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The ecological role of lactobacilli in the gastrointestinal tract: Implications for fundamental and biomedical research

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Bacteria belonging to the genus *Lactobacillus* are members of the Lactic Acid Bacteria (LAB), a broadly defined group characterised by the formation of lactic acid as a sole or main end-product of carbohydrate metabolism. They can be found in plants or material of plant origin, silage, fermented food (yoghurt, cheese, olives, pickles, salami, etc.), as well as in the oral cavity, gastrointestinal tract (GIT), and vagina of humans and animals (31). In particular, the *Lactobacillus* species found in the GIT have received tremendous interest due to their health-promoting properties. They are commonly used as probiotics, which are defined by the FAO/WHO as live microorganisms that when administered in adequate amounts confer a health benefit on the host.

The economic success and exciting prospects of probiotic products have accelerated research on intestinal lactobacilli. Genomics of *Lactobacillus* species is booming, and the genomes of 5 strains that belong to species commonly found in human fecal samples have been recently sequenced, and more genomes are in the pipeline (50). Several comparative and functional genomic investigations have been conducted to gain information about the functionality of lactobacilli in the GIT (69). Unfortunately, a major misconception regarding the ecological role of lactobacilli in the intestinal tract has been embraced by many scientists working in the field. Specifically, there has been a general and persistent assumption that a large number of *Lactobacillus* species form stable and numerically significant populations in the human intestinal tract, especially in the small intestine, where they are presumed to form epithelial associations (101). Considering how widespread

and accepted this perception is, there is surprising little experimental evidence that supports it. Ecological observations on the prevalence and dynamics of fecal *Lactobacillus* populations and the findings obtained with comparative genomics do now indicate that the ecological role of most intestinal lactobacilli, and their relationship with the human host, should be reconsidered.

In this review, evidence is summarized that suggests that only a small number of *Lactobacillus* species are true inhabitants of the mammalian intestinal tract, and that most lactobacilli present are allochthonous members derived from fermented food, the oral cavity, or more proximal parts of the GIT. It is further explained why this knowledge provides valuable information to select strains for fundamental research on the ecological role of lactobacilli in the GIT, for there use as probiotics in foods and supplements, and for pharmaceutical applications.

THE GASTROINTESTINAL MICROBIOTA

The vertebrate GIT, including that of humans, is home to a vast collection of microbial species, mostly bacteria, which is referred to as the gut microbiota. Comparisons of the characteristics of germfree and conventional animals have clearly demonstrated that the gut microbiota has considerable influence on host biochemistry, physiology, immunology and low-level resistance to gut infections (7, 30). Because of the variation in physical and chemical properties in the different compartments of the GIT, specific microbial communities exist in the stomach, small intestine, and large intestine (93). In monogastric

animals, the largest numbers of bacteria reside in the distal gut (colon), reaching densities of around 10^{11} microbes per gram of luminal contents (90). The carbon and energy requirements of the enormous numbers of microbes residing in the colon are met from two sources: complex carbohydrates, proteins and fats that have escaped digestion in the small bowel, and the components of host secretions (mucins) and sloughed epithelial cells. Although nutrient availability is highest proximal to sites of absorption (stomach and the first two-thirds of the small bowel), these sites contain relatively small numbers of microbes in humans. Microbial numbers are restricted in these areas because of the pH of the stomach contents (as low as pH 2), the toxicity of bile salts, and the relatively swift flow of the digesta (93). The population density and diversity increases from the proximal small intestine (10^3 microbes per ml luminal contents in the duodenum), ileum (up to 10^8) to the colon (24). In contrast to humans, however, some animal species have relatively large numbers of bacteria (mainly lactobacilli) in the proximal gut (e.g. forestomach of rodents, crop of chickens, pars oesophagea of pigs)(92, 93). The reason for this special foregut association is likely due to the adherence of lactobacilli to the surface of the non-secretory epithelium lining these sites, enabling the bacteria to form a biofilm-like structure that provides a bacterial inoculum of the digesta (92).

Traditionally, gut microbiota research relied on techniques that required cultivation of the microbes (91). In the last decade, however, culture independent molecular approaches have been intensively applied to study the microbial diversity in the gut ecosystem. The most comprehensive and

probably least biased investigation of microbial diversity within the mammalian gut has come from direct sequencing of 16S rRNA genes (48). The sequences are obtained from DNA extracted from gut samples using PCR in combination with primers that are conserved for large groups of microbes (4, 22, 26). These molecular techniques have revealed that the diversity of the gut microbiota has been greatly underestimated (25). Although a complete catalogue of the members of the collective human gut microbiome is not yet available, more then ten thousand different species are estimated to be present (25), with a large majority of these microbes being resilient to cultivation by the currently available methodologies (90).

WHO'S WHO IN THE GUT

The astounding degree of microbial diversity in the GIT indicates a multitude of ecological niches. Many niches are likely to be determined by anatomical, immunological, and physiological characteristics of the host species. However, many niches are also generated through the development of complex food webs (niche construction) where the product of one microbe becomes the substrate for another (18, 48). Evolutionary theory predicts that in a spatially heterogeneous environment, vacant niches become occupied by organisms, and natural selection favours the emergence of ecological specialists being highly adapted to the available niches (40). During the gradual colonisation of the human GIT in early life, all niches in the gastrointestinal tract are likely to become occupied by well-adapted microbes, many of which are probably maternally acquired (48). Since every ecological niche can only support the existence of one type (according to the niche exclusion theory or competitive

exclusion principle), it is extremely difficult for an organism, accidentally or intentionally introduced into the gut, to gain access (32). These ecological principles explain why the population levels and species composition of the gastrointestinal microbiota remain remarkably constant over time in adult humans, a phenomenon referred to as colonisation resistance or competitive exclusion (7, 82, 112). The bacteria that occupy a niche in the gastrointestinal tract are true residents or autochthonous (found where they are formed) components as defined by Savage more than 30 years ago (80). Other bacteria are just "hitchhiking" through the gut and are allochthonous (formed in another place). An allochthonous organism in one section of the gut may represent, however, an autochthonous member of a more proximal niche that has been dislodged (shed), or it can be derived from ingested food and water (7, 111). Autochthonous strains have a long-term association with a particular host, and they form stable populations of characteristic size in a particular region of the gut (80). It is often difficult to determine whether or not a particular microorganism is truly autochthonous to a particular host (7). However, following the succession and population dynamics of a bacterial group within the gut microbiota does permit the identification of some allochthonous bacteria: they do not persist within the ecosystem and are only detectable for a limited time. As shown below, the identification of the exact ecological status of individual *Lactobacillus* species in the human GIT remains a major challenge.

THE GOOD, THE BAD, AND THE UGLY

At the beginning of the last century, Elie Metchnikoff (1845-1916), a Nobel Prize winner for his work on phagocytosis, proposed that the gut microbiota

produces small amounts of toxic substances that damage the nervous and vascular system and ultimately lead to aging (59). Metchnikoff proposed that administration of bacteria present in fermented milk products would “implant” these beneficial, lactic acid-producing bacteria in the intestinal tract, and would “arrest intestinal putrefaction and must at the same time postpone and ameliorate old age”. His theories were based on two observations. First, Bulgarian peasants with an assumedly long life expectancy, consumed large amounts of fermented milk products (97). Second, the natural fermentation of food by lactic acid-producing microbes prevented the growth of putrefactive organisms. Metchnikoff concluded “as lactic fermentation serves so well to arrest putrefaction in general, why should it not be used for the same purpose within the digestive tube”? Taken as proof of efficacy, milk fermented with the “Bulgarian bacillus” of Metchnikoff subsequently enjoyed considerable popularity in Western Europe (94). Overall, Metchnikoff’s theories remain very influential until today, and have contributed to the conviction that lactobacilli exert important functional attributes in the human GIT that promote health.

Although Metchnikoff focused his theories on lactic acid bacteria that were introduced into the digestive tract through the consumption of fermented food, he argued that these organisms were “able to take its place in the intestinal flora of man” (59). Accordingly, in the era following Metchnikoff, lactobacilli were identified as one of the dominant organisms in the human gut (91). Anaerobic bacteriology was not yet invented, and most gut microbes escaped cultivation due to their strict anaerobic nature. In contrast, lactobacilli (together with clostridia, enteroccci, and *Escherichia coli*) could be cultured with relative

ease due to their higher oxygen tolerance. Consequently, lactobacilli gained a reputation as numerical dominant intestinal inhabitants, and even the advent of anaerobic culture techniques did little to correct this situation. Lactobacilli are still listed as numerically dominant organisms of the gut in current microbiology text books (52, 70, 76), and even researchers working on functional and applied aspects of intestinal lactobacilli have continued to adhere to this dogma (11, 42, 57, 69, 71, 97).

FALL FROM GLORY

It is somehow intriguing how lactobacilli could maintain a reputation as numerically important gut inhabitants, given that the vast majority of experimental studies conducted after 1960 have clearly showed that they form marginal populations in the human gut. When using total anaerobic culturing techniques, lactobacilli form a very small proportion of the cultivable human fecal microbiota and can rarely be cultured at population levels exceeding 10^8 CFU per gram. Most studies report averages of around 10^6 CFU per gram (16, 17, 23, 62, 96, 104). This accounts for only about 0.01 % of the total cultivable counts. Subject to subject variation is significant, and lactobacilli are not detectable in around 25% of human fecal samples (24, 96). The findings obtained by culture are in good agreement with culture independent molecular approaches. In one study, fecal samples of 11 subjects were analyzed by fluorescent *in situ* hybridisation (FISH) in combination with fluorescence microscopy using the Lab158 *Lactobacillus-Enterococcus* targeted probe. Results revealed an average of 4.1 x 10^6 cells per gram of wet feces, which is around 0.01% of the total bacterial counts (33). Quantification of lactobacilli in

fecal samples of three human subjects with a *Lactobacillus*-specific quantitative real time PCR revealed levels between 10^7 and 10^8 target cells per of gram feces (74). In contrast to the studies described above, it was reported that the *Lactobacillus-Enterococcus* group constitute 6.6 % of the human fecal microbiota on average when assessed by dot plot hybridisation using the LAB158 probe (57). Provided that the rRNA abundance measured with dot plot hybridisations correlates with cell numbers, this finding indicates an average presence of 10^{10} lactobacilli and enterococci per gram of human feces. Such a high value is not supported by any finding using alternative methods, and it represents hundreds of times the proportion found by FISH using the same probe (33). It is also ten times higher then the values obtained using dot plot hybridisation with the Lacto722 probe, although this probe also detects streptococci (86). In this respect, it is important to point out that, as the probes used for the quantification of lactobacilli by FISH are not specific for lactobacilli, that the real numbers of lactobacilli could even be less.

High-throughput analysis of 16S rRNA sequences retrieved directly by PCR now allows an comprehensive view on the microbial diversity of the human and animal gastrointestinal tract (25). A quantitative assessment of the results obtained in these studies with a focus on the prevalence and diversity of *Lactobacillus* operational taxonomic units (OTUs), is shown in Table 1. Eckburg and co-workers studied 11,831 bacterial near-full-length 16S rRNA sequences retrieved from cecal, colonial and fecal samples (including biopsies) of three human subjects, and remarkably, found not one single *Lactobacillus* sequence. Lactobacilli were also absent from the libraries generated in several

studies of smaller scale (34, 35, 37, 90). Ley and co-workers studied fecal samples of 12 human subjects and found only six sequences to account for lactobacilli in a total of 18,348 sequences (49). To date, significant proportions of lactobacilli could only be found in two 16S libraries obtained with human samples (26, 36). In a study of impressive scale, Frank and colleagues presented a comprehensive molecular-based analysis of the bacterial diversity of gut tissues samples obtained from patients suffering from inflammatory bowel disease (IBD), as well as non-IBD controls. Around 5% of the sequences obtained from non-IBD patients from the colon were accounted for lactobacilli (Table 1). Hayashi and co-workers found 12.9% of the sequences in libraries generated from jejunal, ileal, caecal, and recto-sigmoidal (luminal) samples of elderly subjects to account for lactobacilli. However, in both studies, the vast majority of the *Lactobacillus* sequences did represent species that are not considered real inhabitants of the GIT (*L. delbrueckii* and *L. mali*), suggesting that these bacteria were introduced through food. Overall, the comprehensive molecular-phylogenetic analysis of the gut microbiota now provides clear evidence for the numerically minor proportion of lactobacilli.

One could now speculate that lactobacilli are underrepresented in 16S rRNA libraries due to a PCR bias that discriminate against *Lactobacillus* sequences. However, this objection is unfounded since *Lactobacillus* sequences are actually overrepresented (when compared to results obtained by culture) in libraries of intestinal samples of mice, rats, pigs, and chicken (see Table 2). Furthermore, it is often argued that the study of fecal samples does not provide accurate information concerning the intestinal microbiota, and that the small

numbers of lactobacilli in human fecal samples might in fact represent remnants of larger populations colonising a more proximal part of the gastrointestinal tract or mucosal sites. In fact, lactobacilli are among the most common bacteria found in the stomach, duodenum and jejunum of humans by cultivation approaches (62, 72). However, as shown in Table 1, molecular investigations of the bacterial populations present in the stomach, small intestine and mucosal biopsies have shown that *Lactobacillus* sequences are only present in small proportions (<1%) in most of these samples. In this respect, it should be considered that *Lactobacillus* populations that can be cultured from the stomach and small intestine are generally rather small ($<10^4$ bacteria per ml) and that most bacteria present are likely to be transients from the oral cavity or from food (7). Taken together, the molecular-phylogenetic characterization of samples taken throughout the human GIT does not support the hypothesis that more proximal or mucosal sites harbour greater populations of lactobacilli, and it appears that lactobacilli are greatly outnumbered by yet to be cultured organisms.

UPS AND DOWNS

Stability is a general characteristic for microbial ecosystems (2). Intestinal ecosystems are no exception, and although they are dynamic, they remain remarkably resistant and resilient to chaotic blooms of subpopulations and pathogens (48). Functional redundancy in the microbiota confers stability, and if perturbed, homeostatic reactions come into place and restore a reasonable stable equilibrium. Molecular fingerprinting of 16S rRNA genes by denaturing and temperature gradient gel electrophoresis (DGGE and TGGE) is a simple

way to show stability of the gut microbiota in healthy adult humans. Several studies revealed that the total bacterial population as well as bacterial groups such as bifidobacteria, *Bacteroides* spp., and clostridia show a high degree of temporal stability down to the species level (82, 96, 100, 112). However, the situation is very different for lactobacilli. DGGE in combination with primers for lactic acid bacteria showed that *Lactobacillus* population in fecal samples of most human subjects show temporal dynamics that are characterized by fluctuations and a lack of stability (82, 100, 104). The temporal fluctuations of *Lactobacillus* populations are also evident when studying the succession of isolates (strains) in human fecal samples. Early pioneering studies, conducted between 1960-1980 by Gerhard Reuter and Tomotari Mitsuoka, showed both persistent and transient *Lactobacillus* strains in human feces (45, 61, 63, 73). Based on current taxonomic criteria, the persistent strains identified in these studies belonged to the species *Lactobacillus gasseri*, *Lactobacillus crispatus*, *Lactobacillus reuteri*, *Lactobacillus salivarius* and *Lactobacillus ruminis* (62, 72). These early findings were confirmed more recently by Tannock and co-workers, who followed the temporal succession of *Lactobacillus* strains by molecular strain typing (41, 96). Human subjects that had a stable and large (>10^6 CFU per gram) fecal population of lactobacilli maintained single strains that predominated throughout the period of investigation (up to 15 months). These strains belonged to the species *L. ruminis* and *L. salivarius*. Although lactobacilli could be cultured from all subjects in these studies, several of the subjects had also periods when no lactobacilli were detectable. Most strains were only detected in one or two fecal sample in the majority of subjects, and then went missing. These sporadic strains belonged to the species *L.*

acidophilus, L. crispatus, L. gasseri, *L. plantarum*, and the *L. casei* group (*L. casei, L. paracasei,* and *L. rhamnosus*) (96).

There are seventeen *Lactobacillus* species that are associated with the human gastrointestinal tract, some of which were only recently detected by molecular techniques using PCR primers specific for lactic acid bacteria (Table 3). However, the studies above show that caution is advised when describing particular *Lactobacillus* species as real (autochthonous) inhabitants. Species such as *L. acidophilus*, *L. casei, L. paracasei, L. rhamnosus, L. delbrueckii*, *L. brevis*, *L. johnsonii, L. plantarum* and *L. fermentum* have so far not been reported to form stable populations in the gut and are likely to be allochthonous. Most of these species are regularly present in fermented foods and they are common inhabitants of the oral cavity (Table 3). The results from feeding studies of lactobacilli indicate that the survival of lactobacilli originating from food during gastrointestinal passage is comparable to that of probiotic strains, and they can be cultured in numbers comparable to that of resident lactobacilli when consumed in cell numbers not uncommon for fermented foods (Table 4). Lactobacilli are present in human saliva in variable numbers but often attain populations exceeding 10^5 CFU per ml (1, 16, 43, 56). The average output of saliva is 1000-1500 ml per day which, when swallowed, potentially introduces doses of oral lactobacilli into the gastrointestinal tract that are comparable to those used in probiotic feeding trials. Interestingly, the species that predominate in the oral cavity, such as *L. acidophilus* (and closely related species like *L. gasseri* and *L. crispatus*), *L. plantarum, L. salivarius, L. brevis, L. rhamnosus*, *L. paracasei*, and *L. vaginalis* are also frequently isolated from

human feces, and the species composition present in the oral cavity and in fecal samples coincides in some humans (16, 60). Dal Bello and Hertel showed that several fecal and oral isolates from three subjects isolated at the same time point were of the same RAPD type, suggesting that these fecal isolates originate from the oral cavity (16). Several *Lactobacillus* species, such as *L. salivarius*, might therefore be allochthonous to the human intestinal tract but autochthonous to the oral cavity (72).

HOW DO AUTOCHTHONOUS LACTOBACILLI PERSIST IN THE GUT?

Most lactobacilli present in the GIT of mice, rats, pigs, and chickens are clearly autochthonous since they form stable populations throughout the life of the animal host, can be cultured in large numbers, and are present in almost all animals (62, 92). As shown in Table 2, clones derived from lactobacilli are among the most common representatives in 16S rRNA gene libraries derived from intestinal samples of these animals. Unlike the human stomach, which is lined with a glandular mucosa, the stomach of pigs, mice, and rats and the crop of birds are lined, at least partly, with a non-glandular, squamous stratified epithelium (92). These regions are densely colonised by lactobacilli which adhere directly to the epithelium and form a layer of bacterial cells. The epithelial associations formed by lactobacilli show characteristics of bacterial biofilms because the bacteria are firmly attached to a surface (epithelium) and are embedded in a matrix of extracellular polymeric substances (27, 81).

Strains closely related to *L. reuteri* and *L. johnsonii* are clearly autochthonous to the rodent and porcine gut because they have been detected there in

several studies in almost all animals (12, 46, 78). These species have recently been used to identify bacterial factors that allow lactobacilli to persist in the gut of mice. These studies have begun to provide mechanistic explanations of the ecological success of lactobacilli as a result of the application of in vivo expression technology (IVET), microarray transcriptome analysis, and the investigation of the ecological performance of isogenic mutants (19, 95, 102, 103, 105, 106). The bacterial factors identified in these studies are summarized in Table 5. Although knowledge about their exact ecological function is still rudimentary, the findings suggest that adherence to the squamous stratified epithelium of the forestomach of mice is a key feature. Proteins such Lsp (Large Surface Protein) seem to initiate adherence, while extracellular polysaccharides (EPS) appear to contribute to the matrix and facilitate cell aggregation. Furthermore, several bacterial factors (MrsB, Dlt, IgA protease) were identified as important allowing the bacteria to overcome adverse environmental conditions generated through high acidity or innate and adaptive host defences (nitric oxide and IgA). Bacterial factors with similar function have recently been shown to be important for *Bacteroides thetaiotaomicron* in the murine gut (68). Peterson and co-workers showed that a capsular polysaccharide (CPS4) with unknown ecological function was essential for the competitiveness of the organism. *B. thetaiotaomicron* avoided CPS4 specific IgA recognition by downregulating epitope expression in vivo. In the absence of IgA (in *Rag1* -/- mice, which lack T and B cells), *B. thetaiotaomicron* triggered expression of inducible nitric oxide synthase (iNOS) in the small intestine, and the organism responded to the oxidative challenge by elevating expression of operons involved in nitric oxide metabolism. Accordingly, MrsB

and the IgA protease of lactobacilli might contribute to ecological performance by counteracting nitric oxide and IgA exposure in the gut. It is striking that all of the factors identified to contribute to the persistence of lactobacilli in the murine gut (D-alanylation of TA, epithelial adhesion, repair of oxidative damage of proteins, *luxS*-dependent production of AI-2, EPS formation, proteolytic degradation of immunoglobulins) are also important contributors to bacterial virulence, thus emphasizing that commensal lactobacilli and bacterial pathogens apply similar strategies to occupy niches within the mammalian host.

It is important to recognize that the ecological cohesions discovered in mice do not necessarily account for persistence in the human gut due to significant anatomical differences. Most importantly, a stratified, squamous epithelium is not present in the human stomach. Still, adherence of lactobacilli to epithelia or mucus is often considered to contribute to the persistence of lactobacilli in the human gastrointestinal tract (69, 101). It has been shown that some lactobacilli have the ability to bind to intestinal mucus and polymers associated with the surface of enterocytes (64, 75), and putative adherence factors of lactobacilli have been identified (as reviewed by Vélez et al. (101)). The ecological relevance of these factors in the human GIT remains to be determined *in vivo*. In this respect, it is important to emphasize that colonisation of mucus associated with tissue surfaces by members of the gastrointestinal microbiota is very limited in humans, and the numbers of bacteria obtained from washed tissue surfaces are considerably lower than those observed in studies of rodents (93). Evidence for significant *in vivo* association of lactobacilli with the

columnar epithelium of the intestinal tract of humans is still inconclusive and more work is needed to determine if association with the epithelium contributes to the persistence of lactobacilli in the human gut. Although not present in the human gut, stratified squamous epithelia seem to be key factors to *Lactobacillus* colonization, as all habitats with high numbers of lactobacilli contain such epithelia (human mouth and vagina, proximal GIT of rodents, pigs, horses, and birds). Adherence to these epithelia appears to be more relevant than adherence to columnar epithelia or mucus present in the intestinal tract, and the identification of adherence mechanisms to squamous cells would therefore teach us a lot about how lactobacilli manage to occupy niches within their mammalian hosts.

In contrast to rodents and pigs, significant epithelial associations of gut bacteria or biofilms have not been described in the human gut. Commensal bacteria appear to live in suspension with limited contact to epithelial cells (99). Rapid generation times are therefore vital for the bacteria to avoid washout. Numerically dominant human gut organisms such as *Bacteroides thetaiotaomicron*, and *Bifidobacterium longum* have highly evolved "glycobiomes" which consist of an elaborate apparatus for acquiring and hydrolyzing dietary and host-derived polysaccharides associated with a large repertoire of environmentally regulated expression systems (83, 110). Complete pathways for the synthesis of amino acids, nucleotides, and some key vitamins were identified. It appears that *Bacteroides* spp. and bifidobacteria base their ecological competitiveness on the utilization of complex nutrients using well-regulated pathways to save energy and assure

high proliferation rates in the lumen of the gut. How lactobacilli facilitate rapid growth in the human intestinal tract remains dubious, as they are fastidious organisms with nutritional requirements one would consider disadvantageous in regions distal to host nutrient absorption. Lactobacilli require amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acid esters, and fermentable carbohydrates for growth, and they have very limited abilities to utilize complex carbohydrates (39). The analysis of genome sequences for several intestinal *Lactobacillus* species (*L. acidophilus*, *L. salivarius*, *L. plantarum*, *L. gasseri*, and *L. johnsonii*) did not reflect an adaptation to the intestinal tract, as the physiology based on genome annotations is in striking contrast to that of the dominant gut inhabitants *Bacteroides thetaiotaomicron* and *Bifidobacterium longum*. (3, 15, 42, 55, 71). It is of course possible that lactobacilli occupy specific niches in the human GIT and have evolved to become ecological specialists, in contrast to *Bacteroides thetaiotaomicron* and *Bifidobacterium longum*, which appear to be generalists with large genomes (40). Lactobacilli could utilize simple carbohydrates that result from the degradation of complex carbohydrates by other microbes. Alternatively, some species such as *L. acidophilus*, *L. plantarum*, and *L paracasei* are able to metabolize complex prebiotic carbohydrates that remain untouched by human enzymes and which could serve as nutrients in the intestinal tract (5, 6, 29, 79). However, these species still lack pathways for the synthesis of most amino acids, nucleotides, and vitamins. The significant auxotrophy revealed by genome characterizations has led researchers to speculate that lactobacilli may inhabit the nutrient rich upper GIT of humans in higher numbers (3, 71). However, as shown in table 1, this view is not supported by recent molecular

characterizations of the microbiota present at these sites. Overall, the findings obtained with the analysis of the currently available *Lactobacillus* genomes provide further support for their allochthony in the human intestinal tract.

ARE THE MAJORITY OF LACTOBACILLI IN THE INTESTINAL TRACT OF RODENTS, PIGS, AND CHICKENS ALLOCHTHONOUS?

As noted above, most *Lactobacillus* species found in the human intestinal tract do not appear to be true inhabitants, and it remains unclear how autochthonous species satisfy their fastidious nutritional requirements in regions distal to host nutrient absorption. Nevertheless, lactobacilli are present throughout the GIT of mice, rats, pigs, and chickens in high numbers, including the large intestine (around 10^8 cells per gram). How do lactobacilli maintain such high cell numbers in the distal GIT of these animals? Like lactobacilli in conventional animals, *Lactobacillus reuteri* colonizes ex-*Lactobacillus* free mice throughout the gut and stably maintains cell numbers of around 10^9 cells per gram in the forestomach, around 10^7 cells per gram in the jejunum, and around 10^8 cells per gram in the cecum and fecal samples (102, 103, 105). These significant numbers do certainly imply that *L. reuteri* does inhabit all these different sites. One could also assume that the different anatomical and physiological conditions present throughout the gut would account for distinct bacterial traits to be required for colonization. Hence, genes that contribute to ecological performance in one compartment would not necessarily affect fitness throughout the gut. However, an unexpected finding in experiments with isogenic *L. reuteri* mutants was that gene inactivation always affected the mutant populations in the entire intestinal tract of mice, independent of gene

function (102, 105, 106). This was especially surprising for bacterial factors involved in adherence and biofilm formation, as significant adhesion of lactobacilli to the columnar epithelia lining the intestinal tract has not been described in mice. So it is unlikely that inactivation of Lsp, a protein involved in adherence to the forestomach epithelium, would result in reduced population levels in the distal intestinal tract (102). Even more puzzling, the proportion of the mutants in the cecum always mirrored that of the forestomach in individual animals in these experiments (Figure 1A-D). As a conclusion, these findings suggest that the cecal *L. reuteri* population is composed of remnants of the forestomach population, and point to the forestomach as the real habitat of *L. reuteri*. *L. reuteri* is therefore likely to be allochthonous to the murine intestinal tract.

It remains to be determined whether this also accounts for other *Lactobacillus* species present in the intestinal tract of rodents, pigs and birds. Comparison of the population composition of the forestomach and cecum of BALB/c mice by DGGE and sequencing of bands revealed that all lactobacilli detectable in the cecum (3 OTU) were also present in the forestomach (58). Similarly, in chickens, DGGE analysis with LAB specific primers revealed that the molecular fingerprint detected in the cecum was virtually identical to that of the crop (Figure 1E). In addition, the *Lactobacillus* succession that has been observed in the crop and the ileum of chicks is remarkably similar (94), suggesting that the *Lactobacillus* microbiota of the intestinal tract of these animals consists of bacteria originating from the crop. In pigs, DGGE analysis with LAB specific primers revealed that the same molecular fingerprint could be

detected throughout the entire GIT, from the distal esophagus to the distal colon (Figure 1F). These findings suggest that numerically dominant *Lactobacillus* populations present in the rodent, pig, and chicken intestinal tract are allochthonous, and that they originate from the forestomach, pars esophagus, and crop, respectively. The identification and characterisation of *Lactobacillus* strains autochthonous to the distal intestinal tract of such animals would be of great interest, since traits that enable their colonisation might be similar to traits of lactobacilli autochthonous to the human large bowel. These bacteria, together with their animal hosts, would provide a good model system to study ecological interactions that are likely to be equivalent in humans.

IMPLICATIONS FOR FUNCTIONAL AND BIOMEDICAL RESEARCH

Lactobacilli offer exciting research opportunities, both in terms of biomedical applications and in acquiring fundamental knowledge of the functionality of gut microbes (94). The tools for genetic modification, identification, detection and functional analysis of lactobacilli have improved tremendously over the last two decades. More and more *Lactobacillus* genomes are becoming available, allowing systematic comparative and functional genomic studies to investigate ecological and probiotic functionality. There is no doubt that the means necessary to carry out detailed and informative studies on gastrointestinal lactobacilli now exist. However, it is important to consider the ecological characteristics of individual species, and their relationship with their host, in such studies. Unfortunately, the ecological status of *Lactobacillus* species in the human gut has generally not been taken into consideration by researchers working in the field despite its important implications. Comparative genomic

investigations to identify colonization determinants require exact knowledge about the origin of strains in order to link genome features to ecological function. The ecological status of most intestinal isolates, including the strains for which genome sequences are available, is at best uncertain. Furthermore, most *Lactobacillus* strains currently used as probiotics are not adequate model organisms to study ecological aspects of gut colonization, as they belong to species that have never been shown to form stable populations in this ecosystem. It would be of great value to include *Lactobacillus* strains, having strong evidence as being autochthonous, in comparative and functional genomic investigations.

The bacteria residing in the mammalian gut and their hosts are likely to have coevolved with each other over a long conjoint history, and by doing so, have developed an intimate and complex symbiotic relationship. The mechanisms underlying these interactions are likely to be specific for a particular microbe and its host, and are probably influenced by other partners of the gut microbiota. Therefore, investigations of the host/microbe interplay in gut ecosystems should be conducted within an ecological context. Most importantly, this research requires the examination of bacterial species proven to be autochthonous in a particular host. This is particularly important when studying the organism's response and behaviour in the gastrointestinal tract by global transcriptome analysis using microarrays. The physiology and expression of phenotypic traits of an autochthonous gut organism colonizing the GIT is a dynamic entity that reflects the microbe's adaptation to the ecosystem and its specific host. In contrast, the response of an allochthonous

organism to the gut environment is likely to be based on signals that are generic (stress response, basic metabolism), and hence will neither reveal much about the environment from which the organism originates nor how autochthonous lactobacilli manage to live in the gut.

It has been clearly shown that gut microbes benefit their host in many aspects (4). Gut bacteria can enhance host immune functions and the mucosal barrier, and they provide protection against incoming microbes (97). These interactions comprise modulation of signal transduction pathways and gene expression in epithelial and immune cells, and their high complexity makes it unlikely that they have emerged by coincidence. In contrast, one would predict that mutually beneficial microbial activities have been shaped by natural selection during co-evolution, as they promote host fitness (4, 48). As a consequence, gut inhabitants that share a long evolutionary history with their host species are likely to possess adaptive health attributes that can be explored when using these organisms as probiotics. It is therefore reasonable to consider autochthonous strains to constitute better probiotic strains for some applications. Indeed, many researchers consider human origin as an important criterion for the selection of probiotics (21, 66, 77). However, although most probiotic strains originate from human gut or fecal samples, they show a poor persistence after administration is stopped (66). This is generally believed to be due to competitive exclusion conferred by the resident gut bacteria and individual differences between human subjects. In addition, human subjects are different, and a strain isolated from one individual would not necessarily compatible with the intestinal ecosystem of another individual. Although these

are legitimate claims, most strains currently used as probiotics do belong to species which are likely to be allochthonous to the human intestinal tract, and their failure to persist might reflect a lack of competitiveness in the gut ecosystem. It would be fascinating to investigate the probiotic characteristics of strains proven to be autochthonous, both in relation to persistence and health benefits. Is a strain autochthonous for one person a better "universal coloniser"? Of course, even autochthonous *Lactobacillus* strains would not be compatible with the intestinal environment and immune system of most individuals. Still, an autochthonous strain is adapted to the GIT, and its ecological fitness, metabolic activity, physiology, and ability to persist and produce microbial products that define its probiotic functionality in the gut should be higher than that of allochthonous strains. It has been shown that lactobacilli and other lactic acid bacteria could be genetically modified so that their cells produced bioactive substances of therapeutic value, and to deliver them upon ingestion to the gut mucosa (85, 89). For this purpose it appears that the utilisation of autochthonous strains makes it more likely that the recombinant organisms will persist, metabolise and produce sufficient amounts of the therapeutic compound at a desired location in the gut.

It is now generally recognized that the health benefits of probiotics are mainly conferred though a stimulation or modulation of the immune system (66). Several animal and human studies have provided unequivocal evidence that specific strains of probiotics are able to stimulate as well as regulate several aspects of natural and acquired immune responses, what opens opportunities to treat or prevent specific diseases that have an immunological aetiology (28).

When targeting host immune functions, it is again likely that the evolutionary history of the probiotic strain is of paramount importance. The autochthonous microbe-immune system relationship in healthy animals is characterized by tolerance, while the immune response to allochthonous bacteria results in a stronger immune response (8, 9). Duchmann and co-workers showed that tolerance selectively exists to intestinal biota from autologous but not heterologous intestinal samples, and the latter resulted in strong responses from blood and mucosal lymphocytes (20). It appears that gut bacteria have evolved properties to avoid an immune response of their host. Indeed, gut bacteria possess factors that induce antigen-specific regulatory T cells which actively contribute to tolerance development (87, 98). As a consequence, autochthonous bacteria might be more promising candidates for probiotics aimed to suppress an inappropriate immune response, desirable in the treatment of inflammatory bowel diseases (IBD). *L. reuteri*, which is autochthonous to rodents and humans, has been shown to modulate macrophage and dendritic cell functions in a way one would expect to favour immunological tolerance (14, 67, 87). Accordingly, strains of *L. reuteri* are especially successful in the prevention of colitis in several animal models (54, 67). On the other hand, the activation of the immune system (such as enhanced phagocytosis and adjuvant effects) observed after administration of some probiotic strains may reflect the allochthonous nature of the bacteria, and these bacteria might be more effective to treat or prevent infectious and rotavirus diarrhoea (53, 84). One would assume that allochthonous organisms are also more successful in the activation of immune responses and the prevention of atopic disease in early life because the immune system will

experience novel antigenic complexes with the encounter of the bacterial strains. It has been shown that virtually all health benefits and effects on host cells reported for probiotics are strain dependent (53). Mechanistic explanations for this strain specificity are so far lacking, but it is likely that the distinct evolutionary histories of currently used probiotic strains are at least partly responsible for their different effects.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The scientific data presented in this review indicate that most *Lactobacillus* species found in the mammalian intestinal tract are in fact not true intestinal inhabitants. They probably originate from more proximal or exogenous sources where the nutrient requirements of these fastidious organisms are satisfied. Future research is needed to identify the autochthonous *Lactobacillus* microbiota of the mammalian intestine. Strains that form stable populations (several months) in the intestinal tract without having significant upstream populations would show clear characteristics of autochthonous intestinal inhabitants. In humans, fecal isolates of subjects fed a diet devoid of lactobacilli could be compared to oral isolates using discriminative strain typing methods to identify strains autochthonous to the GIT. Strains whose ecological status is clearly identified are good candidates to elucidate ecological cohesions that take place within the gut environment, and should be included in functional and comparative genomic investigations to reveal how lactobacilli make a living in the intestinal tract. A better understanding of the ecology of lactobacilli will doubtless help us to more systematically develop probiotic applications.

It has become more and more evident that shifts in gut commensal populations and an aberrant immune reaction towards these microbes are associated with several disease conditions such as allergies, IBD, obesity, and colon cancer. Redress of these ecological and immunological imbalances, for instance by probiotics, has the potential to ameliorate and prevent disease (25). For lactobacilli to become successful in this respect, ecological and functional aspects of the strains should be considered already when screening candidates. As noted by Morelli, there is considerable doubt about the real value of the current selection criteria for probiotics, such as their tolerance to the hostile conditions of the stomach and the small intestine, and their ability to adhere to intestinal surfaces of epithelial cell lines (65). The ecological origin of the probiotic strain remains important, but this requires much more than just picking a fecal isolate. In the future, strains selection could be based on criteria such as ecological performance, persistence, and evolutionary history. Autochthonous strains that naturally persist in human subjects over long periods are tested by nature for their functionality in the gut, and they are likely to possess adaptive traits to benefits their human host. Strain selection should also be directly targeted at the alleviation or prevention of specific medical conditions. This is more difficult, as it requires a mechanistic understanding of the effect one wants to achieve, but ex vivo experiments with immune cells isolated from humans are likely to become very valuable in this respect.

It is important to note that while the majority of traditional probiotic strains are probably allochthonous to the intestinal tract, and they show very little ability to persist in the human gut. These strains might nonetheless be excellent

probiotics with respect to activation of the immune system. As there is no indication that colonization is required for the health benefits of these strains, research on traditional probiotic strains should focus less on the investigation of ecological fitness and the identification of putative colonization determinants, and more on the provision of mechanistic explanations for the health benefits that have been achieved in clinical trials.

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Figure 1. (A-D) Competition experiments between wild-type *L. reuteri* strains 100-23C (A and B), 100-23 (C), and TMW 1.106 (D) and isogenic mutants with insertional inactivations of the *lsp* (A), *msrB* (B), *dltA* (C), and *inu* (D) genes (102, 105, 106). Mixtures of mutant and wild type (1:1) were used to inoculate *Lactobacillus*-free mice, and the percentage of mutants in the total *Lactobacillus* population was determined after 7 days in the forestomach (FS), jejunum (JJ), and cecum (Cec). Data points of individual animals are connected by lines. (E) DGGE analysis of PCR-amplfied 16S rDNA fragments obtained with primers Lac1 and Lac2GC and DNA isolated from the crop, ileum (Ile) and cecum (Cec) of 4 chicken (age: 42 days) that were floor reared at the University of Nebraska (Martínez, Scheideler, and Walter, unpublished). M; Marker representing species isolated from the chickens. DGGE was performed as described by Walter et al., (104). (F) DGGE analysis of PCR-amplfied 16S rDNA fragments obtained with primers Lac1 and Lac2GC and DNA isolated from the esophagus close to stomach (Eso), pars esophagus (Pars), stomach content (Stom), duodenum (Duo) jejunum (JJ), ileum (Ile), cecum (Cec), proximal colon (PrCol), and distal colon (DisCol) of a male, castrated pig (age: 10 weeks) that was reared at the University of Nebraska (Martínez, Burkey, and Walter, unpublished). M; Marker representing species commonly present in pigs. DGGE was performed as described by Walter et al., (104).

Table 1. The representation of *Lactobacillus* sequences in molecular-phylogenetic analysis of the human gastrointestinal microbiota

Sample site	Stomach (tissue)[1]	Small intestine (tissue)[2]	JJ, Ile (tissue)[3]	JJ, Ile, (luminal)[5]	Ile & Col (tissue)[4]	Col & Rec (tissue)[3]	Colon, Rectum (luminal)[5]	Cec, Col, Rectum (tissue) & Fec [6]	Col (tissue)[2]	Col (tissue)[7]	Fecal sample[8,9]	Fecal sample[10]	Fecal sample[11]
Number of subjects	23	20	1	3	2	1	3	3	40	3	4	1	12
Total number of sequences	1,833	1,638	173	545	361	174	545	11,831	3214	110	927	284	18,348
Number of *Lactobacillus* sequences	4	5	0	87*	0	0	54**	0	157***	0	0	0	6****
% Lactobacillus sequences	0.22	0.31	<0.6	16	<0.3	<0.6	9.9	<0.01	4.9	<1	<0.11	<0.4	0.03

[1]Bik et al., (10), [2]Frank et al., (26), [3]Wang et al., (107), [4]Wang et al., (109), [5]Hayashi et al., (36); [6]Eckburg et al., (22), [7]Hold et al., (22), [8]Hayashi et al., (35), [9]Hayashi et al., (34), [10]Suau et al., (90), [11]Ley et al., (49).

*The species detected were *L. mali* (85 sequences) and *L. reuteri* (2 sequences).

**The species detected were *L. reuteri* (27 sequences), *L. mali* (20 sequences), *L. delbrueckii* (7 sequences).

***The main species detected were *L. delbrueckii* (108 sequences), *L. rhamnosus* (38 sequences), *L. reuteri* and *L. animalis* (each 5 sequences).

****Sequence identification was performed using the Classifier tool of the Ribosomal Database Project II (108) with a confidence threshold of 80% with the complete sequence data set kindly provided by Ruth Ley (Washington University, St. Louis).

Abbreviations: JJ, jejunum; Ile; Ileum; Cec, cecum; Col, colon; Rec, rectum; Fec, fecal sample.

Table 2. The representation of *Lactobacillus* sequences in the molecular-phylogenetic analysis of the gastrointestinal microbiota of animals

	Pig[1]	Mouse[2]	Mouse[3]	Rat[4]	Chicken[5,6]
Sample site	Ileum, cecum and colon	Small & large intestine, Fecal samples	Cecum	Fecal samples	Ileum and Cecum
Number of animals	24	Several	Several	several	several
Sample site	Luminal	Luminal & tissue	Luminal	Fecal sample	Luminal
Total number of sequences	4,270	70	5,089	109	1393
Number of *Lactobacillus* sequences	674	8	205*	25	490
% Lactobacillus sequences	15.8	11.4	4.0	22.9	35.2

[1]Leser et al., (46), [2]Salzman et al., (78), [3]Ley et al., (47), [4]Brooks et al., (12), [5]Lan et al., (44), [6]Lu et al., (51).

*Sequence identification was performed using the Classifier tool of the Ribosomal Database Project II (108) with a confidence threshold of 80% with the complete sequence data set kindly provided by Ruth Ley (Washington University, St. Louis).

Table 3. *Lactobacillus* species commonly detected in human feces, saliva, and in food.

Species	Feces	Oral cavity	Food
L. acidophilus	+	+	
L. crispatus	+ (P)	+	
L. gasseri	+ (P)	+	
L. johnsonii	+		+
L. salivarius	+ (P)	+	
L. ruminis	+ (P)		
L. casei	+	+	+
L. paracasei	+	+	+
L. rhamnosus	+	+	+
L. plantarum	+	+	+
L. reuteri	+ (P)		(+)*
L. fermentum	+	+	+
L. brevis	+	+	+
L. delbrueckii	+		+
L. sakei	+		+
L. vaginalis	+	+	
L. curvatus	+		+

(P) reported to persist in some human subjects (62, 72, 96)

**L. reuteri* can only be found regularly in sourdough and in other fermented cereals, such as fermented oat meal. Fecal isolates of these species are therefore unlikely to originate from food.

Table 4. Dose and recovery of allochthonous lactobacilli in human feces.

Bacteria	Daily dose (cells)	Reisolation (CFU/gram feces)	Reference
Probiotics			
L. rhamnosus GG	10^{10}	$10^5 – 10^8$	(38)
L. casei strain shirota	10^{11}	Around 10^7	(88)
L. rhamnosus DR20	10^9	10^5 - 10^6	(96)
Food lactobacilli			
L. paracasei	10^9	$10^7 – 10^8$	(13)
L. delbrueckii	10^{10}	$10^5 – 10^8$	(38)
L. casei	10^{10}	$10^5 – 10^8$	(38)
Oral lactobacilli			
ca. 20% of subjects >10^6 CFU/ml saliva	> 10^{9}*	?	(1) (43)
ca. 40% of subjects >10^5 CFU/ml saliva	> 10^{8}*	?	(16)

*based on a daily saliva output of >1000 ml

Table 5. Genetic factors shown to contribute towards ecological performance of lactobacilli in the gut of mice

Loci	Protein encoded	Strain	Why studied?	Putative function in the gastrointestinal tract	Reference
Lsp	Large surface protein	*L. reuteri* 100-23C[a]	Dominant surface protein	Adherence	(102)
msrB	Methionine sulfoxide reductase B	*L. reuteri* 100-23C	Gene expression specifically induced in vivo	Reduction of oxidized methionine residues, resistance to nitric oxide produced by epithelial cells	(102)
luxS	LuxS	*L. reuteri* 100-23C	Importance of AI-2 on the formation of biofilms by gram-positive bacteria	Quorum sensing (AI-2 prduction/ metabolic importance as part of activated methyl cycle	(95)
dltA	D-alanine-D-alanyl carrier protein ligase (Dcl)	*L. reuteri* 100-23	Importance of *dlt* operon for biofilm formation and adhesion of gram positive bacteria	Resistance against low pH values and defensins	(105)
gtfA	Glycosyltransferase A	*L. reuteri* TMW1.106	Importance of EPS for bacterial biofilm formation	Cell aggregation, biofilm formation	(106)
Inu	Inulosucrase	*L. reuteri* TMW1.106	Importance of EPS for bacterial biofilm formation	Cell aggregation, biofilm formation	(106)
LJ1680	IgA protease	*L. johnsonii* NCC533	In vivo expressed and associated with a long gut persistence phenotype	Degradation of immunoglobulin A	(19)
LJ1654-1656	PTS transporter	*L. johnsonii* NCC533	In vivo expressed and associated with a long gut persistence phenotype	Sugar utilization	(19)

[a]Plasmid free variant of *Lactobacillus reuteri* 100-23