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THE ROLE OF THE VAGINAL FLORA IN HEALTH AND DISEASE

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SUMMARY

The urogenital tract of females is an important microenvironment which, for the most part, is free from infection for much of a person's life. While the introduction of sexually transmitted pathogens is an obvious means to give rise to infection, most cases occur due to organisms originating in the host. Three diseases, bacterial vaginosis (BV), candidiasis and urinary tract infections (UTI), cause many hundreds of millions of infections in the world's female population each year. Depending upon access to proper health care, cures can be achieved through use of antibiotics and antifungals. However, progress needs to be made to better understand how these infections develop. Given the microbial biofilms that exist in the vagina and the 50 or so species of microorganisms that inhabit this niche, the environment is complicated and ever changing. Bacterial interactions, symbiotic or otherwise, and external factors such as antibiotics, spermicides and hormones, can alter the composition and structure of the biofilm. With respect to key organisms which confer a degree of protection against pathogens, lactobacilli have been identified because of their prevalence and ability of some to express factors which inhibit pathogen adhesion and growth. If this knowledge is to be translated into therapeutic regimens, lactobacilli strains must be studied genetically, phenotypically and tested in the clinical setting. The opportunity to restore and maintain a normal flora using exogenous lactobacilli and by stimulating endogenous strains via perbiotic nutrients, is certainly worthy of exploration.

INTRODUCTION

The female urogenital tract is an orifice which is critical to reproduction and to disposal of urinary secretions. The outer skin region comprises the perineum or vulva inside which is the vaginal introitus and urethral opening. In close proximity is the anal opening to the intestine. This milieu is the habitat for a range of microbial species whose emergence comes via the intestine and food intake, the skin and through transfer from a male partner via sexual intercourse. Such a general and basic description, to some, may not appear necessary, but I believe it is critical if we are to truly understand the role of this complex flora in health and disease. While much emphasis has been placed upon the study of pathogens and their virulence factors, comparatively little is

known about the process of health restoration. Some fundamental questions remain to be answered: what is the "normal" flora, how do pathogens survive and emerge to infect the host, what role do the organisms originating in the urogenital tract play in human diseases?

This article will focus on the urogenital flora, particularly the vaginal flora of adults, and how it is influenced by factors other than sexually transmitted bacteria and viruses. The exclusion of sexually transmitted diseases should not be viewed as de-emphasising the enormity of the problem which afflicts hundreds of million women around the globe. The types of complications which result from an imbalanced flora will be discussed with primary focus on urinary tract infections (UTI), bacterial vaginosis (BV) and candidiasis.

WHAT IS NORMAL?

The urogenital flora is established at birth by contact with the mother's vagina at delivery and by intake of milk. This flora appears to be dominated by bifidobacteria and lactobacilli when the new-born receives human milk, and by *Enterobacteriaceae* and streptococci and *Bacteroides* in bottle fed infants (*Edwards*, 1993). The organisms which colonise at birth are referred to as primary and secondary colonisers. As the child grows to puberty, there is an association between oestrogen levels and lactobacilli. During a woman's reproductive phase, lactobacilli are at their peak counts in the vagina. Upon reaching menopause, it has been assumed that lactobacilli were depleted or absent, but a recent study of 73 women has shown that 49% have lactobacilli counts of 100,000 CFU/g of vaginal fluid (*Hillier* and *Lau*, 1997). Many dietary factors influence the colonisation of the intestine, such as casein/whey and phosphate content, oligosaccharides, lactoferrin, iron and proteins and indeed many of these components have now been included in artificial milk formulas. Furthermore, factors as yet unknown, based upon ethnicity of women, appear to influence vaginal colonisation such that higher levels of potential pathogens have been found in black women and lowest levels in Asian-Pacific Islander women (*Goldenberg* et al., 1996a). The intestinal microbial biofilms act as the major source of organisms which colonise the urogenital tract, thus making it important to better understand biofilm structure and function.

Importance of the biofilm concept in understanding the flora

The microbial biofilm is, I believe, a critical component in the balance between healthy or disease in the urogenital tract. Biofilms are composed of organisms in single and multiple layers surrounded by microbial and host matter particularly polysaccharides, in a structure that is invariably adherent to a surface. The first step in biofilm formation is the deposition of a host conditioning film onto cell surfaces. Vaginal epithelial cells are coated in conditioning films containing mucopolysaccharides, glycoproteins and other substances which can act as receptors for microorganisms. For example, Tamm Horsfall protein (THP) present in human urine and likely present in the vagina due to bathing of the area with urinary fluids, acts as a substrate for *E. coli* strains with mannose sensitive adhesins (*Hawthorn* et al., 1991). Another example is an association between elevated foetal fibronectin and BV (*Goffeng* et al., 1997; *Goldenberg* et al., 1996b) implying perhaps that BV organisms bind to the fibrinectin better than lactobacilli whose

numbers subsequently become reduced in the vagina of these patients.

Organisms first adhere to surfaces (including other bacteria already present on a vaginal cell) via physicochemical interactions described in the Derjaguin Landau Verway and Overbeck (DVLO) theory of colloidal stability (*Derjaguin* and *Landau*, 1941; *Verwey* and *Overbeek*, 1948). Basically, the theory describes two boundaries, the primary and secondary minima, which an organism must overcome on its approach towards a surface. At the secondary minimum, separation distances of >50 nm only attractive van der Waal's forces occur (*Busscher* and *Weerkamp*, 1987). At the primary minimum 10-20 nm both van der Waal's and repulsive electrostatic interactions (charge, hydrogen bonding and hydrophobicity) influence adhesion. Irreversible binding can occur when the separation distance is down to <1.5 nm and receptor-adhesin lock and key mechanisms are present. The practical issue here is to illustrate the complicated process of adhesion and the number of ways in which organisms can colonise a surface. Furthermore, it is easy to imagine how fluid alterations (because of hormonal and mucus changes, pH fluctuations, exposure to antimicrobials and semen etc.) can influence these interactions. Having stated that, it is quite remarkable that only about 50 species of organisms colonise the urogenital tract from an intestinal reservoir which contains over 400 species and a pool of 10^{14} bacterial cells (*Tancrede*, 1992). Therefore, there must be a degree of specificity involved in the colonisation and biofilm process.

Once adherent, the organisms growth and multiply and become integrated into a multi-species biofilm. In healthy reproductive aged women, this biofilm is dominated by lactobacilli, while in patients with BV, it is dominated by Gram negative rods, as will be discussed later. The structure of the biofilm is dynamic. While few ultrastructural studies have been carried out on the vaginal flora (*Sadhu* et al., 1989), much progress has been made in understanding biofilms. There is evidence from confocal scanning laser microscopy and differential interference contrast microscopy (*James* et al., 1995; *Sanford* et al., 1996; *Suci* et al., 1997) to show that they can have mushroom-like forms, separated by interstitial spaces filled with surrounding fluid (*Lawrence* et al., 1991; *Lewandowski* et al., 1995). The structure includes a linking film to the surface and a bulk area which can host anaerobic organisms (*Reid* et al., 1998b). The outer layer of the biofilm is the area exposed to host defences, antimicrobials and bacteria entering the system. There is now evidence from cell-to-cell studies which shows that bacteria within biofilms communicate with each other through quorum sensing signalling (*Davies* et al., 1998; *Kolter* and *Losick*, 1998). This might, in part, explain the ability of biofilms to alter metabolic functions and resist antibiotic and host defence attack.

Another feature of biofilms is symbiosis whereby the presence of one species positively affects that of another: for example the growth of BV organism *Prevotella bivia* leads to ammonia production which is utilised by *Gardnerella vaginalis* (*Pybus* and *Onderdonk*, 1997). Also, *P. bivia* produce sialidases which destroy mucins and enhance adherence of other BV organisms and impair the specific immunologlobulin A against the cytotoxin of *G. vaginalis* (*Cauci* et al., 1998).

Based primarily upon *in vitro* data, it is known that many interbacterial interactions take place. Lactobacilli have been shown to produce acids, bacteriocins, biosurfactants and hydrogen peroxide which effect the ability of potential pathogens to adhere, grow and dominate the flora (*Klebanoff* et al., 1991; *Reid* et al., 1998b). However,

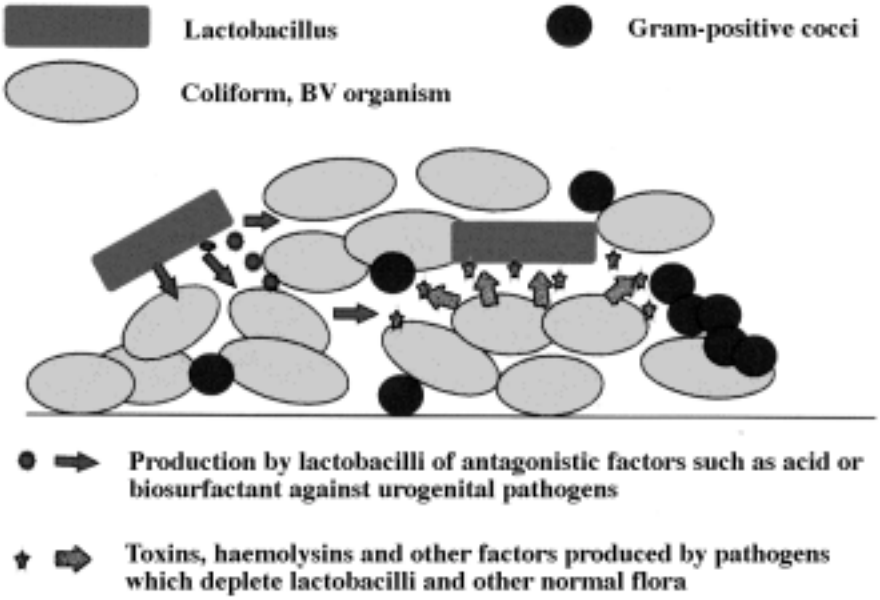

Figure 1: Microbial interactions within the urogenital flora.

there is significantly less evidence of this occurring *in vivo* and studies of this nature are needed.

What factors can influence the flora?

It has been assumed, and occasionally supported by scientific evidence, that urogenital disease occurs because pathogens express certain key virulence factors, such as toxins or haemolysins (*Hughes* et al., 1983; *Cauci* et al., 1996) (Figure 1). While such factors damage the host, the producers have to first to colonise the host (or at least find their way to bare cell surfaces) and presumably resist the competition of the existing flora (Figure 1). In the case of BV, the result is a significant depletion of lactobacilli and substantial increase in numbers of *Gardnerella* and other species causing the disease. Thus, there are competitive bacterial factors which over-ride the normal flora. One such factor is bacteriophage which could kill lactobacilli cells and thereby reduce their colonisation levels (*Kilic* et al., 1996). The extent of phage presence in the vagina and the ability of lactobacilli to resist their action is presently unstudied. Techniques now exist to label phages and follow their infection of bacterial cells within biofilms using scanning confocal laser microscopy (*Doolittle* et al., 1996) and such analysis would be very interesting in relation to urogenital biofilms.

External factors such as spermicidal agents, used more frequently because they can reduce the risk of STDs, deplete the lactobacilli flora especially strains producing hydrogen peroxide (*McGroarty* et al., 1990; 1992; *Cook* and *Rosenberg*, 1998; *Hira* et al., 1997). The effects of semen, douching and the use of oral contraceptives are less clear with respect to the vaginal flora. Certainly, semen increases vaginal pH, thereby reducing the effectiveness of acidity which kills viruses and inhibits the growth of some bacteria (*Kempf* et al., 1991; *Reid* and *Bruce*, 1995). There is new evidence to indicate that uropathogenic *E. coli* can be trans-

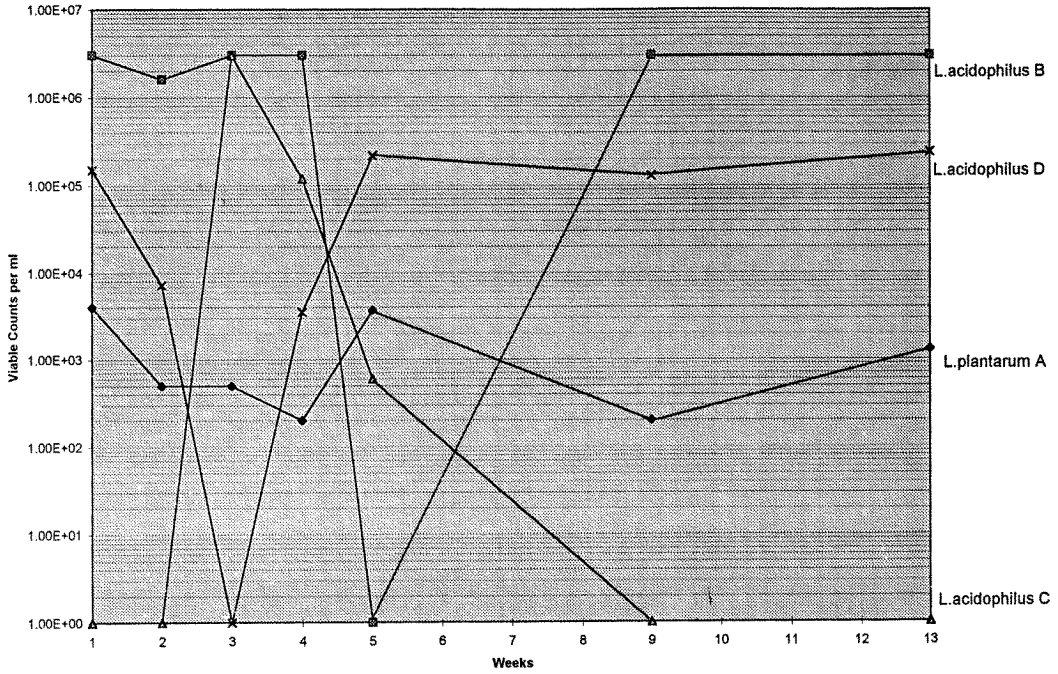

Figure 2: *Lactobacillus* strains from vagina.

mitted sexually (*Foxman* et al., 1997), making the recurrence of UTI a much more complicated scenario to understand and treat effectively.

Micturition has been shown to alter the flora on an hourly basis (*Seddon* et al., 1976), while reduction in oestrogen levels at menopause leads to lower lactobacilli counts and changes to the mucosal environment (*Raz* and *Stamm*, 1993).

The insertion of tampons, diaphragms and intrauterine devices (IUDs) can also disrupt the microflora. The classic example was the use of highly adsorbant tampons to which *Staphylococcus aureus* strains producing toxic shock toxins, attached and caused morbidity and mortality (*Berkley* et al., 1987). It is known that pathogen adhesion to IUDs can be associated with infections, however, when lactobacilli are the dominant organisms on IUDs, infections need not arise (*Reid* et al., 1988).

Once a urogenital infection ensues, the use of antimicrobial agents depletes the normal flora as well as the pathogens and thereby allows new primary and secondary colonisers to take over the microenvironment. The likelihood is that these will include drug resistance organisms, faster growing bacteria than the more fastidious lactobacilli, and intestinal coliforms. Antimicrobial agents are present in many food substances, and therefore the host is likely being exposed to their adverse effects on flora, on a long term basis.

The nutritional environment is clearly important for survival of members of the flora. Studies carried out on the colonic microflora showed conclusively that nutrient modulation can alter the composition of the flora (*Gibson* and *Roberfroid*, 1995). These studies have given rise to the concept of prebiotics which selectively modulate growth of non-pathogens in favour of pathogens. Recently, it has been shown that pre-

biotic compounds can influence the lactobacilli component of the vaginal flora without stimulating pathogens (*Reid* et al., 1995; 1998a). Certainly oligosaccharides have been shown to have utility in altering the flora composition of the intestine (*Tomomatsu*, 1994).

Another interesting feature of the dynamics of the microflora is that some strains appear to remain for a long time while others are transient. This has been shown in the intestine (*McCartney* et al., 1996) and more recently in the vagina (Figure 2). The latter findings as well as unpublished data from our laboratory, shows that new lactobacilli strains ascend into the vagina from the intestine. Perhaps the inability of some new strains to remain in the vagina reflects an inability to integrate into existing biofilms: if that holds true, it has implications for selection of probiotic strains that can survive long after insertion.

WHY ARE LACTOBACILLI IMPORTANT IN HEALTH MAINTENANCE?

There is now extensive evidence to support the claim that lactobacilli can be critical in the host's defence against urogenital infection (*Elmer* et al., 1996; *Hamilton-Miller*, 1997; *Hilton* et al., 1995; *Reid* et al., 1995; *Raz* and *Stamm*, 1993). In order to mimic the effect of the naturally occurring flora, it would appear to be rational to select strains with appropriate characteristics necessary to protect the host from infection. Some characteristics believed to be critical have been identified as early as 1984 (*Chan* et al., 1984; *Reid* et al., 1987, 1992; *Klebanoff* et al., 1991; *Velraeds* et al., 1996). The choice of strain type is perhaps less important although it should be from a species commonly recovered from the urogenital tract (*Reid* et al., 1996). The importance of strain selection has been highlighted by the finding that contents of health food products can be less than reliable (*Hughes* and *Hillier*, 1990) or with strains not named on the label (*Zhong* et al., 1998); very few are based upon lactobacilli expression of factors proven to be protective to the host.

Of all the characteristics which is the most critical? Arguments can be made for adhesion to be important, but factors which compete against other colonising organisms are also likely to be critical for health maintenance. The fact that strains possessing certain characteristics are present even in patients with recurrent infections, would imply that they are not vital in disease reduction. But some controversies exist, for example with hydrogen peroxide. Some studies show a correlation between H_2O_2 and prevention of BV (*Hawes* et al., 1996) while others show that H_2O_2 producers did not appear to protect against BV, UTI, vaginal candidiasis or trichomoniasis (*McGroarty* et al., 1992; *Rosenstein* et al., 1997).

In order to reduce the risk of UTI, it appears that a collection of factors are required including adhesion, production of growth and adhesion inhibitors and an ability to co-aggregate and form a balanced flora. Given the enormity of the microbial competition and the complexity of the host, it is likely that more than one lactobacilli strain will be required to maintain a normal flora.

A European based product, Gynoflor, reported to contain 50 mg of viable, H_2O_2-producing *L. acidophilus* and 0.03 mg oestriol has been tested on non-menopausal women with BV (*Parent* et al., 1996). This design is unusual in that it applies oestrogen to women who have no evidence of reduced oestrogen levels, and it uses

probiotics to actually treat infection rather than prevent recurrences. Six days of therapy with 1-2 vaginal suppositories per day gave a cure rate two weeks out of 77% in the treatment arm and 25% in placebo. The lactobacilli counts increased after treatment, although no DNA probe verification of the strain was used to confirm that it was the colonising organism. Nevertheless, the approach was successful. Likewise in a Japanese study of 11 women aged 20 to 60 years, intravaginal treatment with 5 ml commercial yoghurt reduced vaginal redness, lowered pH and caused a bacteriological cure (*Chimura* et al., 1995). Again, this is not a full proof study and it fails to provide light on mechanisms of action and properties of the probiotic strains (assuming that was what cured the patients), but it is another encouraging sign that this approach to therapy might work.

Delivery of lactobacilli to prevent urogenital infections has primarily involved direct insertion of the organisms into the vagina. However, there is new evidence to indicate that under certain conditions, it is possible to deliver probiotics to the vagina via oral intake (*Reid* et al., unpublished observations). Currently, the use of lactobacilli is arguably the best potential option to antibiotics for prophylaxis of UTI, but some vaccines are in development. A FimH-adhesin-based vaccine, delivery by systemic injection in animals shows promise (*Langermann* et al., 1997) but successful transfer to humans is a long shot and even then the limited target of type 1 piliated *E. coli* makes its' widespread use unlikely.

With respect to bacterial vaginosis, this is certainly a disease which affects a large population. It is defined as a mild infection of the lower female genital tract, characterised by the presence of three of five criteria: release of an amine (one or more of putrescine, cadaverine and trimethylamine)(fishy), release of an amine odour after addition of 10% potassium hydroxide, a vaginal pH greater than 4.5, clue cells in the vaginal fluid, and a milky homogenous vaginal discharge (*Amsel* et al., 1983; *Hillier*, 1993). The examination of clue cells consists of scoring the cell population as to being normal (0 to 3) and dominated by lactobacilli rods, intermediate (4 to 6) with colonisation by small gram-negative or gram-variable rods (*Gardnerella, Bacteroides* and possibly *Fusobacterium, Prevotella, Peptostreptococcus, Porphyromonas* and *Mycoplasma* species) and curved Gram-variable rods (*Mobiluncus*), and BV (7 to 10) with domination by the pathogens (*van der Meijden*, 1984; *Westrom* et al., 1984; *Spiegel* et al., 1980; *Cook* et al., 1989; *Nugent* et al., 1991; *Hillier*, 1993; *Holst* et al., 1994; *Rosenstein* et al., 1996).

There is a clear association between BV, reduced urogenital lactobacilli and increased risk of sexually transmitted diseases, including AIDS (*Biro* et al., 1995; *Cohen* et al., 1995; *Nilsson* et al., 1997; *Sewankambo* et al., 1997; *Paige* et al., 1998), and increased risk of preterm labour (*Chaim* et al., 1997; *Hillier* et al., 1995a; 1995b).

With respect to otherwise healthy pregnancy, the prevalence rates vary between 4.9% to around 7.5% (*Cristiano* et al., 1996; *Fan* et al., 1997; *Martinez de Tejada* et al., 1998) and perhaps vary between different socioeconomic populations and countries (*Llani-Camp* et al., 1996), such as Papua New Guinea where a prevalence of 23% has been reported (*Klufio* et al., 1995). A Danish study of 3,596 pregnant women showed a strong association between BV and *Gardnerella vaginalis, Mycoplasma hominis* and other anaerobes plus a depleted lactobacilli presence (*Moller*, 1998). Indeed, 59.6% of BV cases were associated with a combination of *G. vaginalis* and *M. hominis* again implying some sort of

symbiotic association.

In another study of 18 women over a 10 month period, BV was found to follow candidiasis in 9 of 11 episodes, to occur most often around the time of menstruation, and to resolve spontaneously in mid-cycle after unprotected sexual intercourse (Hay et al., 1997). These findings could be regarded as somewhat controversial and without a rationale, scientific explanation. They remain to be confirmed by others.

If an existing BV, isolated to the vagina, was solely responsible for increasing the risk of preterm labour, it would be expected that treatment of BV would have a favourable outcome on the pregnancy. This can occur (*Steele*, 1996) but it is not necessarily the case, in that metronidazole and clindamycin therapy for BV has been found not to reduce the preterm birth rate (*Joesoef* et al., 1995; *McDonald* et al., 1997). Failure, it could be argued, is due to the infecting bacteria having already ascended into the uterus and infected the foetus or endometrium: there is evidence to support this theory (*Hillier* et al., 1995a; 1996; *Peipert* et al., 1997; *Sweet*, 1995). Interestingly, a study which instilled a single 3% solution of H_2O_2 into the vagina of 23 patients with recurrent BV showed complete clearance of symptoms in 78%, thereby implying that this compound was important in prevention of BV (*Winceslaus* and *Calver*, 1996).

The presence of BV in pregnancy has been found to stimulate inflammatory cytokines in the vagina, in particular interleukin-1 beta (*Imseis* et al., 1997) although the role of these factors in pathogenesis, recurrence and onset of preterm labour remains to be clarified.

With respect to recurring attacks of *Candida* vaginitis, there does not appear to be an association between lactobacilli counts and infection risk (*Sobel* and *Chaim*, 1996). This finding does not rule out an association between onset of candidiasis because of a disrupted flora, nor an ability of lactobacilli with anti-*Candida* properties being able to reduce the risk of infection.

LACTOBACILLI AS THERAPEUTICS

The use of lactobacilli as a mono or mixed culture of live organisms, delivered as dried cells, a fermented product of douche, has been shown to benefit the host and is therefore an excellent example of a probiotic (*O'Sullivan* et al., 1992). As these authors illustrate, there are many so-called probiotic bacteria in commercial products, particularly in Europe and indeed an estimated 90,000 tons of probiotic yoghurt is produced in France each year. However, none of these probiotic organisms have been tested or shown to have any effect in maintaining a healthy urogenital tract which has a reduced risk of UTI. As the evidence provided above indicates, given the proper selection of lactobacilli strains, such therapeutic benefits are certainly possible (*Bruce* and *Reid*, 1988; *Bruce* et al., 1992; *Reid* et al., 1995).

The development of molecular biology tools has impacted upon lactobacilli, providing the potential to create strains which possess added probiotic properties, such as nisin a posttranslationally modified antimicrobial peptide widely used in the food industry as a preservative. Nisin-inducible expression cassettes have been transferred from the producer, *Lactococcus lactis* to *Lactobacillus helveticus* (*Kleerebezem* et al., 1997). In another example, studies have shown that lactobacilli have the potential to be a carrier for oral and perhaps vagi-

nal immunisation, say against *Chlamydia*, because of their adjuvanticity (*Pouwels* et al., 1996). This latter work has immense potential because lactobacilli are naturally occurring and they maintain viability in the vagina. On the negative side, systems will have to be put in place to control or shut down expression of antigens so as not to create the wrong type of host immune response such as anaphylaxis. In the end, it will be government regulatory agencies who will dictate the impact of some of these new developments. For now, most are unlikely to approve use of recombinant organisms and strains possessing plasmids for probiotic application to the human vagina.

The use of prebiotics, functional foods or health supplements, on the other hand, do have a real chance of being approved and made available. The opportunity will be to find the substances and strains of greatest effect in the urogenital tract. With market projections of US $ 100 billion by 2010, (*Stanton*, 1998) and increased scientific interest in the field, there should be an accelerated emergence of natural remedies for the healthy maintenance and restoration of the urogenital tract. Hopefully, this will be led by selection of organisms with proven scientific based characteristics and clinical efficacy.

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