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MINIREVIEWS

Comparative Genomics of *Bifidobacterium*, *Lactobacillus* and Related Probiotic Genera

Oksana Lukjancenko · David W. Ussery · Trudy M. Wassenaar

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Abstract Six bacterial genera containing species commonly used as probiotics for human consumption or starter cultures for food fermentation were compared and contrasted, based on publicly available complete genome sequences. The analysis included 19 *Bifidobacterium* genomes, 21 *Lactobacillus* genomes, 4 *Lactococcus* and 3 *Leuconostoc* genomes, as well as a selection of *Enterococcus* (11) and *Streptococcus* (23) genomes. The latter two genera included genomes from probiotic or commensal as well as pathogenic organisms to investigate if their non-pathogenic members shared more genes with the other probiotic genomes than their pathogenic members. The pan- and core genome of each genus was defined. Pairwise BLASTP genome comparison was performed within and between genera. It turned out that pathogenic *Streptococcus* and *Enterococcus* shared more gene families than did the non-pathogenic genomes. In silico multilocus sequence typing was carried out for all genomes per genus, and the variable gene content of genomes was compared within the genera. Informative BLAST Atlases were constructed to visualize genomic variation within genera. The clusters of orthologous groups (COG) classes of all genes in the pan- and core genome of each genus were compared. In addition, it was investigated whether pathogenic genomes contain different COG classes compared to the probiotic or fermentative organisms, again comparing their pan- and core genomes. The obtained results were compared with published data from the literature. This study illustrates how over 80 genomes can be broadly compared using simple bioinformatic tools, leading to both confirmation of known information as well as novel observations.

Introduction

The first bacterial genome sequences were published in 1995, and within 15 years, over a thousand fully sequenced bacterial genomes have become publicly available [16]. A number of these genome sequences are derived from bacteria used as probiotics or starter cultures in food fermentation, or both. Reid and co-workers [21] defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". A number of bacterial species from various genera are in use as probiotics, including members of *Lactobacillus*, *Lactococcus* and, less commonly, *Leuconostoc*. These Firmicutes are sometimes collectively described as lactic acid bacteria (LAB). Other commonly used probiotic species belong to *Bifidobacterium*, a genus within the phylum Actinobacteria. These genera exclusively contain species that are unlikely to cause disease while colonizing the intestine, and although some species (e.g. *Bifidobacterium dentium*) have been associated with dental disease, these are more commonly members of a normal oral flora. The distinction between normal gut flora (commensals) and

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O. Lukjancenko · D. W. Ussery
Center for Biological Sequence Analysis, Department of Systems Biology, The Technical University of Denmark,
Building 208, 2800 Kgs,
Lyngby, Denmark

T. M. Wassenaar (✉)
Molecular Microbiology and Genomics Consultants,
Tannenstrasse 7,
55576 Zotzenheim, Germany
e-mail: trudy@mmgc.eu

probiotic bacteria having a beneficial effect on their host's health cannot always be made, for which reason we collectively describe them here as 'non-pathogens'. Species belonging to LAB or *Bifidobacterium* are also frequently used in food fermentation, another application where the bacterial load of food is desirably increased. Besides LAB and *Bifidobacterium*, fermentation starter cultures can typically comprise of *Streptococcus thermophilus*, a non-pathogenic member of this genus that mostly contains pathogenic species. Some strains of *Enterococcus* are also in use as starter cultures or probiotics, whereby the used species also contain pathogenic strains. These two genera are therefore of interest, and their species that are used as starter cultures are included in our general description of 'non-pathogens'. Other types of bacteria (particular strains of *Escherichia coli*, *Pediococcus* species and others) or yeasts used as starter cultures or probiotics are not treated here.

For all six genera of interest, multiple genome sequences are publicly available. In many cases, several genomes per species have been sequenced, so that the variation between and even within species can be assessed. One obvious question that could be addressed by comparison of these genomes is: what genes (if any) are common to all genomes of non-pathogens and distinct from genes found in (related) pathogens? Such a comparison requires including multiple species and genera of multiple bacterial phyla (in this case, the phylum of Firmicutes and Actinobacteria). As a general rule, genetic diversity increases with evolutionary distance, so that the genetic variation in such a collection of genomes will be enormous. One way of extracting information from such complex data is by grouping genes into functional groups or families, so that gene families rather than individual genes are compared. Such grouping is based on protein sequence similarity, as this approximately predicts conservation of gene function, ignoring the exceptions resulting from parallel evolution where function similarity does not coincide with sequence conservation. Slight differences in function, resulting from minor differences in sequences, are usually ignored in these groupings, so that fewer but broader groups can be achieved.

In this contribution, 2 approaches were used to compare over 80 genomes from 6 bacterial genera of interest. First, all protein-coding genes from these genomes were grouped into gene families based on sequence identity using a defined similarity cut-off, after which comparisons between and across genera could be performed. Genomes were then compared within their genus for both conserved and variable genes. Second, clusters of orthologous groups (COG) of genes were used to produce functional groups of genes. An attempt was made to identify differences in functional gene distribution between pathogenic and non-pathogenic members of the six genera of interest.

Materials and Methods

Selection of Genomes Used in This Study

Publicly available genomes of the six bacterial genera analyzed here were identified from the NCBI web pages. All completely sequenced genomes (as of July 2010) of 4 *Lactococcus lactis* strains, 3 *Leuconostoc* species and 21 *Lactobacillus* strains from 14 species were included. For *Bifidobacterium*, 11 completely sequenced and 8 incomplete genomes were selected; the latter were chosen when fewer than 70 contigs resulting in 19 genomes from 9 species. Since only 1 complete *Enterococcus* genome was available at the time of analysis, this genome was combined with 10 incomplete sequences, provided they were represented in fewer than 80 contigs, whereby animal isolates were excluded. This allowed inclusion of 2 strains obtained from normal gut flora to give 11 genomes from 4 species. For *Streptococcus*, all *S. thermophilus* genomes were included. All other species of this genus for which genome sequences were available are pathogens, and a selection of these was made of three genomes per species. These were chosen based on their strain characteristics to cover common but diverse serotypes. Animal isolates were excluded, although *Streptococcus suis* (a typical pig pathogen) was included as it has been responsible for a large human outbreak in China. This resulted in 23 genomes from 12 species. All genomes are listed in Table 1, which also provides characteristics such as their size, GC content and the strain description. The latter was extracted from the Genome Project pages at NCBI but checked in the corresponding genome publication when available. This resulted in a few small differences from descriptions listed on the Genome Project Description pages at NCBI. The derived proteomes (protein-coding sequences translated from the DNA sequence) were extracted from GenBank for completed sequences or produced with Prodigal [14] for incomplete sequences.

Definition of Gene Families and Pan- and Core Genome

The pan-genome of a collection of genomes represents all genes encountered in these genomes [27]. In order to define a pan-genome, the criteria to score a gene as 'conserved' or 'novel' were used as previously described [12]. Simply put, two genes are considered to belong to the same gene family and thus 'conserved' when their amino acid sequence is at least 50% identical over at least 50% of the length of the longest gene. All genes of a genome are thus grouped into gene families. Multiple genes per genome can belong to a single gene family, resulting in a lower number of gene families per genome than the reported number of genes. A gene not finding a match with the given criteria is put in its own gene family as a singleton.

An accumulative pan-genome was constructed according to Friis et al. [11], who built on work by Tettelin and co-workers [27]. A resulting pan-genome curve increases in size as more genomes are analyzed, and its shape is order-dependent, though the accumulative pan-genome is not influenced by the order of analysis. Similarly, a core genome is defined as all gene families conserved in all analyzed genomes, and this decreases in size as more genomes are analyzed.

Pairwise pan- and core genomes were calculated for all genome combinations as above, and for each combination, the obtained core genome was expressed as the fraction of the pan-genome. These percentages were visualized in a BLAST Matrix [11].

Core Genome Consensus Tree

Phylogenetic trees were constructed of all core genes that were conserved within the analyzed Firmicute genomes. Multiple alignments of all core sequences were performed with MUSCLE software [7]. PAUP was used to construct a set of core trees [10]. Later, these trees were compared and a best-fit consensus tree was constructed as described by Retief [22].

In Silico MLST Analysis

In silico multilocus sequence typing (MLST) analysis was performed with gene fragments extracted from the genome sequences. For *Bifidobacterium*, gene fragments from *clpC*, *fusA*, *gyrB*, *IleS*, *purF*, *rplB* and *rpoB* were extracted, according to the method proposed for *Bifidobacterium bifidum*, *Bifidobacterium breve* and *Bifidobacterium longum* [6]. For *Enterococcus*, the gene set of *gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt* and *yqlI*, which is advised for use in *Enterococcus faecalis* (http://www.mlst.net), was compared with that designed for *Enterococcus faecium*, which is based on *atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS* and *adk*. For *Lactobacillis*, de Las Rivas and co-workers [4] described an MLST gene set specified for *Lactobacillis plantarum* based on the target genes *pgm*, *ddl*, *gyrB*, *purK1*, *gdh*, *mutS* and *tkt4*. Two alternative combinations of genes have been proposed for *Lactobacillis casei*: *ftsZ*, *polA*, *mutL*, *metRS*, *nrdD* and *pgm* [1] or *fusA*, *ileS*, *lepA*, *leuS*, *pyrG*, *recA* and *recG* (http://www.pasteur.fr). A fourth gene set (*gdh*, *gyrA*, *mapA*, *nox*, *pgmA* and *pta*) has recently been described for *Lactobacillis sanfranciscensis* [20], but since this species is not represented in our dataset, this scheme was not used. For each genus, after concatenation of the gene fragments, a maximum likelihood phylogenetic tree was constructed.

Analysis of Variable Gene Content

The variable gene content of the analyzed genomes was compared using the method by Snipen and Ussery [24]. This method calculates Manhattan distances based on a matrix in which the presence or absence for each gene in each genome is scored with the binary score of 0 (absent) or 1 (present). Core genes and singletons are ignored. BLAST Atlases were produced according to Hallin and co-workers [12].

COG Analysis

COG is a database of proteins where each sequence is assigned to some group. All proteins within a group are believed to have a common ancestor and are likely to share a common function. The various groups are again clustered into some super-groups called functional groups [26]. In this analysis, each found protein was compared to the COG database using BLASTP to identify the functional groups to which they belong. An R-script was used to analyze the protein composition in pan- and core genomes, and the results were visualized in a pie chart. This was done using standard operating procedures [19].

Results

Comparison of Pan-Genomes

After the selection of genome sequences as described in the "Materials and Methods" section, 81 genome sequences were obtained from organisms listed in Table 1. These represented 43 different species and coded for 147,074 protein genes in total. Table 2 summarizes some average findings for each of the analyzed genera. *Enterococcus* has the largest average genome size and *Leuconostoc* the smallest, a difference that is reflected in their average number of genes, since gene density is generally conserved in these bacteria. *Bifidobacterium* has a significantly higher CG content, which was one of the reasons to place this genus in the Actinobacteria [9]. The CG content varied most within the genus of *Lactobacillus*, with a CG content below 37.2% for *Lactobacillus acidophilus*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus helveticus*, *Lactobacillus johnsonii* and *Lactobacillus salivarius*; genomes of the other members of this genus contain at least 38.9% CG. The average number of gene families (as defined in the "Materials and Methods" section) is also shown in Table 2. Since multiple genes per genome can belong to a single gene family, there are fewer gene families than genes per genome, but the difference is small for *Bifidobacterium*. This indicates that there is little gene redundancy in that genus. Lastly, the pan- and core genomes of these genera (based on the analyzed genomes) are quantified in Table 2. The plots resulting in these running totals are shown in Fig. 1, where the average

Table 1 Genomes selected for analysis

GPID	Strain name[a]	Size, bp or Mb	% CG	Contigs	Number of genes	Strain characteristics
82	*Lactobacillus acidophilus* NCFM	1,993,560	34.7	1	1,862	Commercial strain for yogurt, fluid milk production
404	*Lactobacillus brevis* ATCC 367	2,340,228	46.1	3	2,218	Starter culture for beer, sourdough, and silage
402	*Lactobacillus casei* ATCC 334	2,924,325	46.6	2	2,771	Starter culture for milk fermentation and flavour development of cheese
30359	*Lactobacillus casei* BL23	3,079,196	46.3	1	3,044	Probiotic strain
46813	*Lactobacillus crispatus* ST1	2,043,161	36.9	1	2,024	Normal oral/vaginal flora, chicken isolate
16871	*Lactobacillus delbrueckii bulgaricus* ATCC 11842	1,864,998	49.7	1	2,096	Yogurt
403	*Lactobacillus delbrueckii bulgaricus* ATCC BAA-365	1,856,951	49.7	1	1,721	Thermophilic starter culture for yogurt, Swiss and Italian-type cheeses
18979	*Lactobacillus fermentum* IFO 3956	2,098,685	51.5	1	1,843	Not specified
84	*Lactobacillus gasseri* ATCC 33323	1,894,360	35.3	1	1,755	Human isolate, type strain
17811	*Lactobacillus helveticus* DPC 4571	2,080,931	37.1	1	1,610	Cheese culture
36575	*Lactobacillus johnsonii* FI9785	1,785,116	34.4	1	1,737	Competitive exclusion strain in chicken
9638	*Lactobacillus johnsonii* NCC 533	1,992,676	34.6	1	1,821	Probiotic strain
32969	*Lactobacillus plantarum* JDM1	3,197,759	44.7	1	2,948	Probiotic strain
356	*Lactobacillus plantarum* WCFS1	3,348,625	44.4	4	3,101	Human saliva
15766	*Lactobacillus reuteri* DSM 20016	1,999,618	38.9	1	1,900	Type strain, human isolate
19011	*Lactobacillus reuteri* JCM 1112	2,039,414	38.9	1	1,820	Human isolate
32195	*Lactobacillus rhamnosus* GG	3,010,111	46.7	1	2,944	Probiotic strain
40637	*Lactobacillus rhamnosus* GG ATCC53103	3,005,051	46.7	1	2,834	Human isolate
32197	*Lactobacillus rhamnosus* Lc 705	3,033,106	46.7	2	2,992	Probiotic strain
13435	*Lactobacillus sakei sakei* 23K	1,884,661	41.3	1	1,885	Fermenting
13280	*Lactobacillus salivarius* UCC118	2,133,977	33.0	4	2,014	Probiotic strain
18797	*Lactococcus lactis cremoris* MG1363	2,529,478	35.7	1	2,516	Plasmid-cured NCDO712, lab strain
401	*Lactococcus lactis cremoris* SK11	2,598,348	35.8	6	2,504	Cheese production
72	*Lactococcus lactis lactis* Il1403	2,365,589	35.3	1	2,266	Laboratory strain
41115	*Lactococcus lactis lactis* KF147	2,635,654	34.9	1	2,575	Fermenting, non-dairy
16062	*Leuconostoc citreum* KM20	1,896,614	38.9	5	1,823	Kimchi (food, Korea)
40837	*Leuconostoc kimchii* IMSNU11154	2,101,787	37.0	1	2,130	Kimchi? not specified
315	*Leuconostoc mesenteroides mesenteroides* ATCC 8293	2,075,763	37.7	2	2,005	Food fermentation, not specified
70	*Enterococcus faecalis* V583	3,359,974	37.4	4	3,265	Clinical, blood isolate, vancomycin resistant
32949	*Enterococcus faecalis* T11	2,729,089	37.7	49	2,522	Urine isolate
32941	*Enterococcus faecalis* E1Sol	2,853,151	37.5	75	2,737	Faecal isolate, antibiotic-naïve, normal flora
20843	*Enterococcus faecalis* OG1RF	2,739,625	37.7	1	2,515	No info - lab strain?
32919	*Enterococcus faecalis* T3	2,821,089	37.6	40	2,603	Urine isolate
32927	*Enterococcus gallinarum* EG2	3,134,429	40.6	49	2,985	No info
32931	*Enterococcus casseliflavus* EC10	3,423,270	42.5	54	3,243	No info
32935	*Enterococcus casseliflavus* EC20	3,392,502	42.8	57	3,121	No info
46979	*Enterococcus faecium* PC4.1	2,811,160	37.9	78	2,705	Human microbiome, normal flora
32965	*Enterococcus faecium* Com12	2,685,402	38.1	67	2,573	No info
32967	*Enterococcus faecium* Com15	2,771,455	38.3	70	2,698	No info
330	*Streptococcus agalactiae* 2603V/R	2,160,267	35.6	1	2,124	Clinical isolate, common in adults
326	*Streptococcus agalactiae* A909	2,127,839	35.6	1	1,996	No info
334	*Streptococcus agalactiae* NEM316	2,211,485	35.6	1	2,134	Blood isolate
27849	*Streptococcus dysgalactiae* equisimilis GGS 124	2,106,340	39.6	1	2,100	No info
34729	*Streptococcus gallolyticus* UCN34	2,350,911	37.6	1	2,261	Normally rumen flora, this is a clinical human isolate

Table 1 (continued)

GPID	Strain name[a]	Size, bp or Mb	% CG	Contigs	Number of genes	Strain characteristics
						from endocarditis
66	*Streptococcus gordonii* str. Challis CH1	2,196,662	40.5	1	2,051	Causes caries and periodontal diseases
20527	*Streptococcus infantarius infantarius* ATCC BAA-102	1,925,087	37.6	22	1,962	Human microbiome project, normal flora
16302	*Streptococcus mitis* B6	2,146,611	40.0	1	2,018	Clinical isolate
28997	*Streptococcus mutans* NN2025	2,013,587	36.8	1	1,895	Normally oral flora, can cause caries, endocarditis. Clinical isolate
333	*Streptococcus mutans* UA159	2,030,921	36.8	1	1,960	Oral flora, can cause caries, caries isolate
31233	*Streptococcus pneumoniae* ATCC 700669	2,221,315	39.5	1	2,135	Alternative name Spain 23FST81. Pandemic, high prevalence, invasive
29047	*Streptococcus pneumoniae* G54	2,078,953	39.7	1	2,115	Resistant clinical isolate
277	*Streptococcus pneumoniae* TIGR4	2,160,842	39.7	1	2,125	Virulent clinical isolate
269	*Streptococcus pyogenes* M1 GAS SF370	1,852,441	38.5	1	1,696	Group A
16364	*Streptococcus pyogenes* MGAS10270	1,928,252	38.4	1	1,987	Sequenced for comparative genome analysis
286	*Streptococcus pyogenes* MGAS8232	1,895,017	38.5	1	1,845	Serotype M18
13942	*Streptococcus sanguinis* SK36	2,388,435	43.4	1	2,270	Indigenous oral bacteria, causes dental decay, oral plaque isolate
17153	*Streptococcus suis* 05ZYH33	2,096,309	41.1	1	2,186	Causes disease in pigs and occasionally humans
32237	*Streptococcus suis* BM407	2,170,808	41.0	2	2,058	Human clinical isolate
18737	*Streptococcus suis* GZ1	2,038,034	41.4	1	1,978	Causes meningitis, arthritis, pneumonia in pigs human epidemic in China
13163	*Streptococcus thermophilus* CNRZ1066	1,796,226	39.1	1	1,915	Isolated from yogurt for industrial dairy fermentations
13773	*Streptococcus thermophilus* LMD-9	1,864,178	39.1	3	1,716	Used in the manufacture of fermented dairy foods
13162	*Streptococcus thermophilus* LMG 18311	1,796,846	39.1	1	1,889	Isolated from yogurt for industrial dairy fermentations
16321	*Bifidobacterium adolescentis* ATCC 15703	2,089,645	59.2	1	1,631	Normal gut flora
19423	*Bifidobacterium animalis lactis* AD011	1,933,695	60.5	1	1,528	Normal gut flora
42883	*Bifidobacterium animalis lactis* BB-12	1,942,198	60.5	1	1,642	Normal gut flora
32897	*Bifidobacterium animalis lactis* Bl-04	1,938,709	60.5	1	1,567	Normal gut flora
32893	*Bifidobacterium animalis lactis* DSM 10140	1,938,483	60.5	1	1,566	Normal gut flora
32515	*Bifidobacterium animalis lactis* V9	1,944,050	60.4	1	1,572	Normal gut flora
28807	*Bifidobacterium animalis lactis* HN019	1,915,892	60.4	28	1,578	Normal gut flora
17583	*Bifidobacterium dentium* Bd1	2,636,367	58.5	1	2,129	Normal oral and gut flora, can cause caries, caries isolate
20555	*Bifidobacterium dentium* ATCC 27678	2,642,081	58.5	2	2,151	Human microbiome, faeces isolate
18773	*Bifidobacterium longum* DJO10A	2,389,526	60.2	3	2,003	Normal gut flora, probiotic
328	*Bifidobacterium longum* NCC2705	2,260,266	60.1	2	1,729	Normal gut flora, probiotic
17189	*Bifidobacterium longum infantis* ATCC 15697	2,832,748	59.9	1	2,416	Normal gut flora, probiotic
30065	*Bifidobacterium longum infantis* CCUG 52486	2,453,376	60.2	55	2,085	Normal gut flora, human microbiome project
47579	*Bifidobacterium longum longum* JDM301	2,477,838	59.8	1	1,959	Normal gut flora, probiotic
29261	*Bifidobacterium angulatum* DSM 20098	2,007,108	59.4	17	1,586	Normal gut flora, type strain
30055	*Bifidobacterium bifidum* NCIMB 41171	2,186,140	62.8	33	1,810	Normal gut flora, probiotic
30749	*Bifidobacterium catenulatum* DSM 16992	2,058,429	56.1	31	1,720	Normal gut flora
30751	*Bifidobacterium gallicum* DSM 20093	2,019,802	57.5	27	1,580	Human microbiome project
30373	*Bifidobacterium pseudocatenulatum* DSM 20438	2,304,808	56.3	36	1,870	Human microbiome project

[a] The official abbreviation 'subsp.' between species and subspecies name has been deleted throughout this contribution

GPID genome project identification number (NCBI: see http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), *NA* not available

Table 2 Average findings per genus and their pan- and core genome

Genus	Number of genomes included	Number of species	Average genome size (kbp)	Average % CG	Average number of genes (min–max values)	Average number of gene families (min–max values)	Pan-genome[a]	Core genome[a]
Lactobacillus	21	14	2,369	42.4	2,235 (1,562–3,059)	2,071 (1,437–2,873)	13,069	363
Lactococcus	4	1	2,532	35.4	2,465 (2,266–2,504)	2,238 (2,118–2,341)	3,389	1,522
Leuconostoc	3	3	2,025	37.9	1,986 (1,820–2,130)	1,896 (1,724–2,050)	2,927	1,164
Enterococcus	11	4	3,041	36.6	3,078 (2,573–2,515)	2,707 (2,439–3,114)	7,519	1,092
Streptococcus	23	12	1,981	38.9	2,018 (1,696–2,270)	1,923 (1,643–2,180)	9,785	638
Bifidobacterium	19	9	2,209	59.5	1,796 (1,528–2,416)	1,746 (1,497–2,287)	6,980	724

[a] Number of gene families is given

number of gene families present per genome is given as a green line. In all graphs, the pan-genome and core genome curves strongly diverge, indicative of a large variation in gene content between the analyzed genomes within each genus. The largest difference between the pan- and core genome, as a measure for the variance within the analyzed genera, is seen with *Lactobacillus* (21 genomes of 14 species) and *Streptococcus* (23 genomes of 12 species). The variance is larger in four genomes of *Lc. lactis* than in three different *Leuconostoc* species. Thus, intra-species variation in gene content of *Lc. lactis* exceeds inter-species variation of *Leuconostoc*, at least for these analyzed genomes.

The pan- and core genomes of pairwise genome comparisons were also determined to establish the percentage identity for each combination. This identity was expressed as the pairwise core genome divided by its pan-genome and was visualized by colour intensity in a BLAST Matrix. Figure 2 shows the BLAST Matrix for the *Lactobacillus* genomes. The strongest green, indicative of the highest fraction of genes found similar between two genomes, are reported for comparisons within a species, shown at the bottom of the figure. Some species also share a large fraction of genes between them. For instance, the two *Lb. casei* genomes share between 55.5% and 59.3% of their genes with those of the three *Lactobacillus rhamnosus* genomes (represented in the six darker green cells in the upper part of the matrix). An even higher similarity (62.2–62.8%) is found between *Lb. gasseri* and *Lb. johnsonii*. The highest similarity recorded is 93.3%, between two *Lb. rhamnosus* strains, and the lowest is 11.5%, between *Lb. casei* BL23 and *Lactobacillus delbrueckii bulgaricus* ATCCBAA-365.

A similar matrix is shown for *Bifidobacterium* in Fig. 3. In this case, the similarity between the six *Bifidobacterium animalis* genomes is obvious (visible as 15 strongly coloured cells at the bottom right). Two of these genomes reach a similarity of 95.5%. The lowest degree of similarity is seen between *Bifidobacterium gallicum* and *B. longum infantis* strain ATCC 15697 (28.5%).

When a BLAST Matrix was constructed with all genomes included in the analysis, the similarity between *Bifidobacterium* genomes and those of the other genera remained below 3%, illustrative of the difference of *Bifidobacterium* compared to the Firmicutes (results not shown). Thus, despite their sharing of an ecological niche, these bacteria share relatively few genes. A comparison of all Firmicute genomes is provided as Supplementary Fig. S1. As expected, the found percentage identity within any of these genera is much higher than that between genera. For instance, the three *Leuconostoc* genomes produced a similarity of 49.5–52.3% between them, but around 8% to 10% to genomes of other genera. The four *Lc. lactis* genomes gave slightly higher similarities of 16.1–18.4% to all other Firmicute genomes whilst sharing 59.5–66.1% between themselves. An *Enterococcus* and a *Streptococcus* genome typically share 10% to 15% of their genes, and two genomes of *Enterococcus* and *Lactococcus* 14% to 16%. Different *Enterococcus* species share around 30% of their genes, but multiple genomes within one species of this genus have around 70% of their genes being similar.

Comparison of Core Genomes and Conserved Genes

The pan-genomes of all six genera were combined to calculate the core genome shared by all genera. This resulted in only 63 core gene families out of a pan-genome of 37,053 gene families, using the criteria of gene similarity as described in the "Materials and Methods" section. These are listed in Supplementary Table S1. Exclusion of the distinct *Bifidobacterium* genus retained 243 core gene families for the Firmicute genomes that together produced a pan-genome of 30,615 gene families. Since these core genes are conserved in all Firmicute genomes analyzed here, phylogenetic trees could be generated and a consensus tree was generated, as shown in Fig. 4. The consensus core gene tree split all *Lactobacillus* genomes into three main clusters, though *Lb. salivarius* is excluded from these groups. The cluster shown at the top of the figure contains most *Lactobacillus* species

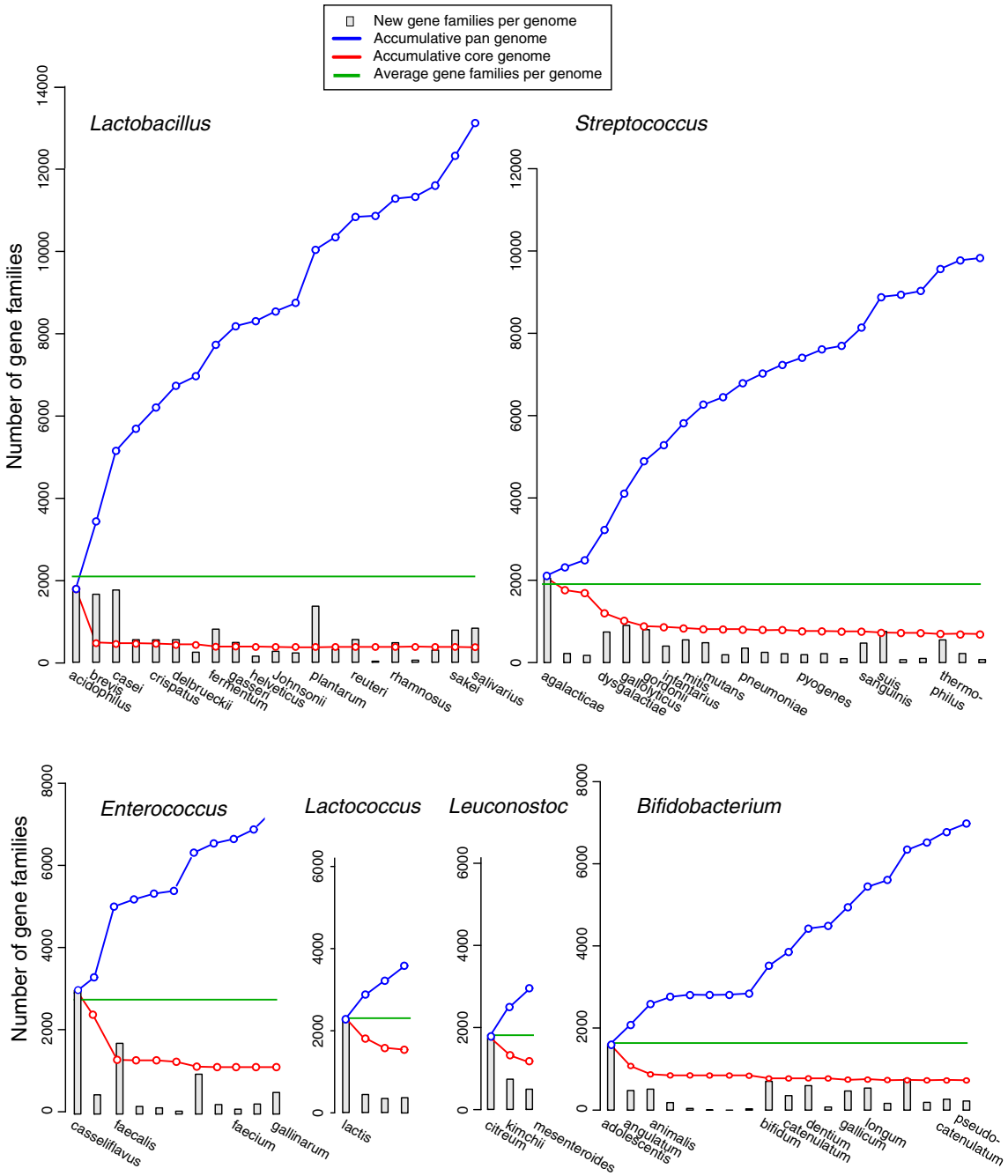


Figure 1 Pan- and core genome plots of the six analyzed genera. The genomes were analyzed in alphabetical order of species names

with lower CG content, though it also includes *L. delbrueckii*, whose CG content is quite a bit higher. This clustering, based on these core genes, corroborates the inter-strain similarities already reported for their complete genomes, as shown in Fig. 2. The *Streptococcus* genus is separated into two large clusters in Fig. 4. Two clusters are also observed for the *Enterococcus* species, while *Lactococcus* is placed outside all other genera.

A more commonly used procedure is to compare only a small subset of core genes. In population biology, MLST of six or seven core gene fragments is frequently used to assess evolutionary distances between isolates within a species. MLST analysis is based on DNA sequences. We adapted this approach to perform in silico MLST for all isolates within a genus, as a measure for evolutionary distance of core genes, and used this for analysis of three genera. Unfortunately, despite the reputation of MLST as being generally applicable and despite a considerable number of gene families being conserved even between Firmicutes and Bifidobacteria

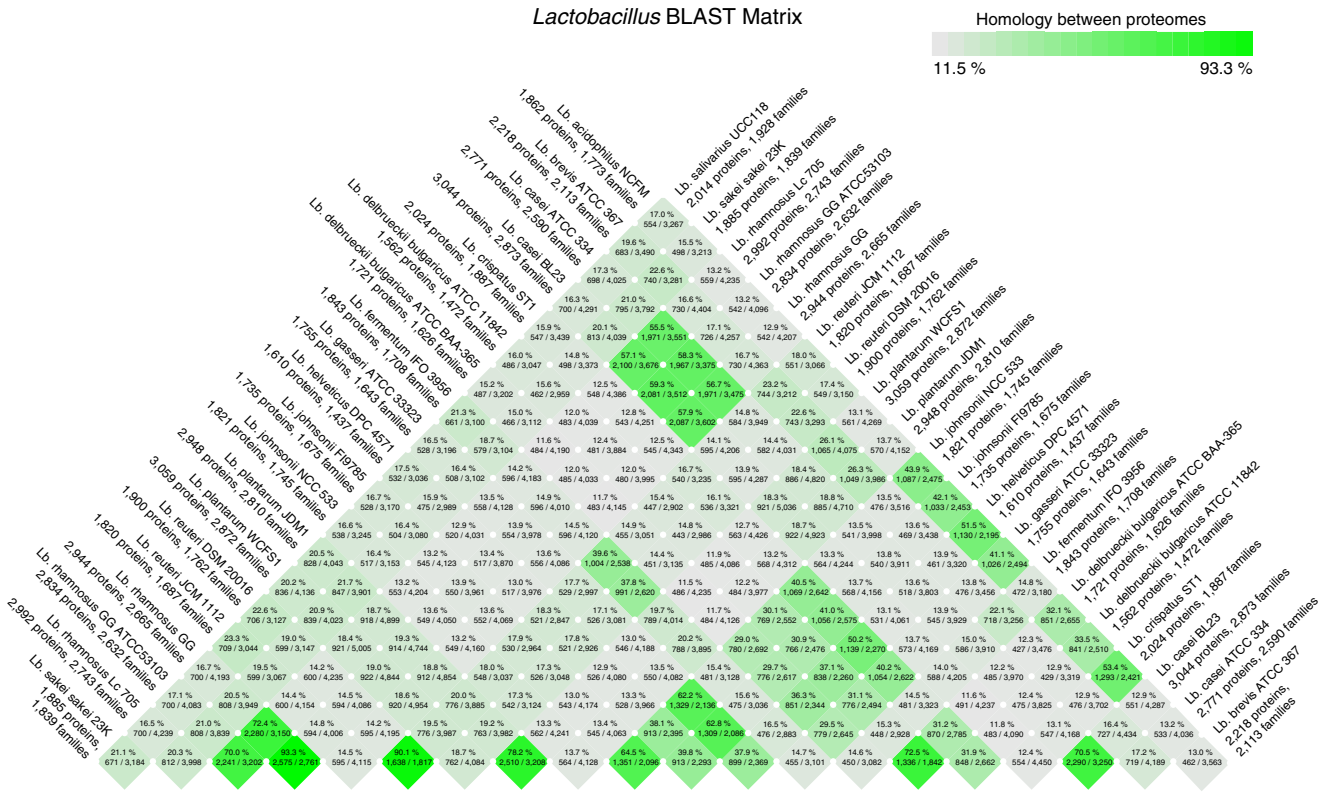


Figure 2 BLAST Matrix for the *Lactobacillus* genomes. To the side, the total number of protein genes and gene families are listed for each genome. In the matrix cells, the shared protein genes are given as a percentage, based on the ratio of the core genome and pan-genome of each pair, as indicated

(63 gene families), different MLST target gene sets have been proposed for various species, and most of these are not conserved between all species (Supplementary Table S1). In order to compare our findings with published data, we have used fragments of various genes depending on the genus, as suggested in the literature.

For in silico MLST analysis of *Bifidobacterium*, 7 gene fragments were extracted according to Delétoile and co-workers [6], as these happened to be conserved in all 19 *Bifidobacterium* genomes analyzed here. A phylogenetic tree of the extracted and concatenated MLST fragments is shown in Fig. 5. Although MLST was not designed for this purpose, the results show that this approach can reveal phylogenetic relationship of these core genes between species within a genus. All multiple isolates per species are correctly clustered, although subspecies are not correctly grouped (see the position of *B. longum longum* and *B. longum infantis*). Three major clusters can be recognized, separated in the figure by green lines. These findings are in accordance to the three groups within this genus recognized by Lee and O'Sullivan [17], based on an extensive 16S ribosomal RNA gene analysis.

The MLST website (http://www.mlst.net) lists two different gene sets to be used for Enterococci. Figure 5 (right side) shows the results obtained with each. Both trees produce little resolution within the species, especially when compared with the consensus tree based on 243 core genes in the previous figure.

For Lactobacilli, four MLST schemes are available: one for *L. plantarum* [4], two for *Lb. casei* ([1], http://www.pasteur.fr) and one for *L. sanfranciscensis* [20], which is not represented in our dataset. The first three MLST schemes were tested, which produced different trees (Supplementary Fig. S2). All three trees clustered multiple strains per species, but the branch positions of these species varied according to the gene set used. It cannot be stated which MLST tree is 'correct' as they all display the evolutionary relationship of the genes analyzed in question—but obviously, the phylogeny of core genes is not always conserved within a genome, as it is affected by recombination. This is also visible from the numbers of core genes producing consensus branches in Fig. 4. With this variation in mind, an MLST tree should be interpreted with caution, as it represents only a tiny fraction of the complete core genome of a strain.

Comparison of Variable Gene Content

The pan-genome of a species or genus comprises both conserved core and variable genes. The latter can also be

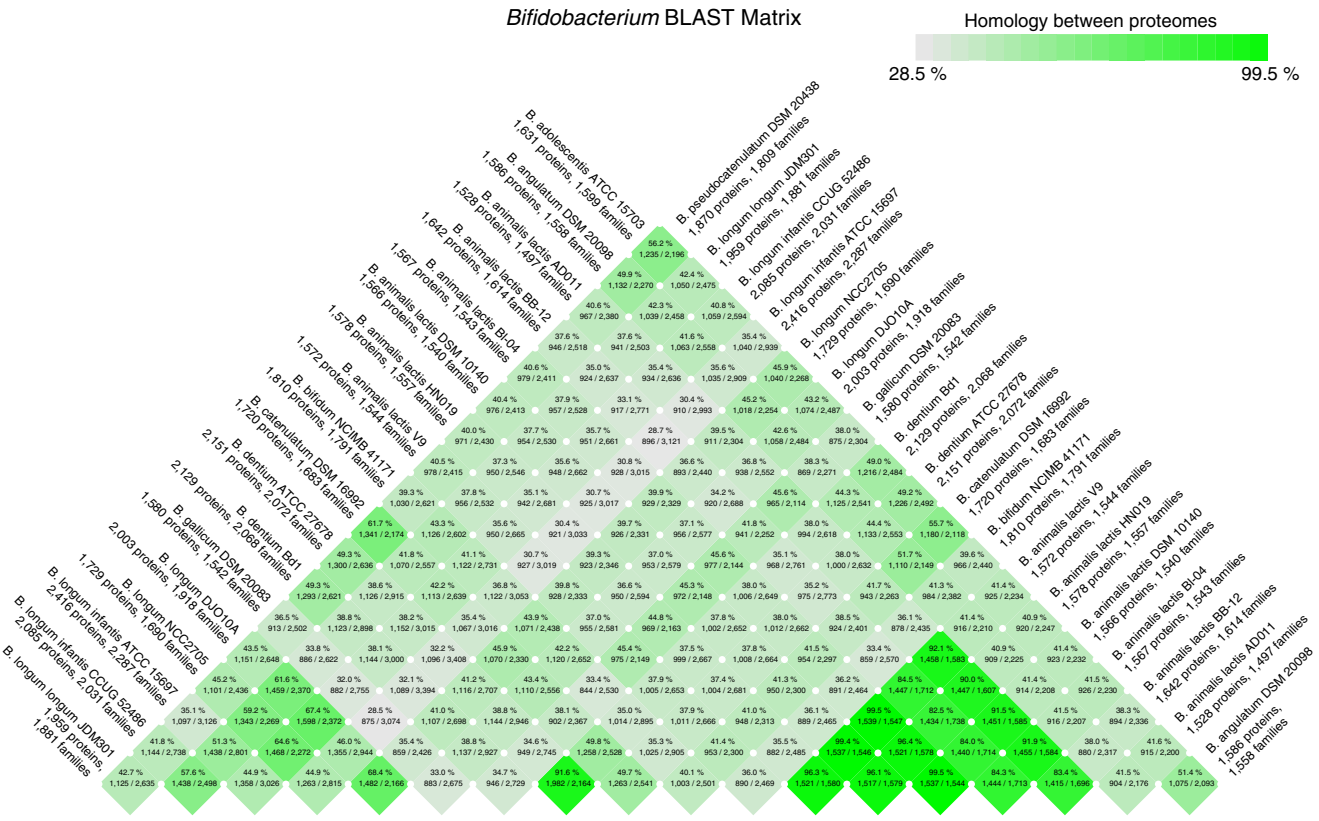


Figure 3 BLAST Matrix for *Bifidobacterium* genomes. Note that the colour scale is different to that of Fig. 2

used to establish inter-genome relationships, although not by phylogeny. Instead, clustering of presence or absence of variable genes can be performed [24]. This method calculates Manhattan distances for genes variably present. Obviously, core genes and genes found present in only one genome were excluded from this analysis, as they cannot identify any correlation between genomes. Thus, only genes whose presence varies, found at least in two genomes but absent in at least one genome, are assessed. The resulting clustering is not a phylogenetic tree, since it is not based on phylogeny of individual genes. Instead, it shows which genomes share more of their variable genes than others.

Figure 6 shows the hierarchical clustering of the *Bifidobacterium* genomes based on their variable gene content. As can be seen, genomes of identical species cluster together and are separated from different species, but the subspecies of *B. longum* are not correctly separated. Since their variable gene content seems to be mixed, this suggests that these two subspecies share the same gene pool for horizontal gene transfer events. The similarity, in terms of variable gene content, between the two species *B. catenulatum* and *B. pseudocatenulatum* is not more than that between various *B. longum* subspecies. A deep division splits *B. animalis* combined with *B. gallicum* from the others, which correlates with the MLST tree shown in Fig. 5.

The analysis of variable gene content can simultaneously be performed with genomes of varying similarity, so that Fig. 6b combines all Firmicute genomes. The 21 *Lactobacillus* genomes are split into two major groups, which match a deep branch in the phylogenetic tree of 16S rRNA genes of this genus [2]. However, the clustering based on variable gene content produces a different picture to the consensus tree based on core genes (compare Figs. 4 and 6b). This probably reflects different evolutionary forces at play. Genes whose presence is variable may be located on mobile elements or may be more frequently subjected to DNA recombination than core genes. The three *Leuconostoc* genomes are placed within the *Lactobacillus* genus; apparently, these share a considerable number of variable genes.

The three major clusters within the *Streptococcus* genus visible in Fig. 6 largely match their taxonomic relationship as defined by 16S rRNA [8], although the distance between *S. thermophilus* and *Streptococcus infantarius*, which are both part of the 'Salivarius group Streptococci', is better captured by variable gene content than by 16S rRNA phylogeny. The discrepancy between this clustering and the consensus core gene tree is even more extensive for this genus.

The four *Lc. lactis* genomes are placed between *Streptococcus* and *Enterococcus*, which reminds of their

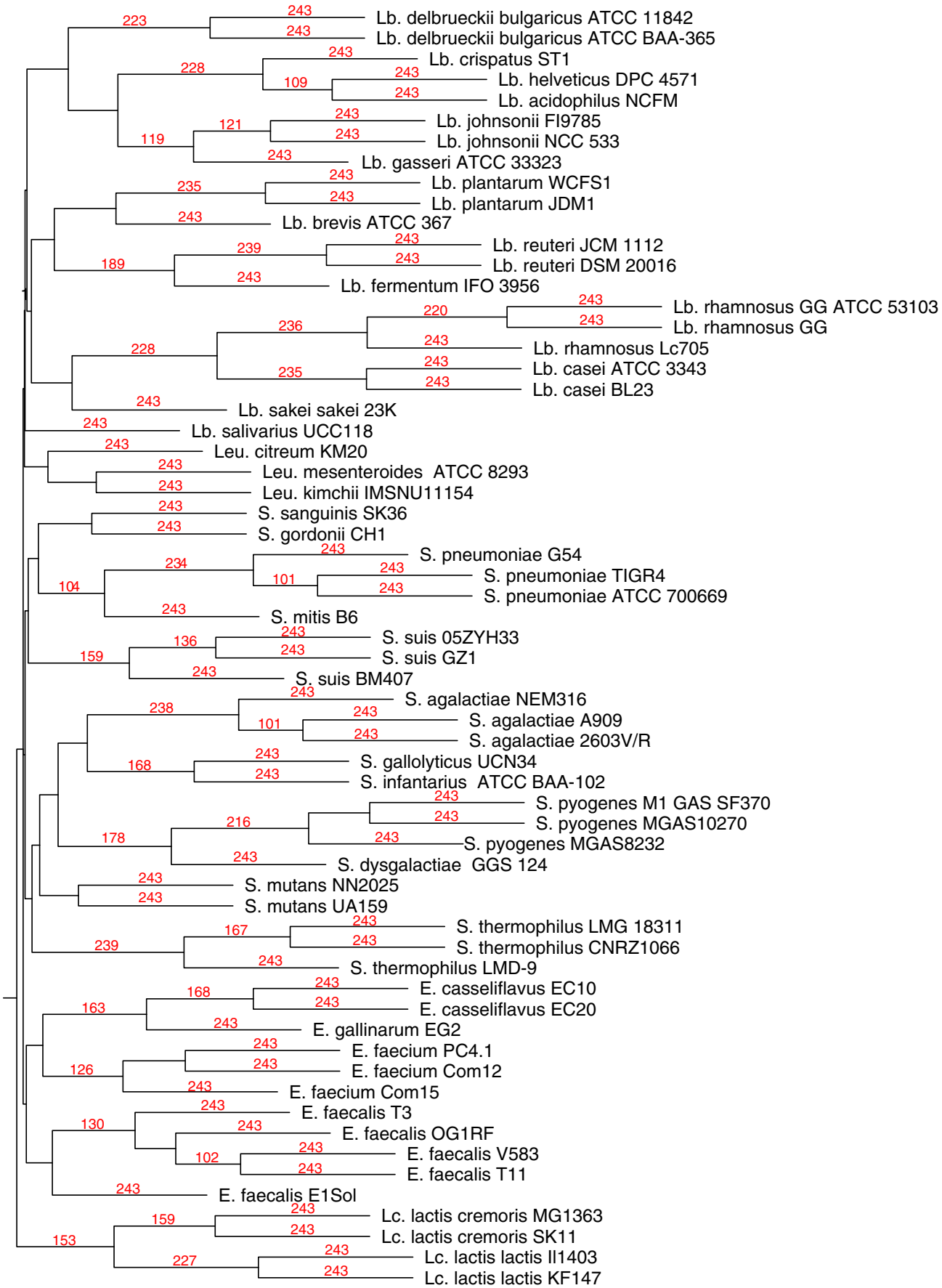

Lb. delbrueckii bulgaricus ATCC 11842
Lb. delbrueckii bulgaricus ATCC BAA-365
Lb. crispatus ST1
Lb. helveticus DPC 4571
Lb. acidophilus NCFM
Lb. johnsonii FI9785
Lb. johnsonii NCC 533
Lb. gasseri ATCC 33323
Lb. plantarum WCFS1
Lb. plantarum JDM1
Lb. brevis ATCC 367
Lb. reuteri JCM 1112
Lb. reuteri DSM 20016
Lb. fermentum IFO 3956
Lb. rhamnosus GG ATCC 53103
Lb. rhamnosus GG
Lb. rhamnosus Lc705
Lb. casei ATCC 3343
Lb. casei BL23
Lb. sakei sakei 23K
Lb. salivarius UCC118
Leu. citreum KM20
Leu. mesenteroides ATCC 8293
Leu. kimchii IMSNU11154
S. sanguinis SK36
S. gordonii CH1
S. pneumoniae G54
S. pneumoniae TIGR4
S. pneumoniae ATCC 700669
S. mitis B6
S. suis 05ZYH33
S. suis GZ1
S. suis BM407
S. agalactiae NEM316
S. agalactiae A909
S. agalactiae 2603V/R
S. gallolyticus UCN34
S. infantarius ATCC BAA-102
S. pyogenes M1 GAS SF370
S. pyogenes MGAS10270
S. pyogenes MGAS8232
S. dysgalactiae GGS 124
S. mutans NN2025
S. mutans UA159
S. thermophilus LMG 18311
S. thermophilus CNRZ1066
S. thermophilus LMD-9
E. casseliflavus EC10
E. casseliflavus EC20
E. gallinarum EG2
E. faecium PC4.1
E. faecium Com12
E. faecium Com15
E. faecalis T3
E. faecalis OG1RF
E. faecalis V583
E. faecalis T11
E. faecalis E1Sol
Lc. lactis cremoris MG1363
Lc. lactis cremoris SK11
Lc. lactis lactis Il1403
Lc. lactis lactis KF147

Figure 4 Consensus tree of 243 core genes conserved in all analyzed Firmicutes. The number of genes supporting the branches is shown in *red*. Values for fewer than 100 genes are not shown

inclusion, prior to the 1980s, into the single genus *Streptococcus* [25]. Within the genus *Enterococcus*, the clustering in Fig. 6 separates each of the analyzed species and confirms that *Enterococcus casseliflavus* and *Enterococcus gallinarum* are more related to *E. faecium* than to *E. faecalis*.

Visualization of Conserved and Variable Gene Content

Conservation and variation in gene content between genomes can also be visualized by a BLAST Atlas [12], which contains information on gene location as well as on gene presence, at least for the reference genome on which a BLAST Atlas is based. Two different *Bifidobacterium* reference genomes were used in the two BLAST Atlases shown in Fig. 7 to which all other *Bifidobacterium* genomes were compared. Only genes present in the reference genome are captured in these atlases as these are used as query, for which the hits in the other genomes are recorded as colour in the BLAST lanes. The more strongly a protein gene is conserved, the more intense the colour is. Different colours are used to separate the different species, and these colours have been kept constant between the two panels, so that it is obvious that genes are mostly conserved within a species. The most inner BLAST lane included in Fig. 7 is that of the reference genome against itself. This shows the maximum colour that can be obtained for each location. Gaps in this 'Blast-to-self' lane where BLAST hits are absent, for instance around 1,700 kb, are due to

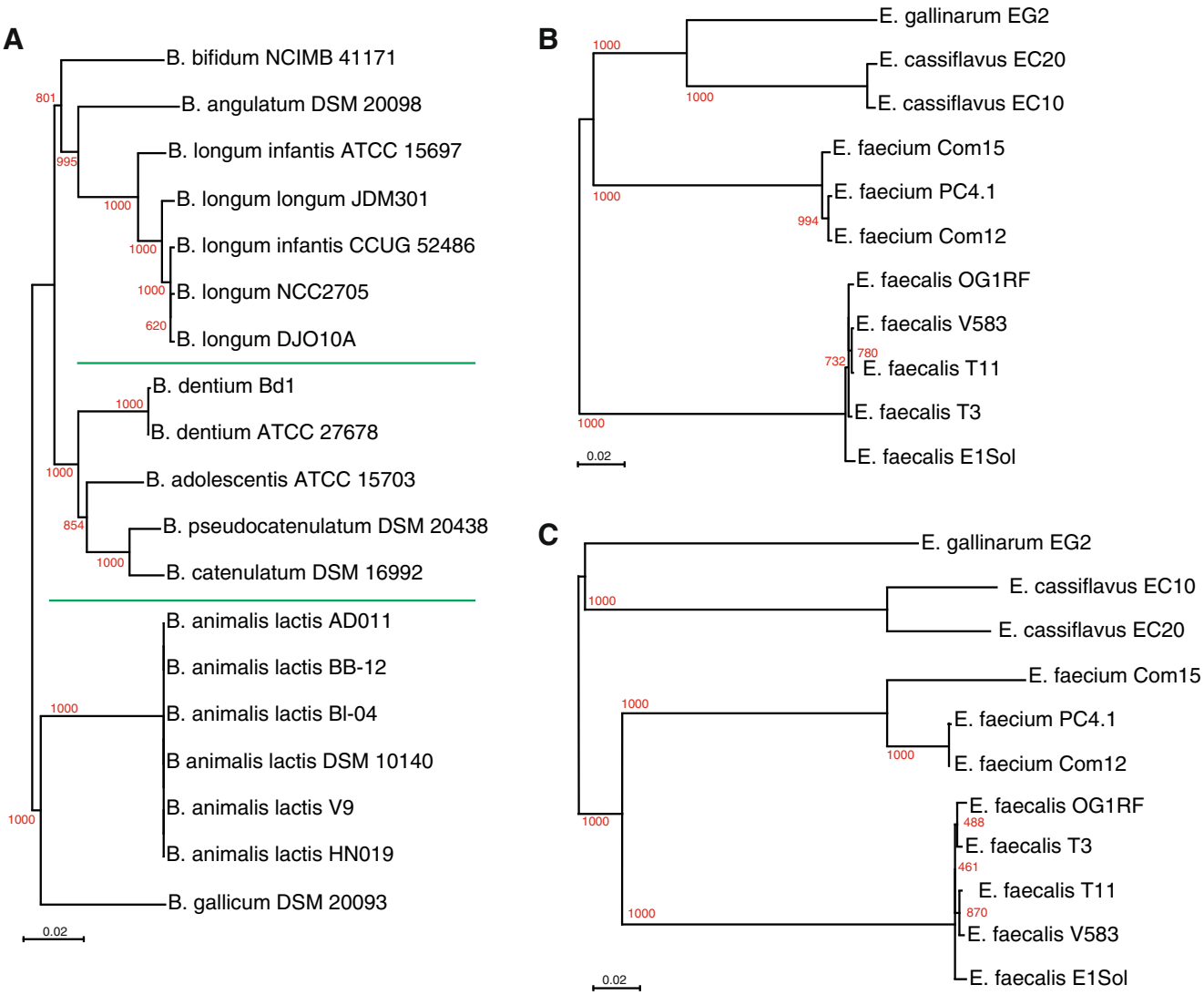


Figure 5 In silico MLST of gene fragments extracted from the genomes of *Bifidobacterium* (**a**) and *Enterococcus* (**b**, **c**). **b** The genes selected for MLST of *E. faecium*; **c** the genes selected for *E. faecalis* were used

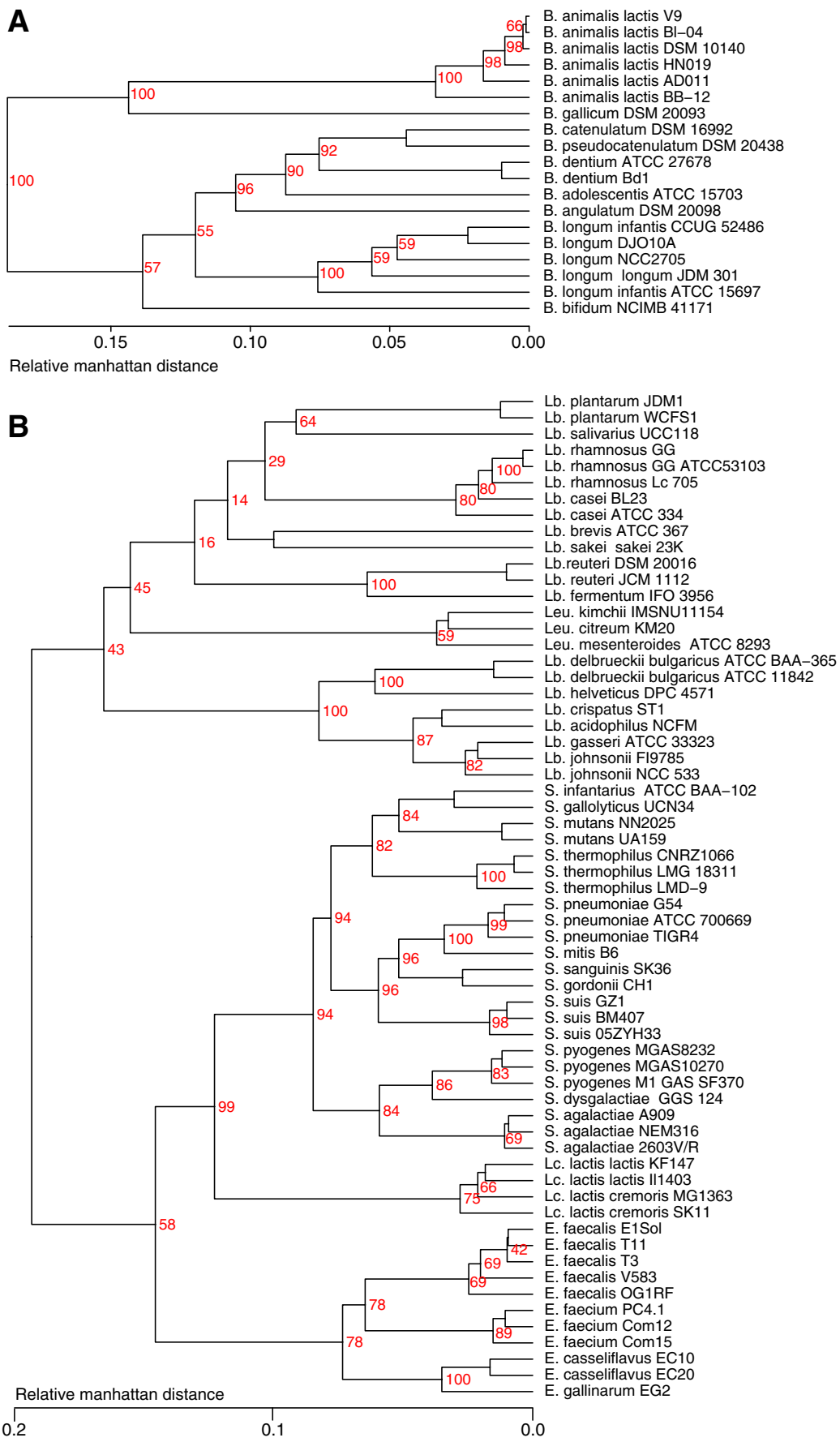


Figure 6 Hierarchical clustering of the *Bifidobacterium* genomes (**a**) and the Firmicute genomes (**b**) based on their variable gene content. The scale at the bottom applies to both trees

non-translated genes such as ribosomal RNA copies. In Fig. 7a, a large region around 350–400 kb appears to produce a gap of non-conserved genes in most *Bifidobacterium* genomes, with the exception of *B. longum infantis* CCUG 52486 and *B. longum* DJO10A. This represents a region with variable genes within the *B. longum* genomes (the red lanes in the atlas), which are completely absent in the other *Bifidobacterium* genomes. Other than that, there appears to be relatively little variation between the *B. longum* genomes. Strong conservation within the species is also observed for *B. animalis* when used as the reference, as shown in Fig. 7b. In that lower panel, the *B. animalis* lanes are far more darkly coloured than in the top panel, whereas the *B. longum* lanes are lighter in colour, illustrating that stronger homology is identified within a species than across species. Note that the large gap of the top atlas is no longer visible now, as the genes that were found in *B. longum* are absent in *B. animalis* and thus are no longer captured when the latter is used as a reference. Taken together, these data suggest that there is relatively strong conservation within a species of *Bifidobacterium*, an observation that has been made by others as well [30].

Figure 8 shows two BLAST Atlases of the *Lactobacillus* genomes. There appears to be considerably less conservation between species of this genus compared to *Bifidobacterium*. Even within the species of the two reference genomes of both panels, there are multiple gaps. This reflects the higher genetic diversity of the *Lactobacillus* genus compared to *Bifidobacterium*.

A BLAST Atlas of *Streptococcus* genomes with *S. thermophilus* LMD-9 as the reference is provided as Supplementary Fig. S3. Two non-pathogenic *E. faecalis* genomes were included as well, since these are normal human flora strains and could be considered to share a similar niche to *S. thermophilus*, at least when colonizing the human gut. There is quite a bit of variation in protein-coding genes between the three *S. thermophilus* genomes, and as expected, there is even fewer conservation in other species of *Streptococcus* or in the two *E. faecalis* genomes. Apparently, similarity in bacterial lifestyle is not necessarily represented by a significant homology in gene content.

COG Comparison of Pan- and Core Genomes

So far, conservation of genes was assessed and reported irrespective of their function, but that information is essential for a biological interpretation. The function of genes is not always known, but a large number of proteins have been assigned to a functional category of orthologous group, based on inference of sequence similarity to functionally characterized proteins. We have extracted the top-level COG groups for the genomes of interest and, in a first step, compared their core and pan-genomes genes. An example of such a statistical analysis for *Bifidobacterium* is shown in Fig. 9. At the bottom, the legend specifies the 3 top-level COG categories: 'information storage and processing', 'cellular processes and signaling' and 'metabolism', which are divided into 18 groups. The pie charts show what the fraction of the complete pan-genome genes of *Bifidobacterium* (left) or of the conserved core genes (right) belongs to each COG group. As expected, genes for which a function is not precise or not at all predicted build a significant fraction in the pan-genome, but these are mostly removed from the core genes, as their presence varies. More surprisingly, the three top categories are more or less similarly distributed in the two pie charts (thereby ignoring the contribution of the grey and black fractions), with a slight overrepresentation only of the information storage genes in the core genome compared to the pan-genome. Within these three broad categories, however, differences are visible when comparing the pan-genome or the core genome of these *Bifidobacterium* genomes. For instance, within 'information storage and processing', class J (translation, ribosomal structure and biogenesis) is enriched in the core genome, at the expense of K and L (transcription and replication, respectively). This means that the gene content related to these latter information storage processes is more variable and is hence captured in the pan-genome but less so in the core genome than the genes related to translation and ribosome biogenesis. Of interest is also the shift within the group 'metabolism' between classes E and G (for amino acid and carbohydrate transport/metabolism, respectively). The results indicate that the gene content for metabolism of amino acids is more conserved than that for carbohydrates, at least between these *Bifidobacterium* genomes. Lastly, enrichment in the core genome of class O, for post-translational modification and chaperones, is apparent within the group 'cellular processes and signaling'.

The *Bifidobacterium* findings can be compared to those of *Lactobacillus*, shown at the top of Fig. 10. The distribution of the three top-level COG categories in the pan-genome of *Lactobacillus* is different to that of *Bifidobacterium*, with more information storage and fewer metabolism genes. This is more obvious from Table 3, which lists the relative fractions of these COG classes when the grey and black fractions are ignored. For the core genes of *Lactobacillus*, the relative increase (compared to its pan-genome) in the fraction of information, storage and processing genes, at the expense of metabolism genes, is far more pronounced than for *Bifidobacterium*. Within the information and storage group, the enrichment of class J genes in the core genome of *Lactobacillus* is also stronger than reported for *Bifidobacterium*.

Figure 10 also shows the plots for *Lactococcus* (middle) and *Leuconostoc* (bottom). Although these last two genera are represented by four and three genomes only, all pan-

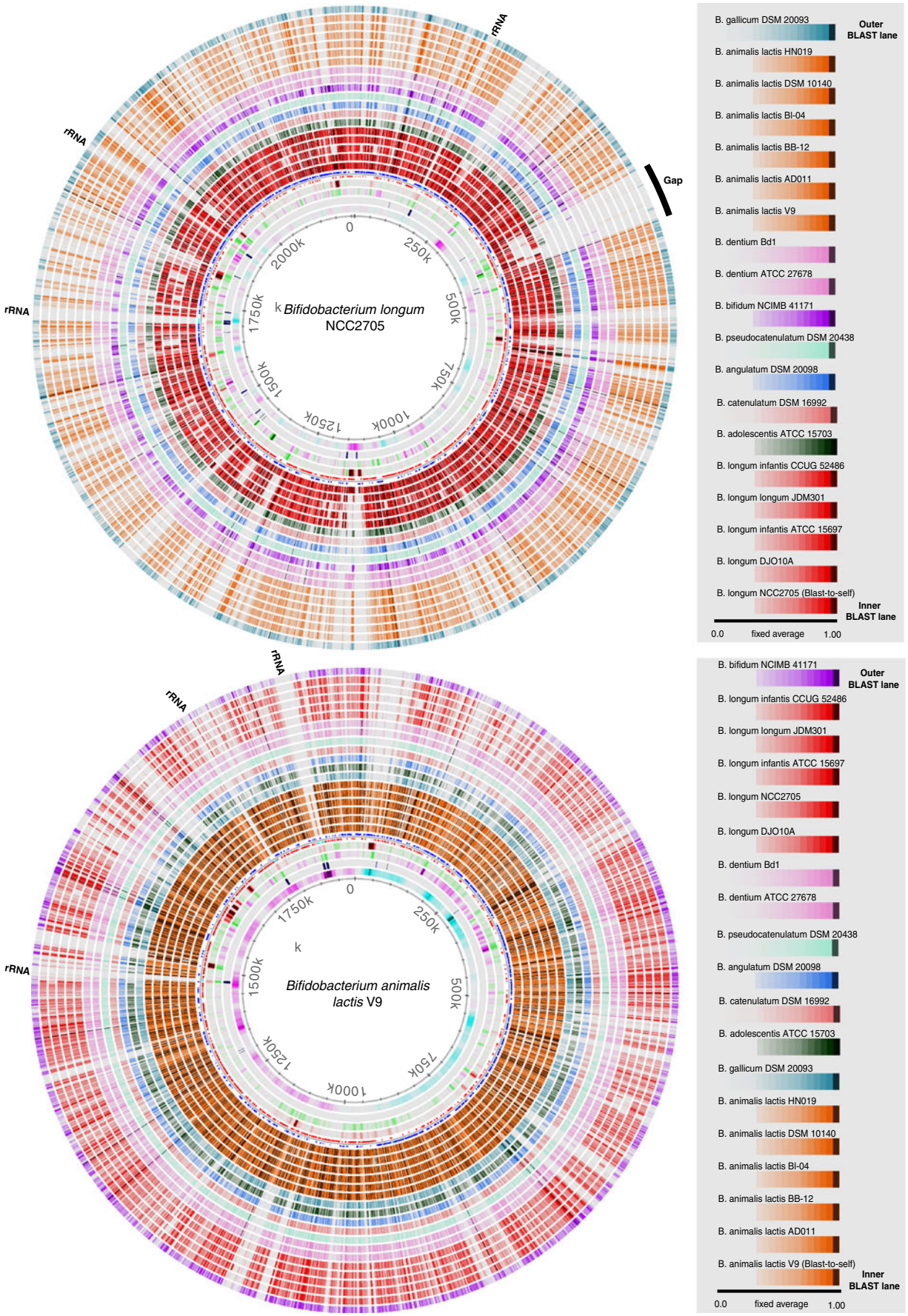

rRNA
rRNA
rRNA
Gap
0
250k
500k
750k
1000k
1250k
1500k
1750k
2000k
k
Bifidobacterium longum
NCC2705
B. gallicum DSM 20093
B. animalis lactis HN019
B. animalis lactis DSM 10140
B. animalis lactis BI-04
B. animalis lactis BB-12
B. animalis lactis AD011
B. animalis lactis V9
B. dentium Bd1
B. dentium ATCC 27678
B. bifidum NCIMB 41171
B. pseudocatenulatum DSM 20438
B. angulatum DSM 20098
B. catenulatum DSM 16992
B. adolescentis ATCC 15703
B. longum infantis CCUG 52486
B. longum longum JDM301
B. longum infantis ATCC 15697
B. longum DJO10A
B. longum NCC2705 (Blast-to-self)
Outer BLAST lane
Inner BLAST lane
0.0
fixed average
1.00
rRNA
rRNA
rRNA
0
250k
500k
750k
1000k
1250k
1500k
1750k
k
Bifidobacterium animalis
lactis V9
B. bifidum NCIMB 41171
B. longum infantis CCUG 52486
B. longum longum JDM301
B. longum infantis ATCC 15697
B. longum NCC2705
B. longum DJO10A
B. dentium Bd1
B. dentium ATCC 27678
B. pseudocatenulatum DSM 20438
B. angulatum DSM 20098
B. catenulatum DSM 16992
B. adolescentis ATCC 15703
B. gallicum DSM 20093
B. animalis lactis HN019
B. animalis lactis DSM 10140
B. animalis lactis BI-04
B. animalis lactis BB-12
B. animalis lactis AD011
B. animalis lactis V9 (Blast-to-self)
Outer BLAST lane
Inner BLAST lane
0.0
fixed average
1.00

Figure 7 Blast Atlas of *Bifidobacterium* genomes with *B. longum* strain NCC2705 (*top*) and *B. animalis lactis* strain V8 (*bottom*) as the reference. To the *right*, the BLAST lanes for each atlas are listed. The *four circles* inwards of the annotation lane of the reference genome represent stacking energy, position preference, global direct repeats and GC skew (from out to in)

genomes look surprisingly similar. However, when concentrating on the functionally annotated genes only (Table 3), some differences become apparent. The core genes of *Lactococcus* and *Leuconostoc* display a similar distribution of the three major COG classes as *Bifidobacterium* (which is taxonomically removed) that is different to the core genome of *Lactobacillus*, to which they are much closer related. Note that, in their pan-genomes, these three COG groups are similarly divided in *Bifidobacterium* and *Lactobacillus*. The shifts observed between pan-genome and core genome within a genus are contrasting between *Lactobacillus* and *Lactococcus*, whereas there is hardly a shift for *Leuconostoc*. From Fig. 10, it can be seen that, in the pan-genome of *Lactococcus*, class L genes make up a relatively large proportion. Within the metabolic gene classes, for *Lactobacillus*, a strong enrichment of nucleotide metabolism genes (class F) is observed in the core genes, whereas genes related to amino acid metabolism (class E) are more favoured in the core genome of *Lactococcus*. A significant increase in the core genes of COG class O (post-translational modification and chaperones) is observed for all analyzed genera. This could be an indication of the importance for such genes in the natural habitat of these gut bacteria.

The COG distribution plots for the pan-genome genes and the core genes of *Enterococcus* and *Streptococcus* is provided as Supplementary Fig. S4; the percentages of the three functionally classified COG top levels are included in Table 3. In contrast to the above examples, these two genera contain both pathogenic and non-pathogenic isolates. As in the previous examples, the large fraction of genes with unknown function is minimized in the core genome, but for both genera. Metabolism genes are neither over- nor underrepresented in the core genome. As before, a strong conservation of genes of COG class J (translation, ribosomal structure and biogenesis) was observed. Carbohydrate transport and metabolism genes (class G) were more frequently found in the *Enterococcus* pan-genome than in the *Streptococcus* pan-genome, though this was less pronounced for their core genomes.

In an attempt to correlate findings with the presence or absence of pathogenicity, all genomes of pathogenic isolates (irrespective of their genus) were combined to collectively compare these with the non-pathogens (probiotic, fermentative and normal gut flora organisms) combined. The pathogenic group consisted of *Enterococcus* and *Streptococcus* genomes only, whilst the non-pathogenic group contained genomes of all genera analyzed. The COG analysis was then repeated for these two phenotypic collections, whereby the pan- and core genomes obviously were recalculated. The pathogenic collection had a pan-genome of 14,209 gene families and a core genome of 508. The pan-genome of the non-pathogenic collection was significantly larger (21,087), and this group produced a core genome of only 278 gene families. The results of the COG analysis are shown in Fig. 11. Surprisingly, the two pan-genome statistics look nearly identical, despite the obvious phenotypic differences between these two groups that both consist of diverse organisms, with a skewed genus distribution. However, the COG distribution between the two core genomes differs dramatically. The fraction of genes for which no homologue could be identified has (nearly) disappeared from the core genome of the non-pathogenic group, but a significant fraction of these genes was retained in the core genome of pathogens. The top level of metabolism genes has decreased in both core genomes, but more so in the group of the non-pathogens. Thus, the core genes of the non-pathogenic isolates are more frequently information storage genes and less likely metabolism genes than the core genes of pathogens (Table 4). Zooming in on shifts in single categories between pan- and core genomes, the enrichment of core genes belonging to class J, already observed for all single genus plots shown above, is even more extensive and far more extreme with the collection of non-pathogenic organisms. An enrichment for class O (post-translational modification and chaperones) within the top-level 'metabolism' is pronounced in the core genome of both groups, but the pathogens also show enrichment of class M genes (cell wall/membrane biogenesis) which is actually reduced in the core genome of non-pathogens.

Discussion

The comparative analysis presented here of 81 bacterial genomes, covering 6 genera and 43 different species, could be performed by grouping their genes into gene families and comparing core and pan-genomes of various subsets of genomes. The findings frequently confirmed taxonomic relationships but could not identify common signatures, in terms of gene content, for all non-pathogenic bacteria included in the analysis. This finding is surprising, as all these species occupy a similar niche. Conserved genes were compared by means of a consensus tree, while genes variably present were analyzed by cluster analysis. The latter indicated that *Leuconostoc* genomes share a considerable number of variable genes with *Lactobacillus*. Functional analysis of the proteins coded by the genes comprising a genus' core genome

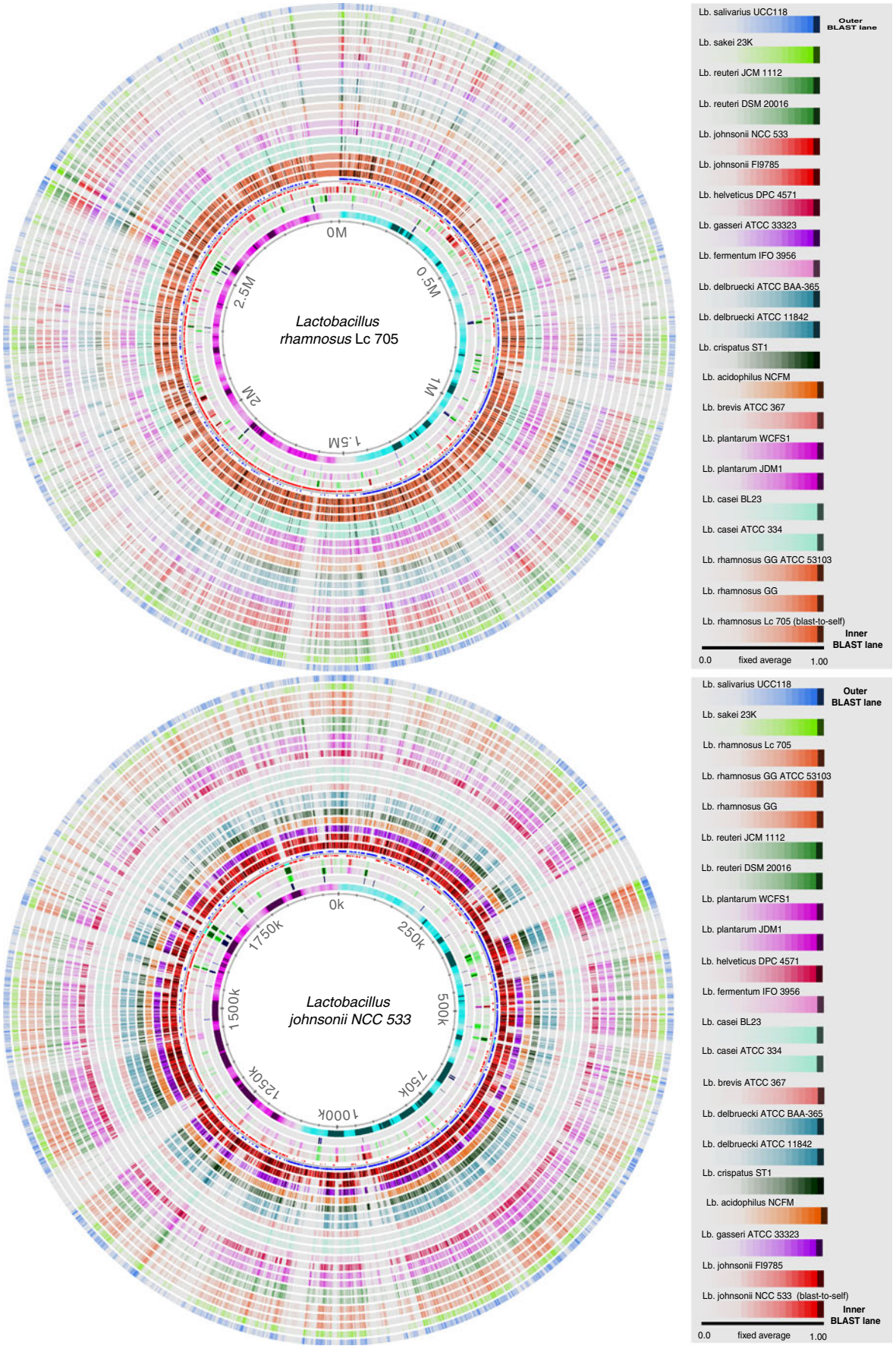

0M
0.5M
1M
1.5M
2M
2.5M
Lactobacillus rhamnosus Lc 705
Lb. salivarius UCC118
Outer BLAST lane
Lb. sakei 23K
Lb. reuteri JCM 1112
Lb. reuteri DSM 20016
Lb. johnsonii NCC 533
Lb. johnsonii FI9785
Lb. helveticus DPC 4571
Lb. gasseri ATCC 33323
Lb. fermentum IFO 3956
Lb. delbruecki ATCC BAA-365
Lb. delbruecki ATCC 11842
Lb. crispatus ST1
Lb. acidophilus NCFM
Lb. brevis ATCC 367
Lb. plantarum WCFS1
Lb. plantarum JDM1
Lb. casei BL23
Lb. casei ATCC 334
Lb. rhamnosus GG ATCC 53103
Lb. rhamnosus GG
Lb. rhamnosus Lc 705 (blast-to-self)
Inner BLAST lane
0.0 fixed average 1.00
0k
250k
500k
750k
1000k
1250k
1500k
1750k
Lactobacillus johnsonii NCC 533
Lb. salivarius UCC118
Outer BLAST lane
Lb. sakei 23K
Lb. rhamnosus Lc 705
Lb. rhamnosus GG ATCC 53103
Lb. rhamnosus GG
Lb. reuteri JCM 1112
Lb. reuteri DSM 20016
Lb. plantarum WCFS1
Lb. plantarum JDM1
Lb. helveticus DPC 4571
Lb. fermentum IFO 3956
Lb. casei BL23
Lb. casei ATCC 334
Lb. brevis ATCC 367
Lb. delbruecki ATCC BAA-365
Lb. delbruecki ATCC 11842
Lb. crispatus ST1
Lb. acidophilus NCFM
Lb. gasseri ATCC 33323
Lb. johnsonii FI9785
Lb. johnsonii NCC 533 (blast-to-self)
Inner BLAST lane
0.0 fixed average 1.00

Figure 8 BLAST Atlas of *Lactobacillus* with *L. rhamnosus* strain Lc705 (*top*) and *Lb. johnsonii* strain NCC533 (*bottom*) as the

identified the relative strong conservation of information storage genes; this was observed for all genera analyzed. When all genomes were divided into a pathogenic and a non-pathogenic group, the pan-genome of both groups showed a surprisingly similar COG distribution; however, their core genome differed considerably. It was observed that, in the core genome of non-pathogenic genomes, genes for post-translational modification and chaperones were enriched.

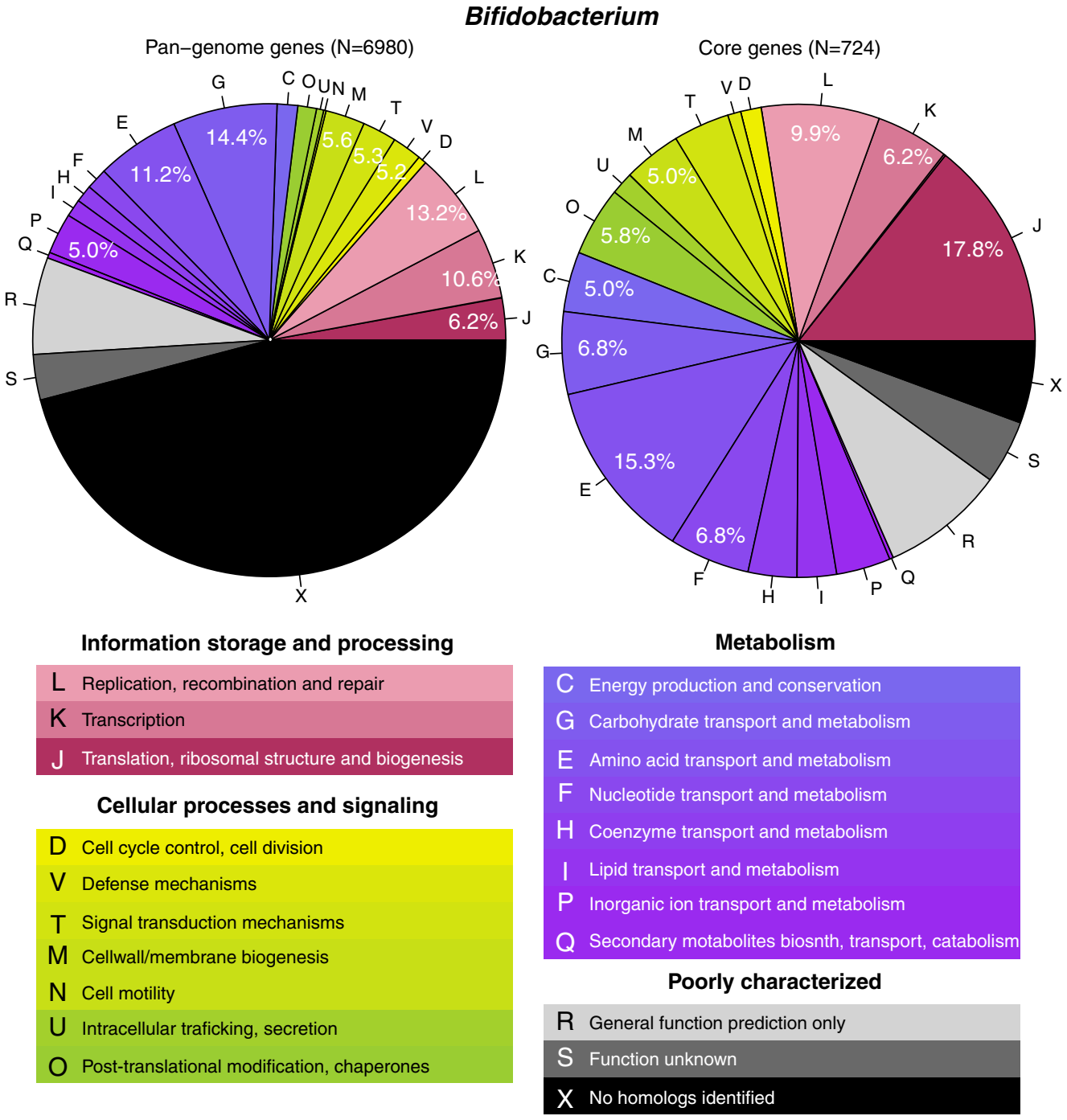
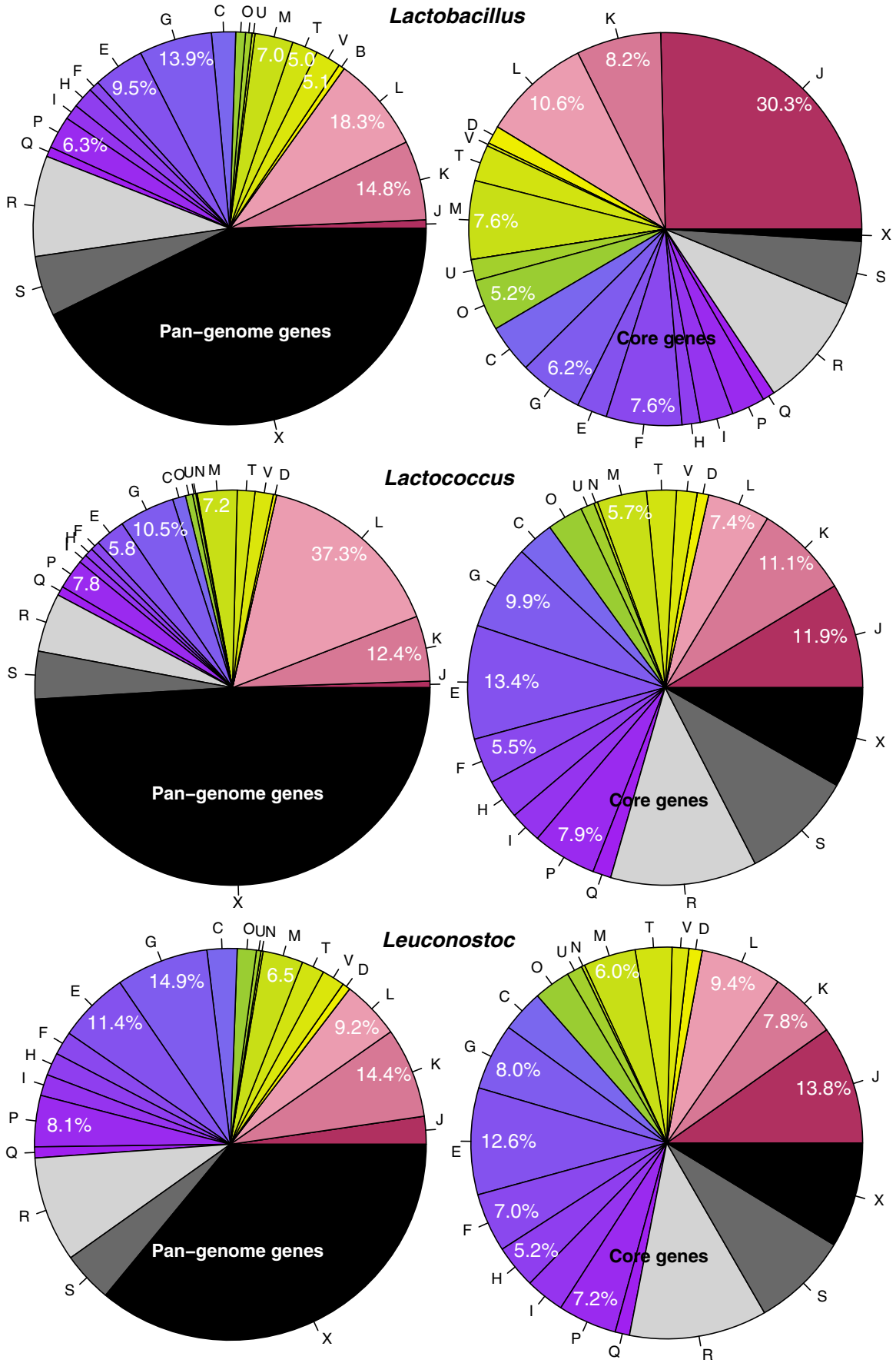


Figure 9 COG statistics for the genes found in the pan-genome (*left*) and core genome (*right*) of *Bifidobacterium* genomes. The key for the COG classes is explained below the pie charts. Percentages given in the pie chart are calculated by exclusion of classes R, S and X. Only values ≥5% are shown


Lactobacillus
Pan−genome genes
Core genes
13.9%
9.5%
6.3%
7.0
5.0
5.1
18.3%
14.8%
8.2%
10.6%
30.3%
7.6%
5.2%
6.2%
7.6%
Lactococcus
Pan−genome genes
Core genes
10.5%
5.8
7.8
7.2
37.3%
12.4%
5.7%
7.4%
11.1%
11.9%
9.9%
13.4%
5.5%
7.9%
Leuconostoc
Pan−genome genes
Core genes
14.9%
11.4%
8.1%
6.5
9.2%
14.4%
6.0%
9.4%
7.8%
13.8%
8.0%
12.6%
7.0%
5.2%
7.2%

◀ **Figure 10** COG distribution of pan-genome genes (*left*) and core genes (*right*) for *Lactobacillus* (*top*), *Lactococcus* (*middle*) and *Leuconostoc* (*bottom*)

A simultaneous comparison of the pan- and core genomes of publicly available genomes of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Streptococcus* and *Bifidobacterium*, as was performed here, has not been published before, but similar analyses have been published for smaller selections of organisms. Canchaya and co-workers [2] performed comparative genomics of the then five available *Lactobacillus* genomes from different species and commented on the high variability within this genus. Schleifer and Ludwig [23] stated that "It is widely recognized that the taxonomy of this genus is unsatisfactory due to the highly heterogeneous nature of its members". Indeed, data presented here illustrate the diversity within *Lactobacillus*. However, the heterogeneity of this genus is not larger than that of other bacteria. Using the same comparison criteria as applied here, the pan-genome of 53 *E. coli* genomes was found to comprise 13,000 gene families, even within this single species [18]. Similarly, an analysis of 27 genomes from 7 *Vibrio* species produced a pan-genome of nearly 15,000 gene families for this genus [31], and 38 genomes of 5 *Burkholderia* species contained as much as 26,000 gene families [28]. Thus, the diversity in gene content within the genus *Lactobacillus*, based on the genome sequences currently available, is not exceptional in the bacterial world.

Our analyses are mainly based on core genomes, an approach that others followed as well [2]. Those authors had defined a core genome for *Lactobacillus* whose size is similar to our findings. However, the fraction of identified orthologous genes in the pairwise comparisons performed by those authors range from 52.3% to 68.9%, which is much higher than our findings of between 12% and 42%, shown in the BLAST Matrix of Fig. 2. The difference may be due to the way these percentages were calculated. Whereas we express these as the fraction of gene families found in the reciprocal pan-genome of the pair of analyzed genomes, their calculations are different, and they do not state the cut-off used to recognize orthologous genes as such. In view of their limited reported range, we believe our way of expressing pairwise homology is more useful, as it gives a more sensitive measure. In a subsequent publication, comparative genomics was performed with a larger set of 12 *Lactobacillus* genomes [3]. Inclusion of 7 more genomes reduced their core genome to 141 genes which indicates they used more strict criteria of inclusion than the 50–50 rule we applied. Similar to our analysis, these authors compared the COG classes of the core genes they had identified, and their findings also reported the largest class represented to be genes involved in translation, followed by replication.

Comparative genomics of both *Lactobacillus* and *Bifidobacterium* was presented in a review [30], which mentioned the ability of *Bifidobacterium* to "synthesize at least 19 amino acids and (…) all of the enzymes that are needed for the biosynthesis of pyrimidine and purine nucleotides". These authors further emphasized the importance of carbohydrate metabolism for *Bifidobacterium* with its capability to degrade complex sugars. Indeed, top-level metabolism genes form a major part of the *Bifidobacterium* core genome (Fig. 9) with class E (amino acid metabolism) as the largest fraction within that category. When we compare this core genome with that of *Lactobacillus* (Fig. 10), our analysis shows that class F genes (nucleotide metabolism) comprise the largest metabolism gene fraction in the *Lactobacillus* core genome. Ventura and co-workers [30] used a known physiological characteristic (*Bifidobacterium* species are known for their prototrophy) and looked for evidence of this in the genomes. In contrast, we have done a neutral analysis of pan- and core genome COG class representation and then compared this between genera. Our approach reveals novel insights that would remain unnoticed when known phenotypes are taken as a start, for instance the conservation of COG class O genes, involved in post-translational modification and chaperones, in both of these genera.

The authors of a recent review on *Bifidobacterium* genomics [17] pointed out that most *Bifidobacterium*

Table 3 Relative fractions of COG groups within the functionally annotated genes for the six genera

COG groups	*Bifidobacterium*		*Lactobacillus*		*Lactococcus*		*Leuconostoc*		*Enterococcus*		*Streptococcus*	
	Pan (%)	Core (%)	Pan (%)	Core (%)	Pan (%)	Core (%)	Pan (%)	Core (%)	Pan (%)	Core (%)	Pan (%)	Core (%)
Information storage	30.0	33.9	34.0	49.1 ↑↑	50.5	30.4 ↓↓	28.1	31.0	26.6	33.8 ↑	34.7	42.6 ↑↑
Cellular process, signalling	21.9	20.2	22.7	20.3	17.1	19.1	19.1	20.0	24.4	18.9 ↓	26.3	20.3 ↓
Metabolism	48.1	45.9	44.3	30.6 ↓↓	32.2	50.6 ↑↑	52.7	49.1	50.0	47.8	39.2	36.9

All percentages are expressed as the fraction of all COG classes C to V. The arrows indicate significant shifts between the pan-genome genes and core genes for a given genus. Percentages do not always add up to 100% due to rounding effects

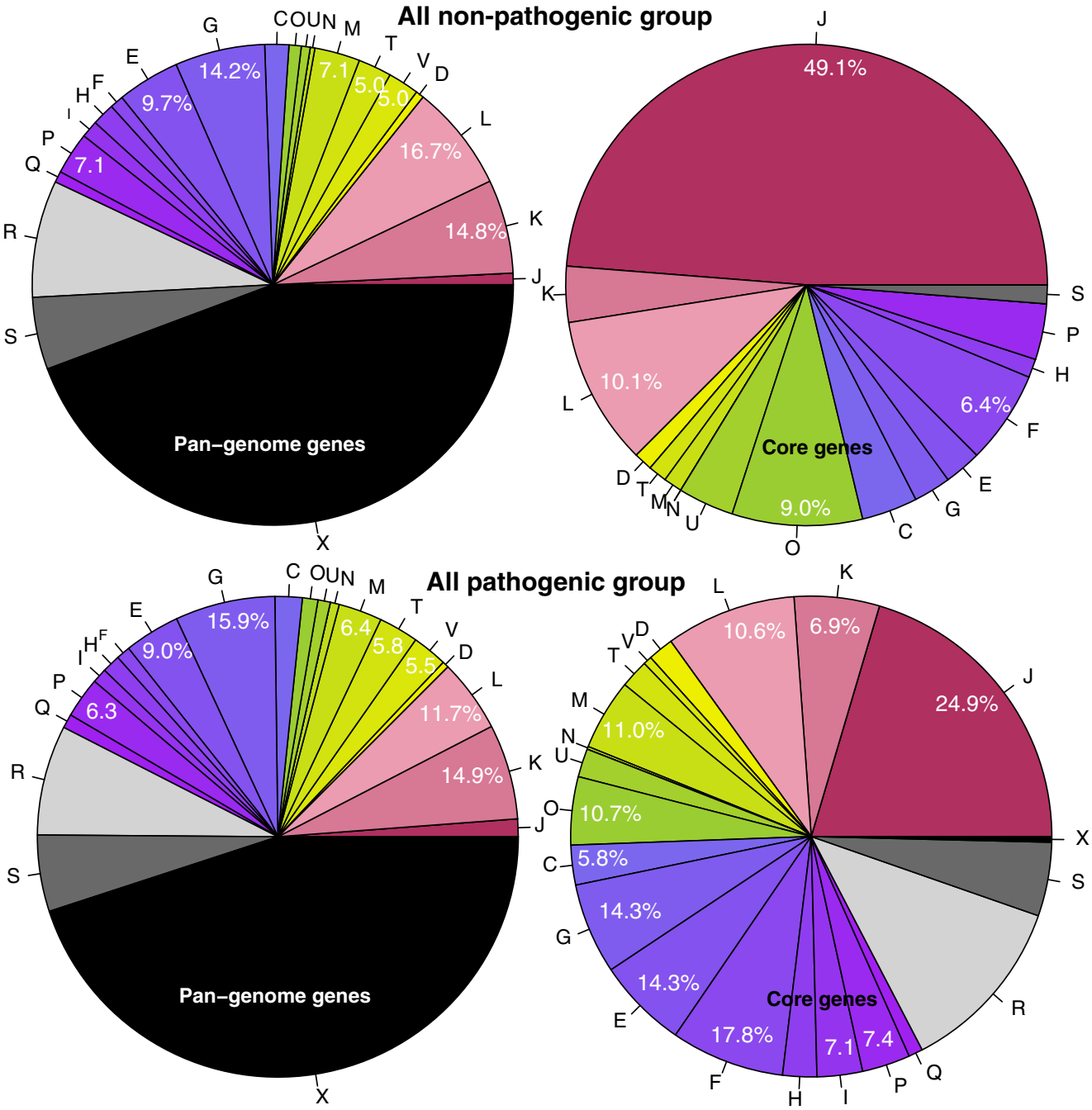


Figure 11 COG statistics for the genes found in the pan-genome (*left*) and core genome (*right*) of the collection of genomes from all included organisms, divided into non-pathogenic isolates (probiotic, fermentative and normal human gut flora) at the *top* and pathogenic isolates at the *bottom*

Table 4 Relative fractions of COG groups within the functionally annotated genes for non-pathogens/pathogens. The arrows indicate how the reported percentages increase or decrease in the core genome compared to the pan genome.

COG groups	Non-pathogens		Pathogens	
	Pan (%)	Core (%)	Pan (%)	Core (%)
Information storage	33.5	64.4 ↑↑	29.3	42.4 ↑↑
Cell. process, signalling	22.0	16.6 ↓	25.7	18.9 ↓
Metabolism	44.5	20.2 ↓↓	44.9	38.7 ↓

genomes have been sequenced from organisms that have a long history of culture outside their natural habitat, the gut, with the exception of *B. longum* DJO10A. There is good evidence that the genome of *Bifidobacterium* is subject to gene reduction to adapt to prolonged culture conditions. This could potentially bias our comparative analysis of *Bifidobacterium* genomes with that of the other probiotic organisms.

The term 'lactic acid bacteria' is commonly used to describe bacteria used as starter cultures and fermentation of foodstuffs. LAB can refer to species from the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Enterococcus*, *Pediococcus* or all of the Lactobacillales, and sometimes includes *Bifidobacterium* as well. However, there are good reasons why these bacteria have been placed into different genera and phyla. The analyses presented here support their current taxonomic positions and stress their differences in gene content. The term LAB incorrectly suggests all these organisms are somehow related; a view that is still being presented in the literature [15]. The use of the term LAB is a bit misleading, as the genetic content from these various genera differ significantly. Moreover, some of the genera within LAB comprise only non-pathogenic species (*Leuconostoc*, *Bifidobacterium*, *Lactobacillus*), whereas other genera are a mixture of pathogenic and non-pathogenic species and strains (*Streptococcus*, *Enterococcus*). It would be better to refrain from the term LAB as there is no common denominator, other than the production of lactic acid (which is not restricted to these organisms) to collectively describe all species and strains supposedly included in this diverse group of organisms.

An extensive comparative study of *Enterococcus* genomes could not be identified from the literature. Most studies concentrate on pathogenicity of *E. faecalis*. Vebø and co-workers [29] compared probiotic and (uro-)pathogenic *E. faecalis* genomes; however, those comparisons were not based on sequence data. The *Enterococcus* genomes we have included were mostly from pathogenic organisms (only two non-pathogenic *E. faecalis* strains whose sequences were nearing completion were publicly available at the time of analysis), which limits the strength of this analysis, as it cannot be used to compare and contrast multiple non-pathogenic with pathogenic *Enterococcus* genomes. The 11 genomes included represent only 4 species, giving a pan-genome of nearly 8,000 gene families. The first four species of *Lactobacillus* or *Streptococcus* genomes in the pan-genome plots of Fig. 1 produce smaller pan-genomes, which could suggest that the diversity of *Enterococcus* could be at least as extensive as that of *Lactobacillus*. The pairwise BLAST comparison within this genus resulted in homologues varying from 24% to 84%, again indicating extensive intra-genus diversity.

Streptococcus and *Enterococcus* are frequently considered as closely related, but the BLAST Matrix comparing all included genomes (Supplementary Fig. S1) does not support this. Instead, somewhat surprisingly, the observed homology between *Leuconostoc* and *Streptococcus* genomes is slightly higher than that between *Streptococcus* and *Enterococcus*. On the other hand, *Lc. lactis* was positioned in between these two genera in the tree based on variable gene content. A shared gene pool between these genera can be hypothesized. Based on the conserved core genes, however, *Enteroccus* is more related to *Streptococcus*, while *Lactococcus* is more distinct.

A small comparative study of *Streptococcus* genomes combined with MLST suggested that *S. thermophilus* is a relatively young clone, evolved by genome reduction which removed or inactivated *Streptococcus* virulence genes [13]. It is possible, however, that the reduced genomes observed are the result of prolonged use as starter cultures, as no fresh human isolates have been sequenced to date. In a short review, Delorme [5] states that "*S. thermophilus* is related to *Lactococcus lactis*…". Indeed, from the all-against-all BLAST Matrix, a similarity between 17.3% and 20.2% is recorded between genomes of these two species, which is higher than that between *S. thermophilus* and any other non-streptococcal genome. However, *Lc. lactis* also shares 16.0% to 18.0% of reciprocal genes with *S. suis*, so these overlapping percentages of gene similarity are no indicator of similarity in (probiotic) phenotype. Within the *Streptococcus* genus, the stated similarity of *S. thermophilus* with *Streptococcus sanguinis* (the only member of the viridans group for which a genome sequence is available) is confirmed in our Matrix, but an even higher similarity is found with *Streptococcu gordonii*.

The COG analysis of the core genomes of separate genera identified both similarities and differences. The three top-level functional COG groups are relatively equally divided over the functionally annotated pan-genes of all species, but their core genomes differ. Notably, *Lactobacillus* and *Leuconostoc* both have a smaller fraction of metabolism core genes than the other four genera and a larger information storage gene fraction. Information storage genes are essential, but redundancy allows so much variation between organisms that they are not all maintained in a core genome of diverse species. In the approach presented here, we first identified the core genomes of groups of bacteria and then sorted the genes in these core genomes for top-level COG categories. As a consequence, genes that were insufficiently conserved based on sequence similarity to be maintained in the core genome are removed despite their possible functional conservation. Using this approach, we found no correlation between the diversity within a genus (using the difference of their pan- and core genome as a measure)

and the fraction of their information/storage COG genes. This lack of correlation is illustrated by the core genome of *Bifidobacterium* (724, or 10% of its pan-genome) and *Leuconostoc* (1,164, or 40% of its pan-genome). These two core genomes contain 34% and 31% information/storage genes, respectively, despite a huge difference in the degree of variation in these two genera.

Of particular interest is the COG analysis where all genomes were divided into a pathogenic and a non-pathogenic group. Virulence genes are not a separate COG category, but from the comparison of the core genomes of the pathogenic group with that of the non-pathogenic group, we can hypothesize that genes belonging to COG categories M (cell wall/membrane biosynthesis) and O (post-translational modification, chaperones) would mostly contribute to virulence. Conversely, it could be assumed that genes highly overrepresented in the core genome of the non-pathogenic group (compared to the core genome of the pathogenic group) most likely contribute to their probiotic or fermentative lifestyle. We observe enrichment for genes belonging to COG class J (translation, ribosomal structure and biogenesis) and again O (post-translational modification and chaperones). The finding that core genes of the non-pathogenic isolates are more frequently information storage genes and less likely metabolic genes than the core genes of pathogens is counter-intuitive. It is generally accepted that commensals and probiotic strains are most adequately equipped to live in the intestine, which would assume they share a large number of (conserved) metabolic genes to do so. Instead, the reduced metabolism gene fraction in their core genome suggests that there is a large variation within these genes, which reflects the diversity of the various commensals, fermentative and probiotic isolates. The vast enrichment for information/storage genes in the core genome of the non-pathogenic organisms is possibly a reflection of the relative poor conservation of all other functional classes in this group, an effect that appears to be less pronounced in the (ecologically more diverse) pathogenic group. The fact that *Bifidobacterium* are not present in the pathogenic group may have skewed these results slightly. A more accurate prediction for conserved genes with an important role in bacteria with a non-pathogenic lifestyle may become possible in the future, when more non-pathogenic *Enterococcus* genomes become available, which allows comparison of gene content within a genus or even species.

Conclusions

This study illustrates the value of comparative genomics of multiple genomes within and between related species and genera. The applied tools are relatively simple to analyze a vast number of genes, and the results can support or contradict existing hypotheses and taxonomic divisions, as well as generate novel hypotheses. We believe the data presented here can assist in understanding the commensal and probiotic relationship of bacteria with their human host. The work presented here demonstrates that the used analyses can be applied to large numbers of genomes, when searching for general mechanisms to predict trends even across genera. The presented analyses can be taken as a test case for comparison of multiple genomes from a largely variable dataset.

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