convert a system lactose mal-digester because that is the clinical relevance, I guess --

DR. SANDERS: Right.

DR. RUSSELL: -- to one who becomes asymptomatic.

DR. SANDERS: Right.

DR. RUSSELL: If they eat X product twice a day, can they then drink a certain amount of milk?

DR. SANDERS: See, most of these studies are done actually delivering milk products. So, it is a certain bacterial load that goes along with the product. So, the yogurt, sort of by definition by formulation standards contains about 10^8 of these viable yogurt cultures per gram. So, if they consume the yogurt that contains this number of lactose-producing organisms there is a reduction in systems and the biomarker for lactose intolerance.

DR. RUSSELL: In that product.

DR. SANDERS: For the consumption of that product. In fact, they have done studies like this where someone will eat a carton of yogurt and then drink milk or take an additional lactose load and it overbears the system. You know, the organisms that are there can help digest the amount of lactose that comes along with the product but not an additional lactose load.

DR. RUSSELL: So, in a way that is kind of a misleading claim --

DR. SANDERS: Well, no products are really marketed -- I mean, if you look at the dietary supplement products that might deliver, in my opinion, the more valuable culture of Streptococcus hemophilus and vulgaricus, not so much the intestinal organisms, probiotic organisms, but if you took a capsule of dried organism along with a glass of milk, you know, there is a chance then that you are delivering an adequate number of those bacteria that would allow you then to drink the milk.

So, you know, whether that specific study has been done -- you know, part of the problem with these claims is that I think, as with many of the products that Roger showed, you would be hard-pressed for at least some of them or many of them to have them pull specific studies on the specific strains that they are selling. They are making, for the most part, generalizations about probiotic research as a whole and as long as it is published about Lactobacillus, they figure they can say it. That, of course, is not an exceptionally rational approach but I don't know that those studies specifically have been done. But I think phenotypically, there is probably enough of a body of evidence on improving lactose digestion that suggests that an adequate dose of about 10^8 organisms can help you digest -- 1 should take that back, maybe 10^{10} organisms can help you digest the lactose that accompanies

LOO or 200 mL of milk.

DR. RUSSELL: Thank you.

DR. BENEDICT: Dr. Cohen?

DR. COHEN: With respect to the primary cultures or organisms, what is generally known about the origin of these? You talked about the Shirota strain, but what about some of the others both in the supplements and the foods? I mean, if I was going to open an operation tomorrow, where would I acquire my organisms?

DR. SANDERS: You would go to ATTC.

DR. COHEN: Is that the source of most of these strains?

DR. SANDERS: You would buy your bugs from them and get someone to grow them for you, or the other thing you could do is go to the culture suppliers. That is their business, to have strains of organisms and, for the most part, those organisms are identified by ATTC as being a source. You know, the sources are many times known. They will list where it came from and whose lab it came out of. The small dietary supplement manufacturer would probably do that. The bigger companies, like Valio, Nestle and all of them, they have isolated their own strains, and they oftentimes will do that from human sources. You know, oftentimes they make a big point that it is from healthy mucosa or fecal sample. I think one of the Lactobacillus

reuteri strains that is commercialized is from a human milk source. So, there are a variety of human sources but I think for the most part the conventional wisdom is that you want it to be a human source. But they are not necessarily traditional food source organisms.

DR. COHEN: I guess you do have some variability with the larger companies, but with the smaller ones then is there generally more homogeneity among strains?

DR,. SANDERS: More homogeneity?

DR. COHEN: In other words, if they are being purchased from common ATTC sources and things like that, are they really using similar strains?

DR. CLEMENS: They are and they aren't. Let's go back to the origin, and Mary Ellen is absolutely correct in terms of they are looking for a human origin, of course. Also, in addition to ATTC you go back to a German counterpart. At the same time, it is important that we actually identify the organisms because methodology which the ATTC and the DSM was categorizing and cataloging -- the methodology has changed since that time. If you go back to a paper, for example, in 1992, if I recall, by Petrov, he indicated that one particular from a German culture actually was six strains based on new molecular techniques. So, again, the supplier doesn't always have those techniques. So, it becomes incumbent upon the end user, whether it is a

food company or a supplement company, to specifically identify and have those genetic markers to know exactly what they are using.

Now, many times taxonomy has also changed. Dr. Archer and myself have gone through a number of strains which, over a very short period of time for example, B. bifidum became B. animalis, became B. lactentis, and who knows where it will go next. So, it is really important that we identify the genetic makeup of these organisms.

DR. SANDERS: In terms of your comment about are these organisms very similar and is there homogeneity between what is being sold, I think that now that we have, over the past ten years or so, the ability to look at DNA chromosomal fingerprints we have a much better sense of how related some of these strains are. In fact, the Lactobacillus acidophilus strain that is sold by Chris Hansen is identical to the one that is sold by -- I shouldn't say identical, but I should say their chromosomal patterns are indistinguishable. So, basically the same genotype is being sold by them as well as by Rodia. That has been published.

In our lab, we haven't published it yet but when we just isolated strains out of product we found that there is a high level of homogeneity between casei product that is in the Dannon product and the Yakult strain that is in their

product. So, you know, you have to say, well, is that because there are common sources of origin, or is it because this is just a dominant genotype that is present in nature? And, you oftentimes have to deal with that question about how many different unique strains really exist, and we really don't know the answer to that question. Are there ten strains of rhamnosus or are there a hundred strains of rhamnosus? I don't know.

DR. BENEDICT: Dr. Buchanan?

DR. BUCHANAN: Clearly, both of your presentations establish a need for understanding what is the actual dose consumed. I guess I have a couple of questions about the degree of sophistication in terms of quantifying those doses. A characteristic of many microorganisms is that if you stress them, like you would stress them in freeze-drying, you might injure them to the extent of anywhere between 90 and 99.99 percent of the population, such that they would look characteristics like bioresistance or resistance to acid. So, what degree of sophistication when you quantify these doses in a product in relation to the actual dose that would survive to reach the active site within the body -- would you be able to say that, yes, we had 10^{10} here but the actual delivered dose to the site in the body was some five or six orders of magnitude less? Is that taken into account when you do these kinds of

evaluations?

DR. SANDERS: No. In terms of what is listed on Labels and whatever, not at all. I think, for the most part, the way products are enumerated is based on the kindest possible methods. So, they are going to be enumerated on media that don't provide selective agents or selective agents that aren't going to be inhibitory to injured cells. It depends on the product though because microbiologically you run into some difficulties because if you have a mixed culture product, like the Actimel product where you have Lactobacillus vulgaricus and a Streptococcus themophilus and a Lactobacillus casei, then you have to have media that can differentiate among those before you can get a count.

So, oftentimes you would have to put some type of selective agent in. In that case, you know, a common approach would be to use media containing bile. Bile has been published many times to show that it inhibits injured cells from growing. For the most part, you know, if you take a group of cells and you plate them on bile or without bile you get much higher counts if they are an injured population on regular media than you would if bile were there. So, if they are forced to enumerate using that type of a medium, then you are going to be getting essentially the strains that are uninjured. If you have a pure culture

product like the Yakult product, then you might not run into that. But, you know, it is really company specific as far as I would know as far as how those enumerations would take place.

DR. BENEDICT: Dr. Hotchkiss?

DR. HOTCHKISS: Yes, a question for you, Dr. Sanders or Dr. Clemens, or maybe both, related to efficacy. Clinical trials are obviously one way to investigate efficacy, but it seems to me that, as you point out, some of these products have such a long history of use -- I think you said Yakult since 1935 in Japan, and they are also very broadly consumed, and consumed repetitively, it seems to me, and raise the issue of epidemiological studies. It seems a fairly straightforward -- well, epidemiological studies are never straightforward but if they were, this is a case for them perhaps. Have the Japanese, through all these years and experience with some of these products, conducted any epidemiological studies? If so, what do they say? If not, why haven't they?

DR. SANDERS: I think that is an excellent question. Yes, that is a population base that is ripe for doing an epidemiological study. I agree. I mean, if you truly have ten percent of the population consuming not just probiotic products but a specific strain of a high level dose going into these people -- I mean, that is a great

opportunity. Have they been done? No. The Yakult company has published one study that was looking at recurrence rates of superficial bladder cancer in patients and what they found was that they could decrease the recurrence rate of this type of cancer by -- I don't know, half a year maybe; yes, it was like 100 and some odd days that they were recurrence-free longer than the population that was not consuming the Yakult product, and that is the only epidemiological study that is very specific on a particular product or a particular probiotic strain that I am aware of.

Now, why don't they do it? Probably partly because of cost and partly because they don't need to do that to sell their product. I mean, even though they are allowed to make these functional food health statements, they don't even have to do it because their product basically seems to -- there seems to be an understanding of what this product does and they maybe don't need the data to do what their mission is.

DR. CLEMENS: There hasn't been any postmarketing surveillance on any of these products to the best of my knowledge either, except for the report that Mary Ellen just alluded to.

In terms of safety, if there had been an issue it would have popped up somewhere in the literature and certainly through government regulatory agencies. But from

an efficacy perspective there has not been any postmarketing surveillance to look, in fact, is it continuing to deliver those respective benefits.

DR. BENEDICT: Dr. Hotchkiss has another comment.

DR. HOTCHKISS: Just as a follow up, you have outlined that there are a number of these items around the world. It seems to me eventually FDA is going to have to make a number of decisions, one of which is efficacy of certain claims that will want to be made around these products, but without the supporting information it is going to be hard for FDA to allow the kind of claims, it seems to me, that people would like to make about these products.

DR. BENEDICT: Dr. Cohen?

DR. COHEN: The issue that you briefly mentioned about the Japanese product and safety I think is an excellent opportunity. If you have ten percent of the nation that is consuming the product, then reviews of the occurrence of these organisms in the population can be done. Aside from making the assumption that it would have been recognized if it was occurring, it would certainly be worthwhile to look at that because then you would have a really measure of risk.

I was thinking, when you were talking about this in the United States, that there might be similar estimates of U.S. consumption but it sounds like there is so much

diversity that it might be hard to, you know, really have the denominators. But the Japanese experience would offer you some denominators that would allow quantification.

DR. SANDERS: Of course, there are many other products on the Japanese market besides Yakult. I mean, Morinaga has quite a large market share in their Bifidobacterium strain. A snow brand is another dairy company that puts out products. But, certainly, if you Looked just at Yakult consumers you would have a nice -- ten percent is a luxury; you know, their population is a huge number of people.

DR. CLEMENS: Yes, you could simply look at the results from their clinical microbiology laboratories for the presence of organisms, or even obtain organisms over a period of time and subtype them. That is certainly something that could be fairly easily done to give some sort of accrued risk estimate for a specific organism.

DR. BENEDICT: Dr. Russell?

DR. RUSSELL: I think this is for Dr. Clemens. Am I correct in saying that for most properties of the probiotics it is assumed or it has been shown that it is important that they reach the active site, for example in the GI tract, as viable organisms?

DR. CLEMENS: The majority of the literature does support that. I think Mary Ellen indicated that, in fact,

you don't always need to have viable organisms to produce activity or desired outcome at the active site-and generally that is the GI tract. At the same time, some really fine work that has been done in Japan by Yasui; if you look at some work that has been done by Bob Yolken at Johns Hopkins, clearly there is some cell-cell communication, and clearly, that is one of the issues. You look at the mucosal lining of other surfaces, for example the respiratory tract, the auditory track, and clearly those cells seem to be responsive to IgA produced in the IG tract or stimulated in the IG tract. So, for the potential benefit, it doesn't necessarily mean it has to only occur in the GI tract.

DR. RUSSELL: Right, but the presumption is that viability is important at least to reach the GI tract so the rest of the processes can take place.

DR. CLEMENS: Over 99 percent of the clinical trials have focused on that attribute, yes.

DR. RUSSELL: And wouldn't that be an important part of the process of making a claim then to show that, in fact, viable organism from something that was eaten, even if it has a lot of organisms when you put it in your mouth, actually still had viable organisms in the intestine?

DR. CLEMENS: As a matter of fact, that is an interesting question. In fact, in many of the studies in which I participate you can recover the various organisms --

what you eat you also recover in the stool samples. But, as you well know, recovering some of these bacteria can be quite a methodological challenge. Bifidobacteria are very fastidious but we have demonstrated that not only do we recover the organisms that we fed but that actually, from genotyping, they are the same organisms that were administered.

DR. RUSSELL: There are in vitro models of the GI tract now that could possibly be used in that, but I am also wondering, if all these things are true, whether the vehicle in which these organisms are and the buffering capacity of the vehicle or the characteristics of the vehicle would greatly alter or change possibly the survivability of the organism, for example, just thinking of gastric acid if you have it in a media of high buffering capacity, it is much more likely that those organisms are going to survive in the small intestine than if you take it as a pill without any buffering capacity.

DR. CLEMENS: You can go in both directions on this, on the buffering capacity example. Clearly, in the stomach you want a high buffering environment so that they can survive the gastric acidity. Yet, in the GI tract, post the valve, you want to have an environment that has a lower buffering capacity because some of the characteristics or properties you wish to have the organism produce -- for

example, short-chain fatty acids, you want to have a lower buffering capacity for their effect against potential pathogens. So, it is a double-edged sword in that regard.

DR. RUSSELL: Except that the buffering capacity, because of the digestive process taking place in the small intestine, would be changing from that quite a bit. I mean, the real buffering issue is probably mostly -- don't you agree -- centered on the stomach?

DR. CLEMENS: Yes, I do.

DR. SANDERS: I was just going to say that is one of the values of consuming probiotics as part of food rather than in a pill, you do have a sort of built-in protective mechanism with consumption.

DR. RUSSELL: Yes, I think it gets back to how important the issue of viability is. If we really think that that is important, shouldn't that be part of a requirement maybe or a regulatory process in order to make legitimate claims? Not only that you start with a viable organism but that somehow it survives in the media that you put it in, in the GI tract?

DR. BENEDICT: And just to follow up on Dr. Russell's question, if viability is important do we need to address potential phenotypic shifting as a result of going from what you are culturing it in to going to the gut? Has that been tested, and is that worth thinking about?

DR. SANDERS: To my knowledge, that has not been systematically tested. There is some work going on at North Carolina State University, in Todd Klaenhemmer's lab to look at the influence of dairy products as a medium of delivery, and their attributes that might enhance or decrease expression of certain gene systems. You know, it is jut the beginning of what could really be a huge investigation because you make a very good point, we really have very little sense -- we have a pretty good sense in industry about what it takes to make these things survive so that when you make your product you have high levels. We know how you can manipulate fermentation conditions. But how that manipulation ultimately affects survival or expression of probiotic function in vivo, I think we know almost nothing about that. In fact, some of the steps we may take to enhance cell population at the front end may put the cells in a physiological state where they, arguably, could be more harmed by the GI tract, and the example of that is exposure, for example, to low pH which, during growth, might in fact activate gene systems to allow them then to survive better through the stomach. Of course, generally speaking the approach in cultivating these organisms is to keep the pH as neutral as possible because you get higher cell densities. So, I think you bring up a very good point and I don't think much is known at all.

DR. BENEDICT: Dr. Buchanan?

DR. BUCHANAN: Thank you, Steve. I just wanted to ask a point of clarification about a comment you made, Dr. Sanders, and I think I am going to ask Dr. Cohen to respond to it. You indicated that enterococci infections are primarily nosocomial in nature, and I believe that is true for the United States but I do not believe that is true for Europe.

DR. COHEN: Well, enterococci occur both as community acquired and nosocomial infections. Common community acquired infections are urinary track infections, maybe endocarditis, potentially post-traumatic. Hospital acquired infections frequently occur in intensive care units, can be blood stream infections; can be heart valve infections; can be surgical wound infections. So, it can occur in both settings.

DR. BUCHANAN: My impression was particularly the antibiotic resistant infections that have been cropping up on both sides of the Atlantic, that the epidemiology associated with those infections was substantially different.

DR. COHEN: Between the parts of the world?

DR. BUCHANAN: Yes.

DR. COHEN: I think the degree to which it has been examined as such is somewhat different. In the United

States the vancomycin-resistant enterococci are primarily a nosocomial problem, and some of the drugs which were used in animals were not used in the United States. In Europe there have been studies that can show vancomycin-resistant enterococci present in people in the community as well as strains in the hospital. When they have tried to look at the different strains of enterococci, they started finding different relationships. There seem to be some species differences, species in animals and humans in the community and in the hospitals. So, it is not totally clear-cut but there does seem to be relationships between some animal species and isolates from humans. Now, in Europe, in the community you are not talking about sick people. In the United States in hospital and in Europe in hospital you are talking about infections occurring with these organisms.

DR. BENEDICT: Dr. Fukigawa?

DR. FUKIGAWA: Thank you. Despite having a Japanese name, I am not familiar with the Japanese experience. So, I have two questions. First of all, could you clarify for me whether the use of probiotics is regulated in Japan or not?

DR. SANDERS: I am not sure what it would take, for example, to put a probiotic product on the market in Japan. I don't know if it is viewed as food ingredients. I don't think they have a dietary supplement type of a

category like we have here. But I do know that if you want FOSHU status, foods for specified health use which is the Japanese functional foods, you do have to go through a very specific petition-filing process and provide documentation for exactly what you are selling; what the active ingredient is; and then the studies that support whatever statement you are asking to be able to make. Then, the Japanese Ministry of Health reviews it and then makes a judgment about whether or not those are allowable.

DR. FUKAGAWA: So, that is essentially paralleling what we are trying to do here in the U.S.

DR. SANDERS: Well, it sounds to me like there is more of a parallel with that and our health claim approach, although I don't think theirs is as extensive and there are many, many more products. I mean, there are 80-100 type of FOSHU foods that are acknowledged in Japan -- ingredients and/or foods. So, their level of approval is a different pace than what we have here, and you can see by what they allow that more general statements are allowable, and they are product specific. So, if Yakult goes in and asks to have their product evaluated, only they can use the statement that is allowable, whereas that is not true here.

DR. FUKAGAWA: The second question I have is in terms of having these products marketed, do they actively use these health claims or is it just a culturally accepted

food ingredient or food stuff, such that it is not an issue to try to convince the public, and so, therefore, what this is leading up to is that perhaps because in the Western world there may be more fear of bacteria -- you know, on the one hand people are buying antibacterial soaps to wash their hands before they feed their child and don't want their child to pick up food from the floor and eat it and yet, on the other hand, we are saying more bacteria are good.

DR. SANDERS; Right.

DR. FUKAGAWA: And perhaps this imbalance is creating the need for us to convene to try to determine whether or not it is something that is potentially bad because we have this uncertainty. Do you think that is part of the dilemma?

DR. SANDERS: It is hard for me to speak for the Japanese culture but the sense I get in terms of what is published and hearing other speakers talk is that there is a more inherent understanding in the Asian culture of what the GI tract is and that, in fact, bacteria are associated with it. I would say that in the U.S. if you asked people if there are bacteria in their intestinal tract they would probably say no. I don't think people are aware of that here. so, there is more of an inherent understanding.

Now, in terms of marketing of the Japanese products, if you get FOSHU status there is a symbol -- I

don't have a picture of it here but it is cartoon almost of a person jumping for joy and that is the FOSHU symbol. So, even if you don't say any of your claims, you are legally allowed to put that symbol on that product and maybe that communicates to the consumer as well.

Plus, with the Yakult ladies, for example, there is an awful lot that goes on just face to face. They do supermarket demonstrations for example where they are providing the product and giving people face to face information about that. But, from what I understand, I would say there is a much greater inherent sense of GI tract health and the role of microflora in the Asian culture than there is here. What you have in Europe is that they like their dairy fermented products there, and we have been very slow to copy that. So, I think that is what their basis is. They are very trusting of fermented dairy products.

DR. BENEDICT: Dr. Russell, did you have another question?

DR. RUSSELL: Sort of a trivial thing just for Naomi. My source is NPR --

[Laughter]

-- but they had a bit report on antibacterial measures in Japan in the general populace, and the antibacterial soaps, and gloves, and everything you can think of is much more advanced in Japan than it is here.

So, I don't think it is fear of bacteria necessarily, but I do think the knowledge of GI ecology and so forth is much more advanced in Japan.

DR. FUKAGAWA: This is probably off the record, but I haven't inherited those genes and I don't know about all this.

DR. BENEDICT: On that note, why don't we break for the long-awaited lunch. It is now 11:47 and we will reconvene promptly at one o'clock. Thank you.

[Whereupon, at 11:47 a.m., the proceedings were recessed, to be reconvened at 1:05 p.m.1

AFTERNOON SESSION

[1:05 p.m.]

DR. BENEDICT: We are going to resume and we will here from Dr. Daniel O'Sullivan, who will talk to us about source organisms and methods of analysis. Please introduce yourself a little more fully.

Source Organisms and Methods of Analysis

DR. O'SULLIVAN: I am essentially a molecular microbiologist. So, that is essentially the viewpoint from which I will be talking to you today.

[Slide]

The areas which I was asked to talk about include source organisms, methods of analysis, genetic drift and strain purity. Essentially this is how I am going to break it down. I am going to give a short overview, and here I was asked to mention some issues, overriding issues; then talk about source organisms and selection of strains. Where do these things come from, and what criteria are used to select them? And then, talk a bit about methods of analysis, the methods which we have for analyzing these probiotic bacteria, and then talk a bit about genetic drift -- what is it and how it impacts probiotic cultures; and then some issues finally on strain purity and safety issues.

[Slide]

So, first of all I will do a short overview. Here

I have just summarized some of the definitions. We all know what probiotics essentially are and, as we heard this morning from Dr. Archer, it is a live microbial feed supplement so it beneficially affects the host's intestinal microbial balance.

A prebiotic is defined as a non-digestible dietary food which essentially targets particular genera in the actual intestine. So, you are selectively feeding what is there.

Synbiotics is essentially when you combine a probiotic and a prebiotic. As you might know, it is known a number of bifidobacteria can actually utilize oligosaccharide, which is non-digestible and very few other organisms can actually do it. So that gives them a selective advantage.

[Slide]

Mary Ellen did a good job this morning and told us all about the potential health benefits, which is good because I don't have to go through all the different health benefits. There is a whole lot of potential ones. Now, if there are so many potential health benefits, how come not everyone is fully agreeable on the benefits of all of these probiotics?

[Slide]

This is a statement that Gerald Tannock, who is a

well-known probiotic researcher from New Zealand, made in a recent book on probiotics. He said, since even some scientists treat the probiotics concept with skepticism, how can the average consumer hope to comprehend the significance of acidophilus or bifidus?

Essentially, a lot of the scientific people looking at the studies, the clinical studies which are done are not fully convinced, and there is a number of reasons for that. And, some of the reasons would be a lot of studies may not be very rigid or stringent. They may not have sufficient controls. Some are much better than others.

Another reason that is being appreciated more and more nowadays is a disregard of the actual species or strain, in some cases not even paying attention to what species are being used. So, that is inconsistency in procedure and you are never going to reproduce an experiment that way.

Then, thirdly, another possibility is the available cultures which are used may be not all that great after all. So, there is a number of different possibilities why everything is not fully accepted.

[Slide]

So, let's look at that probiotic concept. The concept of probiotics, as we have heard, is not brand new. The terminology might be quite new but the concept has been

around for approximately a hundred years, and we heard this morning that at the turn of the century we had Metchnikov, a Nobel prize winner, essentially promoting the use of lactobacilli cultures, fermented dairy products, and Tissier promoting the ingestion of bifidobacteria.

So, essentially the concept has been around for a long time, and the concept is theoretically solid. You essentially have microflora in the intestine, a lot of them, more than cells in a person's own body. So, the balance between good and bad is always a very valid balance and, therefore, the concept is theoretically solid, and no one over the last hundred years can argue very rationally about the whole concept being totally wrong. So, the concept is solid. However, experimentally it is weak when it is done out in practice. That is essentially getting into how do you actually apply this concept.

[Slide]

So, what is essentially needed is scientific validation, and scientific validation comes experimentally. And one of the main reasons, and one of the main features that has to be done is to get a better understanding of what features a probiotic organism should have to elicit a particular effect.

[Slide]

So, now let's look at some source organisms. From

a few talks this morning We got a lot more detail as to what type of species are being sold these days commercially as probiotic organisms. I have separated two groups here. At the top I have Bifidobacterium and Lactobacillus. So, some members of the genus Bifidobacterium and other members of the genus Lactobacillus have been sold as probiotics. Based on all the scientific rationale, these probably should stand a out because that essentially is where the scientific rational should point.

There are other organisms as well. Bifidobacterium has a strong rationale because it is one of the dominant organisms in the large intestine and Lactobacillus is very well suited, and dominant and healthy in small intestines. so, one piece of wisdom is that Bifidobacterium for the large intestine and Lactobacillus for the small intestine.

Now, there are many other general being used commercially as probiotic organisms and these are just some of them here, which include genera like Bacillus. Bacillus essentially is generally not found in the human intestine, so why is it being used as a probiotic? Enterococcus and propionic bacteria are found. The yeast has been used as well, even E. coli and others as well.

A study that was done in Europe was looking at commercial preparations of Bacillus, and these essentially were Bacillus sutilis bacteria for use in probiotics. These

researchers isolated the actual commercial sources, and they characterized them in depth using molecular analysis, and what they found was that neither one of them was Bacillus sutilis. One of them was Bacillus pumulis, which was fairly related to sutilis but not sutilis, and another one was actually alkalophilus. It was an organism that was suited to alkaline environments, which essentially is totally the opposite from a human intestine.

So, then they came up with an interesting question, can any non-pathogenic gram-positive microorganism serve as a probiotic agent? So, that is the concept that essentially has to be looked at as well in terms of what is a probiotic agent.

When you look at it in more detail you can see that in terms of the intestinal population is compromised after antibiotic treatment or other treatments where basically it is not back to normal, then possibly putting any non-pathogenic agent there in high numbers might essentially help out. The rational behind Bacillus is because you use spoors and the spoors can withstand the actual gastric transit, and also possibly they produce inhibitory substances.

However, when you look in more detail, clearly from an ecological point of view, if an organism is going to have an impact then the more suited it is to that particular

environment the more-impact it is going to have.

[Slide]

Now let's look at where these things come from. First of all we will look traditionally. Traditionally, which essentially extends up to today in many incidents, what is required commercially is, okay, we want bifidobacteria; we want a particular Lactobacillus and essentially there is no listed criteria that those particular organisms should have. That is why the sources, as we heard this morning, essentially are multiple or any sources. They come from animals; they come from foods; the environment; culture collections. There is no, or there used not to be any regard to where these things should come from.

Recent understanding of where things are today is that an organism that is going to'be suited to a particular species generally must have evolved in that particular species. Therefore, the accepted rationale today as regards source is that a human probiotic, if it is going to be suited to the human intestine, must have originated there originally. And, also the same for animals. We do know there are a lot probiotics today essentially for human used that have not originated in humans and, therefore, may not be very suitable.

[Slide]

So, these essentially are practical considerations for selecting strains, and these are very important. But traditionally these were the only considerations which were used. One is the ability to grow high numbers in vitro. You must be able to culture it. They must survive in the delivery food, and must have neutral of positive attributes to the food. These are all very practical, very important, but these are not the only ones.

[Slide]

What is also being realized is that there are considerations for efficacy. We heard a bit this morning about acid and bile tolerance. I just want to spend maybe half a minute talking about this. I have listed here tolerance rather than resistance because there is a very distinct difference. If something is resistant to it, then it has a mechanism that is there all the time. Tolerance is when you turn on an inducible stress response.

Now, if you look at biology and these organisms where they live naturally, and just take the large intestine for example, that is essentially a neutral pH environment largely. So, if that is the case, then these organisms will not evolve a resistance naturally. So, why select for an organism that is fully resistant when tolerance is all that is needed?

Now, the general screen that is used for acid, for

example, is just to get a culture, put it straight, say, into pH 3 or lower and test to see how long it can last there. Now, that is a test primarily for resistance rather than tolerance. For a probiotic organism, all you want it to do is to survive the transit so what is probably more relevant is tolerance. So, a lot of good candidates are actually thrown out.

We were screening a lot of bifidobacteria in my lab, and that is essentially what we found. You get some strains which have evolved in the large intestine, dump them into pH 3 and you whack them down 6, 7 or 8 logs. Now, if you would turn on their inducible stress responses by priming possibly by a low acid which is sub-lethal, then essentially they are able to tolerate it. So, that is a rationale that needs to get out there, essentially, what is it you need -- resistance or tolerance? So, a lot of candidate organisms are often discarded because they weren't actually resistant and really you don't need resistance; what you need tolerance.

Similarly with bile. An organism, when it comes in Contact with bile, has gone through the stomach first. So, it is already being stressed. So, its stress responses have already been turned on. We know from biological systems, and we know from lactic acid bacteria that there is cross-protection in stresses. So, essentially, already

having been primed from the stomach, an organism then is better able to tolerate bile. You really don't need bile resistance when its natural habitat, for example, might be the large intestine.

So, essentially the difference between resistance and tolerance is a necessary prerequisite, and this then suggests that basically you need pretreatment of organisms before they are actually ingested.

Another condition, for example will be beta-galactosidase activity and this is very specialized if the actual prebiotic health thing you want to get out is improving lactose mal-digestive symptoms.

Another thing we had a bit about this morning is adherence. It is definitely an accepted fact if you look at microbial ecology that essentially to compete well and to survive long-term in a particular habitat, essentially one with flux like the intestine you need to be able to adhere to the available attachment sites. So, that is very important criteria, however, in practice, as we heard this morning as well, we do not have a complete assay for that. What is generally used essentially is cell lines, generally CAC02 or HD29. These are just two different cell lines, which are single cells, essentially which came from two different individuals.

The conclusions which are drawn from them are

sometimes much, much bigger than what they are. If an organism adheres to that, okay, then it has a potential for adhering at least to some cells. If it doesn't adhere to it, you really can't draw conclusions. So, there are very limited tests, and the only danger in them is that the wrong conclusions are drawn from them. Even if they do adhere to them, it is totally different than what the in vivo situation would be where it has to compete against other organisms as well. So, the only real adherence test is essentially human feeding studies.

Finally, a very important one is essentially niche fitness. This is an ecological term that takes into account all the particular characteristics an organism needs to compete successfully in a particular ecosystem, and that is one where information is only beginning to become available now.

[Slide]

Essentially, if you look at other ecological systems, you must aggressively colonize it to compete. It must compete for nutrients and compete against competitors. So, essentially it is a bacterial competition system.

[Slide]

When we look at bacterial competition in the lactic acid bacteria, we sometimes get fairly narrow and we just look at essentially antimicrobial product. That is one

of the reasons why lactic acid bacteria have been chosen, because they do produce acids, for example, organic acids and they are antimicrobial and that probably is beneficial. They also possibly introduce bacteriocins. People are getting very excited about these now, and these probably do have an impact, although not yet proven, in the composition in ecological situations.

[Slide]

However, the other area which is equally important, if not more so, is competing for available nutrients. How suited is an organism for that particular environment? These can be broken down into carbon/nitrogen, the essential food supplies, plus the other one which is probably more important from a competitive point of view, and that is metals or available cofactors. We talk about that in microbial ecology and we normally talk about essentially iron. We do that when we talk about ecosystems that generally have neutral pH because in many cases the iron is bio unavailable and, therefore, it is a limiting factor for growth, and it has been shown in other systems that essentially it is a major competitive factor.

[Slide]

So, we know it is an essentially requirement for growth, and the only cells on which there is data would suggest that there is no requirement for iron is

Lactobacillus plantarum. That is a 1983 study. Essentially, it is proposed that lactobacilli may be one of the few known organisms that have evolved other cofactors, other than iron. So, iron, essentially, is known as a global element for composition.

So, when I say natural habitats, generally I point to the neutral pH habitats that is the basic chemistry of iron, that it is very insoluble at neutral pH's. It is very soluble at acid pH's but at neutral pH's it is very insoluble and that is why organisms have evolved systems to actively seek out enough iron to grow.

[Slide]

Quickly, let's look at iron uptake. Normally you have two systems. You have a low affinity system and that is a system that organisms use when iron is plentiful. If you have a cell there and you have iron just outside your door, outside your membrane, all you need is a valve system to control iron when you want it.

Then you have the high affinity system. The high affinity system is a system that is evolved in an environment that is insoluble. Then you have to send something outside the cell to solubilize it and bring it back to you. That is a high affinity system. It is high energy. An organism evolves it solely because it gives it a competitive advantage.

So, this is a system that basically we looked in the large intestine for because the large intestine is a neutral pH and iron is absorbed normally in the small intestine. So, essentially, is this a competitive factor? Is this a feature that we should be looking at?

[Slide]

If we look at bifidobacteria as an organism that we are looking for a probiotic, you know it can compete successfully in the large intestine because it can be dominant there, and then the question is, do they express this high affinity system? If they do, then essentially they have evolved it because it is of use to them.

The literature is not very supportive on this because when you look at the study that has been done, it suggests that it does not have this particular system. So, then that would suggest that iron must be plenty bioavailable in that environment. But then when you look at some feeding studies, it seems to contradict that.

Here are infants fed iron fortified milk compared to ones that were not iron fortified and then look at E. coli-bifidobacteria ratios, essentially when you supply iron you get more E. coli. This is just that more iron is getting into the large intestine and taking away that competitive effect. So, this would suggest that bifidobacteria can naturally out-compete E. coli for iron.

[Slide]

So, we actually demonstrated that by essentially looking at some isolates of bifidobacteria in the large intestine, asking the question in low iron conditions can they produce a compound which can inhibit other competitors for iron? You can see E. coli here. You can see a clearing here, and this clearing is bifidobacteria had produced this particular compound but iron essentially negates that, indicating that this is an iron-dependent system. We have shown that bifidobacteria can compete with clostridia as well, Clostridium difficile and Clostridium perfringens, another selection feature that possibly should be considered. I am going to bring this up again in a while when I talk about genetic drift and what can happen in genetic drift.

[Slide]

Before we get there we will talk about some of the methods of analysis, as I was asked to talk about these, and, therefore, what are the methods of analysis which are pertinent to this field? Probably the most important one is identification, accurate speciation. That is where a lot of inconsistency is present in the field. There are organisms being called species; they are actually another species.

We heard this morning that culture companies might get an organism from ATTC and many of those actually have

their own species, and I know some of them are even the wrong genera when you actually characterize them because the systems that were used when they were put there were not taking advantage of what tools are available now. So, this is definitely one of the more important ones.

Traditionally, all that essentially was really available was morphology -- what they looked at, and what biochemical characteristics they had. These are very, very important but essentially they are quite ambiguous and a lot of misclassification can occur if we rely solely on them.

Now we have the advantage of molecular speciation and molecular speciation, in combination with this, gives a much more clear or definitive picture in classification since it is less ambiguous.

[Slide]

Now let's go to molecular speciation and what is it. Essentially, it is picking out a piece of the nucleic acid and doing a sequence analysis on that, and then seeing which ones are closer together than the others. so, you can't just pick out any one, and the piece you pick you call a phylogenetic molecule.

[Slide]

These are the three criteria that a phylogenetic molecule needs to have. It needs to be universally present because you have to be able to compare it to all organisms

and, this is very practical, you must be able to get it into your hands. Essentially, nowadays that means you must universally conserve the DNA regions to allow you to use the technique PCR to pull it out. Then, they must be highly conserved. This is also important for the reliability of a marker. Essentially what that means is that a piece of the DNA does not accept mutations as quickly as others. So, there are some regions that are very conserved; some regions which are not.

[Slide]

So, essentially the gold standard is utilizing the 16S ribosomal RNA gene in bacteria. This is one of the more conserved regions in bacteria, if not the most, and also it is universal, available, and these red arrows point to universally conserved DNA regions which allow you to utilize primers so you can just target this and amplify this out very, very quickly and then do a sequence analysis of that, and that is how we get our present classification system for bacteria.

However, in practice it is not perfect for all genera because in some genera it is so well conserved that it becomes very, very difficult to differentiate between species, which are clearly different species based on phenotypic analysis. So, when you are only talking about one or two changes out of 1500 or 1600, then essentially it

comes down to opening the door for mistakes to be made.

So, to help classify and differentiate other species, other phylogenetic molecules are sought. In the case of probiotics bifidobacteria is a case in point. It is a very well conserved genera from the 16S point of view. So, then alternative marker which has been used in both bifidobacteria and lactobacilli classification is what we call the ITS, which is the internal transcribed space or region, if anyone is interested. It is essentially the region between the two ribosomal genes here, the 16S and the 23S. You are taking advantage of the conserved regions in order to be able to amplify it.

[S l i d e]

So, yes, it is universally present. It is easily obtainable, but that is probably where it falls down a bit, that is, it is not highly conserved. In fact, studies indicate it is one of the least conserved regions, which accumulates mutations a lot more faster. That makes it very, very sensitive but possibly unreliable if, for example, a particular isolate was temporarily, for example, for a long period. It would have accumulated a lot of mutations which would indicate that it might have changed or evolved a lot different. Then you are actually getting an inaccurate picture. But it has been used and, that aside, it can essentially substantiate other speciation system.

[Slide]

Finally, in order to address the highly conserved problem we come up with the region of the recA gene whose protein essentially has been established to be an excellent phylogenetic system because it does have all the criteria that are needed, including being highly conserved. Outside of the 16S it probably is one of the most conserved regions.

[Slide]

Also, the actual region that is between the conserved DNA regions is actually small, which means two sequence reactions can give you the information rather than, say, the approximately 12 that you need to cover the 16 to 16S, and it has the efficient differentiation to differentiate between organisms.

[Slide]

Then when you get your analysis you just draw a tree like that, like a Bifidobacterium tree that we were working on. Essentially, then this would be, for example, an isolate from an individual and that groups, for example, Bifidobacterium infantis. So, essentially you are able to very quickly come up with an accurate molecular speciation.

[Slide]

So, those are the identification tools to improve the ability to accurately find out what the species is. Now, once you have that, then you are going to do feeding

studies, or else you are going to do QC and you must have means for reliably checking authenticity of a culture or for essentially tracking through the intestine such that you can get clinical data. For that, you need essentially DNA fingerprinting techniques, which are reliable and have the ability to differentiate at that sensitivity.

That is what we call tracking tools. The first one I want to briefly go over is pulse-field gel electrophoresis. This essentially might be termed one of the gold standard of prokaryotic DNA fingerprinting because essentially you survey the whole genome.

What you do, you get your culture and you isolate out your genome. The technical problem is you have to do it in a gel matrix such that you don't damage it; it is very delicate. Then, you break it into relatively few pieces with rare restriction enzymes. Then, you can separate these large enzymes on a gel system by pulsing a current at various different angles around it, and then you get your characteristics pattern or RFLP, as it can be called. essentially. This is very characteristic.

Now, if se look at this system, this system is used nowadays in many studies and for characterizing strains, and it is probably one of the more sensitive, meaning it can differentiate at the strain level, not just at the species level, and that is very, very important. A

disadvantage is that it is technically challenging. It takes the best part of a week to actually do this because it takes a long, long time to do this type of manipulations. So, it has excellent applications but, obviously, is not applicable in all cases for quickly checking the authenticity of strains.

[Slide]

Another one which is very well worth talking about because it has been used more and more, and the reason why is because it has now been automated, and that is ribotyping. Ribotyping -- essentially you get your culture, you put it in the machine, come out eight hours later and you actually have a fingerprint. That is why it is gaining more and more popularity in commercial situations since you don't have to think about the science of it. But essentially all that is happening is it is purifying the DNA. It is restricting it, and then it is running out all the actual fragments, which are many, many, so it is like a smear. Then, it is hybridizing them with a probe to the ribosomal RNA, generally to the 16S. As there are generally multiple copies of the ribosomal RNA between 1 and 8, say, in different bacteria, any time where you have a copy you will actually get a light up. This generally is reproducible, however, the danger is that ribotyping is not very sensitive, and basically it differs between different

genera. With some genera it is much more sensitive than with other genera. In some genera it doesn't even differentiate at the species level because you are only surveying a small part of the genome and, therefore, your sensitivity goes way, way down and, therefore, the danger here is that many people are doing what they are calling molecular identification by doing ribotyping, and then running it against a database and there is no database for ribotyping, and then coming up with an identification. That is where the danger lies because it is good for tracking things but not very good for relying on identification.

[Slide]

Then you go into PCR which is very, very quick, technically feasible, and you can do this all within a day and you don't even have to culture something. So, it has many, many uses as regards checking authenticity of cultures on a regular basis.

For example, multiplex PCR where you target two specific regions on a genome, and then you actually get two bands which essentially is like double probing all at once, and this really is quite reliable. The difficulty is coming up with these particular systems to work very well. So, this takes a lot of R&D to get multiplex working, but where it is applicable it is a pretty good and reliable system.

[Slide]

There are other PCR approaches, which is worth mentioning because it is so approachable. Another one, for example, is targeting a unique region. So, you have a whole bunch of'unknown colonies here and you want to determine which one of them is your particular probiotic organism. Well, by just taking a piece of the colony you can essentially target to 16S ribosomal RNA gene and then you can run that out in the gel. So, this can be done in a couple of hours essentially, and then you have a 16S ribosomal RNA gene. But then if you restrict it with an enzyme, then you break it up based on its sequence, and here essentially you will see a tree because it separates quite well, and then you run that out in a gel and you get your RFLP, your pattern. This essentially is a good way of doing a fingerprint.

Again, it should be noted we are targeting a very small region of the genome so sensitivity is low. Therefore, it cannot differentiate too well at the strain level. So, it is only applicable where what you are actually using is sufficiently different from what actually is there.

When you talk about a lot of commercial isolates as they come from sources like animals, they are often very, very different than the human ones. So, when we were doing a commercial feeding study we were able to utilize this low

sensitivity technique because the actual strain, as it turned out, was an animalis strain and that particular strain was not even remotely like anything that is already there, therefore, this will be sufficient for differentiating.

[Slide]

Finally, we look at arbitrary priming PCR, and that is essentially where you survey the whole genome. Here you get into strain sensitivity once again where, essentially you have one particular primer which arbitrarily sits on a region that happens to have homology too, or just enough homology to stick on a piece of DNA here, and if they are arranged fairly near each other you will actually get bands. So, this is a fingerprinting technology that was started ten years ago and had tremendous hype at the time but then quickly, basically, it fell away because the problem is that it is not very reliable. If you try to do this the next day, say this primer, here, was barely sticking on -- under these particular conditions you had chosen it is barely sitting on there and that is resulting in a band coming here, what about the next day? If there was a slight change in conditions, that band would not be there.

That is the main problem with arbitrary priming. However, when you apply the TAP principle to it, which

essentially is a triplicate principle whereby you do a reaction in triplicate and then you deliberately introduce changes to it, like running at three different annealing temperatures, you are putting in fairly significant changes there and that allow you to recognize which bands are reliable and which bands are not reliable. So then essentially you have a triplicate gel, and you see that these bands are reliable in that basically four degrees of temperature here the primers are staying on.

So, this increases reliability. It maintains the actual quickness, the feasibility of using it, and also the sensitivity. It is strain sensitive and essentially rivals pulse-field for differentiating at the strain level.

[Slide]

This is just an example of what a gel would look like in practice.

[Slide]

Finally in tools, I just want to mention in situ tools. These are new tools which are coming out to allow you to actually look at bacteria in the intestine without culturing them. Everything we have learned about the intestine up to recently comes from just plating out samples and seeing what grows and then seeing what is there. Now you can essentially separate bacterial cells directly from fecal sample, and you can isolate total DNA or total RNA,

and then you can target the ribosomal RNA using PCR. Then you get an amplicon which is representative of all the different organisms which are there, all within one amplicon.

Then you can do different things with that. You can just sequence all the different ones by just cloning and sequencing, and you can find out all the different bacteria which are there.

In Europe they have done one of these studies, and what they have found is that most of the organisms that they actually identified were previously unheard of. So, essentially there is a large population in the intestine that we know absolutely nothing about because we can't culture them, or we do not know the right conditions under which to culture them.

Another thing you can do, you can actually get a snapshot using DGGE or density gradient gel electrophoresis. This would be what your amplicon would look like if you ran it out on a non-denaturing gel. Now, if you have an increasing gradient of denaturing, which could be a chemical or a temperature, and then it would be TGGE, and you can separate out all the different ribosomal RNA genes, and they are based on their sequence, and then you would get a pattern and each one of these bands represents different species that actually are in the intestine. You can

actually follow a particular species like this or you can essentially get a snapshot of what the microbial population is at a single time point in a particular individual.

So, this is essentially providing new light or new tools in order to get a better understanding of what is happening there.

[Slide]

Then finally FISH, the fluorescence in situ hybridization, and this is based on the principle that you have now very sensitive fluorescence labels that you can attach to probes, and then you can essentially stick them onto the bacteria directly in a fecal sample. So, you have non-specific probes, which are generally probes to the 16S gene, ribosomal RNA, and then essentially will stick there and light up that cell you see in an actual microscope.

These studies have found that when you look under the microscope, generally the counts might be close to ten times more than what they would be when you are just using total culturing conditions. So, the actual figures that people are coming up with indicate that definitely more than half of the isolates in the intestine -- we have no idea what they are, and possibly it might be 90 percent.

Also, you can get these probes based on the variable regions of the 16S genus or species specific probes, and then you can actual track or get information-

specific species.

So, that is quite technical. I hope I didn't bore anyone with it, but these are essentially the tools which are now available to essentially aid in these probiotic analyses.

[Slide]

Now I just want to quickly talk a bit about genetic drift. This is very pertinent when we are talking about taking organisms out of their particular environment and then putting them in another environment and then asking the question are they going to be exactly the same when you put them back.

Genetic drift -- it is the accumulation of small genetic changes which result in an altered phenotype. These are small genetic changes, generally just mutations. This morning someone was asking about the danger of these gram-positive organisms taking in large pieces of DNA. That is genetic shift, when you actually physically take up genes. That is a genetic shift. That also is a real possibility, getting antibiotic-resistant genes, etc.

I just want to make a comment there as regards the gram-positive organisms. Some gram-positive organisms like Streptococcus and Bacillus have been shown to be naturally competent all the time and will take up naked DNA very readily. However, the probiotic lactic acid bacteria -- no

one yet has been able to find natural competence, and if they had we would love it because it would be very easy to work with them. But, from an ecological point of view, maybe that might be better. But similar to gram-negatives, they do have the conjugation, the transduction, and basically they induce transformation or other artificial transforming abilities.

So what they are open to is genetic changes. Essentially an organism will undergo genetic changes, and the big question is why. Well, that is evolution. Why is evolution there? When essentially climates change, when conditions change, well, then organisms must be in situations where they can actually change, mutations which can be selective for that environment.

[Slide]

That is when we come to the rate of change is very, very important. If you put an organism into a totally foreign environment where it is stressed, then the rate of change goes up because biologically the defenses, the repair mechanisms are brought down to the minimum. So, it wants to change because essentially it wants to now adapt to the new environment. And, that is very, very important when we are talking about probiotics, particularly when we have looked at bifidobacteria because bifidobacteria, in their natural environment, are totally different to what happens when you

take them out because then you are culturing it and it produces these acidic and lactic acids and essentially is getting under stress right away, and it not used to that.

That is where we essentially come to this nice word attenuation. When an organism is attenuated, it means it has lost or changed a particular feature. That is very, very pertinent when it comes to biology. Use it or lose it. That is very, very true. If an organism doesn't need something, especially if it is energetically costly to them, well, then it is going to be the first thing to go.

When I spoke about the high affinity iron uptake systems, that is one of the first things that would actually go because it is energetically costly for an organism to produce these compounds and send them out, have specific receptors to take them up and process the whole thing. That is a very costly system for an organism to have and generally you will find it is one of the early things to go.

Just incidentally, when we were screening commercial isolates for this versus isolates fresh out of the intestine, we found a major difference. You won't find this generally in commercial isolates, which indicates they have been generally cultured outside the natural environment so things do change.

That essentially is one of the prime examples. All the other examples we do not know. We do not know what

features an organism needs to have to essentially attach to particular cells. And, does it produce something that is specific for that, that is not needed when you bring it out into a fermenter? So, these are very important things.

[Slide]

That is one of the things that need to be taken into account in genetic drift. So, clearly, minimum culturing is important because you bring it outside of a habitat that is very, very different than where you want it to perform, therefore, you must not give it a chance to evolve. You must not give natural selection a chance to make it suitable to the new habitat. Frequently, that is what has been done in order to get something to grow better in a fermenter and that might be counterproductive to what is actually needed in vivo.

Mimic in vivo conditions as close as possible. That is almost impossible to do unless you realize what things are important. For example, if maintaining iron compatibility is important, well, then use chelators to get the iron out of your culturing medium.

Very important is dormant storage, as soon as possible when they are isolated to be stored dormant. That means frozen or lyophilized such that no biological changes can occur. Traditionally in culture houses, most of these cultures are stored at four degrees, and then maybe

transferred every month or so. Well, changes occur because it is not dormant and things change very fast. So, having criteria for how these cultures are handled is extremely important if you don't want things to change.

[Slide]

Finally, just a few words on strain purity and safety issues. Clearly, you must know what organisms you are dealing with, and to be fair to the consumer when they read a particular label, it should be accurate.

There was a study done recently looking at bifidobacteria, looking at commercial isolates and then just speciating them. In many cases, it has found that organisms in commercial isolates are primarily Bifidobacterium animalis and they may not be labeled as that. A culture house may just call them Bifidus or Bifidobacterium, or often just give a different species like Bifidobacterium longum. This may be just inadvertent, based on inadvertent classification, but essentially it is important that cultures are identified correctly, otherwise nothing is going to get consistent.

Therefore cultures, when they are handled properly must have good manufacturing practices, and you have to have QC to look for viability. Basically, having 10^8 by the manufacturer, is that any use to the consumer if they are down to zero almost by the time they eat it? So viability

is very important. And culture authenticity, and that is where using accepted molecular fingerprinting techniques for checking authenticity which can be done very fast and cost effectively, will be very important.

Finally, toxicity tests. We heard about safety issues this morning, and this is very important when you are dealing with good, effective colonizers. When you think about it, a very good probiotic organism must have a lot of the criteria that a good pathogen would have as well. Essentially pathogens must have a lot of good criteria because they must be able to compete as well. So, essentially when you are dealing with healthy individuals -- well, that is a different case, but you must also look at other segments of the population which may have problems like advanced diabetes, or immune compromise, etc. So, toxicity tests which are beginning to be done nowadays are, for example, getting mice which have no immune system, which are germ-free, totally susceptible to any bacteria at that time and overload them. And, that is the question essentially, can it have any toxicity or any infection problems. That is an area that essentially needs to be addressed probably in the future.

[Slide]

Finally conclusions, molecular tools for speciation and strain differentiation are now available for

more scientific analysis of clinical trials. If the analysis is scientific, using the same strains and accepted techniques, then consistency will have to come.

Knowledge of the GI ecology, which is essentially growing, will help understand what features of a probiotic bacterium should have. That should be essentially an effective probiotic bacterium. So, essentially these criteria are just coming out.

A company should adopt modern methods to confirm strain authenticity. Then, following isolation of a bacterium from its habitat, it should be cultured minimally prior to use as a probiotic if the intended use is to compete very well in that environment. Thanks.

DR. BENEDICT: Thank you, Dr. O'Sullivan. We will hear first from Dr. Wagner and then open the floor for questions at that time.

While Dr. Wagner is setting up, I want to allay anyone's discomfort with the schedule. The functional schedule is that Dr. O'Sullivan was going to speak for 45 minutes because we asked him to add some methodological considerations into his talk, and he did so and we are grateful. So, Dr. Wagner will speak for 30 minutes, until around 2:15, 2:20, whatever. Then we will open the floor for questions.

We will now hear from Dr. Wagner, from the

National Center for Toxicological Research. He will talk to us about microbial ecology.

Microbial Ecology

DR. WAGNER: I am Doug Wagner. I am from the FDA's National Center for Toxicological Research, where I am a microbiologist.

[Slide]

Before we can really understand the ecology of probiotics we have to understand, or get a better understanding of the ecology of the gastrointestinal flora.

[Slide]

As an example, in the colon there are at least 400 species that can be cultured and identified of bacteria. In fact, there are many more species that are not cultivable that have been observed by the genetic techniques that Dr. O'Sullivan has mentioned. Within the colon population most of the bacteria are obligate anaerobes. These are organisms that generally microbiology has not worked a lot with. The science is not as fully advanced as with the medical bacteria which are mostly facultative anaerobes. So, the facultative anaerobes like the enterobacteria and enterococci really make up a small percentage of the organisms that can be found in the colon. The rest are predominantly bacteroides, bifidobacteria and eubacteria, and then some of the other anaerobes as well.

This is not the typical picture for other parts of the gastrointestinal system however. In fact, this data has been derived from studies of fecal samples which are really not representative of other parts of the gastrointestinal tract either.

[Slide]

To get an idea of how this works, let's see the gastrointestinal tract as a big, long tube here. In the center, if we take a cross-section anywhere along here there is a lumen which contains food material, sloughed off host cells and also a lot of bacterial cells.

Surrounding that is a mucous layer, and it has different characteristics, and its bacterial population can be expected to be different as well. There are sloughed off cells there, and there are mucous filaments, and the bacteria have the ability to adhere to these elements. So, they can persist in this envirdnment longer than they can in the lumen.

Surrounding that then is the monolayer of intestinal epithelial cells. These have on their surfaces microvilli which are covered with molecules such as oligosaccharides that the bacteria can adhere to. On top of that, the enterocytes grow up into villous structures, which are not shown on this simplified drawing, and those villous structures, in fact, fold over themselves.

So, the surface area actually goes from what you would expect from a linear tube to 400 times the surface area that actually occurs in the intestine by the way it is developed.

On top of that there are other microenvironments, such as crypts between villi, and the surfaces of some cells are different., There are epithelial cells that are specialized for various features and they have different abilities to interact with the bacteria in the intestines.

If we look at the length of the intestines, the bacterial concentration is not the same throughout the entire gut. Up around the stomach there are very low numbers of bacteria because of the high acidity. The upper small intestine, only about 10^4 bacteria/ml, but when you get down to the colon there are 10^{11} bacteria/ml. Up in the oral pharynx there are 10^9 bacteria/ml, and a lot of that is the dentition as well, and there are around 200 species of bacteria that have been identified there. So, there are differences in the population of the bacteria all the way through the intestinal tract.

[Slide]

To get a better idea of how these large diverse populations develop, we can look at the development of a child from birth and the development of the enteric flora within the intestines of that child. A fetus has a sterile

gastrointestinal tract and at birth the child is exposed to the mother's flora from the birth canal. The first organisms that can be identified in the feces of the child after it is born are mainly facultative anaerobes such as enterobacteria, especially streptococci. There are some obligate anaerobes observed as well.

These are the founding organisms for this ecosystem. They alter the environment. They produce molecules that are necessary for the colonization of other organisms, and they also change the environment by changing the pH and increasing the anerobicity of the environment.

One thing I forgot to mention in the gastrointestinal tract was that it becomes more anaerobic as you go down the length of the tract. That is why there are a lot more obligate anaerobes in the colon than up higher in the intestinal tract.

These organisms make it possible for the next group of organisms to colonize in the intestinal tract. A lot of the same organisms stay around and more are added. We start to see lactobacilli and propionibacteria and the bifidobacteria. During breastfeeding bifidobacteria are preferentially grown because the breast milk contains the fructo-oligosaccharides and other nutrients that are specifically utilizable by these organisms. So, these organisms also change the environment and the succession

continues to a climax population which is larger in number of different kinds of organisms and has more of the obligate anaerobes. This then is around ten months, after the child is starting to eat diverse groups of foods and there are more specific nutrients for these individual genera when we see this large population. It becomes relatively stable, although it is a dynamic system and even through adult life it changes over time.

[Slide]

Probiotics are probably going to be applied to infant formulas, and there may be good reasons for it as the medical system changes. Under a normal vaginal delivery, a child gets colonized with his mother's flora, and then during breastfeeding the child is nourished with specific nutrients that are preferential for healthful bacteria in the intestines, especially bifidobacteria which are really the wonder bugs. They do a lot of things. They appear to be very good at preventing pathogens from colonizing, and they produce vitamins B and folic acid, and a number of other beneficial factors are known to occur.

When a child is born by cesarean section, he gets some of the mother's flora but he also gets a lot of bacterial contaminants from the environment and this, of course, occurs in a hospital environment where there are a lot of organisms that we really don't want to be colonized

with. So, it is possible that probiotics could be used in this respect to assist in the colonization of infants that have been delivered abdominally.

Also, formula feeding generally does not provide a lot of the specific nutrients for bifidobacteria and other organisms, and can also lead to the colonization by uncharacterized bacteria.

[Slide]

So, developmental stage, of course, is a part of the host that affects microbial ecology. The diet is believed to have an effect on microbial ecology as well as drugs, especially antibiotics. These do perturb the microbial ecology. There are environmental factors that have been noticed between people in the Third World versus people who live in our kind of environment. Genetics and the host defenses are important in determining what kind of organisms are going to populate that large population of bacteria as well, and stress responses of the host can actually change the population dynamics as well.

[Slide]

I was going to talk about methods of analysis but Dr. O'Sullivan did a very good job of it. I would just like to point out that really we look at analytical techniques in two different veins, that is, one, we can look at individual organisms, identify the species and the strains of the

organisms, and study their metabolites. We can evaluate them in animal models. This helps us to understand what the organisms might be doing in the intestinal population, but as individuals we don't really see everything that occurs in the population.

In population studies, it is very difficult to isolate 400 species of bacteria and identify each one, and characterize them fully. It is more useful, anyway, to look at total effects of groups of bacteria, such as metabolic markers. For instance, the lactic acid bacteria that produce a lot of lactic acid and other short-chain fatty acids -- we can evaluate effects on those bacteria by drugs or by the addition of probiotics to the community on how they affect that group of bacteria.

The new technology of DNA can be useful for identifying bacteria within the population because a gene shift can be made with probes for the specific DNA markers of a lot of different organisms, and they can all be screened at once.

Continuous culture models are useful for studying populations. It is very difficult to study all the different ecosystems in the human intestines because we really can't sample much of the intestine, usually just the fecal samples. So, it is helpful to use animal models and in vitro models to study the enteric flora. We can also

look for effects on indicator species, whether they disappear or appear given a particular treatment of the flora. I am not going to talk about the techniques since Dr. O'Sullivan already mentioned them.

[Slide]

He also talked a lot about strain purity and genetic drift. I just want to point out that right now the taxonomy is undergoing a lot of change as we go from identifying these organisms by conventional techniques to the genetic methods that are now in general used to identify organisms. So, there have been a lot of changes in the nomenclature of the bacteria.

Also, it has already been mentioned that there has been some controversy about the host sources of a lot of the bacteria and whether they really came from human sources or animal sources, and this is believed to be an issue.

In the gastrointestinal tract there are 10^{14} bacteria and it has been calculated that there are 10^{6} mutations per every 20-minute division of those bacteria. This allows for a lot of adaptability by the gastrointestinal flora.

This idea can also be applied to large cultures of bacteria that are used for making probiotics. There are mutations that occur in those populations of bacteria as well. So, over time a starter culture, a fermentation

culture can change because of mutational events.

In probiotics, if there are any transferable gene cassettes that contain virulence factors or antibiotic resistance markers, these might be transferred to some of the bacteria within the host's intestinal flora and, thus, the probiotic could be introducing a real problem to the enteric flora as well.

[Slide]

So, how do the enteric flora and also probiotics protect hosts from infection by pathogens? It has been believed that adhesion is very important, and we have talked a lot about adhesion. In bacterial pathogenesis adhesion is the first step to invasion of a host cell by bacteria. So, adhesion may not necessarily be a good thing because the opportunistic pathogens would start their process of pathogenicity by that technique as well by adhesion.

Another of the issues is competition for nutrients. Dr. O'Sullivan talked about the competition for iron and other micronutrient. Even though the gastrointestinal tract is a nutrient-rich environment, micronutrients are in short supply and the organisms that compete the best for those have a niche and stay present. In fact, the reason the flora maintains itself as well as it does, given all the challenges it has, is because each organism that is in there somehow has its own specific

niche, either provided by other organisms or by its own specific capabilities.

Bacteria can produce antimicrobial substances, such as bacteriocins and other things that can prevent pathogens from growing. They also modify their environment. They change the pH. They tend to lower the pH and lower pH is a difficult environment for a lot of pathogens. They also are known to imrnunomodulate the host. So there is an idea that there is a priming of the host immune system by the enteric bacteria that make the immunity more rapid and a better response against pathogens.

[Slide]

To illustrate how probiotics can work against an invading pathogen, I did some experiments with imtnunodeficient mice that had both the beige and the nude defects, which makes them immunodeficient in both innate and acquired immunity. These mice, as germ-free mice, when they are colonized with Candida albicans, which is a pathogenic fungus -- 75 percent of the mice show evidence of dissemination of the fungus from the gastrointestinal tract into the internal organs which are generally sterile.

When the mice are colonized first with a probiotic organism and then challenged with the Candida albicans there is a marked reduction in the ability of the Candida to disseminate even in these very immunodeficient animals. In

heterozygous mice, which are less immunodeficient, the change is not as striking but it is in these animals.

[Slide]

Of course, immunodeficient animals brings up the issue of safety concerns. It is not so much a problem with the probiotics that are used for humans, but if a probiotic mixture contains occult pathogens, that can be a problem, but there could be opportunistic pathogens. For instance, a lot of the probiotic preparations do have enterococci, and enterococci are growing in our concern for opportunistic pathogenesis. We don't know how much probiotics can really upset the microbial population balance. There have not been a lot of studies on this issue and more need to be done.

We are concerned about the transfer of virulence determinants and antibiotic resistance. One of the issues with the enterococci, of course, is that some strains of enterococci carry antibiotic resistance markers on conjugated transposons. These are like pathogenicity islands or transferable cassettes that can be moved from one organism to another. In vitro it has been shown that enterococci can transfer vancomycin resistance, for instance, to other gram-positive organisms, such as Listeria and Staphylococcus aureus.

Immunodeficiency of the host is a concern. We don't have a lot of information, although there have been

immunodeficient people using prabiotics that are available right now and have not reported a lot of cases of infections.

We need to be also concerned about genetically engineered bacteria, if they are engineered to treat a specific condition the organisms are not transferred to healthy individuals because the product of the engineered bacteria, being a biological product, may be harmful to normal hosts.

[Slide]

With our immunodeficient mice, we wanted to find out if any of these bacteria had any potential to be harmful to mice right after they are born and as they grow. So, mice that were born to mice that were germ-free mice that were then associated with these different organisms were allowed to grow up to 4-8 weeks of age or 8-12 weeks of age where we evaluated their survival. For instance, with Lactobacillus reuteri there were 79 percent survivors out of the 28 mice that were born. With Lactobacillus GG -- the name has changed, but there were 64 percent that survived out of the 53 mice that were born.

So, with these two organisms there was a little bit of infant mortality and we need to be a little concerned about this. Interestingly, in vitro experiments and also other experiments, I noticed that these organisms were more

adherent to epithelial cells than the acidophilus or the bifidobacteria. So, there is a case where there may be a link between adherence and the capacity to be pathogenic.

[Slide]

So, to summarize all this then, we need to know whether the probiotic is going to be dietary supplement or a drug. That is going to determine how it is going to be regulated.

The manufacturers are going to have to make sure that the products don't contain what we know to be opportunistic pathogens. We are going to be always one step behind, I think, not knowing what the next new opportunist is going to be. There are always up and coming new pathogens out there.

We want to avoid though having organisms that are carrying transferable elements for virulence or antibiotic resistance, and we also want to be very careful about genetically modified organisms.

That is all I have.

DR. BENEDICT: Thank you very much. So, now if we could ask both of our previous speakers, Dr. O'Sullivan and Dr. Wagner to migrate close to the microphones, we can --

DR. HOTCHKISS: Colonize the microphones.

DR. BENEDICT: Colonize the microphones. Thank you, Dr. Hotckiss, I like that. We can begin to pepper you

with questions.

Questions and Answers

DR. FUKAGAWA: Dr. Wagner, just a point of clarification, in your slide with the candidiasis and the heterozygous mice who received the Lactobacillus casei had 26 percent dissemination. Everything else was about zero. What is the significance of that?

DR. WAGNER: It is statistically significant. You know, there was still some dissemination with the Candida even in the presence of the Bifidobacterium. It wasn't protecting as well as the lactobacilli.

DR. BENEDICT: Yes, Dr. Cohen?

DR. COHEN: There have been a number of studies looking at people who get low white cells during chemotherapy to try to look at interventions, most of which have been giving them various kinds of antibiotics, putting them in protected environments, and such. Are you or anybody aware of any studies where people have actually tried to prevent bacterial or fungal infections by using probiotics in this population?

DR. WAGNER: What population? I am sorry.

DR. COHEN: This would be people who would be undergoing chemotherapy for hematologic malignancies or bone marrow transplant, or something like that. Particularly since these products have been so widely used in Europe, I

was curious as to whether or not in any of these trials anyone has ever tried to prevent infections in those populations by using these agents.

DR. WAGNER: I don't know of any studies where they have done that.

DR. RUSSELL: I was wondering, these tracking systems that you have described, are they used also in picking up bacteria once they have been fed to an individual to see if they are in the stool? In other words, rather than culturing the stool to see whether or not that organism is present, are you using fingerprint techniques on the stool to see if those organisms that were fed actually appeared in the stool?

DR. O'SULLIVAN: You mean non-culturing techniques?

DR. RUSSELL: Non-culture techniques, simply to Look at whether or not the fed organism is coming out sithout culturing.

DR. O'SULLIVAN: Yes, using the in situ methods, people are now doing that. You can actually get probes which are strain specific. If you have a strain specific probe that can differentiate between the exogenous strain you are feeding from the natural flora that are actually there, then essentially you can follow it using, for example, density gradient gel electrophoresis by just

running out the actual amplicon and then hybridizing. So, yes, people are using that now for tracking.

DR. RUSSELL: And the sensitivity of that as compared to culturing, for example, is much, much higher?

DR. O'SULLIVAN: The sensitivity is higher if you are using fluorescent probes. It is higher than culturing because when you are culturing it must be essentially quite dominant because you don't have selective media. If you are using, for example bifidobacteria, most reach very high dominance because you can only analyze so many colonies. So, it must be very, very high. When you are using culture-independent techniques the sensitivity is several logs higher but I do not know -- 1 mean it has not been fully calculated essentially because it has only been used by a few labs presently.

DR. RUSSELL: I was just theoretically wondering whether these techniques might be so sensitive that they would pick up amounts of bacteria that were really there but were insignificant. I don't know the answer. I was wondering whether these techniques might certainly be able to show the appearance 'of bacteria that have been fed.

DR. O'SULLIVAN: That is a good question. Unfortunately, they are not that sensitive yet. But, if that was the case, you can make these semi-quantitative by doing dilutions. Then you can find out are you dealing with

maybe ten or a trillion bacteria there, or are you dealing with, say, a thousand or a million.

DR. HOTCHKISS: A question for Dr. O'Sullivan to help me understand a couple of points that you made. As I understand, you laid out at least four criteria that you suggested were important to determine efficacy, at least from the microbial viewpoint not from the epidemiologic or disease but at least from the microbial, tolerance, beta-galactosidase activity, adhesiveness and niche fitness. Then, I got the impression that when you went through those, kind of your general conclusion was that in at least some of those, if not most of those areas that you put out we really don't have, or don't understand or haven't applied modern tools to understand each of those points. For example, for adhesiveness you said we don't really have a test.

So, am I right in getting the impression that at least from the viewpoint you have put out about efficacy that we really haven't determined the efficacy very well?

DR. O'SULLIVAN: That's correct. It is a very complex ecological system to study, adherence in any ecological system, it is a multifaceted event and what features are required; there are multiple features required. It is not really solved for any ecological system. The human intestine is probably in its infancy as regards when studying that particular feature.

It would be nice if there was a whole list of criteria that essentially, when you add them all up, would equal the optimum strain. If that was the case, then we would have a lot of organisms that could complete naturally with the resident flora.

The resident flora are not superbugs. They are essentially bugs which have adapted to that, and they have adapted certain criteria.

The other thing is essentially getting a single strain from a single person and then having a single inoculum, as it were, to feed everyone. In the future, when more and more is understood about the diversity of the strains with different types of intestinal conditions, the likelihood is that there will be multiple strains of a single species being utilized in the probiotic culture such that it would have a potential impact on the greater population, or a greater percentage of the population.

DR. BENEDICT: Dr. Hotchkiss?

DR. HOTCHKISS: Just a quick follow up. Did I understand that either most or the majority or the vast majority of organisms in the lower GI tract are really unknown?

DR. O'SULLIVAN: That is the very interesting thing which is coming out of these studies, especially when you clone the individual amplicons and sequence them. The

majority are essentially unknown, unclassified. That opens the question of when we have a handle on what these are, maybe there are a whole bunch of other bacteria which essentially should be used in combination for effectiveness.

When you have an environment that is populated with such a diverse amount of strains and species and genera, maybe it is bit naive to think we just take one of them that essentially is going to obtain dominance. They all have evolved to interact with each other, not in pure culture, but essentially to interact with other strains.

So the likelihood is that they feed off of each other. That is a very, very complex thing to understand. That is going to take many years, probably, of studying. That is, hopefully, where the whole thing will get better.

DR. BENEDICT: Dr. Montville?

DR. MONTVILLE: I just wanted to reemphasize that point because we forget sometimes that we only look at the organisms that we can culture. In most ecosystems, where they started using molecular ecology, they find out that about 90 percent of the inhabitants, we can't see or handle in a traditional sense.

DR. BENEDICT: Let me just ask a methodological--I beg your pardon; Dr. Buchanan?

DR. BUCHANAN: This is a question directed to Dr. Wagner. We heard from several of the speakers this morning

about the desirability of having human isolates, whatever that was. However, in your presentation, you have indicated that the intestinal tract of the human is essentially sterile at birth and that your human isolates are the environment that you are exposed to consequently.

Would you care to comment or follow up on this recommendation earlier this morning that we use "human isolates" as a criterion for what would be a good probiotic?

DR. WAGNER: At birth, the infant gets the mother's flora and that is the closest flora, genetically speaking, to being accommodated to that individual because the child is genetically closely related to the mother. So it still shows that, perhaps, the host is still important. We really don't have enough information yet to know whether organisms that come from other sources can gain any kind of advantage.

Right now, probiotic organisms, a lot of them don't come from human sources and they don't persist--they don't compete against the normal enteric flora. They disappear after you stop giving the probiotic. So the question could be answered both ways. We think it is good but we don't know how important it is.

DR. BENEDICT: Dr. Sigman-Grant?

DR. SIGMAN-GRANT: I am curious, Dr. Wagner, when you talked about the mutations and the rate of mutation that

would occur, what kind of implication might that have to changing the strain?

DR. WAGNER: The implication is--I guess what I am implying is that, in a fermentation environment, the organisms that are being fermented could become modified to the point where they no longer have the probiotic effect. I think the manufacturers are going to have to test their culture vats every so often and make sure that their organisms don't drift away from activity.

DR. SIGMAN-GRANT: Would that be in culturing it as the mutations also occur within the GI tract?

DR. WAGNER: Mutations are occurring in the G.I. tract all the time and, for the most part, we can observe that they don't have that negative of an impact, the system being dynamic and, of course, most mutations are detrimental also. So it is not too often that one of the bacteria gains great advantage over the other organisms.

DR. SIGMAN-GRANT: By mutating.

DR. WAGNER: Right.

DR. BENEDICT: Let me just follow that a little bit. Don't you think that it is at least possible that, over the lifetime of a human, the mutations will allow the organism to evolve to be almost person-specific such that if you take an organism from me, it might not be universally beneficial to someone else or at least not universally

colonizing or some other factor.

Perhaps, if either of you could comment on that, how do you pick the person who is the prototypic host?

DR. WAGNER: That is a really good point and I think that is part of the problem with trying to use a probiotic to reconstitute someone's intestinal flora. The best intestinal flora for them is their own or someone closely related.

DR. O'SULLIVAN: I think that is a very good comment as well. We do not know, essentially, how specific it is. Is this specific to every individual? Or is it based on age, race, sex, gender, ethnic original and, obviously, diet as well has a bit effect. That is where a lot of studies need to be done because when you just put your hand into a whole species and pull out one strain and use it at as probiotic, it is like putting your hand down on earth and pulling out five people and saying, "We have got a basketball team here."

Essentially, you must know what criteria you are looking for. If you know what a basketball team is made up of, well, then, you know they must be closing on seven feet and good athletes and things like that. And then, essentially, you have the makings of a good team there.

It is the same with probiotics. If you know what criteria a successful colonizing strain has and,

essentially, if you know what things are impacting it such as diet, age, and we do know, especially the work that Gerald Tannock is doing in New Zealand, that, generally, as a person gets into adult life, gets older, probably beyond 30, then, essentially, their flora is often more stable.

From studies that they have been doing, looking possible a year *over* an individual's flora, essentially his own flora is very stable. He is up there now. And, essentially, the younger people's flora was essentially much more sensitive to changes.

So, obviously, organisms do evolve over a person's lifetime and therefore, those organisms are particular suited to that environment. It is essentially unknown yet whether it is all individually specific--if that was the case, then, you must just go after your own probiotic.

But essentially it is not known yet how specific.

DR. BENEDICT: Dr. Fukagawa?

DR. FUKAGAWA: My question was answered.

DR. BENEDICT: Dr. Buchanan?

DR. BUCHANAN: The comments on not knowing necessarily what is in the microflora of a system brought a question to mind which was, of the discussions we have heard this morning, we have talked primarily about probiotics for which it is either monocultures or a very limited number of cultures.

Historically, fermented products were actually made by back slopping where you just took part of the last batch and put it back in. But it does bring the question, is one of the prerequisites that we will need to be looking at in terms of probiotics is that it be a defined culture, or a limited number of defined cultures, in order to be able to achieve the quality assurance and quantity control that you are both discussing in terms of the products that are available in the marketplace both in terms of safety and efficacy.

DR. O'SULLIVAN: I definitely think you have to have defined cultures, defined strains. It must be either at present working with single defined strains or essentially a combination of the defined strains because you have to control it. Otherwise, essentially, you are opening the door for unknown bacteria to be in there.

So it must be defined if you want to have some handle on the safety of it. How many strains? That is open to interpretation.

DR. BENEDICT: Dr. Cohen?

DR. COHEN: Of the most common probiotic strains, what do we find in surveys of intrinsic antimicrobial resistance? I sort of ask this on the basis of the experience that we have had with the Enterococci where intrinsic cephalosporin resistance has probably been very

critical in the emergency of the vancomycin-resistant Enterococci in hospitals.

To what extent have people examined these strains for resistance to a variety of both antibiotics that have been produced as well as other potential resistances such as heavy metals and other types?

DR. O'SULLIVAN: That is a very interesting area. What you will indicate is natural isolates in the intestine because they are exposed to antibiotics over a person's life will have evolved some resistance. For example, that is how we make selected media for the bifidobacteria. We largely rely on their resistance, intrinsic resistance, to different antibiotics.

Now, the difference comes from is this intrinsic resistance or is this actually on a transferrable cassette. That is where the issue really needs to be looked at. If it essentially has changed the target site of a particular element in its cell wall or in its ribosome or one of its enzymes such that it has de-evolved the target site for than the antibiotic.

That is not a transferrable antibiotic resistance and that is where the distinction needs to be made. In the vancomycin-resistant case, yes, there is a vancomycin-resistant gene that essentially can be mobilized and picked up by other bacteria. That is where horizontal gene

transfer is a big issue.

Obviously, if a culture is going to be used in large scale and all the known cassettes most essentially be accounted for--but then, again, what you really need is a complete sequence of the whole genome so you can examine it co see are there any cassettes there carrying genes which we do not have probes for, as if yet.

Maybe that puts an argument for the actual strains which are undergoing genome sequencing. We know that Lactobacillus acidophilus is just about being finished in North Carolina and San Diego. Then, from just looking at that genome, then we can see how many mobile elements are actually there.

No matter which bacterial complete genome you actually look at, you will see a whole load of elements which are potentially mobile. What you want is to make sure that in the vicinity there is no virulence gene or there is no antibiotic resistance gene, per se, that is important that can be mobilized.

DR. WAGNER: Responding to, say, lactobacilli and vancomycin 'resistance, a lot of the lactobacillus are innately resistant to it because they have an DEE gene which is just in their nature and they are effectively resistance. But that gene is not known to be mobilizable. We could say that every gene is mobilizable, possibly, because

transposons are fairly random.

But it doesn't really seem to be the case in the real world and so we mainly need to be concerned about those resistance genes that we know are transferable as opposed to just every antibiotic resistance. We won't find bacteria that are susceptible to every antibiotic anymore.

DR. BENEDICT: Dr. Buchanan; go ahead.

DR. BUCHANAN: In taking your two presentations together, it would appear that we have the horns of a paradox. We have 10^6 mutational events occurring every twenty minutes in the large-scale population, so, by definition, we have a heterogeneous population.

We have a product that requires that the organism be provided in fairly high doses. I think the doses we heard this morning were 10^8 to 10^{10} would be an effective dose. We have the recommendation that reproduction of the organism should be minimized to avoid the mutational events. I am trying to figure out how you optimize that kind of a system where you grow it up to large numbers but you don't grow it up.

Any comments on how you match those two? Or just one other thing; are there some mutational events that we shouldn't be worried about?

DR. O'SULLIVAN: Correct. That is the comment I want to make is essentially, yes, 10^6 changes can occur.

But that is over a massive number of nucleotides. Now, if you look at an actual genome, and look, for example, at all the actual genes which are required for that organism to grow, they will essentially accept mutations a lot less frequently.

You also have repair systems happen. Those changes will be repaired. When you have nonconserved regions such as the ITS I was talking about, a lot of that is nonessential so that will accumulate more mutations. Now, when you look at essential genes, what is necessary is the actual phenotype, the protein.

Each of the amino acids is coded by three nucleotides. There is always a nonsense nucleotide, meaning you can change that particular nucleotide and it will not affect the actual protein, itself. When you look at essential genes, like the rekA protein and look at the accumulation of these mutations, they are always nonsense mutations.

So, essentially, that is how evolution actually works. Then, essentially, you must look at, now you are actually going to open the fermenter so what is important, what genes are important in the fermenter, might not all be the same as the genes which are important in the actual in vivo intestine and, therefore , you may get mutations being accepted in those important genes in the fermenter.

So you cannot, essentially, have a situation where you are going to take it out of the intestine, grow it up in a fermenter over many generations to high numbers, and guarantee you haven't changed anything.

At present, that is impossible to guarantee. But you can minimize, essentially minimize the actual growing it up in the other environment where, essentially, mutations in genes which are essential for the intestine will be accepted.

DR. BENEDICT: Dr. Hotchkiss?

DR. HOTCHKISS: I am glad you raised that point, Bob, because I worked in the fermentation industry and, in the real world, what you do is you take a very small culture and you grow it up. You make a product out of that and you take a small bit of that culture and grow it, regrow it and regrow it.

So you go through tens of years of generations of the same organism. That is the economics of business and so forth. So this business of genetic drift seems like a real issue if that is, in fact, something you have got to worry about in something you are calling a probiotic.

DR. BENEDICT: Let me just flagellate the same equine that I did this morning. This is maybe a ten- or fifteen-year question, but let's assume that you are able to conquer culture so that we don't have adverse genetic drift.

The same question comes to mind that we still have the phenotypic changes that can be induced by culture conditions without mutations, just differential expression of genes.

I am actually asking this more to get it on the record, but I would also like to have your comments. Is it not going to be better, ten or fifteen years from now, to know the appropriate genes and use array technology not to really much care what organisms are there, but we want to know that certain genes have been expressed--that is not true; that is an exaggeration--but array technology to determine gene expression is going to be pretty important, I would think, to show what the characteristics are you are going for are actually expressed in the human.

Maybe it is quicker than I am thinking, but do you have comments on that?

DR. O'SULLIVAN: That is a pretty good insight right there and that is essentially where, in the future, I can see it going as well.

At present, we know of some criteria, some features, which essentially are likely to be important in the intestine. But we do not all of them and possibly array technology will allow us to pick out what genes are actually expressed in the intestine.

So, using these in situ things, be it FISH or be it, essentially, array technology, a bigger picture will

probably come about as to what genes are expressed preferentially in the actual intestine. Then you can essentially use your array technology on a culture after you have prepared it for commercial use and determine are these still expressed.

So I agree. We can see that the seeds of the technology are there to develop these particular assays. But it will probably take quite a few years before they are at fruition. That is a very good point.

DR. BENEDICT: Thank you. Why don't we take the break now. Wait; we have two minutes. Are there additional questions? I misdid the time. Okay; let's take the break until 3 o'clock.

[Break.]

DR. BENEDICT: We have heard already from many folks talking to us about the organisms, themselves, and questions appertaining thereto. We are going to begin to look at the effects on the host and, also, I trust, by the host. So Dr. Rex Gaskins is going to speak to us on probiotics and the immune system.

Dr. Gaskins?

Probiotics and the Immune System

DR. GASKINS: Thank you.

[Slide.]

Being a silent participant all day, it seems clear

that, perhaps, we won't be able to decide if eating bacteria is a natural and a healthy activity, but I bet you will all agree that sitting around in a gloomy hotel room with coats and ties and dresses on talking about it is unnatural and unhealthy.

So what I hope to do is try to illuminate, nighlight, some of the questions that we have encountered in an effort to try to understand host-microbe interactions at the intestinal epithelium.

I was asked to talk about probiotics in the immune system, and I think one of the first points that I would like to make is that if that is what we want to understand, it would seem that, indeed, we must focus on host-microbe interactions at the intestine epithelium.

That is what my group is trying to do. We are not working, per se, in the probiotic area. There are a number of additional challenges associated with probiotics and most of those challenges, I think, have already been mentioned this morning.

When I look at the probiotic research field, I find it encouraging that, indeed, there is more and more focus on intestinal questions, trying to understand mechanisms operative at the epithelium relative to probiotics and their effects on the immune system.

However, historically, I think if one surveys the

literature, a problem with the probiotic research field is sometimes either improper *or* the lack of biological context. For example, it is not uncommon to encounter studies in which investigators were comparing animals dosed either with or without probiotics, taking T-cells, plasma blood T-cells, stimulating those in vitro with conventional T-cell mitogens, comparing their ability to proliferate in response to, for example, concanavalin A and finding, perhaps, a few thousand count increase from the animals that *were* dosed with probiotics and then drawing sometimes sweeping conclusions about the ability of probiotics to "boost the immune system."

I actually find those data and that approach somewhat hard to interpret. I think one challenge, one problem, associated with that is that it leads to what can be a misleading concept of good versus bad bacteria. Frankly, if one tries to view bacteria as the epithelium views bacteria, I am not sure that you can distinguish bacteria as being either good or bad.

That is the concept I want to talk about today. What is clear is the fact that the epithelium is extremely sensitive to bacteria and, perhaps, most sensitive to changes in microbial community structure.

In fact, it is our view that most of the structures and many of the functions of the intestine

evolved in response to the persistent antigenic challenges associated with the normal gastrointestinal microbiota.

[Slide.]

I am always telling the students that framing the question is the most important part of research and, in an effort to try to address, ask the right question, then sometimes it is a good idea to start with generalizations. The one that we have taken is that, in a simple sense, the intestine represents two complex cellular communities, one comprised of, mainly, bacteria, mainly anaerobic bacteria, and another comprised of a very complex buried host-cell compartment comprised of epithelial cells and wide variety of immune cells.

So, really, when we are talking about interactions between the normal microbiota and the host, we are talking about interactions between two complex cellular communities. Unfortunately, we don't understand very well the regulatory cues by which these two compartments interact.

However, it is clear from a lot of phenomenological research and anecdotal evidence that these structures that we have talked about and we will talk a little bit more about now almost certainly had to evolve in response to the persistent challenge offered by the normal microbiota, not by periodic encounter with pathogens because, as you will see, it is just such an exquisite and

beautiful system that the major driving, the major stimulus, must represent normal gut bacteria.

[Slide.]

First, with all the gastroenterologists in the audience, I want to make sure that you don't get concerned. This individual actually suffers from "powerpointitis" and is meant to represent a normal intestine. Really, the information has been already communicated and I don't want to belabor the point.

The major point, though, is, indeed, we are talking about a very complex system. Doug Wagner talked about the new ideas or the new evidence that is emerging from molecular approaches to try to understand this complex system. I think I am aware of a couple of instances in which cloned ribosomal RNA libraries were generated. Indeed, it is at least this complex as the numbers represent here; in other words, perhaps 400 to 500 different species.

Interestingly, some of the molecular work demonstrates the preservation of what is referred to as higher taxonomic structures; in other words, numerous species or strains, even within individual genera. Obviously, that complexity will be quite difficult to understand.

In other words, these bacteria are most likely occupying the same niche. However, there is some reason,

at

there is some advantage, of having multiple organisms in that niche rather than a few organisms. Indeed, there are a number of important ideas, a number of important observations, that are coming from molecular approaches.

I think the objectivity associated with taking a molecular approach to try to understand this complex system cannot be overemphasized.

Another important point it regards to host-microbe interactions is the fact that, in addition to regional gradients of bacteria density and regional gradients of bacteria composition, one finds regional gradients of immune structures and functions. In other words, the intestinal immune system varies along the gastrointestinal tract just as does the microbiota.

Currently, those are just parallel observations. In other words, we do not understand that if a regional differences in immune structure and function relate, somehow, to regional differences in bacterial density and composition. That is something that we would like to understand.

[Slide.]

This slide violates all of the rules of a good slide, but it, effectively, illustrates the point I would Like to make and that is that, just as for the normal nicrobiota, the host side is equally complex. So here we

are looking at the epithelium. We have talked about it quite a bit today. And then just a wide variety of immune cells that are residing underneath the epithelium in the lamina propria compartment, for example.

This is a very dynamic compartment. These cells are talking to one another and they are talking to one another via a variety of bioactive cytokines and other mediators, bioactive lipids and so forth. The degree to which we understand that currently most often reflects various in vitro studies in which we have removed subsets of these cells and tried to ask questions how one subset of cells modifies the phenotype of another cell subset.

So, by removing these cells, we also introduce significant bias just as we do when we remove bacteria and ask questions about single organisms out of their normal environment.

But I think this slide, again, represents what the host thinks about the normal microbiota, not what the host thinks about a probiotic that one introduces or about what the host thinks about periodic encounter with a pathogen. I think this level of complexity reflects the fact that the host has invested heavily in trying to protect itself from the normal microbiota.

[Slide.]

One way to try to bring some resolution to the

complexity is to consider that there are both compartmentalized immune structures and diffuse immune structures. I think the one that has received the most attention over the years is the compartmentalized secretory IgA system.

You are all familiar with Peyer's patches. We like to think of them as B-cell universities in the intestine. Remember, that the epithelium is covered with a specialized type of epithelial cell here that is sampling the contents of the lumen and essentially passing off the antigens that it encounters to macrophages that are residing here in the dome area of Peyer's patches.

Macrophages then activate T-cells which educate the B-cells. It is the collective cytokine soup that seems to stimulate B-cells to differentiate toward an IgA isotype. So that is reasonably understood.

So here is an opportunity in which, indeed, the host can learn or encounter the probiotic organisms that you dose the animal or human with. So this is an organized lymphoid compartment generating active or acquired immunity.

[Slide.]

This just reviews the structure of Peyer's patches which I just did. Another important point, I think, that was mentioned this morning is the fact that although these cells go to school here--in other words, they learn which

antigens to make antibodies against--they actually leave the B-cell university through the lymph and the blood.

[Slide.]

They take up residence in all epithelial tissues of the body and essentially are silent until they encounter, once again, those antigens that they first encountered in the intestine.

This is the phenomenon referred to as the common mucosal immune system, a very beautiful effective strategy to protect the epithelium and the body from the persistent challenges associated with normal gut bacteria.

Why do I say "normal" gut bacteria? Greater than 50 percent of secretory IgA is directed against normal gut bacteria. The development of this system is delayed in animals that are nursing, in breast-fed animals, for example, because the secretory IgA provided in the milk prevents or delays interactions between bacteria in the intestine and the host secretory IgA system of the developing animal or human.

Therefore, the development of the secretory IgA system is delayed. Again, additional evidence that the major stimulus that drives development of this very complex and elegant immune structure are antigens associated with the normal microbiota.

[Slide.]

In addition to organized lymphoid compartments, then one encounters diffuse lymphoid compartments illustrated here by the epithelium and the lamina propria. I think the very fascinating observation here is the multilayered or multi-tiered nature of defense.

For example, the mucus layer covers the epithelium. The mucus layer is much more than goo. It is actually-- the chemical composition of mucins varies along the gastrointestinal tract and we, and others, have made observations that, indeed, the host is able to change the chemical composition of mucins in response to inflammatory stimuli in a rather acute fashion.

I think that is consistent with the idea that, indeed, the host uses mucins again in a protective fashion, mainly, and, in addition to chemical composition being important for the protective barrier, secretory IgA sticks to mucus. So you have a very sticky type of surface that is consistent with the idea that this whole system evolved simply to keep all bacteria away from the epithelium.

However, if this protective physical barrier becomes'compromised, then the host is very sensitive to and responsive to translocation of bacteria. It is really the epithelial cells that have to orchestrate this interaction among immune cells.

Indeed, if one finds translocation through tight

junctions, for example, then very quickly epithelial cells become activated. The cytokines synthesize and secrete a change and they change in a manner that assures that, indeed, the right immune cells--for example, neutrophils in response to IL-8, expand in the lamina propria compartment.

So you find this type of inflammation in response to compromises in the innate physical barriers. There seems to be a very dynamic interaction between those compartments.

[Slide. 1

One of the points I wanted to make is that we feel that, indeed, this complex innate and acquired immune system that is found in the intestine evolved in response to normal bacterial antigens. I think the best evidence for the idea comes from the rather expansive germ-free research.

So, back in the late 60's, early 70's, there was an awful lot of very good germ-free research mainly with mice, sometimes with rats, sometime with pigs. But this slide summarizes kind of the outcome. I think, perhaps, you are familiar with the outcome and that is that both innate and acquired immune components in the intestine are vastly underdeveloped in germ-free animals relative to conventional animals and that, indeed, if you conventionalize a germ-free animal, sometimes with a single bacteria group, then most any of the compartments, the immune compartments, are quickly activated, so represented here by, for example, an

increase in Peyer's patch size and function, responsiveness and so forth.

Epithelial-cell renewal rate also contributes a very important innate barrier. Epithelial-renewal rate is increased in response to conventionalization of a germ-free animal.

The chemical composition of mucins changes in response to conventionalization with germ-free animals with bacteria. The paper that you were made aware of that we contributed to a book by Gerald Tannock on probiotics, on Probiotics of Critical Review, in that paper, we went through and tried to survey all of the germ-free research, particularly studies in which germ-free animals were conventionalized with various types of bacteria.

You will find, in that chapter, a complete table or at least as complete as we were able to generate, describing host changes in response to various bacterial groups. In response to questions this morning about appropriate animal models to try to study the complex responses to nonpathogenic bacteria, I think the germ-free animal offers one reasonable model.

[Slide.]

So we wanted to understand, we would like to understand, what is the nature of the signals that the host perceives that leads to these rather profound or dramatic

changes in both innate and acquired immune compartments in response to normal bacteria.

In an effort to do that, one paper appeared in Gut--perhaps this is a little bit hard to read, but this is in the journal, Gut. It appeared in 1997 and it has really been influential for us in trying to formulate the right question regarding the nature of signals that are stimulating immune responses or development of active and innate immunity in the intestine.

This paper was communicated by a group of Japanese researchers in which their interest was in trying to devise fixation protocols that preserve the mucus layer. It was really an eye-opener for us. Indeed, with conventional dehydrating fixatives, such as formaldehyde, the mucus layer is lost.

So then one's view of the epithelium, of course, is influence by your histological preps, and then we begin to, perhaps, think erroneously about host-microbe interactions. But with the use of nondehydrating fixatives such as acetic acid and ethanol, one can reasonably preserve the mucus layer.

What these researchers describe--this is a beautiful paper; I would highly recommend it for everyone in the room--is, indeed, a continuous mucus layer throughout the intestine, increasing in density in those regions of the

intestine that are most heavily colonized with bacteria, reaching up to 300 microns in thickness in the rectum.

The continuous mucus layer that you can see, I hope, represented here and then, of course, the crypts here in the colon are also, in effect, plugged with mucus. So this must represent the medium by which the host communicates with normal bacteria.

I think it is very important, of course, if we want to understand the signals, we have to think about where are they living and what is the medium by which the host recognizes these bacteria.

[Slide.]

So we have tried to replicate their findings. Just to illustrate the power of using these techniques as far as trying to formulate the right question, here are simple histological preps from the colon or normal inbred mice that were fixed either with conventional 10 percent neutral buffered formalin or Carnoy's, which is an acetic-acid alcohol-based fixative.

You can see that, also, we were able to preserve this continuous mucus layer here. Also, we stained these tissue samples with high-iron diamine which will distinguish sulfated mucins from sialated mucins. What one finds is that, indeed, there is an illuminated array of mucins in this layer.

In other words, one finds a layer of sialated mucins followed by a layer of sulfated mucins and then another layer of sialated mucins. Sulfated mucins, because of their polyanionic charge, prevent bacteria from digesting the oligosaccharrides that comprise the mucins. So, in that sense, the terminal sulfate residues represent an additional barrier.

So there, again, the host over time has mounted responses that enable it to preserve that very important mucus barrier. So probably we are talking about a very dynamic evolution that is occurring. This represents the battleground, if you will, of the dynamic evolution between the host and microbes.

It is the sulfated mucin compartment that we have observed in two animal models that changes very acutely in response to inflammation. We think the reason for that is that the sulfated residues, again, protect the mucins from mucolytic activity which appears to be rather widespread among normal gut bacteria.

Also, we would like to understand which communities are living in the mucus layer and, of course, the in situ methods have been described.

So, one of the activities we are involved in is simply trying to combine fixation protocols that will preserve the mucus layer with protocols necessary for in