Microbiology Spectrum
American Society for Microbiology Press

The Genomic Basis of Lactobacilli as Health-Promoting Organisms

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ABSTRACT Lactobacilli occupy a unique position in human culture and scientific history. Like brewer's and baker's yeast, lactobacilli have been associated with food production and preservation for thousands of years. *Lactobacillus* species are used in mixed microbial cultures, such as the classical *Lactobacillus bulgaricus*/*Streptococcus thermophilus* inoculum for yogurt fermentation, or combinations of diverse lactobacilli/yeasts in kefir grains. The association of lactobacilli consumption with greater longevity and improved health formed the basis for developing lactobacilli as probiotics, whose market has exploded worldwide in the past 10 years. The decade that followed the determination of the first genome sequence of a food-associated species, *Lactobacillus plantarum*, saw the application to lactobacilli of a full range of functional genomics methods to identify the genes and gene products that govern their distinctive phenotypes and health associations. In this review, we will briefly remind the reader of the range of beneficial effects attributed to lactobacilli, and then explain the phylogenomic basis for the distribution of these traits across the genus. Recognizing the strain specificity of probiotic effects, we review studies of intraspecific genomic variation and their contributions to identifying probiotic traits. Finally we offer a perspective on classification of lactobacilli into new genera in a scheme that will make attributing probiotic properties to clades, taxa, and species more logical and more robust.

BENEFICIAL EFFECTS ASSOCIATED WITH LACTOBACILLI

The genus *Lactobacillus* includes 177 species (http://www.bacterio.net/lactobacillus.html): they are non-spore-forming, mostly nonmotile, and rod-shaped (although coccobacilli are observed). They generally have a fermentative metabolism (although genome sequence analysis has provided evidence of potential for respiration [1]) with lactic acid as the main fermentation product.

Lactobacilli grow in rich carbohydrate-containing substrates such as food (dairy products, grain products, meat and fish products, beer, wine, fruits and fruit juices, pickled vegetables, mash, sauerkraut, silage, and sourdough), water, soil, and sewage; they are part of the microbiota associated with the mouth and gastrointestinal and genital tracts of humans and many animals (2).

With regard to their beneficial and protechnological properties, 35 *Lactobacillus* species have Qualified Presumption of Safety (QPS) status from the European Food Safety Authority (EFSA) (3) and 12 species are Generally Recognized as Safe (GRAS) by the FDA (http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices). Lactobacilli constitute 43% (84 species) of the total number of microorganisms with certified beneficial use (195 species representing 28 genera of phyla *Actinobacteria*, *Firmicutes*, and *Proteobacteria*) (4), with 22 of them represented by strains that are patented in Europe due to their potential probiotic properties (E. Salvetti and P. W. O'Toole, under revision).

Given the rising importance of lactobacilli as beneficial microbes, the focus of the present review is the genetic and genomic basis of health promotion by lactobacilli. A comprehensive survey of the discovery research that first identified these features is beyond the scope of this review, and the reader is referred to excellent recent surveys on this topic including Lebeer et al. (5) and Papadimitriou et al. (6).

Received: 12 January 2017, **Accepted:** 21 February 2017, **Published:** 23 June 2017
Editors: Robert Allen Britton, Baylor College of Medicine, Houston, TX, and Patrice D. Cani, Université catholique de Louvain, Brussels, Belgium
Citation: Salvetti E, O'Toole PW. 2017. The genomic basis of lactobacilli as health-promoting organisms. *Microbiol Spectrum* 5(3):BAD-0011-2016. doi:10.1128/microbiolspec.BAD-0011-2016.
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Beneficial effects associated with lactobacilli, which have been the subject of decades of research, may be classified into three broad categories—*in vivo* survival mechanisms, *in vivo* colonization mechanisms, and direct effects on the host (see Table 1 for representative examples). Arguably, only effects on the host are true "probiotic" traits, as defined recently by Hill et al. (7), but because surviving intestinal transit is probably required to exert beneficial effects, work aimed at developing probiotic strains and identifying probiotic features has traditionally included survival and colonization analysis, as well as metabolic adaptation to nutritional substrates typically available in the mammalian intestine. A fourth category, effect on the intestinal microbiota, has recently been formalized (8), although exerting a direct effect on other microbes such as pathogens has always been a recognized potential probiotic trait (5).

A preponderance of the literature on probiotic mode of action in lactobacilli is based on studies performed *in vitro* or in preclinical models, as distinct from in humans or in animals. As a consequence, there are many features and gene products whose actual contribution to probiotic function in lactobacilli is unclear. This is reflected in the lack of success in obtaining EFSA approval for probiotics and functional foods, mainly linked to insufficient characterization of the food and poor scientific support for the claimed effect (9), the absence of a beneficial physiological effect based on the scientific evidence assessed (10), and the nonrecognition of the property of preventing, treating, or curing a human disease with food (11).

In reviewing the literature at this juncture (Fig. 1), a clear stalling in investment in *Lactobacillus* probiotic research is apparent, reflected in the large knowledge gap in molecular mechanisms that still needs to be filled.

GENOMIC DIVERSITY OF LACTOBACILLI

The genome sequences of almost all *Lactobacillus* type strains and some historically associated genera were recently determined (12, 13), providing the definitive genomic framework for mining all relevant phylogenetic and functional information and corroborating the genetic basis of what had been described for nearly a century, namely the extreme phenotypic diversity of lactobacilli (2, 14).

Comparative analysis (12, 13) uncovered the extraordinary level of genomic diversity of the lactobacilli: the sizes of the genomes, in fact, range from 1.23 Mb to 4.91 Mb (four times larger) and the DNA GC content range is from 31.93 to 57.02%. The overall level of genome difference among the members of the *Lactobacillus* genus was found to be comparable to that between members of a bacterial family (12). Even more astonishing is consideration of the pairwise average nucleotide identity values that are comparable for those between members of taxonomic orders or classes (12). The genus as currently defined is polyphyletic, meaning it encompasses the descendants of several most recent common ancestors (MRCAs), specifically those of *Pediococcus*, *Weissella*, *Leuconostoc*, *Oenococcus*, and *Fructobacillus*. The overall significance of this unusually complex phylogenomic landscape is that it makes for a challenging research context. In comparison with studies of pathogens, for example, where comparative genomics has led to discovery of pathogenicity islands

TABLE 1 Selected examples of probiotic traits in lactobacilli

Category	Host benefit	Representative examples and effectors	Reference
In vivo survival	Viable bacteria reach site of action in the gut.	Acid resistance in *L. plantarum* by ATPases	93
		Bile resistance in *L. salivarius* by bile salt hydrolases	94
	Bacteria can metabolize dietary ingredients.	Metabolic diversity of intestinal lactobacilli	12
In vivo adherence	Maintaining bacterial numbers and close host cell association	Biofilm formation in *L. reuteri* 100-23	76
		Aggregation of *L. crispatus* cells during murine colonization	95
		Aggregation and adhesion protein allowing colonization by *L. gasseri*	96
		Production of bacterial surface adhesins	31, 97
Direct effects on host	Altered cellular or organ functions	Alteration of signal transduction, apoptosis and barrier function in epithelial cells by *L. rhamnosus* LGG proteins	98, 99
		Alteration of innate immune cell function by *L. acidophilus* S-layer protein	100
		Suppression of inflammation in colitis by histamine produced by *L. reuteri*	101
		Degradation of proinflammatory cytokines by *L. casei*	32
		Altered cytokine production due to *L. rhamnosus* pili	36
Effect on microbiota or pathogens	Restores normal microbiota or excludes pathogens	Anti-*Listeria monocytogenes* activity of *L. salivarius* bacteriocin	88
		Controversial effects of probiotics on the human gut microbiota	102

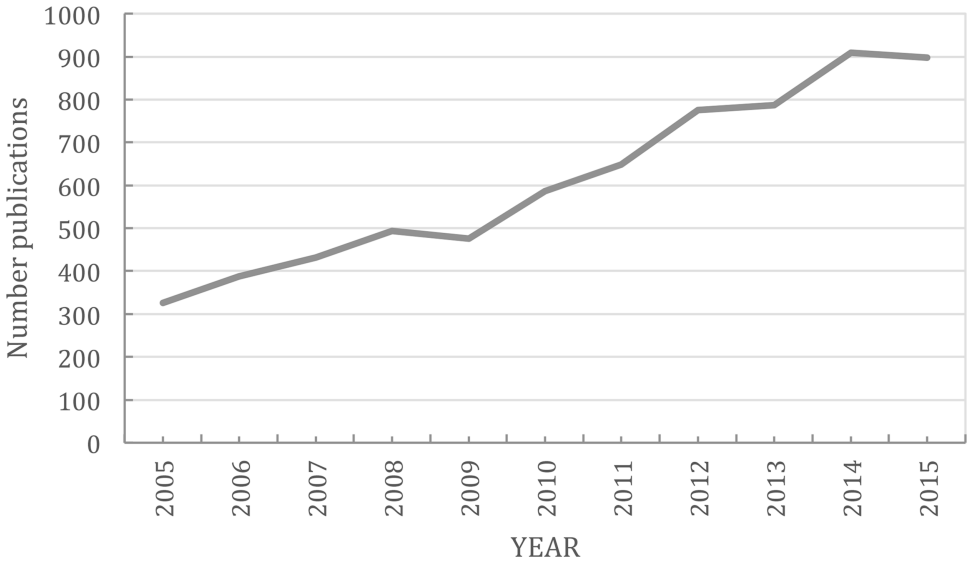

FIGURE 1 Publication numbers by year using search terms (lactobacillus probiotic) in PubMed. Search performed 13 July 2016.

and converting bacteriophages, such discovery paradigms are less helpful in lactobacilli, with the exception of the cobalamin biosynthesis/propanediol utilization island of *Lactobacillus reuteri* (15, 16). The imminent reallocation of the members of the current genus *Lactobacillus* across a number of smaller, genomically more cohesive new genera (Salvetti et al., in preparation) will provide a more sensible context for genotype-phenotype matching in the lactobacilli.

It is reasonable to expect to be able to understand the interaction of *Lactobacillus* species and their respective environments in the context of their genomic relatedness and genome content. A simple hypothesis is that the MRCAs of all the lactobacilli had a large genome with diverse metabolic capability, and that adaptive radiation to the range of niches occupied by contemporary species was marked by gene loss in those species whose niche is nutrient-rich like the mammalian body or fermented foods, and clade-specific gene acquisition in some clades.

DISTRIBUTION OF PROBIOTIC-RELATED TRAITS IN LACTOBACILLI

Despite these challenges, we and others have been mining the wealth of genomic data for lactobacilli to catalog the number and distribution of traits linked with probiotic function including those in Table 1. These analyses are ongoing (E. Salvetti et al., in preparation), but our initial observations are already instructive. The data provide a framework for testing the well-recognized phenomenon (17) of strain specificity of probiotic effects, and for using comparative genomics and genome annotation mining to uncover probiotic traits in other species. The outcome of analyses based on comparative genomics within species is discussed further below.

Carbohydrate Metabolism

Lactobacilli are saccharolytic but devote a lower proportion of their coding capacity to carbohydrate degradation than, for example, bifidobacteria (18) or *Bacteroides* spp. (19), even in the larger-genome species such as *L. plantarum* (20). High-throughput annotation of 213 genomes identified glycosyl hydrolases corresponding to 48 of the 133 families of glycoside hydrolases (GHs) in the CAZy database (http://www.cazy.org) (12). Newly identified enzymes included an endo-α-*N*-acetylgalactosaminidase in *L. brantae* and *L. perolens* that may be involved in mucus utilization and could thus be a colonization factor. Glycosyl hydrolase family 95 (GH95) enzymes had not previously been identified in the lactobacilli, but were identified in *L. harbinensis* and *L. perolens* (12), which were isolated from traditional fermented vegetables in China and spoiled soft drinks, respectively. GH95 is a fucosidase, a type of enzyme that is well recognized in bifidobacteria (21), *Bacteroides* spp. (22), and *Akkermansia muciniphila* (23). Although used by bifidobacteria for breaking down human milk oligosaccharides, most of the evidence suggests that these fucosidases are used by gut commensals or pathogens for metabolizing fucose residues on intestinal mucus (reviewed in reference 24). The unusually high

proportional gene count encoding glycosyl hydrolases in six *Lactobacillus* clades (12)—*L.* (*par*)*alimentarius*, *L. perolens*, *L. plantarum*, *L. rapi*, *L. fructivorans*, and *Carnobacterium* spp.—suggests adaptation of these clades to the selective pressure typically encountered in the gut. Members of these clades have been isolated from the gut of animals (i.e., goat, poultry, and honey bee stomach) and are used as feed additives or probiotics.

Genomics of Surface Carbohydrate Decoration

The major form of surface carbohydrate in Gram-positive bacteria, exopolysaccharide (EPS), contributes to technological features like product viscosity and texture, but also to host interaction (25–27). In *Lactobacillus*, EPS production is strain specific and growth medium dependent, and the EPS can be bound or released by the bacterial cell (e.g., 28, 29), making translation of *in vitro* findings to probiotic effects *in vivo* very challenging. Genus-wide genome comparison identified a number of glycosyltransferases that show restricted presence across species and that may be relevant for probiotic function. For example, the *L. gasseri* genome harbors a gene encoding GT11 (galactoside α-1,2-L-fucosyltransferase), and the *L. delbrueckii* DSM 15996^T encodes GT92 (*N*-glycan core α-1,6-fucoside β-1,4-galactosyltransferase). Production of fucose-containing lipopolysaccharide is an immune-evasion/antigenic mimicry strategy of pathogens such as *Helicobacter pylori* by virtue of the fucose-containing structures present in Lewis-type blood group antigens (30), and it will be interesting to know if lactobacilli use such a strategy to modulate innate immune interaction. The GT11 enzyme is predicted to be encoded by the *A. muciniphila* genome, so perhaps this trait is present in other gut commensals.

Surface Protein Repertoires

Surface proteins are the next topological interaction layer below EPS on *Lactobacillus* cells, and they thus represent an important interface with the external environment (31). As noted above (Table 1), an extracellular protease of the subtilase type produced by the *L. casei* strain present in the commercial probiotic cocktail called VSL#3 degrades IP-10, an inflammatory mediator involved in colitis (32). This appears to be the only published example of a surface-anchored enzyme contributing to a probiotic trait in lactobacilli (which is distinct from probiotic-derived soluble proteins such as p40 and p75, which have been detected in *L. casei* group members [33]). However, cataloging of the repertoire of this class of protease (lactocepin) in the lactobacilli identified 60 genes among 213 genomes, which were proportionally overrepresented in members of the *L. delbrueckii*, *L. casei*, *L. salivarius*, and *L. buchneri* clades, as well as the carnobacteria. The high level of sequence divergence found between the predicted lactocepin proteins (12) makes it at least plausible that the uncharted specificity of some of these proteases could include human proteins as targets.

A major class of surface proteins in Gram-positives is those that are covalently attached to peptidoglycan by a sortase transpeptidation reaction (34). Searching the translated protein data for the target motifs of the sortase enzyme identified the repertoire of sortase-anchored proteins in the lactobacilli. This identified 1,628 predicted LPXTG-containing proteins and 357 sortase enzymes in the 213 genomes. Species known to contain strains identified as being probiotic did not harbor unusually high numbers of sortase-anchored proteins; in fact, the greatest absolute number or genome-size-normalized number was in the milk isolate *Carnobacterium maltaromaticum*. The fact that sortase-anchored proteins may have almost any biological properties that cannot always be identified from their primary sequence (such as fibrinogen binding, exemplified in reference 35) makes this a difficult bioprospecting approach. A more productive screen identified 67 *pilus* gene clusters in 51 *Lactobacillus* strains, whereby the *pilus* gene search was based on the surface structures identified as mucin binding and immunomodulatory in *L. rhamnosus* GG (36). These clusters were present in clades and species not known to be probiotic, such as *L. thailandensis*, *L. ruminis*, and *L. koreensis*, broadening the avenues of exploration for new beneficial strains.

INTRASPECIFIC DIVERSITY OF *LACTOBACILLUS* SPECIES HARBORING PROBIOTIC STRAINS

Although a number of studies have addressed the similarity and the differences within species of the *Lactobacillus* genus through comparative genomics, knowledge of the evolutionary history and the genomic diversity below the species level is still incomplete. Unraveling the intraspecific diversity of lactobacilli is, in fact, fundamental for the regulatory perspective, for the development of identification tools for tracking isolates during industrial processes, and for the commercial standpoint to differentiate probiotics or starter cultures (37).

The effects of microbial strains that are marketed as established probiotics along with their clinical evidence assessed in clinical studies were recently reviewed by Di

Cerbo et al. (38) and Salvetti et al. (39), and they are summarized in Table 2.

Data regarding the evolutionary genomics and population structure of species harboring probiotic strains shown in Table 2 are outlined and reviewed below.

Lactobacillus acidophilus

First described by Moro in 1900, *L. acidophilus* is one of the most commonly used microorganisms for dietary applications and it is available in several foods such as milk, yogurt, formulas, as well as in dietary supplements with reported probiotic effects. The probiotics effects associated with *L. acidophilus* strains are resistance to bile and low pH, adhesion to human colonocytes in cell culture, antimicrobial production, and lactase activity, which contribute to the mediation of host immune response, lowering of serum cholesterol, improving host lactose metabolism, and preventing or treating infection (37).

The first genome sequence published was from strain NCFM (40) which led to the identification of several mucus- and fibronectin-binding proteins, implicated in the adhesion to human intestinal cells, several classes of transporters which were found to be finely regulated by carbohydrate source (induced by their respective substrates but repressed by glucose), likely contributing to the competitive ability of *L. acidophilus* in the human gastrointestinal tract, and nine two-component regulatory systems, some of them associated with bacteriocin production and acid tolerance (40, 37).

An updated population structure of *L. acidophilus* based on the comparative analysis of genomic sequences from 34 isolates showed that this species is monophyletic and characterized by a low rate of intraspecific diversity, with the commercial isolates identical at the genome sequence level (41). Although the phenotypic features of the isolates were diverse (i.e., the effects on the immune response following oral vaccination in healthy adults or differences in oxalate depletion), less variation is detected at the genomic level, in accordance with what was unraveled by other genotypic analyses as PCR-fingerprinting assays, randomly amplified polymorphic DNA analysis, and multilocus sequence typing (MLST). This suggests that commercial use has domesticated *L. acidophilus*, with genetically stable, invariant strains being consumed globally by the human popula-

TABLE 2 Species for which strains have been ascribed probiotic properties and related applications (according to references 38 and 39)[a]

Trait	*L. acidophilus*	*L. brevis*	*L. casei*	*L. crispatus*	*L. delbrueckii*	*L. fermentum*	*L. gasseri*	*L. johnsonii*	*L. paracasei*	*L. plantarum*	*L. reuteri*	*L. rhamnosus*	*L. sakei*	*L. salivarius*
Gastrointestinal mucosa adhesion	×						×				×	×		
Cancer	×											×		
Vaginal and urinary tract disorders	×	×		×		×	×			×	×	×		×
Hypercholesterolemia	×									×	×			
H. pylori treatment	×	×	×				×	×	×		×			
Oxaluria	×	×								×				
Mastitis						×	×							×
Immunomodulation	×		×			×	×		×	×	×	×	×	×
Gastrointestinal diseases	×		×		×					×				
Survival in the gut	×		×							×		×		
Diarrhea treatment	×		×		×				×		×	×		
Periodontal disease	×									×	×	×		×
Type 2 diabetes mellitus	×		×		×							×		
Muscle, bone, and cartilage diseases											×	×		
Skin disease	×							×	×		×	×	×	×
Ear, nose, and throat diseases	×		×						×					
Respiratory diseases	×		×			×			×	×	×	×		
Behavior/mental illness	×													
Other	×		×						×	×		×		×

[a]An × means the respective trait has been recorded in that species.

tion. A limited level of diversity is governed by the variable presence of three prophage remnants, designated as Potentially Autonomic Units (PAU, observed for the first time in *L. acidophilus* NCFM genome [40]), and a region of three contiguous loci with phage-related functions, whose distribution was linked to the isolate history, as commercial or culture collection derived. No active prophages were identified in the panel of strains, according with the absence of the recent description of phages active on *L. acidophilus* strains.

The remarkable genetic stability, supported also by the absence of extrachromosomal DNA, along with their effective phage resistance, has likely contributed to the commercial success of *L. acidophilus* strains, allowing manufacturers to maintain quality control of the cultures for probiotic production and dairy fermentations (41).

Lactobacillus brevis

L. brevis is an obligate heterofermentative Gram-positive organism that produces CO_2 as a side product from glucose metabolism. Strains of this species were isolated from plant materials, fermented beverages, and the human intestinal tract (2).

To date, all genome-based information regarding the intraspecific diversity of probiotic features in *L. brevis* mainly relates to two strains, ATCC 367 and KB290.

L. brevis ATCC 367 was the first *L. brevis* strain studied for probiotic features and its genome, sequenced in 2006 (42), revealed the presence of mucus-binding proteins and other surface layer proteins, which contribute to the adhesion to epithelial cells and extracellular matrices as fibronectin (31, 43).

The probiotic properties of strain KB290, isolated from a traditional Japanese fermented vegetable (*suguki*), include tolerance to gastrointestinal juices, immune system modulation, and gut health improvement (44, 45), and its genome sequence was reported in 2013 (46).

At the genomic level, the main difference between the two strains is the presence of nine plasmids in KB290, which constitute, to date, the highest number of plasmids ever detected among *Lactobacillus* species. The KB290 genome harbors genes for 375 unique proteins, while the ATCC 367 genome harbors genes for 169 unique traits. The majority of unique ATCC 367 genes encode for hypothetical proteins, while, among the unique genes in the KB290 genome, 177 encode proteins of known functions such as putative cell surface proteins, which might enhance the utilization of plant material, and proteins involved in the biosynthesis of cell wall-associated polysaccharides, which could contribute, on one hand, to form a protective shield against host complement factors in the gastrointestinal tract, and, on the other hand, trigger the host differential mucosal responses. The genomic regions harboring these genes in KB290 have a different DNA GC content compared to the genome average, indicating that this strain has undergone events of lateral transfer. This is also supported by the whole-genome alignment between ATCC 367 and KB290, which revealed huge rearrangements generated by homologous recombination between mobile elements, extensively distributed in both genomes.

The nine plasmids in KB290 together carry 191 predicted protein-coding genes (7% of the genome total). Although harboring plasmids constitutes a metabolic/fitness cost for host cells, no strains were found lacking all nine plasmids after plasmid curing attempts, suggesting that the plasmids impart a range of beneficial features to the host. Genes detected on the plasmids were involved in conjugation, presumptive cell wall polysaccharide biosynthesis and stress response (such as multidrug resistance transporters, which possibly confer bile resistance, or DNA protection proteins with ferritin-like domains that could enhance tolerance to oxidative stress and reduce lipid oxidation). Stress-inducible proteins contribute to the survival of probiotic bacteria in the harsh conditions they encounter in the host and they are effectively considered probiotic factors (6).

Based on the data reported by Fukao and colleagues (46), *L. brevis* is considered as a multiniche bacterium which, like other lactobacilli, contains genomic regions of laterally transferred genes; further research is needed to understand the role of *L. brevis* plasmids in the gut (46).

Lactobacillus casei/Lactobacillus paracasei

L. casei and *L. paracasei* are two phylogenetically closely related species, both members of the normal human gut microbiota, used in the food industries as starter cultures for dairy products or beneficial microbes, and reported to improve nutrition and to aid disease prevention and therapy (47).

The high genomic relatedness between these two species is the reason for the ongoing misidentification of strains belonging to *L. casei* and *L. paracasei*: according to the current valid nomenclature, the majority of the sequenced *L. casei* and *L. paracasei* strains would be allotted to *L. paracasei* subsp. *paracasei*, because all of them showed >99% identity with *L. casei* ATCC 334, which is currently the type strain of *L. paracasei* subsp. *paracasei* (48).

Given the interchangeable use of *L. casei* and *L. paracasei* names in many publications, the data presented below will refer to both species.

The evolutionary history of these species has been visualized through MLST, which showed the strains radiating into three distinct lineages (49), and comparative genomic hybridization (CGH), which revealed adaptation of these strains to cheese environment through genome decay of genes involved in carbohydrate utilization and transcriptional regulation (50).

A first comparative genomics analysis of 21 strains representative of the genetic, ecological, and geographical diversity of these species revealed a high level of synteny across the genomes and no major rearrangements (48). Strains of dairy origin had a prevalence of accessory genes with high homology (at protein level) to those detected in *L. fermentum*, another species commonly found in milk, while plant isolates showed the most diverse repertoire of genes coding orthologs with high amino acid identity to species commonly found in other plant isolates. These observations suggested the contribution of niche-associated gene exchange to the composite nature of *L. casei/L. paracasei*, also supported by the detection of a polycistronic region associated with lifestyle adaptation with high nucleotide identity with genomic regions in *L. plantarum* and *L. brevis*, and a polycistronic cluster for L(+)-tartrate catabolism and malate transport in *L. casei/L. paracasei* wine isolates with high identity with the same cluster in *L. plantarum*. Similar to other lactobacilli, horizontal gene transfer has been the dominant force in adaptation of these two species to new habitats and lifestyle in combination with the evolution of genetically distinct clusters shaped by extensive decay of genes associated with carbohydrate utilization (48).

Focusing more on probiotic factors, the analysis of the genomic intraspecific diversity of 34 other strains (dairy, plant, and human isolates) revealed the presence in the *L. casei/L. paracasei* core genome of several factors associated with host-microbe interactions such as cell-envelope proteinase, hydrolases p40 and p75, and the capacity to produce short branched-chain fatty acids (*bkd* operon), which could have an active part in the complex cross talk between bacterial strains and human or animal gut. A particular interest derived from the *bkd* operon, because the branched-chain fatty acids contribute to the preservation of the integrity of the colonic epithelium, inhibition of inflammation, and modulation of energy metabolism; the "fitness advantage" coming from this feature for the strains was provided by the generation of ATP from amino acid metabolism under anaerobic conditions in protein-rich anaerobic environments (51).

Lactobacillus crispatus

Strains belonging to *L. crispatus* have been isolated from the gastrointestinal tract of humans and animals, from the oral cavity, and, above all, from the urovaginal tract, where it counts for more than 80% of all vaginal bacteria (52). As the major component, *L. crispatus* contributes to the maintenance of the healthy vaginal microbiota, and its absence is correlated with several vaginal diseases (i.e., bacterial vaginosis). The beneficial effects described for this species include reduction of recurrent urinary tract infections and bacterial vaginosis in women and the inhibition *in vitro* of the growth, viability, and adhesion of uropathogens, suggesting a role for *L. crispatus* in protecting the vagina from invading pathogens (53, 54).

The intraspecific diversity of *L. crispatus* was investigated by mining the genome sequences of 10 strains (nine vaginal isolates and one from chicken cecum) (55), which revealed a general collinearity and synteny interrupted only by 5 to 21 genomic islands. These regions were rich in metabolism and EPS biosynthesis genes, prophages, and adaptive immunity traits, pointing to a role for these acquired elements in the adaptation of *L. crispatus* to varying habitats. The genomic fitness related to the adaptation to the vaginal environment was also reflected in the type of CRISPR/Cas systems, which were different between the vaginal isolates and the chicken isolate, and also by the presence of genes encoding enzymatic pathways for the utilization of carbohydrates (such as mannose) available in the vagina (56).

A total of 103 proteins with adhesion- and host colonization-related domains and 30 putative S-layer protein-encoding genes were also identified, along with six strain-specific adhesins, and a sortase-anchored protein with multiple mucus-binding domains.

All the strains under investigation were found to possess genes for antimicrobial substance production, including three to four L-lactate dehydrogenase *loci*, hydrogen peroxide-encoding genes, and sets of putative bacteriocin gene clusters, including two regions coding bacteriolysins. The promotion of the vaginal health may also benefit from the presence in the core genome of components with the same mucin- and fibronectin-binding domains of their counterparts produced by vaginal pathogens such as *Gardnerella vaginalis*, thus actively interfering with the adhesion of these pathogens to the vaginal mucosa.

The comparative genomics analysis of the 10 *L. crispatus* strains provided novel information on their adaptation to the vaginal environment as well as the factors for the competitive exclusion of pathogens, this unveiling the mechanisms at the basis of the role of this species in the maintenance of vaginal health (55).

Lactobacillus delbrueckii

L. delbrueckii is one of the most used lactic acid bacteria related to dairy food production, where, among others, the subspecies *bulgaricus* has been historically applied for yogurt production in protocooperation with *Streptococcus thermophilus*, while the subspecies *lactis* has been traditionally used for cheese making (57).

Yogurt is considered a nutritious, natural, and safe component of a healthy diet and it is at the basis of probiotic concept. Up to now, yogurt is the only functional food product for which a health claim has been validated in Europe, related to the attenuation of lactose intolerance. In addition, both *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were found to be correlated with immune modulation and diarrhea-alleviating effects (58).

The first complete genome sequence of *L. delbrueckii* subsp. *bulgaricus* showed a high number of rRNA and tRNA genes, a signal of a phase of genome size reduction, a higher GC content at codon position 3, supporting an evolution toward high GC content genome, and the loss of superfluous amino acid biosynthesis functions, which could be correlated to the adaptation to the protein-rich milk habitat (58).

The comparative genomics analysis of three strains of *L. delbrueckii* subsp. *bulgaricus* revealed that the three genomes shared a high number of genes encoding putative proteases or peptidases, which are essential for efficient utilization of environmental proteins, along with an aminotransferase, contributing to the transfer of branched-chain amino acids into corresponding α-keto acids, which are known to have cheesy flavors. Among stress tolerance genes, which are essential for industrial fermentation adaptation, a thioredoxin system (composed by two thioredoxin reductases and two thioredoxins) was found, along with a peptide methionine sulfoxide reductase, the genes associated with cell membrane biogenesis and extracellular housekeeping proteases, which confer stability at low pH and are assumed to play an important role in *L. delbrueckii* subsp. *bulgaricus* oxygen tolerance and acid response (59).

The subsp. *lactis* is distinguished from subsp. *bulgaricus* by its more extensive carbohydrate-metabolizing capability, such as sugars of vegetal-origin like maltose, mannose, saccharose, and trehalose.

The comparative genomic analysis of 10 *L. delbrueckii* strains (5 belonging to subsp. *lactis* and 5 to subsp. *bulgaricus*) revealed that *L. delbrueckii* subsp. *bulgaricus* genomes were smaller (1,810 to 1,872 kb) than those of subsp. *lactis* strains (1,844 to 2,125 kb) (57). This difference was linked to the presence of a higher number of IS elements in subsp. *lactis* than in subsp. *bulgaricus*. The genomes of both subspecies showed an aberrant GC content at the third codon position in coding sequences (already observed for subsp. *bulgaricus*), a high number of pseudogenes, and a tendency toward elimination of genes involved in amino acid biosynthesis and carbohydrate metabolism, thus reflecting an ongoing evolution and the adaptation to a protein-rich environment. The analysis of genes related to carbohydrate metabolism revealed that, in contrast to subsp. *lactis*, subsp. *bulgaricus* can only metabolize mannose in addition to the milk sugar lactose, thus showing a more advanced adaptation to the milk medium. A key adaptation to the milk environment is the presence of the major cell wall-bound protease PrtB responsible for the first step in the degradation of milk proteins in both subspecies, while it is not found in closely related lactobacilli.

An acquired lactose-galactose antiporter to import the milk sugar lactose was another important feature in all the strains examined (which is the transport system of choice in a lactose-rich environment), while the ancestral dedicated phosphotransferase system (PTS) (which excels in conditions where the substrate concentration is low) was detected only in *L. delbrueckii* subsp. *lactis* (57). This suggests the evolution of the ancestral organism in the mammalian digestive tract, an environment where both conditions are met. This observation is also consistent with the fact that most of the known closely related lactobacilli are gut isolates and with the presence of genes coding for putative mucus-binding proteins in the majority of *L. delbrueckii* subsp. *lactis* strains under study.

Since subsp. *bulgaricus* is historically faster and more reliable in milk fermentation than the subsp. *lactis*, the similarity of their genomes indicates that the industrially relevant differences between the two subspecies are likely found in gene regulation rather than gene content (57).

Lactobacillus fermentum

L. fermentum is an obligate heterofermentative lactic acid bacterium that is usually isolated in fermented food and in the gastrointestinal tract of humans and animals.

To date, two *L. fermentum* strains are commercially available, namely CECT 5716 and ME-3, which were shown to reduce inflammation and intestinal damage *in vivo*, to improve the effects of influenza vaccination in healthy volunteers, and to have antioxidative properties (60–62).

To date, no extensive analysis of the genomic intraspecific diversity related to the probiotic traits has been performed for this species. However, interesting information can be collected from the study by Archer and colleagues (63) where, in the framework of a project aiming to study the probiotic potential of a panel of acid- and bile-tolerant strains, 12 *L. fermentum* strains (three from infant feces and nine from homemade curd) were found to harbor genes coding for a bile salt hydrolase, a fibronectin-binding protein, a mucin-binding protein, a sortase, and an ATP-binding substrate protein, which also showed 100% similarity both in fecal and dairy strains (63).

In addition, the description of four *L. fermentum* genomes (strains 3872, MTCC 8711, CECT 5716, and F-6) allowed the detection of genes coding for mucus- and collagen-binding proteins, bile salt hydrolases, and proteins involved in EPS production, likely involved in the adhesion mechanisms (64, 65).

A recent picture of the general genomic diversity of this species can be depicted by the MLST derived from 203 *L. fermentum* isolates from different regions and products, which indicated that this species had a clonal population structure and its evolutionary history is not correlated with geography or food type (66).

Lactobacillus gasseri

L. gasseri belongs to the *L. acidophilus* complex and it is usually found in several sites of the human body, such as the mouth, intestines, feces, or vagina. Strains of this species are considered as members of the human intestinal "probiome," which includes commensal intestinal bacteria with beneficial effects on human health (67).

Genome sequencing of strain ATCC 33323^T, of human origin, allowed the detection of a high number of genes coding for proteins predicted to be essential in the gastrointestinal tract, such as bile salt hydrolases, bile transporters and drug resistance traits, cell surface structures, 2CRSs and other transcriptional regulators, *luxS*, bacteriocin and restriction/modification systems and traits involved in sugar transport and metabolism, oxalate degradation (reducing the incidence of disorders related to high levels of oxalic acid in the urine), and stress resistance.

Recently, the genome sequences of four vaginal *L. gasseri* strains were compared, showing a total of 122 protein families shared by all four strains but absent in other vaginal species. Traits reflecting organismal interactions were elucidated, such as an addiction module toxin, a toxin-antitoxin addiction module regulator, and a protein of the toxin-antitoxin system AbrB family; the genomes also harbored a pediocin immunity protein, a signal transduction histidine kinase regulating citrate and malate metabolism, and strain-specific aminotransferases, transcriptional regulators, and inner permeases. The presence of unique proteins not detected in other vaginal lactobacilli suggested that this species has experienced lineage-specific gene gain and loss.

Because this comparative genomics is only based on four vaginal strains, the combined analysis of an increasing number of genome sequences of strains within the same species will help to delineate species-specific genes that influence the ecological and evolutionary dynamics of this species (68).

Lactobacillus johnsonii

L. johnsonii is a natural inhabitant of the gastrointestinal tracts of several hosts, including humans, mice, dogs, poultry, pigs, and honeybees.

The probiotic-associated activities reported for this species are, among others, pathogen inhibition in the chick gut, alleviation of diabetes symptoms, reduction of serum cholesterol levels, immunostimulation, and adhesion to intestinal epithelial cells (69)

The genome sequence of *L. johnsonii* NCC533, a human isolate extensively studied for its probiotic properties, showed that this commensal strain was deficient in biosynthesis of amino acids, purine nucleotides, and cofactors, but it harbored, in compensation, an impressive array of transporters, peptidases, and proteases, along with PTS sugar transporters and β-galactosidases, indicating a reliance on mono-, di-, and trisaccharides for its fermentative metabolism and a major adaptation in the upper gastrointestinal tract, where amino acids, peptides, and lower-order oligosaccharides are abundant. Further metabolic cassettes for saccharide metabolism, cell surface proteins, bile salt hydrolases and bile transporters were identified (69).

Phenotypic analysis and CGH analysis between this strain and the type strain of the species, *L. johnsonii* ATCC 33200^T, showed a lower intestinal persistence in ATCC 33200^T and allowed the detection of 233 NCC533-specific genes associated with the long-gut-persistence phenotype including surface proteins and translocases, PTS transporters, bacteriocin, and proteins involved in EPS synthesis (26).

Although an extensive comparative genomic analysis between *L. johnsonii* strains is yet to be reported, a large survey of 39 isolates from fecal-bacterial populations of a few host species was performed with the Simple Sequence Repeats assay and MLST analysis that resolved the isolates into three clusters, according to their hosts (chickens, humans, or mice) (70). These data suggest a phylogenetic separation paralleling host specificity that arose as a result of coevolution of the host and its gastrointestinal tract microbiota.

The bacterial-host specificity identified in *L. johnsonii* constitutes an interesting element to be considered for the selection of health-promoting specific strains based on the microorganisms and host genetics (70).

Lactobacillus plantarum

L. plantarum is one of the most widely known *Lactobacillus* species because of its distribution in a variety of environmental niches (many types of fermented foods, and human body), its versatility, and its metabolic capacity, which facilitates its use in several industrial settlings, either as starter cultures or probiotics. The probiotic properties related to this species are mainly linked to health promotion in humans and animals, and members of this species were found to reduce the concentration of cholesterol and fibrinogen and the risk of cardiovascular diseases and atherosclerosis (71).

L. plantarum WCFS1 was the first sequenced *Lactobacillus* genome (20). It harbored traits for stress response and gastrointestinal tract survival, substrate utilization and respiration, quorum sensing and bacteriocin production, host interaction (with the epithelial barrier as well as with the immune system), modulation of cell shape or surface properties, and interaction with food components and other microorganisms (72).

The comparative genomics analysis based on six strains showed a very high conservation of gene order and sequence identity of orthologs; however, a variety of highly variable regions were detected which mostly included (i) prophages, IS elements and transposases (highly diverse both in gene content and insertion position); (ii) the plantaricin (*L. plantarum*-associated bacteriocin) biosynthesis cluster (composed by highly conserved genes together with less conserved traits); (iii) the CPS/EPS biosynthesis genes; and (iv) the sugar lifestyle cassettes (accumulated within their lifestyle adaptation region). An additional extent of diversity was provided by the presence of numbers of repeated domains, particularly in extracellular proteins (such as adhesins or membrane-anchored protein), which play a role in the interactions between strains and their environment.

These data suggested that the genome diversity of *L. plantarum* is high and explain its flexibility and versatility, which allow this species to succeed in diverse niches and applications. In particular, the presence of genomic islands containing mosaic cassettes of (likely laterally acquired) carbohydrate-metabolism genes indicates the development of a "natural metabolic engineering approach" by *L. plantarum* strains that allows them to optimize their genomes for growth in specific niches (73).

The genome sequences of 54 *L. plantarum* strains isolated from different food sources and natural hosts (as human and insect) (74) revealed a high genetic conservation for orthologous groups involved in energy metabolism, or biosynthesis or degradation of cellular structural components, such as nucleotides, proteins, lipids; however, high variability was shown in regions including genes involved in EPS biosynthesis, restriction modification, sugar-importing PTS and other transport functions, sugar metabolism, and bacteriocin production, as well as elements like prophages, insertion sequences, and transposases. The analysis of the gene content related to the origin of the strain indicated that gene distribution poorly reflected strain origin, different from what was already observed for other lactobacilli such as *L. reuteri* and *L. rhamnosus*.

The absence of niche specialization (74) showed that *L. plantarum* did not undergo the process of bacterial adaptation to specific environments, because it acquired and retained functional capabilities independently of its niche, representing a typical example of a "nomadic" bacterial species.

Lactobacillus reuteri

L. reuteri is autochthonous to several vertebrates because members of this species are isolated from mammalian and avian gastrointestinal tracts, human urogenital tract, and breast milk. It showed several strain-specific beneficial properties relevant to human health including, for example, the production of essential B complex vitamins (folate, cobalamin, thiamin, and riboflavin) and antimicrobial compounds (i.e., reuterin); in addition, *L. reuteri* is considered a model organism for studying host-symbiont interactions as well as microbe-host coevolution. Lineage-specific genomic differences were revealed by the multilocus sequence analysis (MLSA) of more than 100 strains, reflecting the niche characteristics in the gastrointestinal tract of respective hosts. Interestingly, human-derived *L. reuteri* strains clustered in two distinct MLSA clades; one of them (namely clade II) is related specifically to humans, while strains in

the other (clade VI) are closely related to isolates from chickens (75).

To gain insight into the distinguishing features of human-derived *L. reuteri* strains, comparative genomic analysis performed on 10 strains from three host origins (human, rat, and pig) unveiled two distinct populations in *L. reuteri* and, among human isolates, the same two clades (clades II and VI) observed with the MLSA. This observation led to the hypothesis that the two human-derived clades had been shaped by different evolutionary forces, since they were as dissimilar to one another as they were to clades that contained rodent- or porcine-derived strains. The two clades, in fact, were characterized by (i) the presence of clade-specific mobile genetic elements (as two complete prophages in clade II genomes and clade-specific transposase families), (ii) distinct metabolic functions and probiotic phenotypes (clade-specific order and composition of genes related to arginine catabolism mechanism and folate production); (iii) diverse rate of production of reuterin, which was enhanced in clade VI strains; (iv) the presence of clade specific of the transcriptional PocR (which gene cluster showed only 80% of identity between clade II and clade VI); (v) the differential effects on cytokine production by human myeloid cells exposed to strain supernatants; (vi) the histamine production by *L. reuteri* clade II strains that corresponded with anti-inflammatory properties.

These differences reflected the distinct ecology of these strains and the symbiotic relations they establish and maintain with their hosts. Clade II strains were all from human fecal samples and they did not cluster with isolates from other hosts, suggesting these members as part of the autochthonous *L. reuteri* population in the human intestinal tract; conversely, strains in clade VI clustered with poultry strains, and thus they might be allochthonous to humans originating from poultry.

As for the non-human isolates, the host specificity of *L. reuteri* in the mouse gut is mediated by specific adhesins and other adaptation factors including the urease cluster, an IgA protease, and genes involved in biofilm formation (76). This specificity was supported by experiments in gnotobiotic mice, which demonstrated that only rodent strains colonized mice efficiently.

These data provided new hints related to host-microbe relationship, and they also highlighted the impact of distinct evolutionary paths within the same species, which determine how microbes act on the fitness of their hosts (77).

Lactobacillus rhamnosus

L. rhamnosus is commonly found in a variety of ecological habitats, including artisanal and industrial dairy products, the oral cavity, the intestinal tract, and the vagina. This species includes the allegedly best-characterized probiotic strain, namely *L. rhamnosus* GG, which displays a wide array of probiotic properties including the reduction of diarrhea, atopic eczema, and respiratory infections (78).

Its genome sequence, released in 2006, showed the presence of genes for three secreted LPXTG-like pilins (*spaCBA*) and a pilin-dedicated sortase that is essential for mucus interaction, likely explaining its ability to persist in the human intestinal tract (79).

An extensive comparative and functional analysis based on 100 strains showed the presence of two geno-phenotypic groups (namely A and B): group A clustered strains which lack of *spaCBA* pili, a different carbohydrate metabolism profile (they could assimilate D-lactose, D-maltose, and L-rhamnose) and a distinct CRISPR system, indicative of the adaptation to a dairy-like environment; conversely, group B included strains characterized by a specific set of traits that confer more competitive fitness to the intestinal tract, such as bile resistance, pilus production, and L-fucose metabolism. Based on these data, strains of group B, which were also very similar to *L. rhamnosus* GG, are likely to be autochthonous in the gastrointestinal tract, and thus actively exert beneficial effects on it (80).

Further information derived from the comparative genomic analysis of two phylogenetically related marketed probiotic strains, *L. casei* BL23 and *L. rhamnosus* GG, unveiled a high degree of synteny, interrupted only by genomic islands with prophages, transposases, and sugar transport systems, confirming again the role of horizontal gene transfer in bacterial evolution. Shared proteins included the identical *spaCBA-srtC* gene cluster, which was also found in other *L. casei* strains. Conversely from *L. rhamnosus* strains, none of *L. casei* strains produced pili, despite the high level of conservation and sequence identity. This could be explained by the transcriptional start site of the *spaCBA* operon, which was characterized by the presence of an IS element in *L. rhamnosus* strains (but absent in *L. casei* strains) that triggers the expression of pili, conferring on *L. rhamnosus* strains a beneficial trait to colonize and persist in mucosa-associated niches (81).

A further comparative genomic analysis based on 40 strains of *L. rhamnosus* from various niches (mostly fermented foods and human-associated niches) provided a better understanding of the variome-associated genes

and their distribution in terms of metabolic and regulatory diversity. Furthermore, horizontal gene transfer events were detected in some strains or clades that involved genes related to carbohydrate transport and catabolism functions, EPS biosynthesis, bacteriocin production, restriction modification systems, and bacterial defense systems (CRISP-Cas) together with other elements that reflect niche adaptation such as the diversity of extracellular functions putatively involved in host interactions (i.e., cell adhesion or host immune system modulation) (82).

Lactobacillus sakei

L. sakei is a psychotrophic lactic acid bacterium found naturally on fermented plant material, meat products, and fish. This microorganism is widely known for its biotechnological potential in biopreservation and food safety rather than probiotic properties (which were assessed for strains isolated from the human gut [83]), and it is used as a starter culture for the controlled production of fermented meats.

The analysis of the first genome sequence produced (*L. sakei* 23K) showed a combination of several features used by the organism to adapt and grow in meat products rather than in the gastrointestinal tract, such as the ability to exploit purine nucleosides, abundant in meat, for growth and energy production, and to degrade arginine when carbon sources are lacking; a versatile redox metabolism, combined with iron and heme acquisition and the capability to produce biofilm, allows this microorganism to withstand oxidative stresses and proliferate on meat surfaces (84).

A CGH approach in combination with fermentation profile analysis of 10 and, more recently, 18 strains of *L. sakei* (taking strain 23K as a reference) mainly revealed that the features observed in 23K are distributed also in the other strains of different origin, and they constitute the common gene pool invariant of this species. Interestingly, the clustering based on carbohydrate-fermentation patterns divided the panel of strains into two phenotypic groups that were not consistent with the two genetic groups that emerged with the genome hybridization. In addition, several *rrn* clusters were observed in all strains and they can be related to the ability of an organism to achieve faster doubling times, suggesting the rapid adaptation by this microorganism to changing environmental conditions. No differences were detected between the strains belonging to the two *L. sakei* subspecies, suggesting that niche-specific genes are components of *L. sakei* pangenome (83, 85).

Lactobacillus salivarius

L. salivarius is a natural resident of the oral cavity and the gastrointestinal tract of both humans and animals, and it has been also isolated from human breast milk. The probiotic properties of members of this species include the immunomodulatory effects in cell lines, mice, rats, and humans and the ability to inhibit pathogens, alleviating intestinal disease and promoting host well-being (86).

The first genome sequence available for this species was that from strain UCC118 in 2006, a strain isolated from the terminal ileum of a healthy patient that has been extensively studied for its beneficial properties both in human trials and animal models. The genome comprised a circular chromosome, a megaplasmid, and two plasmids. Genes responsible for the synthesis (*de novo* or by interconversion) of nine amino acids and exopolysaccharides were identified both on the chromosome and on the plasmids, as well as genes related to the central carbohydrate metabolism and transport, including also those of the pentose phosphate pathway. This indicated for the first time that *L. salivarius* should be grouped among the facultatively heterofermentative instead of homofermentative lactobacilli (87), a feature that was also confirmed phenotypically. In addition, the megaplasmid harbored the genes encoding a two-component class IIb bacteriocin, namely Abp118, which is protective against the invasive foodborne pathogen *Listeria monocytogenes* (88). Taken together, all these data indicated how the presence of a multireplicon genome architecture contributed to the metabolic flexibility and adaptation of *L. salivarius* UCC118 to dietary fluctuations and the varying environments encountered in the gastrointestinal tract of different hosts (89).

The genome diversity of *L. salivarius* was explored applying MLST and CGH on a collection of 33 strains derived from different ecological niches, with diverse plasmid content and phenotypic traits. The hybridization signals identified 18 regions characterized by variable traits mainly related to niche adaptation and survival, and they included transposases, bacteriophage genes, CRISPR loci, EPS biosynthesis, and carbohydrate metabolism. Interestingly, the pseudogene number was very different among the panel of strains, suggesting genome decay and an ongoing adaptation within the species. Three major clusters were observed, but they were not consistent with the isolation sources: however, most of the animal-associated isolates clustered together by hierarchical analysis of EPS cluster I and II, whose distribution in the genomes added an additional extent of diversity among the strains (28).

Data reported by Raftis and colleagues showed that the level of diversity in *L. salivarius* was higher than that in *L. plantarum* and *L. casei*, which was also related to the limited clustering of strains from the same origins, as well as the poor correlation with complex phenotypes (as EPS production) (28).

THE CONTROVERSY IN *LACTOBACILLUS* TAXONOMY

Understanding and ascribing the beneficial effect of particular strains of lactobacilli to the species level is challenging because of the poor correlation between the phylogenetic relationship and the physiological properties of *Lactobacillus* species (13). Since its description by Beijerinck in 1901, the genus *Lactobacillus* has dramatically expanded in membership, often resulting in significant taxonomic changes, causing confusion and leading to the misidentification of lactobacilli (90).

As already mentioned, the most updated phylogenomic analysis based on 73 core proteins of 175 *Lactobacillus* species showed a high molecular diversity that is far too broad to encompass a single well-defined genus, reflected by the DNA GC content and phenotypic diversity (12).

An ongoing multilocus sequence typing and network analysis in our laboratory based on 29 ribosomal proteins and 12 established phylogenetic markers in 238 genomes of *Lactobacillus* and related genera (namely *Pediococcus*, *Leuconostoc*, *Weissella*, *Fructobacillus*, and *Oenococcus*) confirms that genus *Lactobacillus* is polyphyletic, intermixed with the other genera of family *Lactobacillaceae* and *Leuconostocaceae* and characterized by a complex evolutionary history (Salvetti et al., in preparation). The combination of sequence-based (phylogeny) and distance-based methods, namely, the average nucleotide identity (ANI), the average amino acid identity (AAI) and the percentage of conserved proteins (POCP), reveals the presence of 10 consistent subclades whose suitability to be nuclei of novel genera is being substantiated through the ongoing investigation of clade-specific genes and other conventional taxonomic data.

Members of these groups have been shaped by similar evolutionary events and are characterized by patterns of presence/absence of specific sets of genes that may be used as novel tools for their characterization. The absence, in fact, of a discriminative phenotypic feature supports the description of novel genera starting from the genotypic subclusters. This represents the most coherent driving force available to improve the taxonomic description of the genus and to prevent *Lactobacillus* from a never-ending expansion.

The creation of more uniform taxonomic nuclei within the *Lactobacillus* genus will also prevent misidentification issues that are still the major cause of mislabeling of probiotic food products reported worldwide (91). The determination of the genus, the species, and the strain contained in a probiotic product is the first essential requirement for a novel food marketing authorization and a health claim submission (92). Taxonomic characterization provides, in fact, information regarding the main physiological, metabolic, beneficial, and safety properties of the organism.

In addition, unravelling the taxonomic relatedness of health-promoting lactobacilli together with the analysis of the mechanisms by which they adapt to specific environments will provide a new framework for the selection of innovative beneficial microbes.

ACKNOWLEDGMENTS

This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No [659801]. Microbiome research in P.W.O'T's laboratory is supported by Science Foundation Ireland through a Centre award to the APC Microbiome Institute, and by FIRM awards to the ELDERFOOD and IMMUNOMET projects from the Dept. Agriculture, Fisheries and Marine of the Government of Ireland.

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