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(MahenthiralingamE@
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Lactobacillus acidophilus is a Gram-positive lactic acid bacterium that has had widespread historical use in the dairy industry and more recently as a probiotic. Although *L. acidophilus* has been designated as safe for human consumption, increasing commercial regulation and clinical demands for probiotic validation has resulted in a need to understand its genetic diversity. By drawing on large, well-characterised collections of lactic acid bacteria, we examined *L. acidophilus* isolates spanning 92 years and including multiple strains in current commercial use. Analysis of the whole genome sequence data set (34 isolate genomes) demonstrated *L. acidophilus* was a low diversity, monophyletic species with commercial isolates essentially identical at the sequence level. Our results indicate that commercial use has domesticated *L. acidophilus* with genetically stable, invariant strains being consumed globally by the human population.

The lactic acid bacteria (LAB) are a group of Gram-positive bacteria united by their ability to produce lactic acid as a major end product of carbohydrate metabolism¹ which has resulted in their artisanal and industrial use in dairy fermentations². A history of safe consumption of lactobacilli is widely acknowledged, but more recently specific LAB strains associated with health benefits have been sold as probiotics, establishing a market sector in excess of \$100 billion annually^{2,3}. Most of the marketed strains belong to the genera *Bifidobacterium* and *Lactobacillus*. The lactobacilli are a highly heterogeneous taxonomic group, encompassing species with a wide range of genetic, biochemical and physiological properties⁴. The number of validly named *Lactobacillus* species has considerably increased in the last 10–15 years, with 201 species currently described^{5,6}. The standards for the designation of new *Lactobacillus* taxa have also been recently updated, underlining the importance of applying the same rigorous standards to previously described and taxonomically assigned isolates⁶.

Lactobacillus acidophilus is added to commercial yoghurts and dairy formulations both for its flavour and for probiotic effect, and is one of the most commonly selected *Lactobacillus* species for dietary use^{7–9}. Taxonomically, *L. acidophilus* has undergone multiple revisions concurrent with changes and enhancements in techniques to investigate taxonomic relationships, from biochemical tests, through DNA sequence-based approaches, to comparisons utilising the whole genome sequence (WGS)¹⁰. The phenotypic and biochemical characteristics of individual *L. acidophilus* isolates show evidence of diversity^{11,12}; however, recent genotypic analyses indicate less variation is present within the *L. acidophilus* genome. PCR fingerprinting demonstrated that five independent *L. acidophilus* isolates grouped as a single strain genotype by Randomly Amplified Polymorphic DNA (RAPD) analysis¹³. A seven-locus Multi Locus Sequence Typing (MLST) scheme revealed that the *L. acidophilus* isolates examined encompassed just two MLST allelic profiles, with distinct isolates differing by a single nucleotide¹⁴. Recent studies independently examining the genomic sequences of three (NCFM, ATCC 4796 and La-14)¹⁵ and five (CIP 76.13^T, CIRM-BIA 442, CIRM-BIA 445, DSM 20242 and DSM 9126)¹⁶ *L. acidophilus* genomes, have also shown remarkable levels of genetic identity.

The limited infraspecific level diversity of *L. acidophilus* warrants further investigation in light of its taxonomy, worldwide commercial use and food-based consumption, and the increasing clinical role of probiotics. High throughput DNA sequencing enables rapid acquisition of bacterial WGS allowing researchers to comprehensively interrogate phylogenetic relationships between groups of bacterial isolates at taxonomic levels from domain to strain¹⁷. Here, we elucidate the infraspecific phylogenetic relationships between *L. acidophilus* isolates using reference-free, *de novo* assembly of whole genome sequence data, combined with hierarchical gene-by-gene analysis of whole genome sequences (WGS)¹⁸. Examination of a collection of *L. acidophilus* isolates spanning

Table 1 | *L. acidophilus* isolates and genome sequences examined in this study

Isolate	Strain aliases	Year	Isolate source	Genome status	Genome source/reference
Culture collection isolates					
LMG 9433 ^{T a,b}	ATCC 4356; LMG 13550	1964	Human	draft	This study
LMG 11428 ^{a,b}	ATCC 832; Rettger 4B	1922	Rat	draft	This study
LMG 11466	ATCC 13651; DSM 9126	1960	National Institute for Research in Dairying (Reading, UK)	draft	This study
LMG 11467	ATCC 314; L. F. Rettger 43	1920	Human	draft	This study
LMG 11469	ATCC 4355; Kulp R-1-1	1924	Rat	draft	This study
LMG 11470 ^{a,b}	ATCC 4796	1980	-	draft	This study
LMG 11472	ATCC 9224	1950	-	draft	This study
LMG 13550 ^{T a}	LMG 9433 ^T ; ATCC 4356	1964	Human	draft	This study
LAB 283 ^a	LMG 11430; ATCC 4357; Kulp strain PAK	1963	U Gent	draft	This study
LAB 66	LMG 11428	1922	U Gent	draft	This study
LAB 69	-	-	U Gent	draft	This study
LAB 76	LMG 11428	1922	U Gent	draft	This study
CIP 76.13 ^T	LMG 9433 ^T ; ATCC 4356	1964	Human	draft	¹⁶
DSM 20242	ATCC 4357; LMG 11430; LAB 283	1963	-	draft	¹⁶
DSM 9126	ATCC 13651; LMG 11466	1960	-	draft	¹⁶
ATCC 4796	LMG 11470	1980	Human Microbiome Project (HMP)	draft	NCBI RefSeq Assembly [ACHN01000000]
ERR203994 ^T	ATCC 4356 ^T	-	-	draft	NCBI Short Read Archive
Commercial isolates					
C21		2008	Commercial isolate	draft	This study
C46		2008	Commercial isolate	draft	This study
C47		2008	Commercial isolate	draft	This study
C49		2008	Commercial isolate	draft	This study
CUL 21 ^{a,b}	NCIMB 30156	2004	Commercial isolate	draft	This study
CUL 60 ^{a,b}	NCIMB 30157	2004	Commercial isolate	draft	This study
CuT2		2008	Commercial isolate	draft	This study
HBCA		2008	Probiotic product	draft	This study
NCFM ^{a,b} (isolate)	Derived from LMG 9433 ^T	1975	Commercial probiotic	draft	This study
Rm 344 ^{a,b}		2012	Commercial isolate	draft	This study
Rm 345 ^a		2012	Commercial isolate	draft	This study
NCFM (Reference genome)	Derived from LMG 9433 ^T	1970	Derived from LMG 9433	complete	NCBI RefSeq [CP000033] ²³
CIRM-BIA 442		-	Dairy product	draft	¹⁶
CIRM-BIA 445		-	Dairy product	draft	¹⁶
La-14		-	Danisco/DuPont (Brabrand, Denmark)	complete	¹⁵
Unknown isolate					
ERR256998	FLI007	-	-	draft	NCBI Short Read Archive

^aIsolate phenotype examined by MALDI-TOF analysis.^bIsolate growth kinetics examined by Bioscreen C analysis.

a timeframe of 92 years, multiple geographic locations and distinct sources, demonstrated an absence of genetic diversity within the species and WGS identity for strains in current commercial use.

Results

***L. acidophilus* lacks genomic diversity compared to other *L. acidophilus* group species.** Within the bacterial genus *Lactobacillus* multiple phylogenetic groups have been defined based on analysis of 16S rRNA gene sequences, with the most recent analysis placing *L. acidophilus* within a cluster designated as the *Lactobacillus delbrueckii* group¹⁹. The same group of closely related *Lactobacillus* species has also been referred to as the *L. acidophilus* group in multiple previous studies^{14,20,21} and this terminology is adopted herein. Despite the widespread use of *L. acidophilus*, the number of polyphasically identified isolates of this species deposited within well characterised collections of LAB was limited and previous analysis had shown very little genetic diversity within this species^{9,13,14,16}; 24 isolates and 10 *L. acidophilus* genomes were collected as representative of the available diversity of this species (Table 1). To contextualise the genetic diversity of *L. acidophilus* isolates at the

species level, ribosomal MLST (rMLST)²² was used to evaluate the genomic diversity present in species representative of the *L. acidophilus* group. Fifty-three genes that encode ribosomal proteins (rps) were identified in all 32 *L. acidophilus* genome sequences examined (Table 1), and among 108 reference genomes available from species within the *L. acidophilus* group¹⁹ (Table S1). The *L. acidophilus* group (represented by nine species) encompassed 99 rMLST types, with no two isolates for an individual species other than *L. acidophilus*, sharing a single allelic profile. A phylogenetic network of rMLST gene nucleotide sequence variation within the *L. acidophilus* group was constructed using the Neighbour Net algorithm (Figure 1). All species were resolved and rMLST gene sequence diversity was evident at the infraspecific level in all species except for *L. acidophilus*, *Lactobacillus iners* and *Lactobacillus ultunensis* (Figure 1). Of the 53 rps loci examined in the rMLST analysis, just 13 showed sequence variability in *L. acidophilus* isolates. In contrast, all other *L. acidophilus* group species showed within-species variation at 41 or greater rps loci (ranging from 41 in *L. iners* to 51 in *L. helveticus*) (Figure 1). While all 9 *L. acidophilus* group species demonstrated clear

Figure 1 | *L. acidophilus* lacks genomic diversity compared to other *L. acidophilus* group species. A NeighborNet graph of 32 *L. acidophilus* (Table 1) and 108 *L. acidophilus* group (Table S1) genomes was generated using the sequence of 53 concatenated rMLST loci, providing a least-squares fit of 99.99%. The number of genomes from each species included in the network are given in brackets adjacent to the species name label. The scale bar indicates uncorrected P distance measured as number of nucleotide differences over 9338 parsimony informative nucleotides.

separation in the rMLST gene Neighbour Net, *L. acidophilus*, *L. crispatus*, *L. ultunensis* and *L. helveticus* were linked by a polytomy, suggesting they had all evolved from a common ancestor (Figure 1).

The intraspecific diversity of *L. acidophilus* using whole genome MLST (wgMLST). Further analysis of *L. acidophilus* genome sequences was conducted using DNA sequence from all complete protein-coding regions to improve resolution and enable intra-specific genetic diversity to be mapped. Of 1,864 loci defined in the *L. acidophilus* NCFM genome sequence²³, Genome Comparator analysis¹⁸ identified 1,815 (97.4%) complete loci present in all the *L. acidophilus* genomes examined (Table 1). Of these 1,815 core loci, 972 (53.6%) showed sequence variation in at least one isolate. A NeighborNet analysis¹⁸ of allelic variation within all shared loci encoded in the *L. acidophilus* NCFM genome sequence was able to resolve each isolate within the *L. acidophilus* cluster and demonstrated the presence of a notable, highly conserved, sub-group comprised of commercial isolates (Figure 2).

Genome sequences generated from commercially used *L. acidophilus* strains (Table 1) and isolates cultivated directly from current probiotic products¹³ formed a tight cluster centred on the widely used *L. acidophilus* NCFM (Figure 2, labelled in blue). Only one isolate in commercial use within a dairy product (strain CIRM-BIA 445) placed outside the commercial isolate cluster (Figure 2); the phylogenetic relationships between commercial *L. acidophilus* isolates are shown at higher resolution in Supplementary Figure S1 and the variable loci within these genomes are listed in Supplementary Table S2. The published *L. acidophilus* NCFM genome sequence (accession number CP000033) was generated in 2005 using Sanger sequencing²³. This reference genome formed a distinct arm deriving from the central commercial isolate node and separated from a duplicate isolate of *L. acidophilus* NCFM that was re-sequenced as a control for this study (Figure 2; see asterisk). When the two NCFM genome sequences were compared, all loci defined in the published reference sequence²³ were present in re-sequenced genome. However, 89 loci showed sequence differences and 26 of these variable loci from the NCFM re-sequence were found to have identical sequence in all other *L. acidophilus* isolates. This suggests that errors

in the original NCFM genome²³, that were corrected by the massively parallel sequencing reads used for the duplicate NCFM isolate examined, were the most probable source of this variation.

Comparison of the total number of loci with variable sequence between the commercial and type strain cluster isolates provided a measure of the limited variation within the industrial isolates. Within the commercial isolate cluster, 118 loci were found to have variable sequence in at least one isolate. By comparison, isolates from the type strain cluster, representative of a single strain deposited in duplicate locations or under a different alias, such as *L. acidophilus* LMG 9433^T and *L. acidophilus* LMG 13550^T (Table 1), variation in at least one isolate was observed at 337 loci. Additional evidence of genetic conservation of the commercial isolates was also seen in 6 loci constituting the prophage remnant designated Potentially Autonomic Unit 3 (PAU3)²³; these were not detected in any of the type strain cluster sequences, yet this region was fully intact in all commercial isolates.

The genome sequences generated from other *L. acidophilus* isolates taken from different culture collections (Table 1) were also diffuse in their placement in the wgMLST NeighborNet (Figure 2). No major sub-clusters other than the type strain cluster were apparent in the culture collection genomes examined (Figure 2). However, in analogous fashion to the type strain cluster, close placement of identical isolates sequenced in different studies using distinct sequencing and assembly technologies was seen. For example, *L. acidophilus* LMG 11470 (Illumina HiSeq2000 and Velvet assembly, this study) and ATCC 4796 (454-GS-FLX, Newbler assembly, Human Microbiome Project) (Figure 2, Table 1).

Conservation of the *L. acidophilus* genome and limited protein-coding locus variation. To map the conservation of the *L. acidophilus* genome, a pairwise comparison of selected isolate sequences was carried out against the *L. acidophilus* NCFM annotated reference genome²³ (CP000033; Figure 3). This analysis corroborated the wgMLST data (Figure 1 and 2) and demonstrated that genetic variation in the core genome of *L. acidophilus* was primarily comprised of single nucleotide polymorphisms (Figure 3). Novel DNA that did not map the NCFM reference genome was not found in any of the *L. acidophilus* isolate sequence assemblies.

Figure 2 | Whole genome MLST analysis of the infraspecies diversity of *L. acidophilus*. A Neighbor Net plot was generated using wgMLST. The scale bar indicates distance measured in number of allelic differences over 1815 genomic loci conserved across all *L. acidophilus* genomes examined. Isolate numbers are coloured to represent their commercial (blue) or culture collection (green) history (Table 1), with other notable groups circled. High resolution analysis of the 14 commercial *L. acidophilus* genome sequences in the circled region of the neighbour net plot is provided in Supplementary Figure S1. The NCFM isolate genome sequenced as part of this study is indicated by an asterisk. The GenBank accession of the NCFM reference genome sequence is given brackets. Numbers given in superscript indicate CRISPR sequence types assigned in Supplementary Figure S2.

Only limited evidence of genetic loss was detected in the pairwise analysis, with the absent genomic regions confined to genes encoding phage-related, mucus binding and sugar metabolism functions (Figure 3).

Three prophage remnants, designated as Potentially Autonomic Units (PAU; PAU1, PAU2 and PAU3) within the *L. acidophilus* NCFM genome²³ and a novel region of three consecutive loci (LBA0058 to LBA0060) with phage related functions, demonstrated variable presence among the *L. acidophilus* isolates. Differences in the distribution of the three PAU regions was evident when the isolate history, as commercial or culture collection derived (Table 1), was considered. The PAU1 locus was widely distributed across all isolates with exception to the commercial isolates CUL21 and C47 (Figure 3). The remaining PAU regions, 2 and 3, were intact for all commercial isolates. However, the culture collection isolates demonstrated variable presence of loci within PAU2, PAU3 and the phage-related LBA0058-60 region (Figure 3). A region corresponding to *L. acidophilus* LBA1019-LBA1020, encoding mucus binding proteins, was also absent from culture collection isolates *L. acidophilus* LMG 11469, LMG 11472, LAB 69 and ATCC 4796/LMG 11470. Additionally, a region encoding functions related to cellobiose metabolism (LBA0871-LBA0883) was identified as absent from the commercial isolate sequence, *L. acidophilus* CIRM BIA-445 (Figure 3).

***L. acidophilus* clustered regularly interspaced short palindromic repeats (CRISPRs).** CRISPRs regions provide a unique insight into

the evolution of bacterial phage resistance and have also been recently proposed as a means to identify industrial isolates²⁴. One CRISPR region was identified within the *L. acidophilus* NCFM reference genome²³ and at the genome scale this showed considerable synteny with all other *L. acidophilus* sequences investigated (Figure 3). To further interrogate small sequence changes in this region of the *L. acidophilus* genome assumed to be polymorphic as a result of historical phage attack, 20 genomes that contained a complete CRISPR region on a single sequence contig were compared (Supplementary Figure S2). The *L. acidophilus* NCFM CRISPR was defined as the archetypal reference sequence for this analysis and was composed 32 units of a repeat region and a spacer region²³. The shortest CRISPR sequences were present in 5 culture collection isolates (*L. acidophilus* CIP 76.13^T, LMG 13550^T, ERR203994^T, DSM 20242 and LAB 283), each of which were missing 3 spacer sequences (CRISPR types 4 and 6, Figure S2). CRISPR sequences from isolates re-sequenced under different aliases (DSM 20242 and LAB 283, CRISPR type 6 and 3 representing the type strain, CRISPR type 4; Figure S2) were conserved within-isolate, with the exception of *L. acidophilus* LMG 9433^T, which possessed spacer sequences 2 and 3 (these were not present in CIP 76.13^T, LMG 13550^T or ERR203994^T sequences; Figure S2). Nine of the 11 commercial isolates examined had identical CRISPR regions with no evidence of absent or duplicated spacers in relation NCFM (CRISPR type 3; Figure S2). The two commercial isolates with variant CRISPRs

Figure 3 | *L. acidophilus* genome sequences compared to *L. acidophilus* NCFM. The black, innermost ring represents the published genome sequence of *L. acidophilus* NCFM (accession CP000033). Further concentric rings correspond to isolates according to the key. Co-localising isolates from Figure 2 that could be linked by alias (Table 1) are combined into a single ring. Ring presence indicates 98% sequence identity. Regions of interest are annotated.

both possessed differences in relation to spacer 7. This spacer was duplicated after its first occurrence and absent at its third occurrence for isolate CulT2, while *L. acidophilus* C46 just lacked the third occurrence of spacer 7 (Figure S2). CRISPR sequence types (Figure S2) did not fit parsimoniously onto the whole genome phylogenetic network (Figure 2).

Conservation of *L. acidophilus* phenotypic traits. The lack of diversity seen within the *L. acidophilus* genome sequences suggested that the phenotypes of the corresponding isolates would also be invariant. Biochemical assessment (API 50CHL) of the carbohydrate fermentation profile of the *L. acidophilus* isolates (Table 1) was diagnostic of the species, but did not show significant differences between the commercial or culture collection isolates. The growth kinetics of selected commercial and culture collection isolates (Table 1) was also examined and no significant differences in lag phase, maximal growth rate, and maximum culture density was seen (Figure S3). Finally, to examine the isolate phenotype at the protein level, Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS) was carried out as a high-resolution analysis (Figure 4). While separation of *L. acidophilus* protein profiles generated by nine isolates (Table 1) from other LAB species –as shown in other studies²⁵– was observed (Figure 4, Panel A), there was no differentiation by MALDI-TOF of the commercial and culture collection isolates (Figure 4, Panel B).

Discussion

Numerous studies have used comparative genomics to identify similarities and differences within the LAB^{26–28} and for comparing species level diversity within the *L. acidophilus* group²⁰, but to date no study has conducted a comparative genomics analysis encompassing a large number of LAB isolates below the species level. This represents a fundamental gap in knowledge concerning probiotic bacteria, as their beneficial characteristics may be unique to a single strain and

Figure 4 | Diversity of *L. acidophilus* MALDI-TOF profiles. MALDI-TOF profile distance scores were plotted in two dimensions as described in the Methods. Panel A shows the profiles of *L. acidophilus* (isolates NCFM, LMG 9433^T, LMG 11428, LMG 11470, LMG 13550^T, Rm 344, Rm 345, CUL 21 and CUL60; Table 1) compared to 6 other lactobacillus control species. Panel B shows the profiles of commercial and culture collection *L. acidophilus*. Distances were calculated using the Pearson correlation similarity coefficient and position tolerance optimisation was set to 2%. Coordinates were calculated using multidimensional scaling.

encoded within their WGS. Understanding the genomics of LAB is also important from the regulatory perspective to accurately identify isolates, and from the commercial standpoint to differentiate specific probiotic or fermentation traits. We have used WGS combined with a functional gene-by-gene diversity analysis approach to assess the infraspecies diversity of *L. acidophilus* as a single probiotic species. *L. acidophilus* was found to be a monophyletic species and isolates in global commercial use were clonal. The industrial significance of isolates with probiotic characteristics may have driven re-isolation and re-naming of the same isolate from environmental samples, and little information is available concerning the history of proprietary commercial strains. Similar levels of genomic conservation were observed in the probiotic subspecies *Bifidobacterium animalis* subsp. *lactis*, where isolates from disparate commercial products were found to have highly conserved genome sequences and assumed to be an entirely monomorphic taxon, until the genome sequence of a culture collection isolate was found to represent a genomically unique strain²⁹. These findings raise a number of questions relating to industrial strain identification, the commercial success of a single strain and whether human domestication has directed the evolution of *L. acidophilus* towards a narrow bottleneck.

Accurate identification of microbial content has been proposed as one of the most important product labelling criteria to support probiotic health claims³⁰. The level of genetic conservation seen between probiotic *L. acidophilus* in this study, and in previous analyses of two commercially distinct *L. casei* probiotic isolates³¹, could mean that probiotic health claims formulated from functional studies of one isolate could be applied to other, genetically monomorphic probiotic isolates. Multiple genetic and phenotypic identification strategies have been proposed as suitable to identify probiotic species, but no recommendations on how to analyse and interpret whole genome sequencing data have yet been proposed³². Although the 16S rRNA gene sequence has been widely used to classify *Lactobacillus* species¹⁹, given its conserved nature compared to other functional genes, traditional phylogenies drawn from the 16S rRNA gene sequence alone are often unstable and require addition of functional genes to improve resolution^{21,33}. Our use of rMLST²² and wgMLST, implemented within BIGSdb¹⁸, was able to place *L. acidophilus* in the context of other *L. acidophilus* group members (Figure 1) and resolve strain differences within this essentially clonal species (Figure 2). Since multiple probiotic products are composed of mixtures of microorganisms³⁰, the utility of rMLST to resolve phylogenetic differences across domains²² makes it an ideal approach to bring unity and standardisation of strain identification to the probiotic field³⁰.

Isolates investigated in this study showed no evidence of extrachromosomal DNA such as plasmids. Indeed, there was no evidence of assembling DNA beyond that homologous to the *L. acidophilus* NCFM reference sequence. Previously published *L. acidophilus* genomes^{15,16,23} also reflected this lack of plasmid DNA, although a single isolate – *L. acidophilus* 30SC – has two reported plasmids³⁴. The anomalous presence of extrachromosomal DNA in the *L. acidophilus* 30SC genome sequence is due to the mis-identification of this strain; phylogenetic analysis of 30SC genome clearly demonstrates that it should have been classified as *Lactobacillus amylovorus*^{33,35}. For this reason, the *L. acidophilus* 30SC genome sequence was excluded from analysis in this study. The correct identification of probiotic isolates, not only to satisfy product health claims and labelling guidelines, but also to maintain a rigorous standard for taxonomic assignment of genome sequences, is of particular importance for accurate downstream analysis.

Genome sequence analysis revealed that *L. acidophilus* as a bacterial species has remarkable genetic stability, especially when compared to other closely related *Lactobacillus* species (Figure 1). Going beyond this, the lack of variation among *L. acidophilus* isolates in commercial use is striking. One possible explanation for this is the global propagation, storage and repeated re-use of commercial pro-

biotic isolates of *L. acidophilus* from within the commercial isolate cluster. In a similar case in a different probiotic species, two commercial isolates of *L. casei*, isolated directly from probiotic products produced by different companies were found to share a virtually identical genome sequence and encode a comparable exproteome³¹. The *L. casei* data³¹ and our data suggest that human practice in terms of the use of probiotic LAB or dairy starter cultures may restrict the “natural” evolution of these bacteria, leading the widespread distribution and ultimately human consumption of highly clonal strains.

The commercial success of *L. acidophilus* may also be attributed to its genomic stability, allowing manufacturers to maintain good batch-to-batch quality control for probiotic manufacture and dairy fermentations. Phage spoilage of bacterial starter cultures is a major problem for the food industry²⁴, but remarkable lack of variation within the *L. acidophilus* CRISPR may suggest it has not recently undergone substantial phage attack, although functional degradation of genes associated with the CRISPR region has been documented in *L. acidophilus* NCFM³⁶. This may explain why CRISPR sequence types do not fit parsimoniously onto the wgMLST phylogenetic network. While the *L. acidophilus* genome contains phage-like remnants (eg. PAU regions)²³, it does not encode an active prophage, and while bacteriophage interactions with other *L. acidophilus* group species are widespread³⁷, there are no recent report of phages active on validated *L. acidophilus* strains. The effective phage-resistance of *L. acidophilus* may also be a reason for its commercial success and widespread usage as stable and reliable commercial LAB species.

Bacterial pathogens such as *Yersinia pestis*³⁸ and *Mycobacterium tuberculosis*³⁹ show low genetic diversity concomitant with reaching an evolutionary bottleneck once within the human host. Our data suggest that *L. acidophilus* reached a similar evolutionary constriction that was associated with its historical human use in dairy fermentation and that is now being propagated by commercial and widespread probiotic use. Despite considerable effort to isolate *L. acidophilus* from non-human associated sources (MB, PhD Thesis Cardiff University), isolates from outside the dairy and probiotic industry were not identified, except from animals such as rats (Table 1), which are implicitly linked with human waste and activity. The lack of diversity within the *L. acidophilus* genome also suggests that genotyping methods that only sample a portion of the genetic content such as 16S rRNA gene sequencing¹⁹, PCR-fingerprinting¹³, or multilocus sequencing typing¹⁴ will not have sufficient resolution to support a specific health claim and its association to a given probiotic strain³⁰. From a clinical perspective, the availability of WGS data has already advanced our understanding of pathogen population biology¹⁷. Continued development of analysis techniques, widespread availability of cost-effective sequencing and dissemination of bioinformatics expertise will assist in translating what we have learned from pathogenic systems into probiotic systems, potentially transforming the ways in which we regulate the manufacture and commercial use of microorganisms.

Methods

***L. acidophilus* isolates and genome sequences.** *L. acidophilus* isolates and genomes were drawn from the following sources: 12 isolates of *L. acidophilus* were obtained from the culture collections of Belgian Coordinated Collection of Microorganisms (BCCM/LMG) and the University of Gent Laboratory for Microbiology; 5 genome sequences representative of culture collection strains were also obtained from the databases (Table 1; 17 genomes representative of culture collection isolates); 12 commercial isolates were obtained from probiotic products¹³, a probiotic supplier (Cultech Ltd., Port Talbot, UK) and T. Klaenhammer (North Carolina State University, Raleigh, NC, USA; a duplicate isolate of NCFM); the genomes of 4 additional commercial isolates were obtained from the databases (Table 1). Sequencing reads from one *L. acidophilus* genome sequence of unknown provenance were obtained from the NCBI short read archive and assembled into a draft genome sequence (Table 1) as described. The date of isolate recovery, its source and strain aliases were investigated and crosschecked where possible using the Strain Information Database (www.straininfo.net). Culture, storage and identification of *L.*

acidophilus isolates was carried out as previously described²³. Additional genomes from *L. acidophilus* group species were drawn from the DNA databases (Table S1).

Whole genome sequencing. Genomic DNA was extracted from the growth of single-colony inoculated *L. acidophilus* cultures with a Wizard genomic DNA purification kit (Promega, Southampton, United Kingdom). Genome re-sequencing was performed by the Oxford Genomics Centre, Wellcome Trust Centre for Human Genetics; www.well.ox.ac.uk/ogc. Briefly, Illumina multiplex libraries were generated from genomic DNA acoustically sheared to 200 to 300 bp using a Covaris E210 device. DNA fragments were end repaired, and a 3' nontemplate adenosine residue was ligated to the Illumina multiplexing adaptor oligonucleotide for sequencing. Libraries were pooled and analyzed together, in equimolar amounts, in a flow cell lane of the Illumina HiSeq 2000, generating 100-bp paired-end reads, which were deposited in the NCBI sequence read archive (SRA) with run accessions ERR386024 – ERR386044 and ERR386051 – ERR386052. Genome sequence data were assembled using Velvet version 1.2.10 shuffle and optimization scripts to create contigs with optimal parameters, with k-mer lengths between 83 and 95 bp⁴⁰. Assembled data were deposited in the rMLST genome database (<http://rmlst.org/>), implemented with Bacterial Isolate Genome Sequence Database (BIGSdb) software¹⁸. The BIGSdb autotagger automatically identified rMLST loci, assigned alleles, and tagged the sequences for future reference. The database automatically provided a report of the rMLST allelic profiles¹⁸.

rMLST and whole genome analysis. Relationships among *L. acidophilus* group isolates were established using phylogenetic networks based on rMLST sequences²². The 53 *L. acidophilus* group rps loci identified in the automated annotation process were compared among all isolates using the BIGSdb Genome Comparator module¹⁸. The aligned sequences were visualized with the Neighbor-net algorithm implemented in SplitsTree version 4.13.1⁴¹. The vector graphics editor Inkscape 0.48.4 (www.inkscape.org) was used to annotate Neighbor Net images. *L. acidophilus* isolates were further analysed using whole genome MLST (wgMLST)¹⁸ at 1,864 loci defined in the genome sequence of *L. acidophilus* NCFM²³ with the Genome Comparator. A distance matrix based on shared alleles was generated and visualised with NeighborNet¹⁸. Whole genome alignments were visualised and annotated using BLAST Ring Image Generator (BRIG) v0.95³⁷.

CRISPR identification and analysis. *L. acidophilus* NCFM CRISPR sequence was used to search other *L. acidophilus* genome sequence data using BLAST+ tools implemented via the BIGSdb Web-interface¹⁸. The CRISPRtary: Dictionary Creator tool at the CRISPRdb (<http://crispr.u-psud.fr/crispr/>)⁴² was used to identify direct repeat and spacer sequence in genomic regions containing CRISPR sequences, assigning a numerical value to each new spacer sequence encountered. The numerical profiles generated by CRISPR spacer sequences were compared. Each unique CRISPR sequence was assigned a CRISPR sequence type. Incomplete or partially assembled CRISPR regions were excluded from further analysis.

Phenotypic analysis. The phenotype of the *L. acidophilus* isolates (Table 1) and control species (*L. brevis* LMG 6906^T, *L. casei* 6904^T, *L. gasseri* LMG 9203^T, *L. johnsonii* LMG 9436^T, *L. paracasei* subsp. *paracasei* LMG 7955, *L. plantarum* LMG 6907^T and *Enterococcus faecium* LMG 14205) was examined by API50 CHL biochemical analysis following the manufacturer's instructions and using their profile database (BioMérieux Marcy l'Etoile, France). A Bioscreen Microbiological Growth Analyser C (LabSystems, Finland) was used to determine the growth kinetics of selected *L. acidophilus* strains (Table 1) and a *L. casei* LMG 6904^T control as described⁴³ and the specific growth parameters calculated using the R statistical software module grofit⁴⁴. MALDI-TOF analysis of the cellular proteins was carried out as described^{25,45}, using a 4800 Plus MALDI TOF/TOFTM Analyzer (Applied Biosystems, Framingham, MA, USA). Quadruplicate cell extracts were evaluated for each isolate (Table 1) and the profile data exported to BioNumerics 6.0 (Applied-Maths, Sint-Martens-Latem, Belgium) to enable normalisation and cluster analysis as described⁴⁵. Multi-dimensional scaling (MDS) was used to visualize the matrix of data similarities generated by BioNumerics 6.0.

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Author contributions

All authors contributed to the work presented in this paper as follows. M.B. performed the majority of the research and data analysis. K.A.J., J.B. and M.C.J.M. assisted with genome sequencing and BIGSdb analysis, and M.A. and P.V. contributed to the MALDI-TOF phenotype assessments. M.B., P.V., J.R.M., and E.M. contributed to the study design and strain resources. M.B. and E.M. wrote the first draft of the manuscript and all authors contributed to is revision, further analysis and written development.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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