Biophysical Interactions of vaginal microorganisms

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Chapter 1.1

General Introduction

Microbiota Restoration: Natural and Supplemented Recovery of Human Microbial Communities

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Abstract

In a healthy host, a balance exists between members of the microbiota, such that potential pathogenic and non-pathogenic organisms can be found in apparent harmony. During infection, this balance can become disturbed, leading to often dramatic changes in the composition of the microbiota. For most bacterial infections, relatively non-specific antibiotics are used, killing the pathogens as well as non-pathogenic members of the microbiota and leading to a substantial delay in the restoration of a healthy microbiota. However, in some cases, infections can self-resolve without the intervention of antibiotics. In this review, we explore the mechanisms underlying microbiota restoration following insult (antibiotic or otherwise) on the skin, oral cavity, gastrointestinal and urogenital tracts, highlighting recovery by natural processes and following probiotic administration.

Introduction

Humans live in symbiosis with a diverse community of microorganisms, the composition of which has evolved to perform many specific tasks that benefit the host, as well as to survive and thrive in sites that provide these microorganisms with a suitable nutrient-filled habitat. The task of identifying specific roles for these 100 trillion symbionts, whose gene pool far exceeds that of their host, is currently elusive, but the composition of this microbiota is now being deciphered.

As far as can be determined, before birth the body is sterile. However, during and following birth the body becomes colonized by a vast array of microorganisms that originate from the mother's birth canal, from the living environment and from handling by other individuals. A recent study of vaginally- and Caesarian-delivered babies showed that despite highly differentiated maternal communities, the neonate microbiota were undifferentiated across multiple body habitats, regardless of delivery mode[1]. Vaginally delivered babies acquired bacterial communities resembling their own mother's vaginal microbiota, dominated by *Lactobacillus*, *Prevotella*, or *Sneathia* spp., whereas infants delivered by Caesarian harbored bacterial communities that were more similar to the general skin surface and dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. Furthermore, the composition of the microbiota can differ between infants that are fed with either breast milk or formula[2], however, it seems that after weaning there is a degree of maturation and homogeneity of the microbiota[3]. Indeed, a study of the gut microbiota of twins has identified a 'core microbiome' with a wide array of microbial genes that are shared from individual to individual, although with substantial diversity in bacterial lineages[4]. This functional homogeneity is surprising given the infant's primary colonizing microorganisms, nutrient intake and macro-environment.

The extent to which such differences in the infant microbiota influence health later in life will also be of great interest. For instance, it is known that bacterial strains can prime the immune system and alter susceptibility to respiratory ailments and metabolic syndrome[5-7], while strains embedded in the microbiota, such as *Clostridium spp.*, uropathogenic

Escherichia coli, or perhaps sulfate-reducing bacteria, might later be associated with disease[8-10]. The crucial part played by the microbiota in human health has only relatively recently become widely recognized, in large part because of studies that have led to, and stemmed from, the Human Microbiome Project[19].

The human microbiota

To date, the most comprehensive analysis of the human microbiota looked at 27 distinct sites on the body and revealed the presence of 22 bacterial phyla, with most sequences (92.3%) related to just four phyla: *Actinobacteria* (36.6%), *Firmicutes* (34.3%), *Proteobacteria* (11.9%), and *Bacteroidetes* (9.5%)[11]. These sites included the oral cavity, gut and skin (three of the four areas discussed in this review). Each habitat harbored a characteristic microbiota and a relatively stable set of abundant taxa between individuals and over time (Figure 1), though the rare taxa varied immensely. Although there are differences in the abundance of phyla between sites, such as sebaceous, moist and dry skin sites colonized by *Actinobacteria (Corynebacterium, Propionibacterium, Microbacterium, Micrococcus*), *Firmicutes* (*Staphylococcus, Clostridium*), *Proteobacteria* (*Pseudomonas, Janthinobacterium, Serratia, Halomonas, Stenotrophomonas, Delftia, Comamonas* genera) and *Bacteriodetes* (*Sphingobacterium, Cryseobacterium*)[12,13], the distributions are collectively much more similar than those found in the gut or vagina. In fact, the variation in microbial community constituents between body sites are much greater than those between different free-living communities (e.g. soil and water) [11]. Indeed, bacterial distributions differ between the tongue and

faeces far more than between faecal samples from different individuals[14], and samples from the same individual taken three months apart, though dissimilar, resemble each other more than samples from different individuals.

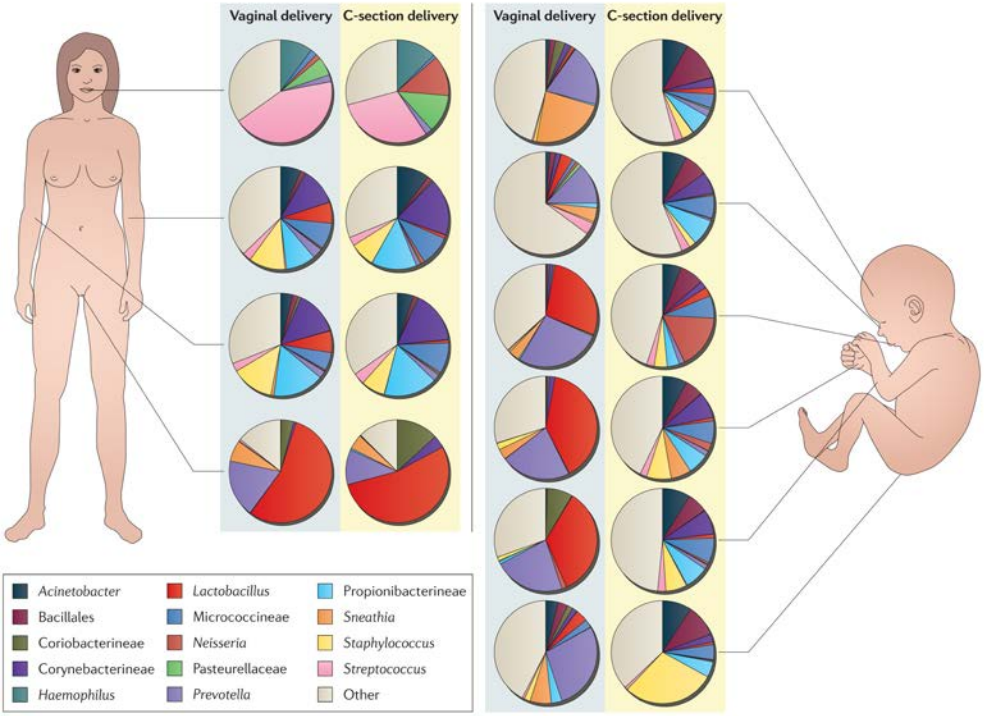

Figure 1 | Taxonomic distribution of microorganisms in mother and baby.

Immediately after birth, humans become colonized by a range of microorganisms, particularly on the skin, oronasopharyngeal area, gastrointestinal tract and urogenital tract. The host and its microbiome have developed mechanisms to not only protect against infection, but also restore microbial homeostasis, even without pharmaceutical therapy. The genus-level distributions are shown for a sample of mothers and their babies, grouped according to the delivery method of the babies (vaginal or by Caesarian section (C-section)). The data shown are averaged for each group. Data from Reference 1.

Studies on the vaginal microbiota show a different pattern; at this site the bacterial type[15] and abundance[16] in the microbial community can change rapidly in a matter of weeks and sometimes days. In addition, differences in vaginal microbiota exist between individuals, even those who are reportedly healthy[17]. In ecological terms, a more diverse microbiota with adequate nutrients would be expected to equate to a more stable ecosystem[18], although relationships between diversity and stability have long been debated in the ecological literature. The vagina provides a marked contrast: here, increased diversity, especially decreased dominance of lactobacilli, equates with an aberrant microbiota and infection[16].

Natural and artificially aided restoration

The past sixty years have seen the introduction of antibiotics to treat bacterial infections. Yet, before and since this era, countless individuals have recovered from dysbiosis and infection through mechanisms not yet fully understood. With dwindling antibiotic options, and growing bacterial resistance, it is timely to examine the mechanisms of natural restoration of eubiosis, with a view to developing new methods to prevent and cure disease. Furthermore, the recognition that antibiotics also eradicate non-pathogens and disrupt the natural microbiota for some time after their administration[21,22] has been one reason for the growing interest in administering beneficial microorganisms (probiotics) to aid in recovery from infection[23-25]. In this review, we examine how indigenous microorganisms and probiotics are able to help the host overcome infection and restore the microbiota to a state associated with health. Four body sites, the skin, oral nasopharyngeal cavity, gastrointestinal and vaginal tracts, will be discussed

(Figure 2), as the complex microbial communities that occupy these sites carry a broad range of functions indispensable for human health, while at the same time being constantly challenged by potentially pathogenic organisms.

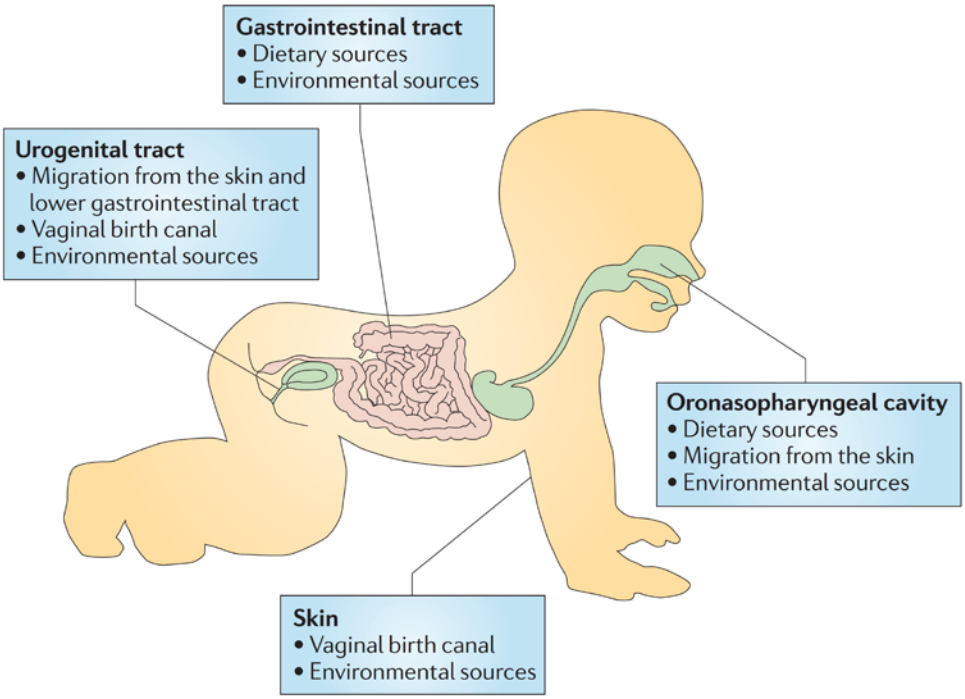

Figure 2 | The sites discussed in this review.
The figure illustrates the main areas discussed in this review that are colonized by bacteria, and for which there is some evidence for natural restoration after pathogen insult.

Perturbation of the human microbiota

The ability of microorganisms to selectively colonize a niche reflects their evolutionary adaptation. In many instances, it is when an organism from one niche migrates to another that problems for the host ensue. For

example, uropathogenic *E. coli* strains emerge from but do not infect the gut en route to infecting the bladder. The switch from homeostasis to a disease state can occur through various means, including physical insult to the mucosa, disruption of the indigenous microbiota by the use of antimicrobials, expression of specific virulence factors, and access to the site by excessive numbers of pathogens.

Microorganisms at wound sites

The main functions of the skin are to provide mechanical strength, regulate water and salt loss and protect the body from environmental damage, including that caused by microorganisms. The outermost layer of the skin, the stratum corneum consists of corneocytes surrounded by lipid regions containing long chain ceramides, free fatty acids and cholesterol[26]. Interspersed among keratinocytes in the epithelium are specialized antigen-presenting Langerhans cells, of the dendritic cell family, which serve as sentinels of the immune system by sampling antigens that pass through the stratum corneum. These cells then migrate to draining lymph nodes and relay antigenic information to the adaptive immune system[27]. Diseased skin is often characterized by a reduced barrier function and an altered lipid composition and organization[26]. Although some *Staphylococcus* species, such as *Staphylococcus epidermidis,* are consistently found on the skin surface, other species such as *Staphylococcus aureus* may only transiently colonize intact skin. However, when this surface is penetrated both species can colonize the damaged area to exploit the nutrient-rich bloodstream[28]. Staphylococci cause a range of cutaneous and systemic infections, including impetigo, furuncle, subcutaneous abscess, staphylococcal scalded skin

syndrome, toxic shock syndrome and neonatal toxic shock syndrome-like exanthematous disease. The virulence factors produced by *S. aureus* can disrupt the epithelial barrier, inhibit opsonization by antibody and complement, interfere with neutrophil chemotaxis, cytolyse neutrophils, and inactivate antimicrobial peptides[29]. In a recent study of skin wounding in a diabetic mouse, there was a selective shift noted in colonizing bacteria accompanied by transcriptional changes indicative of mobilized defence and immune responses[30]. After wounding, there was a shift towards an increased abundance of *Firmicutes* species (including *Staphylococcus*) along with a prolonged immune response.

Skin can be damaged by burns owing to fire, heat/cold, electricity, radiation, or caustic chemicals. The extent of the burn is measured by the depth to which the skin layers are damaged. Partial thickness burns (first and second degree) usually heal well because new skin can grow upward from the dermis. However, when the dermis is destroyed (full thickness or third degree burn), skin growth is repudiated and deep scarring develops. In the case of heat causing partial thickness damage, some of the microorganisms on the skin are likely to survive, and, during handling of the patient, other microorganisms will contaminate the site, providing them with access to the blood through the permeated barrier. Burn infections are relatively common, with *S. aureus* the most common pathogen, followed by *E. coli, Pseudomonas aeruginosa*, coagulase-negative staphylococci, and a range of aerobic and anaerobic pathogens[31,32]. During the initial 48 hours following injury, Gram-positive bacteria heavily colonize the burn wound. By 5–7 days, a shift occurs with Gram-negative opportunists, armed with invasive and virulent properties, superseding the initial colonizers[33].

Antibiotics are often administered locally with a view to reducing the pathogen count and preventing infection.

The ability of the non-pathogenic indigenous microbiota to outcompete the pathogens and restore bacterial and immune homeostasis (defined in this case as maintaining stability associated with health of the host) to burn wound sites has not been studied to our knowledge. But, given the competitive nature of indigenous organisms, and the availability of progeny that survive at the burn site and that are present on adjacent skin, it is reasonable to suggest that the indigenous microbiota may help prevent infection in many instances, assuming that immune defences are intact.

The process of wound healing necessitates repair of the skin barrier and revascularization, as well as a return of normal immune function. It is not uncommon for wounds to become with persistently infected with *Bacteroidales, Corynebacterium* spp*., Peptoniphilus* spp., *Staphylococcus* spp., *Serratia marcescens, Prevotella* spp. and *P. aeruginosa*[32]. The persistence of these infections can have several causes, including the ability of the organisms to form biofilms[34]. However, in most otherwise healthy people, skin repair mechanisms allow the reparation to be completed, where after the indigenous skin microbiota are restored to the surface. To better understand the natural restoration process and determine the role, if any, of non-pathogenic microorganisms in this process, further studies are warranted to examine sequential changes in the skin microbiota after insult and during healing.

Recovery following antibiotic therapy

In the vagina, the microbial profile can change quite rapidly and extensively from one in which *Firmicutes*, especially lactobacilli, dominate to one with high abundance of *Bacteroidetes* and *Actinobacteria* and an aberrant condition termed bacterial vaginosis (BV) (Figure 3). BV occurs in an estimated one-third of women at any given time, with rates over 70% in sex workers and HIV infected individuals[35-37]. In about one third of cases, BV is accompanied by vaginal discomfort and homogeneous, malodorous vaginal discharge. It is diagnosed most commonly by one of two methods. The Amsel criteria requires three out of the following four parameters: homogenous, milky discharge; vaginal pH greater than 4.5; presence of "clue cells" on microscopic examination of a vaginal smear; and/or positive amine or "Whiff" test[38]. The Nugent scoring system involves performing a Gram-stain on a vaginal smear and enumerating lactobacilli versus Gram-negative rods and other bacterial morphotypes[39]. Irrespective of symptomatology, BV has been associated with an increased susceptibility to preterm labour perhaps owing to inflammatory processes[40], sexually transmitted infections including HIV infection, perhaps owing to a damaged epithelial layer or altered expression of protective compounds like elafin[41,42], and pelvic inflammatory disease caused by pathogens[43].

When treated with the nitroimidazole antibiotic metronidazole, the microbiota recovers to a state whose composition is close to, but not necessarily exactly the same as the healthy, pre-infection state, even up to 25 weeks later (Figure 3). Notably, the lactobacilli abundance increases with recovery, coinciding with a decrease in *Proteobacteria, Bacteroidetes* and *Actinobacteria*, perhaps because lactobacilli are resistant to metronidazole.

In terms of organisms and host site, it is intriguing that *Prevotella* can persist in the mouth in some patients without any major pathological adversity though in others it is causative of destructive periodontitis, but in the vagina they become part of an aberrant microbiota causing BV[44]. Such a change in mucosal site exposes *Prevotella* to a new community of microorganisms, some of which may activate dormant virulence factors or provide a substrate for the production of amines that are responsible for malodor[45]. Although *Prevotella* colonization and an increase in bacterial diversity are key features of BV, the exact trigger(s) for the dysbiotic state has yet to be identified. However, when the abundance of *Prevotella* drops or disappears and *Lactobacillus crispatus* or *Lactobacillus iners* increases, the host no longer has BV[16]. In some women this process can happen without treatment, illustrating a natural restoration. Alternatively, probiotic lactobacilli can be administered to aid the restoration process. In healthy women, the cure rate for antibiotic treatment of BV would be expected to be higher than for HIV-positive individuals, but it still varies from 27% to 63% depending on the cohort and regimen[46,47], whereas intra-vaginal use of probiotic lactobacilli or oral antibiotic plus oral lactobacilli have been shown to give a cure rate of around 88%[23,48]. Interestingly, in a study following individuals for two months, BV self-resolved partially or completely in 31% individuals[49].

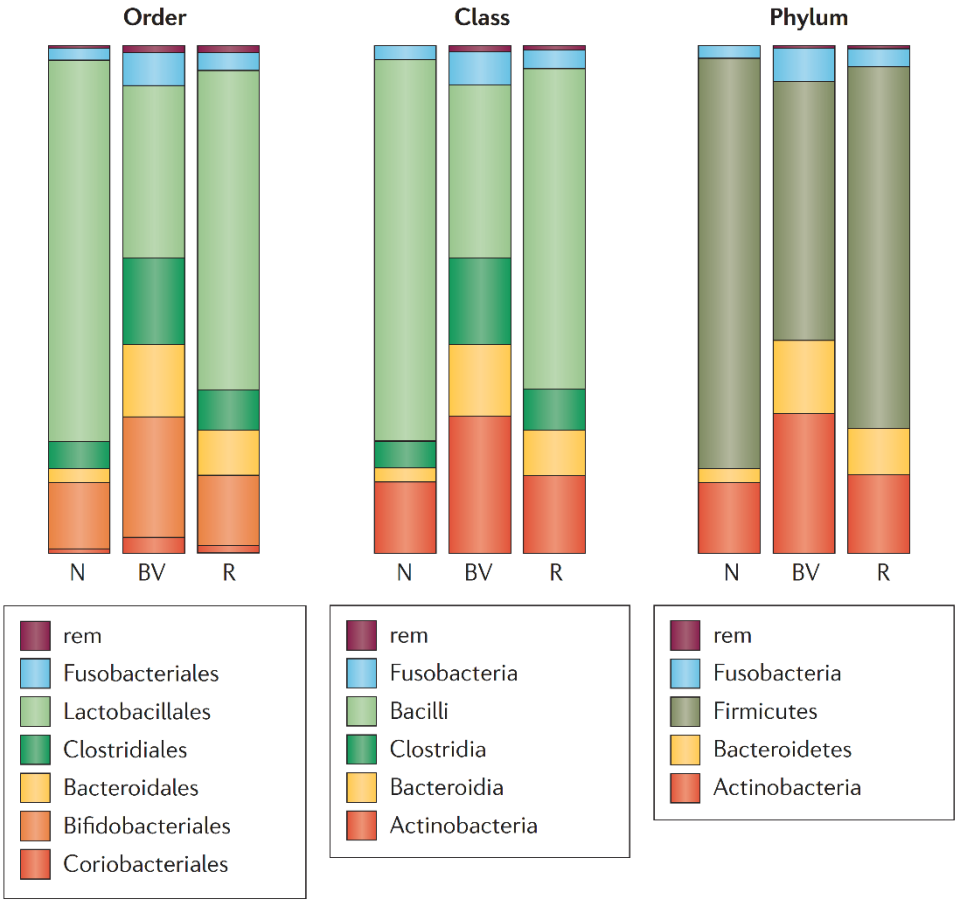

Figure 3 | Changes in the vaginal microbiome before and after bacterial vaginosis.

Vaginal samples from HIV-positive individuals in Mwanza, Tanzania, were processed by Illumina[16]. One group (n=60) was regarded as normal (N), or healthy, at day 0 of recruitment, with a Nugent score of ≤3 at screening. A second group had bacterial vaginosis (BV) on recruitment, with a Nugent score of ≥7. Individuals with BV were then treated with metronidazole, and those who subsequently returned to health at any time in the 25 weeks following treatment were designated as restored (R). The plots show the proportion of the microbiota in each clade at the level of order, class and phylum; the remainder (rem) groups contain all groups of organisms that made up less than 0.5% of the total in any of the three patient groups. Data from Reference 17.

Recovery of the intestinal microbiota

Another example associated with a shift towards an increased microbiota diversity comes from celiac disease, an autoimmune disorder of the small intestine in which dietary gluten ingestion leads to chronic enteropathy. In a study of the dominant duodenal microbiota of children with celiac disease, differences were noted not between individuals but between active and remission states, with *Bacteroides vulgatus* and *E. coli* detected more often in patients than controls[50] and a potential decrease in abundance of *B. vulgatus* and *E. coli* in individuals in remission. Another study suggested that the change from a microbiota dominated by *Lactobacillus curvatus, Leuconostoc mesenteroides* and *Leuconostoc carnosum* to one with *Lactobacillus casei* and *Bifidobacterium adolescentis* coincides with a return to health[51]. The ability to alter the phylum-level abundance of microorganisms in the gut[52], for example by microbiome transfer[53], demonstrates the power of microorganisms to influence health and disease. In addition, the fact that different microbiota compositions can be associated with a healthy state[16] suggests that microbiome functionality, rather than precise species abundance, may be key maintaining health. By understanding how indigenous and probiotic organisms respond to different conditions *in vivo,* it should become possible to understand the mechanisms by which pathogens are displaced leading to a return to homeostasis.

Mechanisms of restoration

Restoration of a healthy microbiota is driven by multiple species, and requires substantial reorganization after insult. There are numerous mechanisms whereby the normal microbiota can return as depicted in Figure 4.

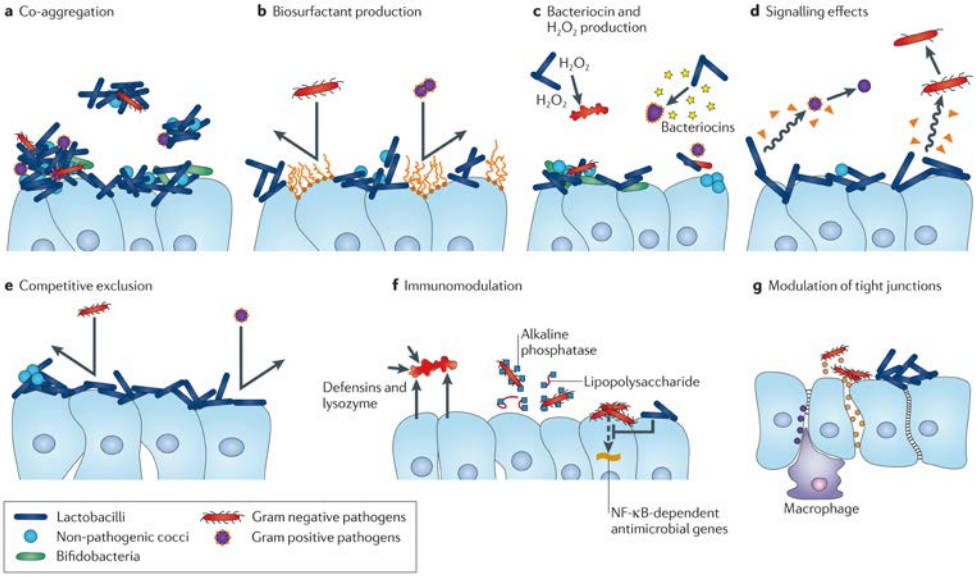

Figure 4 | Possible mechanisms contributing to microbiota restoration.

A | Coaggregation of non-pathogens and pathogens interferes with their ability to infect the host.

B | Biosurfactants produced by lactobacilli help to prevent pathogen adhesion to mucosal surfaces.

C | Bacteriocins and hydrogen peroxide produced by lactobacilli can inhibit or kill pathogens.

D | Signalling between bacteria can lead to down-regulation of toxin production in pathogens.

E | By competing for nutrients and surface receptors, bacteria can competitively exclude pathogens from surfaces.

F | Regulation of immune responses by microbiota constituents can result in; (1) the production of host factors such as lysozymes and defensins, which can kill pathogens; (2) the production of alkaline phosphatases which bind to LPS and negate its toxicity; (3) deregulation of NF-κB signaling in host epithelia (whether this is influenced by lactobacilli as indicated remains to be confirmed).

G | Upregulation of tight junction proteins might help to limit damage caused to epithelia by inflammatory processes or pathogens.

Coaggregation

The phenomenon of coaggregation is one in which microbial communities assemble in distinct, inter-linked structures[54]. In the oral microbiota, following disruption by antibiotic administration or mechanical cleaning, the reorganization of a non-pathogenic biofilm with specific subsets of species derived from the planktonic phase, is a key component in a return homeostasis. A problem arises if the antibiotic has depleted levels of non-pathogenic species to the extent that recovery of their normal levels is delayed and the new biofilm that forms is essentially made up of pathogenic species.

In the vagina, *L. iners* survives antibiotic therapy for BV, and it is possible that *L. iners* and other lactobacilli coaggregate with BV constituents as a key step to restoration of homeostasis. If lactobacilli are depleted and planktonic drug resistant pathogens are in the vicinity, a recurrence of BV is more likely. A genomic study of *L. iners* has identified potential surface adhesins that could bind other bacteria[55] but differential binding of non-pathogens versus pathogens has not been investigated.

Pathogens that inhibit interleukin-8 (IL-8) and disrupt epithelial-cell homeostasis or block access by other species to toll-like receptors (TLRs) can more easily infect the host[56]. Potentially, non-pathogens could compete with pathogens and enhance host defences, or the pathogenic biofilm could reach a state in which pH levels or certain metabolites stress the cloistered structure and lead to its disintegration or detachment[57]. The administration of probiotic lactobacilli may simply accelerate these pH and metabolic changes[58].

Biosurfactant production

There is evidence from two body sites that biosurfactants, surface-active compounds synthesized by microorganisms, may have a role in restoration and maintenance of microbial homeostasis. In the oral cavity, home of unexpected microbial diversity[59,60], regular toothbrushing and use of antimicrobial mouth rinses disrupt the microbiome, while dietary components, particularly sugars, increase bacterial growth. Despite these insults, the oral cavity in most individuals maintains a degree of microbial stability for long periods of time. One reason for this appears to be indigenous non-pathogens such as *Streptococcus mitis* which produces biosurfactant molecules that can substantially reduce the presence of pathogenic species such as *Streptococcus mutans*[61]. When adsorbed onto surfaces, these rhamnolipid-rich compounds can prevent *S. mutans* adhesion[62] through weakening adhesion forces between the pathogen and enamel, yet other early colonizing members of the oral microbiota are not blocked, suggesting selective activity against cariogenic strains.

Vaginal lactobacilli produce biosurfactants, made up of a mixture of proteins, lipids and carbohydrates, that help displace dense mixed cultures of uropathogenic *E. coli, Enterococcus faecalis* and *Gardnerella vaginalis*[63-65]. These effects occur even with sparse numbers of lactobacilli, suggesting that the secreted low-molecular-weight biosurfactants spread out over the surface of the vaginal mucosa and the subsequent alteration in surface tension helps repel hydrophobic pathogens[66]. The biosurfactants therefore provide a means of restoration towards lactobacilli-dominated microbiota and displacement of the pathogenic species.

Bacteriocin and hydrogen peroxide production

Once in contact with pathogens, the ability to produce substances that kill or inhibit their growth could be an important factor in restoring homeostasis. Bacteriocins are ribosomally synthesized molecules with a relatively narrow killing spectrum whose modes of action include interfering with cell wall structure and biosynthesis, forming pores in their target bacterial membrane, and permeabilizing membranes[67]. However, the organisms producing bacteriocins are not simply indiscriminate killing machines. Rather, they likely sense the bacterial dynamics of their niche, and produce bacteriocins to retain a competitive presence. This is illustrated by *L. acidophilus* La-5, whose bacteriocin expression is controlled by an auto-induction mechanism involving the secreted peptide IP-1800: bacteriocin production increases when the organism senses its target[68]. The ability of bacteriocin-producing strains to make immunity proteins, such as the product of the associated Abi gene (skkI) found in *Lactobacillus sakei,* that protect the organism from its own bacteriocin, is crucial for the survival of the organism and retention of the microbiome structure[69].

Several studies have strongly suggested that bacteriocins could have a role in restoring homeostasis. An *S. mutans* strain in which the lactate dehydrogenase gene had been deleted (making it entirely deficient in lactic acid production), produces mutacin 1140, a peptide antibiotic, that gives it a strong selective advantage over most other strains of *S. mutans*[70]. Experiments in gnotobiotic rats have shown that this strain is genetically stable and less cariogenic than wild-type *S. mutans*, and possesses strong colonization properties such that a single application can result in its permanent colonization and displacement of indigenous, disease-causing *S.*

mutans strains. The lantibiotic mutacin 1140 was shown to remove lipid II from the target's septum and block cell wall synthesis.

In arguably the most elegant *in vivo* verification of how a bacteriocin functions in the gut to date, Corr *et al*.[71] showed that the Abp118 bacteriocin produced by *Lactobacillus salivarius* UCC118 was essential to protect the host against infection by invasive *Listeria monocytogenes.* This does not prove that the bacteriocin restores homeostasis, but it shows at least one mechanism whereby pathogenesis can be interfered with in the gut.

Hydrogen peroxide (H_2O_2), a powerful oxidizing agent produced by lactobacilli, is able to kill pathogens in the vagina through the production of free radical[72]. Lactobacilli appear able to protect themselves from toxic accumulations of H_2O_2 through the production of $Fe^{(3+)}$ activated extracellular peroxidase[73]. Hydrogen peroxide induces peroxisome proliferator activated receptor (PPAR)-gamma nuclear translocation and enhances transcriptional activity in gut epithelial cells, directly modulating epithelial cell responsiveness to inflammatory stimuli[74]. The use of H_2O_2 as an antibacterial agent and host signaling transduction molecule, makes its contribution to homeostasis two-fold.

Signaling effects

In the 1960s, a number of studies on urinary tract infections (UTI) showed that in a portion of women, the infections resolved without treatment. More recently, in a study of 1143 women in northern Sweden who had symptoms suggestive of UTI, of the 288 individuals that were treated with a placebo for seven days, 28% showed a spontaneous curing of

symptoms after the first week, and 37% after 5-7 weeks[75]. After evaluating dropouts, the overall spontaneous cure rate of symptoms and bacteriuria was calculated as 24%. In a study of 50 women, the median time for spontaneous remission was reported to be 4 weeks with placebo treatment, substantially shorter than the 7 months for those who required concurrent antibiotics[76].

One possible mechanism for these effects is that host and/or bacterial signaling factors induce a down-regulation in the expression of virulence factors, forcing the pathogens to retreat deeper into the bladder epithelium[77] or back to the vagina. For uropathogenic *E. coli*, this process might involve a two-component response system composed of a histidine kinase activated by extracellular signals in the host environment and a response regulator that, in turn, modulates the expression of genes that induces cell invasion[78]. Whether the clinical subsidence of the infection is influenced by indigenous bacteria in the vagina is debatable, but anti-virulence signaling molecules have been identified in some strains of lactobacilli, known to be present in the vagina and intestine, as well as in some probiotics. These include *Lactobacillus reuteri* RC-14 which produces signaling molecules that inhibit toxic shock toxin (TSST-1) expression in multiple strains of *S. aureus*, and interferes with the P2 and P3 promoters of the staphylococcal global regulatory system *agr*[79]. Presumably *in vivo*, the ability to repress toxins could reduce host damage and inflammation, and thus symptomatology, but whether it would help restore microbiota homeostasis remains to be seen.

Competitive exclusion

The ability of indigenous bacteria to compete with transient pathogens requires that the former have attributes that strengthen their ability to colonize the host. For instance, *Bacteroides fragilis* produces multiple capsular polysaccharides that are essential for colonization of the gut[80]. These polysaccharides not only aid in persistence but also function in immune regulation helping to exclude pathogens and restore homeostasis.

Arguably, the most extreme assessment of competitive exclusion of pathogens comes from whole stool implantation to treat individuals with chronic gut infections. In this process, a sample of homogenized faeces from a healthy relative or friend is instilled through a nasogastric tube into the recipient's stomach or through enema infusion or nasoduodenal tubes[81,82]. For a cohort of 159 individuals, the overall reported success rate for restoring normal homeostasis to the gut microbiota was 91%[83]. In one patient infused with her husband's microbiota through colonoscope, there was a rapid and prolonged change in bacterial composition with *Bacteroides* dominating, *Clostridium difficile* absent and defecation frequency returning to normal[84]. This was a particularly interesting case, as it showed that colonization with exogenous bacteria is possible, an end result that has so far eluded probiotics. The implantation appears to re-establish colonization resistance whereby the dense population of infused microorganisms blocks the pathogens from causing a relapse of enteric disease.

This treatment appears to competitively exclude *C. difficile* and prevent potentially fatal symptomatic relapse. The recipients had received extensive antibiotic treatments during their *C. difficile* management and just prior to faecal transplant, thus much of their indigenous microbiota would

have been eradicated. In future, it may be possible to create a healthy microbiota in a chemostat or other complex multistage continuous cultures that simulate the large bowel, and use this as an inoculum rather than human stool.

Immunomodulation

Antimicrobial factors such as defensins, lysozyme and hemocidins can have a role in restoration of microbial equilibrium. The host's immune system must properly calibrate responses to pathogens and differentiate commensal and exogenous non-pathogenic organisms[85], and appears to do this through pattern recognition receptors, which mediate the detection of bacterial antigens and activate signaling cascades that regulate the immune response. The homeobox-containing protein Caudal has been shown in to regulate the interaction between Drosophila and its indigenous microbiota by repressing nuclear factor kappa B (NF-κB)-dependent antimicrobial peptide genes, thereby maintaining homeostasis[86] (Figure 4). These studies illustrate that immune regulation, to a large extent instigated or controlled by microorganisms, can target pathogens and thus help indigenous organisms to re-emerge.

The microbiota can have a role in intestinal inflammatory autoimmunity, but perhaps they also counter systemic immune aberrations such as allergy, rheumatoid arthritis (RA) and type I diabetes (T1D)[87]. Polysaccharides purified from microbiota constituents can suppress pro-inflammatory interleukin-17 production by intestinal immune cells and protect the host from inflammatory disease through a functional requirement for interleukin-10-producing $CD4^+$ T cells[88]. Short-chain fatty

acids produced by intestinal commensals also appear able to counter immune aberrations, as illustrated by their binding to G-protein-coupled receptor 43 (GPR43) in models of colitis, arthritis and asthma[89]. In relation to restoration after skin injury, staphylococcal lipoteichoic acid can act selectively on keratinocytes triggered through TLR3, which then inhibits inflammatory cytokine release from keratinocytes and inflammation triggered by injury through a TLR2-dependent mechanism[90].

These examples illustrate some of the molecular mechanisms used by commensal bacteria to help prevent disease and restore equilibrium. Intriguingly, the effects can be conferred by species that under other conditions may be pathogenic. The interface between the microbiota and the host's immune system is changing with dietary modifications and the widespread use of antimicrobials[91]. Likewise, our categorization of pathogens versus non-pathogens needs to be carefully considered, as clearly some so-called pathogenic or virulence factors are used by commensal and perhaps even probiotic organisms to convey benefits to the host. For example, capsules, adhesins, biofilm capacity, production of toxins and the ability to induce inflammation are regarded as pathogenic properties, yet many commensals also have capsules, use adhesins, and have the capacity to induce antimicrobial inflammatory processes depending on circumstances. Indeed, part of the reason that *E. coli* Nissle 1917 is considered to be a probiotic and useful for treating some inflammatory bowel disease cases, is the part that its capsule plays in chemokine induction[92]. As Blaser and Falkow illustrate, there is a need to increase our focus on the rules that govern the evolution of cooperation[91]. In terms of ecology, equilibrium requires boundaries and penalties for transgressors,

and maintenance of homeostasis requires inputs from multiple sources, including the commensal microbiota. In a state defined clinically as being healthy or devoid of disease, the equilibria form a continuum structure that permits the co-evolution of competing organisms with sufficient elasticity to cope with invaders. Studies are needed to not only understand these phenomena, but to investigate aberrant conditions in which equilibria either is not functioning, has never been attained, or is unable to be recalibrated.

Modulation of tight junctions

The integrity of the epithelial lining of the mouth, gut and vagina is crucial to maintaining health. When this is disrupted or breached, the microorganisms on the outer surface gain access to the tissue and bloodstream and induce disease. For example, HIV can decrease trans-epithelial resistance through disruption of tight junction proteins (claudin 1, 2, 4, occludin and ZO-1) and thereby allow viral entry into the host[93]. Potentially, lactobacilli could counter this effect as they up-regulate the scaffold protein zonula occludens (ZO)-1 and transmembrane protein occludin[94,95], as well as elafin (unpublished data), a molecule associated with HIV resistance[42]. However, there is no evidence to date that the sparse coverage of vaginal cells by indigenous lactobacilli or the presence of specific bacterial species can protect against HIV, and it remains to be determined whether probiotic application could confer any degree of protection.

In the intestine, various sensory pathways can influence homeostasis and recovery from, or prevention of, inflammatory processes[96]. For example, Paneth cells whose function can be regulated by the unfolded

protein response and autophagy, have a crucial role in limiting barrier breaches of the epithelial lining through their ability to sense the microbiota and produce antimicrobial molecules through cell-autonomous MyD88-dependent activation of TLRs[96].

Probiotics to augment microbiota restoration

One rationale for the use of probiotics is to help restore and maintain homeostasis. Although the commercial field of probiotics is sadly still lined with too many undocumented products making unproven claims, the pending implementation by regulatory agencies of some or all of the guidelines published on what constitutes a probiotic[97], will hopefully focus attention on strains proven to confer health benefits (exemplified in Table 1). Not all probiotic applications have been successful. For example, attempts at using probiotics to treat Crohn's disease met with failure, although this may not be unexpected given that there is only sparse evidence to indicate that microbial imbalance is its primary causation[98]. In other cases, such as treatment of *Helicobacter pylori* infections, the inability of probiotic organisms to colonize and displace deeply embedded pathogens is also anticipated. Nevertheless, probiotic therapy has been shown to reduce some of the side effects of pharmaceutical treatment of *H. pylori* and potentially decrease pathogen density[99].

Several studies have shown that probiotic bifidobacteria and lactobacilli may help prevent or reduce caries rates[100]. This is somewhat counter-intuitive, as both species can actually be involved in deep carious lesions. However, it seems when supplemented as a probiotic, these

organisms displace and reduce salivary levels of mutans streptococci and that by failing to colonize, they do not induce acidic damage to the teeth.[101,102]

The recent interest in the potential for probiotics to influence obesity through altering the gut microbiome is fueled by studies showing that a higher abundance of certain species, such as *Bacteroides* and *Staphylococcus,* can be found in obese individuals[103]. However, to date no interventions have been tested for obesity, although a randomized study of 256 pregnant women showed that ingestion of probiotic *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 reduced threefold the frequency of gestational diabetes mellitus (GDM)[104], potentially through alteration of energy uptake. Furthermore, a combination of *L. rhamnosus* GG and *B. lactis* Bb12 improved glucose tolerance in non-obese pregnant women, suggesting that this approach is worthy of consideration for obesity[105].

One of the most chronic inflammatory skin diseases is atopic dermatitis (AD), the hallmark of which is dry, red, itchy skin. The prevalence of AD is about 18% to 25% in children[106]. Risk factors for AD include sensitizations to ingested and inhaled allergens. Unlike microorganisms on healthy skin, the microorganisms colonizing the skin of AD patients, *Staphylococcus, Corynebacterium*, and *Candida*[107], are characterized by profound polymorphism of cells. AD is accompanied by destructive changes on the skin and bacteria on the upper layers of epidermis. Potentially, the maturation of the intestinal microbiota has a role in the spontaneous resolution of AD that occurs by age two in many children. To test this theory, probiotic lactobacilli and/or bifidobacteria have been administered

to pregnant women and newborns at high risk of AD. In one study of 68 infants followed for a year, the prevalence of eczema was significantly lower in the probiotic group versus placebo (18.2% vs. 40.0%, p=0.048)[108]. In another study, children aged between 2 and 10 years old who had received a *L. sakei* supplement[109] showed significant clinical improvement, suggesting that the effect may not be specific for a probiotic strain. However, the failure of *L. reuteri* to remediate eczema[110] contradicts this theory, and the lack of clinical effect on AD or asthma-related events using *L. rhamnosus* GG in a prospective double-blind study randomly assigned trial of 131 children (6-24 months old)[111], indicates that more studies are needed. In terms of how the gut microorganisms might influence the skin, several mechanisms have been proposed including immune modulation and improving tight junction barrier function.

The high prevalence of an aberrant microbiota does not necessarily translate into symptomatic infections in all cases. This is certainly the case with BV. A major reason for a natural return to homeostasis is likely the ability of indigenous lactobacilli to ascend from the rectum into the vagina and dislodge BV biofilms, using mechanisms outlined in Figure 4. Several studies have provided conceptual proof. Ingested probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 have been shown to reach the rectum, ascend to the vagina, reduce yeast and bacterial pathogen ascension and increase the number of women whose BV resolved[49,112]. This approach holds great promise to interfere with dysbiosis before symptomatic BV or UTI recurs.

In closing

Chau *et al*.[113] speculated that probiotic species may have arisen by a selection process on peptidoglycan that can down-regulate the effects of toxic virulence factors produced by pathogens. The proposal was that pathogens might rather be commensal and live in an environment that is not pathologic to the host. This implies there are triggers that can sway the balance of the microbiome away from homeostasis to pathogenesis, and similarly factors that return the balance from an infected state. Many such triggers of disease have been identified, yet far less known are triggers of a natural return to homeostasis. Studies that provide insight into this area will be challenging, but may lead to new methods to treat and prevent disease. The alternative challenge will arise if we let the current, often ineffective arsenal of therapies run out.

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Chapter 1.2

General Aims of this Thesis

The general aim of this thesis is to develop a better understanding of the biophysical interactions that exists between vaginal microorganisms and probiotic strains. The specific interactions that have been investigated in this thesis are coaggregation behavior and adhesion forces, changes in virulence markers, fluctuation patterns of inflammatory cytokines, and changes in artificial biofilm morphology and composition. This research may help to give insight on how commensal and pathogenic vaginal strains physically influence each other and the epithelial cells to which they adhere and whether a probiotic strain can be helpful either to reduce virulence/target the epithelium and when. Furthermore, the bacterial adhesion forces obtained may yield potential target therapeutic binding sites on the epithelial cells.