

MINIREVIEWS

A Special Fondness for Lactobacilli

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John B. S. Haldane (1892-1964), noted British geneticist, physiologist, and popularizer of science, established new paths of research in population genetics and evolution. Emphasizing the immensity of the Milky Way in the night sky and the fact that there were 400,000 species of beetles but only 8,000 species of mammals, he is reported to have said, "If one could conclude as to the nature of the Creator from the study of his creation, it would appear that God has a special fondness for stars and beetles." In a similar vein of thought, perusal of the issues of American Society for Microbiology journals and others published in recent years might cause the reader to conclude that microbiologists have a special fondness for lactobacilli. Fifty-two publications concerning lactobacilli (with "lactobacillus" or "lactobacilli" appearing in the article title or abstract) have appeared in *Applied and Environmental Microbiology* alone during 2003. It is no wonder: these are fascinating and useful bacteria.

Lactobacilli are members of the lactic acid bacteria, a broadly defined group characterized by the formation of lactic acid as a sole or main end product of carbohydrate metabolism. The lactobacilli are gram-positive, non-spore-forming rods or coccobacilli with a G+C content usually below 50 mol% (22). Eighty species of lactobacilli are recognized at present (55). They are strictly fermentative, aerotolerant or anaerobic, aciduric or acidophilic, and have complex nutritional requirements (carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, vitamins). Using glucose as a carbon source, lactobacilli may be either homofermentative (producing more than 85% of fermentative products as lactic acid) or heterofermentative (producing lactic acid, carbon dioxide, ethanol, and/or acetic acid in equimolar amounts). The nutritional requirements of lactobacilli are reflected in their habitats, which are rich in carbohydrate-containing substrates: they are found on plants or material of plant origin, in fermented or spoiled food, or in association with the bodies of animals (22).

Lactobacilli are important in the production of foods that require lactic acid fermentation, notably dairy products (yogurt and cheese), fermented vegetables (olives, pickles, and sauerkraut), fermented meats (salami), and sourdough bread. The use of lactobacilli in the food industry has a long history, and the functions of the bacteria in the industrial setting have

been well studied (28). Lactobacilli that inhabit the bodies of animals, however, are much less known, despite an almost continuous interest by scientists spanning about 100 years.

Elie Metchnikoff (1845-1916), winner of a Nobel prize for his pioneering descriptions of phagocytosis, was interested in the ageing process. While modern research on this topic concentrates on the maintenance of nonmutated DNA sequences, Metchnikoff focused on the gut microbiota as a source of intoxication from within (40, 41). According to Metchnikoff, the bacterial community residing in the large bowel of humans was a source of substances toxic to the nervous and vascular systems of the host. These toxic substances, absorbed from the bowel and circulating in the bloodstream, contributed to the ageing process. Gut bacteria were thus identified as the causative agents of "autointoxication." The offending bacteria were capable of degrading proteins (putrefaction), releasing ammonia, amines, and indole, which, in appropriate concentrations, were toxic to human tissues. Metchnikoff inferred that low concentrations of toxic bacterial products could escape detoxification by the liver and enter the systemic circulation. His solution for the prevention of autointoxication was radical: surgical removal of the large bowel. A less frightening and more popular remedy, however, was to attempt to replace or diminish the number of putrefactive bacteria in the intestine by enriching the gut microbiota with bacterial populations that fermented carbohydrates and had little proteolytic activity. Oral administration of cultures of fermentative bacteria would, it was proposed, "implant" the "beneficial" bacteria in the intestinal tract. Lactic-acid-producing bacteria were favored as fermentative bacteria to use for this purpose, since it had been observed that the natural fermentation of milk by these microbes prevented the growth of non-acid-tolerant bacteria, including proteolytic species. If lactic fermentation prevented the putrefaction of milk, would it not have the same effect in the digestive tract if appropriate bacteria were used? Eastern Europeans, some of whom were apparently long-lived, consumed fermented dairy products as part of their daily diet (40, 41). This was taken as proof of efficacy, and milk fermented with the "Bulgarian bacillus" of Metchnikoff subsequently enjoyed some vogue in Western Europe: the birth of probiotics. First coined in an entirely different context by Lilley and Stillwell (34) to describe substances secreted by one type of microorganism that stimulated the growth of another (probiotic to contrast with antibiotic), the term "probiotic" was subsequently used to describe "organisms and substances which contribute to intestinal microbial balance" (44). Fuller's definition (13), "a live microbial feed supplement which beneficially af-

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fects the host animal by improving its intestinal balance," has been widely used. "Living micro-organisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition" has been suggested (20), as well as the formulation "Probiotics contain microbial cells which transit the gastrointestinal tract and which, in doing so, benefit the health of the consumer" (63). So, too, have the following: "defined, live microorganisms administered in adequate amounts which confer a beneficial physiological effect on the host" (49); "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (52); and "microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host" (51).

Probiotic products, many of which contain lactobacilli, are actively promoted by the dairy, food, and "self-care health" industries and have been accepted uncritically by food scientists as well as the general public. However, claims of the efficacy of probiotics in relation to human health benefits do not result from rigorous, unbiased evaluations such as would be required by the U.S. Food and Drug Administration for pharmaceutical products (60). In other words, these claims have not been subjected to the usual four phases of assessment of efficacy (47).

Metchnikoff's view that consumption of bacterial cells in food would alter the proportions in which certain populations were present in the gut microbiota overlooked one of the most powerful forces in nature: homeostasis. Put in simple terms, homeostasis is the force in nature by which, although everything changes, everything stays the same (2). Homeostasis of bacterial communities is represented by a steady state that is generated by the organisms themselves. Competition for nutrients and space, the inhibition of one group by the metabolic products of another group, and predation and parasitism all contribute to the regulation of populations in particular proportions, one to the other. Because all of the ecological niches are filled in a regulated bacterial community, it is extremely difficult for allochthonous (formed in another place) microbes, accidentally or intentionally introduced into an ecosystem, to establish themselves. This phenomenon is referred to as "competitive exclusion" (2). The newly introduced bacteria have no way of earning their living in the ecosystem, since all possible niches have been filled. The composition of the human gut microbiota, as shown by the examination of fecal samples, has a remarkable stability (58, 69). The genetic fingerprint (denaturing gradient gel electrophoretic profiles) of this bacterial community remained constant in samples collected during long-term studies, even of 18 months' duration (63). For many of the humans who have been studied, this stability extended beyond genera and species, even to the level of bacterial strains (30, 37). Competitive exclusion is relevant to the introduction of probiotic bacteria into the gut. These bacterial cells are allochthonous to the bacterial community of the bowel, and as demonstrated in several studies, they have only a transient existence in the gut ecosystem (1, 11, 54, 57, 63). To take one study as an example, *Lactobacillus rhamnosus* DR20 was administered in milk to human subjects daily for 6 months (63). The probiotic strain was detected only while the probiotic product continued to be consumed. Once consumption of the probiotic product ceased, so too did excretion of the bacteria in

the feces. Moreover, levels of the probiotic strain were relatively low (10^5 to 10^6 organisms per gram of feces), and it was detected only irregularly in samples collected from about 40% of the subjects who had preexisting, stable *Lactobacillus* populations resident in their guts. The remainder of the subjects did not have stable *Lactobacillus* populations, and the probiotic strain could be detected in all of their fecal samples during the period of probiotic consumption, because the probiotic cells were not outnumbered by those of resident lactobacilli.

Allochthonous lactobacilli are commonly introduced into the gut ecosystem because they are ubiquitous in nature. They are part of the microbiota of many foods, and these food-derived *Lactobacillus* species can be detected transiently and unpredictably in human feces (7, 66). In contrast, as noted above, a proportion of human subjects harbor autochthonous (formed where found) lactobacilli (63). First postulated in relation to the gut ecosystem by Dubos and colleagues (9), the concept of autochthony was subsequently defined by Dwayne Savage: "Autochthonous microbes are characterized as indigenous microorganisms that colonize particular regions of the tract early in life, multiply to high population levels soon after colonization, and remain at those levels throughout the lives of healthy well-nourished animals. Autochthonous microorganisms should be found in essentially all individuals of a given animal species, irrespective of their geographical location" (56).

As a result of further reflection on observations made in recent studies of *Lactobacillus* ecology, the following concise definition could be proposed: "An autochthonous species has a long-term association with a particular host species, forming a stable population of characteristic size in a particular region of the gut, and has a demonstrable ecological function." This definition could be considered as a working hypothesis and a basis for further discussion.

Autochthonous *Lactobacillus* species can be clearly identified in the case of broiler chickens raised under commercial conditions (19, 31). Lactobacilli become established in the crops of the birds soon after hatching and persist throughout the life of the host despite the common administration of antimicrobial drugs in the poultry feed (long-term association with a particular host species). At least some *Lactobacillus* strains adhere to the crop epithelium and proliferate to form a biofilm. The metabolic activities of the lactobacilli that persist in this way influence the pH of the digesta, which, in turn, inhibits the proliferation of enterobacteria (demonstrable ecological function) (14). Shed from this site, *Lactobacillus* cells provide an inoculum of the digesta, which is then rich in lactobacilli throughout the remainder of the gut (stable populations of characteristic size) (14, 31). A major proportion of the microbiota of the ileal contents, for example, is composed of lactobacilli (35). Moreover, species succession is detectable within the total *Lactobacillus* population of the chicken gut. While members of the *Lactobacillus acidophilus* group and *Lactobacillus reuteri* are early colonizers, *Lactobacillus salivarius* is consistently detected only in older birds (19, 31). The mechanistic regulation of this succession would be fascinating to study, because it would appear that prior conditioning of the habitat by other lactobacilli, or by changes in chicken physiology or dietary composition, is required for *L. salivarius* to become established and persist in the avian gut. A similar

Lactobacillus succession occurs in the crop and the ileum, suggesting that colonization of the crop determines the composition of the microbiota of the ileal digesta with respect to the *Lactobacillus* population.

L. reuteri is autochthonous to the rodent gut, as evidenced by the facts that it has been detected there in several studies; adheres to the nonsecretory epithelium of the forestomach, thus forming a biofilm; persists at constant population levels throughout life in the guts of formerly *Lactobacillus*-free mice inoculated by mouth with a pure culture on a single occasion; and influences small bowel biochemistry (23, 38, 42, 64, 67). *L. reuteri* and the gut ecosystem of mice therefore provide an excellent paradigm for study of the molecular basis of autochthony. In the past decade, promoter-trapping technologies have been developed to overcome the limitation of in vitro models for study of the traits that enhance ecological performance in complex ecosystems. For example, in vivo expression technology (IVET) was developed by Mahan and coworkers to study gene expression by *Salmonella enterica* serovar Typhimurium during infection of mice (36). IVET has also been used to identify in vivo-induced (*ivi*) genes for a number of other pathogens, and mutations within a subset of these *ivi* genes resulted in a decrease in virulence (46). IVET recently identified *L. reuteri* strain 100-23 genes that were specifically induced in the murine gut (65). A plasmid-based system was constructed containing '*ermGT*' (which confers lincomycin resistance) as the primary reporter gene for selection of promoters active in the guts of mice treated with lincomycin. A second reporter gene, '*bglM*' (encoding beta-glucanase), allowed differentiation between constitutive and in vivo-inducible promoters. Application of the IVET system using *L. reuteri* and formerly *Lactobacillus*-free mice revealed three genes induced specifically during colonization. Sequences showing homologies to xylose isomerase (*xylA*) and methionine sulfoxide reductase (*msrB*) were detected. The third locus showed homology to a protein of unknown function. Xylose is a plant-derived sugar commonly found in straw and bran and is introduced into the gut via food. Xylose in the gut could be derived from the hydrolysis of xylans and pectins by other members of the gut microbiota. The selective expression of xylose isomerase suggests that *L. reuteri* 100-23 meets its energy requirements in the gut at least partly by the fermentation of xylose or isoprimeverose (the main component of xyloglucans) (4). Methionine sulfoxide reductase is a repair enzyme protecting bacteria against oxidative damage caused by reactive nitrogen and oxygen intermediates. Nitric oxide is produced by epithelial cells of the ileum and colon and possibly acts as an oxidative barrier, maintaining intestinal homeostasis, reducing bacterial translocation, and providing a means of defense against pathogens (25, 50). This pioneering IVET study showed the utility of the technology in investigating the molecular basis of autochthony and identified bacterial properties that may be essential for *L. reuteri* persistence in the gut (65). Indeed, there is now a strong case to be made for carrying out genomic comparisons between *L. reuteri* 100-23 and a strain of the same species that does not colonize the murine gut. Strain 100-23 clearly has properties that allow it to form a biofilm and to persist on the forestomach epithelia of mice. Moreover, this strain can be manipulated genetically and will express heterologous genes introduced in vitro (by electrotransformation) or by horizontal

gene transfer into the gut ecosystem (24, 38). Genomic comparisons of *L. reuteri* strains in relation to the ecological phenomena with which they are associated in the murine gut could reveal the molecular bases of autochthony.

It has been the hope of some microbiologists that lactobacilli could be genetically modified so that their cells would produce substances of biotechnological, and perhaps therapeutic, value. Rather than use these recombinant bacteria in industrial fermentors, the aim has been to use the bacterial cells in the gut as in situ factories that would deliver a bioactive substance to a particular region of the gut (39). This work has been impaired by the use of allochthonous species of lactobacilli, resulting in little progress in achieving the overall goal. The recognition of autochthonous species associated with different animal hosts makes it more likely that recombinant lactobacilli that will have at least some likelihood of metabolizing, and perhaps persisting, in the gut can be produced. The work of Lee and colleagues, in which recombinant vaginal lactobacilli that synthesized and secreted the first two domains of human CD4 were developed and shown in vitro to competitively block infection of target cells by the human immunodeficiency virus, provides a good example of a rational approach to this type of research (5). Although an autochthonous *Lactobacillus* species was used in these experiments, whether the recombinant bacteria have the ability to persist after instillation into vaginas remains speculative.

The interactions of lactobacilli with their hosts and their impact on host characteristics continue to fascinate microbiologists (59). Clues as to the influences of bacteria on the mammalian host have been obtained from comparisons of the biochemical and physiological characteristics of germfree and conventional mice, but comparative research of this type can now be performed at a sophisticated level because of the advent of genome sequencing of animals and the consequent manufacture of DNA microarrays that feature sequences representative of the entire genome of the animal. The potential for obtaining exciting knowledge of mechanistic influences of the microbiota on the host by this approach has been demonstrated by the pioneering work of Hooper and colleagues, who studied the impact of colonization of formerly germfree mice by *Bacteroides thetaiotaomicron* (26). But monoassociation experiments with formerly germfree mice are not representative of what occurs in the natural ecosystem. A single bacterial strain colonizing the gut of a gnotobiotic usually attains a much higher population level than it does in a conventional animal, where the microbe is faced with intense competition from the other members of the microbiota. Physiological differences between germfree and conventional animals can also influence colonization patterns. The wash-out effect of small bowel motility confines the bacteria to the more static terminal ileum or large bowel of conventional animals, but this restriction disappears in the monoassociated animal because of the slower peristalsis characteristic of the gnotobiotic host (18). Additionally, in the complex conventional ecosystem, the up-or down-regulation of host gene expression induced by the presence of one bacterial species could be negated by the impact of another species (26). Thus, a more ecological view would favor abandoning the additive approach (germfree animal plus bacterial species) and adopting a subtractive approach (conventional animal minus bacterial species). Mice that lack lactoba-

cilli yet are colonized by a complex microbiota functionally equivalent to that of conventional mice have been produced and would appear to offer the ideal model in which to determine the impact of both allochthonous and autochthonous lactobacilli on the regulation of expression of host genes (61).

From a pragmatic point of view, the impact of *Lactobacillus* metabolism on the nutrition and physiology of farm animals is an important area of study. Although antimicrobial drugs have been added to the food of farm animals for several decades, the precise mechanism by which the growth rate of the animal is augmented and feed conversion is improved is unknown. Feighner and Dashkevich reported that antimicrobial supplementation of the food of broiler chickens resulted in decreased bile salt hydrolase activity in the ilea of the birds (12). This may have been a particularly important observation because, at least among members of the gut microbiota of mice, lactobacilli are responsible for much of this enzyme activity (62, 64). Bile salt hydrolases catalyze the cleavage of an amino acid from the steroid nucleus of conjugated bile salts. It is not clear why lactobacilli produce an enzyme with this property, because they would not gain energetically from the deconjugation process, but it may be an essential property enabling the bacteria to survive transit through the small bowel, into which relatively high concentrations of conjugated bile acids are released (8). The deconjugating activity of the lactobacilli could be important to the host, because deconjugated bile salts are less effective in emulsification of dietary lipids and micelle formation. Thus, the bile salt hydrolase activity of lactobacilli in the small bowel could impair lipid digestion and absorption by the host and could have implications in the poultry and pig industries, where rapid growth and efficient feed conversion are required for profitability. Much attention has recently been paid to the phylogeny of the gut microbiota, but little has been paid to the microbial physiology of complex bacterial communities or their individual components (16, 17, 32, 33, 35, 68). It is time that this imbalance was rectified. Lactobacilli could provide model bacteria for such physiological studies because their relationship with the farm animal host (chickens, pigs) is much better defined than that of other members of the microbiota (3, 14, 19).

A large proportion of the immune cells of the body are associated with the gut. In the healthy host, the presence of the microbiota is tolerated by the immune system, although the mechanisms involved are not precisely known (10). Nevertheless, it can be inferred that tolerance toward the microbiota exists, because human patients with inflammatory bowel diseases and experimental animals with dysfunctional immune systems suffer from chronic, immune-mediated inflammation of the bowel mucosa (45, 53). Much evidence points to the presence of the microbiota as the fuel for this smoldering inflammation. The autochthonous microbe-immune system relationship in healthy animals must therefore be one of tolerance and requires mechanistic investigation. The allochthonous microbe-immune system relationship is presumably quite different, at least initially, because the immune system will experience novel antigenic complexes with each encounter with a different bacterial strain. Continuous close encounters with the same strain, either serendipitous (food microbiota) or intentional (probiotic), could, one supposes, eventually engender tolerance. Lactobacilli have been shown to invoke re-

sponses from immune cells, but much of the research reported has failed to establish a natural consequence for the host of such responses should they occur in vivo (6, 21, 27, 43). Specifically, we do not have measurements of the impact of lactobacilli on the immune systems of healthy humans in the community with respect to resistance to disease, apart from preliminary studies on the prevalence of diarrhea in high-risk groups (48). While probiotics seem not to have a major effect in altering the composition of the gut microbiota, they may have a role in manipulating the immune system in relation to specific diseases that have an immunological etiology, such as inflammatory bowel diseases and allergies. It must be noted that the titillating reports that have appeared in this respect are reports of small studies emanating from single research groups (15, 29). Where medical outcomes are involved, there is a need for large, comprehensive trials to prove efficacy in very well defined patient groups, in varied geographical locations with different ethnic mixes and cultural values.

Lactobacilli clearly offer microbiologists exciting research prospects, both for biomedical applications and for acquiring fundamental knowledge of how bacterial cells function in the gut ecosystem. As model gut bacteria, they may provide lessons in the molecular mechanisms that define autochthony as well as in understanding bacterial physiology in relation to host welfare. For these reasons, lactobacilli are set to remain the fond favorites of many microbiologists.

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