PROBIOTICS: the Potential for a Live Microbicide

Satellite Symposium at Microbicides 2010

May 22, 2010
David L. Lawrence Convention Center
Pittsburgh, Pennsylvania
USA
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BV</td>
<td>Bacterial vaginosis</td>
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<tr>
<td>BVAB 1, 2 and 3</td>
<td>Bacterial vaginosis associated bacteria</td>
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<td>CBER</td>
<td>Center for Biologics Evaluation and Research</td>
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<tr>
<td>cfu</td>
<td>Colony forming units</td>
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<td>CMC</td>
<td>Chemistry Manufacturing &amp; Controls</td>
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<td>CV-N</td>
<td>Cyanovirin-N</td>
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<td>CVL</td>
<td>Cervico-vaginal lavage</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>FAO</td>
<td>Food and Agriculture Organization</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FDC</td>
<td>Food, Drugs and Cosmetics</td>
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<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
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<td>GCM</td>
<td>Global Campaign for Microbicides</td>
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<td>GMO</td>
<td>Genetically modified organisms</td>
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<tr>
<td>GMP</td>
<td>Good Manufacturing Practices</td>
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<tr>
<td>GRAE</td>
<td>Generally Recognized As Effective</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally Recognized As Safe</td>
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<tr>
<td>GusA</td>
<td>ß-glucuronidase</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<td>IgG, IgM, IgA</td>
<td>Immunoglobulin G, M, A</td>
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<td>IL-6, IL-8, IL-10</td>
<td>Interleukin-6, interleukin-8, interleukin-10</td>
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<tr>
<td>IMPT</td>
<td>Initiative for Multipurpose Prevention Technologies</td>
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<tr>
<td>IND</td>
<td>Investigational New Drug</td>
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<tr>
<td>MBA</td>
<td>Masters of Business Administration</td>
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<td>MPT</td>
<td>Multipurpose prevention technology</td>
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<tr>
<td>NGO</td>
<td>Nongovernmental organization</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>RANTES</td>
<td>Regulated on Activation Normal T Cell Expressed and Secreted</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SHIV</td>
<td>Simian human immunodeficiency virus</td>
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<td>SLPI</td>
<td>Secretory leukocyte protease inhibitors</td>
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<tr>
<td>STI</td>
<td>Sexually transmitted infection</td>
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<tr>
<td>STD</td>
<td>Sexually transmitted disease</td>
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<tr>
<td>TFF1</td>
<td>Trefoil Factor 1</td>
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<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
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<td>WHO</td>
<td>World Health Organization</td>
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This report summarizes the proceedings of the Microbicides 2010 Satellite Symposium ‘Probiotics: the potential for a live microbicide’, held on May 22nd, 2010 in Pittsburgh, Pennsylvania (USA).

The planning committee would like to take the opportunity to thank the organizers of Microbicides 2010 for allowing us to host this event during the conference. We are very grateful to our funders – the Office of AIDS research at the NIH, the UCSF-Gladstone Institute of Virology & Immunology Center for AIDS Research, the United States Agency for International Development (USAID), the UCSF Bixby Center for Global Reproductive Health, the Mary Wohlford Foundation, the Public Health Institute, and the Coalition for Advancement of Multipurpose Technology (CAMI). We also want to extend our gratitude to the members of the planning committee, to all of the speakers, the moderator and the rapporteur who donated their time and expertise to make this event a success.

Although research on probiotics has been conducted for decades, the field is currently receiving increased attention due to recent advances in developing enhanced human lactobacilli strains that can deliver anti-HIV drugs and function as a live microbicide. The Microbicides 2010 Conference provided a real opportunity to bring together researchers, industry representatives and also potential donors to discuss the science, current stages of development, gaps in our knowledge and potential next steps.

This report attempts to give readers an overview of the field and invites you to participate in this multidisciplinary alliance as we move ahead with the research and development of probiotics for HIV prevention.

The video recordings of the event are posted online at www.cami-health.com/probiotics.html

Craig R. Cohen
UCSF Bixby Center for Global Reproductive Health

Anke Hemmerling
UCSF Bixby Center for Global Reproductive Health

Bethany Young Holt
Coalition Advancing Multipurpose Innovations (CAMI)
University of California, Berkeley
New technologies such as molecular detection of vaginal bacteria using PCR can identify and quantify bacterial species in vaginal fluids. These techniques enable researchers to detect novel bacteria, and to follow changes of the vaginal microbiota over time. Researchers discovered that while healthy women have many lactobacilli of a few predominant strains, they have only few species of other potentially harmful bacteria commonly associated with BV. In contrast, among women with BV, a plethora of species linked to BV were found, including some species never before identified. Analyzing vaginal swabs, frequently collected from healthy women as well as women with BV over several months, revealed cyclical changes in bacteria levels in relation to menses and hormonal changes. Using this new PCR technology to longitudinally follow women’s vaginal flora is crucial to accurately assess the influence of treatments for BV, including use of probiotics, on the reconstitution of the highly dynamic vaginal microbiota.

Throughout the last two decades, clinical research to assess the safety and efficacy of first generation probiotics has been steadily increasing. Study designs improved as more data about vaginal microbiota and new technologies became available. Although vaginal lactobacilli strains show a considerable geographical diversity across the globe, several strains are present in most populations: L. crispatus, L. gasseri, and L. iners. Early studies on probiotics for genital health were primarily conducted in Europe and North America, using strains like L. acidophilus, later introducing L. reuteri (former fermentum) and L. rhamnosus (former casei), all

Probiotics in fermented milk, yogurt and cheese have been used for centuries, and the market volume for probiotic foods reached $16 billion in 2008. However, there are few rigorous scientific studies assessing the benefits of probiotic foods, and the regulatory oversight for food products is less stringent than the approval pathway of probiotics used as drugs or drug delivery systems. Research interest in probiotics for genital health, particularly bacterial vaginosis, has increased significantly over the last 10 years.

Probiotic lactobacilli are self-replicating bacteria in normal vaginal flora. The first generation of probiotics used selected human lactobacilli strains for the prevention of recurrent bacterial vaginosis (BV) or urinary tract infection (UTI) following standard antibiotic treatment. Recently, the potential of genetically enhanced lactobacilli strains as delivery agents for anti-HIV drugs is generating hope for the development of a live and self-renewable microbicide.

The normal vaginal environment is dominated by lactobacilli species that are critical for maintaining an acidic pH which inhibits the growth of other pathogens. The vaginal microbiota is complex, and an imbalance can both lead to overgrowth of commensal anaerobes, often manifest as BV, and support establishment of pathogens. Conventional antibiotic therapy for BV is often insufficient and results in high recurrence rates. BV has been linked with preterm labor in pregnant women, pelvic inflammatory disease, and an increased risk for sexually transmitted infections including HIV.

New technologies such as molecular detection of vaginal bacteria using PCR can identify and quantify bacterial species in vaginal fluids.

Executive Summary

1.0
predominantly colonizing in animals. The newest studies also investigate L. crispatus and L. gasseri.

Earlier research (1980-2000) testing probiotics for BV prevention without prior antibiotic treatment were often plagued by problematic study designs, small sample sizes, short trial periods, combination with other agents, incomplete blinding, and somewhat unspecific outcome measures. Over the past decade, study designs have improved and outcome measures have become more standardized, including use of Amsel’s or Nugent’s scoring systems for detection of BV, and measuring vaginal colonization of the exogenous strain following administration. Dose levels of administered lactobacilli strains increased to approximately 10^9 colony forming units (cfu) per dose.

In addition, current research (2000-2010) has investigated the use of probiotics in women with a healthy vaginal flora. For prevention of recurrent BV, newer studies (2005-2010) combine probiotics with an initial standard antibiotic treatment. These studies enrolled a larger number of women subjects, measured colonization of the exogenous lactobacilli species, and administered probiotics either orally or vaginally. They often found a sizable difference in BV cure rates between placebo and the tested probiotic.

Future clinical studies of probiotics must include measures of protocol adherence in the study design, and test the expansion of antibiotic treatment to more effectively destroy bacterial biofilms prior to administration of probiotics. They also need to investigate the influence of sexual intercourse and of changing hormones throughout the menstrual cycle on vaginal colonization of lactobacilli. As we embark on clinical testing of bioengineered strains, support for the research will depend on the development of transparent safety standards for bio-containment, product accountability and reliable antibiotic clearance, as well as effective communication of those measures to the public and other stakeholders.

The next generation of probiotics is based on naturally occurring human lactobacilli strains that are engineered to contain genes that produce potent antiviral compounds. Once colonizing the vagina, these genetically enhanced self-renewing bacteria have the potential to continuously produce highly potent HIV inhibitors. MucoCept developed by Osel, Inc. is such a product. Many steps were completed for the development of these enhanced probiotics. Not only was it necessary to select an optimal Lactobacillus strain out of hundreds of existing strains, but a bacteria preservation technology that can produce large quantities of stable dry powder of high pharmaceutical grade quality had to be developed. An enhanced strain such as L. jensenii 1153, engineered to produce the HIV entry inhibitor Cyanovirin-N, must be capable of colonizing the vagina. In addition, in situ protein expression and bioactivity and immunological responses of the host to the strain need to be carefully monitored first in animal models and later in humans.

Experiments in Chinese rhesus macaques showed consistent lactobacilli colonization of CV-N-expressing L. jensenii for up to 90 days, at high levels of 10^6-10^7 colony forming units (cfu) per swab collected. No evidence for immunogenicity against the recombinant CV-N was observed, and no antibodies against recombinant L. jensenii were detected. The strain was not able to survive in the environment outside the macaques, and was easily cleared from the colonized macaques using antibiotics such as azithromycin as a vaginal suppository.

Recently, this product was tested for potential efficacy against simian-HIV (SHIV) in a non-human primates proof-of-concept study. A repeated low dose challenge model administering the genetically enhanced lactobacilli L. jensenii 1153 expressing CV-N to macaques was used to test the efficacy for HIV prevention in Chinese rhesus macaques. Such a repeated challenge model closely mimics the typical setting of numerous incidents of exposure and natural transmission and can optimize the number of animals enrolled while still reaching adequate
levels of power. Remaining uninfected animals in both the active and the control group were re-challenged with the SHIV virus until they seroconverted. *L. jensenii* 1153 expressing CV-N continuously produced inhibitory levels of $10^5$ cfu and caused a 57% reduction in the rate of SHIV acquisition in comparison to the control animals ($p=0.037$). Additionally, the few infected macaques that received *L. jensenii* 1153 expressing CV-N had lower plasma viral loads than the untreated infected animals. In the future, the stability of the strain and the durability of protection over time in non-human primates need to be assessed.

With FDA approval, a pre-Phase 1 safety clinical trial using bioengineered bacteria with a surrogate marker called GusA (β-glucuronidase) will be conducted in 2011 to evaluate colonization, clearance and biocontainment in a small group of volunteers. In addition, future development of the *Lactobacillus*-based delivery platform will study the co-expression of additional HIV inhibitors to develop it as a future multipurpose microbicide active against HIV and other STIs.

ActoGenix in Belgium is also researching genetically modified bacteria for drug delivery. This company developed the technological platform to mass produce enhanced *Lactococcus lactis* strains, derived from the food industry, for use as oral capsules delivering the bacteria to the gastrointestinal tract. In mouse models, a modified *L. lactis* can be used to deliver anti-inflammatory cytokines like Interleukin-10 to downregulate inflammatory bowel disease. A clinical phase 2a study in humans successfully demonstrated that such a product is safe and tolerable, and confirmed the biocontainment in humans. *L. lactis* producing Trefoil Factor 1 (TFF1) could prevent oral mucositis, a painful side effect of radio- or chemotherapy that leaves patients unable to eat or drink, often leading to curtailment of lifesaving cancer treatments. Animal models using hamsters have been completed, and preliminary data from trials in humans show promising results. *L. lactis* could also serve as a delivery platform to produce pro-insulin to treat juvenile diabetes. Animal data showed that diabetes was reversed in 60% of the mice. In the future, this technology could be used to deliver multiple therapeutics by combining *L. lactis* strains engineered to produce different therapeutics.

The regulatory approval process for probiotic drugs faces unique challenges. First, pharmaceutical grade drugs need to be produced in facilities complying with Good Manufacturing Practices (GMP). Second, drugs that deliberately release genetically modified organisms (GMO) must follow special guidelines addressing biocontainment and clearance. Scientists and small companies involved in drug development often lack the expertise necessary for the successful completion of the regulatory approval process, and may not have the capacity for large scale manufacturing. Regulatory agencies in different countries may have unique requirements which need to be taken into account when designing animal experiments and clinical studies. Different animal data may be requested, GMO regulation may differ, and the application process may involve differing steps in Europe and North America.

Since probiotics as drugs or drug delivery systems fall into a category different from food and are not generally recognized as safe or effective, the regulatory burden at the US FDA is much higher. The sponsoring company needs to file an Investigational New Drug (IND) application and demonstrate the safety and effectiveness of the product before marketing to the FDA Center for Biologics Evaluation and Research (CBER). The development to probiotics to prevent HIV infection will likely require testing and marketing of these products in sub-Saharan Africa. The regulatory procedures in many African countries for probiotics remain unclear, but many regulatory agencies there follow regulations set forth by the US FDA.

In 2002, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) released guidelines for research on probiotics as foods to lay out a minimum consensus. However,
specific guidelines for probiotics as drugs (including the genetically modified strains) are still needed, and a guidance document by the WHO or FDA could facilitate international collaboration between agencies in order to streamline such regulation. Such regulatory guidelines will be crucial to educate and guide researchers designing future studies, especially those testing bioengineered products and combination strains.

Using lessons learned from the microbicide field and from the research on female condoms, research needs to move from a product-oriented approach to a more end-user oriented approach that focuses on women’s needs. From the beginning, strategies for reaching and engaging key audiences need to be developed carefully to gain their support for the development of these products, including the clinical research and future marketing and distribution process.

Current demand and acceptability of probiotic products need to be assessed for different regions of the world. A US-based study targeting women and health care providers found that women are familiar with probiotics, often have used them in the past, and would be willing to buy them if their beneficial effects were scientifically proven. The fact that such a product is perceived to be “natural” was of high importance to some women. The concept of second-generation genetically modified products was met with cautious optimism by most women, and they requested detailed information on safety and possible side effects. Providers also expressed interest for using probiotics in patients, but were adamant about the need for evidence based benefits and the equitable accessibility of products they would recommend.

Probiotics could become a key component of future multipurpose prevention tools (MPTs) for sexual and reproductive health that prevent multiple adverse health outcomes simultaneously, including sexually transmitted infections such as HIV, unplanned pregnancy, as well as reproductive tract infections.

Women in sub-Saharan Africa continue to bear the burden of HIV and have the highest need for a female controlled HIV prevention tool. As in Europe and America, probiotics as food supplements are available in Africa, but awareness of the potential for probiotics as drugs is still very limited. Creating demand for probiotics as drugs needs to start with the end user and must also engage other stakeholders such as the women’s partners, and community leaders and health providers, throughout the research process. While the community can best identify and address barriers in product delivery, the support of community leaders is essential for the development and implementation of study protocols. Support from health care workers is critical for the uptake of a method, but these providers have often not been properly exposed to new technologies and consequently may not be able to advocate for them.

The context in which a technology will be introduced needs to be considered, and daily realities of the end users such as storage, sanitary requirements and privacy when administering the product, disposal options, cost of product acquisition including time and travel, as well as perception of HIV risk and benefit from probiotic use must be addressed. In addition, acceptability studies of probiotics are needed in Africa alongside and in preparation for designing clinical trials.

The development of probiotics will face complex challenges similar to those seen in previous microbicide work. These include issues of general product development, financial support, regulatory hurdles, manufacturing and logistical barriers and effective branding and commercialization of the product. Community involvement in clinical trials needed more than ever before. It is critical to forge multidisciplinary alliances that include scientists, advocates, funders, government agencies, regulators, health care providers and the community of end users. Only through building on these synergies will we be able to successfully move probiotic drugs from the test tube to the community in order to prevent HIV and other STIs.
List of Speakers

Craig Cohen, MD MPH
University of California, San Francisco (USA)

Samukelisu Dube, MD MPH
PATH (South Africa)

Anke Hemmerling, MD PhD MPH
University of California, San Francisco (USA)

Jeanne Marrazzo, MD MPH
University of Washington, Seattle (USA)

Laurel Lagenaur, PhD
Osel Inc., Santa Clara (USA)
National Institutes of Health, Bethesda (USA)

Renee Ridzon, MD MPH
Bill and Melinda Gates Foundation, Seattle (USA)

Lothar Steidler, PhD
ActoGeniX NV, Zwijnaarde (Belgium)

Jim A. Turpin, PhD
NIAID, National Institutes of Health, Bethesda (USA)

Qiang Xu, PhD
Osel Inc., Santa Clara (USA)

Bethany Young Holt, PhD MPH
Coalition Advancing Multipurpose Innovations (CAMI)
University of California, Berkeley (USA)
Craig Cohen, MD MPH  
*University of California, San Francisco*

Craig R. Cohen is a professor in the Department of Obstetrics, Gynecology and Reproductive Sciences at UCSF and an Attending Physician at San Francisco General Hospital. He received his undergraduate degree from University of California, Berkeley, his medical degree from the University of Louisville, and his master’s of public health degree in epidemiology from the University of Washington. He served as an Intern, Senior Research Fellow, and Assistant Professor in the Department of Obstetrics and Gynecology at the University of Washington prior to joining the faculty at UCSF in 2003.

Dr. Cohen conducts medical research in two major areas: HIV and sexually transmitted infection prevention and HIV/AIDS care and support in developing countries.

In collaboration with the Kenya Medical Research Institute (KEMRI) he established the Research Care and Treatment Program (RCTP). He has been the Director of the Family AIDS Care and Education Services (FACES) since its creation in 2004, a CDC PEPFAR-funded program serving Western Kenya and Nairobi. As part of FACES, Dr. Cohen has also developed the Student Training Elective Program (STEP), which allows medical students and residents to do clinical and research electives at FACES sites. Additionally, Dr. Cohen directs the UCSF Reproductive Infectious Disease (RID) Fellowship, which aims to provide Ob/Gyn and post-doctoral trainees with the tools and experience necessary to develop into independent investigators and launch productive careers in the field of RID. Furthermore, in 2005, he began the Infectious Disease Research Training Program (IDRTP), a program for Kenyan masters and doctoral students and a joint effort between KEMRI and UCSF.

Samukelisu Dube, MD MPH  
*PATH, Durban, South Africa*

Dr. Samukeliso Dube is the Program Director of the Global Campaign for Microbicides (GCM) based in Johannesburg, South Africa. She is a public health physician with more than ten years experience in clinical management, policy, programming and advocacy of public health programs in Southern Africa, especially HIV and AIDS. She has worked in the HIV/AIDS field with a number of international NGOs, including Oxfam UK and Action AID International. Before joining the Global Campaign for Microbicides(GCM) , Dr. Dube joined GCM from the Infectious Disease Research Unit at the University of Limpopo, Medunsa Campus, as a senior lecturer and held other responsibilities including being co-investigator on various HIV prevention trials, conducting TB molecular and classic epidemiology research, STI surveillance and research and vaccine preventable diseases. Dr Dube currently oversees GCM’s program work in Africa on accelerating availability of HIV prevention tools for women, with research interests in TB and in multipurpose prevention technologies.

Dr Dube holds a degree in Medicine and Surgery, a Masters in Public Health, Tropical Medicine and Infectious Diseases and is currently completing an MBA.
Anke Hemmerling, MD PhD MPH
University of California, San Francisco

Dr. Hemmerling is a Project Director at the UCSF Bixby Center for Global Reproductive Health since 2007, implementing clinical trials to study probiotics for the prevention of genital infections. She received a MD (1999) and a PhD in Medical Sciences (2003) from Humboldt University Berlin and earned a MPH (2004) from the University of California at Berkeley. During medical school and residency in Obstetrics & Gynecology she has worked in numerous health projects and hospitals throughout Latin America. From 2004 – 2007, she worked as a postgraduate research fellow for the Bixby Program at the UC Berkeley School of Public Health and as a Director of Special Health Projects for Venture Strategies for Health and Development, being involved in a number of global women’s health projects.

Laurel Lagenaur, PhD
Osel Inc. & National Institute of Health

Dr. Laurel Lagenaur received her undergraduate degree from Indiana University, where she focused her interest in Microbiology. She worked as a clinical and research microbiologist at the University of California, Davis. She received her Ph.D. from Stanford University in the department of Microbiology and Immunology with a focus on molecular virology. She did postdoctoral training at UCSF in the department of Stomatology, examining viruses involved in the oral manifestations of HIV, and was briefly an adjunct faculty member at UCSF. She was the first employee of Osel Inc and has worked at Osel for 12 yrs. She is currently a visiting scientist at the National Institutes of Health, and is working there under a cooperative research agreement to examine the feasibility of using a live Microbicide (based on human vaginal Lactobacillus as a delivery system) to prevent HIV infection.

Jeanne Marrazzo, MD MPH
University of Washington, Seattle

Dr. Marrazzo is Professor in the Division of Infectious Diseases at the University of Washington in Seattle, Washington. She is the Medical Director of the Seattle STD/HIV Prevention and Training Center, president of the American STD Association, and an Associate Editor of the journal Sexually Transmitted Diseases. She serves on several national committees related to control of infectious diseases, particularly in the area of women’s health, and is the Protocol Co-Chair of the VOICE Study, a large study implemented by the Microbicides Trial Network that is evaluating HIV pre-exposure prophylaxis administered vaginally and orally to women at high risk for HIV infection in sub-Saharan Africa. She is a Fellow of the American College of Physicians and of the Infectious Disease Society of America, and has been the Principal Investigator for numerous studies of clinical and epidemiologic aspects of genital chlamydial infections, vaginal infections, and cervicitis. Major areas of research interest include molecular epidemiology, pathogenesis, and management of bacterial vaginosis, diagnosis and screening of chlamydial infection and epidemiology and management of cervicitis. She is also the Principal Investigator on several web-based educational projects on STD management and an interactive site on viral hepatitis.
Renee Ridzon, MD MPH
Bill and Melinda Gates Foundation

Dr. Renee Ridzon is a physician trained in internal medicine and infectious diseases. Since 2002, Dr. Ridzon has worked at the Bill and Melinda Gates Foundation as Senior Program Officer in HIV Prevention. In this role, she has been responsible for oversight of research on microbicides, pre-exposure prophylaxis, male circumcision, and other HIV prevention interventions. She serves on the Office of AIDS Research Microbicide Research Working Group and the Scientific Advisory Committee of the International Partnership for Microbicides. She often serves as a liaison between the Foundation and the WHO, NIH, USAID, CDC, Global Fund, and PEPFAR.

Prior to working at the Gates Foundation, she served for 10 years at the Centers for Disease Control and Prevention, first in the Epidemic Intelligence Service, then as a Medical Epidemiologist in the Division of Tuberculosis Elimination and lastly in the Division of HIV/AIDS Prevention. During her tenure at the CDC, Dr. Ridzon remained clinically active as Adjunct Assistant Professor of Medicine at Emory University and as Consultant in Infectious Diseases at Brown University. Dr. Ridzon’s service in the public health service was acknowledged by her promotion to the rank of Captain (O-6) and the awards of the US Public Health Service Outstanding Unit Commendation, Commendation Medal, and the Secretary’s Award for Distinguished Service, among others.

She has served as a member of the Board of The International Union against Tuberculosis and Lung Disease and is a current board member of the World Lung Foundation. She has contributed over 50 articles and chapters to the peer-reviewed scientific literature and serves on the editorial board of the International Journal of Tuberculosis and Lung Disease.

Lothar Steidler, PhD
ActoGeniX NV, Belgium

Dr. Lothar Steidler is a molecular biologist who obtained his PhD from Ghent University in Belgium. He has invented and pioneered “TopAct” technology: the use of recombinant microflora for topical and active delivery of proteins. The development of a robust environmental containment system enabled his team to advance into the first clinical studies ever, using genetically modified microorganisms as therapeutics.

Dr. Steidler has published 35 papers and is the main inventor on 9 patent families that cover TopAct technology. He received the Biogent-Plant Genetic Systems Award (Belgium, 1987), the BBSRC Underwood Award (U.K., 1995) and The William Grant&Sons Young European prize for Invention and Discovery (U.K., 2001). Dr Steidler previously held positions at Ghent University (Ghent, Belgium), Flanders Interuniversity Institute for Biotechnology (Ghent, Belgium) and Alimentary Pharmabiotic Center (Cork, Ireland). Dr. Steidler’s is one of the founding members of ActoGeniX, where his department engineers novel recombinant Lactococcus lactis, designed for in vivo production of therapeutic proteins (cytokines, peptides, allergens).
Jim A. Turpin, PhD  
**NIAID, National Institute of Health**

Dr. Turpin was awarded a Ph.D. in Biological Sciences with specialization in Immunology in 1988 from the University of Texas Graduate School of Biomedical Sciences at Houston (UT-GSBS) graduate program in Biological Sciences at the University of Texas MD Anderson Hospital and Centers.

From 1988 to 1992 he worked at Walter Reed Army Institute of Research (WRAIR) in Washington, DC, where he began working with HIV. In 1992, he joined the National Cancer Institutes Frederick Cancer Research and Development Center (NCI-FCRDC), and in 1994, moved to the Laboratory of Antiviral Drug Mechanisms (LADM). While at the LADM, Dr. Turpin was involved in the discovery and development of anti-HIV inhibitors, several of which went on to be candidates for topical microbicide development.

In late 1998, he joined Southern Research Institute (Southern) in Frederick, MD as the manager of the Retrovirus Research Laboratory in the Infectious Disease Research Department. While at Southern, he was the PI for the NIAID Topical Microbicide screening contract and was responsible for the development and expansion of the Southern HIV topical microbicide program.

In March 2002, he joined TherImmune Research Corporation as the Associate Director of Infectious Disease and Immunology where he directed drug discovery programs for candidate anti-microbials (virus, bacterial and fungal), HSV and HIV topical microbicides.

In May of 2003, Jim joined the topical microbicide program in the Division of AIDS at NIAID. Dr. Turpin is the Preclinical Team Leader for the Microbicides Research Branch and the Program Officer responsible for the Integrated Preclinical/Clinical Program for HIV Topical Microbicides (IPCP-HTM) and the Microbicide Innovation Program (R21/R33) (MiP), which are the topical microbicide branch’s primary tools for discovery, development and advancement of topical microbicide compounds and strategies to clinical testing.

Qiang Xu, PhD  
**Osel Inc., Santa Clara**

Dr. Xu has over 18 years of experience in academia and industry from biotechnology start-up to pharmaceutical companies, with goals to develop therapeutics for the prevention and treatment of infectious and immunologic diseases. He received his Phd from Kansas State university and completed his postdoctoral training at the university of California, Berkeley. Dr. Xu started his biotechnology career at Bayer, and worked at Pan Pacific Pharmaceuticals and Scios, Inc. prior to joining Osel in 2001. He is currently Vice President of Research at Osel, Inc., where he has served as the principal investigator on 8 grants funded by NIH and other Foundations.

Bethany Young Holt, PhD MPH  
**CAMI & University of California, Berkeley**

Bethany Young Holt is with the Public Health Institute and is on the faculty of the University of California at Berkeley School of Public Health. She directs the Coalition Advancing Multipurpose Innovations (known as CAMI), a consortium of biotech developers, researchers, health advocates, and clinicians working in the area of reproductive and sexual health. CAMI is helping launch the Initiative for Multipurpose Technologies (IMPT), an initiative that raises awareness about and support for new and existing technologies that can be used in various combinations to address multiple reproductive and sexual health needs.

Dr. Young Holt received her PhD in epidemiology and master’s in maternal and child health from UC Berkeley, and her bachelor’s in biology from the College of Wooster. She has more than 20 years experience working in the area of reproductive health and HIV/STI prevention in the U.S. and internationally, including traineeships at the National Cancer Institute, the Centers for Disease Control and Prevention, and the Institute Pasteur in Dakar, Senegal; Peace Corps volunteer work in Mauritania, West Africa; and relief work with the U.N. High Commission for Refugees (UNHCR) in Senegal and Ethiopia.
4.0 Presentations
Probiotics have been used for centuries and probiotic foods reach a significant market volume. The regulatory oversight for food products is less stringent than for probiotics as drugs or drug delivery systems. Rigorous scientific studies assessing the benefit of probiotic lactobacilli for genital health have increased significantly over the last 10 years. Lactobacilli are self-replicating bacteria in normal vaginal flora helping to prevent infections. The first generation of probiotics used selected, unmodified human strains for the prevention of genital-urinary infections. Recently, the potential of genetically-enhanced lactobacilli strains to be used as delivery agent for anti-HIV drugs is generating excitement and hope for the development of a live, self-renewable microbicide.

I would like to welcome the audience to our symposium and thank our sponsors - the Office of AIDS research at the NIH, the UCSF-Gladstone Institute of Virology & Immunology Center for AIDS Research, the United States Agency for International Development (USAID), the UCSF Bixby Center for Global Reproductive Health, the Mary Wohlford Foundation, the Public Health Institute, and the Coalition for Advancement of Multipurpose Technology (CAMI).

We have invited a great speaker panel and are fortunate to have Dr. Rene Ridzon from the Bill & Melinda Gates Foundations with us as the moderator for this symposium. The idea for the symposium emerged approximately 6 months ago during our work at UCSF conducting clinical research on probiotics. Many of you gathered here today with a background in the field of microbicides may agree that, for a variety of reasons, this new field has not received the attention we believe it deserves.

Consequently, we thought that this Microbicides 2010 Conference in Pittsburgh could be a real opportunity to bring together researchers, industry representatives and also potential donors to discuss the science, current stages of development, gaps in our knowledge and potential next steps. Also I hope that this group will serve as a launching squad for promoting a further discussion following this symposium and throughout the conference. Building today’s agenda we included plenty of opportunity for discussion and hope that this will be a highly interactive and productive afternoon.
Increasing numbers of women infected with HIV around the world, most of who are in sub-Saharan Africa, are still left without a microbicide that reduces women’s risk of HIV acquisition. The currently tested third generation of microbicides uses antiretroviral drugs, and is now in clinical phase 1-3 trials in sub-Saharan Africa and other parts of the world. For the future, I would like to introduce the notion of probiotics as potential live microbicides and to think of them as a potential fourth generation microbicide.

Research interest in probiotics for genital health, especially bacterial vaginosis, increased remarkably during the last 10 years. Their potential benefit for other conditions like yeast or recurrent urinary tract infections has also been explored. Lactobacilli are bacteria that exist in abundance in the normal vaginal flora, they produce hydrogen peroxide, lactic acid and other bacteriocins which inhibit pathogens and function as antiretroviral or antibacterial compounds. The advantage of probiotics is the ability of lactobacilli to self-replicate and self-replenish. One of the disadvantages of early generation microbicide candidates was that those gels had to be applied closely timed to sexual intercourse. In contrast, periodically replenished self-replicating probiotics are administered coitally independent.

Probiotics are all around us and have been used in foods like fermented milk, yogurt and cheese for centuries. Research on health benefits of probiotics started in the early 1900 in Europe, later in the US. The term “probiotics” was introduced in the 1950s. Any supermarket today in the US, and especially in Europe, has a large dairy section dedicated to probiotics. These food products are marketed to improve general wellbeing, especially gastrointestinal health. The market volume reached $16 billion in 2008 and is projected to grow impressively.1

On the other hand, you may also be aware of the recent lawsuit against the Dannon Company2, forcing them to moderate their claims about health benefits. As we will learn later today, the regulatory pathway for probiotic food is very different from the one regulating probiotics as drugs or drug delivery agents.

The vaginal flora is complex and an imbalance can lead to overgrowth of pathogens and subsequent infection. Conventional antibiotic therapy is often insufficient, especially against biofilms of bacteria. The optimal environment for lactobacilli growth is still not fully explored. We will be hearing more from Dr. Jeanne Marrazzo about some recent discoveries around vaginal microbiota in healthy women and women with bacterial vaginosis.

The first generation of probiotics (also called bacterial therapeutics) refers to the non-engineered naturally occurring strains. Different strains are currently being explored by several groups around the world, predominantly in North America and in Europe. Research is investigating the use of these strains following antibiotic therapy for the prevention of recurrent bacterial vaginosis. Other studies have looked at reducing the risk for recurrent urinary tract infection. In addition, we also know that women who harbor sufficient levels of lactobacilli and have a normal vaginal flora have a decreased risk of acquisition of other sexually transmitted infections (STI) including HIV.

However, the next generation of probiotics will be the focus of today’s discussion. As Drs. Qiang Xu and Laurel Lagenaur will explain, these second generation bacterial therapeutics are genetically engineered and based on naturally occurring lactobacilli strains, where genes that produce potent antiviral compounds (for instance, single chain antibodies or antiviral compounds like cyanovirin) are inserted into the Lactobacillus genome. Once colonizing the vagina, these genetically enhanced self-renewing bacteria continuously produce highly potent antiviral compounds.

We will also be hearing today from Dr. Lothar Steidler, the founder of ActoGeniX in Belgium, a company that has been developing a genetically enhanced Lactococcus strain for use in the
gastrointestinal tract. They have completed the first human trials of that organism here in the US and in Canada.

As already mentioned, clinical research in probiotics has increased over the years. But, as a study by Hibberd in 2008 points out\(^1\), only a small fraction of this research has been conducting randomized clinical trials and published the results in peer-reviewed literature.

This leaves us with an urgent need to move ahead. As Dr. Anke Hemmerling will show us later, different strains have been tested around the world, administered orally or vaginally, as single or combination strains. The newer studies are looking at a wide array of outcomes, including colonization as a proxy for potential effectiveness. Most of these studies unfortunately included only fairly small sample sizes, and part of those limits in study design is owed to lack of the level of financial support required to run larger trials.

In summary, probiotics have several mechanisms of action. One is through the production of hydrogen peroxide and bacteriocins, prompting researchers to select strains that produce high levels of these substances. Another mechanism of action is the production of antiviral compounds by genetically enhanced strains. In addition, a vaginal microbiota with high levels of lactobacilli has a decreased risk to be overgrown with pathogens that are associated with bacterial vaginosis as well as other STIs which are known cofactors for HIV infection.

The goal of today’s symposium is to have a lively discussion around the potential of probiotics as a live microbicide, with a special focus on HIV prevention. Another goal is to forge alliances that we need to advance this field and to make smart decisions about next steps. We would also like to receive input from the donor community and the regulatory experts.

A final report will be written and widely disseminated. We are also video recording this event and will post it online for colleagues that were not able to attend today’s meeting: www.cami-health.com/probiotics.html
The vaginal environment is complex and highly dynamic. The vaginal microbiota of healthy women has many lactobacilli and few harmful bacteria. Imbalances can lead to overgrowth of bacteria and subsequent infections, like bacterial vaginosis (BV), which often poorly responds to antibiotic therapy.

New molecular technologies using PCR can identify novel bacteria linked to BV and reveal cyclical changes of bacteria levels in relation to menses and hormonal changes. Using new PCR technology to follow women’s vaginal microbiota over time is crucial to accurately assess the influence of the treatments, including the use of probiotics.

As the first speaker today, I will talk primarily about the vaginal microbiota and less about probiotics to give you the background for your understanding of the approaches of new therapies in this environment. Specifically, I will update you on developments in the field of cultivation-independent technologies. I will briefly define the scope of our research and try to make it accessible to this very diverse audience. I will explain how we use molecular detection of vaginal bacteria without traditional cultivation methods.

Broad range 16S ribosomal DNA PCR was developed to identify bacterial species in vaginal fluids. These organisms can then be visualized and confirmed with fluorescent in situ hybridization (FISH). Bacteria specific PCR assays allow quantification of the species present in the vagina. These techniques have been incredibly useful to follow women longitudinally over time, to monitor their treatment for bacterial vaginosis (BV), and to assess what is prompting the BV. They have also enabled us to detect novel bacteria in women with BV. Finally, I will close with some thoughts on how these discoveries might change our thinking about BV, not just in understanding its pathogenesis, but also how the new knowledge might change our discussion of designs for clinical trials, particularly involving probiotics and new antibiotic therapies.

The normal vaginal environment is dominated by lactobacilli species that are critical for maintaining a fairly acidic pH (<4.7) by producing lactic acid, which buffers the normal pH down. This low pH favors the growth of other lactobacilli and inhibits the growth of other pathogens. Cultivation
methods identified the major species of human lactobacilli to be L. crispatus and L. jensenii. Ideally the Lactobacillus strains present also produce hydrogen peroxide for maximal protective benefit.

Bacterial vaginosis (BV) is the most commonly diagnosed cause of vaginitis and also the most common reason for women to seek evaluation of vulvovaginal symptoms. BV is strongly associated with race as well unprotected sex. It is exceedingly prevalent in areas of the world where HIV incidence is very high, and very common in Sub-Saharan Africa, where BV prevalence is between 60-80% in many settings. In our STD clinic in Seattle the BV prevalence is about 25%. The classic signs and symptoms are abnormal discharge and amine odor, which is the result of volatile byproducts of the anaerobic metabolism produced by glycosidase activity of anaerobe pathogens. Women initially respond to anti-anaerobic treatment for BV in about 80% of the time, but recurrence rates very high, making recurrence the rule rather than the exception. When following women who had been initially successfully treated for about a year, about three quarters of them will experience a new episode of BV within the subsequent year. This illustrates our limited knowledge on BV treatment; we really just know how to manage it as an epiphenomenon. We temporarily alleviate the symptoms, but often fail to reconstitute a Lactobacillus predominant flora.

In healthy women, the gram stain vaginal smear will show predominantly lactobacilli resulting in a Nugent Score of 0-3 on the Nugent scale. In women with BV, the picture looks very different. We see a profound loss of H2O2-producing lactobacilli and an overgrowth of anaerobes or facultative anaerobes, often clustered around the vaginal epithelial cell, the so called “clue cells” visible in microscopy. Those pathogens produce sialidase which destroys IgA, as well as glycosidase and volatile amines.

For a long time researchers assumed that BV was a fairly straightforward process. But a closer look into the immune environment reveals interesting processes like the surge of IL-1Beta and IL-10 and a decrease of IL-8 and the defensin secretory leukocyte protease inhibitors (SLPI). This loss of defensins may be one of the reasons that women with BV are vulnerable to superinfection by other sexually transmitted diseases like HIV, chlamydia, gonorrhea or trichomoniasis.4,5

BV in and of itself is an unpleasant condition, but really attracted researchers’ attention in the late 1980s and early 1990s, when it became clear that these anaerobes dominating the vaginal flora was particularly dangerous for pregnant women. It was estimated that up to 11% of preterm deliveries in the US are associated with BV. A number of other conditions are also associated with these anaerobes, particularly infections of the upper genital tract like pelvic inflammatory disease. BV has also consistently been linked with an increased risk of STI acquisition, including HIV. 6,7

In a meta-analysis by Atashili8, the overall increase in HIV susceptibility attributable to BV is approximately 60%. There are a number of theories how this may be mediated. The hydrogen peroxide produced by lactobacilli is virucidal and has a direct effect on HIV. Consequently, the characteristic absence of lactobacilli in women with BV decreases H2O2 production. The elevated alkaline pH and predominance of anaerobes also may activate CD4 cells. Additionally, cytokines are up-regulated to differing degrees, depending on the BV-associated anaerobes present. We also know that some of these BV-associated
bacteria directly stimulate HIV expression from T-cells and monocytes. The existence of multiple theories indicates that the process of enhancing HIV susceptibility is probably multifactorial.9

In the early stages of BV research BV used to be called *Gardnerella* vaginitis, because substantial amounts of *Gardnerella vaginalis* were found in all women with BV. The bacteria was detected using the classic cultivation studies of the past on agar plates. The *Gardnerella* bacterium is a key component of the Nugent Scoring, as the ample presence of this pathogen is reflected in a high Nugent score. Swidsinski et al.10 also showed *Gardnerella vaginalis* to be heavily represented in biofilms associated with BV. Their study used vaginal biopsies to show that the presence of *Gardnerella* in biofilm predicted treatment failure. However, *Gardnerella* is that it is also present in 35-40% of women who don’t have BV. Consequently, although *Gardnerella* seems to be highly represented during BV episodes, the pathogenesis of BV is a multifactorial process. Other bacteria identified in cultivation studies like *Mobiluncus mulieris* and *curtisii*, *Mycoplasma hominis* and various anaerobes (Porphyromonas, *Prevotella*, Peptostreptococcus, Veillonella) are also important players in BV.

Unfortunately, anaerobes are very hard to cultivate, even under excellent laboratory conditions; some of them have not even been cultivated at all. New estimates looking at the microbiota of the gut estimate that probably less than 1% of the total bacterial burden in the human body has actually been externally cultured.11

Using molecular analysis offers a great step forward toward cultivation independent identification of bacteria. Researchers have taken advantage of the 16S ribosomal DNA gene that is present in all bacteria. It codes for a small subunit of ribosomal RNA that is needed for protein synthesis. Molecular techniques can detect this 16S rRNA gene in conserved regions of the genome, allowing for a non-specific broad range quantification of bacterial mass. This step is then followed by a differentiation process where variable sequences in between the conserved regions are detected to define specific bacteria.12,13,14,15

Applying these techniques, as described in one of our first papers15, we enrolled 13 women without BV (Nugent Scores 0-3) and with a healthy Lactobacillus predominant flora, and performed broad range PCR on their vaginal fluid samples. We identified 16 species in 13 subjects, with an average of only 3 species per subject. By far the most strongly represented species was *L. crispatus*, with *L. iners* as a close second, and various other lactobacilli. This was a surprising finding, as *L. iners* accounts for a large proportion of the bacterial biomass, but its function remains fairly unknown. Overall, the biology was actually not as diverse as we had expected. These findings confirmed the domination of lactobacilli in the healthy vaginal environment, demonstrated that the number of species was relatively small, and showed no BV-associated bacteria in detectable numbers using this approach in these healthy women.

The remaining questions focused on the origin of these bacteria. Do they exist in small quantities below detection level or are they coming in from other niches outside the vagina, like the rectum or the oral cavity, or are they being transmitted from the sex partners? Using molecular technology, we enrolled 20 women with BV (Nugent score 7-10) and we identified 46 species, an average of 14 species per subject. Some of the detected species were novel phylotypes, and had never been found or cultivated before, comprising 58% of the overall bacteria we found. *Gardnerella vaginalis* was almost always present. We also found sizable quantities of BVAB 1, 2 and 3, as well as *Atopobium vaginae*, Leptotrichia, *Megasphaera* and more obscure species not well described in the BV literature before.15 *Atopobium vaginae* was shown to be associated with metronidazole resistant tubo-ovarian abscesses in earlier studies.12

The microbiology of BV is much more diverse than we currently know. How can these findings enhance our diagnosis and understanding of
At 30 days. The presence of BVAB1, 2, 3 or Peptoniphilus lacrimalis and Megasphaera predicted a 2 – 9 fold increased risk for treatment failure at 30 days. Independent effects are difficult to assess for interrelated multifactorial processes, but the sole presence of BVAB3, also associated with cervicitis, seemed to strongly predict treatment failure (3-fold increased risk).16

The emerging data characterizes BV as a heterogeneous dynamic disorder with great variability in the microbiology as well as the immunology, suggesting that some bacteria either contribute directly to the BV pathogenesis or they are markers of host susceptibility to BV. All these new findings have implications for the design of future studies. BV studies of the past often have been cross-sectional, which is insufficient to understand the dynamic of the condition. One study17 collected daily vaginal swabs of two healthy women over one month to quantify particular bacteria over time. We found that Gardnerella vaginalis spiked during menses, when Lactobacillus levels decreased. It is known that BV frequently occurs after menses, and a change in pH caused by alkaline blood was thought to contribute. Additional factors may contribute to this surge of BV during and right after menses: the iron levels in blood, or hormonal modulations during the cycle, especially changing estrogen levels may be involved. Another important point is that had this study only been looking at 2 time points (Day 0 and 30), all the longitudinal variation would have been missed.

These findings are especially important when considering whether and where to include a probiotic into the mix as a part of BV treatment. A better knowledge of the precise effects of lactobacilli on the microbiology of the vagina will be critical for the design of future BV treatments. Another study18 following a women with recurrent episodes of BV with daily and later weekly swabs during each new BV episode. The results indicate that she initially responds well to treatment each time, all the BV
associated bacteria decrease, but then always resurge shortly afterwards. *L. crispatus* also reconstitutes each time, but never to very high levels. Interestingly, *L. iners* remains at fairly high levels throughout the study, and it is unclear how its presence impacts the reconstitution of *L. crispatus* or *L. jensenii*. In summary, longitudinal follow ups with frequent swabs will have to become a cornerstone of future trials to accurately assess the influence of our treatments on the reconstitution of the highly dynamic vaginal microbiota.

We have some answers. The new studies have demonstrated that BV is a syndrome that is associated with the acquisition of complex vaginal bacterial communities that include many novel and uncultivated species. Heterogeneity in the microbiota, in addition to behavioral factors, may explain differences in treatment outcomes, relapse rates and incidence of adverse sequelae. It needs to be considered in evaluating responses to treatment, especially in clinical trials.

But the data also raised more questions. We still don’t know what makes an individual BV associated bacterium particularly harmful. Are some species inherently more pathogenic? Is resistance to the antibiotics a factor? Are some bacteria just representatives of a more deranged microbiologic environment or for a host factor allowing pathogenic bacteria to proliferate? Is it transmitted with other pathogens? Is it part of a biofilm like *Gardnerella* and is that the reason for the persistence? Have we defined all the BV associated bacteria we can? Our future success in advancing BV treatment and prevention will hinge upon our dedication to account for the complexity of this microbiota and monitor and interpret the response to trials. Thank you for the opportunity to talk to you today.

I would like to acknowledge my colleagues David Fredricks, Kathy Ringwood, Tina Fiedler, Kathy Thomas, Sujatha Srinavasan, Congzhou Liu, Kathy Agnew, Nancy Dorn, Dana Varon, Lauren Asaba, Duyn Dithmer and Susan Heideke.
Q & A

In women with BV associated bacteria after treatment, do you also find those bacteria in mouth and gut?

We have collected yet unpublished data of oral and rectal swabs. We found some of the key species in all those environments, particularly in the oral environment. This may explain why women having sex with other women and more receptive oral sex have higher BV rates. Extravaginal niches will be important to look at in the future, especially for probiotics, because a combined vaginal and oral delivery to colonize the rectum may be critical for vaginal colonization and sustained cure.

Did you also look into the impact of other behavior such as anal intercourse, smoking cigarettes, and oral contraceptive use that could impact the cytokine immune profile?

Yes, we have looked at behavioral factors. Douching was not an issue in our cohort. Clearly, unprotected sex was – we found that after unprotected sex with men women are more likely to be colonized with Megasphaera 2 and we are looking at the male reservoir specifically for that bacterium, but there are other bacteria as well to look at. Smoking was a risk factor for prevalent BV in our studies, but not so much for the incidence of BV, because of the low sample size of smokers in our study. But the postulated mechanism is that it reduces secretory leukocyte protease inhibitor (SLPI) in the respiratory tract (also reason for smokers to get more bronchitis). The theory for BV is that smoking affects the vaginal SLPI levels.

Have you looked at candida species? Have you seen a negative association between candida and BV? Have you looked at the interactions?

We looked for candida in this group and we did not see a lot. The rates of candida colonization by culture in the group that we studied are quite low, less than 10%. In our STD clinic it is a little bit higher. Someone needs to look at the microbiota in women with BV and candida and women with candida or BV alone, because they will probably be distinct. In my clinical experience, women with a dual diagnosis are much harder to treat.

How specific are your findings for Seattle, or do they translate around the world?

Other groups have also looked at this, namely Jacques Ravel’s group in Maryland, and Dave Martin’s group at LSU. Dave Fredericks works with Scott McClelland on samples from Kenya to look at this question. There are probably some subtle differences, but the cultivation studies showed that BV associated bacteria are represented across these communities. I think there are going to be differences that are more related to sexual behavior and probably what extravaginal niche you are exposed to (oral, anal sex). More work needs to be done before we can say that with confidence.

Regarding BVAB3 and cervicitis: how much do we know about the local geography? When you swab in the in posterior fornix, on the cervix or near the introitus in the same woman, do you find a different prevalence of microflora?

We are just designing this very study, and we are modeling it after a Herpes study that Anna Wald has led, using an anatomical map of the vaginal geography where they tried to determine the shedding and the neurological involvement. Possibly the biofilm is not across the whole vagina, but maybe certain areas of the cervix are more important for the more inflammatory bacteria like BVAB3.

Among the L. iners that you recovered, what was the percentage of H2O2 producers?

Not many. We detected a lot more L. iners through the PCR. The cultivation of L. iners is much more difficult, it is called L. iners because it is inert. This is the reason why its magnitude has not been appreciated until now, because in the past people really relied on culture. When you go back to the cultivation data, most of the research says that L. iners does not produce H2O2. When we looked at it, it has definitely been the minority of species that do produce H2O2, less than 10%, but I am not confident that cultivation is a very good way to represent this species.

Some papers in the late 1970s and early 1980s by Glucks who looked at lactobacilli and a number of other organisms that were cultured out and showed that during the menstrual cycle they peaked at time of ovulation, so they appear to be estrogen controlled. Any place from 5-fold to 2 logs, in animals and humans. Is their data maybe off in terms of the sensitivity of the assay that you have now. What is the minimal change that you can detect?

Probably 1-fold. When you use the specific qPCR, according to Dave Fredericks you can detect at least 100 organisms, so you really can get down very low.

It will be important to know what is going on during the menstrual cycle to understand when would be the best time to administer a probiotic.
Bioengineered Lactobacilli as Next Generation Probiotics

Qiang Xu, PhD

The next generation of probiotics is based on naturally occurring human lactobacilli strains, and is engineered to contain genes that produce potent antiviral compounds. Once colonizing the vagina, these genetically enhanced self-renewing bacteria have the potential to continuously produce highly potent HIV inhibitors. Many steps needed to be completed for the development of such a probiotic, like the selection of an optimal Lactobacillus strain capable to colonize the vagina, and the development of a bacteria preservation technology for mucosal delivery.

I would like to take the opportunity to share our experience in the development of bioengineered lactobacilli as the next generation probiotics, termed MucoCept.

The proceeding talks about the commensal vaginal flora and its role in the protection against vaginal infections have shown that the decrease of Lactobacillus in vaginal flora is associated with an increased risk of acquisition of BV, and the acquisition of BV is a cofactor in HIV infection. Osel is interested in bioengineering commensal vaginal lactobacilli to deliver HIV inhibitors. For this purpose, an optimal strain needs to be selected to express highly potent HIV inhibitors such as proteins or antibodies, and the bacteria preservation technology needs to be developed to produce a dry powder for storage in ambient temperature as a pharmaceutical product. In addition, persistent vaginal colonization of this strain at high levels is a key requirement for the protective effect of such a product.

Our research team has been focusing on the following key issues:

- to optimize administration of bioengineered bacteria to colonize and compete with native flora;
- to address the in situ protein expression and bioactivity and the immunological responses of the host after the bioengineered bacteria is administered;
- to develop the preservation of lactobacilli technology;
CV-N (APVT-CV-N) retained the same anti-viral activity with low mitogenic potential.

Additional studies conducted in collaboration with Dr. Raina Ficherova’s lab using a co-culture model of human epithelial cells and several CV-N expressing *L. jensenii* strains showed that all strains expressed high levels of CV-N in both vaginal cells and cervical epithelial cells. Importantly, the expressed CV-N binds to gp120, indicated it is biologically active. There is also no evident upregulation of proinflammatory markers like IL-6.

In collaboration with Drs. Dean Hamer at NCI and Brigitte Sanders-Beer at BIOQUAL, we have established an animal model for vaginal *Lactobacillus* colonization, which allow us to collect data for safety and efficacy prior to dosing in humans. The established Chinese rhesus macaque model is particularly interesting because these animals harbor endogenous vaginal lactobacilli, mostly *L. johnsonii*. The model allows the clearance of vaginal lactobacilli with azithromycin and subsequent restoration of the endogenous *L. johnsonii* colonization. The model also supports a persistent colonization of the human vaginal isolate of *L. jensenii*, at a level of 10⁵-10⁷ cfu per vaginal swab collected. As an example, two macaques that were vaginally inoculated with CV-N-expressing *L. jensenii* were followed over time. These macaques showed consistent lactobacilli colonization for up to 90 days, at levels of 10⁵-10⁷ cfu per swab collected. In the non-human primate model, CV-N produced in situ can also be detected when CV-N-expressing *Lactobacillus* strains were introduced into the vaginal cavity of macaques. In several independent studies, as shown in this exemplary picture, cervical-vaginal lavage was collected over a time period of 6 weeks post inoculation. We were able to consistently detect the expression of APVT-CV-N in full length. We also investigated the immunogenicity against this modified *Lactobacillus* and its product. We did not observe any evident immunogenicity against the recombinant CV-N. Similarly, we did not detect any antibodies against *L. jensenii*.
Regulatory considerations are especially important when investigating a genetically modified bacterial strain. We have assessed possible environmental persistence of this bioengineered Lactobacillus strain in collaboration with Dr. Dorothy Patton. This bacterium is not able to survive outside the macaques, and we did not observe any persistence of the engineered bacteria in the environment, including the animal facility, near the cage, or in our laboratories. We have also assessed the stability of the bioengineered Lactobacillus. The expression cassettes that had been integrated into the chromosome of Lactobacillus were genetically stable in the bacteria recovered from the animals. In addition, there was no sexual transmission of the bacteria from female to male macaques. Furthermore, we established a rescue therapy by confirming that the engineered bacteria contain no antibiotic resistance markers and can be easily cleared from the colonized macaques using azithromycin as a vaginal suppository.

To optimize the manufacturing process of the CV-N-expressing Lactobacillus, we investigated the issues of cell viability and cell recovery upon rehydration, appropriate bacterial dosage form, and shelf life at room temperature storage during the development of the product. We focused on optimizing the fermentation process, the formulation of the preservation matrix and determining the optimal dosage so as to maximize the shelf life at room temperature. These efforts resulted in the development of a preservation matrix that includes trehalose, skim milk, and sodium ascorbate. This matrix, in combination with a transient buffering step, ensures good cell recovery upon rehydration of the dried powder. The produced Lactobacillus powder contained up to $10^{10}$ cfu/100 mg. This significant advancement in formulation and preservation resulted in a viability rate of over 90% upon rehydration.

Because of the high potency of this bacterium, we are able to consider other potential dosage forms as well. To optimize the dosage of the bacteria, we investigated different delivery modes in a non-human primate model. In one experiment, we administered the dried Lactobacillus powder in 2 different ways, in capsules or reconstituted the powder in 1 ml of MRS, to 6 macaques (3 for each group). Vaginal swabs were collected 3 and 10 days after the final bacterial inoculation. Results showed that the bacteria can be easily recovered at both time points from all 3 animals receiving lactobacilli in MRS. But using the capsule for delivery, only one animal showed significant bacterial recovery at the first time point, while Lactobacillus level was beyond detection limit for the other two animals. With time, we could recover lactobacilli from one more animal, indicating improper dosage forms resulted in a delay in colonization and recovery of lactobacilli.

Currently, we are focusing our work on several issues. We are conducting pre-IND consultations with the FDA on the use of bioengineered Lactobacillus. With agency approval, we will move forward to evaluate bacterial colonization, clearance and biocontainment in human volunteers together with our collaborators at UCSF. To be cautious and to minimize exposure to human subjects, we are going to use bioengineered bacteria with a surrogate marker called GusA (ß-glucuronidase). In addition, we are working to expand the platform to co-express additional HIV inhibitors, deliver either by single or multiple strains, so as to develop a multipurpose microbicide against HIV and other STIs. We are also seeking funding to develop mucosal delivery of antigens for a vaccine program, such as for the rectal route. We need to further refine the dosage form to optimize vaginal colonization.

The field is growing and is getting more diverse. For instance, Bharat Ramratnam is working on CV-N-expressing Lactobacillus strains in yogurt for feeding and gut CV-N expression. Richard Markham investigates alpaca antibodies to inhibit HSV by targeting integrins. Lin Tao studies mannose binding Lactobacillus. Ruth Ingrid Connor researches antiviral factors in Lactobacillus. Jane Hitti assesses the impact of Lactobacillus on genital HIV shedding.
I would like to thank our funding agencies (NIH, USAID and the Gates Foundation), our team and all our collaborators for their dedication and contribution. Many colleagues over many years have supported us to drive this program forward.

Bioqual, Inc: Brigitte Sanders-Beer
Southern Research Institute: Carol Lackman-Smith, James Cummins
Advanced Bioscience Laboratories, Inc.: Deborah Weiss, Jim Treece
University of Pittsburgh: Sharon Hillier, Lorna Rabe
Brigham and Women’s Hospital: Raina Fichorova
UCSF: Craig Cohen, Anke Hemmerling
University of Washington: Dorothy Patton, Yvonne Cosgrove Sweeney
NIH: Dean Hamer, E. Berger, A. Gronenborn, S. Rao, Mario Roederer
CDC: Laura Barrientos
University of South Alabama: Lewis Pannell
NCI: Barry O’Keefe
CAMI & UC-Berkeley: Bethany Young Holt
IPM: Joe Romano, David Fairhurst
LBL, Broad Institute, JCVI
Stanford University: Gary Schoolnik, Mark Holodniy
Planned Parenthood Mar Monte: Jill MacAfee
Aaron Diamond AIDS Research Center, David Ho
San Raffæle Institute: Paolo Luasso, Luca Vangelista
Profectus Biosciences: Timothy Fouts, Antony Dimitrov
Chinese CDC: Zhiqing Zhang

Q & A

A technical question based on Jeanne Marrazzo’s discussion on the use of pyrosequencing and new technologies – what technology are you using to detect the colonization of these bacteria?

We have used culture-dependent methods to monitor and recover our lactobacilli. We are also using qPCR targeting 16S rRNA to monitor the flora. We are very interested to expand our use of these new technologies to monitor the microflora.

Most bacteria in the gut and other mucosal sites do get antibodies. Our pilot data in the human vagina show that lactobacilli are frequently developing antibodies. I am surprised that your data does not support this. However, I am not so surprised that it is not against the cyanovirin-N. What methods did you use to look for antibodies?

We were concerned about the development of antibodies against the bioengineered strain, not only against the recombinant cyanovirin-N, but against recombinant strains in general. But our findings are consistent with the general believe that the vaginal mucosa is a poor immunoinduction site. By a simple delivery of antigen in the absence of adjuvant you will not expect to see much immunological response. We monitor the production of IgG and IgM and we did not see any evidence of immunogenicity against recombinant cyanovirin-N and recombinant L. jensenii.

How did you detect whether they were antibodies against your strain?

We immobilized biotinylated lactobacilli to Avidin coated ELISA plates. CVL collected prior to exposure to L. jensenii/1153 or its derivatives were used as baseline samples for each macaque. Anti-L. jensenii/1153 and anti-CV-N sera were generated in rabbits and serial-diluted to be used as pseudo-positive controls for the ELISA. Antibodies (IgA, IgG and IgM combined) specifically against L. jensenii/1153 in non-diluted Chinese rhesus macaque CVL samples were detected by ELISA.

You mentioned IL-6 and IL-8 as cytokine measures and I was wondering whether you looked at quantitative levels and at other cytokines and defensins and SLPI?

We have also looked at SLPI as well, and we did not observe a significant upregulation. We also looked for other apoptotic markers, and we did not see evidence for upregulation. Dr. Raina Fichorova’s group is going to give a talk on this topic during this M2010 conference.

My other question is about quantity: What is the quantity of the bacteria in the preparation that you used to inoculate the macaques, and what kind of levels do you end up with when you established persistent colonization?

We used 10⁹ cfu for inoculation, and were able to recover 10⁵ to 10⁷ cfu per swab. Below 10⁵ cfu we considered it to be below detection level.
Testing Proof of Principle in a Repeated Low Dose Challenge Model using a Live Lactobacillus Microbicide

Animal studies with Chinese rhesus macaques showed consistent lactobacilli colonization of CV-N-expressing *L. jensenii*. No evidence for immunogenicity against the recombinant CV-N was observed, and no antibodies against *L. jensenii* were detected. The strain was not able to survive in the environment outside the macaques, and was easily cleared with antibiotics. First animal studies demonstrated efficacy of CV-N-expressing *L. jensenii* with a significant reduction in the rate of simian-HIV acquisition. In the future, the stability of the strain and the durability of protection over time in non-human primates need to be assessed.

I will talk today about testing proof of concept in a repeated low dose challenge model using genetically enhanced lactobacilli as a delivery vehicle the potent entry inhibitor cyanovirin. First, we developed a non-human primate model for testing a repeated vaginal challenge. We needed to have a model that could support vaginal colonization to test this live prevention strategy. We selected the Chinese rhesus macaque model, because these animals have a menstrual cycle similar to humans of about 28 days with an overt menstruation lasting about 4 days. Captive animals menstruate every month. Contrary to earlier studies, we found that macaques harbor endogenous lactobacilli, primarily *L. johnsonii*, which is phylogenetically close to *L. gasseri* in humans. We consistently found lactobacilli in 90% of the animals that we sampled, from multiple animal colonies, indicating that this model is suitable for live microbicide testing.

However, this is not a perfect model. The flora of the macaques fluctuates greatly with the menstrual cycle, and the *Lactobacillus* levels of normally $10^9$- $10^7$ CFU drop precipitously from 1-4 logs during menstruation, presenting a challenge to the model. The human vaginal flora is more stable in healthy women. Women have about 10-100 fold higher levels of lactobacilli than macaques. Humans also tend to have less of a drop during menstruation. David Eschenbach reported no significant drop, and Jeanne Marrazzo mentioned a study earlier today by Srinivasan that showed about a half a log drop in *Lactobacillus crispatus* in women with stable flora. In order to detect lactobacilli in situ in the macaque vagina after inoculating the animals
with Lactobacillus producing cyanovirin we took cervical vaginal lavages and the subsequent protein production could be seen as early as 24 hrs after inoculation and up to 3 to 6 weeks. The detection range for the protein starts at approximately $10^5$ cfu. During menstruation, the cyanovirin levels in macaques were low (as were the Lactobacillus levels). We could detect cyanovirin in biopsies and it was colocalized with the lactobacilli on the mucosal surface and in the vaginal lumen.

To evaluate the cyanovirin producing live microbicide we selected the low dose repeated challenge model because it more closely mimics the typical setting of exposure and natural transmission. People are exposed to HIV more than one time. In humans at peak viral load with greater than 100,000 copies/ml of viral RNA in blood the transmission of HIV vaginally is approximately 1 in 30, dropping to 1:300 if the viral load is 10,000 copies/ml blood. In comparison, our low dose repeated non-human primate model showed a 1 in 3 infection rate, making this a fairly stringent model.

We designed the study trying to optimize the number of animals enrolled while still reaching adequate levels of power. This is an example of how a small study can reach significance employing the repeated challenge design. In this example, after one challenge you should find half of your animals in the control group infected, compared to a 20% infection rate in the experimental group. A single dose challenge model would not reach significance level even when using 20 animals per group (40 animals, 40 challenges, 60% efficacy comparing 10 vs. 4 infected animals, p=0.1)

However, using a repeated low dose challenge model, re-exposing the animal remaining uninfected after previous challenge(s), a significant difference in the infection rates between control and experimental group can be seen after 5 rounds of challenges (20 animals, 50 challenges, 60% efficacy, p=0.03). It is important to note that even with a 50% infection rate, all of the animals will eventually succumb to infection after enough challenges. In summary, the repeated low dose challenge model allows you much greater power in a small study.

The implemented low dose challenge protocol enrolled two study arms, 12 animals in our experimental group that were treated with Lactobacillus expressing cyanovirin CV-N, and 8 control animals. Before exposure to SHIV all

**Repeated Low Dose Challenge**

**Control group: n = 10**

- 10 challenges
- 5 infections
- 60% infection rate

Challenge each animal

- 16 challenges
- 7 infections
- 47% infection rate

- 18 challenges
- 8 infections
- 45% infection rate

- 20 challenges
- 10 infections
- 50% infection rate

* p = 0.35

**Experimental group: n = 10**

- 10 challenges
- 2 infections
- 20% infection rate

Challenge each remaining uninfected animal

- 18 challenges
- 3 infections
- 17% infection rate

- 25 challenges
- 5 infections
- 20% infection rate

- 30 challenges
- 6 infections
- 20% infection rate

* p = 0.03

**Summary:** 20 animals; 50 challenges; 60% efficacy, p = 0.03
animals received antibiotic treatment with low dose azithromycin to remove their endogenous lactobacilli. The female macaques had regular menses. We challenged the animals up to 6 times (once a week) or until they became infected with SHIV SF 162p at 300 TCID50. We started challenging 24 hours after the last Lactobacillus inoculation and frequently measured Lactobacillus levels and viral load. We monitored Lactobacillus levels each week. Most animals were colonized throughout the study unless they were menstruating (wk1: 11/12 animals, wk2: 9/12, wk3, wk4: 10/12, wk5: 12/12, wk6: 8/12), and we challenged the animals, whether or not they were colonized.

Colonization with Lactobacillus expressing cyanovirin CV-N was usually measured at levels around $10^6$ cfu. Seven of the 8 control animals were eventually infected after 23 total challenges, resulting in an infection rate of 30.4%. Among the 12 animals treated with Lactobacillus expressing cyanovirin CV-N, eight of 12 animals were eventually infected after 61 challenges (13.1% infection rate). Four animals remained uninfected, even after six challenges. This difference in infection rates between both groups translates into a 57% reduction in the rate of SHIV acquisition, which reaches a significant p-value of $p=0.037$. An unexpected finding was that among animals with breakthrough infection, the macaques receiving Lactobacillus expressing cyanovirin CV-N had lower viral loads by one log as compared to untreated infected animals. This phenomenon continued after peak viral load throughout the study, and the mechanism is unclear at this point.

In summary, the repeated low dose challenge model demonstrated the ability of Lactobacillus expressing cyanovirin CV-N to colonize and continuously produce inhibitory levels of $10^6$ cfu. In the SHIV challenge, Lactobacillus-treated animals showed a 57% final reduction in the rate of acquisition. Lactobacillus-treated animals with breakthrough infection showed a one log reduction in SHIV viral loads. This is the first successful demonstration of a live anti-HIV microbicide in vivo.

In the future, we need to determine the stability of our Lactobacillus expressing cyanovirin CV-N. The durability of protection over time needs to be assessed by reducing the frequency of dosing in future repeated challenge protocols. A larger study would enable us to improve precision of the degree of inhibition and to better define the correlates of protection, and to define whether menstruation confounds protection through decreased cyanovirin production. We believe that Lactobacillus levels above $10^5$ cfu/swab are a correlate of protection, we need more data to confirm this assumption. We are continuing to express other potent inhibitors such as the newly described single chain antibodies like scFVs (VRC01).

The best candidates will be selected to proceed toward clinical development. We recognize that in some cases combinations of antibodies and cyanovirin may be the best approach. The 57% reduction rate of infection is very promising, especially seen in perspective with other investigated methods such as the current vaccine trials that reached 31% efficacy. Epidemiological modeling shows that a reduction of this rate could have a dramatic impact on the epidemic. We have the potential for an even higher efficacy in humans, because of the stable Lactobacillus levels at least 10-100 fold higher than in macaques and the smaller decline in Lactobacillus levels during menses. This approach has the potential to have long lasting traction. Our data showed persistent colonization for up to 89 days. Additionally, the time gap of 24 hours between last inoculation with Lactobacillus expressing cyanovirin CV-N and SHIV challenge indicates that the product does not have to be administered immediately before coitus. Finally, this approach is inexpensive, the product has a good stability, and it is fairly easy to administer. Our main goal is to move the best product forward into the clinic as rapidly as possible.
I would like to thank the Osel team that helped to move this into animal testing (Letong Jia, Wenjun Huang, Peter Lee, Xiaowen Liu, Yang Liu, Thomas Parks, Kimberly Smith, Qing Xia, Qiang Xu, Rosa Yu), as well as our funders (IATAP, NIH, IPM/USAID, CONRAD Foundation/GMP, GATES Foundation GCE) and collaborators (Dean Hamer at NIH NCI, Brigitte Sanders at Bioqual Inc., Gary Schoolnik at Stanford University, Ranajit Pal at Advanced Bioscience Laboratories Inc.). I also would like to extend my gratitude to my former colleagues Chia-Hwa Chang, Teresa Chang, Kirsten Essenmacher, Courtney Frasier, Pat Martin, David Simpson and Yonghong Zhu.

Q & A

I have two questions regarding the lower viral load lower among infected animals treated with lactobacilli:

How far does your product get absorbed across the mucosa? For the human transmission we think that most transmissions are a single founder virus that establishes infection, but what do we know about the SHIV model? 

Those are my two theories:

• In this challenge model we have a high challenge with a 1:3 infection rate, possibly resulting in multiple founder viruses getting across. Our model was a SHIV, so it is a clonal envelope for this experiment possibly the animals colonized with lactobacilli had a slightly lower number of viruses getting across which then led to lower replication. (Because the envelope is clonal, it would be hard to detect multiple founder populations).

• The other possibility is that somehow the cyanovirin is present in the vicinity of the initial sites of replication thus affecting the first round.

I don’t know if I believe either one of those theories.

I am not quite assured by the lack of immune activation of the Lactobacillus species itself. Since this was in animals, have you looked at the presence of target cells?

No, we have not done that yet. We selected cervicovaginal lavages at every time point and we are currently looking into antibody production. We did not collect tissue in this experiment.

This was just completed two months ago. It is possible to do that, but we have not done it yet.

What target cells do you suggest we look for?

CD4 cells, for SHIV.

Since there is no complete protection, did you check for any lesions when you took the vaginal swabs?

No, we did not check for lesions.

Did you look for a dose response effect? Did you look at quantitative culture results?

We did look at quantitative cultures each week. We dosed the animals with as much as we could put in a 1-3 ml volume, which is somewhere between 10⁴ - 10⁵ cfu of bacteria. They establish at a certain level, and there is a limit of how much Lactobacillus the animals will support, making it hard to choose a dosing range. We looked at the animals every week, we used the speculum to take a clean swab.

In terms of the difference in the log of the viral load, was that sustained over time? Did you follow these animals or was that a one time assessment?

The viral load difference was maintained until they became undetectable. The animals are now about 12 weeks past their challenges and their viral loads are fairly low, the SHIV is well controlled (initially anyway).

And regarding the control of dosing. As shown in Qiang Xu’s presentation about Brigitte Sanders-Beer’s experiments, we are increasingly able to control the dosing and we learned how to deliver the powder to achieve a certain dose level.
Clinical research assessing safety and effectiveness of probiotics has been steadily increasing. Study designs improved as more data about the vaginal microbiota and new technologies became available.

Future clinical studies of probiotics must include measures of protocol adherence into the study design, and test the expansion of antibiotic treatment to more effectively destroy bacterial biofilms prior to administration of probiotics. They also need to investigate the influence of sexual intercourse and of changing hormones throughout the menstrual cycle on vaginal colonization of lactobacilli.

The clinical testing of bioengineered strains must employ safety standards for biocontainment, product accountability and reliable antibiotic clearance, as well as the effective communication with the public and other stakeholders.

My task today is to give you a very brief update on clinical research on probiotics for vaginal health, focusing on the first generation of probiotics since the genetically enhanced second generation of probiotics is currently still in preclinical stages. In order to consolidate the sheer volume of studies with different focus and scientific rigor I will only include studies published in peer-reviewed literature and focus on randomized control studies.

There is an impressive diversity of predominant vaginal lactobacilli populations in different regions of the world. In summary, *Lactobacillus crispatus* seems to be the dominating strain in North American and European populations, whereas in a Nigerian cohort *Lactobacillus iners* is more prevalent. In the future, these regional differences need to inform our research in order to select the most suitable strains for a population. The research focus shifted over time. While in the 1980s all virtually all studies were investigating *Lactobacillus acidophilus*, the 1990s saw the introduction of strains like *L. reuteri* (*former fermentum*) and *L. rhamnosus* (*former casei*). In the last ten years, additional strains used in probiotic studies were *L. gasseri* and *L. crispatus*.

Clinical research, often in cross-continental collaboration, was conducted in many parts of the world. Reid’s group out of Canada have done a large amount of work, other studies came out of Europe (especially Italy and Scandinavia), the US, Nigeria, Brazil, China and the Philippines.
Early clinical trials from 1986-1996 investigated probiotics used without adjacent antibiotic treatment. They were usually randomized, but not all of them double-blind, some of them being prospective open label trials. All of them tested *L. acidophilus* at dose levels of $10^7 - 10^9$ cfu/dose and with fairly small sample sizes over 1-6 months. Predominantly, the product was administered vaginally. The randomized studies often used other remedies believed to have an effect on vaginal health at the time, such as acidifying agents or acidified tampons instead of a placebo group. Some of the studies additionally administered Vitamin B supplements and estrogen, making the interpretation of the results with regards of the actual effects of the probiotic impossible. The outcome measures of these early studies are often fairly undefined, but instead used vague terms like flora improvement. Only one trial used the Nugent Score to measure BV cure.

In the last five years, fewer trials still investigated the use of probiotics without antibiotic.

### Clinical Trials 1980-2000 – Testing Probiotics Only in Women with Bacterial Vaginosis

<table>
<thead>
<tr>
<th>Year</th>
<th>Author</th>
<th>Study Design</th>
<th>Tested Strains</th>
<th>N</th>
<th>Administration Route/ Dose</th>
<th>Study Duration</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1986</td>
<td>Friedlander</td>
<td>P</td>
<td>Unspecified Lactobacillus</td>
<td>38</td>
<td>Vaginal douche twice daily for 1-2 weeks, plus daily oral VHRB supplement</td>
<td>6 months</td>
<td>Increased normal flora from 13% to 76% one week post treatment, and 55% at 3 &amp; 6 months</td>
</tr>
<tr>
<td>1989</td>
<td>Fredricsson</td>
<td>R, OB, AC</td>
<td>L. acidophilus</td>
<td>61</td>
<td>Fermented Milk at 2.5 x $10^9$ cfu/dose vaginally for 7 days, repeat after 1 week OR: Oral metronidazole for 7 days OR: Acetic acid jelly vaginally OR: Estrogen cream vaginally</td>
<td>28 days</td>
<td>BV cure: probiotics 7%, metro 93%, Acetic jelly 18%, Estrogen 6%</td>
</tr>
<tr>
<td>1992</td>
<td>Hallen</td>
<td>R, OB, PC</td>
<td>L. acidophilus</td>
<td>60</td>
<td>Twice daily vaginal suppository with 10^8 cfu/dose for 6 days OR: Placebo</td>
<td>20-40 days</td>
<td>Flora improvement with probiotics (p&lt;.005) at Day 10, no significant improvement at Day 20-40</td>
</tr>
<tr>
<td>1993</td>
<td>Neri</td>
<td>R, OB</td>
<td>L. acidophilus</td>
<td>84</td>
<td>Yogurt 10^8 cfu/dose vaginally for 7 days, repeat after one week OR: Untreated OR: Vaginal acetic acid soaked tampon</td>
<td>60 days</td>
<td>BV cure: probiotics 87%, placebo 5% (women in 1. Trimester)</td>
</tr>
<tr>
<td>1996</td>
<td>Parent</td>
<td>R, PC</td>
<td>L. acidophilus</td>
<td>32</td>
<td>Tablet at 10^7 cfu/dose and 0.03 mg estradiol vaginally for 6 days OR: Untreated OR: Vaginal acetic acid soaked tampon</td>
<td>28 days</td>
<td>BV cure (Nugent Score): probiotics 88% placebo 22%</td>
</tr>
<tr>
<td>1996</td>
<td>Shalov</td>
<td>R, OB, CU</td>
<td>L. acidophilus</td>
<td>46</td>
<td>Daily oral yogurt for 1 or 2 months</td>
<td>2 months</td>
<td>Increased vaginal cultures for L. acidophilus (p=.05), decreased BV episodes (p&lt;.001)</td>
</tr>
</tbody>
</table>

*P=randomized OB=observer-blind PC=placebo-controlled AC=Active-controlled CO=cross-over P=prospective N=sample size*
adjacent antibiotic therapy, now often testing combination strains (L. rhamnosus/fermentum and L. reuteri/casei) and others.

Studies designs improved, all studies now being randomized, double-blind and placebo controlled, occasionally compared against another active substance like the antibiotic metronidazole (active-controlled). The small sample sizes and short trial durations still prevailed, but both vaginal and oral applications were tested at dose levels of 10^9 cfu/dose, the latter usually administered over longer periods of time (at least 14 days) than the former (5 days). The selected outcome measures start to standardize, now almost all studies using the Nugent Score, and some including general Lactobacillus colonization.

During the last decade a number of studies investigated the use of probiotics in healthy women, seeking to advance the knowledge about reactions of the normal vaginal microbiota to the introduction of exogenous strains into the

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<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005</td>
<td>Nishijima</td>
<td>R, DB, PC</td>
<td>L. johnsonii/La1</td>
<td>24</td>
<td>Daily fermented Milk 10^9 cfu/dose orally for 14 days OR: Placebo</td>
<td>14 days</td>
<td>Increased vaginal Lactobacillus colonization (p=.025) [in pregnant women]</td>
</tr>
<tr>
<td>2006</td>
<td>Anukam</td>
<td>R, DB, AC</td>
<td>L. rhamnosus GR-1, L. reuteri RC-14</td>
<td>40</td>
<td>Vaginally daily at 2 x 10^9 cfu/dose for 5 days OR: Vaginal metronidazole twice daily for 3 days</td>
<td>30 days</td>
<td>BV cure (Nugent 0-3; Sialidase neg, no discharge) Day 6: 80 vs 45% Day 15: 85 vs 45% Day 30: 90 vs 55%</td>
</tr>
<tr>
<td>2008</td>
<td>Petricevic</td>
<td>R, DB, PC</td>
<td>L. rhamnosus L. reuteri</td>
<td>72</td>
<td>Tablets orally daily at 2.5 x10^9 cfu/dose each for 14 days OR: Placebo</td>
<td>14 days</td>
<td>Nugent Score decreased by 3 points in probiotic group, no decrease in placebo group [Postmenopausal women]</td>
</tr>
<tr>
<td>2009</td>
<td>Mastro marine</td>
<td>R, DB, PC</td>
<td>L. brevis CD2 L. salivarius FV2 L. plantarum FV9</td>
<td>39</td>
<td>Tablets vaginally at 10^9 cfu/dose for 7 days OR: Placebo</td>
<td>21 days</td>
<td>BV cure (Nugent) probiotics 61%, placebo 19%</td>
</tr>
</tbody>
</table>

Clinical trials 2000-2010 – Testing Probiotics Only in Healthy Women

<table>
<thead>
<tr>
<th>Year</th>
<th>Author</th>
<th>Study Design</th>
<th>Tested Strains</th>
<th>N</th>
<th>Administration Route/ Dose</th>
<th>Study Duration</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>Reid</td>
<td>R, DB, AC</td>
<td>L. rhamnosus GR-1, L. reuteri RC-14, L. rhamnosus GG</td>
<td>42</td>
<td>Oral daily at 10^8 cfu/dose of L. rhamnosus GR-1 plus L. reuteri RC-14 for 28 days OR: Oral once daily 10^9 cfu/dose of L. rhamnosus GG</td>
<td>28 days</td>
<td>Better Nugent Scores in GR-1/RC-14 regimen (p=.017), healthy flora in &gt;90%</td>
</tr>
<tr>
<td>2003</td>
<td>Colodner</td>
<td>P</td>
<td>L. rhamnosus GG</td>
<td>42</td>
<td>Orally once or twice daily for 1 month</td>
<td>28 days</td>
<td>78.6% GI colonization, only 9.5% vaginal colonization [Postmenopausal women]</td>
</tr>
<tr>
<td>2003</td>
<td>Reid Charbonneau</td>
<td>R, DB, PC</td>
<td>L. rhamnosus GR-1, L. reuteri RC-14</td>
<td>60</td>
<td>Oral daily at 2 x 10^9 cfu/dose of L. rhamnosus GR-1 plus L. reuteri RC-14 for 60 days OR: Placebo</td>
<td>90 days</td>
<td>Significant decrease in vaginal coliform counts (p&lt;.05), better Nugent scores in GR-1/RC-14 regimen</td>
</tr>
<tr>
<td>2009</td>
<td>Antonio</td>
<td>R, DB, AC</td>
<td>L. crispatus STV-05</td>
<td>90</td>
<td>Vaginal capsules twice daily at 10^8 cfu/dose for 3 days OR: Vaginal capsules twice daily at 10^9 cfu/dose for 3 days</td>
<td>2 months</td>
<td>Colonization with STV-05 at follow-up: 90% if Lc neg at baseline 51% if Lc pos at baseline impeding colonization: Unprotected sex [Odds ratio 75] [women age 14-21]</td>
</tr>
<tr>
<td>2009</td>
<td>Hemmerling</td>
<td>R, DB, AC</td>
<td>L. crispatus STV-05</td>
<td>12</td>
<td>Vaginal applicator daily up to 2 x 10^9 cfu/dose for 5 days OR: Placebo</td>
<td>14 days</td>
<td>Good safety and acceptability outcomes</td>
</tr>
</tbody>
</table>
vaginal environment. These studies had sound designs, being randomized, double-blind and placebo controlled. They used already familiar strains, and in addition a new strain emerged: Lactobacillus crispatus. The small sample sizes (<100) and limited time period (1-3 months) continued, and the administration remained a mixture of vaginal or oral application at dose levels of $10^8$ cfu/dose. The comparison groups were either placebo or a lower dose of a different strain. Outcome measures used Nugent score to determine BV at the end of the study and the vaginal colonization of the administered strains. The results regarding colonization are interesting: one study in particular found high gastrointestinal but very low vaginal colonization. Antonio found a significantly increased probability of colonization with the exogenous L. crispatus strain if women had no endogenous Lactobacillus flora at baseline. Also emerging was the fact that sexual intercourse, especially with exposure to semen, dramatically reduced the chance of colonization with exogenous lactobacilli. These results from studies with healthy women illustrate that colonization will have to become a standard outcome measure in future trials.

Studies in the last five years have increasingly started to combine probiotics with an initial standard antibiotic treatment, either metronidazole, clindamycin or tinidazole. Study designs all featured randomization, double-blinding and placebo control. Sample sizes now reached several hundred participants followed up to 6 months. A newly emerging strain was Lactobacillus gasseri. Dose levels remained at $10^8 – 10^9$ cfu/dose, either as oral or vaginal administration. Most of the studies measured the rate of BV cure after 1 month of treatment, using Nugent’s and/or Amsel’s scores. All studies showed a sizable difference in cure rates between placebo and active product. More trials were also looking into colonization.

In summary:
- Most recent trials give antibiotics before the administration of probiotic lactobacilli.
Probiotics: the Potential for a Live Microbicide

- The dose level has established at $10^8 - 10^9$ cfu/dose.
- Vaginal and oral administration prevails, the oral dosing usually being longer and at a higher dose.
- Colonization is measured in few trials, and the factors impacting colonization are rarely explored.
- Outcome measures are increasingly standardized.
- Most study designs do not include measures of protocol adherence.

Many factors can impact colonization of lactobacilli in the vagina, some of them are better explored than others. In order to optimize the level and frequency of dosing we need to better understand the hormonal changes during the menstrual cycle, the role of (un)protected sexual intercourse and semen exposure, the influence of preexisting endogenous flora on exogenous strain colonization. In light of new discoveries regarding persistent pathogenic biofilms in the vagina, the effectiveness of current antibiotic regimens needs to be reassessed. Measuring protocol adherence is a crucial addition for future study designs. Future clinical research will also have to include at-risk populations like pregnant women, who could greatly benefit from a product preventing bacterial vaginosis which is highly associated with preterm labor.

Additionally, need to explore the viability under unrefrigerated conditions, as we are moving into future trials in African sites. And finally, as we are embarking on clinical testing of bioengineered strains, the support for the field will depend on the development of transparent safety standards for biocontainment, product accountability and reliable antibiotic clearance, as well as the effective communication of those measures to the interested public.

Q & A

**What kind of behavioral messages do we need to develop?**

We had some acceptability focus groups in our study to assess whether moving ahead with a vaginal applicator was really something that worked for women. Bethany Young Holt and Samu Dube are going to elaborate on acceptability with bioengineered strains, and what we need to give women, especially as we moved forward into this new field.

**Have you been able to detect any big differences between yours and Gregory Reid’s results?**

If you look at the same strains investigated by different groups you receive different results and I don’t know if that is because of the geographic diversity of dominant strains. Their Nigeria cohort has very high rates of high *L. iners* and very low *L. crispatus* rates, which may explain their different success rates treating BV.

In Gardner’s early experiments he tried to transmit BV, which failed when using *Gardnerella* alone, but successful when transmitting vaginal secretions of women with BV and putting them into healthy women. How about turning that around? Instead of isolating specific lactobacilli for administration, couldn’t we use secretions from women with healthy flora to put them into the women with BV? This would raise various issues around ethics and concurrent diseased possibly transmitted in such an experiment. However, a combination of several lactobacilli strains is already explored in some clinical trials, but the design is more complicated for these cases. Also, a possible negative interaction between strains, such as the known interaction between endogenous and exogenous strains, needs to explore in the future.
Another example of genetically modified bacteria for drug delivery is *Lactococcus lactis*, producing anti-inflammatory cytokines to treat inflammatory bowel disease, ulcerative colitis and oral mucositis. First clinical trials have demonstrated that such a product is safe and tolerable, and confirmed the biocontainment in humans. In the future, this technology could be used to deliver multiple therapeutics by combining *L. lactis* strains engineered to produce different therapeutics.

The regulatory approval process for probiotic drugs faces unique challenges and the requirements may differ in different countries. Pharmaceutical grade drugs must be produced in facilities complying with Good Manufacturing Practices (GMP). Biocontainment and eradication of drugs that are deliberately releasing genetically modified organisms (GMO) needs to be ensured.

First of all I would like to thank the organizers for giving me the opportunity to present our work to you. I am a co-founder of Actogenix in Belgium and we have been developing bacterial therapeutics for a long time, calling them ActoBiotics, which are high technology medicinal products with a very straightforward manufacturing process.

We are using genetically modified organisms, mostly focusing on *Lactococcus lactis*. This bacterium is derived from the food industry, safely used worldwide in large quantities (for instance in mozzarella cheese), and it is easy to produce on a large scale using fermentation and freeze-dry processes. The formulation encoated in capsules is taken orally and the active *Lactococcus lactis* is released in the gastrointestinal tract. The most challenging part about employing this technology is the initial choice of the right therapeutic to incorporate into the delivering *Lactococcus lactis*, the processing is fairly generic and easily reproducible on a large scale. New therapeutics can then use the established infrastructure and delivery mechanism.

At Actogenix we run several clinical and preclinical programs.

One older study assessed the use of *Lactococcus lactis* for the delivery of Interleukin-10, one of the major anti-inflammatory cytokines, to the gut.
mucosa in order to down regulate inflammatory bowel disease (or a model of it in mice) and has since been successfully repeated by several groups and encouraged us to move ahead into clinical phases.

The following clinical study (AG011) was a Phase 2a safety and efficacy study in subjects with moderately active ulcerative colitis used a dose escalation design. In three cohorts we delivered high doses of bacteria ranging up to 2 x 10^{12} cfu/day over 28 days. We tested two formulations — oral capsules and enema. The first and second cohort consisted of 15 patients each (10 active and 5 placebos), the third cohort included 20 active and 10 placebo. The trial was a randomized, double blind and placebo controlled multi-center and multinational trial. To have mastered the logistics, production and regulatory requirements around that was a major success for our small company. The major endpoints of the study were safety and tolerability. We measured pharmacodynamic biomarkers for AG 011 in fecal excretions to validate our containment strategy. We developed an assay to determine both the total amount as well as the viable amount of AG 011 in stool samples. At baseline before exposure no AG011 should be found, and after exposure to Lactococcus lactis the total as well as the live counts were measured throughout the trial. The results confirmed the suitability of our containment system in humans.

Another product, which we are currently developing and testing in the US, is a Lactococcus lactis producing Trefoil Factors to prevent oral mucositis. This painful inflammation with necrosis and ulceration of the oral cavity is a consequence of chemotherapy or radiotherapy or a combination of those, especially in patients treated for head and neck cancer. It severely affects the patient’s ability to eat or drink and increases the risk for sepsis and mortality. Patients need to receive parenteral nutrition and IV analgesics to control pain. Often an interruption of the cancer therapy becomes necessary, reducing the number of administered courses of chemotherapy and negatively impacting the effectiveness of the treatment. Trefoil Factor 1 (TFF1) is a protein secreted by human salivary glands and contributes to the structural density of the mucus layer covering the oral mucosa. The mucus layer acts as a physical barrier against bacteria and noxious environmental agents. Its potential for therapy of oral ulcers has been established in validated animal models of intestinal mucositis. LL-TFF (1, 2, 3) promote protection and healing of mucosal tissues and circumvents efficacy issues. In animal models, we successfully demonstrated that Lactococcus lactis delivering TFF1 can cure ruptured epithelial lining when given orally to mice. Subsequently, we used a hamster model developed by Dr. Steve Sonis at Harvard to monitor the effects of TFF1 on live hamsters exposed to radiation, scoring the severity or the mucositis. The results in hamsters showed that a treatment with TFF1 can maintain the condition at a critical level and avoid the development of ulcerations.

We are now starting the clinical research on TFF1 (AG013 protocol) and nearly completed the first dosage group. All our patients will eventually develop oral mucositis, but not all of them during the early cycles of radio- or chemotherapy. In our cohort about 50% of the patients developed mucositis during the first cycle. We enrolled patients developing oral mucositis in cycle 1 and started the treatment before cycle 2 was initiated. In this open label trial we are testing 3 different dose levels (2 x 10^{11} cfu/dose once, three or six times daily) over 14 days.

Another preclinical program that we are very enthusiastic about aims to treat juvenile diabetes, using our Lactococcus lactis delivery platform to produce IL-10 and pro-insulin. Our animal data in mice suggests that diabetes can be reversed in 60% of the diabetic mice.

In the future, our technology could also be used...
to deliver combinations of multiple therapeutics by engineered Lactococcus lactis strains.

For the second part of my talk I would like to share some of my knowledge on regulatory issues that I acquired along the way.

We faced specific regulatory challenges in the clinical development of ActoBiotics™.

First, to guarantee the safety and efficacy of a product, a Good Manufacturing Practice (GMP) compliant manufacturing process needs to be established and product quality and consistency needs to be demonstrated, including testing of identity, purity, stability, potency and animal toxicology studies. Second, the novelty and GMO status of the recombinant Lactococcus lactis therapy is considered by regulators as a deliberate release of GMO in the environment and needs to demonstrate appropriate risk assessments and functioning containment systems. We need to communicate to the society that what we do is safe and that we know what we are doing. Our small research operations need to be elevated toward the standard of the pharmaceutical industry, and not the standard used by the food industry.

We need to engage regulators in order to educate us on issues of documentation and product approval according to GMP standards. As trained scientists, we are very proficient in the laboratory. We sought support from experts for designing the clinical studies. The step in-between is the Chemistry Manufacturing & Controls (CMC), and for our product this was the most elaborate part. For a genetically modified product, the process of genetic engineering, strain selection and maintenance of a research cell bank is straightforward and by definition a non-GMP process. However, everything following those initial stages needs to comply with GMP standards. This includes the establishment of a master cell bank, the development of the processes for fermentation and biomass concentration as well as lyophilization to produce a drug substance in bulk.

The production process has to be robust and should not overly depend on any of the variable parameters; it needs to be scalable to finally treat thousands of patients. All the used raw materials have to be of clinical grade and everything has to be animal-product free. During the product development the first audit by regulatory authorities takes place and includes evaluation of efficacy and toxicology data in relevant animal species. The used product in animal pharmacodynamic and toxicology studies needs to be identical to the GMP product eventually used in clinical trials, but at the animal study stage does not require GMP standard documentation.

The whole GMP documentation focuses on 4 aspects: identity, purity, stability and potency at any of the relevant stages during processing. Most parts have to follow the GMP protocol. This requires a considerable financial investment by the company, and often this process will need to be outsourced to a subcontractor. Consequently, the company developing such products has to set up a GMP facility for testing all these aspects, for instance sequencing, monitoring viability of the engineered bacteria, measuring residual moisture and residual lactate of the product.

Interacting with regulatory agencies in different countries can pose its own challenges. For example, mouse and monkey are both responsive to human IL-10 and are suitable to gather relevant animal data. In our case, the Canadian authorities asked us to provide much more extensive toxicology documentation in monkeys after we had started out with mouse models only.

In addition, regulation on what data has to be presented to regulatory authorities may differ. The Canadian authorities have very concise regulations, and they will move all documentation to the study sites. European authorities request an overview on strategy and a product description, but the data supporting your strategy is kept at the company and has to be presented upon request. US authorities
require having all of the documentation in their files. One of the advantages of the IND process in the US is that it is open, and the sponsor company continues to submit new data and the FDA continues to archive it as it is submitted. This may seem more cumbersome in the beginning, but actually the comprehensive and flexible US procedure is a good system.

Differences between regulatory agencies also extend to the approval process itself.

Initially, all systems afford the developers a certain period of scientific advice during which they can be approached to discuss future steps of product development. Such a consultancy phase allows adding supporting studies to produce the additional data the agency finds necessary, like additional animal studies. Without this window of initial communication before the filing of the final product, a late discovery of gaps in the data in a more advanced phase could derail an entire project.

The US system encourages developers to very early communication with the regulatory authorities. Another peculiarity about the US system is the existence of recombinant DNA advisory committee to supports local authorities when overseeing a project using recombinant technology.

Specific for the European review and approval process is the scrutiny for products involving recombinant DNA, which is regarded as much more problematic than elsewhere. Contained use is less of an issue as long as a complete physical barrier is provided.

But products with a deliberate release strategy like in the outdoors trials of engineered maize and soy beans are scrutinized and may be judged by reviewers not necessarily familiar with clinical programs and testing of medicinal products.

Clinical trial dossier – Requirement per regions

USA (IND)
Sections are populated throughout the clinical development of the product
The full reports have to be provided within the INDs

EU (CTA)
High variability of module 1 in the different EU MS
Module 2 ➔ IMPD
Full CTD needed for MA

Canada (CTA)
Focus is made on the CMC section of the CT
The preclinical and clinical sections are limited to the IB
I would like to thank the team of Dr. Chantal Matthieu at Leuven University, Belgium, for animal experimentation on type 1 diabetes. Many thanks also to Dr. Steve Sonis and his associates at Harvard University in conducting oral mucositis studies. In the end I am much obliged to my team at ActoGeniX. We were founded in 2006 and have raised more than 35 million Euros since for our research. We have 20 employees and a few actobiotic products in clinical development.

Q & A

This was an amazing presentation on what is needed on the regulatory end to move our products forward. The big question is how long did it take you for your first product from inception to phase 2?

This process was much like jumping out of a plane. Sometimes it is better if you have a parachute, but it really goes faster if you don’t. It took us less than a year for the first clinical trial to set up all the logistics in Canada, Sweden and Belgium. We really were fortunate during the production process because we were able to use the same development line. But it was a tremendous effort, even with the 50 employees we still had before the financial crisis.

For the second product, the whole expertise for a new technology and the production infrastructure and all the subcontractors were already set up and we had our analyses. Often the lack of expertise and capable collaborators for such cutting edge technologies and who also GMP requirements can slow a project down. So the only thing we had to do the second time around was to use the same validated assay for the different product.

How long is the time from rehydration of the bacteria once administered in vivo until they produce the cargo?

_Lactococcus lactis_ does not divide, it does not multiply, they are just a delivery agent.

Once of the main reasons why we chose _Lactococcus lactis_ is that it is a non-colonizer. That comes with a lot of draw backs — it is not robust and you have to do a lot to preserve it. But on the other hand, it has a profile which is better suited for regulatory processes than any colonizing bacteria.

_Lactococcus_ lives inside the body, it just does not divide. This platform has to provide about 10,000 fold lower concentrations of protein (such as TFF1) than through conventional delivery (injected or oral purified protein) in order to get that active compound closer to the receptors.
To help ensure the success of the field the research community needs to integrate the community of end-users from the beginning to ensure their support for the development of these products. We need to think about the audiences we want to reach and how to best engage them, and we need to understand current demand and acceptability of probiotic products.

Probiotics could become a key component of future multipurpose prevention tools (MPTs) for sexual and reproductive health that prevent multiple adverse health outcomes simultaneously, including HIV, STIs, unplanned pregnancy, as well as reproductive tract infections.

Today Dr. Samu Dube and I will be talking about building the demand for probiotics that improve sexual and reproductive health as well as provide an overview of some of the acceptability data we have for sexual and reproductive health probiotics.

Following our overview, we will initiate a discussion around what is needed to ensure support and acceptability for such products. We will draw upon the lessons learned from the microbicide field as well as other experiences such as the promotion of female condoms to help us think about how we, as researchers and prevention advocates, should move forward from here, the audiences we need to reach and how to best engage them.

Probiotics represent an exciting opportunity to develop a multipurpose prevention technology or tool (known as MPTs) for sexual and reproductive health. MPTs, also referred to as “combination” technologies, are designed to address multiple sexual and reproductive health needs, such as STIs, including HIV, unplanned pregnancy, and other reproductive tract infections such as bacterial vaginosis urinary tract infections.

The new Initiative for Multipurpose Prevention Technologies (IMPT) is promoting awareness and support for innovative prevention strategies, including probiotics, that can prevent multiple adverse sexual and reproductive health outcomes. The mission of the IMPT is to raise awareness about and support for new and existing multipurpose prevention technologies.
When looking deeper at the data we found that acceptability was greatest in women who had experienced a vaginal infection like BV or UTI in the past. Those women were also more willing to pay out of pocket for such a product.

The data suggest that women were interested in the following:

- How would such a product improve their overall vaginal health?
- How often would they have to use the probiotic and would they have to use it every day in order to maintain the effect?
- What would be the cost of such a long-term preventative treatment and could it be covered by insurance and/or be available over-the-counter?
- Safety and a low side effect profile was a prerequisite for use. Some women were very interested to learn more about the formulation and the bacterial strains used in the product.

The fact that such a product would be “natural” was of high importance to some women. When we explained the concept of second-generation genetically modified products, the participants asked whether these probiotics could still be considered natural, how the bacteria would reside in the body, and whether the side effects would be different.

Providers expressed a more cautious interest in probiotics for disease prevention and were more hesitant to prescribe such products to their patients, aware about the current lack of evidence base for many products available in health food stores. Other concerns included the equal access to these products on a global scale, safety and potential side effects, the compatibility and interaction with existing treatments such as antibiotics and resistance after long-term use.

So where do we go from here?

Awareness and support for probiotics is still very preliminary. More needs to be done to
understand consumer demand and acceptability among women and providers in diverse settings and regions of the world.

Similar to the development of other multipurpose prevention technologies and strategies, the development of probiotics that reduce the risk for disease and improve sexual and reproductive health is going to face a variety of complex challenges such as product development, financial support, regulatory hurdles, manufacturing and logistical barriers and effective branding and commercialization of the product.53

In order to advance multipurpose prevention technologies (MPTs) for sexual and reproductive health including probiotics we must plan deliberately and early to increase awareness and collaboration across disciplines. It is critical that we work to achieve multidisciplinary support and pursue opportunities to collaborate with partners from a variety of disciplines that will ultimately ensure the success of product development, clinical trials, access, and community acceptance. Building upon experiences with microbicides and the female condom, we need to identify ways to collaborate and find synergies between funders, government agencies, private donors, regulators and health care providers. Collaborations between scientists from multiple disciplines are also critical, such as engineers, behavioral scientists, social scientists, lab scientists. For example, product developers can collaborate with social and behavioral scientists to help ensure that products are not only effective, but are acceptable and women will use them. Learning from our experiences with microbicides, we need to involve the community early on to ensure there is understanding and support for the research, including the clinical trials.

A multidisciplinary cadre of advocates is essential and each of us here today can be advocates in our own way.

Thank you!

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I would like to acknowledge our funders, the Mary Wohlford Foundation and the US Agency for International Development (USAID). I would also like to thank the following colleagues for their input: Wayne Shields (ARHP), Polly Harrison (AVAC), Marianne Callahan and Matt Reeves (CONRAD), Kathryn Stewart and Tracy Salkowitz (CAMI), Alan Stone (MEDSA Ltd), Kevin Whaley (Mapp Biopharmaceutical), Jess Cohen and Maggie Kilbourne-Brook (PATH), Craig Cohen and Anke Hemmerling (UCSF), and Judy Manning (USAID).
Women in sub-Saharan Africa bear the burden of HIV and as end users need to be engaged from the beginning to ensure the final product meets their needs. The support of community leaders is essential for the development and implementation of study protocols. The community can best identify and address barriers in product delivery. Creating demand is a complex process that includes generating attention, build desire for the product and inspire active demand for it. Awareness and support for probiotics are still limited in Africa and acceptability studies are needed in preparation for designing clinical trials. To successfully navigate the complex challenges for the development of probiotics, it is critical to forge multidisciplinary alliances that include scientists, advocates, funders, government agencies, regulators, health care providers and the community of end users.

I am excited to talk to you today about strategies to create demand for probiotics. I will highlight some potential opportunities and challenges that the probiotics field can learn from our experiences with microbicides.

Women continue to disproportionately bear the burden of HIV in Southern Africa. The need for more options is unabated; hence we need to discuss the potential of probiotics today. As in Europe and America, probiotics are available to consumers on many shelves in Africa as dietary supplements, and they are used as complementary and alternative medicine.

The private sector creates demand with the end user in mind, targeting individuals with messages about products that will benefit them. If a probiotic is positioned for HIV prevention, the target market is a woman who wants to protect herself from HIV and other sexually transmitted infections. In order to effectively reach these female end users, we need to initiate a parallel process of community engagement. If you are a product developer you need to find out whether a product is beneficial, safe and desirable. As an advocate, the cherry is whether you can use it as well. I believe that advocates need to be able to convince other women to use the product, and demand creation is about identifying such champions.

Who else needs to be engaged: We also need to reach decision makers and health care providers of the community to learn about and support the introduction of the product.
Until recently, we have not succeeded very well at including the community from the very beginning because of challenges in managing expectations. When a product is still far off and early in the product development process, we don’t want to raise unnecessary hopes. However, enthusiasm and anticipation that are finally needed for a product uptake are also needed in this early phase. Thus early on you may not need massive population engagement for effective end user involvement, you need just a few individual champions. Reaching out to populations only during product launch may be too late for public health products. Take a female condom for example, and go to some clinics in a township in South Africa. You will meet a substantial number of health care workers and female nurses who have never seen or touched a female condom. These nurses are the first line health care providers where women get their information.

We also need to understand that the answer to who best represents the community is always changing. It is not enough to include a community representative into our research advisory board, and to assume that we will get all the relevant information about the community from this one person. We always need to ask: who else needs to be in the room? Recently in Zambia, community chiefs were demanding that they be included in the process of protocol approval, and indeed some men demanded that there is need for them to give consent on behalf of their partners in order to participate in a clinical trial. This brings to the fore the issue of who needs to be involved in the product development process, and whether this has impact on future trial participation. The involvement of the community early on is also important to identify and address barriers in product delivery and maintaining a functioning supply chain.

We can learn a lot from our experiences in advocacy for microbicides. In the beginning, we supported the idea that a microbicide could work and advocated for financing microbicide research. Governments had to be engaged, including other national scientific bodies. As research continued, community involvement in the research became a very important dimension of HIV prevention trials.

The advocacy message shifted towards addressing regulators’ perspectives for trial approval and to prepare a successful licensure of such products. It is important to note that what happens in one country does have an effect on what would happen in another country. We have seen through anecdotal evidence that what happens in Zimbabwe will impact what happens in Zambia for example, thus regional perspectives are crucial in the product development process.

Commercial marketers have drifted from a product-oriented approach to an end user oriented approach; but does this transition apply to public health products? For probiotics, clearly we have the end user in mind. The perceived benefits are obvious - a potential to maintain a healthy vaginal environment and protect against HIV - and hence the product is fulfilling an unmet need. But creating demand is more complex than just verifying an existing need for such a product. To guide a successful creation of demand is a process following the AIDA model:

1. **Attention:** Identifying an underserved market segment. Raise awareness through involving communities in research.

   Probiotics already exist in many places in Africa and many women already use them. We need to draw attention to the increased potential benefit of new probiotics under the umbrella of multipurpose prevention technologies.

2. **Interest:** Making communities interested in identifying and solving their problems (“not for us without us”).

   We need to generate their interest by explaining how probiotics could address their need for HIV prevention.
3. **Desire:** Communities and target populations need to then desire and anticipate the particular product.

I witnessed at a conference how a group of women invented and sang a song about how they want a microbicide, a song of hope that one day a microbicide would be available, which was a very powerful example of community engagement and demand creation.

4. **Action:** Managing expectations as target populations start to demand the product. Ensuring manufacturing capability and effective supply chain.

We also need to be prepared to effectively communicate if the research results do not support the hopes that have been raised. Supply needs to be uninterrupted and sufficient to meet demand. If public health agencies do not do it well, we then need to give it to the experts in the private sector who do it well.

In order to successfully use the AIDA model, the daily realities of the end users need to be considered. How does one incorporate a probiotic into a woman’s life? What are her daily routines and preferences? Indeed there are many barriers that can prevent a successful introduction of a new probiotic. A closer look at some of these realities has guided packaging and formulations of a microbicide. For example, how would a woman living in a shack in an African informal settlement dispose of a vaginal ring or applicator? Where will a woman wash her hands afterwards? Can women wash their hands, and how can they insert a product privately? How could she keep the product or unused applicators out of the reach of children?

Pricing of the product and its production cost, whether to the manufacturer, the government or to the women, will affect demand. This includes the actual price of the probiotic, the cost of time and travel to the health center, possible HIV testing etc.

People assess perceived benefits of HIV prevention products in relation to their perceived personal risk. Research shows that the majority of people underestimate their own risk of HIV infection. In light of this, the positioning of the product becomes important. In order to reach as many users as possible, should probiotics be introduced primarily as a product for vaginal health and prevention of bacterial vaginosis, or for HIV prevention? We need to think differently and be innovative in this regard.

We need to think carefully about the context in which a technology will be introduced if we want the women to actually use them and we need to involve them to answer these questions while developing the final product. Only if a product fulfills a need and suits the end users life styles, demand will be sustainable. In later stages, a successfully introduced product use can be improved (for example, simplified dosing) or its use can be expanded to a multipurpose product, then including a contraceptive component. This is the total augmentation that hopefully sustains demand.

To conclude, we do not need to reinvent the wheel. We can draw synergies from existing community structures when building a movement for probiotics. I believe that if we succeed in creating a network of people, avoid a “silo approach” and learn from previous experiences, we can successfully introduce probiotics in Africa successfully move from the “test tube” to the community.

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**Q & A**

**Since there are probiotics available, could a trial be done using these products to see if people would use them as intended?**

Yes, the probiotics studies done at UCSF by Cohen et al. included an acceptability component. However, we need more studies looking at acceptability in different regions of the world. We have some data from the U.S., but as far as I know we don’t have much elsewhere.

**The idea of product providing both pregnancy and HIV prevention is a good idea from the perspective of the reluctance of people to acknowledge their risk for HIV, but perhaps being interested in birth control.**
Navigating Regulatory Requirements for Probiotic Microbicides

The regulatory burden at the US FDA for probiotics as drugs or drug delivery systems is higher than for probiotic foods. The sponsoring institution needs to file an Investigational New Drug (IND) application and demonstrate the safety and effectiveness of the product before marketing. The development to probiotic microbicides to prevent HIV infection will require testing in sub-Saharan Africa, where regulatory procedures for probiotics remain unclear and may follow the regulations set forth by the US FDA. Specific regulatory guidelines for probiotics as drugs (including the genetically modified strains) are needed to inform future research. A guidance document by the WHO or FDA could facilitate international collaboration between agencies in order to streamline regulations.

I would like to give you an overview about regulatory requirements for the development of probiotics as drugs. It is crucial for the timely progress of the field that we communicate with regulatory agencies early on and shape our research designs according to these requirements.

As we heard earlier in Dr. Steidler’s presentation, there are different regulatory requirements mandated by the different regulatory agencies in Canada, Europe and the US. In addition, the regulatory framework employed by many African regulatory agencies may again be different. However, many African regulatory agencies follow to some degree recommendations set forth by the US regulatory authorities.

In the US, the regulatory framework is laid out in the Food, Drugs and Cosmetics (FDC) Act. This congressional judgment rules that certain products must be subject to scientific scrutiny and the claimed benefits must be substantiated. The intended use of a product will govern which regulatory category is appropriate and determine the regulatory burden at the FDA.

Regulation of probiotics faces special challenges, because the content is derived from nature and often an unpurified heterogeneous mixture highly dependent on manufacturing processes. The challenges of correct classification also complicate regulation. Probiotics can be categorized as food or medical food, as dietary supplements, or drugs.

If, as often in the past, probiotics are marketed as established foods, the FDA Center for Food Safety and Applied Nutrition as the overseeing agency allows
This important difference also needs to be communicated to the community that already has experience with over-the-counter probiotics marketed as food. In the past, the predominant strategy to market probiotics as foods has not only left a dearth of reliable clinical data, but also resulted in a current situation when manufacturers of probiotic foods often could not produce pharmaceutical-grade drugs, because they do not meet the requirements for Good Manufacturing Practices (GMP).

Considering all these regulatory requirements, a timeline for drug research and development is usually a 10-year process. The preclinical testing must lay a logical foundation for clinical studies, must test appropriate dose, use appropriate animal models, address biocontainment and clearance (for genetically modified drugs), and show how to prevent a reversal to toxicity.

Even with this fairly new research field of probiotics regarded as drugs, efforts to streamline regulation for probiotics as foods are several years old. The FAO/WHO guidelines marketing without prior FDA approval and the evidentiary burden lays with the FDA in post-marketing control. On the contrary, if probiotics are categorized as food additives, new drugs or biological products, the evidentiary burden is on the developer of the product and has to be demonstrated prior to marketing to the FDA Center for Biologics Evaluation and Research (CBER). Further, new products intended for prevention, treatment or cure can be categorized as not-generally-recognized-as-safe (non-GRAS) and/or not-generally-recognized-as-effective for the intended use (non-GRAE).

For new drugs, the FDA requires an Investigational New Drug (IND) application to demonstrate safety and efficacy of the new drug. If the appropriate category is not a drug, a premarketing approval is only required if the product is not-generally-recognized-as-safe (non-GRAS). No rigorous clinical studies substantiating efficacy claims (non-GRAE) are required, just theoretical concepts. Since our research clearly focuses on probiotics as drugs, a much higher regulatory burden is required.
from 2002 lay out a minimum consensus on what producers need to address:

- Adoption of a standard definition of probiotics
- Identification of genus, species and strain
- In vitro tests to establish antimicrobial activity, adhesion, resistance to spermicides and antibiotics
- CONSORT guidelines for clinical trials
- Good Manufacturing Practices (GMP) for a non-contaminating manufacturing process to ensure consistency, quality, and safety of products
- Labeling requirements including the strain, viable CFUs, dose and route of administration, health claim, storage conditions, corporate contact
- Regulatory framework that allows health claims on labels only to be made if scientific evidence exists
- Standardized shelf-life conditions

In order to further facilitate international collaboration and use research data for drug approval in different places overseen by different regulatory agencies, it would be helpful to establish agency-wide working groups on probiotics in order to provide leadership for regulation and standardization. The development of an FDA guidance document on probiotics, alike the 2004 ‘Guidance for Industry - Botanical Drug Products’\(^5\), would be extremely useful for drug developers to provide an outline of the requirements for the developmental pathway for probiotics as drugs. A harmonization of international regulation of probiotics research could encourage global collaborations for product development. A high degree of regulatory clarity is even more critical for bioengineered probiotic strains.

In order to use resources for research and development of probiotic microbicides economically, clarification and regulatory consensus for clinical research would enable us to streamline clinical research, avoid duplication, and employ smarter study designs. To enable such a process, a few questions need to be agreed upon between researchers and regulators.

When can individual strain characteristics be generalized with regards to safety and efficacy, avoiding redundant clinical studies?

What extent of animal, preclinical and phase 1 studies are necessary to provide a sufficient biological basis before clinical studies can be implemented?

What extent of additional preclinical toxicity studies of widespread probiotics generally-regarded-as-safe (GRAS) is necessary?

How will combination strain products be evaluated? The currently favored strategy to approve each drug separately before testing a combination of strains in a co-formulation would prolong the approval process for combination strain products tremendously.

The WHO-sponsored Symposium on Regulatory Considerations for the Review of Microbicide Clinical Trials and Product Registration at the Microbicides 2010 conference here in Pittsburgh brought together FDA representatives, including from their office for communication with foreign sponsors. They are currently developing a document on combination products and on microbicide development. Ideally the field of probiotic microbicides would be incorporated in such a document. We also learned that the FDA does accept non-IND data for licensure from trials outside the US as long as these trials are well-conducted and follow guidelines of Good Clinical Practices (GCP), and the database can be reviewed by the FDA auditors.

Several experts have published on this topic; please see the reference list to find more detailed information.\(^3\)\(^6\)\(^7\)\(^8\)
Probiotics have developed into a budding research field that is actively moving forward toward the attainable goal of creating a live microbicide. This symposium took a close look at the steps needed to reach this goal. Understanding the complexity of the vaginal environment will be the foundation for sound research designs. The first live anti-HIV microbicide is in the pipeline. Data has established the proof of concept that a live probiotic microbicide can prevent SHIV virus transmission in monkeys. As the field moves forward from the preclinical to clinical testing, designing future research according to the requirements of the regulatory approval process is critical, and lessons learned from other fields can help to guide this process. For the success of a bioengineered live probiotic microbicide messaging and outreach are of crucial importance. Microbicide research has identified the need to engage the community early to win the support of potential users, funders, and institutional leaders. The goal of creating a live probiotic microbicide is achievable.

I was asked to present some closing remarks at the end of this symposium to sum up what we have learned today about the potential creation and impact of a probiotic live microbicide.

Your first impression when looking at the title of this symposium ‘Probiotics as a live microbicide’ could have been one of a field just starting to develop basic concepts. However, at the end of this afternoon you see a budding field and may even sense an enthusiasm not unlike the one of the early days of microbicide research. Probiotics as a research field has really come a long way in a short time. Evidence was presented today that it is actively moving forward, toward the goal of creating a live microbicide and that this goal is attainable. Today, we took a close look at the steps needed to reach this goal.

First, Dr. Jeanne Marrazzo gave us a solid background on the vaginal environment and urged us to take that knowledge seriously. We were shown the microbiota in the vagina is a highly complex multifactorial problem with many open questions. Why is this important to us? Because understanding the complexity of the vaginal environment will be the foundation for sound research designs as we move live probiotic microbicides forward.

We heard from Dr. Qiang Xu and Dr. Laurel Lagenaur that the first live anti-HIV microbicide is in the pipeline. They presented data that establishes the proof of concept that a live probiotic microbicide can prevent SHIV virus transmission...
In addition to regulatory uncertainties we are proposing to advance bioengineered bacteria as a microbicide. Bioengineered products are often misunderstood and feared by the public. Consequently, messaging and outreach are of crucial importance for the success of live probiotic microbicides. Dr. Bethany Young Holt and Dr. Samukeliso Dube outlined the need to engage all stakeholders and to learn from the lessons microbicide research can offer. Microbicide research has identified the need to engage the community early on and to manage the high expectations of people, going into the community and engaging potential users, funders, and institutional leaders for their support. This will be critical in gaining acceptance of bioengineered live microbicides, as well as, assuring it can meet expectations of impact on reducing HIV transmission and acquisition.

Dr. Lothar Steidler took us one step further. Having the most experience to date with taking bioengineered Lactobacillus strains into clinical testing and approval, he laid out the steps necessary to transition from the preclinical to clinical testing and regulatory approval. He also urged us to think ahead about the requirements to make a live probiotic microbicide a reality. He shared his and his companies’ regulatory experience in developing their bacterial therapeutics, outlining regulatory issues which determine the next steps in development for any live probiotic microbicide. And, he told us something else very important: it can be done and there are efficiencies gained with experience. As Drs. Xu and Lagenaur pointed out there are other live probiotic microbicides in the pipeline. Dr. Steidler reminded us that with the proper preparation we do not have to start over from zero for each new microbicide. We can reuse not only our bioengineering technology, but also the development processes and regulatory knowledge gained to facilitate advancement of additional live probiotic microbicides.

Dr. Craig Cohen’s talk expanded on the US specific requirements for regulatory approval and showed that the regulatory framework for probiotics is still developing. We will need to move forward with some unknowns, but in many cases these unknowns are similar to the ones the microbicide field has faced. These uncertainties will have to be resolved as we move forward, but as we have learned from microbicides and other drug development endeavors, i.e. cancer, the challenges are not insurmountable.

In addition to regulatory uncertainties we are proposing to advance bioengineered bacteria as a microbicide. Bioengineered products are often misunderstood and feared by the public. Consequently, messaging and outreach are of crucial importance for the success of live probiotic microbicides. Dr. Bethany Young Holt and Dr. Samukeliso Dube outlined the need to engage all stakeholders and to learn from the lessons microbicide research can offer. Microbicide research has identified the need to engage the community early on and to manage the high expectations of people, going into the community and engaging potential users, funders, and institutional leaders for their support. This will be critical in gaining acceptance of bioengineered live microbicides, as well as, assuring it can meet expectations of impact on reducing HIV transmission and acquisition.

Dr. Anke Hemmerling illustrated in her overview on completed clinical research of unmodified probiotic strains that we already have an extensive clinical history with probiotics that we can draw from. And pointed out, we should use this experience and the outcomes as lessons learned and in conjunction with other lessons learned develop the most efficient clinical studies to test live probiotic microbicide. During this symposium, the potential for a live probiotic microbicide has been discussed. The speakers have given us an overview of what has been done and how we need to proceed in the future. A big step in the development of a live probiotic microbicide was taken today. This symposium brought together a group of people with strong interests in moving the field of live probiotics microbicides forward. In summary, the goal of creating a live probiotic microbicide is achievable. All of us participating in this symposium are motivated by the common goal of fewer HIV infections. Today, we came together and laid out the road map of achieving this with a live probiotic microbicide.
## Symposium Agenda

### PROBIOTICS: the Potential for a Live Microbicide

**Satellite Symposium at Microbicides 2010**

Saturday, May 22, 2010  |  1-5 PM  
David L. Lawrence Convention Center  |  Pennsylvania

**AGENDA**

<table>
<thead>
<tr>
<th>Time</th>
<th>Session Title</th>
<th>Speaker</th>
<th>Affiliation</th>
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<tbody>
<tr>
<td>1:00 PM</td>
<td>Overview of the Field of Probiotics</td>
<td>Dr. Craig Cohen</td>
<td>University of California, San Francisco</td>
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<tr>
<td>1:20 PM</td>
<td>Molecular Investigation in the Study of Genital Microbiota: Implications for Probiotic Approaches</td>
<td>Dr. Jeanne Marrazzo</td>
<td>University of Washington, Seattle</td>
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<tr>
<td>2:00 PM</td>
<td>Bioengineered Lactobacilli as Next Generation Probiotics</td>
<td>Dr. Qiang Xu</td>
<td>Osel Inc. Santa Clara</td>
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<tr>
<td>2:20 PM</td>
<td>Testing Proof Of Principle In A Repeated Low Dose Challenge Model using a Live Lactobacillus Microbicide</td>
<td>Dr. Laurel Lagenaur</td>
<td>Osel Inc. &amp; National Institutes of Health</td>
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<td>2:40 PM</td>
<td>Coffee Break</td>
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<tr>
<td>3:00 PM</td>
<td>Update on Clinical Research</td>
<td>Dr. Anke Hemmerling</td>
<td>University of California, San Francisco</td>
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<tr>
<td>3:25 PM</td>
<td>Clinical Application of Genetically Modified <em>Lactococcus lactis</em></td>
<td>Dr. Lothar Steidler</td>
<td>ActoGeniX NV (Belgium)</td>
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<tr>
<td>3:55 PM</td>
<td>Demand for and Acceptability of Probiotics</td>
<td>Dr. Bethany Young Holt</td>
<td>CAMI &amp; University of California, Berkeley</td>
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<td>Dr. Samukelisu Dube</td>
<td>PATH (South Africa)</td>
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<td>4:35 PM</td>
<td>Regulatory Requirements for Probiotic Microbicides</td>
<td>Dr. Craig Cohen</td>
<td>University of California, San Francisco</td>
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<td>4:45 PM</td>
<td>Closing Remarks</td>
<td>Dr. Jim A. Turpin</td>
<td>NIAID, National Institutes of Health</td>
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<td>Moderator</td>
<td>Dr. Renee Ridzon</td>
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<td>Bill and Melinda Gates Foundation</td>
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