Comparative genomics of *Fructobacillus* spp. and *Leuconostoc* spp. reveals niche-specific evolution of *Fructobacillus* spp.

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**Abstract**

**Background:** *Fructobacillus* spp. in fructose-rich niches belong to the family *Leuconostocaceae*. They were originally classified as *Leuconostoc* spp., but were later grouped into a novel genus, *Fructobacillus*, based on their phylogenetic position, morphology and specific biochemical characteristics. The unique characters, so called fructophilic characteristics, had not been reported in the group of lactic acid bacteria, suggesting unique evolution at the genome level. Here we studied four draft genome sequences of *Fructobacillus* spp. and compared their metabolic properties against those of *Leuconostoc* spp.

**Results:** *Fructobacillus* species possess significantly less protein coding sequences in their small genomes. The number of genes was significantly smaller in carbohydrate transport and metabolism. Several other metabolic pathways, including TCA cycle, ubiquinone and other terpenoid-quinone biosynthesis and phosphotransferase systems, were characterized as discriminative pathways between the two genera. The *adhE* gene for bifunctional acetaldehyde/alcohol dehydrogenase, and genes for subunits of the pyruvate dehydrogenase complex were absent in *Fructobacillus* spp. The two genera also show different levels of GC contents, which are mainly due to the different GC contents at the third codon position.

**Conclusion:** The present genome characteristics in *Fructobacillus* spp. suggest reductive evolution that took place to adapt to specific niches.

**Keywords:** *Fructobacillus*, *Leuconostoc*, Comparative genomics, Fructophilic lactic acid bacteria, Niche-specific evolution, Metabolism

**Background**

Lactic acid bacteria (LAB) are found in a variety of environments, including dairy products, fermented food or silage, and gastrointestinal tracts of animals. Their broad habitats exhibit different stress conditions and nutrients, forcing the microbe to develop specific physiological and biochemical characteristics, such as proteolytic and lipolytic activities to obtain nutrients from milk [1], tolerance to phytoalexins in plants [2], or tolerance to bile salts to survive in the gastrointestinal tracts [3]. *Fructobacillus* spp. in the family *Leuconostocaceae* are found in fructose-rich environments such as flowers, (fermented) fruits, or bee guts, and are characterized as fructophilic lactic acid bacteria (FLAB) [4–6].

The genus *Fructobacillus* is comprised of five species: *Fructobacillus fructosus* (type species), *F. durianis*, *F. ficulneus*, *F. pseudoficulneus* and *F. tropaeoli* [6, 7]. Four of the five species formerly belonged to the genus *Leuconostoc*, but were later reclassified as members of a novel genus, *Fructobacillus*, based on their phylogenetic position, morphology, and biochemical characteristics [8]. *Fructobacillus* is distinguished from *Leuconostoc* by the preference for fructose over glucose as the carbon source and the need for an electron acceptor (e.g. pyruvate or oxygen) during glucose assimilation. *Fructobacillus* is further differentiated from *Leuconostoc* by the production...
of acetic acid instead of ethanol when glucose is metabolized. We previously compared these microorganisms with special attention to the activities of alcohol and acetaldehyde dehydrogenases; *Fructobacillus* lacks the bifunctional acetaldehyde/alcohol dehydrogenase gene (*adhE*) [9] and its enzyme activities. They are the only obligately heterofermentative LAB without *adhE* to date, suggesting that niche-specific evolution occurred at the genome level. Recent comparative genomic studies also revealed niche-specific evolution of several LAB, including vaginal lactobacilli and strains used as dairy starter cultures [10–12].

This is the first study to compare the metabolic properties of the draft genome sequences of four *Fructobacillus* spp. with those of *Leuconostoc* spp., with a special focus on fructose-rich niches. Results obtained confirm the general trend of reductive evolution, especially metabolic simplification based on sugar availability.

**Methods**

**Bacterial strains and DNA isolation**

*Fructobacillus fructosus* NRIC 1058T, *F. ficulneus* JCM 12225T, *F. pseudoficulneus* DSM 15468T and *F. tropaeoli* F214-1T were cultured in FYP broth (1%: 10 g D-fructose, 10 g yeast extract, 2 g polypeptone, 0.5 g Tween 80, 0.2 g MgSO4·7H2O, 0.01 g MnSO4, 4H2O, 0.01 g FeSO4·7H2O, 0.01 g NaCl; pH 6.8) at 30 °C for 24 h. Genomic DNA was isolated by the method of a combination of phenol/chloroform and glass beads as described previously [13].

**Draft genome sequencing and de novo assembly**

Whole-genome sequencing was conducted by Illumina Genome Analyzer II system, with insert length of about 500 bp. Total 6,060,140, 1,904,646, 2,474,758 and 13,680,640 reads with average lengths of 60 to 91 bp were obtained from *F. fructosus* NRIC 1058T, *F. ficulneus* JCM 12225T, *F. pseudoficulneus* DSM 15468T and *F. tropaeoli* F214-1T, respectively. *De novo* assembly using the Velvet Assembler for short reads with parameters optimized by the VelvetOptimizer (Version 1.2.10) [14] resulted in 57, 28, 15 and 101 contigs each (Length: 1,489,862, 1,552,198, 1,413,733 and 1,686,944 bp; N50: 89,458, 226,528, 283,981 and 226,443 bp). The k-mer sizes for the strains were 81, 45, 51, 63 bp each. The genome was annotated using the Microbial Genome Annotation Pipeline (MiGAP) [15] with manual verification. In the pipeline, protein coding sequences (CDSs) were predicted by MetaGeneAnnotator 1.0 [16], tRNAs were predicted by tRNAscan-SE 1.23 [17], rRNAs were predicted by RNAmer 1.2 [18], and functional annotation was finally performed based on homology searches against the RefSeq, TrEMBL, and Clusters of Orthologous Groups (COG) protein databases.

**Genomic data of Fructobacillus durionsis and Leuconostoc spp.**

Draft genome sequence of *Fructobacillus durionsis* DSM 19113T was obtained from the JGI Genome Portal (http://genome.jgi.doe.gov/) [19] and annotated using MiGAP in the same way as other *Fructobacillus* spp. Annotated genome sequences for nine of the twelve *Leuconostoc* species were obtained from the GenBank or RefSeq databases at NCBI. Of *Leuconostoc* spp., genomic data of *Leuconostoc holzapelli*, *Leuconostoc miyukkimchii* and *Leuconostoc palmae* were not available at the time of analysis (December 2014) and were not included in the present study. When multiple strains were available for a single species, the most complete one was chosen. GenBank accession numbers of the strains used are listed in Table 1.

**Quality assessment of the genomic data**

The completeness and contamination of the genomic data were assessed by CheckM (Version 1.0.4) [20], which inspects the existence of gene markers specific to the *Leuconostocaceae* family, a superordinate taxon of *Fructobacillus* and *Leuconostoc*.

**Comparative genome analysis and statistical analysis**

To estimate the size of conserved genes, all protein sequences were grouped into orthologous clusters by GET_HOMOLOGUES software (version 1.3) based on the all-against-all bidirectional BLAST alignment and the MCL graph-based algorithm [21]. The conserved genes are defined as gene clusters that are present in all analyzed genomes (please note the difference from the definition of specific genes). The rarefaction curves for conserved and total genes were drawn by 100-time iterations of adding genomes one by one in a random order. From this analysis, two genomes (*L. fallax* and *L. inhae*) were excluded to avoid underestimation of the size of conserved genes, since they contained many frameshifted genes, probably due to the high error rate at homopolymer sites of Roche 454 sequencing technology.

For functional comparison of the gene contents between *Fructobacillus* spp. and *Leuconostoc* spp., CDS predicted in each strain were assigned to Cluster of Orthologous Groups (COG) functional classification using the COGNITOR software [22]. Metabolic pathway in each strain was also predicted using KEGG Automatic Annotation Server (KAAS) by assigning KEGG Orthology (KO) numbers to each predicted CDS [23]. The numbers of genes assigned to each COG functional category were summarized as a table (Table 2). In the present study, *Fructobacillus*-specific genes were defined as those conserved in four or more *Fructobacillus* spp. (out of five) and in two or less *Leuconostoc* spp. (out of nine). *Leuconostoc*-specific genes were defined as those conserved in seven or more *Leuconostoc* spp. and one or less *Fructobacillus* spp.
The Mann–Whitney U test was applied to compare genome features and gene contents of Fructobacillus spp. and Leuconostoc spp. The p value of 0.05 was considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics for Windows (Version 21.0. Armonk, NY: IBM Corp.).

Phylogenetic analysis
Orthologous clusters that were conserved among all Fructobacillus spp., all Leuconostoc spp. and Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842 (as the out-group) were determined by GET_HOMOLOGUES as described above. For phylogenetic reconstruction, 233 orthologs that appeared exactly once in each genome were selected. The amino acid sequences within each cluster were aligned using MUSCLE (version 3.8.31) [24]. Poorly-aligned or divergent regions were trimmed using Gblocks [25], and conserved regions were then concatenated using FASconCAT-G [26]. A partitioned maximum likelihood analysis was performed to construct the phylogenetic tree with RAxML (version 8.1.22) [27] using the best-fit evolutionary models predicted for each alignment by ProtTest [28]. The number of bootstrapping was 1,000 replicates.

Polysaccharides production and reaction to oxygen
Polysaccharides production from sucrose were determined by the methods as described previously [29]. Briefly, the strains were inoculated on agar medium containing sucrose as sole carbon source and incubated aerobically at 30 °C for 48 h.

To study reaction to oxygen on growth, the cells were streaked onto GYP agar [8], which contained D-glucose as the sole carbon source, and cultured under anaerobic and aerobic conditions at 30 °C for 48 h as described previously [4]. The anaerobic conditions were provided by means of a gas generating kit (AnaeroPack, Mitsubishi Gas Chemical, Japan). These studies were conducted for the type strains of five Fructobacillus species, Leuconostoc mesenteroides subsp. mesenteroides NRIC 1541T, Leuconostoc citreum NRIC 1776T and Leuconostoc fallax NRIC 0210T.

Data deposition
Annotated draft genome sequences of F. fructosus NRIC 1058T, F. ficulneus DSM 15468T, F. pseudoficulneus DSM 15468T and F. tropaeoli F214-1T were deposited to the DDBJ/EMBL/GenBank International Nucleotide Sequence Database with accession numbers BBXR01000000, BBXQ01000000, BBXS01000000 and BBXT01000000, respectively. Unassembled raw sequence data were also deposited to the database with accession number DRA004155. The phylogenetic tree and associated data matrix for Fig. 6 are available at TreeBASE (Accession URL: http://purl.org/phylo/treebase/phylows/study/TB2:S18090).

Results and discussion
General genome features of Fructobacillus spp. and Leuconostoc spp.
Draft genome sequences of four Fructobacillus spp. were determined by the Illumina Genome Analyzer II system. The sequence coverage of F. fructosus NRIC 1058T, F.
Table 2 Gene content profiles obtained for Fructobacillus spp. and Leuconostoc spp.

|                        | F. fructosus NRIC 1058<sup>T</sup> | F. durionis DSM 19113<sup>T</sup> | F. ficulneus JCM 12225<sup>T</sup> | F. pseudoficulneus F214-1<sup>T</sup> | L. mesenteroides ATCC 8293<sup>T</sup> | L. carnosum KB16 | L. citreum KM20 | L. hallii KCTC 3537<sup>T</sup> | L. delthio KCTC 3774<sup>T</sup> | L. inhae IMSNU 11154<sup>T</sup> | L. kimchi IMSNU 11154<sup>T</sup> | L. lactis KACC 91922 | L. pseudomesenteroides 1159<sup>T</sup> |
|-----------------------|-----------------------------------|----------------------------------|----------------------------------|-----------------------------------|----------------------------------------|-----------------|-----------------|------------------------|------------------------|--------------------------|--------------------------|----------------|---------------------|
| (C) Energy production and conversion | 40 34 41 36 43 69 49 66 39 67 50 68 56 61 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (D) Cell cycle control, cell division, chromosome partitioning | 35 36 41 37 43 37 33 40 24 33 23 45 30 38 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (E) Amino acid transport and metabolism | 112 106 159 137 160 192 152 129 110 136 116 179 139 152 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (F) Nucleotide transport and metabolism | 64 61 77 74 73 91 88 85 71 88 78 97 82 100 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (G) Carbohydrate transport and metabolism | 61 61 69 63 74 168 123 155 80 172 138 156 120 162 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (H) Coenzyme transport and metabolism | 51 49 54 49 64 91 73 80 52 72 64 98 78 78 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (I) Lipid transport and metabolism | 40 43 44 43 51 62 56 71 40 71 59 64 58 57 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (J) Translation, ribosomal structure and biogenesis | 180 175 188 180 190 193 191 185 162 193 166 198 186 191 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (K) Transcription | 93 84 89 87 115 133 128 129 93 150 132 153 100 151 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (L) Replication, recombination and repair | 110 86 97 86 115 110 100 105 57 92 95 119 96 125 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (M) Cell wall/membrane/envelope biogenesis | 84 77 73 74 84 110 92 105 81 98 75 102 93 94 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (N) Cell motility | 10 7 6 4 11 11 12 14 7 12 5 17 13 12 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (O) Posttranslational modification, protein turnover, chaperones | 46 37 47 40 49 63 59 59 39 54 44 67 46 58 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| (P) Inorganic ion transport and metabolism | 49 48 51 54 54 81 70 77 46 61 56 83 63 70 |                                  |                                  |                                   |                                        |                 |                 |                        |                        |                          |                          |                |                     |
| Column | Fructobacillus spp. | Leuconostoc spp. |
|--------|---------------------|------------------|
| [Q] Secondary metabolites biosynthesis, transport and catabolism | 10 | 7 |
| [R] General function prediction only | 67 | 55 |
| [S] Function unknown | 111 | 100 |
| [T] Signal transduction mechanisms | 31 | 27 |
| [U] Intracellular trafficking, secretion, and vesicular transport | 15 | 12 |
| [V] Defense mechanisms | 34 | 23 |
| [X] Mobilome: prophages, transposons | 44 | 12 |
ficulneus JCM 12225<sup>T</sup>, F. pseudoficulneus DSM 15468<sup>T</sup> and F. tropaeoli F214-1<sup>T</sup> were 329-, 55-, 90-, and 513-fold, respectively. Genome sequences of nine Leuconostoc spp. and Fructobacillus durionis were obtained from public databases (see Methods). The genome features of the strains used in the present study are summarized in Table 1. The genome sizes of Fructobacillus ranged from 1.33 to 1.69 Mbp (median ± SD, 1.49 ± 0.30 Mbp) and are significantly smaller than those of Leuconostoc (<i>p</i> < 0.001), 1.69 to 2.30 Mbp (median ± SD, 1.94 ± 0.21) (Fig. 1a). Accordingly, Fructobacillus strains contain significantly smaller numbers of CDSs than Leuconostoc strains (median ± SD, 1387 ± 132 vs 1980 ± 323, <i>p</i> < 0.001) (Fig. 1b). The DNA G + C contents of both species are also significantly different (<i>p</i> < 0.001): median ± SD is 44.4 % ± 0.30 % in Fructobacillus and 38.1 % ± 2.05 % in Leuconostoc (Fig. 1c). The difference in G + C contents is caused by the composition at the third codon (GC3): 46.0 % ± 1.02 % in Fructobacillus and 30.9 % ± 4.12 % in Leuconostoc. The low GC3 value in Leuconostoc spp. shows a good contrast with the high GC3 value in Lactobacillus delbrueckii subsp. bulgaricus [11]. In L. delbrueckii subsp. bulgaricus, the changes in GC3 are attributed to ongoing evolution [11], and similar selection pressure might be responsible here. Overall, these distinct genomic features strongly support the reclassification of Fructobacillus spp. from the genus Leuconostoc.

Since most of the genomes analyzed in this study were in draft status, quality assessment of the genomes was conducted using CheckM. The average completeness values for Fructobacillus and Leuconostoc genomes were 94.3 and 98.7 %, respectively (Table 1). Except for the genome of <i>L. inhae</i>, which exhibited the contamination value of 5.4 %, all genomes satisfied the criteria required to be considered a near-complete genome with low contamination (≥90 % completeness value and ≤5 % contamination value) [20]. The lower completeness values for Fructobacillus genomes might be attributable to insufficiency of the reference gene

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**Fig. 1** Genome sizes (a), number of CDSs (b) and GC contents (c) in Fructobacillus spp. and Leuconostoc spp. The line in the box represents the median, with lower line in the 25 % border and the upper line the 75 % border. The end of the upper vertical line represents the maximum data value, outliers not considered. The end of the lower vertical line represents the lowest value, outliers not considered. The separate dots indicate outliers.
markers used by CheckM, for which the genomic data of *Fructobacillus* spp. were not reflected at the time of writing this paper (December 2014), rather than the lower quality of these genomes. In addition, the lower completeness may indicate specific gene losses in the genus *Fructobacillus* since the closer investigation of CheckM results showed that seven gene markers were consistently absent among five *Fructobacillus* genomes while on average, 14.6 markers were absent out of 463 *Leuconostocaceae*-specific gene markers.

**Conserved genes in *Fructobacillus* spp. and *Leuconostoc* spp.**

The numbers of conserved genes in the nine genomes of *Leuconostoc* and five genomes of *Fructobacillus* were estimated as 1,026 and 862, respectively. They account for 52 % and 62 % of average CDS numbers of each genus (Fig. 2a). The difference in the average CDS numbers reflects their genomic history including ecological differences between the two genera. A previous study also reported 1162 conserved genes in three genomes of *Leuconostoc* species [30]. The smaller number and the higher ratio of fully conserved genes in *Fructobacillus* spp. is probably due to a less complex and consistent habitat with specific sugars only, such as fructose. It is a major carbohydrate found in habitats of *Fructobacillus* spp., e.g. flowers, fruits and associated insects. On the other hand, *Leuconostoc*, that are usually seen in wide variety of habitats, including gut of animals, dairy products, plant surfaces, or fermented foods and soils, possess a larger number of conserved genes. Figure 2b shows the distribution of gene clusters in two genera. The frontmost peak (721 gene clusters) represents conserved genes that are shared by both *Leuconostoc* and *Fructobacillus* spp. Genus-specific conserved genes are indicated as leftmost and right peaks in Fig. 2b. The leftmost peak (159 gene clusters) represents genes that are present in all *Leuconostoc* genomes, but absent in all *Fructobacillus* genomes, and the right peak (24 gene clusters) represents *vice versa*. The much smaller peak of the right compared to that of the left indicates that *Fructobacillus* spp. have lost more genes or have acquired less genes than *Leuconostoc* spp. during diversification after they separated into two groups. In addition, the number of gene clusters located near the center of the figure was small, which indicates that the exchange of genes between the two genera is not frequent and that they share distinct gene pools. This supports the validity of the classification of *Fructobacillus* as a distinct genus [8].

**Comparison of gene contents between *Fructobacillus* spp. and *Leuconostoc* spp.**

The identified genes were associated with COG functional categories by COGNITOR software at the NCBI. The sizes of COG-class for each strain are summarized in Table 2, and for each genus in Additional file 1: Figure S1. In addition, ratio of genes assigned in each COG category against the total number of genes in all COGs were determined for each genus and shown in Fig. 3. *Fructobacillus* spp. have less genes for carbohydrate transport and metabolism compared to *Leuconostoc* spp. (Class G in Fig. 3 and Additional file 1: Figure S1): Class G ranked 9th largest in *Fructobacillus* whereas it ranked 3rd in *Leuconostoc*. Similarly, the number of genes in Class C (energy production and conversion) was significantly less in *Fructobacillus*.
spp. than in Leuconostoc spp., suggesting that energy systems in Fructobacillus spp. are much simpler than those in Leuconostoc spp. The smaller number of CDS and conserved genes in Fructobacillus spp. could have resulted from metabolic reduction caused by scarce availability of carbohydrates other than fructose.

When compared based on the ratio of genes (Fig. 3), Class D (cell cycle, cell division and chromosome partitioning), Class J (translation, ribosomal structure and biogenesis), Class L (replication, recombination and repair) and Class U (intracellular trafficking, secretion and vesicular transport) were overrepresented in Fructobacillus spp. than in Leuconostoc spp. However, the numbers of genes classified in the four classes were comparable between the two genera (Additional file 1: Figure S1). The conservation of genes in these classes against the genome reduction may indicate that their functions are essential for re-production, and the class names roughly correspond to housekeeping mechanisms.

To understand gene contents involved in metabolic/biosynthesis pathways in more detail, ortholog assignment and pathway mapping against the KEGG Pathway Database were performed using the KAAS system. The number of mapped genes was significantly less for Fructobacillus spp. as compared to Leuconostoc spp. (Table 3). Firstly, Fructobacillus spp. lack respiration genes. Whereas oxygen is known to enhance their growth [8], the strains have lost genes for the TCA cycle, and keep only one gene for ubiquinone and other terpenoid-quinone biosynthesis (Table 3). Presumably they do not perform respiration. This characteristic is not applicable to certain Leuconostoc species: L. gelidum subsp. gasicomitatum [31], formerly classified as L. gasicomitatum [32], has been reported to conduct respiration in the presence of heme and oxygen [33].

Secondly, Fructobacillus spp. lack pentose and glucuronate interconversions (Table 3). They lost genes for pentose metabolism, unlike other obligately heterofermentative LAB that usually metabolize pentoses [34]. They do not metabolize mannose, galactose, starch, sucrose, amino sugars or nucleotide sugars, either [7, 8]. Moreover, the species possess none or at most one enzyme gene for the phosphotransferase systems (PTS), significantly less than the number of respective genes in Leuconostoc spp. (13 ± 3.13, average ± SD). This validates the observation that Leuconostoc spp. metabolize various carbohydrates whereas Fructobacillus spp. do not [8] (Fig. 4.) However, the genome-based prediction does not
always coincide with observed metabolism: *Fructobacillus* species do not metabolize ribose [8], against its metabolic prediction (Fig. 4). The discrepancy is due to an absence of ATP-dependent ribose transporter. On the other hand, some *Leuconostoc* spp. have the transporter and metabolize ribose.

Thirdly, *Fructobacillus* spp. have more genes encoding phenylalanine, tyrosine and tryptophan biosynthesis compared to *Leuconostoc* spp. (Table 3), although this difference is statistically not significant (p = 0.165). The difference is mainly due to presence/absence of tryptophan metabolism, and the production of indole and chorismate. This is important to wine lactobacilli [35]. The reason of the sporadic conservation of indole biosynthesis in *Fructobacillus* remains unknown.

### Comparison of genus-specific genes

To further investigate their differences, we defined genes as *Fructobacillus*-specific when they are conserved in four or more *Fructobacillus* species (out of five) and two or less in the nine *Leuconostoc* species. On the other hand, genes are *Leuconostoc*-specific when they are possessed by seven or more *Leuconostoc* species (out of nine) and zero or one in the five *Fructobacillus* species. According to this definition, 16 genes were identified as *Fructobacillus*-specific and 114 as *Leuconostoc*-specific (Additional file 2: Table S1). These numbers are smaller than the numbers of fully conserved genes in each genus (24 for *Fructobacillus* and 159 for *Leuconostoc*), because we defined genus-specific genes after mapping them to the KEGG Orthology (KO) database; genes without any KO entry were excluded from the analysis.

Interestingly the *adh* gene coding alcohol dehydrogenase [EC:1.1.1.1] was characterized as *Fructobacillus*-specific whereas *adhE* gene coding bifunctional acetaldehyde/alcohol dehydrogenase [EC1.2.1.10 1.1.1.1] was characterized as *Leuconostoc*-specific. There was no alternative acetaldehyde dehydrogenase gene in *Fructobacillus*. These results are consistent with our previous study reporting the lack of *adhE* gene and acetaldehyde dehydrogenase activity in *Fructobacillus* spp. [9] and their obligately heterofermentative nature with no ethanol production [6, 8]. No production of ethanol is due to an absence of acetaldehyde dehydrogenase activity, but it conflicts with the NAD/NADH recycling. Therefore, there must be a different electron acceptor in glucose metabolism [4, 6, 9].

NAD(P)H dehydrogenase gene was found as *Fructobacillus*-specific (Additional file 2: Table S1). This is the only gene used for the quinone pool in *Fructobacillus* spp., suggesting that the gene does not contribute to respiration. Rather, it is used for oxidation of NAD(P)H under the presence of oxygen. This helps to keep the NAD(P)/NAD(P)H balance, since their sugar metabolism produces imbalance in NAD(P)/NAD(P)H cycling as described above. Indeed, *Fructobacillus* spp. can be easily differentiated from *Leuconostoc* spp. based on the reaction to oxygen [8]. In our validation study, *Fructobacillus* spp. grew well under aerobic conditions but poorly so under anaerobic conditions on GYP medium (Fig. 5). Presence of oxygen had smaller impacts on growth of *Leuconostoc* spp., but they generated larger colonies under anaerobic conditions than under aerobic conditions.

### Table 3 Discriminative pathways between *Fructobacillus* spp. and *Leuconostoc* spp.

| Pathway                                             | *Fructobacillus* spp. Mean (SD) | *Leuconostoc* spp. Mean (SD) | p   |
|-----------------------------------------------------|---------------------------------|-------------------------------|-----|
| Glycolysis (map00010)                               | 12.2 (0.84)                     | 19.5 (1.72)                   | 0.001|
| TCA cycle (map00020)                                | 0                               | 4.2 (0.79)                    | 0.008|
| Pentose and glucoronate interconversions (map00040) | 3.2 (1.64)                      | 7.9 (2.80)                    | 0.008|
| Fructose and mannose metabolism (map00051)         | 2.8 (0.84)                      | 9.4 (2.12)                    | 0.001|
| Galactose metabolism (map00052)                     | 5.8 (0.84)                      | 11.6 (2.72)                   | 0.003|
| Ubiquinone and other terpenoid-quinone biosynthesis (map00130) | 1 (0)                           | 7.6 (0.97)                    | 0.001|
| Oxidative phosphorylation (map00190)                | 9.2 (0.45)                      | 12.7 (1.57)                   | 0.001|
| Valine, leucine and isoleucine degradation (map00280) | 2 (0)                           | 4.4 (0.84)                    | 0.001|
| Starch and sucrose metabolism (map00500)           | 6.4 (1.52)                      | 12.9 (2.28)                   | 0.001|
| Amino sugar and nucleotide sugar metabolism (map00520) | 11.2 (0.45)                    | 19.5 (2.17)                   | 0.001|
| Pyruvate metabolism (map00620)                     | 12 (1)                          | 19.8 (1.99)                   | 0.001|
| Carbon metabolism (map01200)                       | 30.6 (3.21)                     | 37.4 (3.20)                   | 0.005|
| ABC transporters (map02010)                        | 33.8 (3.11)                     | 50.6 (8.34)                   | 0.003|
| Phosphotransferase system (map02060)               | 1 (0)                           | 13 (3.13)                     | 0.03 |

Map numbers shown in parenthesis correspond to the numbers in KEGG

a The values indicate means and standard deviations of number of genes used for the pathways

Interestingly the *adh* gene coding alcohol dehydrogenase [EC:1.1.1.1] was characterized as *Fructobacillus*-specific whereas *adhE* gene coding bifunctional acetaldehyde/alcohol dehydrogenase [EC1.2.1.10 1.1.1.1] was characterized as *Leuconostoc*-specific. There was no alternative acetaldehyde dehydrogenase gene in *Fructobacillus*. These results are consistent with our previous study reporting the lack of *adhE* gene and acetaldehyde dehydrogenase activity in *Fructobacillus* spp. [9] and their obligately heterofermentative nature with no ethanol production [6, 8]. No production of ethanol is due to an absence of acetaldehyde dehydrogenase activity, but it conflicts with the NAD/NADH recycling. Therefore, there must be a different electron acceptor in glucose metabolism [4, 6, 9].
but were found in *Leuconostoc*. *Fructobacillus* also lack TCA cycle genes. This suggests that, in *Fructobacillus*, pyruvate produced from the phosphoketolase pathway is not dispatched to the TCA cycle but metabolized to lactate by lactate dehydrogenase. The lack of pyruvate dehydrogenase complex was also reported in *Lactobacillus kunkeei* [35], which is also a member of FLAB found in fructose-rich environment [4, 36].

The levansucrase gene was also characterized as *Fructobacillus*-specific (Additional file 2: Table S1). The enzyme has been known to work for production of oligosaccharides in LAB [36, 37] and for biofilm production in other bacteria [38]. However, production of polysaccharides was unobserved in *Fructobacillus* spp. when cultured with sucrose. The reason for this discrepancy is yet unknown. Incompetence of sucrose metabolism, including no dextran production, in *Fructobacillus* spp. has been reported [7, 8], and systems to metabolize sucrose, e.g. genes for sucrose-specific PTS, sucrose phosphorylase and dextranucrase, were not detected in their genomes. On the other hand, *L. citreum* NRIC 1776T and *L. mesenteroides* NRIC 1541T produced polysaccharides, possibly dextran. Production of dextran from sucrose in the
The genus *Leuconostoc* is strain/species dependent [39], and a dextranucrase gene was identified in six *Leuconostoc* genomes (out of nine) in this study. A number of genes encoding peptidases and amino acids transport/synthesis/metabolism were also found as *Leuconostoc*-specific genes (Additional file 2: Table S1), suggesting that *Leuconostoc* spp. can survive various environments with different amino acid compositions. Several PTS related genes and genes for teichoic acid transport were also characterized as *Leuconostoc*-specific. LAB cells usually contain two distinct types of teichoic acid, which are wall teichoic acid and lipoteichoic acid. The identified genes are involved in biosynthesis of wall teichoic acid in *Bacillus subtilis* [40]. Few studies have been reported for wall teichoic acid in *Leuconostoc* spp. and none in *Fructobacillus* spp.

### Phylogenetic analysis

To confirm the phylogenetic relationship between *Fructobacillus* spp. and *Leuconostoc* spp., a phylogenetic tree was produced based on concatenated sequences of 233 orthologous genes which were conserved as a single copy within the tested strains. The tree showed a clear separation of the two genera (Fig. 6), indicating that *Fructobacillus* spp. have distinct phylogenetic position from *Leuconostoc* spp. This agrees well with the previous reports using 16S rRNA gene or house-keeping genes [7, 8].
Conclusion

Genome-based analysis on conserved genes and metabolic characteristics clearly indicated the distinction between Fructobacillus spp. and Leuconostoc spp. Fructobacillus spp. possess smaller numbers of CDS in smaller genomes compared to Leuconostoc spp. This is mainly due to the absence of carbohydrate metabolic systems. Similar genomic characteristics have been reported for L. kunkeei [41], a member of FLAB found in fructose-rich environment. Since they are known as poor sugar fermenter in the group of LAB and always inhabit in fructose-rich niches, the characteristics could have resulted from an adaptation to their extreme environments. Niche-specific evolution, usually genome reduction, has been reported for dairy and vaginal LAB [10–12], and the present study reconfirms such niche-specific evolution in FLAB. These findings would be valuable to know a link of diverse physiological and biochemical characteristics in LAB and environmental factors in their habitats.

Additional files

Additional file 1: Figure S1. Comparison of gene content profiles obtained for the genera Fructobacillus and Leuconostoc. The Mann–Whitney U-test was done to compare Fructobacillus spp. and Leuconostoc spp., and significant differences (P < 0.05) are denoted with an asterisk (*). (PPTX 941 kb)

Additional file 2: Table S1. Genus-unique genes for Fructobacillus and Leuconostoc. (XLSX 15 kb)

Abbreviations

CDS: protein coding sequences; COG: Clusters of Orthologous Groups; FLAB: fructophilic lactic acid bacteria; KO: KEGG Orthology; LAB: lactic acid bacteria.

Competing interest

The authors declare that they have no competing interests.

Authors’ contribution

AE, SO and LD designed the study. NT, YS and HY conducted draft genome sequencing and analysis. AE, SO and LD designed the study. NT, YS and HY conducted draft genome sequencing and analysis. AE, SO and LD performed the data analysis. AE prepared the draft of the manuscript. AE, YT, LD, JM and MA contributed to the revision of the manuscript. All authors have read and approved the final manuscript.

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