Old Herborn University Seminar Monograph 22:
Biological consequences of host-microbe interactions.
Editors: Peter J. Heidt, Lars Å Hanson, Helena Tlaskalová, and Volker Rusch.
Old Herborn University Foundation, Herborn-Dill, Germany: 105-115 (2009).

HOW PROBIOTICS *LACTOBACILLUS* GR-1 AND RC-14 IMPROVE UROGENITAL HEALTH IN WOMEN

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SUMMARY

The urogenital tract of females is important for reproduction as well as urine excretion. The anatomical nature of the area, so close to the anal skin and open to the exterior, makes it particularly susceptible to microbial colonization and infection. In addition to innate immune factors, mucins and epithelial barrier function, the indigenous microbiota, especially lactobacilli, help protect the niche from disease. A capsule product containing *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 is the world's most documented probiotic designed for women and shown in clinical studies to repopulate the vagina, displace pathogens and reduce the risk of infection. The mechanisms of actions continue to be uncovered but include modulating host immunity, altering the micro-environment to be less receptive to pathogens through production of acid, biosurfactants, hydrogen peroxide and signalling compounds, and dislodging pathogen biofilms. With urogenital infections inflicting an estimated one billion women each year, the use of probiotic lactobacilli to augment or replace antimicrobial agents represents an important addition to the options available for women.

INTRODUCTION

The health of the female urogenital tract is like any other part of the body, it falls under the World Health Organization's 1946 definition: "Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity." In today's technologically and medically advanced society, this definition is clearly out of date, if only for the fact that it is almost impossible for a human to have 'complete physical, mental and social well-being'. Indeed, so much attention has been paid to disease and so little to health, that there are few indicators of health per se.

The 'health' of the urogenital tract of females is influenced by a number of factors so much so that it is rarely 'healthy'. For example, at any given time large numbers of women have bacterial vaginosis (BV), a condition not believed to represent health, due to elevated pH, dominance of pathogens and potential pathogens, modulated immune profiles, and an increased risk of acquiring sexually transmitted infections, urinary tract infection (UTI)

and having preterm labour (*Allsworth* and *Peipert*, 2007; *Cauci* et al., 2003; *Chaim* et al., 1997; *Cherpes* et al., 2003; *Klebanoff* et al., 2004; *Sewankambo* et al., 1997; *Sharami* et al., 2007). Vulvovaginal candidiasis (VVC) and UTI are common in females, and the menstrual cycle often causes pain, discomfort and mental and physical disruption that are not consistent with optimal well-being (*Sobel*, 2007; *Sobel* and *Chaim*, 1996). Thus, the 'health' of the urogenital tract is a relative term and discussing the ability of probiotic lactobacilli to 'improve' urogenital health must be viewed in perspective. For the record, probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). This definition therefore requires that products be tested in humans and shown against placebo or standard therapy to provide measurable benefits.

THE ORIGINS OF LACTOBACILLI PROBIOTICS

The basis for application of lactobacilli to the urogenital tract comes from studies performed at least 35 years ago which showed that women who had recurrent urogenital infections had severely depleted lactobacilli counts in their vagina, while healthy women were densely colonized by these indigenous bacteria (*Bruce* et al., 1973). This association was not well understood at the time, nor was the actual composition of the vaginal microbiota, the species of *Lactobacillus* that dominated, or the nature of the benefits accrued by these bacteria. Nevertheless, Bruce and subsequently Reid, pursued this line of enquiry with a view to understanding the role of lactobacilli in female health, and in devising novel strategies to restore and retain urogenital health.

The hypothesis was that lactobacilli strains could be identified and administered to the vagina to improve health and reduce the risk of infection.

In order to select appropriate strains for this challenge, one must understand what species are present in the natural state, how they function to counter infection, then make sure that they can be scaled up for human studies and potential commercialization (to make them available to as many people as possible). This selection process is by no means an easy step. In the 1980s when this research began, and even until recently, it had been assumed that lactobacilli needed to adhere to the vaginal surface and inhibit the growth of pathogens (*Reid* et al., 1997). This was based upon pathogen studies showing that infection was often causes by adherence to cells and subsequent growth and production of virulence properties.

In fact, as we now appreciate, adherence of pathogens is not a pre-requisite for infection, and completely inhibiting their adhesion is all but impossible. In many cases of where a female is 'healthy', pathogenic bacteria or yeast can be found in the vagina (*Devillard* et al., 2004; *Hyman* et al., 2005). Likewise, the need to inhibit growth or kill pathogens is based upon chemotherapeutic concepts where the aim is to wipe out pathogens. As we now appreciate, this is not always a necessary outcome in curing infections of the urogenital tract. Pathogens can be displaced, disarmed through inhibition of expression of virulence factors, or eradicated by priming of the immune system (*O'Garra* et al., 2004; *Laugh-*

ton et al., 2006; *Saunders* et al., 2007). Thus, the selection of interventional strains is somewhat complex.

A common approach by the few companies with lactobacilli products ostensibly of use for females, has been to select a *Lactobacillus acidophilus* strain in the belief that this species was the most commonly found in the healthy vagina. Or, for a strain to produce hydrogen peroxide (H_2O_2) as the main antagonistic compound (*Al-Mushrif* et al., 1998). However, it is now clear that *L. acidophilus* is not the most common species in the vagina, it is *L. iners*, an organism that is fastidious (*Burton* et al., 2003) and problematic to upscale for commercial use. In addition, while H_2O_2 appears to have a role in disease prevention, based upon epidemiological observations that strains expressing it are more common in healthy women, there are other factors that clearly play a role in disease control.

Another common misconception promoted by some companies is that the more lactobacilli delivered in a product, the better the effect. Thus, some clinically untested products contain six or more strains of lactobacilli and bifidobacteria (chosen for reasons not disclosed) and claim to deliver tens of billions of viable cells. There are several concerns with this approach, not the least of which is lack of clinical data, mechanisms of action, properties of the strains, and viable counts at end of shelf-life. The vaginal milieu is not designed to harbour tens of billions of bacteria. On the contrary, the vagina and the organisms present modulate the microbial count, at least through quorum sensing, as well as other means yet to be fully understood. The organisms invariably form biofilms, and send out and receive signals to determine the extent to which they can multiply. This method of self-controlling an environment is necessary, in essence so that the species do not run out of nutrients or fall foul to detection and eradication by the immune system (*Quadri* et al., 2002; *Swidzinski* et al., 2005). Thus, one rarely finds more than 10^9 cfu/g in the vagina (*Delaney* and *Onderdonk*, 2001) and to add $20x10^9$ in a product makes little sense, unless the viable count drop-off is rapid, or the hope is that one of the strains might have an effect.

A further problem with multi-strain applications is that no apparent due note has been made of the potential for one strain to counter the activity of another. Studies have shown that some strains induce a particular immune response, and others induce the opposite effect (*Diaz-Ropero* et al., 2007; *Fink* et al., 2007).

LACTOBACILLUS RHAMNOSUS GR-1 AND *LACTOBACILLUS REUTERI* RC-14 AND MODE OF ACTION

In selecting *Lactobacillus rhamnosus* GR-1 (originally *L. casei* subsp. *rhamnosus*) and *Lactobacillus reuteri* RC-14 (originally published as *L. acidophilus* then re-defined as *L. fermentum*), a stepwise process was involved.

Initially, the GR-1 strain was selected for its antagonistic properties against uropathogens and for its ability to adhere to uroepithelial cells (*Chan* et al., 1984; *Reid* et al., 1987). In addition, its ability to coaggregate with uropathogens was believed to be useful in potentially blocking the ascension of these organisms to other areas of the vagina and into the bladder (*Reid* et al., 1988). The strain was able to be propagated into a form that could be deliv-

ered to humans, either in a milk product (*Gardiner* et al., 2002; *Reid* et al., 2001b) or dried capsule (*Reid* et al., 2001a). Subsequently, *Lactobacillus* GR-1 has been shown to modulate host immunity in a way that enhances antimicrobial activity yet down-regulate the potent inflammatory processes associated with discharge and symptoms and signs of infection (*Kim* et al., 2006; *Lorea Baroja* et al., 2007; *Kirjavainen* et al., 2008). The strain produces by-products, likely acid, that kill bacteria and viruses rapidly including HIV (*Reid* et al., 2006), quantities of lactic acid that stress uropathogenic *E. coli* cell surfaces (*Cadieux* and *Reid*, 2008), and quorum sensing molecules that can also play a role in interference with pathogenesis (*Elwood* et al., 2008). The organism was not found to produce H_2O_2 when originally tested (*Tomeczek* et al., 1992), but recently, using TMB agar it has been shown to be a producer (Schellenberg, J. unpublished). In short, this organism has a number of properties suitable to vaginal and distal urethral activity and restoration of health to this region.

The reason for adding a second strain to the probiotic formulation was to have better activity against Gram-positive pathogens. The *Lactobacillus* GR-1 strain has been shown to have bacteriocin-like properties against *E. coli* (*McGroarty* and *Reid*, 1988), but when used in a small pilot human study, there was evidence that enterococci were not displaced (*Bruce* and *Reid*, 1988). Enterococci are becoming more and more recognized as urinary pathogens, as well as being part of a disrupted flora associated with BV and infection (*Kelly* et al., 2003; *Jahic* et al., 2006). Furthermore, Group B streptococci are problematic in the vagina of women about to give birth, as they can infect and potentially kill the newborn (*Bayo* et al., 2002).

The screening for a second strain focused primarily on inhibition of growth of Gram-positive cocci. This resulted in selection of *L. fermentum* B-54 (*Reid* et al., 1987) to accompany strain GR-1 in clinical studies. The combination showed great promise based upon a series of clinical studies, three of which showed reduced incidence of UTI recurrences (*Bruce* et al., 1992; *Reid* et al., 1992, 1995). This formulation would have been retained but for the 1996 discovery of biosurfactants produced by lactobacilli (*Velraeds* et al., 1996). These compounds altered the microenvironmental surface tension and produced adverse conditions for the adhesion of a wide range of pathogens (*Velraeds* et al., 1998). Studies using polymer substrates showed that even low numbers of lactobacilli could significantly reduce pathogen colonization, biofilms and also displace these organisms (*Reid* and *Tieszer*, 1993, 1995; *Reid* et al., 1995b). The most potent activity was found in *Lactobacillus* RC-14, and therefore it replaced B-54 in the combination with strain GR-1 with a view to determining if these two strains were clinically compatible and able to improve vaginal health. Conjointly, studies were performed on RC-14 and various proteins and peptides were discovered which played a role in the strains anti-Gram positive coccal activity (*Heinemann* et al., 2000; *Howard* et al., 2000; *Reid* et al.2002; *Laughton* et al., 2006), as well as against E. coli virulence expression (*Medellin-Pena* et al., 2007). Most recently, we have shown that the mode of action of RC-14 is not reuterin (*Cadieux* et al., 2008), the antibiotic described as being critical for *L. reuteri* probiotic activity (*Dobrogosz*, 1998).

The concept of delivering lactobacilli for urogenital health had historically involved direct implantation into the vagina. However, recognizing that

pathogens ascend into the vagina from the woman's own intestine, and these organisms then infect the host, we hypothesized that lactobacilli also originated from the woman's own gut, and thus probiotics could be delivered to the vagina by oral intake. The first step in proving this concept was to show that *Lactobacillus* GR-1 and RC-14 could pass through the intestine. This was shown by *Gardiner* et al. (2002) who recovered the strains in faeces of volunteers who ingested the organisms suspended in milk. In order to produce a product that could be more widely used and therefore had longer shelf-life, a capsule was produced by Chr. Hansen (Horsholm, Denmark) with these strains in dried form. Using a technology that improved shelf-stability and increased passage of the organisms beyond the stomach, the capsules were then used in a series of clinical trials.

Upon a successful proof-of-concept study (*Reid* et al., 2001a) and independent verification of the results (*Morelli* et al., 2004), the strain combination was shown to reduce pathogenic bacteria and yeast ascension into the vagina from the rectum (*Reid* et al., 2003), and produce a more consistently normal lactobacilli-dominated vaginal microbiota (*Reid* et al., 2001a, 2003, 2004), with a dosage of one billion or more bacteria (*Reid* et al., 2001a). The vaginal counts of lactobacilli increased, including indigenous strains, indicating that the treatment itself encouraged recovery of the host's microbiota. These studies fulfilled the definition of a probiotic and have led to the strains being the first and most documented probiotics for urogenital health in the world.

The potential for probiotic lactobacilli to reduce the risk of preterm labour is based upon displacement of an aberrant microbiota and interference with the inflammatory pathway that leads to cyclooxygenase (COX)-2 and prostaglandin production. Having shown that *Lactobacillus* GR-1 and RC-14 can reach the vagina after oral intake, the next step was to determine if this could restore the vaginal microbiota in pregnant women with BV. In a study of 22 pregnant women with BV given the probiotics once daily for 30 days, the vaginal pH returned to normal in 73% and no safety issues arose (*Oleszczuk* et al., 2008). Further studies are needed to confirm the findings, but *in vitro* experiments strongly support the potential for these strains to lower the risk of preterm labour. Using trophoblast and placental cells in tissue culture, we have shown that *L. rhamnosus* GR-1 can significantly down-regulate COX-2 and TNF-α and up-regulate the protective prostaglandin dehydrogenase (*Yeganegi* et al., 2008).

The disruption of the vaginal microbiota can have other consequences, such as chronic vulvovaginitis. In a pilot study in Russia where vulvovaginitis is common amongst young girls, the use of *Lactobacillu*s GR-1 and RC-14 daily for one month in four 7-10 year olds and nine 11-19 year olds was found to restore the vagina to having no clinical signs of infection (*Uvarova* et al., 2007).

Another concern is the potential for BV to increase the risk of squamous metaplasia. In other Russian study, 30 women of reproductive potential aged 18 to 40 with diagnosed intraepithelial squamous cell cervical lesions were studied. Histology revealed mild (CIN 1/condyloma) intraepithelial lesions in 24 patients and severe (CIN2-3) intraepithelial lesions in 6 patients. All study patients had Human Papilloma Virus infection confirmed by RT-PCR: high oncogenic risk viruses in 17 patients, low oncogenic risk viruses in 7 patients, and both high and low oncogenic risk

HPV in 6 patients. The control group was comprised of 20 healthy women of reproductive age. Following once daily oral administration of two capsules of *Lactobacillus* GR-1 and RC-14 for 15 days, no BV was found and there was a significant reduction in pathogen counts in the vagina (*Minkina*, 2007).

NEXT FOCUS FOR CLINICAL USE

In recent times, several studies have been performed to explore the breadth of usefulness of these probiotic strains. Given that antibiotics and antifungals are widely used to treat urogenital infection, and that side effects and drug resistance rates are increasing, it was hypothesized that *Lactobacillus* GR-1 and RC-14 could augment the cure of infections through their ability to displace pathogens, modulate immunity, and reduce drug side effects.

Three studies have now shown support for this hypothesis. In the first, 106 patients diagnosed with BV were randomized to receive metronidazole and either lactobacilli or placebo. Thirty-day follow-up showed significantly improved cure of BV with probiotic supplementation (*Anukam* et al., 2006a). The precise mechanisms were not investigated in the trial, but subsequently, it has been shown that the lactobacilli are able to resist metronidazole and even grow in its presence (Anukam and Reid, 2008). A second study, this time performed on 64 women in Brazil, showed almost identical findings, whereby use of *Lactobacillus* GR-1 and RC-14 improved cure of BV in patients treated with 2g tinidazole (88% versus 50%) (*Ruiz Martinez* et al., 2009b). This is the first series of studies to show augmentation of antimicrobial cure, and it provides hope that the longevity of antibiotics and antifungals usefulness may be extended by adding probiotics. Most importantly, given the adverse effects of urogenital health on quality of life (*Ellis* and *Verma*, 2000; *Lowe* and *Ryan-Wenger*, 2003), any supplemental or alternative approaches must be welcomed.

Regulatory agencies do not extend the approval or food or dietary supplements to applications other than oral. Thus, the insertion of a *Lactobacillus* capsule into the vagina constitutes a drug therapy. Previous studies showed the potential for intravaginal use of lactobacilli (*Reid* et al., 1995; *Cadieux* et al., 2002), so in order to determine if *Lactobacillus* GR-1 and RC-14 have the ability to cure BV, a study of 40 women was undertaken in which they inserted lactobacilli capsules vaginally for five days. At 30-day follow-up, the cure rate with the probiotics was superior to using metronidazole on its own (*Anukam* et al., 2006b). A larger trial is warranted, but the signs are very encouraging that probiotic lactobacilli have the potential in at least this application, to replace antibiotics to cure an infection.

A misconception with lactobacilli in the urogenital tract is that they should be used to treat VVC, because this infection is caused by loss of lactobacilli. In fact, this is not the case, and lactobacilli are often present when VVC arises, and there is little evidence to indicate that lactobacilli alone can cure VVC. Nevertheless, we explored the question of whether *Lactobacillus* GR-1 and RC-14 could augment the cure of VVC by antifungal. In a randomized, placebo controlled study of 68 women diagnosed with VVC, a single dose of fluconazole (150mg) was administered supplemented every

morning for the following four weeks with either two placebo or two probiotic capsules containing *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14. At four weeks, the probiotic treated group showed significantly less vaginal burning and itching (11.8% versus 32.4%; p=.04), lower presence of yeast detected by culture method (14.7% versus 38.2%; p=.03), and less vaginal discharge (14.7% versus 44.1%; p=.008) (*Ruiz Martinez* et al., 2009a). The mechanisms of action have to be determined, but is not likely associated with H_2O_2 (*Ruiz Martinez* et al., 2008), and may comprise increased displacement of the yeast by the lactobacilli as shown *in vitro* (*Velraeds* et al., 1998; *Koehler* and *Reid*, 2006), reduced re-ascension of yeast from the rectum to vagina (*Reid* et al., 2003), or up-regulation of antifungal host defences (*Kirjavainen* et al., 2008).

CONCLUSION

The use of lactobacilli for urogenital health in women requires careful consideration of the strains to be used, the properties they confer, how they are manufactured, stored and delivered, and clinical evidence must be obtained that they are beneficial before they should be termed probiotic. The success of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 demonstrates for the first time that this approach to restoration and retention of a healthy vaginal microbiota is possible. As more genomic and functional data become available on these strains, we will better understand how they work, and under which circumstances. Other strains will also become available, possibly selected for different scientific or clinical reasons. As long as women are the beneficiaries of such probiotic regimens, we will have achieved a laudatory goal as scientists.

ACKNOWLEDGEMENTS

Our research is supported by NSERC, AFMnet, and the Ontario Neurotrauma Foundation.

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