystander effect of acute infection is an effect then we change our microbiota dramatically in response to inflammation. And this can be seen here in which in the naïve mice in the small intestine, these mucous layers and barrier defense really prevent the tight contact within the microbiota and epithelial cells. If you actually have an infection, what you can see in that the microbiota here seen by 16S fish is now in tight contact with epithelial cells and there is a massive increase of the density of this microbe. And importantly this microbe not how shifted

completely in a small number of E. coli that when barely detectable prior to infection become the dominant population in these mice.

If you actually do a cross-staining to look at the E. Coli population you can actually find this microbe now present inside the villi, there is a translocation and you can grow this microbe from the periphery. As you can see they're not detectable in the non-infected mice. But you can grow this E. coli population from the mediastinal lymph node, liver and the spleen.

So, clearly an acute infection can have bystander effect by changing the kind of microbe we have, by impairing regulatory pathway and by inducing translocation of those commensal microbes. So, everything was in place to look at the potentiality then in these settings you can develop an adaptive response against these commensal. And these questions actually asked in laboratory by Tim Hand, Post-Doctoral Fellow.

So, the model he utilized to test this possibility was actually very great model

that was developed by Chuck Elson in the University of Alabama that the transgenic mice in which all the T cells are actually specifically for one commensal antigen that is a CBir antigen. And this CBir antigen is actually expressed by some commensal that are present in human but also in mice. And importantly for the clinicians in this room you can organize this antigen as one that is actually highly organized in the context of Crohn's disease.

So, the first experiment indeed to look at this point was to actually take the CBir transgenic cells and translating them to a mice that is naïve. And as you can see, as you do that in a naïve mice the cells don't proliferate. This is a proliferation marker and they don't become Th1 cells capable to produce interferon-gamma. If you do that in the context of an infection you can see that now those cells that are specific for commensals become highly activated. They become Th1 cells able to produce gamma but also proliferate massively.

So, clearly in the context of an acute infection the immune system is no longer able to discriminate the commensal and the pathogen and is able to develop a full-blown adaptive response against commensal antigen. This is actually summarized here where you can see then there is a high frequency of cells that are able to recognize CBir antigen when you really stimulate them with antigen. And the CBir transgenic cells are able to just produce gamma to a level that is actually comparable to the response against the pathogen per cell base. So, clearly a Th1 response can be induced against commensals in the context of an acute infection.

To the first point I would like to make is the fact that when you have an infection at a various site and this could be the GI tract, the skin, the lung, the immune system is not going to be able to discriminate because a high level of inflammation, because translocation of the microbe and actually because of impairment of regulatory pathway. Which means that we could actually propose

then most of the response of course is against the pathogen which is going to be here in the important to control the infection. But you can have actually some bystander effect.

And we have shown a few years ago that if you can an oral antigen during an acute infection you can actually now induce a strong Th1 response against this oral antigen, this food-derived oral antigen. But at the same time, acute infection can also disrupt the relationship with the microbiota by inducing a full-blown Th1 response against commensal antigen.

So, the next point I wanted to make is we could speculate then all of that is very transient. Maybe you develop all these responses but they disappear very quickly because, of course, we don't to just maintain high level of reactivity against commensal. So, Tim actually asked the possibility then these cells could actually develop as memory cells or not. So, when actually Tim looked at what happened to the cell that actually specific for commensal that have been

developed during an acute infection, he find that the cells do expand during the infection which has been shown before and for CD4 T cell memory. And then they contract and then they slowly decay and it has been classically shown for CD4 T cell responses but they are still present three months post-infection. In low number but still detectable.

And what is actually important is the fact then if you try to recall these and do a classical recall these cells to look at memory potential and capacity re-expand upon antigen exposure, he was able to find that actually if you challenge these mice with a CBir peptide you can now recall the capacity of these cells to proliferate and to expand in the lamina propria of the small intestine but also systemically. So, these cells are really full-blown memory cells with the capacity to be recalled upon secondary infection.

So, the point I would to make here is that during GI infection you're clearly going to have a disruption of the barrier integrity and you're going to have destruction

of a large fraction of the regulatory pathways which has regulatory T cells. A fraction of the response that occur in these settings is actually likely to be directed against the microbiota. And the microbiota responsive cells have actually the phenotype and the capacity to be recalled exactly like memory CD4 T cells that are generated against the pathogen.

So, what is the model we're working with? Working with the potentiality of primary infection inducing immunity against pathogen but also having bystander effect and potentially inducing immunity against commensal. And this actually could mean that when you have a subsequent infection et cetera, et cetera you can actually keep building this pool of cells that are reactive to commensal. And this actually means that you never really see a pathogen in the GI tract without having this constant recall response against the microbiota.

And in fact, you are never naïve in your capacity to see a pathogen because it's

always going to be parallel with immunity to commensal. So, what could be the consequences? Of course it's a model. The first consequence could be maybe positive. Maybe having an immunity to commensals help the immune system by creating an environment that is actually more capable to control infection by boosting innate responses.

But the other potentiality of course is the one you are interested by is the fact that if you have actually genetic disruption of barrier integrity, of failure to develop regulatory pathway in human, this could build up and could be a trigger of inflammatory disorder such as IBD. And you could actually speculate and maybe (inaudible) could actually obey to a similar pattern of a development. We could also maybe speculate that increasing the pool of activated cells in the periphery and because of the large number of cross reactivity between the microbiota and cells; these now have influence on the development of disorders.

Of course, this is a model we are

working on trying to test hypotheses but I hope then this could be point of discussion and for this audience. So, just in case I have gone too fast, I just wanted to remind you the two points I wanted to highlight which is the fact then the microbiota is extremely important to promote immunity to pathogens. And this is not only true at the level of the GI tract but also the skin. And I think what remains to be explored is really how other tissues are controlled by the microbiota and how we can actually harness the mechanistic understanding of this relationship to boost immunity against a potentially pathogen microbes and especially the one that emerge in the context of antibiotic treatments.

Importantly, pathogens themselves can actually create bystander response of the microbiota. And this also could have a pathogenic consequences. In the context of fecal transplant maybe some that may be important to try to take in account because patient that have inflammatory disorders may actually may develop immunity to new commensal

antigen. May be interesting to see how these actually developed in the context of these transplantation.

So, just the final point is again we are a mosaic of a different community of microbes and I think much remain to be learned experimentally to try to understand how this microbe communicate with the immune system. But I think really trying to just understand how the fine control of these microbe interaction at certain state in disease with each tissue is going to be an important area of research for us in the experimental side. And we hope to be very much informed by you that are actually doing the treatment.

So, this work was actually done by my group at the NIH. I'm actually an internal investigator. Essentially I have shown some of the work of Shruti Naik that is a graduate student and Tim Hand, a Post-Doctoral Fellow. We have actually really great collaboration in particular with a group of Mark Jenkins. Fantastic collaborations Julie Segre at the NIH and with clinicians that are actually in

the campus and collaborations with (inaudible) and of course we'd like to thank the funding agencies and all of you for your patience. Thank you very much.

MR. RAMESH: (inaudible) Ramesh for Infectious Disease. Does microbiota transplantation can it cause T cell exhaustion?

DR. BELKAID: Yeah, I mean and I think it's a very important question in the context of what's going to be done than actually what is -- which is transfer a new microbiota and transfer of potentially a new array of antigen, millions of antigens. What's going to happen actually to the peripheral repertoire? Do we develop new response with these commensals and I think it's an open question. At this stage I don't think anyone has an answer. But I think it will be interesting to see what happened to the immune response of those hosts and the specificity of this response in the periphery upon transfer, yes.

MR. BRANDT: Larry Brandt, New York.

I'm a gastroenterologist. You showed very nicely some work that you did on infection with parasites and how that changed the immune response to commensals. Do you have any data on infection with bacteria or things that we as gastroenterologists see more commonly in our daily work?

DR. BELKAID: Yes. You mean the real pathogen for you. So, those actually are real pathogens but they're not the one you clearly have an interest for. So, we have actually now done studies on Yersinia. Tuberculosis and looking at actually the effect on the immunity of commensals and have exactly the same kind of finding. And actually the Yersinia induce a beautiful immunity to commensals. I should not say beautiful. That's a terrible problem with basic researcher.

The other thing is we have actually now started to develop studies looking at the role of the skin microbiota in controlling staph aureus. And actually we have preliminary data that suggests that very much

like we have seen leishmania major using staph ep is a very important, interesting strategy to prevent colonization but also to develop appropriate immunity against this pathogen.

So, yes, we exactly going in this direction. I mean we have used those parasites as a principle but I think it's important now to move to different kind of pathogen that are of high interest to clinicians.

DR. MCDONALD: Cliff McDonald, CDC. It's interesting that the hygiene hypothesis is one that has been around for some time suggesting that as we reduce early exposure to pathogens we're seeing more allergic phenomenon. And there's some evidence to support that from more microbiome type of work I think is increasing. But this sort of goes to the opposite direction at least if you believe the stimulation of response to commensals could lead to allergies.

I think here in America many of these early exposures to parasites have been markedly declined in the West and yet allergic

type phenomenon are increasing. So, does that make you think that maybe this is more of a beneficial effect actually?

DR. BELKAID: So, I think it's not the infection per se it's the kind of infection you have been exposed with. I think the major driver of human immune response has been worms infection which have been very much depleted from all countries but in a very recent manner. So, I think parasites, the kind of parasite I'm working with are acute parasites. They are that are going to create inflammation and tissue damage.

The reality is we have eliminated the worms from this picture which I think have been of the most important controller really of the tissue metastasis. So, I think I agree with you then this could go against what you were saying but we actually postulate and it's absolutely true maybe those worms were in place to prevent those kind of responses. And maybe aberrant reactivity to commensals has developed as a bystander response to remove this major regulatory response in this tissue.

The other thing I wanted to actually tell you which I haven't discussed with but if you have certain acute infection what is quite remarkable is you eliminate certain bacterial strains that never come back. Like, for example, one infection with *T. gondii* and you never have *Lactobacillus* again unless you actually give it back.

So, certain infections are going to really completely deprive and make the microbiota more poor over time. And this combine with antibiotic would become really a problem.

MS. MANSFIELD: I had a question. Linda Mansfield, Michigan State. So, if these T reg cells are reexpanded as memory cells, do they ever then home away from the gut and are found in lymph nodes and spleen?

DR. BELKAID: So, in this particular infection, no. So, in this particular infection the regulatory T cells are gone from everywhere. They basically die. And then eventually will come back when the mice actually just have less inflammatory

responses.

But what is really, really surprising and we're testing now different kind of pathogen especially Yersinia for example, is the fact that strangely enough they induce elimination of regulatory T cells in very defined tissue. For example, Yersinia shut down regulatory T cells in the liver and the bone marrow for a very long period of time. And this could have really massive consequences for the physiology of the host.

So, some of these acute infections sometimes are not very pathogenic per se but they can dramatically destroy certain compartment for a long period of time. And the liver, for example, is an important site of tolerance induction. So, I think this elimination of regulatory population defense in different tissue in response to acute infection or all infection could have long-term consequences in the way that we have not predicted.

MS. MANSFIELD: Thanks.

MR. KHORUTS: Alex Khoruts from

University of Minnesota. I'm curious as to your definition of immunity toward commensals. You've showed expansion of CBir specific cells. Those T cells of course could have a variety of phenotypes from T regs to all time producers, et cetera. What is the phenotype of T cells? What is the fate of the CBir antigen quantitatively, does it actually go down with this immunity? And if you take these, sorry to load you with questions, if you take these T cells that are antigen specific that are memory-like and put them into new mice do they stimulate some sort of inflammatory response in naïve animals that have that antigen and have not seen the parasite?

DR. BELKAID: So, your point is actually very important. So, when you have an infection you can develop different kind of immunity. In the context of a strongly Th1 responses, all the CBir cells become Th1 which is very surprising because under normal circumstances because the antigen in the gut should induce T regs of TH-17, if you have an

acute Th1 responses they all become Th1.

They processed for the long term as Th1 like cells. They can express T bet. They can reexpress gamma. But your point is actually can we transfer disease which is really the important point? Can actually accumulation of these cells be causative?

We don't know that yet. And the reason we don't know that yet is CBir is not a very dominant antigen. So, what we're actually developing right now is actually tools to look at E. coli specific responses because in the context of an acute responses what you have is 90 percent of your gut is now E. coli because you have all this bystander inflammatory responses and E. coli become really the dominant stage. It's the one percent before infection and really the majority after infection.

So, we're now developing a tool to track these responses and we hope to be able to just address exactly the question you asked. Is it causative? Is it going to create disease by itself? We don't know that

yet.

MR. KHORUTS: So, the response you are giving seems more in favor of microbiota transplantation. And have you actually after giving leishmania major to the mice, have you actually tried skin microbiota transplantation on that mice and see what happened?

DR. BELKAID: Yes, we actually promote immunity against it. So, if you basically increase the amount of staph epidermidis on the mice that is already infected, you can make the mice control infection faster after they have developed the infection. Yeah, I mean I think the point I wanted to make is you know, all of you are working on the possibility of having fecal transplant but there may be actually transplantation of microbiota in different sites, in the lung or in the skin. And I think that could be an important area to prevent staph areas infection others. So, that was the point.

MR. SCHENTAG: Jerry Schentag, University of Buffalo. Your thesis that we

have a regulatory system on a massive population perhaps that errs on the expense of not killing some minor component until it's too late is interesting. I'm just wondering what would happen if you changed the massive population a lot by an antibiotic for instance, then does it then autoregulate its immune system response to the minor selected components of that population effectively? Like, for instance, the *C. difficile* we're talking about today.

DR. BELKAID: So, yes your question which I understand well is basically if we eliminate the microbes via antibiotics do we also eliminate antigen-specific regulatory cells. I don't think anyone knows that. I don't think anyone knows what's going to happen to the repertoire of regulatory T cells when you have massively changed your microbiota. And maybe the repertoire of these cells is extremely variable and actually can adapt very quickly to new population of regulatory T cells. And maybe that could have an issue if you just don't these cells that

are specific from certain commensals. So, we don't know that.

MS. MILLS: We have time for one more question.

MS. HAYS: This is a little bit off target but our hospital it just getting, Ann Hays from UVA, we're starting a bathing regimen for patients hospitalized with chlorhexidine gluconate and to decrease the incidents of catheter infections and central line infections and carbapenem-resistance.

What do you think the result of that is going to be on the commensals, the response to infection, any thoughts?

DR. BELKAID: So, are you sequencing also at the same time the microbiota before and after?

MS. HAYS: No, this is going to be across the board for patients.

DR. BELKAID: Because I think that would be fascinating to actually what has happened to you and the antigen's microbiota upon these treatments. I know that at NIH they have this massive, they treat actually

very, very severely sick patient with this bathing strategy where they basically wrap them in steroids and actually maintain them with a bath. So, now they are actually in the process of looking what happened to the microbiota. Is some of the positive effect of this treatment also due to a massive change and reset of the microbiota of these patients? I think it's an open question. It would be fascinating to look at that.

MS. MILLS: All right, everyone. We're ready for a break. It's 10:37. If you look at the clock up there and use your time to that we'll be back here and starting up at 11:00. Thank you.

(Recess)

MS. MILLS: Okay. Our next speaker is Dr. Eric Pamer from the Sloan-Kettering Institute and he'll speak to monitoring and manipulating the intestinal microbiota to prevent systemic infections.

DR. PAMER: Okay, great, thank you. My talk should be coming up in a sec. So, this is without my hands. Great. So, I'm

going to talk about some work that we've done at Sloan-Kettering where I'm the Chief of the Infectious Disease Service and we are a cancer hospital where we take care of patients with no immunocompromise to probably some of the most immunocompromised people you could ever encounter. And I'm going to tell you a bit about the microbiota in some of our most immunocompromised patients. And also some mouse studies that we have done.

I'll start with presenting a case that is a very, very typical run of the mill case for us. It might not be at most hospitals but it's a pathogen that we're encountering I think increasingly across the country. And in this patient was a patient with acute myelogenous leukemia who was newly diagnosed and started on intense chemotherapy which resulted in a loss of neutrophils and neutropenia. Patient developed fever and with very broad spectrum antibiotics, a quite typical cocktail that can be anticipated to affect many members of the microbiota, the patient then became afebrile which is good.

But remained neutropenic and then returned with a new fever.

And at that point we often assume that there may be a fungal cause for the fever and so an antifungal agent was initiated. And the fever continued and the patient grew out enterococcus faecium from the blood. And the blood remained positive for this organism for nine consecutive days. We treated the patient with two antibiotics that we have left to treat this highly antibiotic resistant organism and the patient developed some other issues as well but actually resolved the bacteremia when the neutrophils came back.

So, Vancomycin-resistant to enterococcus is a gram positive coccus. It forms chains. It is in our leukemia and in our bone marrow transplant population the most common cause of bacteremia now. So, every time I go on service for my two week stint I will encounter one or two people with bacteremia and then many others which VRE infections in other sites.

It is an organism that is of

particularly high risk for people who have been previously treated with broad spectrum antibiotics. In fact, we almost never see it in patients who haven't been treated with broad spectrum antibiotics previously for some other reason.

So, a few years back Carlos Ubeda, who was working work with me, got interested in looking at how antibiotics affect the microbiota. Now, there are many studies of this that are out there. We decided to look in mice and we treated mice with ampicillin and then performed 454 16S sequence analysis of these mice pretreatment, post treatment and then after stopping antibiotic treatment. And what this shows is that if you look in the ilium or if you look in the cecum, first of all, an important thing to remember when we're talking about the fecal flora is that we're really not getting a good idea of what's going on in the ilium of our patients.

Here you can see in mice that the flora that is inhabiting the ilium which is just the ileocecal valve away from the cecum

has a very different microbiota. Most of you know that but it's kind of an unknown in our patient populations what's going on in the ileum.

Antibiotics dramatically change the composition of the flora, ampicillin in this case. And then when we stop ampicillin the residual flora that comes back is different than the flora that's present under antibiotic pressure but it doesn't come back to the state that existed prior to antibiotics. In particular, we've lost the Bacteroidetes here and in fact, an interesting loss of lactobacillus from the flora.

Now, we were interested in knowing how ampicillin treatment affected colonization with Vancomycin-resistant enterococcus. If you take mice that have never been treated with antibiotics and give them a ten to the eighth Vancomycin-resistant enterococcus orally, over the course of 15 days the enterococcus disappears from the gut flora and becomes undetectable. And this is colonization resistance mediated by the normal

flora.

If you treat with ampicillin the story is completely different. There is now a very high density of VRE in the gut up to ten to the tenth organisms per gram in the cecum and about ten to the eighth in the small intestine. Now, if you stopped the antibiotics and asked whether the residual flora that's there can push the VRE out, it does a little bit but it's really, you know, once VRE takes hold in the gut it becomes very hard to eliminate. And it is still present at very high inocula or very high densities in the colon.

And of course this is, in a hospital environment, one of the major problems we deal with. The persistent shedding of these highly antibiotic resistant organisms even after patients recover from whatever underlying disease they had, they continue to shed it at very high levels.

We wondered, yes, the densities is great but what proportion of the microbiota is composed of VRE in these animals. And so, we

sequenced these mice that were treated with ampicillin. Again we show a marked change in the microbiota after ampicillin treatment. Now, if you give ampicillin and VRE at the same time, the gut essentially fills up with VRE. 99, 98 percent of the bacteria in the gut are VRE. So, it has a remarkable ability to just take over if you destroy the microbiota. And this is what things look like weeks after stopping the ampicillin. It still is 50 percent of all the bacteria that are present in the cecum.

So, we have very close colleagues in bone marrow transplant, in the bone marrow transplant program, Marcel van den Brink and Rob Jeng and they were interested along with us in what was going on in our patient population that were so susceptible to VRE. And so, we decided to look at our allo-HSCT patient populations and just to bring everybody up to speed on what's involved in getting an allogeneic bone marrow transplant, patients often receive total body irradiation, intense cytotoxic chemotherapy. We give

prophylactic antibiotics because we know that patients are highly susceptible to certain infections.

Patients develop mucositis so there is a loss of epithelial integrity. They develop prolonged and profound neutropenia and monocytopenia. They develop fevers in which case we add on top of the prophylactic antibiotics very broad spectrum antibiotics. Then to top it all off, when the bone marrow and grafts, they can often develop graft versus host disease which often involves the intestinal mucosa.

So, in the roughly 560 million year relationship that microbes have had with guts, we think that this is probably one of the biggest insults to that relationship. It has, as might be anticipated, a profound effect on the microbes that are living with the lumen of the gut.

So, we started to look at sort of an explorative level at the composition of the microbiota of patients during the course of transplant. So, here on a timeline we're

looking from pre-transplant, the minus numbers, to days post stem cell infusion. And we collected fecal samples. We performed sequence analysis. Each bar represents around 3-5,000 16S sequences. And the different colors are different bacterial taxa.

And one of the things you'll note, A, B, C, D and E are five different patients. They all came in with a diverse flora prior to transplant and in three of the patients post-transplant their microbiota underwent changes that perhaps are unique to bone marrow transplant patients but they are, I think, some of the most dramatic shifts in the composition of the microbiota that have been described. You see a loss of diversity in these patients. Once diversity is lost we see very rapid fluctuations in the composition with domination by different species. This beige here or whatever you call this color is *step viridans*. In this case this is followed by coag negative staph or staph epidermidis.

These two patients developed intestinal domination by Vancomycin-resistant

enterococcus and in both cases it preceded the development of VRE bacteremia. In this case by about 20 days and in this case by a few days. Prior to the red down here describes the duration of VRE bacteremia. This patient actually we couldn't clear because the VRE actually developed antibiotic resistance to both daptomycin and linezolid and the patient succumbed to this infection.

So, with this data we moved on to do a larger longitudinal study, 94 consecutive allo transplant patients. We collected lots of fecal samples and sequenced them. This is just an overview. Each box represents an individual patient and the samples that we obtained, the blue lines here, you can't read them represent the antibiotics that were administered during the course of their transplant. We're looking from day minus 15 to day plus 35. So, it's a restricted look at what's going on in the microbiota. One thing that you may detect is that there's sort of a greenness to the whole thing. That reflects VRE.

So, we have VRE in many of our patients and I'll show you that over half of our patients end up getting dominated by Vancomycin-resistant enterococcus during the course of their transplant. This is work that we recently published in Clinical Infectious disease where we characterized all the samples that we looked at and to help us with our analyses we decided to establish states of domination. This is patterned after Jacques Ravel's approach also to establishing states of the microbiota, in his case the vaginal microbiota.

And here we can see that things cluster. These are all the samples where VRE became a predominant member. Streptococcus, we have lots of samples that were biodiverse. Down here we're showing, if it's light it means it's post-transplant. If it's dark it means it pre-transplant and you see that most of our biodiverse samples were from periods of time that preceded the transplant. And then we lose and we establish all these different states of domination.

Another way of looking at this is with the Circos plots. This work was done by Joao Xavier and our computational biology program where one can look on these plots. This color here represents biodiverse state. This is the enterococcus dominated state, streptococcus and then the others. And the lines that connect from here to there, for example, represent state transitions.

And so, we can use this approach to look at state transitions pre-transplant, from pre-transplant to post-transplant and then exclusively post-transplant. And what this shows is that state transitions rarely occur pre-transplant. So, most of the consecutive samples remain in the biodiverse state. From pre-transplant to post-transplant most of the transitions that occur go to states of domination. And then post-transplant there are lots of consecutive samples remain in the same state of domination that they were in.

This is just showing over time how many, what percentage of patients developed domination by VRE. It's over 50 percent by

day 35. Streptococcal domination comes in second and interestingly proteobacteria domination is only around 20 percent. We think that that reflects the fact that we are giving quinolone prophylaxis to many of our patients to prevent gram negative bacteremias.

What leads to domination? We looked at pre-transplant disease, transplant type, conditioning regimen. None of those things predicted a development of domination by any bacterial species. What did predict it was the antibiotic that was administered prior to the state of domination. And the one that jumped out at us and we didn't expect this was metronidazole which increased the rate of VRE domination by a factor of over three. And it was the only significant association we could find with VRE domination.

Interestingly Vancomycin did not but that's because Vancomycin is given systemically and it actually doesn't achieve levels in the gut that would drive expansion of VRE. Other interesting finding here was that fluoroquinolone administration reduced

the incidents of proteobacteria domination by a factor of roughly 10. And that's good. And that was what we had hoped we might see because proteobacteria are often some of the biggest problems in our transplant population.

We also looked at how domination affects the risk of bacteremia. Not surprisingly enterococcus domination almost tenfold increased risk of VRE bacteremia. And if you did slip through the cracks and did get proteobacteria domination there was a sixfold increased risk of gram negative bacteremia in this patient population.

So, now we're going to go back to the mouse and look at whether or not we can reverse intestinal colonization by VRE. And this was work done by Carlos Ubeda that was recently published in Infection and Immunity. And what he did was to treat mice with ampicillin for a week, give them VRE, stop the ampicillin after a day. And if you just follow them that's what you see in the blue dots. They remain highly colonized for 15 days with Vancomycin-resistant enterococcus.

He then also had a group that received three inoculations with normal fecal flora from a normal mouse that has never seen antibiotics. And followed those as well and that's the red dots. And what you see is that over the course of 15 days there's a roughly a seven log decrease in colonization in fact to the level of most of these mice having no detectable VRE anymore; a remarkable effect that would be very hard to achieve with any sort of antibiotic treatment.

If we looked at what's occurred to the microbiota, you see by giving a fecal transplant like this, we reestablish a normal flora here that looks very much like the untreated flora whereas these blue dots here still have, harbor a lot of VRE and are lacking many of the organisms that are there.

So, Carlos went on to try to fractionate the flora to see which component of the flora was driving VRE away from the gut. He used aerobic culture and anaerobic culture and was able to show that anaerobically cultured colonic contents were

much more effective at reducing CFUs of VRE than aerobically cultured flora. And if you look at the composition, you can start to see patterns where in this case a mouse that continued to harbor lots of VRE did not get reconstituted. And in this case there's one mouse that still harbored some VRE and its missing some components that are present in the mice that cleared it well.

This is another way of looking at that. This is a heat map where we've now stratified all the mice according to how much VRE they continue to harbor against plots here of all the different bacterial taxa that we could identify and their density is color coded here. And one thing jumped out that there's a particular genus *Barnesiella* that correlated almost perfectly with elimination of VRE from the gut. It pops out in the (inaudible) correlation as a standout, a negative correlation with VRE colonization.

And so, we wondered whether or not we might find a similar correlation in humans. So, we went back to our transplant patients.

These are -- each vertical line represents a patient at the moment. We detected their VRE domination, that's why it's all green. And then we looked at the preceding sample which is shown here. And in these preceding samples you see that there's biodiversity. And what we wondered was whether these biodiverse samples were missing a species like *Barnesiella* in comparison to all the samples we have from patients who never developed VRE domination.

And that appears to be the case if we look at all the never enterococcus dominated patients we see quite commonly a pretty high proportion of VRE, I'm sorry of *Barnesiella* in them, these are all the patients who were dominated by VRE and we rarely see it. And if we see it, it's only at very low levels. So, we think that there's probably a correlation there between the presence of *Barnesiella* and protection from VRE domination.

I want to talk a little bit about some work we've been doing recently on

Clostridium difficile. I'm sure everybody in this room is familiar with this organism. This is an experiment that a student in my lab, Charlie Buffie, did. It was a simple experiment of giving antibiotics for three days, stopping them and then inoculating mice with spores of C. difficile one, six, 10, 14 or 21 days later. And we wanted to see what was the duration of susceptibility after a short course of these antibiotics.

Clindamycin left mice vulnerable for six days. All three mice, each dot represents a mouse. By day 10, 14 and 21 two mice had developed resistance but one still remained susceptible. Ampicillin shorter duration susceptibility to C. diff infection.

Enrofloxacin did not render mice susceptible at any time point to C. diff infection. I find this is the most interesting. This is a combination of metronidazole, neomycin and vancomycin. And the interesting thing, there are a couple of interesting things. One is that these mice appeared not to be susceptible to C. diff infection on days one and six. We

think that's due to the coprophagia that mice practice and the fact that they were reintroducing vancomycin into their gut for the first six days.

But then if we waited 10 days, 14 days and 21 they were highly susceptible. So, metronidazole, vancomycin and this just showing vancomycin alone also for six to 10 days high level susceptibility are although treatments for *C. Difficile* are devastating the microbiota in ways that render the gut highly susceptible either to re-exposure, upon re-exposure or to reemergence of the infection. Probably accounting for the very high rate of recurrence after treatment.

Here is again another heat map approach that we used looking in all of these mice trying to identify organisms that were associated with susceptibility or resistance. And using a Spearman rank correlation, that's what Charlie was able to find was that there were eight bacterial operational taxonomic units that fell into these families that correlated highly and statistically

significantly with resistance to C. diff. You'll see that three of them belong to the lachnospiraceae which I'm sure you'll be hearing more about from Vince Young in the talk following this.

So, we have quite a high incidence of Clostridium difficile infection particularly in our bone marrow transplant patients. And we've been working with one of our colleagues, Juliet Barker who is the leader of the umbilical cord blood transplant program. And we've followed 172 cord blood transplant patients and they develop Clostridium difficile infection in two different, at two different time points.

There's a C. difficile infection that occurs before engraftment. A lot of times that is diagnosed right in the periconditioning period where there's already a lot of diarrhea to begin with and it gets diagnosed a lot because we find it by PCR. But then there's also a C. diff infection that occurs after engraftment. That's when the neutrophils return. And those seem to be more

typical of the C. difficile infections that we see in other patient populations as well with high incidence rates of recurrent infection.

So, we have followed these patients and what we have particularly been interested in is identifying predictors at the time of engraftment for the development of C. difficile infection. And so, we've performed deep sequence analysis on their fecal samples at the time of engraftment and have identified bacteria belonging to the Bacteroidetes phylum as being predictive of resistance to the development of C. difficile infection.

And here's some data on some of the samples that we looked at. We see that patients come in and in the initial sample that we get, 32 out of 38 patients will generally be Bacteroidetes positive on sequence analysis. But if you look at the risk of C. diff infection in either of these groups, it's quite high but equal between the groups.

If we look post engraftment and look at C. Difficile infection afterwards, we see

that there's about a 50/50 split. So, half of the patients now completely lack Bacteroidetes in their fecal samples. And they have an over 50 percent incidence of C. difficile infection. And so, this is a patient population that we're going to target for the reintroduction of their own pre-transplant of flora as we move forward here.

So, here are just some concluding points. Complex microbial networks provide colonization resistance. We think there are many direct mechanisms but like Yasmine Belkaid indicated there are also going to be indirect mechanisms where the microbiota is stimulating the immune system. And the bacterial populations acting for resistance can be identified. We think many of them may belong to the Porphyromonadaceae family and reconstitution I think is, of microbial populations is going to play a big role potentially in dealing with our antibiotic response problem. And I don't think it's going to be limited to C. difficile infection but it's going to be relevant to many of the

other organisms that we're encountering.

And these are my collaborators. I mentioned them all, transplinters, ID, computational biology, MDPHVs in the lab and funding, NIH supported a good deal of this work but also a lot of support from the Tow Foundation which has supported the Castori Center at Sloan-Kettering. Thank you.

SPEAKER: What about multidrug-resistant gram negative pathogens? Can we use similar strategy to or are you working on it?

DR. PAMER: Yeah, we're working on that. So, we're looking at the carbapenemase producing enterobacteriaceae. And there's no question that they are capable of dominating the intestine in much the same way that VRE can. And the mechanisms by which they are suppressed by the commensal flora, I think, is likely going to differ and there probably will be different bacterial taxa that drive resistance to that then to VRE.

DR. MCDONALD: You mentioned, Cliff McDonald, CDC. You mentioned possibly giving

back pre-transplant microbiota to these patients for the C. diff. You're targeting those that are depleted in micro --

DR. PAMER: Yeah.

DR. MCDONALD: -- Bacteroidetes. Do you have any thoughts about maybe trying to deplete those microbiota of the proteobacteria or the enterobacteriaceae first? I mean, some of these things, although we say that everything can be a pathogen in the wrong setting. I mean, these people that are profoundly neutropenic, which is probably what you're dealing with, and now the standard I think is often to give them a floracol and to try to keep the -- I mean there's rationale that in those people probably the enterobacteriaceae are organisms they'd be better off without. And it's a small part of the microbiota anyway.

DR. PAMER: Right. First of all, we wouldn't do this until the profound neutropenia is resolved and that's why we're targeting post engraftment. Prior to contemplating any of this I think the mucosa

needs to heal and the neutrophils and the monocytes need to be circulating in the blood again.

At that point, I think it's an interesting thought. Would it make sense to deplete the enterobacteriaceae from the sample? We're not planning that in our protocol at this point. We're reasoning that patients, if it's there at the beginning it's probably still there and so, we feel much more comfortable, obviously, doing it autologously than to go in with a new microbiota. Because I think many of the issues that were raised earlier this morning would be very relevant in this setting.

DR. TARR: Phil Tarr, Washington University. Obviously VREs are fearsome. Do you any epi with VSEs and do you see the same gut ecology with the VSE surge prior to bacteremia?

DR. PAMER: Yeah, that's an interesting question. We see a little bit of it but it's an interesting epidemiologic shift that has occurred over the last 20 years or so

where those of us who trained in the '80s in infectious disease saw lots of enterococcus faecalis. It's switched now to what we're seeing predominantly here as enterococcus faecium which is mostly vancomycin-resistant. So, we do see some faecalis infections but they're a minor part.

MR. ORENSTEIN: Bob Orenstein from Mayo Clinic. I was interested in the work that you had done previously looking at some of the role of the innate immune system in VRE protection. I'm wondering if you have some correlates of the presence of *Barnesiella* and measurement of things like flagellin or reg three gamma.

DR. PAMER: Right. So, that's interesting. So, you're pointing out some work we've done where the commensal microbiota, and this is also from Laura Hooper's lab predominantly, that the commensal flora can drive the expression of antimicrobial factors like reg three gamma in the gut. We suspect that some of these commensals and some of these obligate

anaerobes will be the organisms that are driving the expression.

We're right now growing many of these anaerobic organisms. We have a panel of over 50 now bugs that most people have never heard of but that in some form or other correlate with resistance to either C. diff or VRE that we're working with. And we just started a germ-free colony to be able to colonize them with either individual bugs or with consortia of these. And among the things we'll be looking at is the induction of the innate immune system and protection.

DR. BAKKEN: Johann Bakken, Duluth, Minnesota. Are you aware of any human data on patients that have been permanently dominated with VRE employing FMT to try to raise or to get rid of that dominance?

DR. PAMER: Yeah. No, I'm not aware of it. A big difference of course with VRE and C. diff is that VRE carriage is asymptomatic and it's not an enteropathogen per se. It becomes a problem in our patient population when it achieves a density that

enables it to get into the blood. So, I'm unaware of anybody doing it.

MR. SLATER: Jay Slater, FDA. I'm curious about the diversity that you observed pretreatment, pre-transplant in your patients. I mean clearly going back to our first presentation this morning this isn't a normal population. They've been exposed to chemotherapy before perhaps or broad spectrum antibiotics maybe even sepsis. Do you have any sense as to how that diversity compares to the diversity in a normal population? Any instinct about that? How much recovery did they really experience after they were first --

DR. PAMER: So, our patients when they come in for a transplant many of them come from the leukemia service and have undergone extensive chemotherapy already and prolonged neutropenias and antibiotics treatments. However, remarkably, they're mostly in remission when they come for a transplant. And so, many of them have been away from antibiotics for a while and they

come in with a remarkably diverse flora. Some of them are precolonized for us with VRE which is a problem but they often have a good complement of anaerobes and when we do measures like the Shannon diversity, they come close to the normals that of course, 80 percent of people apparently are not normal, so I'm sure most of us in my lab are -- have Shannons that are in the same range.

MR. BRITTON: Rob Britton, Michigan State University. You brought up a really interesting point about the difference between the ileal communities and colonic communities. And most of the transplants of course you're only going to be using what's coming out in stool. Are you working on getting any ileal bugs to also include in your transplants so you can also correct the upper GI dysbiosis?

DR. PAMER: So, we're not and that's not an easy thing to do. We are looking though in mice at how effectively do you reconstitute the ileum when you give a fecal pellet to a mouse. And it seems to be partial at best. So, there are things that belong to

the erysipelotrichaceae family that we're not seeing reconstituting very well in the ileum after a fecal transplant. So, I think that's just going to be an issue that we'll have to deal with.

MR. BRANDT: Thank you, Eric. That was a very interesting talk. Larry Brandt, New York. You told us how VRE is of great concern and importance in your immunocompromised patients treated with metronidazole. What about the non-immunocompromised patients treated with metronidazole because we use this very often in treatment of C. difficile of course. And I did a study on sepsis in C. Difficile patients and I never saw VRE as an organism in that group of people. It was primarily E. coli and staph and klebsiella.

So, what is the effect of metronidazole in a non-immunocompromised patient and in patients with C. diff on VRE?

DR. PAMER: I think it's a great question. We don't really know. I mean our analyses have been, at this point, restricted

to bone marrow transplant patients. We've recently extended it but don't have any data yet on leukemia patients. We are really focusing on the immunocompromised host and that is also where we see the most VRE bacteremia. Those are the floras where we screen for VRE in order to isolate patients.

We probably have VRE in many of our other patients as well who are also being treated with metronidazole but I think in the absence of profound neutropenia and the damage that goes on to the immune system and the gut epithelium, that VRE domination probably occurs but it just is not translating into bacteremias that we're detecting.

MS. MCCLANAHAN: Sarah McClanahan from Thomas Memorial Hospital. I'm just curious to know are you maintaining these patients in strict protective environments and what are you cleaning the environment with?

DR. PAMER: So, most of the transplant patients during their neutropenia are in contact isolation. They're in individual rooms and the infection control

folks are doing a variety of things. We've recently studied the benefits of copper on bed railings which seems to be remarkably effective. So, we're doing I think pretty standard things. Thanks.

MS. MILLS: We need to move on.

DR. YOUNG: Thanks. I know I'm standing in the way between you and feeding your microbiota so I will try to move things along. And we're going to stay with C. Difficile. And I'm going to talk about what we've been doing in animal models. And this is prefaced by the fact that I'm not going to be talking about the human work that we're doing at the University of Michigan but we're trying to look at these two systems and as humans are often bad models for what we are finding in the mice, we are going to focus on the mouse model for this lecture. But a couple of disclosures quickly. I am on a clinical advisory board for the use of non-tox C. diff, for the prevention of C. diff and also received some money from Pfizer under an ASPIRE grant to study the effects of the

antibiotic tigecycline on the gut microbiota but I will not be talking about these.

So, as an infectious disease Doc, Eric and I, we work under Koch's postulates on how we look at causality for infection and that we have to find a pathogen. And in particular the third postulate, we have to recreate disease in a susceptible host is an essential part. And for *C. Difficile*, as most of you know, this was fulfilled in 1977 or at least published in 1977 using a hamster model. And this is work by John Bartlett and his group.

But in the setting of *C. diff* we're starting to think differently because we know that antibiotics are a key part and we wanted to start understanding how rather than always finding the bad bug that there are good and bad communities. And this is why we are thinking about things like fecal transplantation to restore a good community and replace a bad community. And this is a novel way of thinking of treating infectious diseases.

But to review this in *C. difficile*, what we think happens is most people have at least what we'd like to consider a normal microbiota and the standard insult that we have is antibiotics. And this results in this loss of colonization resistance. We've heard this a couple of times already today. At that end of the talk, I'll try to describe functionally what might mediate colonization resistance. But needless to say, we changed the structure of the microbiota to this functionally susceptible one where is now you're exposed to the infectious form, the *C. Difficile* spores, they'll germinate perhaps under the influence of bias. It has become the vegetative form which produces the toxin that causes the mucosal damage in all that we see as far as clinical *C. difficile*.

Nothing is like throwing good money after bad; we'll give more antibiotics to treat the *C. difficile* infection and hopefully the patients. We do have this cycle of recurrence where when you stop the treatment for *C. Difficile* you can get recurrence in a

growing unfortunate subset of patients up to say certain series 25-40 percent of patients. And that is the case where at least some people are recommending the use of fecal transplantations to try to restore this normal microbiota to break that current cycle.

This is not a new idea, this idea that the microbiota can protect you. Rolf Freter, when he was at the University of Chicago as a Post-Doc, interestingly publishing without his primary advisor, I told my Post-Docs never to do that. Published this paper where he was looking at this concept that the normal human microbiota or flora as you refer to it at times is a factor in the resistance of human to enteric diseases. And he even says that he's not the first to propose. That 40 years before he published that Nissle also proposed this.

So, when we're studying the gut microbiota in C. Difficile infection, it's nice to study it in humans. There's unfortunately a lot of clinical material that we can look at but I'm going to tell you about

the animal models. And using the animal models and also exploiting 16S as Eric had talked about to look at how the community changes and what is the interaction between the pathogen and the indigenous microbial community in the gut.

And as I said Koch's postulates were initially fulfilled for *C. difficile* using the hamster model. And I mentioned that John Bartlett was the first to publish in 1977 and a few months later the group by Bob Feckety at the University of Michigan, have to plug the Wolverines as much as possible, they also recreated this same and actually they were working on it at the same time. And Dr. Bartlett in his retrospective 30 years after publishing this was very gracious to say he knew that the group by Bob Feckety was actually hot on their heels. And so, they were just basically doing the same type of work.

And a lot of people have used this hamster model to look at the pathogenesis, to look at what antibiotics can treat, to look a

little bit about recurrence, to look at what might be protective in terms of innate and adaptive immunity. But Bob Feckety also had Ken Wilson working with him. And Ken, who had also worked with Bob Freter when he eventually moved to the University of Michigan, brought up this idea shortly after Koch's postulates were fulfilled. He said that the normal hamster sequel microbiota could actually prevent the antibiotic cecitis.

So, basically doing fecal or in this case cecal transplantation into hamsters he was able to protect antibiotic treated animals. And so, the data are here. This is the only data in the whole paper. He used a cecal homogenate and if that was administered to mice that were challenged with clindamycin and *C. difficile*, it could protect 24 out of 30 whereas a broth control was unable to detect or one out of 20 was protected. This protective activity in the cecal homogenates was destroyed by heating, destroyed by filtration. Interestingly enough, it was also destroyed by treatment with clindamycin which

is what used to trigger it anyways. And given what we know now, not surprisingly vancomycin preserved that activity in the cecal homogenates.

And so, again, this is probably one of the first ones that I know of of specifically trying to treat this particular infection. There are reports from the '50s of using fecal transplantation for colitis resulting after antibiotics. And this was done in the surgery literature in the 1950s but they hadn't known about *C. difficile* specifically at that time.

But because what we can do with immunology, with knock out animals and just the general availability of lab mice, you know, it'd be nice to be able to look in a murine model for *C. difficile*. Interestingly enough some of the work initially was unable to recreate what was found in hamsters. And to review, hamsters given a single dose of clindamycin and then infected with *C. difficile* would rapidly succumb to the disease.

If you did the same thing to most laboratory mice at least at that time, you were not able to get infection. You were not able to get disease. If the animals were germ free you could infect them with *C. difficile* and they would die but that really doesn't tell you what's going on with respect to the microbiota. You've basically eliminated it.

So, in 2008 Ciaran Kelly had revisited the mouse model and published this particular model which has been used by a number of people including several in this room where he gave an antibiotic cocktail for a minimum of three days. And this is the cocktail listed here, switched them to plain water for two days and then gave a single dose of clindamycin challenged with *C. difficile*. And through the magic of alchemy these five antibiotics would basically turn a mouse into a hamster. Because now if you give a single dose of clinda challenged with *C. difficile* they'll rapidly succumb to the disease.

And kind of liking alchemy myself, I challenged my technician to recreate this.

And she kind of broke down the antibiotics into the preconditioning regimen, into the clindamycin alone. And remember this cause I'm going to go over some of Eric's data later that'll say that not all microbiota are the same in all mice. But only when you gave the preconditioning antibiotics and clindamycin followed with *C. difficile* infection would you get lasting colonization.

In our hands, we got temporary colonization for about a day or two if we only gave clinda but never got colitis. But we got bad colitis if we gave both the preconditioning antibiotics and the clindamycin. And if you titer the dose of *C. difficile* that goes in you can actually get a kind of basically a binary response. All the animals are infected with *C. difficile* but about half of them when we give 10 to the fifth vegetative cells in this particular setting, about half of them would stay well.

They wouldn't lose weight. They would still run around the cage. They'd eat just fine. Another set within 48, 96 hours

would rapidly lose weight, would actually be found dead or have to be sacrificed. And this kind of recapitulates what Ciaran Kelly's group had shown initially as well.

What does this look like histologically? Here's a normal mouse colon. And there's one where we've given the antibiotics and the C. difficile. This is the equivalent of a pseudomembrane there. There's fibren, there's red blood cells, there's polys, there's a lot of sub-mucosal edema. There's a lot of damage. The animals that were actually controlling the infection somewhat didn't have as much epithelial damage. They still had inflammation, not as much edema. But the ones that succumbed to the disease had a lot of destruction to the epithelial and a lot of edema which probably explains the weight loss.

So, we were interested in comparing these two groups of animals. Both infected with C. difficile but some having a more mild clinical course than others. Sure enough, when we actually scored the histopathology,

the ones that were doing well had less histopathology in terms of both inflammation and edema than the ones that succumbed to their disease. The ones that were more ill had more of actually the pathogenic factor, the *C. difficile* toxin itself, than the ones that were well. And this was directly correlated with the amount of *C. difficile* that we saw in the tissue. The ones that were well had less *C. difficile* than the ones that were ill.

We looked at the microbiota using the 16S techniques that Eric has already introduced. And if you look at the animals that were well, these are the filled stars here, their microbiota was similar returning to what we saw in our un-antibiotic treated uninfected mice. And in our mouse colony we mostly see these gram positives, these Firmicutes belonging to the lachnospiraceae family. When you give the antibiotics, you change it mostly to one that's dominated by enterobacteriaceae. Interestingly enough, the animals that were sick continued with that

enterobacteriaceae domination there. And that's what we saw in this particular case as opposed to the ones that seemed to be controlling the infection where the lachnospiraceae at least partially were coming back to more baseline levels.

We've modified this model a little bit. We can give actually a single antibiotic and we've tried several antibiotics as similar to what Eric had described. And we found out that this cephalosporin cefoperazone which we had studied previously is able to, in a long-lasting manner, reduce the diversity of the microbiota. It was able to substitute for both the antibiotic cocktail and the clindamycin. So, if we give cefoperazone for a minimum of five days, wash it out for two days, challenge it with *C. Difficile*, the animals rapidly succumb to *C. diff* and yellow in this case is *C. diff*.

I didn't point in the previous one but *C. diff* is a minor player. If these animals are cefoperazone treated it can become the dominant member of the microbiota and

these animals actually die very quickly from overwhelming *C. Difficile* colitis regardless of the dose.

So, we've exploited this particular model of using cefoperazone treated mice to look at the differential virulence of different strains of *C. difficile*. And we're going to -- I'll just describe the results from two different strains that are commonly used. VPI 10463 is one that's been used on a lot of the hamsters' models. It was used in the original description of the mouse model and it causes a very severe rapid disease but 630 which interestingly enough shares an almost identical toxin to that of the VPI but produces somewhat less of in vitro.

Actually it's been used also in the hamster models, been used to study adaptive immune responses. What do we see when we put this into animals? And Eric had introduced the idea of germ-free animals as platforms for hypothesis testing. So, based on our preliminary results up there we wanted to see what would happen if we isolated one of these

lachnospiraceae. Those are the ones that were correlated with somewhat protection or at least a milder disease, not complete colonization resistance but milder disease. As opposed to E. coli which we knew we were able to grow out of our mice here, what would happen if we were basically monoassociating germ-free mice with either a lachnospiraceae or with one of these E. colis and then challenged with C. difficile.

And we used the two strains. As I said, 630 in a germ-free mouse as well would cause colonization and moderate disease but the animals wouldn't succumb. Strain VPI 10463 in a germ-free mouse caused rapidly fatal disease, animals dying within two days. So, what we did is we isolated the lachnospiraceae using the 16S as a guide to guide our culture results. There are ways and I can describe offline to you if you want, ways that you can use 16S data to find specific needles in the haystack as was described earlier.

What did we see? Well, as I said,

the germ-free mice are rapidly colonized with *C. diff* to very high levels. If you precolonize the animals with *E. Coli* and then about a week later try to challenge them with *C. difficile*, there's really no change for either the strain 630 or the VPI. I'm sorry this is just 630 right here. In terms of 630 there's no difference in colonization. There's no difference in the amount of toxin being produced.

But if you precolonize with *lachnospiraceae* by around two logs or so you decrease the levels of *C. diff* colonization and you also correspondingly decrease the amount of toxin that was produced. These animals though don't succumb in either case cause these animals survive.

And it's not just the amount of the organism that's there cause it's interesting. The *E. coli* would reach levels of about ten to the tenth per gram of cecal contents and really wouldn't change the colonization by *C. Diff* at all. The *lachnospiraceae* only colonizes to about ten to the seventh per gram

of cecal contents but is able to reduce by two logs the growth of *C. difficile*.

How about when we use the virulent strain? Well, what we saw is that if, these are the germ-free animals, if you give them *C. diff* I said they succumb within two days if they're just monocolonized with *C. difficile*. If you put *E. Coli* in they actually die a little bit faster and that's presumably because of the amount of damage that's happening to the colon. You're probably getting either translocation of the bacteria itself or at least exposures to a lot of LPS that would be in the gut.

However, the weight and this is how we're measuring health, is the baseline weight of the animals that were precolonized with this lachno strain that we call D4 followed by *C. difficile* actually maintained. And the animals were well. Three animals out of about 14 succumbed. And interestingly enough, when you look at that those three animals that succumbed were the ones that actually stochastically had the highest levels of

colonization in spite being precolonized with the lachnospiraceae and correspondingly had the highest levels of toxin. But in the other animals, the lachnospiraceae was able to reduce quite significantly in some cases, the amount of colonization by *C. difficile* and result in the amount of toxin producing the disease in the gut.

Now, other people have looked at this. Trevor Lawley recently published a paper where he found six, a cocktail of six organisms from his mice that were able to completely restore colonization resistance in his mouse model of *C. difficile*. And this is similar, we're going to hear from Emma Allen-Vercoe about a group of bacteria that have been isolated from people that are also has a role in treating, they didn't look at protection, but in treating patients with *C. difficile*.

So, this idea of mining the microbiota to find organisms that have functions that you're interested in, may represent something that may be an alternative

or the next generation of where we can go following fecal transplantation. But not everything is the same. Eric has also been using a mouse model of *C. difficile* and he uses the exact same genetic background of mouse, the C57 black 6, one of the work horses. What he finds though, as I mentioned earlier, if I gave a single dose of clindamycin I get transient colonization but it would be rapidly eliminated.

In his colony, and we assume, we haven't done it yet, but we assume that the differences in the baseline microbiota results in the fact that if he gives a single dose of clindamycin there's sustained susceptibility of these animals to *C. difficile* and induced colitis. So, it's not just the host. It is the microbiota. The microbiota does give you clues about functions of the system. And it's that function of the system that's quite important.

Rob Britton, who you'll hear from after lunch, and I wrote a review where we kind of speculated reviewing the literature

what are some of the mechanisms by which the indigenous microbiota can mediate colonization resistance against *C. difficile*. Is it just basically competition for nutrients? Or is converting some nutrients into something like for example short chain fatty acids that at least in vitro may be somewhat inhibitory to *Clostridium difficile*?

Could it be other things? Other things that the indigenous microbiota metabolites like bile acids producing germinants or in some cases producing secondary bile acids that are actually inhibitory to the vegetative form of *C. Difficile*? Some bacteria will produce bacteriocins. There's been other published reports that certain types of bacillus species will produce bacteriocin that's inhibitory to *C. difficile*.

But we can't forget about the host in spite of the fact that we're microbiologists. And that the immune system, we've already heard, we've heard this morning how the microbiota can signal to the host in

the immune system and actually modulate the host immune system. Is some of this what's going on in this particular system to mediate colonization resistance to *C. difficile*?

So, the final thing I'd like to talk about is we like to move from this idea of microbiome structure, looking at 16S, looking what the community overall shape is like, membership. But really getting at that idea of function that Lita Proctor talked about this morning, we have to understand that these bugs have millions and millions of genes that can carry out many, many different metabolic functions. How do these metabolic functions impact the function of the microbiota itself and in this particular case we're interested in colonization resistance.

So, with cefoperazone, let me explain a little bit more that we've done. If you give cefoperazone for 10 days, we've already looked and we've published papers that shows that when you change the microbiota from one structure to another structure. And that structure is also associated with a different

function, that is, you're very susceptible to colonization and disease by *C. difficile*.

We further published before that if you take the animals off of antibiotics for six weeks you get colonization resistance restored. Even though the community structure, and I'll show that in just a bit, is different you can restore this function of colonization resistance even though you have a different community structure. And if you just let animals hang out without doing anything you actually have a very similar structure.

So, what do these data look like? This is kind of revisiting some data that we published before. But the susceptible state, the original one here, when you treat with antibiotics is quite different from the baseline state here. Which is what was listed here as no antibiotics, the green symbols here. And if you allow the animals to recover for six weeks after treating with antibiotics they're in a different state. They segregate differently than the animals that were either

treated with the antibiotics, looked at two days after antibiotic treatment.

Six weeks, the community has changed back to something closer to normal, the control here, but not completely back to normal. And interestingly enough, or not surprisingly hopefully, if you don't do anything to animals for eight weeks their microbiota pretty much stays stable.

So, now we have two different functions, susceptible or resistant and at least three different community structures. Well, what happens if we begin to look at function? And to look at function we began to do metabolomics analysis. I won't go into the details but basically but using mass spec techniques we can either look at in an untargeted fashion or in a targeted fashion what are the metabolites that arise in the gut ecosystem from the activities of the microbes presumably?

And what we find out again, we have this susceptible state and the resistance state. And just remember that this resistance

state is the one of the animals that were treated with the antibiotics and then allowed to recover for six weeks. Where there was segregation by community structure there's not segregation of the three resistant states by the treatment, in other words related to community structure. Susceptibility and resistance was quite different in terms of both carbohydrates and bile acids by looking at untargeted metabolomics.

We confirm these results in vitro. We looked at some of the carbohydrates that went up. We show that actually *C. difficile* in vitro on that carbohydrate is the sole carbon source. Actually does much better than if you're using amino acids which are some of the other things that you see going down in the particular untargeted metabolomics. And also, the bile acids, we see increase in bile acids that are known to be good germinants for the *C. Difficile* spores.

Again supporting the idea that the function that happens when you treat the loss of function in particular but as far as gain

of function, as far as *C. difficile* is concerned you have a good environment to germinate in and you have a good environment to grow rapidly in. So, that's just a glimpse of what we're trying to do. You know, I can't give the untargeted and targeted metabolomics full weight of what we're trying to do but we're looking at specific organisms now that can carry out specific functions.

Can we build in the germ-free animal a particular type of metabolic profile based on the indigenous microbiota that we basically build up from scratch by taking organisms where we know the genome, we know their metabolism in vivo, or mostly in vitro, can we recreate that in vivo and then challenge with *C. difficile*? Again using these germ-free mice as a test bed for our hypothesis about function and the dynamics of the microbial ecosystem, understanding maybe a little bit more how we can modulate it to try to treat and prevent *C. difficile* infection.

You know, I'm part of a large group. My program officer is here. My grant number

is there. I think everything should be okay right now. But this is an ERIN project that's looking completely at *C. difficile*, looking at host responses, looking at immunology, looking at basic bacterial pathogenesis, looking at microbial ecology and clinical studies. The PIs here are all the people who are doing all of those different studies.

I feel a little bit like the Lorax. I'm just speaking for a lot of the people who have done all of this work and I'm grateful for the funding from Allergy Infectious diseases to be able to do this work on *C. Difficile* and I'd be happy to take any questions at this point. Thanks.

Eric?

MR. MARTENS: That's a great talk. Thanks for showing a picture of our paper.

DR. YOUNG: I didn't show the data.

MR. MARTENS: I'm wondering whether you've tried to put in anything other than *lachnospiraceae* into your germ-free mice? There's quite a bit of literature or it's been brought up that *Bacteroidetes* may confer

resistance or at least it seems to correlate with recovery. Did you try to put like B. theta in there?

DR. YOUNG: Yeah, Eric Martens is also at the University of Michigan. We've bandied the idea of using B. Theta especially since he can modify the carbohydrates used and change the fatty acid production profile. We haven't done that directly.

Interestingly enough, you noticed our mice are pretty much Firmicutes dominated. And so, we were thinking of trying to look for a Bacteroidetes that would be associated but we don't have any that are associated with colonization resistance either at the baseline state or after you kind of pulse with various antibiotics. But the B. theta one is one that we're going to try to do going forward. I have a new Post-Doc who is interested in trying that. Who's talked with Eric in doing that?

SPEAKER: One of your slides puzzles me because, and this may just show my ignorance in infectious disease, you were

talking about C. difficile infection of the Golden Syrian hamster but you showed a plot of verotoxin. What is relationship between verotoxin and C. difficile toxin?

DR. YOUNG: Oh, yeah, I'm sorry. That was C. Difficile measured on vero cells. I'm sorry. We use vero cells as the cells for the testing.

SPEAKER: Okay, thank you.

DR. YOUNG: You can use hela cells and others and I hope it didn't say verotoxin. I should look at that.

SPEAKER: It might not have but some work that I had done with Phil before and programmed --

DR. YOUNG: Right, right. No, it's the vero cell cytotoxin assay. The vero cell is one of the classic ones used for the cytotoxicity assay.

SPEAKER: I'm wondering if you looked at the role of immunization of the mice to see what the impact of that might be on the microbiota and whether those organizations that look protected might be associated with

immunization rather than just a local immune response.

DR. YOUNG: With immunization. Meaning that the host develops an immune response against the indigenous microbiota and that protects or?

SPEAKER: Well, we think that immunization may stimulate antibodies that might be protective against C. Diff. I wonder if it actually drives changes in the microbiota. I'm wondering if you looked at that in your mice and whether it was protective.

DR. YOUNG: We haven't looked at it that way but interestingly enough and these are data that we've just gotten recently. If you infect with 630 first, the one that allows long-term survival and doesn't cause illness in the animals, those animals develop a very robust immune response against the toxins that are in 630. And as I mentioned VPI shares almost a virtually identical toxin.

If we try to retreat with clindamycin or cefoperazone and challenge with

the VPI we get a good degree of protection. So, it is true it's already been shown in the hamsters that if you immunize against toxin and you have a good antitoxin response and we have good responses both measured by serum antibodies as well as fecal IGA that is associated. Now, whether or not you'd be able to immunize an animal that had an altered microbiota, we haven't done that yet.

Okay, thanks. Time to feed the microbiota.

MS. MILLS: I can't believe it everybody but we're completely on schedule. So, if you'll be back here at 1:30. Just to let you know there is a cafeteria downstairs. If you walk out the front door, go straight down the street and cross the next street you'll be at the Natcher Building. There's a larger cafeteria there.

So, there's a couple of options for not great food. See you at 1:30.

(Recess)

MR. RELMAN: All right. Good afternoon. I'm David Relman from Stanford

University. I'm honored and pleased to moderate the afternoon session. I did not have the wonderful benefit of being here this morning. I know many of you who spoke and I certainly am fond of the work that leads to this workshop. And so, will certainly try to provide whatever thoughts I can or responses to comments made, et cetera. I know that everyone wants this to be useful and helpful in moving this discussion forward.

So, the proceedings are going to continue this afternoon with several presentations having to do with tools and models. And I think the focusing on the concept that in fact what this drug or agent is is in fact a complex entity. It's a community and it's not a random assortment of organisms but a particular configuration of organisms with certain joint net properties that no one organism alone may have. And to get to a better understanding of what all that means is I know the subject of what all of you are thinking hard about, working hard on and what our sponsors have to try to address.

So, without further ado, Robert Britton is the first speaker this afternoon. He's from Michigan State, the first of two Michigan State speakers and is going to talk a little bit about some of the tools that he and his coworkers have been using to try to understand what it means to transplant a community and the effects it might have. So, Robert, thanks.

MR. BRITTON: All right, well thank you. Thanks, Melody, for the invitation to tell you all about my work. And so, we're really interested how microbial communities resist pathogen invasion and unlike the talk you heard earlier this morning when they were really focused on the immunology side of things, we're really just looking at how the bacteria provide a way of basically keeping pathogens at bay.

We're also interested in how pathogens are evolving to more easily invade these communities. And this is actually along with Vince, Michigan State also has another one of these ERIN grants and it's been very

instrumental in us getting this work off the ground. And as you can see we really are thinking about uses bugs as therapeutics which is really the subject of this workshop. And very excited to tell you about what we're doing.

First, for disclosures, I have a number of industrial partners that support research in my laboratory mostly on the development of probiotics and essentially none of the work I'll talk about today is one of these projects.

So, the overall question that we're really trying to address is how do microbial communities resist pathogen invasion? And so, we've of course chosen *C. difficile* for obvious reasons this is, I think, the best model to understand this question. And but the overall goal is rather than just basic science it's also understand can we actually identify protective microbes or communities that could be used as therapeutics. And of course, this is not a novel question in my lab. There's a lot of people who are after

this around the world.

But what I wanted to do today is to share with you the tools that we're using and how we're applying them to get at these answers. And then finally, I would just like to then discuss some of the key challenges that, you know, we've really have to come to consider in my laboratory and so, I'm really excited about this workshop. I'd really love to have some input from you guys about what you think about some of the challenges that I observe as a microbial physiologist coming at this from that standpoint rather than a clinician. And I think there's some interesting concepts and key points that you out there who are clinicians really need to think about when you're using tools such as fecal transplants.

So, we use two different models that we've developed in the lab and one is these mini bioreactors. And I'll describe these in a minute. We also are using in vitro, in vivo studies with humanized microbiota mice. So, Eric and Vince, nicely introduced you into the

animal models that we have for studying *C. difficile* infection. I won't have time to really discuss any of the work we've done in the mouse yet other than to say that our humanized mice, and so these are animals that actually have, they were germ-free and we gave them basically human stool. They behaved differently than both Vince's and Eric's humanized mice. And so, the antibiotics that develop or introduce disease in these animals is different.

So, again, just highlighting that communities are different. Something that we're all going to have to take into account both at the basic science level but also in medicine.

Now the bioreactors, the reason we developed these is that they're actually relatively simple and they're a higher throughput tool to study communities and how they impact invasion. Whereas, of course, we don't have any host here so we want to take communities that we identify in these reactors that are protective against *C. diff* and then

test them against human bacteria in these humanized microbiota mice. And the hope is that once we can establish a good defined community that doesn't have any toxic effects or adverse outcomes in these animals that that would be than an indication that we should move into humans with these bacteria.

So, just to give you an overview of sort of our study design, we collect human fecal samples from healthy donors. We didn't have anywhere near the criteria that the Human Microbiome Project did. I'm pretty sure everybody had oral disease and zits and things like that so.. But we screened for C. diff and probably not in a way that Phil would like us to so we're learning a lot here. But we processed these samples, we pool and freeze them and we wanted to do this so that we could have a bit more of a standardized system. We can also do this with individual samples as well and we have done that.

But essentially what we do is we have these mini bioreactors and essentially we treat them with an antibiotic or not and we

add C. diff. And all we're going to really do now is just ask okay what's different? We basically invade these communities here. So, we look at the community analysis. We look at the metagenomes, metabolomes and then we also culture out of these to identify but also then have these bacteria in pure isolates so we can go in and utilize these as potential therapeutics. And so, the hope is that we'll identify one or more strains that are key inhibitors and then go back and test these into animal models and also in the bioreactors.

So, there's a lot of different human bioreactors out there and a lot of them are geared towards trying to be a colon. And this is one of them. So, you can see this is actually a system that's been widely used and it's developed by Glenn Gibson and George MacFarlane. And it basically has these three different vessels, different pHs to I think basically moving their way through the colon. You have all this automated pH control, circulating water bath. It's a very complex

system.

You can use this to study *C. difficile* invasion and Mark Wilcox's lab in England has done this quite extensively. But you might imagine that this is quite a tricky thing to set up. And in fact, most of the papers that describe using this usually only have one or two replicates of the experiment. And we thought that was going to be a very difficult way for us to really test lots of different bacteria for their ability to suppress disease.

So, what we developed were basically these mini bioreactors. And so, essentially what we've done is we've designed basically a set of six chambers here which hold about 25 mills. We run about 15 mills of culture continuously through these. These are basically watershed plastic and so, they're fabricated via stereolithography. So, they basically bore the hole out of the middle and the block stays completely sealed and that helps us a lot with keeping things sterile throughout the experiments. And so, we just

come in and drill holes in the top of these. You have your influent, your effluent and then a sample port.

In doing this I learned a lot about tools that I never knew that I had. So, in fact this is a 60 spot stir plate. So, essentially this would cost you \$4,000 to get 60 magnets that spin a little bar. And so, essentially we designed this so that would fit over this so that we can keep our communities continuously stirred. And so, this is what they look like up close. Again, here you can see these individual chambers and our tubes flowing in and out.

So, of course, this isn't, we're going quite the opposite way of everybody else who's trying to basically rebuild a colon. But really what we really only wanted to have was a community that could resist *C. difficile* invasion and we've been able to successfully do that.

To give you a little more information about the basic bioreactor running of the samples, so basically we have a fresh

media port. We dump this into our bioreactors and then we pump the waste out. They're basically done at a constant volume. We have an eight hour turnover in our bioreactors that's about three times faster than the previous setup I showed you from Glenn Gibson. We can do this all inside the anaerobic chamber which is really nice for us. So, we have a 37 degree glove bag and essentially we can do all the manipulations in there.

We've got the pH buffer to six point eight. We were constantly monitoring this when we initially did this but we've found that our system was buffered to a point where we really don't have to follow this cause it doesn't really change. We use a very complex but a low carbohydrate media to support the growth of the fecal samples when we put them into the chambers.

And so, this just gives you an idea of what one of these looks like. These are 24 reactors that are now being continuously run via peristaltic pumps than then pump the medium through into the chambers and then back

out into the waste bottles. Pretty challenging to do when you have these big rubber gloves and when you're trying to manipulate things very small. And I have to say that the people in the lab have done a great job getting this up and running.

So, to tell you a little bit about the stool samples that went into the chambers, we basically collected fecal samples from 12 healthy anonymous donors. They were screened for *C. diff* and if they were negative they were allowed to go in. These were processed and frozen. And then what we do is we simply take a little chip out of the slurry and resuspend that and pool it. We inoculate reactors. We've used the same pool to inoculate our germ-free mice and then we also then use this to culture out isolates.

We now have I'd say maybe 12 or 14 generations of these mice. And surprisingly their microbiota is very stable. In fact, we don't need to keep these in a germ-free facility anymore. We've now moved them up to Michigan State and over time this progeny

that's passed on doesn't get invaded. We keep them under reasonably clean conditions but not quite germ-free.

And so, just to give you an idea of what our criteria were is we just wanted people that were at least two months without antibiotics, at least two days without probiotics and we had a wide range of age. A lot of *C. Difficile* in vitro bioreactor experiments use only elderly patients. And so, basically we had people donate at their homes. They basically transported their stool samples back to the lab within the 24 hours. They were asked to collect their samples and then put them immediately on ice. And we also gave them basically an anaerobe pack to keep the samples anaerobic. And so, then of course we've then screened them for *C. diff.*

So, to give you then an idea of how are we actually then using these reactors to study *C. difficile* invasion? So, we take a bioreactor, we inoculate this with a fecal slurry at day zero. Because they're coming out of frozen we let them sit for a day

without any flow. We let the bacteria wake up and start dividing again.

At day one, we give them 24 hours of flow and then we either treat them with clindamycin or we mock treat where we just add sterile water. And then after we treat for a period of three days, we give them a day to let the clindamycin concentrations drop low enough so that they wouldn't inhibit *C. difficile* and then we add *C. diff* strains. And then we monitor these over time. And so, essentially we're just asking can *Clostridium difficile* evade these reactors.

In addition to that, you've already been introduced now several times to community analysis. And so, we do a number of microbial community analysis looking at pyrosequencing of the 16S ribosome RNA genes. As we've done this over a number of times to the initial slurry and then basically every other day. And so, one of the really nice things about the system is we can do a lot of replication.

So, this just shows you the *C. difficile* abundance in the reactors. They're

spiked in around ten to the fifth, ten to the sixth in this experiment. The mock treated cultures you can see they basically wash out at the exact rate that you would expect this dilution so there basically is no replication of *C. difficile* if we don't treat them with antibiotics. However, if we treat them with clindamycin you can see that they basically stay up at a nice level and we can take this out 14 days and they'll just basically stay within the community. So, they have a niche now. They've invaded and they're staying there.

We also can use this to also test how far down can we go down on our inoculum. So, we can even go down to around ten to the two cells that are inoculated into this and you can see that we basically get invasion. But it's also invasion and actually expansion. And so, the media we're using obviously has a niche for *C. diff* around ten to the sixth. And this is about roughly point one to one percent of the entire community that's in there.

But again, the fact that we've got these n equals ten, nine, seven, we can do 48 of these reactors at a time. So, I think this is going to give us a really nice tool to be able to address basically what communities can we develop in vitro that can suppress C. difficile infection.

One other side note I wanted to just tell you about, these reactors have a lot of different applications and so, certainly if you have any interest in this, I'd love to hear about how you might want to apply our technology to your study. But one of the questions we were interested in addressing is the fact that these hypervirulent strains that have been talked about now for about a decade and sort of these 027 ribotypes, there's been some indication that maybe they're not as hypervirulent as once thought. We actually thought maybe they're hyperendemic.

So, we've tested competitions between 027 strains and other ribotypes that are not associated with hypervirulence. And you can see then here at day zero when we

inoculate our human fecal bioreactors this is the ratio of 027s to non-027 strains. You can see that in all cases the 027 strain always wins out against the 002. And we've done this now with four independent 027 strains and four independent non-027 strains which are all clinical isolates isolated at the Michigan Department of Community of Health.

And so, it suggests that these 027 strains may have some advantage of being able to invade a community. And that may be why we see them a little more often in hospitals, at least in certain outbreaks.

So, but of course what we really want to know is who's basically disappearing when we give an antibiotic that allows *C. difficile* to grow. And so, this is my one microbial ecology slide I'll show you where we basically look at the principle coordinate analysis of the Axis I and Axis II. Essentially, we're just plotting the distances between these individual communities in two dimensions here. And so, every one of these triangles or circles or squares essentially

are a single community plotted against each other. So, you can see at day two most of the bioreactors cluster together. And then once we start treating for clindamycin for day two through five and then after even we stop, these communities that have now been invaded by C. Diff now cluster together suggesting they've had a fairly similar shift in their microbial community. And the ones that are mock treated, of course, are elsewhere.

So, knowing this we'd like to know okay who's missing from this? And so, essentially what we've done is we've done metastats which is just basically a statistical tool to ask which members of the microbiota are now missing from this sample. And so, we have a whole number of organisms here that are listed and these would be things, these would be the candidates just like the *Barnesiella* that was talked about before, these would be candidates for things that would actually suppress C. difficile invasion. And we've very actively trying to culture some of these and we've been

reasonably successful with some classes and not very successful with others.

We can also learn about what other organisms in our system, of course, here's *C. difficile*, we can learn about what other organisms are also then associated with *C. Diff.* At least in our model that may also be assisting *C. Diff* for invading this community.

Now, one of the things you might notice is that I don't have any species names up here. And so, there's a number of things that I want to talk about for the rest of my talk that are really important considerations not only for the sciences but also the clinicians and I think most importantly maybe for the regulatory agencies in trying to understand how we should be addressing both fecal transplants or next generation probiotic communities if you will.

And so, I want to say that if you haven't been paying attention till now, wake up cause this is one of the back take home points. Is that the function of bacteria are

strain specific. They're not species specific. So, you know, if you pick out a lactobacillus acidophilus at the store and then you go and get another one from a different brand, they could have very different functions. And I'm going to give you a couple of examples of why this is.

But really knowing just the species is not going to help you in knowing what the function of that organism is. And I think that's something that's commonly missed, at least in some of my interactions with some of the clinicians I've worked with. Many of the bacteria that we are identifying haven't been cultured before so we really don't know anything about them. And in fact, we don't even know how to culture some of them. Some of them we may not be able to culture as individual organisms but only in tandem. So, there's big challenges in just getting out the good bugs that we want to get if we actually want to pull them out into pure culture and have a more defined microbial community to treat people with.

And so, the last point I want to make today, too, is that many of our next generation probiotics are organisms that are mutualists but in fact, they're opportunistic pathogens in the wrong setting. And I think just like Lita Proctor discussed earlier today, you know, a number of the HMP strains that have been identified are in fact, things that we may not want to put into people. But I think that's food for thought for the end of the day.

So, the way we look at these communities and I think this is pretty clear now from the talks earlier this morning is we use the 16S gene to essentially infer evolutionary relationships between different bacteria. And it's very useful for that. I mean, it's been I think a very groundbreaking tool first developed by Carl Woese. But it's not really very useful for inferring the function at a species or a strain level because the 16S gene is a ribosomal RNA and it basically does the exact same thing in every organism.

One really important point that taxonomists, microbial ecologists will tell you is that you cannot identify species based on 16S ribosomal DNA sequence. We do it. We're not supposed to but in fact, you know, if you really want to classify a species you have to have a lot of different ways of detailing that. And so, it's important to note that if you do a blast of your 16S ribosomal RNA sequence and it comes back with *Bacteroides fragilis*. Maybe it's going to be that in the end but you have to do more work in the end to figure that out.

And metagenomics will help get it function that the 16S gene can't but the really, I think the functional characterization doing things such as using our bioreactors and then interacting with the host to see how the immune system is functioning, you know functional characterization is going to be very important for future applications. And this is true in all fields of microbial ecology when you're talking about medicine or soil or marine

environments.

So, just to give you an example then, let's take this example here about the why knowing the sequence of the 16S gene is really of limited use in your function, so you find that your intestinal community that you're studying has a bloom of Escherichia Coli. So, is that going to be good or bad?

Anybody want to hazard a guess? Huh, bad? Okay. It could be bad. Okay, so let's look under door number one. Well, hey, it's actually just a human commensal. So, this is something that you always find there. It's probably not going to cause any problems. However, that 16S gene could also be describing E. coli 0157:H7. So, that would be bad and these are basically a picture of kidneys that had to be removed because of HUS. Or maybe you're talking about E. Coli Nissle which was probably one of the first identified in the marketed probiotics. But the point is is that the 16S gene doesn't tell you the function of that E. coli. It doesn't tell you if it's got shiga toxin, if it's got some

other toxin or if it actually has a beneficial property in stimulating your immune system.

But you might say well, hey, I would know as a physician if my person had a 157:H7. I would agree with that. So, let me give you another example just from my own lab and this is in collaboration Jim Versalovic at Baylor College of Medicine. Jim's been interested in immunomodulatory lactobacillus reuteri strains for about a decade. And so, we've identified some of these strains with him. And so, this graph is just showing you the relative level of tumor necrosis factor, a common proinflammatory cytokine that's associated with infection and also IBD. He has a strain of lactobacillus reuteri which greatly turns down TNF in both cell culture monocytes and also primary monocytes isolated from pediatric Crohn's patients.

But we have another strain of lactobacillus reuteri which at the 16S level is a hundred percent identical. Essentially has no effect on these. And we actually even know what the genetic basis is of this. Is

that we know that this strain here contains a three gene cluster which converts histidine into histamine and it's actually the production of histamine by the strain which actually has its anti-inflammatory properties. And he's going on to now study these in mouse models of colitis.

So, again highlighting that knowing the 16S gene you couldn't -- so, if you thought say if fecal bacter presnutia is an anti-inflammatory strain because of all the press it's received, just because the one that you isolate in your system is not necessarily going to be that way. And you're going to have to go in and show it.

So, and then finally one I think of the other important points that I'd like to make is that a lot of the bacteria that we're identifying in other projects that we're isolating from both human fecal samples and also from intestinal tissue samples with Tom Schmidt who's been looking at the colon and the ileum, a lot of these bacteria has sort of scary names. *Bacteroides fragilis*,

streptococcus milleri, prevotella bivia, we isolate these over and over, yet these are all associated with anaerobic infections. This is actually, I think, the most common cause of anaerobic infection.

So, the question is do we just throw all these out because we don't want to use them? Well, they're really mutualists. I mean, that's what they're there for originally so if we actually exclude these organisms in next generation probiotics, I mean we may be missing key members of the intestinal microbiota. If we're going to try to rebuild a "normal" microbiota we may have to consider how do we distinguish the pathogenic ones from the non-pathogenic ones. And I'm not sure that that information's out there for all of these.

The other thing is if you believe that these organisms and many others are what's being transplanted during fecal transplants. So, if you don't like them you'd better get over it because that's what's going in with the rest of the feces.

So, just to finish up, this I think is the real crux for me about where fecal transplants need to be discussed. So, we have recurrent CDAD and we know that fecal transplants and perhaps next generation probiotics in the future with isolated communities will be good. And this is hopefully now going to generate some happy people who are going to now be healthy. But if you believe that these things can actually cure disease, I think you also have to accept that they can probably cause disease. And we've been talking mostly about pathogenesis in the morning and infections but there's a lot of literature now that obesity, fatty liver disease, cardiovascular disease, autoimmunity and there's a lot and even more in mouse models are actually influenced by the microbiota.

And so, we have to figure out a way to be able to identify which of these communities can go this route because we don't want to cure C. diff but then give somebody diabetes or obesity. And I think that this is

a real big area that we have to discuss.

So, with that let me just thank the people who did the work. Jenny Auchtung and Cathy Robinson here holding their little babies. They were the two people who built the bioreactors. James Collins does the humanized microbiota mice and Lily Jensen is an undergrad working with us and Bob Stedtfeld helped. He was the guy who actually, the engineer who designed the reactors. I'd like to thank Linda Mansfield, our next speaker Shannon Manning who are my co- PIs in the ERIN. And I'd like to thank Melody Mills. She's a very supportive and enthusiastic program officer. So, that's always good. She's always upbeat. And I'd like to thank Kate Eaton for the germ-free mice and Vince and his laboratory. They've been very instrumental in getting us going with our germ-free mice.

And so, here's springtime in Michigan this year. So, with that I'll take questions.

MR. RELMAN: Thanks, Robert.

Question, Eric?

DR. PAMER: In your bioreactors did you inoculate them with C. diff spores and is there any role for biosalts in the process?

MR. BRITTON: Yeah, so we tried that initially. And so, we didn't have success with spores even though we have taurocholate in the reactors and so, they're right now inoculated with vegetative cells.

Now, the three chamber setup kind of gives you some time for sporial or germination to happen. And so, we think that what's happening is we're not giving the cells a long enough time to actually germinate and then actually invade the community. So, one of the things we're going to try in the next couple of months is to actually take the spores, give them some taurocholate before we put them in. Kind of get the process going and see if that's why we're not missing. But certainly in the three chamber reactor they do get germination invasion but of course they're going from vessel A to B to C. And they only see invasion in C so there's probably a time

period where the cells have been able to wake up and start their physiology.

We're forming them kind of fast and so I think that if we slowed down the flow rate we might also be able to -- but at this point when we want to study sporulation we're going to tweak that aspect of it.

MR. TRIPLETT: Hi, Eric Triplett, University of Florida. I agree with everything you say. I think however --

MR. BRITTON: Wow, nobody ever does that.

MR. TRIPLETT: -- 16S RNA is still useful because it broadly tells us about the physiology of the organism. It may not tell you specific host microbe interactions but it can broadly anaerobic, anaerobe whatever.

MR. BRITTON: Absolutely. I do agree that.

MR. TRIPLETT: So, then but the problem with studying function and we all want to get the function. But the problem is our primitive knowledge of bacterial genome annotation. One example of that is that 40

percent of the well characterized enzymes for which we have EC numbers have no known gene identified yet.

And even if we have function then there's you have to look at expression and even if you have that is it really made to protein. So, we're primitive in a lot of levels but we'll see if we can make progress.

MR. BRITTON: So, you are preaching to the choir because I agree. I think one of the things we've done a great job of is sequence a lot of stuff. And we have not spent very much time trying to annotate those things. And I think there was a recent call, I think at NIAID, where they were asking people to now start characterizing these genes. But they were asking people to do 10 genes a year, is that right Melody?

MR. TRIPLETT: And they stopped funding CEED and other things where they were making a lot of progress. The problem is it's not a sexy thing to do so the agencies don't consider it transformative but it's really, really important.

MR. BRITTON: And I also think the issue, too, is that a lot of people believe that we do know the functions of a lot of these things. So, for example, E. coli you'll see reviews where 85, 90 percent of the genes are known, the function's known but unfortunately a lot of things that you find in Pfam are DUF domains which are, for those of you who don't know, are domains of unknown function. So, you can't use Pfam annotation as a way.

So, it's really probably about 40 or 50 percent of E. coli one of the most well studied organisms that we actually know the genes. But that is a limitation, I agree.

MR. RELMAN: If I could just maybe ask a question and you can let it be until the end of the afternoon if you wish. You began by talking about, and this is the strain or the organism --

MR. BRITTON: It's off cause I'm on. Here.

MR. RELMAN: There were sort of two ways of looking at this issue from a strain

specific manner which is to say what might be missing without which I've lost this property of colonization resistance. So, that's asking what's necessary. But then at the end you talk about the use of organisms and testing their properties in isolation and asking are they sufficient. How do you see trying to merge those two different kinds of approaches which may be giving you very different kinds of answers?

MR. BRITTON: Yeah.

MR. RELMAN: Necessary versus sufficient.

MR. BRITTON: So, that's a very challenging question. That's one of the reasons we developed the bioreactors. Because, so a lot of probiotics that originally developed on the market are individual strains. And I think it's pretty clear now that it's going to be cocktails and communities that are really going to be what the next generation of probiotics are going to be.

So, that's why we developed the

reactors. We were thinking how are we going to, if we get a model going how are we going to be able to test 20, 30, 50 different combinations of communities to really hopefully get at the best one? At this point now if you're going into mice, I mean, what are you going to test? Maybe a handful if you're lucky because it's a lot of work. So, yeah, I actually think that it'll take both though because you know understanding the individual activity say anti-inflammation you kind of probably have to do that a little bit at that strain level or single strain level. So, it's going to be a challenge.

MR. RELMAN: Thank you. Our next speaker is also from Michigan State, Linda Mansfield. Linda is going to take us to the world of veterinary science and share some of the insights that have been learned from large animal experiments and medicine.

MS. MANSFIELD: As they say, now for something completely different. So, actually I think for those of you that have been very interested in fecal transplants that this will

be commonplace and you've probably been very familiar with a lot of this literature.

So, what I've been asked to do today, I'm actually not going to present my research data. I've been working in this Enteric Research Investigational Network and with the BEACON Center for evolution and action. But today I've been asked to actually review some of the evidence based approaches in veterinary medicine for using, for fecal transplantation.

The first thing I found out when I started to deeply review the literature is that they do not call it fecal transplantation in veterinary medicine. And it's actually termed transfaunation and we also know that re- ingestion; microbial re-ingestion is also an important aspect that's occurring in animals. And so, I meant to tell you what is in this talk. What we're going to talk about a little bit are how microbial transfer contributes to health and well-being in animals. Animals have been transferring their microbiota for a long period of time.

We're going to talk a little bit about fecal ingestion in animals and then clinical applications that are in regular practice in veterinary medicine. And a selected ruminants because they've been worked on extensively. There's a really huge literature. I found the first paper in the 1920s but I hear from someone here in the audience that they go back even further than that. And then I selected horses and dogs to talk about because it's used to treat a lot of antibiotic associated diarrheas and some other infectious diarrheas.

And so, we know that microbiota contributes to health and well-being of animals including humans. But a lot of this data was originally generated in animal systems. So, we know that the microbiota produces volatile fatty acids. It enhances water uptake into the bowel. It reduces alkaline phosphatase and degrades cellulose in herbivores and it also produces a wide range of vitamins including vitamin K. And we've heard already this morning about its ability

to stimulate both innate and adaptive responses and certainly all the cellular development of the GI tract particularly the cells that come into the lamina propria is all occurring after one acquires one's microbiota.

We know from animal work that the microbiota can help to exclude pathogens and interestingly there's been a lot of work on how it enhances social communications. And I thought any of you that have had dogs have understood how avidly dogs seek the tea leaves of the fecal pat but there's other animals that do this as well.

So, some of the first work to look at fecal transfer was actually looking at fecal re-ingestion as a natural process in some animals. And this is actually most common in some herbivorous animals like microtine rodents and rabbits. And it's, the knowledge of it is quite good. But it's also been documented in domestic animals, in piglets, foals, dogs and non-human primates as well where somewhat less is known about it.

But there is a relationship between

changes in diet quality and use of this cecum coprophagy system in rodents. And as the value or the nutritional value of the diet decreases, there's an increase in the fecal coprophagy and they even were able to document that rats that were prevented from reingesting their feces have reduced growth rate. So, it's a significant source of both vitamins and amino acids for these animals.

And certainly these nutrients that are unavailable in the diet that are supplied by the endoflora like vitamins and amino acids are better utilized by animals that have digestive tracts where they have fermentation vats at the anterior end rather at the posterior end. So, this allows for more utilization of the nutrients. So, re-ingestion is a mechanism for compensating for dietary changes and it may involve coprophagy and post gastric fermentation.

So, this is an unusual example but this is a study in hyenas that was done by Kay Holekamp in Africa. But Kay is at Michigan State University and she worked with Tom

Schmidt for this project. And here she looked at adult spotted hyenas and their ability to actually communicate with other hyenas through a pasting behavior. And these glands, these scent glands are actually with the anus of the animal and they accumulate a paste. They can be everted by animal and I don't know if you can see this but the hyenas will actually paste the environment usually pasting this on grass stalks and this will leave a strong musky odor near their dens and borders of their territories.

And so, in this study they hypothesized that the microbiota in these anal sacs contributed to paste odors that varied among clans. And they did a typical 16S RNS gene survey and they were able to show that the scent secretions had dense communities of fermentative bacteria that were odor producers. And so, the other that was interesting is that the bacterial communities were more similar among hyenas from the same social group than from distal or different groups.

And so, when they cultured these scent pouches they did quite a lot. This was work from Tom Schmidt. They showed a number of genera of bacteria that were present and scanning EM you could look at bacteria on the surface of the pouch. They had both gram negative and gram positive organisms but the relative abundances of these OTUs were more similar among clan mates than among hyenas from different clans.

And so, this was a form of communication. And in this non-metric multidimensional scaling plot you can see these relationships. So, this was one clan of hyenas. Another and here their territories did not overlap so the pasting was separate. Whereas these were more similar to one another and their territories did overlap and there was some probably cross-pasting going on in these animals.

And so, the other thing that they found was that in these infant hyenas they were likely to inoculate themselves with this clan-typical bacteria using pasting bacteria

even when they didn't have any paste in their anal glands. And so, some of these things are natural behaviors in animals and ingestion of feces or doing some behavior like pasting is a way to acquire the normal microbiota as an infant and it apparently is protective.

So, now I'm going to get into some of our domestic animals where transfaunation has been done relatively extensively. And in ruminants it was done largely because ruminants in our society are food animals and they wanted to enhance their growth. And we know that they're herbivores and they depend on symbiotic associations with their gut microbes to obtain energy from structural components of plants. So, these animals are herbivores. And what we also know is that many domestic animals they have either fore- or hind gut fermentation.

And so, there are some differences that come from that kinds of systems. So, I gave you a little anatomy here of the GI tract. So, when a cow eats something the food will first go into this gigantic fermentative

vat that's called the rumen. This then the food will then go into the reticulum, the omasum and the abomasum which you can't see and then it goes into the small intestines and the large bowel and out. So, cows have four stomach compartments in a fore-gut and what we know is that the fore-gut in cows allows for volatile fatty acids, vitamins and proteins from microbes to be absorbed.

So, there has been attempts to engineer the rumen microbial ecosystem. And some of the indications for them are prolonged anorexia in cows or acute indigestion. And here there would be increased acidity of the rumen and decreased appetite and growth loss. There's also some surgical indications for this like left displaced abomasum which is the true stomach twisting and displacing. And then just simply for growth promotion.

So, they define transfaunation as transfer of ruminal fluid orally to the cow from a normal animal that has rumen bacteria, protozoa and volatile fatty acids. And so, to get a donor it's a little more sophisticated

in cows than in some other animals that I'll show you later. They actually will place a ruminal cannula so that they can into that rumen repeatedly and they could even feed the cow or do whatever they want, take a sample. It's non-painful and they can sample it repeatedly and then to give the transplant they can simple paste a tube, pass a stomach tube and very easy and not painful for the cow.

So, what is interesting though is similar to what's been said earlier today is that from all animal species there's an assumption that a normal animal has beneficial contents. And that there hasn't been a lot of interrogation in some animal species as to what's in that normal animal's contents. But the idea is to transfer good versus bad components. And in ruminants this has actually been addressed.

And so, there have been extensive studies doing fractionation studies where they've actually done intentional additions of different parts of the microbiota. And I'll

tell you about that a little bit later. So, there's such a large history of transfaunation and domestic ruminants that I really, I think you would have glazed over if I tried to present any more than this but they have -- these are some of the processes that have been demonstrated. They found, first of all, that the rumen is anaerobic and that these anaerobic organisms will actually promote more anaerobicity and enhancement of the diversity of the community.

They also know that there's been isolation in mechanisms of acetogens demonstrated, specialist succinate utilizers, highly active amino acid deaminators and other microbial components that are degraders of hydrolysable tannins. And more recently they've been looking at competitive interactions between community members mediated by bacteriocins.

And then finally, there's been work to show that anaerobic fungi and anaerobic ciliate protozoa are also important in this community and can help to regulate proportion

of the community. And then finally, the nutritional interactions of the microbial food web are important.

And so, this is taken from an article by Flint in Trends in Microbiology that demonstrates an example of microbial food web in the rumen of a ruminant. And I think we won't go into the details but it's easy to see that it's complex and it's interactive. And they've demonstrated cellulolytic population of bacteria as well as a non-cellulolytic population. And you can see the main organisms that are here. But there's a flow between these community members and these fermentation products then go on to support other members of the community.

So, if we think about these communities, if there is selective removal of certain members of the community it's going to have an impact. And sometimes, you know, that may mean that a substrate builds up and doesn't get utilized. Or it may mean, in fact, that a product that would normally have been broken down will be present and then can

go on to damage the mucosa. And so, these things potentially could be important to think about.

And then this, I'm not going to talk about this extensively, but the functional studies were actually done on fauna free animals. In this case if you're using a ruminant you don't want to use a cow for these kinds of things because they're big and expensive. So, they've used these little goats and they're able to caesarean rederive them and then add back different members of the community and that's how they've established some of those functional processes of the different components of the community. And so, I'll just leave it at that there.

And then the last thing I wanted to say about cows is that cows are not calves. And but there has been some success in using transfaunation to cure chronic indigestion in calves. And so, this is a process in ruminants that you can use in adults and you also use in the very young. So, calves with chronic indigestion become depressed. They

have poor body condition, decreased appetite and they go into metabolic acidosis. And they can collapse and die from this and the treatment really is mainly transfaunation. And really it just consists of giving intravenous administration of sodium bicarb, doing the transfaunation and then giving electrolytes and these calves will return to normal and increase significantly in their growth rate.

And so, in cows and in ruminants transfaunation has been highly successful and it's in regular use. Transfaunation's also been used for decades in horses. And it's almost difficult in some regards to try to read primary articles because it's all in the medical books, textbooks and you don't get many of the details there. But what we know about horses is they have a large component of hindgut fermentation that encompasses a cecum and a colon. So, they're monogastrics unlike the cow and then the ingesta goes into the small intestine and then into the hindgut and their natural diet is grass. So, in the hindgut they have fibrinolytic bacteria that

produces short chain fatty acids and these provide the majority of energy requirements for horses.

And so, there's been some fairly recent work by Daly and another group headed by Scott Weese where they've been able to show that both infectious colics and non-infectious colics in the horse can be driven by changes that mediate changes in the microbial community. And so, this in fact is data about a non-infectious cause. So, in horses that are highly susceptible to colic they frequently die from colic and it's a major health problem. And we know that carbohydrate fermentation has been associated with colic in horses.

And so, horses are normally on a grass diet and if they're switched to a carbohydrate diet we do see shifts in bacteria like lachnospiraceae from the grass to the concentrate. And then this shift is even more pronounced in animals that have this type of colic. And we also see a decrease in obligate fibrolytic acid-intolerant bacteria and here

we have a decrease on concentrate diet and that's also mirrored in the horses with colic.

Scott Weese shows that colic is a form of colonic dysbiosis in horses and this group of horses had infectious colics. And he simply used 16S just to show that healthy horses had a different distribution of taxa than horses with colitis. And there was a decrease in the Firmicutes group and an increase in the Bacteroidetes group and you can see on the NMDS plots that horses that had colic in red here had communities that were significantly different than those in blue.

So, this establishes that colic is really driven largely by the changes in the microbial community. So, the response to that was for both infectious causes of diarrhea and non-infectious causes the use transfaunation. And I'll point out to you that horses are susceptible to antibiotic-associated diarrhea, salmonellosis, *Clostridium difficile* and *Clostridium perfringens* among others. And in fact, they have been shown by Scott Weese to transfer pathogens between human and horse.

And so, the treatment here is fairly static and always done the same way where there's fluid replacement, control of inflammation, reduction of fluid secretion with antidiarrheal agents and then control of endotoxemia cause that's what they die of in the end. But an important aspect of this is reestablishment of the normal flora which either transfaunation or probiotics. And actually transfaunation has been the favored way to go here and then finally, afterwards feeding to reestablish short chain fatty acid colon content.

So, transfaunation in the horses might be a little bit graphic for this audience but this is typical. The horse will be examined and then fauna is harvested from the large bowel of a healthy horse and here we have somebody doing this. So, veterinarians do this kind of thing every day where you're going to do a rectal exam and there may be a number of organs you can examine through the rectum of a horse. And we will not say more about that but at any rate the clinician will

go in get a fresh sample of distal bowel contents and then basically this is harvested, filtered and made into a liquid slurry and then there's testing of the donor material to prevent transmission of infectious agents.

And that's been done in a rather standard by first culturing and now using some molecular tests. So, the horse is given antacids, antimicrobials are discontinued and then they're gavaged by passing a nasal gastric tube into the stomach and I'll show the previous picture. Horses will stand nicely when you pass a nasal gastric tube. It's not painful to them and you can pass a really big tube into a horse. So, you can pretty much put anything you want down there.

Okay, and then the last thing that I'll say about horses is that there is a move and a push to go to probiotics instead of transfaunation even in the horse. And so Weese and Rousseau did this study. It was a safety and efficacy trial where they looked at healthy foals that were 24-48 hours old and they assigned them to either treatment or

control groups. And then they gave them lactobacillus pentosus WE7. This was a strain that had been tested as safe in a safety trial in adult horses. And unfortunately these were given once a day for seven days but the probiotics were significantly associated with signs of depression, anorexia, diarrhea and colic in these foals. And in fact, there were some heroic measures that were needed to actually rescue the foals.

And so, the conclusions in this paper were that probiotics and horses are not currently supported in a science-based fashion. And some probiotics can act as pathogens in a young aged animal. And in fact, one thing that was very important was that administration of the probiotic actually blocked absorption of colostrum which is extremely important in horses. So, they don't live well if they don't get that colostrum. And so, you know there was a call for safety and efficacy testing for all equine probiotics.

So, there is some work on fecal

transplantation that's been done in dogs. Many of you may have dogs and we know they're monogastrics. They crave human food. They're known to ingest feces as a natural process. Some of them are worse about this than others. It can be quite disgusting and there is only a small component of hindgut fermentation. So, they have a very small cecum. So, they're much analogous to the human situation than some of the other animals that I mentioned.

So, they also get antibiotic-associated diarrheas and they do have a number of bacterial pathogens that are similar to humans. And so, fecal transplants have been successful and you can place a nasal gastric tube in a dog to do this but when I was in practice basically we would make the slurry, test it and then put it into a little capsule, pop it down. And that would allow it to go through the stomach so that it would start to disintegrate after that. And that worked pretty well.

But I will point out that in a study that we did in my lab in the veterinary

hospital we simply tested a number of animals that came into the clinic who were either normal or they came in for diarrhea and found that if you look at a panel of pathogens, certainly not all of them, that many of the normal dogs were walking in with, you know, that were positive for pathogens. And when this particular dog one, when he got diarrhea pretty much almost had the same pathogens as before and then was treated with antibiotics and still had pathogens after that.

And these are just a few of the other dogs that we tested. And there were some dogs living in a research colony that had an inherited disease. They had quite a few positives and yet were perfectly healthy. We followed that dog number one, we followed a number of dogs with diarrhea and we found that there was a change in fecal microbiota with diarrhea and antibiotic treatment. And that's not surprising but there's some interesting things about this.

So, this was the normal state of the dog, the dog during diarrhea and after and if

we look at this data another way we see there was a bloom of clostridia and E. Coli that resolved even before the antibiotics were given. And if we look at the relationships between these microbial communities from different times we see that in pet one on day one these samples in these early samples before diarrhea clustered together, whereas during diarrhea and treatment they clustered differently. And then after resolution of the diarrhea they were in an entirely different group. And so, it's likely that there's a new normal associated with this.

The last animal that I'll mention and I won't say much about it because Rob Britton has already covered it is that fecal transplantation has been performed experimentally in mice now by a number of people. But Rob's work is particularly interesting because it's using germ-free mice and giving normal human microbiota. And what's interesting about this is the mice are really normal after they are given this.

And so, these are animals that have

a pretty undeveloped immune system in the bowel. And I think Rob talked about this a little bit but these fecal communities demonstrated some stability over time within the germ-free facility and now the mice have been taken out to actually observe how they do in a specific pathogen-free facility.

So, in terms of the lessons learned from veterinary medicine, we know that resident microbiota in animals have been shown to harbor pathogens that can be transmitted to recipients that molecular testing can be used to declare animals free of both colitogenic bacteria but you wonder about those unstudied microbes in that population. We know that the germ-free mice had no adverse consequences despite a pretty undeveloped immune system but critically ill animals had many more adverse consequences with transfaunation. And probiotics need much more testing.

So, the loss of gut microbial community diversity in animals or shifts in the community taxa have been associated with disease. But there's evidence for

transfaunation enhancing health in both ruminants and horses and even dogs. So, we know that many domestic animals can return to stability of the GI microbial community after transfaunation but it's likely to be a new normal. And there is a big need for evidence-based probiotics even in veterinary medicine.

So, I'll just thank the people that have been involved with this in my lab and Kate Eaton our collaborator, Rob Britton and James Collins. Thank you.

MR. RELMAN: Thanks Linda.

Questions? Yeah, go ahead.

MR. KHORUTS: Alex Khoruts from University of Minnesota. In this indigestion in cows which is I'm sure different from human indigestion, what are the precipitating causes and what happens to the microbial community structure in that?

MS. MANSFIELD: That's a good question. Well, metabolic acidosis is one of the main components of that and cows are put under tremendous stress because they're asked

to grow so fast and they're actually asked to lactate. And often this can push that kind of situation. The other thing that is associated with it is feeding of higher levels or carbohydrates. And so, all of those things can produce that kind of syndrome.

MR. KHORUTS: So, given that this dates back centuries there was no probably high fructose corn syrup diet for cows back in 17th century.

MS. MANSFIELD: Not a lot of high fructose but I can tell you that when a cow produce -- I don't remember the numbers but hundreds of pounds of milk from one cow in a year and a lot of our production animals will go from one to 220 pounds in some amazingly short period of time. And so, we push them metabolically to the limit. And that does have a stress on the microbial community.

MR. BRANDT: Larry Brandt, New York. I was under the impression that when horses develop diarrhea that you could actually do a fecal transplantation into the cecum of the horse and that that would correct the problem.

Am I mistaken about the route or is either route successful in a horse?

MS. MANSFIELD: You are correct about it. And in fact, there are horses -- colic is so common and there's some very, very severe colics that are surgical colics where they simply have to remove the devitalized bowel. And so, for some of those horses transfaunation has been a lifesaving thing. And they'll actually take them into the surgical suite, you know, tip them down onto the table. They'll open the bowel and they put in to this stuff called DTO-Smectite which basically will sop up endotoxins and other toxins and then they give them the transfaunation. And that actually can be rescuing.

MR. BRANDT: Well, on your slide though did you not say that you gavaged them? I sort of understood that to mean you gave it by the oral route rather than the rectal route.

MS. MANSFIELD: And that's right. It can be done either way.

MR. BRANDT: Okay.

MS. MANSFIELD: And in fact, most veterinarians are not setup to do surgery so.

SPEAKER: I'm a gastroenterologist but my father's a veterinarian so we've had a lot of discussions about these things. So, I have two different questions for you. One is my understanding and this is rudimentary is that dogs actually harbor *Helicobacter pylori*, for instance, and it's not pathogenic. And there's been some suspicion that that may be actually transmission to some human hosts. But the second one is and my dad didn't know this either, do dogs or horses get *Clostridium difficile*? I saw *Clostridium* listed there but is it the same organism?

MS. MANSFIELD: They can. And horses can get some of the same strains that humans do. I don't think we know that in dogs unless there's somebody in the audience that knows that.

DR. BRANDT: Actually dogs can get *C. difficile*. The strains that occur in dogs can be passed to humans. There was a study

that was reported out of Canada in which dogs that were part of the visitation program, they visited patients in ICUs, actually contaminated the patients in the ICUs. I think the number was, I thought it might be 73 percent of the dogs actually had C. diff and in a couple of the dogs it was a NAP1 BI strain. So, it was particularly virulent but there is this shared pathogen with man's best friend.

MS. MANSFIELD: They've also shown that Clostridium difficile in horses is more likely to be hospital acquired in an equine hospital now. And so, when they're setting up for transfaunation they'll use a horse that's been out on the farm and test them. And the other question is that there is Helicobacter felis that's been found in the dog and the cat. And to my knowledge I haven't heard about Helicobacter pylori.

MR. RELMAN: I had a question for you. I'm interested in the long-term follow up of some of these animals and their transfaunation experiments. There may be some

important lessons for human medicine having to do with stability but also the maintenance of properties that we associate with a healthy microbiota and you probably have a lot more, you know, your community has a lot more time on the topic than well we do in large numbers.

MS. MANSFIELD: They are followed pretty vigorously after transfaunation especially the animals that were critical when they received it. And I know one example was that dog number one that I told you, showed you some data from. Up to day 134 but we actually came back and sampled him later. He continued to have bouts of spontaneous diarrhea and he eventually lost diversity and he died actually.

MR. RELMAN: All right. Thanks again, Linda. The agenda calls for a break at this point. We're going to reconvene at 10 after three and then have a third talk and a discussion. So, thank you.

(Recess)

MR. RELMAN: Okay, if you could all take your seats, we're going to get started

again. What's happening here? All right, welcome back. I'm delighted to introduce our third speaker, Emma Allen-Vercoe who is from the University of Guelph in Canada. And she's going to give us her perspective on therapeutics, ecosystem therapeutics and the questions and the possible approaches. So, thank you, Emma.

MS. ALLEN-VERCOE: Okay, thank you very much. Can you hear me or do I need to?

MR. RELMAN: I'm not sure that's -- it's on right?

MS. ALLEN-VERCOE: Can you hear me? Okay, so thank you very much to Melody for inviting me to come and speak to you and maybe give a little bit of a Canadian perspective. It's been a bit of a whirlwind year with lots of things happening and it's just really becoming a very interesting area of research and I've been really fortunate to have been sort of right in the fray from the get-go.

So, I'm here to talk to you about something that we've coined microbial ecosystem therapeutics or defined microbial

ecosystem therapeutics, the sort of the next generation of fecal transplant if you like. So, taking individual species from poop and mixing them together to make a defined therapeutic ecosystem to use in such diseases as *C. difficile* infection.

So, the caveat is that I'm not a clinician. So, please don't me any hard clinician questions. My clinician counterpart is Dr. Elaine Petrof at Queens University in Kingston, Ontario and she can't be here today unfortunately because her husband's on service. So, she has to look after the kids. So, she's told me that if anyone has hard questions then they're more than welcome to email her and hopefully she can answer them.

So, my background is more in microbiology and I'm reinventing myself as a microbial ecologist of late and so my talk is really going to be focused more of the microbial ecology and some of the things that we're doing in my lab.

So, I'm going to start off by sort of stating the obvious that human health

depends on microbiota health as we've all seen. And we are the vessels for our microbiota. As I say to my students we're the spaceships for our microbiota and like it or not they control us more than we give them credit for.

Now, when you're talking about microbial ecology of course the human gut microbiota is a very complex microbial ecosystem. But what is really key to understand is that you have to, in order to understand the function and behavior of this ecosystem you need to study the whole thing. So, it's very, very easy to imagine and kind of what's been done in the past, break things down into their component parts and try and make an average of what you see. That's really not what happens in microbial ecology.

Usually microbes, they work together. There's some synergy there as I'll be mentioning in a little while. And I wanted to just sort of make an analogy here because I'm a parent of a teenager. This is Phoebe, my teenage daughter. She's 13. And this is

her typical kind of expression and body language at home, sitting there on the sofa, kind of slouching, scowling at people and really answering people in monosyllables. And this is what Phoebe looks like when she's with her friends. And so, you can see there's a big difference there. And I'd like to say -- I wouldn't like to say that my daughter is like a microbe because she's not but just like teenagers, microbes prefer to be with their friends. And they behave very differently when you take them out of context.

And so, when I take Phoebe out of context and I put her in a room on her own she's pretty miserable. But when she's in a room full of her friends, she's pretty happy. And so, just keep that in mind when you're thinking about microbes because microbes are pretty similar. And I know I'm not supposed to anthropomorphize microbes very much but I do it all the time because I actually think it helps me understand how they work.

So, when ecosystems are in equilibrium, another analogy, you can imagine

them as being like a rainforest. So, when you have a high diversity of species like you have in a rainforest, you have a healthy ecosystem. You have balance in the system and you have some resistance to disease. When you have a low diversity of species and this is a forest that has been ransacked by a beetle that's a particular problem in Alberta at the moment, then you end up with a sick ecosystem. You get imbalance and susceptibility to disease.

Now, microbial ecology, one of the things that really fascinates me about microbial ecology is it's just the same in terms of the principles as macroecology. And so, we know an awful lot about macroecology. So, we just have to apply those principles to the microbial world and we see a lot of similar things.

Now, what's interesting is that everyone is sort of saying save the rain forest, save the rain forest. But nobody is until recently even started to say save our gut microbiota rain forest. And I think that's kind of where we've been going wrong

for a little while now.

So, I'm going to give you a little bit of, for those of you who aren't microbial ecologists, a little bit of background on basic ecological principles of complex microbial communities. So, and these are just very, these are just generalizations and there is some nuances but I won't go into them. I don't really have time to go into them today. But in general these are some principles that you should know.

So, first of all, if you have a balanced, complex microbial community that means that it has high diversity and rich functionality. So, that means there might be some redundancy as well in that functionality. Function redundancy means that you have greater resilience in the face of stress. Now you can imagine these or you can conceptualize these ball and cup diagrams very easily. So, in this ball and cup diagram on the left-hand side here we have this is sort of my depiction of a complex microbial community with a lot of diversity in it. So, lots of different

microbes, all the different colors and shapes. And that microbial community, you can imagine, is a ball that's sitting inside this domain of attraction is what we call it, which is this sort of like cup. And when it's in this domain of attraction if it's a nice healthy microbial community and the ecosystem and the environment that it's in is setup to support it then that cup will be quite a deep recess.

And so, what that means is that it's going to take an awful lot of stress to push it out of that recess. Now, when you have a low diversity, so you can see here we've taken out some of the shapes. This is again my conceptualization, my terrible drawing. But you can see that some of the microbes are now like these yellow ones here, they're in there in more abundance. There's a lot less diversity there. And the domain of attraction is shallower. And that means that it takes less stress to push this ecosystem out of its equilibrium.

So, the other point that I've already mentioned is that microbial ecosystems

display synergism. So, a microbial ecosystem is not just a simple sum of its parts and functionally that's true, very much so. So, you can't just take each individual component and figure out what it does and then imagine that you can add that all together and that will tell you what the functional output of that ecosystem will be because it's going to be more than that.

The other important point to take home is that if you have repeated stressors on a system, so again starting off with the diagram that I had here, you can imagine these repeated stresses are something like antibiotic pressure, for example, if this was a gut ecosystem. And you can see how it's being pushed out of its ecosystem into a new domain of attraction with a slightly more shallow recess. And if that keeps happening repeated stresses are going to eventually push this ecosystem out until it's completely out of whack and it has no more functional stability.

Now, the ecosystem might actually

never recover from that situation without some intervention. And I think the reason I'm putting this up is because this is very relevant to C. difficile infection I think. Now, extinction events might impact health and we've heard about this as well in this workshop already about the hygiene hypothesis that we're preventing proper colonization of our bodies by being generally too clean.

The missing microbiota hypothesis is a slightly newer hypothesis which I think actually resonates very well with what might be going on right now, what we're seeing that we're disturbing proper colonization that's occurring across generations through, for example, things like antibiotic overuse. And antibiotic overuse especially in early childhood might be particularly problematic. The reason that I bring up childhood there is because there is a period of time that appears to be in early childhood around the time of weaning that the ecosystem is particularly susceptible to stress and perturbation. And after that time it seems to set itself. We

don't really understand how or why that is at the moment but once its set itself it's quite difficult to change it.

So, why in my lab are we interested in human gut microbial ecosystems since everyone's interested in this these days? But I am particularly fascinated with poo and I have been for a while from the perspective of the bugs, not necessarily from the perspective of the humans although that is obviously a big part of what I do.

Now, most studies of human gut microbes are reductionist in nature. In other words you take one or two microbes and you'll study them in detail. And you'll do maybe make up some simple ecosystems of one or two or three or four microorganisms and put them together. If you think about it this history of the study of infectious disease has mostly been done this way. Most infectious diseases, the microbes that cause infectious diseases have been looked at in a very reductionist fashion. And there's nothing wrong with that. I think it's very valuable. But sometimes

putting it back into the perspective of the whole ecosystem can be very valuable as well.

So, many diseases have been shown to be associated with this kind of amorphous term called gut dysbiosis which we haven't actually been able to define particularly well yet. We're still getting to the gut of that if you'll excuse the pun. Defining gut dysbiosis as I said is very difficult but defining a role for it in disease is even harder. And so, we really have to get to grips for that to be able to understand what might be the underlying problems here.

Now the first step in understanding dysbiosis is to understand how gut microbial ecosystems function. So, what we need is a simpler model of the human gut because it's not very convenient to take people and to expect them to be little experiments for you. I think ethics regulations would have something to say about that as well. And so, what we need is a model system that we can work with. And we've been doing that as well in mice and we've been doing that in animal

models. And again, they have their ethical stipulations as well. I think there's benefit to combining all types of models together and getting an output from that..

We have chosen to go the route of in vitro studies. And Rob's done a great job of explaining about bioreactors and so, that's great. That's set me up for what I'm going to talk about. The reason that we do that is because they're generally inexpensive compared to say gnotobiotic mouse studies. They're easy to set up and I think my graduate students might disagree with me there. It depends on how you look at it but sometimes easier to set up than say a gnotobiotic mouse experiment.

You can frequently sample the bioreactors as many times as you want and you don't have to ask it permission. So, that means that you can take lots of samples over a longer period of time. And you can very, very strictly control the factors that influence the environment in the ecosystem and you can change that again without any ethical

considerations. They're useful for mechanistic studies and as I said they lack some ethical considerations.

And now, although Rob's kind of gone into this in detail I'm going to just explain about our system that we use. Cause we don't use anything nearly as cool as the mini bioreactor system. We've kind of gone back to using the older system that George McFarlene and Glenn Gibson had sorted out. But we've kind of taken a bioreactor that's available off the shelf and we've converted it to use it as a chemostat system.

So, a chemostat looks something like this. You have a vessel in which you have a stirrer. You have a bubbler, the bubbler is there. It's basically connected to a nitrogen supply which runs through a filter to keep it sterile. And we run nitrogen through the system to keep it under positive pressure all the time and to keep the oxygen out. That's how we keep things anaerobic. We have a pH probe obviously to maintain pH and that's connected to a computer which is in turn

connected to some pumps. And if the computer senses that the pH is off then it turns on the acid or the base pump to add some acid or base to keep it at a regular pH.

We have a temperature probe, obviously, to keep it at 37 and we put in media and we take media out at the same rate. And that's how we end up with what we call a steady state. Our system setup is 400 mills as a running volume and our tension time or the time that it takes for one whole media change is 24 hours which mimics the transit time across the distal gut. And I should say that this is a system that we're set up to mimic the distal part of the colon, okay?

This is what it actually looks like in the lab. We seed it with fresh feces so we've got a lot of funny looks in the lab. And actually we offer an honorarium to our students so it's surprising how many students we have turning up expecting 25 bucks in order to give us a fresh poop sample. And they'll happily volunteer. It's a host-free system so that has its benefits and its drawbacks.

So, like I said I think that whatever we get from this kind of analysis we should always remember that it has to be looked at in combination with other studies where we maybe do human studies or maybe do mouse studies or whatever.

But the key thing is that it can be used to support growth of fastidious gut anaerobes and I'm sure you've heard about the unculturable majority of the human gut. I don't actually believe in that too much as I'll come to in a minute. And I think a lot of the problem is that we're trying to culture these microbes in isolation of their friends and they don't like that. So, we've actually been able to culture quite a lot of these unculturable microbes in vitro in this kind of setup. Just to orientate you that's where the vessels are sitting.

So, we actually in our lab we're a bit old school. We use denaturing gradient gel electrophoresis to measure ecosystem parameters. That's really because we've been struggling for a long time to get the funding

to actually do this properly with 16S and I'm happy to say that that's starting to resolve itself. But DGGE actually does have some benefits. You can follow things longitudinally and you see a much bigger, broader picture. If you look in too much detail you could get caught up in the little details and it becomes a much choppier thing to be able to see. Whereas DGGE is a much higher level view of what might be going on.

So, what we do is we run our gels. Denaturing gradient gel electrophoresis for those of you that don't know is just a way to separate DNA based on its GC content not on its size. So, what we do is we amplify up again our favorite 16S ribosomal RNA gene and then we run it on these special gels that allow us to separate the bands out according to GC content. And basically what you'll end up with then is a gel that looks something like this and each band is more or less representative of a taxon or a species within that community.

So, we can actually run that through

a specialist software program that we have and we can start to get an idea of temporalities going on in this ecosystem here. So, here we have an ecosystem at time zero this is inoculated with feces and you can see that there's similarity in this here. It drops quite a bit in the first few days but then it reaches steady state. This drop is actually representative of the fact that feces is not actually truly representative of the ecosystem in the distal gut because feces contains a lot of dead bacteria from things that you might have ingested or things that have died and further up, from further up the gut.

So, I don't necessarily think that that's a bad thing and it's been seen before by other people. So, we really do think that we have a good model. Now using the Robogut we've shown just give you a very high level view of what we've done in the past four years. So, this is four years in one slide.

We've shown that ecosystems in independent vessels seeded with identical communities and run under identical conditions

reach an identical equilibrium. Now, that sounds obvious okay and to a certain extent it is but we had to prove that this was a system that was reproducible. Secondly, ecosystems seeded from different donors retained distinct characteristics in vitro and I'll show you a little bit about that in a minute. And third, defined communities retained functional elements of the native communities from which they were derived. So, in other words if you define a derived community from a native community then there are elements of that that seem to be conserved as you go on in terms of its functional output and its behavior.

So, one of the things that we've done is that's sort of relevant to the C. diff world I guess is we've been looking at modeling ecosystem perturbation. And what we've done here is we've taken ecosystems grown up in the chemostats that have been seeded from fresh feces from two donors. We call them donor 1 and donor 2. And what we do is we bring that up to steady state. It takes about 36 days, quite a bit longer than DGGE

setup. Unfortunately it does take a while and it smells quite a bit so it's a shame this isn't Smell-o-Vision. I'm sure that you'd get a lot out of that.

So, what we did is we took a control vessel that we added nothing to apart from PVS and we added clindamycin to another vessel over a period of time. And we wanted to see and we did that according to a clinically relevant regime. So, in other words we used a concentration of clindamycin that has been shown to be pharmacologically cumulative in the gut in the average person's treatment regime. And then what we would do is we would sample daily. We extract the DNA, we run DGGE and then we do the analysis. And in a nutshell this is kind of what you see.

So, the green line here is where we added the clindamycin and the red line here is where we stopped the clindamycin. So, you can see that donor 1 reached steady state and steady state is defined above this blue line. If the black stays above the blue line then that's steady state. You can see there's a

bit of a dip there but that's not significant.

And this donor had a rapid change very, very quickly after clindamycin was introduced but after clindamycin was stopped, popped right back up again to the sort of baseline if you like. Whereas donor 2 is more, is interesting. Clindamycin treatment meant that the ecosystem did change and it dropped in sort of a two-step model there, a two-step phenotype. But interestingly, after clindamycin was taken away and the system was left to wash out it never quite returned to baseline. So, that was quite interesting and just shows that patterns of perturbation differ between donors.

So, not only is it really, really complicated to look at different ecosystems but it's even more complicated to try and compare different donors as well. There's a level of complexity there.

I just wanted to point out that microbes, as I said about my daughter Phoebe, they usually prefer to grow with their friends. Microbiologists however prefer to

grow microbes on their own because it's easier, cheaper, it's much simpler. Now, there is no such thing as an unculturable microbe in my world and I teach this to my students as well. But there is such a thing as an unimaginative microbiologist. I think we're only limited by our ability to sort of translate what we think about the way that microbes grow into the laboratory.

The key to growing unculturable microbes is to grow them with their friends or to trick them into thinking that their friends are there. So, back to the analogy of my daughter, if I'm going to stick her in a room on her own, she'd be much happier if she had a laptop or her phone or something so she could FaceTime with her friends even if her friends aren't exactly there.

So, we have become fairly good at growing microbes or unculturable microbes or any microbes from the human gut. And we can't grow them all yet but we are learning and the more we try the more we get. So, Elaine Petrof is pictured up here, asked me, this is

a few years back now in 2010, called me up one day and she said she has this clinic full of C. diff patients and she was starting to do fecal transplant but finding it really gross and I'm sure that many of you do. And so, she knew that I could culture a lot of these microbes. And she said, can I just use some of these cultured microbes to make fake poop? And her idea was to just sort of go into my freezer which is fairly expensive and pick out half a dozen things and stick them together and put them in the patient.

Now, in the past I believe that the barrier to this was this perceived unculturability. But if we can overcome that then the idea here was to develop this fecal transplant concept further by using pure bacteria. So, it's a bit more like a probiotic approach. But it's not your average probiotic and thanks to Gregory we've called it rePOOPulate and the name's kind of stuck and it kind of went out there with the paper as well. And some editors had an issue with that but I'm glad we kept it because it keeps

it stuck in your mind. If we did that then it should mitigate fears about safety, reproducibility, delivery and shelf- life. And so, we call this a microbial ecosystem therapeutic.

Now, our healthy donor was a healthy female in her early 40s. She had an average BMI, very healthy lifestyle. Interestingly she was born and raised in India. She'd had very, very few or no antibiotic exposures in childhood. It was incredibly difficult to find a donor who had no antibiotic exposures. She'd had one reported exposure to antibiotics in the last 10 years. We actually took a fresh sample of feces and we put in a Robogut to allow us to culture as many microbes as we could. We've actually got about 70. We've done better than that since but for this particular donor we've done about 70 strains.

We profiled them all for antimicrobial resistance and we theorized that we should one donor for one ecosystem. In other words, I shouldn't just go to the freezer and pick out a handful of bugs but I

should actually think about how nature has made this ecosystem in this patient, in this donor, and so if we derive all the microbes from her ecosystem and put them together then that should function as a proper ecosystem.

So, this is what rePOOPulate looks like. Two things I want to point out. The first one is that you'll see that these are closer species by full-length 16S alignment. I have made a point of not calling these the species just like we heard Rob say I don't believe that we should be speciating things because it doesn't tell us very much. It tells us a broad range about their physiological attributes but not much else.

The second thing is that you'll see that for a couple of strains we had two or more that identified with a particular species. Well, we keep being asked why did we put them both in? And the reason is that they were both in the original ecosystem so there must have been some force that was shaping that ecosystem for some reason with those two or more strains in there. So, we didn't see

any reason to leave them out for now until we know more about functionality.

The next thing I want to point out is that a lot of these ones in red they're all lachnospiraceae family species. Why is that important? Well, you've heard Vince talk about lachnospiraceae. Happen to think they're one of the most important groups of bacteria in the human gut. They're extremely oxygen sensitive. Many members are very, very hard to culture but we've had a lot of success with that thankfully. So, we think that stability, prevalence and potential functionality of lachnospiraceae make them pivotal in maintaining gut homeostasis and health.

Now, interestingly when we've done the same experiment where we've derived experiments with ecosystems from ulcerative colitis and Crohn's disease patients, we're never able to get a lot of lachnospiraceae out of there. This is, I'm going to really say this very quickly cause it's in our paper but we actually put this ecosystem that we made of

33 species into two patients with severe refractive C. difficile infection. In the first patient, now both of these patients their inciting antibiotic was Cefazolin, the first patient was through a hospital procedure for a knee arthroplasty. And she had several rounds of C. difficile relapse before she was referred to the rePOOPulate study. And then you can see that very shortly after she was referred here at this point to the rePOOPulate study, we took samples and we did some testing of the toxins. And that's how it's done in Canada.

And Elaine actually she did this testing two or three days after we'd done the procedure and it came back negative. She didn't believe it so we did it again. And it came back negative again.

Now, the patient is fine. She was done in April 2011. So, we are more than two years out now. She's still doing really, really well. What's interesting is that after she had had her procedure and she came back for referral, that's when Elaine learned that

her GP had actually given her several rounds of antibiotics for UTI infections which are quite common in elderly ladies. And yet, she still remained free of C. difficile.

Now, what was interesting as well is when we did the same treatment for patient two who had again C. Difficile disease brought on by Cefazolin treatment for cellulitis, a sort of similar period of events here and after rePOOPulate treatment she also had several rounds of antibiotics for cellulitis again that did not reincite her disease, C. diff disease. And she was done in June 2011 so we're nearly two years out on her, too.

This is in the paper so I'm going to breeze over it very quickly. All I want to point out is that here is rePOOPulate here in these two columns. This is patient one, patient two. This is the pretreatment sample that we got from each patient. You can see it's quite different and you can see that by day two, week two, week four, month six, you can see how things are changing at the time.

Now, when we first did this work we

couldn't understand why there was so much chaos here and here. Now, we know that there was subsequent antibiotic exposure so that must have something to do with it.

The last thing I want to point out about this work is that in patient one but not patient two, if you look at the patient profile at day 14, at the same time we also put the rePOOPulate ecosystem into the Robogut. And at day 12 in the chemostat you can see that these two profiles match pretty well. So, that shows that after 14 days you can see this matching. That wasn't the same for patient two but it does show that the chemostat represents a good surrogate for in vivo work.

This is my favorite blog that came out after we did this study. I do love that title. I think that's really good. Just wanted to show that.

So, how does rePOOPulate work? I don't really have time to show this because I see my red light's come up. So, I'm just going to point out that what we did is we did

an experiment where we added two ecosystems together, two healthy ecosystems together in the Robogut and we showed that one displaced the other quite rapidly. So, I'm not going to show you that experiment and I'm just going to flip through to where are we now?

Well, we're waiting to continue our clinical trial. We've only done two patients but we have another 20 to do over the summer. It's difficult because there's no drug class yet for rePOOPulate and Health Canada has actually been very, very helpful and supportive and trying to work with us to determine our safety parameters. We're tweaking the formula. So, we're including bacteria that have anti-C. Diff activity in vitro. We're doing animal and Robogut studies mimicking ecosystem destruction and subsequent repopulation.

RePOOPulate II is now being prepared from the donor that gave us the ultimate fighting chemostat, the better ecosystem that displaced the first one, new and improved, methink. We're doing metagenomics and

metatranscriptomic studies and we're looking ahead at the potential for using MET to treat other diseases. We have got dysbiosis as a key feature including IBD, obesity, regressive autism, et cetera.

So, I'll finish with this. What should an ideal therapeutic ecosystem be? We think it should be safe, defined, controllable, reproducible, stable and deliverable, acceptable, available, and effective. And I think you can see that standard probiotics although they fit all of that perhaps the most important one being effective, they can be a bit iffy.

Fecal bacteriotherapy has some definite issues with reproducibility, controllability, although we'll hear from Dr. Kuritz tomorrow. I'm sure he'll tell us otherwise but there's still some issues about it being acceptable. Whereas we think MET is really ticking all of those boxes.

I'm going to leave that and I am just going to finish with this. I foresee a time when gut microbial ecosystem functional

screening will be a critical component of all comprehensive medical checkups. It will be possible to enhance ecosystem functionality to maintain health by manipulating the microbiota. And broad spectrum antibiotics will not be used without measures to protect the microbiota.

I think the symbiontology will become a new medical specialty. Okay, so with that I'd just like to acknowledge the people in my lab, the people that have helped me and all the people that have funded me. Thank you very much.

MR. RELMAN: Are there questions for Emma before we start the panel discussion? Eric?

DR. PAMER: One of the things that we know from pathogens is that as they get passaged in vitro they can eventually lose their virulence. And so, often we passage them through animals again so that they maintain that. And I'm wondering if you think that might become an issue with commensals that are passaged for prolonged periods of

time in vitro and will they potentially lose their effectiveness?

MS. ALLEN-VERCOE: Yeah, that's a really good question and the answer is yes they will become lab adapted. And I think that in many ways, one of the things that I didn't say is that when we were doing this experiment putting the ecosystems into humans we were told by our IRB that we had to scrape biomass off plates after we'd grown each ecosystem individually.

Well, I took issue with that because as an ecologist I think that's a really bad idea because the minute you try and grow microbes on their own they start to drift genetically. They start to change because they're adapting to a lifestyle that's not necessarily what they're used to. And so, I think what would be a better way of doing it is if it was the whole ecosystem was grown in a chemostat, that is with their friends and where the pressure to conform to a different state of being is released, then perhaps that genetic drift will be I'm sure it will still

be there to a certain extent but perhaps it won't be quite so bad.

And the other thing is that when you're working with these ecosystems, if you take each individual strain you can actually freeze it down and keep it so that you can always go back to something that hasn't drifted if you needed to in the future.

MR. RAY: Hi, Arnab Ray, gastroenterologist at Oxner Clinic in New Orleans. Thank you for that very informative talk. In your quest to find the ideal ecosystem do you take into account things like how someone was born, how they were breastfeed? You know if you were born vaginally versus C-section, if you were breastfeed versus formula feed as we think that those adjustments seem to make possibly a healthier microbiome?

MS. ALLEN-VERCOE: Yeah. And when Elaine and I first had this idea how to do this we thought great, this is going to be a great idea. And then the first hurdle was how do we pick someone who's really healthy to

make our super donor? And that was something that we wrestled with. And actually we were more focused at that point on antibiotic use and we really didn't want to find anyone who'd had any prior antibiotic use. And as it turned out that was impossible. We couldn't find anyone that had had no exposure to antibiotics and we were very lucky with our donor that we had that she'd had so few.

I've since given a lot of thought to the post birth, the period of being an infant and I think we were just supremely lucky that our donor was breastfeed. She was born vaginally and she was born in rural India. Who knows that could be, you could think of it as good or bad. It depends what she's been exposed to but certainly when we looked in her microbes that we recovered from her there were some that were species that could be considered to be opportunistic pathogens.

And for all of the people that we have cultured we have always found those kinds of things. And I don't think we'd find an ecosystem that has no opportunistic pathogens.

But then that begs the question what is an opportunistic pathogen cause you can't just decide on what that is based on a name as we've been told.

So, I think that in the future as we go through we're trying to find our sort of super donor. The way that we're approaching it now is to maybe look functionally at the ecosystems and how they interact with each other, maybe finding our dominant ecosystem. And although I didn't really a chance, I just kind of breezed through it, I think if we can actually look at the ecosystem and how powerful and how, powerful is the wrong word, how resilient it is, how functionally redundant it is and maybe there's an ecosystem hierarchy. And what we want to do is we want to find those ecosystems that sit right at the top of that diagram that I showed you in a very, very deep recess which are very kind of set and very stable. And so, that's kind of where we're going.

MR. RELMAN: Vince.

DR. YOUNG: Vince Young, University

of Michigan. Great talk. As you were isolating these organisms, you were isolating them alone, literally isolating it. And so, have you -- there are ways in which people try to look at dependent organisms, cross-feeding, you know advanced culture using that these two only grow. Was that ever tried and do you think that's useful? And sort of related to that is just from counting, when I first read the paper, there are a lot more Firmicutes than Bacteroidetes. Is that reflected in the microbiome of that -- in the fecal microbiota of that patient?

MS. ALLEN-VERCOE: Okay, so I'll answer the second part of that question first because it's easier. Actually we got a lot more Bacteroidetes out but Elaine was not happy about putting some of those Bacteroidetes strains back into the patient. So, she went by, I mean we were kind of going blind here. She wasn't quite sure how to do it so she decided she would look at the list. And we'd done all the antibiotic profiling so we'd made sure that we got ones which were

sensitive.

And then she would, based on the name which now we now is probably not the best thing, but based on the name she would decide if it was something that she would put in her mum, then it passed. And if it was something that she wouldn't put in her mum then she would leave it. So, there were other Bacteroidetes. There was Bacteroidetes fragilis, vulgatus, thetaiotaomicron and they're all in there. So, we took them out but I did draw the line at her taking them all out because I think that it's quite important to have some in there.

The first part of your question, just remind me.

DR. YOUNG: Organisms that are depending on the other one necessarily for us to cultivate.

MS. ALLEN-VERCOE: Yeah, so we actually have got a lot better at cultivating things since we did this first ecosystem using that principle exactly. What we do is we actually take the waste material from the

chemostat which we call liquid gold cause we don't believe that it's waste. We think it's actually very valuable stuff and a bit like rumen fluid is used in sort of microbiology of old. We put it back in and then we're able to cultivate a lot of other organisms.

And so, it is a lot of hard graft but we're also as we're learning things and we're getting genomes and things we can actually start to improve the ways that we culture these things.

MR. RELMAN: Okay, I'm sorry was there another question. Yeah, sorry.

MR. HENN: Thanks, yeah. Great talk, Emma. I'm curious of your thoughts on how reductionist you can actually be in your systems. Cause you know the response is quite variable.

MS. ALLEN-VERCOE: Yes.

MR. HENN: And I'm curious how far back you think you could pull that.

MS. ALLEN-VERCOE: And I'm always asked that question. So, I am not against going more reductionist, don't get me wrong

but I think that before we do that we have to kind of evaluate what the functional output of the ecosystem is and the damage we do by taking things out. Because there will come a point when we'll just be using another probiotic, you know? And so, we're trying to make an ecosystem that is therapeutic here not just a bunch of beneficial microbes.

So, I'm not sure. I mean the first target is obviously those species where we have more than four, more than two isolates. We put them in and we put in more than four, as I said, because we think that the redundancy is quite important. Then that they might because they've been maintained in that original ecosystem there must be a reason for that. But now we have, we've now got genomes for all of them. We literally just got them. So, we're just going through to see how much overlap there is.

And but the beauty of doing this kind of thing is that we can set up, we can make hypotheses and we can set up ecosystems in the Robogut and see how they function. And

we can see maybe at what the point things will start to collapse and that's kind of the limit of what we've reached.

MR. RELMAN: Yeah.

MR. BRITTON: So, I had a question about how you define antibiotic resistance. So, and the reason I'm asking is so a lot of lactobacilli which are commonly used as probiotics are actually vancomycin resistant but they're not vancomycin resistant because of the plasma. It's because they have a change in their active site of their DDL ligase. So, they put lactate in their animal peptides instead of alanate.

So, were you only looking for antibiotic resistance that was horizontally transferred or did you just as this point go for everything cause you're having complex --

MS. ALLEN-VERCOE: What we were looking for was sensitivity to vancomycin and imipenem, at least one of those at least. Because those would be the two antibiotics that Elaine reasoned that she would use to clear a patient if everything went wrong. But

you're right. There are some microbes that are intrinsically resistant. And in the case that they're intrinsically resistant we weren't worried about that. Like the Bacteroidetes, the fact that they're vancomycin-resistant that's okay. And we would only use them if, in fact it turned out that most of the microbes that we isolated were sensitive to most antibiotics. So, that was a good thing too.

MR. HWANG: Chuhern Hwang, University of Virginia. So, I know you went over this very briefly. You had a slide in which you talked about how when you introduce a new bacterial ecosystem, a new microbiome, that essentially instead of having an average there was essentially the introduced one replaced the existing one. And I was just wondering whether did you use a more diverse and robust, I suppose ecosystem to replace the original one? And if so, have you tried it the other way around?

MS. ALLEN-VERCOE: Yes. And I apologize. I rushed over that very, very

quickly. But yes we are getting a complete displacement. So, we have done some sequencing work. Actually we've done phyla chip experiments as well with Willem DeVos at Wageningen University that has shown that. We are definitely getting a displacement of one ecosystem with another. So, we're not getting an average mix.

Sorry, the last part of your question?

MR. HWANG: Is it usually the more diverse ecosystem that replaces the other or?

MS. ALLEN-VERCOE: It's -- we don't know yet. So, the next experiment that we're doing which is actually planned to be done over the summer is to take the defined ecosystems that we derived from each of those people, each of those donors and to do the same experiment. Now, when we do that we'll try to match phyla and to match the diversity so that we can really test that. But we haven't done that experiment yet.

MR. RELMAN: Maybe one more, Alex and then Vince, two more.

DR. KHORUTS: Maybe this is a related question. But does this simplified microbial community, do you think it acts as some sort of scaffolding to build up the entire fully diverse set? Or have you looked at later time points? Does it keep growing?

MS. ALLEN-VERCOE: You know, I'd love to look at the later time points but our IRB has only allowed us in the patients anyway to go up to six months. In our next application that we write we'll try and see how far they'll allow us to push that.

My own thoughts are actually that what we're making here is a kind of a long-lasting Band-Aid. And if there is anything functionality left in the ecosystem of the patient, it depends on how trashed it is before you go in there to sort of fix it. Then over time things might equilibrate and if the microbes have evolved with their host, you could argue that they're better suited to that host and they might have, given the chance they might come back. But we don't know the answer to that yet.

MR. RELMAN: Vince, did you have one?

DR. YOUNG: Yeah, just sort of as a final comment and maybe I'd get your opinion on this. As we deal with these complex systems, you know we always talk about this idea of emergent properties. And the question is about antibiotic resistance and this will dovetail into this idea that we're talking about how to regulate things like fecal transplant or these complex communities.

When it comes to antibiotic resistance what we see in the community is often different than what we see in the individual organism on a plate. And a couple of things that come to mind that I've seen several times in the literature giving vancomycin and you see a depression in Bacteroidetes which you'll never see if you try to treat B. frag with vanco on a plate. It doesn't care. Or these blooms of enterobacteriaceae, I have grown several out of patients and out of mice and put them on Biolog or Vitek and they're "pan" sensitive

and they come up under the pressure of eight antibiotic.

So, I think that's something we need to think about as we talk about how are we going to test, regulate, validate, QC things like this. The standard ways that we have to do may not hold true when we're talking about communities and I don't know if I -- you're nodding your head so I think you sort of agree with that.

MS. ALLEN-VERCOE: Yeah, I concur, yes. No, I do agree with that. I think that we're going to have, however this falls out we're going to have to define a whole new way of thinking about things and we can't go back to our set of fallbacks because they're just not relevant to looking at ecosystems, yeah.

MR. RELMAN: If you don't mind I think what we want to do is transition to the panel. Hold your question because I think this last issue might be a nice beginning point for a group discussion. And so, if we could call upon the morning speakers and the afternoon speakers to come down here, we'll

have a chance to entertain more questions and talk about some of these same issues.

All right, we almost have more people down here than we have up in the audience. No dearth of interesting questions. I'm afraid that we're going to have many more of those than answers. Among the general broad issues that have been discussed at least this afternoon there are issues of properties that we wish to restore and maintain and how to measure those and whether properties ought to be measured individual isolates and then somehow understood from the collection of those isolates or whether there are different kinds of properties that can only be measured with a true community as the substrate. And if that is the case then how does one go about developed assays to measure the kinds of properties that we might really care about such as colonization resistance or resilience. That might even be an easier thing to measure than some of the others.

And really to understand what is it that we're trying to restore in someone who

has any of these various disorders that are associated with altered communities that's a very hard thing to know. I'm a little bit sanguine about the breadth and kinds or properties that we really do need to be cognizant of and monitor before we can be assured that someone's been restored to health or not despite what they tell us. Despite the fact that they say I'm having a normal bowel movement and I'm feeling pretty good and I want to get up in the morning and do my thing.

We don't know whether they have in fact because of a transplantation or a facilitated recovery of their own microbiota whether they have lost something that they will only know when they encounter salmonella next week or a water supply with a lot of selenium next year or a very different diet. And I think one of the interesting questions that we can begin to monitor now given that there's already a lot of growing experience with these transplantation events is post market monitoring of all of the various kinds of features that are associated with the

microbiota that we care to try to understand and follow and see whether these things really have been maintained now over some increasing periods of time.

I've just heard through the grapevine through folks here and others not here that some of the C. diff patients who have been cured of their diarrhea don't necessarily have such a stable new state when they get a whiff of an antibiotic six months later or when they travel or are subjected to some kind of stress that has some ill- defined effect.

So, the property issue is one. Another is going to be safety and we've heard a lot about different ways of thinking about safety. The one I kind of like the best is what would you do to your mother? You're going to get a lot of answers to that one. So, I think we probably need something a little more standardized and easily measured.

SPEAKER: What would you do to your horse?

MR. RELMAN: What would you do to

your horse or your dog? Maybe that's really the hardcore question. But again, we've got short-term safety issues and we have long-term safety issues that get to some of the comments I just made. We haven't really talked much about those but a few people have already mentioned well what if there is some host associated physiologic phenotype that can be cotransferred with the microbiota to some degree?

Although I'm a little bit cautious about thinking that that can be done so easily with the microbiota alone. But suppose that the person who gets the microbiota from somebody who had a proclivity towards metabolic syndrome also for some reason adopted that kind of donut, diet of the donor and started living that kind of lifestyle. Well, then maybe that phenotype would be transferred and that is something that we have to really be cognizant of.

So, there's the safety issue and then maybe with both of these the whole issue of how to standardize and develop reproducible

data at the least so that we can at least have some confidence in whatever it is that we think we've answered with either of these kinds of questions. So, that's my little tirade.

Comments from the table here or comments from elsewhere or other questions you'd like to raise, the stage is open. If nothing else we do know there was a question until we battered him into submission. Up there.

SPEAKER: Anybody that knows me knows I'm not easily suppressed. This is an ecological question. It's got to do with the whole extinction thing that we're beginning to study more as we extinguish species from the planet. I'm a little worried about the idea that we just replace four or five and eventually they will take and change the ecosystem back to what it was.

I think like many of us here in the audience we're thinking that the only thing that really gives the patient a complete shot at restoring their previous ecosystem is to

give every bug that's there or that could possibly be there and then let the ones sort it out that fit in the environment. And they'd be the ones that would stay long-term. I mean, to me it's all the organisms even realizing that in some people some of them will survive and some of them won't but they survive because somehow they're compatible with that person from the time that that person starting getting exposed to organisms.

So, we need to do that. I don't think we should feel like we can replace two or three species and maybe the probiotic experience has already taught us that. That we've got to actually give all of those bugs even if they sound bad because if they are things that we need in that balanced ecosystem at the end and they've been extinguished, you've got to put them back before it's going to go back to balance, I think. I mean that's just an opinion at this point but it's a hypothesis that should be discussed further.

DR. BAKKEN: A counter argument to the point you're making would be the published

report by Michael Tvede in Copenhagen in Lancet 1989 where they published six patients that were treated with synthetic stool, 10 selected organisms from the stool of some 200 healthy children were instilled and all the patients responded well in a durable fashion. Now, the follow-up to that study is that it is the only treatment modality that is currently being used in Denmark. They have more than 30 patients on record all the majority being successfully treated. I think their success rate is in the neighborhood of 80 percent.

There is an ongoing multinational study in Scandinavia using Michael Tvede's cocktail. And so, 10 organisms is more than five but it's still a very limited number based on what lives in the colon. So, just that as a counter argument.

MR. RELMAN: Thanks, Johann. That was Johann Bakken from the University of Minnesota. I was just reminded that if you could please identify yourselves when you make a comment. So, Larry.

DR. BRANDT: Larry Brandt, New York.

The problem that I see that one balanced ecosystem is not the same as another. And we will do a fecal transplant on someone using a healthy donor, balanced system and we won't get a positive result. So, we'll change donors and use another healthy donor, another balanced ecosystem and will get a positive result. Is it because it was the second and had we used the first one again maybe that would have worked? Or is there a particular organism that alone or in combination with the other organisms is responsible for the success of that particular mission?

I don't really know how to solve that problem but I think that in some patients 33 species or 50 species will work. And in another patient it may not work.

MR. BRITTON: To just address Johann's point, I think that it could be either way especially with the point brought up in the back. It depends on what the patient really needs. If it needs, the person just needs to be tweaked a little bit and maybe the 10 species can at least get the

disease out of there and let their normal microbiota reestablish then it probably can be a small collection. Whereas people and in some of Vince's studies and some of the people with recurrent C. diff, I mean they really have a very low diversity and it's almost like they'll never come back.

And that group might need a more diverse community. And I don't think we know enough to know what that would be but --

MS. MANSFIELD: Linda Mansfield. I think there's a little experience that we could gain from germ-free mice that have been colonized with Schaedler flora which is a limited flora. And they tend to be fine and to live stably. But they often acquire additional community members and they seem to be very susceptible to that. And so, they would likely be more susceptible to pathogens coming in I would guess. Maybe Vince could talk about that because he's used them.

MS. PROCTOR: Well, I'm actually trying to address some of the common ideas that are floating around. So, before I was

the manager for the HMP I actually was a card-carrying microbial ecologist. And way before there was any sequencing tools at hand the environmental microbiology or microbial ecologist field were actually measuring community level property. So, I didn't really know who the players were.

And examples would be measuring hydrogen production or hydrogen consumption or some kind of community level property so you could assess how the different members of the communities were interacting. Now, the idea is that the aerobes consume the oxygen and that when anaerobic metabolism kicks in then many kinds of metabolic byproducts are produced that then become substrates for the rest of the population. There was this sort of concept called geochemical zonation where microbes conditioned their own environment to actually then allow the growth of microbes that grow in more and more reducing conditions.

And we knew that. But we had no real tools to assess who the players were.

So, I think by default in the early field of microbial ecology the ecologists had to actually measure community level functional properties. So, probably this is where the field is going and we could probably gain quite a bit from this earlier literature measuring community level properties of microbial communities. Looking at things like hydrogen production or methane production or other kinds of geochemical end member products as a way to maybe define what is stability, what is resilience and what is an ecosystem property that we can measure reproducibly without getting into the nitty gritty of who's necessarily the membership. So, that might be something, that's just sort of an idea I want to plant and I think maybe if we go the microbial ecological literature we could learn quite a bit especially from that earlier literature.

MR. RELMAN: I agree with you, Lita. And I think that probably many of those community level are system-wide features that are probably important. And it also touches

on a point made earlier about the individualized kinds of needs and the individualized features of each host as a particular environment in which one system may have a proclivity for doing one thing and less of a proclivity for doing something else.

MR. SCHENTAG: Yeah, David. Jerry Schentag from the University of Buffalo, by the way. I think that it might be possible to divide this argument a little bit though and some of your comments reflect that. If our only goal is to replace something which is overgrown like a C. Difficile that's been knocked out, been expressed because we've knocked out all the bacteria that'd ordinarily suppress it with an antibiotic, you could theoretically just go in and replace the bacteria that are sensitive to that antibiotic, were therefore killed, restore the balance and then you'd probably get rid of the C. difficile. And I think that might be why we're having more limited success sometimes with smaller numbers of organisms.

But if the goal remains replace the

entire flora that's been extinguished you still have to give all the flora a chance to be reestablished by giving them all I think. And that's maybe how I divide the argument. It depends on the goal; long-term correction of dysbiosis may be a lot harder target than simply getting rid of something that's overgrown.

MR. RELMAN: Yeah, I agree completely. In fact, I think that this scenario of intractable *C. difficile*, recurrent *C. difficile* disease is a very particular kind of problem whose clinical outcome we're measuring in a very simple way which is resolution of clinically evidence disease. And there's a lot more that we hope comes along with the elimination of disease features and the restoration of health. But we're just hoping that that all kind of comes along for the ride.

Johann?

DR. BAKKEN: Yeah, I just wanted to add that restoring the healthy microbiome is something that takes a long period of time.

And it certainly takes longer than the resolution of the diarrhea state with C. diff. Whether it's by 10 organisms like Michael Tvede or rePOOP or through fecal transplantation, when you do serial studies analysis after the patient has restored normal bowel habits you will see that the diversity is still reduced and the phylotypes are still in lower numbers than what you have in health.

So, in a sense, we are what we eat and we restore our microbiome by what we eat over time through vegetables, through grain products, through the food we put in our mouth.

MR. RAY: Arnab Ray, Oxner Clinic, New Orleans. Just to try to flip the conversation a little bit and bring it a little clinically, Emma you mentioned basically creating this deforestation of our environment. And in clinical practice we have these patients who are placed on these pulse tapered doses of vancomycin over months at a time, not ever been shown to be clinically superior but it's been done all the time.

I'm wondering whether we're perpetuating at state of dysbiosis creating worse situations for ourselves and whether we start clinically arguing against this because we're creating this depleted state. Cause when we do our fecal transplants in recurrent patients I'm not seeing pseudomembranes and severe toxins. I'm seeing normal looking colons and I feel like I'm just treating dysbiosis as much as I'm treating maybe recurrent C. diff.

MS. ALLEN-VERCOE: Yeah, I suppose the way that I look at this and I'm not a clinician but the way I look at this use of antibiotics to treat a disease that's caused by antibiotics is I'm a gardener. So, I look at my lawn, right and I think, okay, if I've got dandelions in my lawn I don't set fire to my lawn. And yet, that's exactly what is being done when you use an antibiotic to treat a disease that's caused by antibiotics.

And so, I agree in many ways. But I also can see the clinical side of things that sometimes it works and it just depends on, I

guess, we don't know this but it depends on the extent of ecosystem destruction. And so, I think, you know in an ideal world, if you had all the money and time in the world then each patient should be looked at -- that there shouldn't be a one size fits all treatment. And each person should be looked at in terms of, you know, if it was possible to do to actually spend the time to look at their ecosystem and see what's lacking and then tailor an ecosystem replacement to kind of act as a Band-Aid for that.

But I don't see that happening in the short-term cause it's going to take a lot of time. I don't think we know how to do that yet. But I do think that's coming in the future and I think maybe that's where we have to have an eye to.

DR. EISENSTEIN: Barry Eisenstein, Cubist. To put a maybe regulatory spin on which perhaps we're going to get to tomorrow but at least from an industrial clinical perspective I spend a lot of my time thinking about getting drugs registered and have gotten

off several discussions about what we call endpoints. Given the extraordinary complexity of the microbiome and given the complexity in knowing what reconstitucional events are going to be associated with what potential later-term events including chronic diseases like, well obesity is not really a disease but leads to potential disease states.

How do we understand what we could say these surrogate markers of biological measurements in the GI tract, how do they reflect what the clinical endpoints we really care about later which Bob Temple from the FDA has described as feelings, functions and survives as the key elements, how do we make the connection between the surrogates that have never, as I understand, been fully validated in this extraordinarily complex system with endpoints that patients and caregivers care about?

DR. YOUNG: That's a tough one, right? I guess I wear a stethoscope sometime and so do you and a number of us do so. I mean some of the endpoints with regards to C.

diff specifically, I mean obviously resolution of diarrhea, those are the things that you can do. You can look at, you can get a little fancier say you want to look endoscopically look for healing of the mucosa, loss of pseudomembranes, et cetera.

But it's hard for us to know what are the surrogate markers of the "healthy" microbiome because those are functional probably. But the one that has been bandied about and I am guilty of it because I put this in the title of a couple of my papers is this idea of diversity, right? Ecosystem diversity. And just for everyone in the room, I've been, I guess what's the politically correct -- lectured by macroecologists. There is still some debate and I like using the macroecologic kind of surrogates when we're talking about the microbial communities.

But some people say that the principles that they worked so hard to study there don't necessarily apply to the microbial communities because of differences in how microbes are versus the "charismatic"

mega-fauna and mega-flora that we have based on our ecology on. So diversity is one that we use and we generally say that higher diversity is better but that also can be somewhat of a misnomer, too.

And so, we just don't have these simple surrogates for what is a healthy microbiome but probably the more we look at function that's the type of thing we need to do. But we're asking a lot too. For example, from cardiology, if someone is going to the FDA to approve a stent for someone who has coronary artery disease, it's going to be evaluated on how long it should open that blockage before re-stenosing. It's not going; you're not going to ask that stent to change the person's cholesterol and high blood pressure.

But in some ways that's what we're asking the microbiome to do. Because the microbiome influences everything, we have to be careful that we don't give them diabetes, inflammatory bowel disease, autism, et cetera, et cetera. So, maybe in some ways we're

asking for too many surrogate markers. I don't know. That's not an answer. It's actually raising more problems but I think that's what we're doing.

DR. EISENSTEIN: No, I think you are pointing the complexity and I completely buy the acute care issues with C. diff. It's the later aspects that become much more difficult to get one's hands on particularly at a time when the science of the microbiome is still early.

MR. RELMAN: Yeah, Barry, I'll just say it's a really good question and deserves a lot more time. But I think one way to look at it in a general way is to ask well, if there is this close relationship between these communities and the host, you could in essence ask the host are you seeing what you would consider you? This particular host would consider a healthy set of companions. You know, a community that resonates with the normal set points in this host tissue.

So, you might someday soon maybe look at host response factors following

transplantation and say, am I now seeing the signaling and sort of gene expression and metabolic features that are associated with the normal homeostatic state where my normal indigenous microbiota is there and I'm recognizing it as such. And I'm making more questions.

DR. EISENSTEIN: Yeah, I'm sure we're going to get to this tomorrow when we hear from the FDA but again a lot of those are surrogate that may or may not be relevant.

MR. RELMAN: Yeah, yeah. Yes, in the middle. There's a lot in the middle.

DR. GRAHAM: I'll do. David Graham, Houston, gastroenterologist. You know I think that we're a little off where at least the clinicians live. I mean the horse is clearly out of the barn. People are taking somebody's stool and giving it to somebody else.

And so, the concern that I have is not whether they get fat but whether they have another disease that those of who lived around long enough and know that when we used to endoscope and didn't sterilize our instruments

we know we passed a lot to helicobacter. And we know we probably passed hepatitis C and whatever else was out there. And they're still actually doing that when the sterilization breaks down with the instruments.

And so, when you're giving somebody stool I'm more worried about what else they're getting that's going to make a major effect on their life later. When I was a student long ago and we were treating infectious diseases, I mean we were of course told not to use antibiotics unless we needed them. And then when to use antibiotic use the narrowest spectrum for the shortest time. And now as you know our current house staff use like three antibiotics for the longest time. And we were told not to do that because we were screwing up the body's protective bacteria which was protecting their lungs and their other parts of the body and that when you followed those patients that you could actually measure super infections and worse outcomes.

So, we're worrying about poop. And yet in our emergency rooms and in our hospitals they need to worry about everybody get treated for white count and fever and the fact that these other microbiomes is much more prevalent and important than the few patients who happen to have C. Diff. Where what we need is an effective therapy when a patient walks in the door, we have something we can give them that makes them well and that has low relapse rate and doesn't cost very much.

And we have none of that right now which is why physicians and patients have gone to this new technique of getting feces taken in any way they want. So, I think this is neat to talk about but it's going to happen and I think we need to worry about how to make it happen efficiently and then we'll worry about the details of the science later if there's people still interested. There may not be.

It's just like my favorite example is the guys that did serum therapy for pneumococcal. When penicillin came along they

said, I have questions but it took a while before those questions now are relevant again.

MR. RELMAN: Any comments?

SPEAKER: (off mic)

MR. RELMAN: Yeah and I think to a large degree we get that too and there's a very acute need problem here. The only reason we're actually sitting here in part is not just cause it's an interesting questions but because there are a lot of very sick people out there for whom there was really very little and now there is potentially something. And it seems to be a lot more complicated and potentially carrying some costs that we just don't understand.

And yet, everything's a tradeoff. And in this particular acute situation the tradeoff may be at least for the short-term positive until we figure out the reasons why it might not be.

MR. BRITTON: But yeah, so as one of my colleagues put it though, and I do recognize that yes we don't necessarily to know how they work to use them. A lot of

drugs we don't really know how they work and we still use them quite a bit. But the first time somebody comes in for a fecal transplant, gets cured let's say of C. diff and a year later gains 60 pounds and has Type II diabetes, it doesn't really matter if the fecal transplant caused it or not. The first call to the attorney is going to I need \$100 million for my pain and suffering. And not to really joke about it, I mean that's going to probably have hospital administrators up in arms. You just cost us \$100 million because you put in an unproven therapy.

So, I think that that's an issue that we have to think about.

DR. GRAHAM: I have a good friend in Japan, I do helicobacter and so I've gone to Japan every year for 30 years. And about 20 years ago I told him he needed to get his helicobacter cured. And he had heard that if he got his helicobacter cured he may get gastroesophageal reflux and Barrett's esophagus and die.

And so, the year before last I saw

him and he said, every year you see me you tell me to get it cured. I don't need to get it cured anymore. I'm cured. And I said, what did you? Did you finally take therapy? He said, no I had a total gastrectomy for early gastric cancer.

So, when you tell me about somebody gets fat and I have somebody lying in the hospital dying of this disease I think that's a small price to pay if it occurs. And the likelihood that it's going to occur I think is just because somebody said it doesn't make the likelihood that it's going to occur. And we're talking about people and mice. And every time we've studied mice we've found out they're crappy little people.

MS. KAHN: Stacy Kahn, from University of Chicago. I think people are raising a lot of interesting questions about the safety -- what? Oh, sorry. Stacy Kahn, University of Chicago. I wanted to make a comment about the safety. I think people are raising excellent questions and clearly we need to have long-term studies. We need to

question how long the impact of the microbiome transplant is going to work. But I'd like to remind everybody that the antibiotics have a lot of long-term consequences. And other medications we use that help treat one disease, for example, proton pump inhibitors which we commonly use to treat acid reflux and gastritis which have been a wonder drug are also causing side effects.

And we won't have a crystal ball as somebody said earlier. And we need to be mindful about weighing the costs and the balance and the timing of everything.

MR. RELMAN: Thank you. Yeah.

DR. COOK: David Cook, (inaudible) Health. Eric, I have a specific question for you. You've got a set of patients who are undergoing this dramatic community change due to a whole bunch of factors including chemotherapy and antibiotics and potentially radiation and their underlying disease. Do you have plans to follow the development of their restorative community over a long enough period of time that we can actually learn

something about the dynamics of how you establish or reestablish a stable community in that setting? Cause then it could teach us something about transplantation of new communities into dysbiotic states.

DR. PAMER: I think it's a great question. We have been able to collect fecal samples quite regularly as long as our transplant patients are in the hospital. When they go home it becomes more difficult to regularly get samples from them. But some of them do come back for a variety of reasons. And we see that some leave with a very monotone flora. They might be dominated by enterococcus and come back with a flora that looks very much like the flora they had when they came in for their transplant.

We don't know how they reacquire the diversity but they do. And others come back with a flora similar to what they left with. So, I do think there are ways and it's been postulated that there are ways that we reacquire flora just by going out into our regular environment. I've read that just

going into a public bathroom and somebody flushing the toilet next to you and the aerosolization from that can potentially reexpose you to flora.

And so, how these people are getting it we haven't really looked at closely but obviously --

SPEAKER: (inaudible) joke about venereal disease --

DR. PAMER: So, I think one question while I have the microphone here that I wanted to sort of raise and it kind of addresses something that Lita brought up in her talk this morning was pathogens that we can find in our normal flora. And if you go through the literature there are case reports that will describe arthritis by prevotella and brain abscess with porphyromonas.

And I think some of these end up in the PATRIC database as pathogens. But they're really bugs that belong in the lumen of the gut and they're just fine there. And it's really just when they end up getting it to bloodstream for whatever reason, a

diverticulitis or even just brushing your teeth can lead to a transient bacteremia and if you're very unlucky it'll end up as a brain abscess.

I think it's inevitable that transplants are going to be done and there's going to be a bacteremia and there's going to be an outcome that is going to suggest that it was a pathogen that was transmitted. And I think -- I don't have an answer to how to get around that but I think it is an issue that needs to be anticipated and that it wouldn't necessarily kill a program that's helping 99 percent of people because one adverse event came that was fairly predictable.

MS. PROCTOR: I actually wanted to sort of address that but in just preparing for this workshop I was curious how fecal transplants are being done now. There's certainly a huge grassroots movement and I think DIY fecal transplants gave me 45,000 hits, different clubs and people have YouTube videos and all that. But I also -- the other thing that I found was there are a lot of

medical facilities that are providing fecal transplants as a fee for service.

And which is a little shocking to find that out. On the other hand that might be a real opportunity to somehow partner with facilities that are already doing it, to follow those patients on some level. I think I counted a half a dozen facilities around the country that are providing fee for service, more than that but just that first couple of pages on the Google page.

So, I'm wondering if it's already being provided to people around the country if there wouldn't be some way to partner with them. Now, I don't know how they handle their liabilities when they provide the fee for service transplants. But there might be an opportunity there.

MR. RELMAN: Alex and then here.

DR. KHORUTS: I was actually going to extend on Eric's point. Alex Khoruts from Minnesota. Given, for example, *Bacteroidetes fragilis*, it's been mentioned a couple of times so I thought I'd stand up for B.

fragilis. But yes, it does cause abscess but it could be the least of the possible evils because it doesn't really go systemic. It stays localized. The ammenthem goes around it and so, you have a relatively protected perforated gut because of that abscess that formed.

One could say, well, you know, a bad thing happened but it's not the worst thing that could have happened. So, maybe it's not such an evil pathogen as, for example, some proteobacteria that goes just systemic and septic shock. So, there is a range of from really true evil like cholera to pathobionts that could be in different parts of the spectrum.

MR. SUN: This is Wellington Sun from FDA, Division of Vaccines. So, I'd like to pose to the panel a question that I think we might get to tomorrow but maybe we can get a head-start today which is when we approve a drug or vaccine or biologic, one of the things we look at is what is the adverse event profile for this product? And the way we get

at that is through randomized control, placebo, double-blinded studies and where we have a good comparator.

So, the panel, given your expertise and your knowledge of the microbiome and we've heard potential long-term effects, but how would you characterize your concerns at this point of the state of knowledge of the microbiome? What are -- what may be potentially this profileable adverse events that you are concerned about for fecal transplantation?

DR. TARR: Short-term I think we have some usual suspects, sepsis, viral infections probably would show up. I'm going to propose liver and CNS and fever syndromes. The intermediate and long-term I think it's speculation and I think we need excellent registries starting now so that we can begin to profile, admittedly without a control group, what is occurring in this wild type group of humans getting these therapeutics. This is just a first pass estimate of what we might see.

MR. RELMAN: There are two folks here who have certainly seen a lot of, at least, clinical side effects. Larry or Johann?

DR. BRANDT: So, Phil, as a dominant figure in the field what your answer was in a word is speculation. And we've now had a huge -- that's the wrong word. We've had a substantial number of case reports, small case series, one randomized trial detailing essentially, pretty essentially, a zero complication rate.

We also have a situation that I'm certain we're going to discuss in great detail tomorrow where the FDA has, at this point, called stool a drug. It therefore is an unapproved drug at this moment. As a result of which it really is illegal to do fecal transplant. And most people that are doing fecal transplant now don't know that this is illegal to do.

So, in a sense, the FDA did what they had to do. In a sense, they've placed the physician who's doing the fecal transplant

at risk for using a drug that they don't know has not been approved cause they don't know it's really a drug. And yet, this unapproved drug is every day saving lives from a disease that nothing else, for the people with recurrent C. difficile, can save them.

So, I think that at this particular time it's absolutely critical to start to get a codified registry where every single one of these cases are entered and followed for a very long period of time where the data can be obtained periodically to evaluate it. But I think that it is unconscionable that this procedure not be allowed to go forth based upon the evidence to date so far recognizing we don't everything about this. We probably are closer to knowing nothing about this but it does a damn good job of saving people's lives for something that heretofore we don't have a solution for.

So, we're really trapped and we need a way out of this trap. And we need to get on this road to move forward so we can then have a lot of these being done, observe the data

and see exactly what it is we're dealing with.

MR. RELMAN: Scott, I hate to put you on the spot or maybe someone else. Could you clarify what the current status is of from the FDA's point of view of the use of feces for transplantation? Is it illegal per se?

DR. STIBITZ: I'll pass it over to Jay.

DR. SLATER: I'm Jay Slater from the FDA. And I'm an MD and I'm not a lawyer, so, I'm not going to use words like legal and illegal.

FDA has determined, after a fair amount of internal discussion I would want to tell you, that fecal microbiota for transplantation is an unapproved drug and therefore its use should be under IND. I think as an MD I'd like to divert the conversation slightly at this point. Just reacting to some of the questions that have come up during today's session, I don't think anybody in the FDA or outside of the FDA is saying FMT yes or no, should it be happening or should it not be happening?

I think the question that we're grappling with and that you're all grappling with is how should it be done and how does it actually work? How we can figure out the way to do it best?

Nobody is saying that when you have a critically ill person lying in front of you, you should worry about consequences 10, 15, 20 years down the line. Pediatric oncologists confront this and have confronted this for a very long time. But I think it's quite clear that if you have a choice between two or three or four different approaches that are equally efficacious you should choose the one that has the least long-term consequences. And the only way to learn about that is to do these studies in an organized manner.

There are many different ways to approach organized manners and I think FDA has something to contribute to the process that will be positive, both short-term and long-term, and I'll discuss that more tomorrow. I think we might consider the experience, for instance, of the Children's

Cancer Oncology Group which was not mandatory and was not mandated by FDA or anyone else. Was actually a scientist and physician driven organization that did a great deal for taking a very sick group of patients and really determining in a very good, large scale prospective way what the best ways were to treat them.

And as a pediatric resident that participated in some of these oncology trials, I can tell you that these were the sickest imaginable people and the trials were carefully thought out, slavishly adhered to and ended up really determining some of the best successes in modern medicine. And I think all of our hopes is that this is where we're going to be five and 10 years from now but nobody is talking about yes or no, should it not happen.

MR. RELMAN: Thank you. Johann, you had your hand up.

DR. BAKKEN: Just extending the question that Dr. Brandt raised. For those of us that have administered FMT to very sick

patients, none of us, I believe, would advocate FMT as first-time therapy or treatment of choice for newly diagnosed C. diff. The category of patients we're dealing with are those that have had anywhere from three, four, up to a dozen relapses over a period of time that may extend from a few months up until several years.

They have tried these other therapeutic approaches and continued to relapse and have miserable lives and my question is, in this time when the FDA is requiring an IND to administer FMT what would be the consequences for the un -- or for the physician who is unaware of this now being defined as a drug and requiring an IND when he or she administers FMT to a very ill patient?

DR. SLATER: I honestly don't think I can answer the question. I mean I, you know, it's not in my area of expertise within the Agency.

MR. RELMAN: Would it be reasonable though to take from this discussion the sense that the FDA is duly concerned but willing to

allow prudent and thoughtful approaches to clear clinical problems as physicians best see appropriate for the circumstance?

DR. SLATER: Well, I think what should be clear to anyone that's approached the FDA with the question about FMT patients is that we are working to develop solutions. And sometimes those solutions come extremely quickly as the situation warrants. And I'll talk more about that tomorrow.

MR. RELMAN: So, I think one thing that we, as a group, then can be thinking about is what are the kinds of science that will directly serve the need to understand all of these issues in the short-term as well as in the longer term? Can we learn from these experiences as Lita already pointed out given that they are happening? And can we learn in a thoughtful, effective, efficient manner? Yes?

MS. HAYS: Yes, Ann Hays, University of Virginia. I think everybody's right to a certain extent. However, I think that we are overlooking some of the more salient issues

about this. This is being driven -- FMT has been driven by our patients. They are doing -- if we do not treat them, they are going to treat themselves. They are not going -- then there's no examination of donor stool. And there's no registry and there's no long-term data that's going to come out of it.

Yes, we need long-term data. I would point out that most of the drugs that we use that are approved by the FDA, and I'm a general clinician, a gastroenterologist, but sometimes I read these reports and I just go what kind of crappy science is this that allowed this drug to get approved when it only is working in -- is giving maybe a 10-

Percent in response rate? Our number one drug for C. Difficile, metronidazole, is not approved by the FDA and currently is only beneficial in approximately what, 70 percent of patients.

I think that we now have guidelines just this month from the American College of Gastroenterology. I think that in terms of using FMT and so we have very specific

guidelines for the clinicians in the world. I cannot see Dr. Jeff Hill down in Danville, Virginia getting an IND to treat his patient who's sick. So, and that patient probably won't get treated because that's not a particularly progressive community and most communities in the United States are not particularly progressive communities.

If you go out to Oregon, patients are going to treat themselves if their physician doesn't tell them to and probably their physician will. It's just going to happen. So, I would love to see the FDA and all of our other federal regulatory bodies get onboard, give us clinicians who are taking care of the patients good support, give us good guidelines and help us help our patients at the same time we look for what the long-term consequences are as well as learn the science.

DR. YOUNG: I agree with the passion there cause I know the -- I've taken care of these patients who are absolutely desperate and things like that. And it's interesting to

talk about it in the setting of C. diff but I also, and it was brought up about metronidazole, when something is "approved" by the FDA and has an indication then there comes the whole idea of off-label use. I don't know what the label would look like on this but I'm -- that was just an aside.

But I know that people are proposing also FMT for the list is very long and in some case distinguished and in some case not so distinguished. What do we do with regards to that? Do we apply the same thing -- is this registry for the use of FMT as the cure-all for -- and we can give the list of however long we want to make it. Or are we going to have to have this meeting for every single indication which we come up for FMT where there's a loud enough call for the use of it.

And you know, everyone in the room knows some of the other diseases that are being proposed for which FMT may be useful. So, it's something to think about. What are we starting by this particular meeting where ostensibly we are looking at FMT? Although

it's not in the title the Public Workshop, a lot of it is stemming from C. diff but is it really FMT for whatever FMT might be used for?

DR. MCDONALD: Vince, I think you just touched on something, you know, there's an element of this that --

MR. RELMAN: If you could just --

DR. MCDONALD: Oh, yeah. Cliff McDonald, CDC. I'd already introduced myself this morning a couple of times. People are giving me a hard time about that.

The decision to rule this as a drug or biologic had a lot of sequelae that come from that. And the challenge of that, of course, is that this is very accessible and we've just heard that as are probiotics that are sold over the counter as nutritional supplements which is also really essentially unregulated. It's approved as nutritional supplements. They're being used -- they're actually probably from a biologic sense more dangerous because they are single organisms given in high doses and I think FDA does have some intention to go into that a little bit

more carefully.

But I don't know, I doubt that they're going to be able to pull back nutritional supplements, probiotics. I just can't see that and so this is going to continue. This is different group. It's not CBir but you know, so this is sort of like trying to grab a ball of mercury. And I think that what we've heard is as soon as we and I'm talking we, the three federal agencies represented here but also professional organizations that are in the room, will get together and say this constitutes what is within the realm of reasonable science and safety.

Until we get -- we need to move will all due speed to that I think. And that's going to do the most good for the most people. Anything else is just going to alienate important grassroots organizations who we need to be partners in all of this and as soon as we can get to a partnership role with them the better. And, you know, I think that probably for FDA and just a bug in their ear, but I

realize that there is strict frameworks that you can't make this stuff up. But, you know, where compounding pharmacies should have been these last 20 years is probably where this should be now.

That is, these are some -- stool is there. It's off the shelf. It's a generic already? It's already generically available. Yeah, there's no patent on it, generically available. You know, what the key is is that what's going to do the most damage from a public health standpoint probably is if someone started shipping one big back which was contaminated with Hep. B and who knows what else out, and you see all these infections occur, that's when I know my division will be called and be involved. That's what we don't want to see happen.

That's where it has to rise to the level of regulation definitely where you start looking like a manufacturer. And getting away from this thing of meeting a need that's right in front of you, and that's with compounding, I mean we can't say compounding all has to

stop. But there's clearly a kind of compounding that needs to stop which looks like manufacturing and starts having those public health impacts when something goes wrong.

Now, I think there's other concerns, too. There's very much more theoretical stuff. But at the end of the day risk is defined by outcomes. It's not defined by the basic science and most of you are basic science. It's not defined by that. I mean, that's the potential for risk and it's a place to look but it's going to be rates and outcomes of things, of real disease and real outcomes. Right now people are dying of C. difficile. People with multiply recurrent disease are dying of it when they get another bout and it comes back severe.

Only take a couple of those with where people did not have access to fecal transplant to just put all of us in the wrong position. And I'm saying the collective we and the benefit of all patients. So, I just want to just encourage that type of reasonable

latitude if we can find that in the regulatory code to look for that.

MR. RELMAN: I appreciate your comment and another way to frame that is simply to point out that all of these discussions have to be context dependent and we're losing sight of some of the local context. Maybe one last comment because this, I think, is the end of the time we had allotted and it's exactly where this whole workshop is going to pick up in the morning on clinical experience and then the path forward.

So, maybe one comment since you were waiting up there.

SPEAKER: Yeah, a short one but maybe something that we could think of as we start talking about the regulatory framework for this. Let me bring back the analogy of just simply banking blood and testing it and making that model work for stool. I mean, it's the same problem. You've got to make sure it's pathogen free. Got to do some standardization. You've got to test it and it's just a really clean way to handle

something like this if you think that it's beneficial. And clearly we think this is beneficial. We should talk about it more tomorrow, of course.

MR. RELMAN: Thank you. I think we will draw this to a conclusion for the day. Thank you all for your attention, your interest and thank you to all the speakers.

(Whereupon, at 4:55 p.m., the PROCEEDINGS were adjourned.)

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CERTIFICATE OF NOTARY PUBLIC

STATE OF MARYLAND

I, Christine E. Allen, notary public in and for the State of Maryland, do hereby certify that the forgoing PROCEEDING was duly recorded and thereafter reduced to print under my direction; that the witnesses were sworn to tell the truth under penalty of perjury; that said transcript is a true record of the testimony given by witnesses; that I am neither counsel for, related to, nor employed by any of the parties to the action in which this proceeding was called; and, furthermore, that I am not a relative or employee of any attorney or counsel employed by the parties hereto, nor financially or otherwise interested in the outcome of this action.

(Signature and Seal on File)

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