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Original article

Zooming in Into the Taxonomic Identification of a Human Breast-milk Derived *Bifidobacterium* sp. 2450

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Abstract

Exact species/subspecies identification is essential in understanding human-associated microbiota and the practical application of a particular bacterial isolate. With the fast evolution of DNA sequencing technology, however, there is a transition in molecular identification methods from single gene sequence- to whole genome sequence analysis, resulting in subtle changes in bacterial taxonomy. Here we report the application of three different species identification molecular methods and species delineation concepts to identify a human breast milk isolate *Bifidobacterium* sp. 2450 - classical 16S-rDNA sequence analysis, multilocus sequence typing (MLST) and digital DNA:DNA hybridization (dDDH). Comparison of the partial 16S-rDNA sequence of *Bifidobacterium* sp. 2450 and type strains of the *Bifidobacterium* genus positioned the new isolate in the *B. longum* cluster. At subspecies level, including the four subspecies of *B. longum (longum, infantis, suis* and *suillum*), the partial 16S-rDNA sequence derived from *Bifidobacterium* sp. 2450 was >99.5% (1490 bp) identical to *B. longum* ssp. *infantis*. MLST was based on the concatenated partial sequences of the *clpC*, *dnaG*, *dnaJ1*, *hsp60, purF*, *rpoC* and *xfp* genes. Unlike16S-rDNA sequence analysis, MLST situated *Bifidobacterium* sp. 2450 closer to the *B. longum* ssp. *longum* cluster, separately from *B. longum* ssp. *infantis*. Next, dDDH was performed to compare the draft genome of *Bifidobacterium* sp. 2450 to complete genomes of type strains. Hybridization values with the type strain of *B. longum* ssp. *longum* DSM 20219 were 75.8 against only 62.4 for *B. longum* ssp. *infantis* DSM 20088. With a species delineation threshold for dDDH of 70, this method identified *Bifidobacterium* sp. 2450 as *B. longum* ssp. *longum*. Although the results from the three methods disagreed at intraspecies level, they all confirmed the new isolate to belong to the *B. longum* species, an identification level that is satisfactory for practical purposes.

Keywords: *Bifidobacterium longum,* Digital DNA:DNA Hybridization, Multilocus Sequence Typing.

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INTRODUCTION

Human breast-milk (HM) microbiota and bifidobacteria in particular became a focus of research due to their potential role in preserving the infant's health. *Bradyrhizobium*, *Corynebacterium*, *Propionibacterium*, *Pseudomonas*, *Ralstonia*, *Serratia*, *Sphingomonas*, *Staphylococcus* and *Streptococcus* were found to be the core genera in HM (Notarbartolo et al., 2022). On a bacterial species level bifidobacteria are of particular interest because of their health-beneficial properties (Arboleya et al., 2011; Selma-Royo et al., 2021), abundance in HM and the gastrointestinal tract of healthy infants (Zuo et al., 2016; Kordy et al., 2020) and evidence that bifidobacterial communities are inherited by the infant from their mother by a vertical transfer (Duranti et al., 2017). The health effect of HM microbiota is related to establishment of immune homeostasis in the infant, enhancing digestive processes or prevention or correction of dysbiosis (Dogra et al., 2020; Selma-Royo et al., 2021). Consequently, supplementation of breast milk or the mother's or infant's diet in general with bifidobacteria is regarded as potential means of positively influencing and protecting the health of infants (Oshiro et al., 2020). However, precise identification of a new isolate intended for functional food development is required in order to relate it to the safety- or health-beneficial record of a particular species. In the case of bifidobacteria, *Bifidobacterium breve* and *B. longum* are the most common species found in HM (Solís et al., 2010; Arboleya et al., 2011).

Exact taxonomic identification is complicated by the fast evolution of molecular methods and species delineation concepts. The first species identification methods based on genotyping were DNA-DNA hybridization and 16S-rDNA gene sequencing. For DNA-DNA hybridization it was postulated that 70% or more relatedness is required to assign a new isolate to a particular bacterial species (Wayne et al., 1987), while more than 97% identity in 16S-rDNA sequences will be recognized for cultures within a single species (Janda & Abbott, 2007). A myriad of other genotyping methods such as ribotyping, RAPD-PCR (Sakata et al, 2002), BOX-PCR (Masco et al., 2003) and multi-locus sequence typing (MLST) (Yanokura et al., 2015) permit further clustering at intraspecies level, yielding different results. And finally comes the digital DNA:DNA hybridization (dDDH), relying on comparison of whole genome sequences of unknown bacterium to that of a particular type strain, adopting the 70% threshold for species delineation from the classical DNA-DNA hybridization and suggesting a 79% similarity dDDH cut-off value for the subspecies (Meier-Kolthoff, 2014). All these methods offer different resolution and may not necessarily align perfectly in the process of species/subspecies identification.

The aim of the presented study was the identification of a human breast milk isolate *Bifidobacterium* sp. 2450 using 1) a classical 16S-rDNA sequence analysis; 2) multilocus sequence typing (MLST) for seven house-keeping genes (*clpC*, *dnaG*, *dnaJ1*, *hsp60, purF*, *rpoC* and *xfp*) and 3) digital DNA:DNA hybridization (dDDH).

MATERIALS and METHODS

Bacterial strain

Isolate *Bifidobacterium* sp. 2450 was obtained and purified from a human breast milk sample in 2020 (Michaylova et al., 2022) and further maintained at the LBB Culture Collection (LB Bulgaricum PLC, Sofia, Bulgaria).

Whole-genome sequencing

Total DNA from *Bifidobacterium* sp. 2450 was extracted with the E.Z.N.A. Bacterial DNA Kit (Omega Bio-tek Inc, Norcross, Georgia, USA) according to the producer's instructions. Sequencing was performed on an Illumina NovaSeq6000, reads were assembled with the SOAPdenovo assembler (Li et al., 2010), SPAdes (Bankevich et al., 2012), AbySS (Simpson et al., 2009) followed by CISA (Lin & Liao, 2013) for a final integration of contigs. Identification of coding genes was performed with GeneMarkS (Besemer et al., 2001). The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JBHEEA000000000. The version described in this paper is version JBHEEA010000000.

16S-rDNA sequence analysis

For the purpose of identification by 16S-rDNA sequence analysis, the gene sequence of *Bifidobacterium* sp. 2450 was extracted from the draft genome sequence and clustered against type strains of the *Bifidobacterium* genus as described by Ventura et al. (2006) or against a selection of 25 *B. longum* strains (Yanokura et al., 2015). The clustering and the resulting tree were generated by the CLC Sequence Viewer software ver. 6.6.1 (www.clcbio.com, CLC bio A/S) by the unweighted pair group method with arithmetic mean (UPGMA) algorithm.

Multilocus sequence typing

Multilocus sequence typing (MLST) was based on concatenated partial sequences of the *clpC*, *dnaG*, *dnaJ1*, *hsp60, purF*, *rpoC* and *xfp* genes. The sequences of the genes in *Bifidobacterium* sp. 2450 were extracted from the draft genome sequence, while sequences for a selection of 25 *B. longum* strains were derived from the work of Yanokura et al. (2015). The clustering and the resulting tree were generated by the CLC Sequence Viewer and the UPGMA algorithm.

Digital DNA:DNA hybridization

Digital DNA:DNA hybridization (dDDH) was performed based on the draft genome of *Bifidobacterium* sp. 2450 with the TYGS platform (Meier-Kolthoff & Göker, 2019), which performs comparison with a large number of type strain genomes and retrieves the closest match. dDDH values along with their confidence intervals (C.I.) were calculated following formula 2 of the Genome-to-

Genome Distance Calculator (Meier-Kolthoff et al., 2013). Additionally, a phylogenetic tree of the best matches and the query strain was generated with FastME 2.1.6.1 (Lefort et al., 2015).

RESULTS

Whole-genome sequencing

The sequencing of *Bifidobacterium sp.* 2450 resulted in the assembly of 37 contigs, covering over 2,43 Mbp, containing 2151 genes (84 % of the genome) with the number of protein coding sequences, tRNA genes and rRNA operons being 1982, 55 and 5, respectively. The GC-content of the genome was 59.8%.

Identification by 16S-rDNA sequence analysis

As a first step in the identification the partial 16S-rDNA sequence of *Bifidobacterium* sp. 2450 was aligned and clustered with the respective sequences in the genomes of type strains of the *Bifidobacterium* genus. The resulting tree clearly demonstrated the power of 16S-rDNA sequence discrimination of bifidobacterial species and clustered *Bifidobacterium* sp. 2450 unequivocally in the *B. longum* cluster (Figure 1). This cluster currently contains four subspecies, that of *B. longum* ssp. *longum*, *B. longum* ssp. *infantis, B. longum* ssp. *suis* and *B. longum* ssp. *suillum* and therefore to zoom in the identification at intraspecies level, partial 16S-rDNA sequence of *Bifidobacterium* sp. 2450 was further compared to a selection of strains from these subspecies. With this analysis the new isolate clearly clustered into the *B. longum* ssp. *infantis* cluster (Figure 2) with a sequence >99.5% (1490 bp) identical to that of other *B. longum* ssp. *infantis* strains.

Identification by Multilocus sequence typing

Unlike 16S-rDNA sequence analysis, MLST offers higher discriminatory power as it relies on sequence diversity within multiple loci in the genome. Clustering of the concatenated sequences of the *clpC*, *dnaG*, *dnaJ1*, *hsp60, purF*, *rpoC* and *xfp* genes identified in *Bifidobacterium* sp. 2450 with 25 strains from the *B. longum* ssp. *longum*, *B. longum* ssp. *infantis, B. longum* ssp. *suis* and *B. longum* ssp. *suillum* subspecies positioned the new isolate closer to *B. longum* ssp. *longum* cluster (Figure 3). MLST placed *Bifidobacterium* sp. 2450 separate from the *B. longum* ssp. *infantis* strains in contradiction with the result obtained form 16S-rDNA sequence analysis. Meanwhile, the selection of the 25 strains derived from Yanokura et al. (2015) clustered in the expected *longum*, *infantis* and *suis*/*suillum* clusters in good correlation between 16S-rDNA sequence analysis and MLST (Figures 2 and 3).

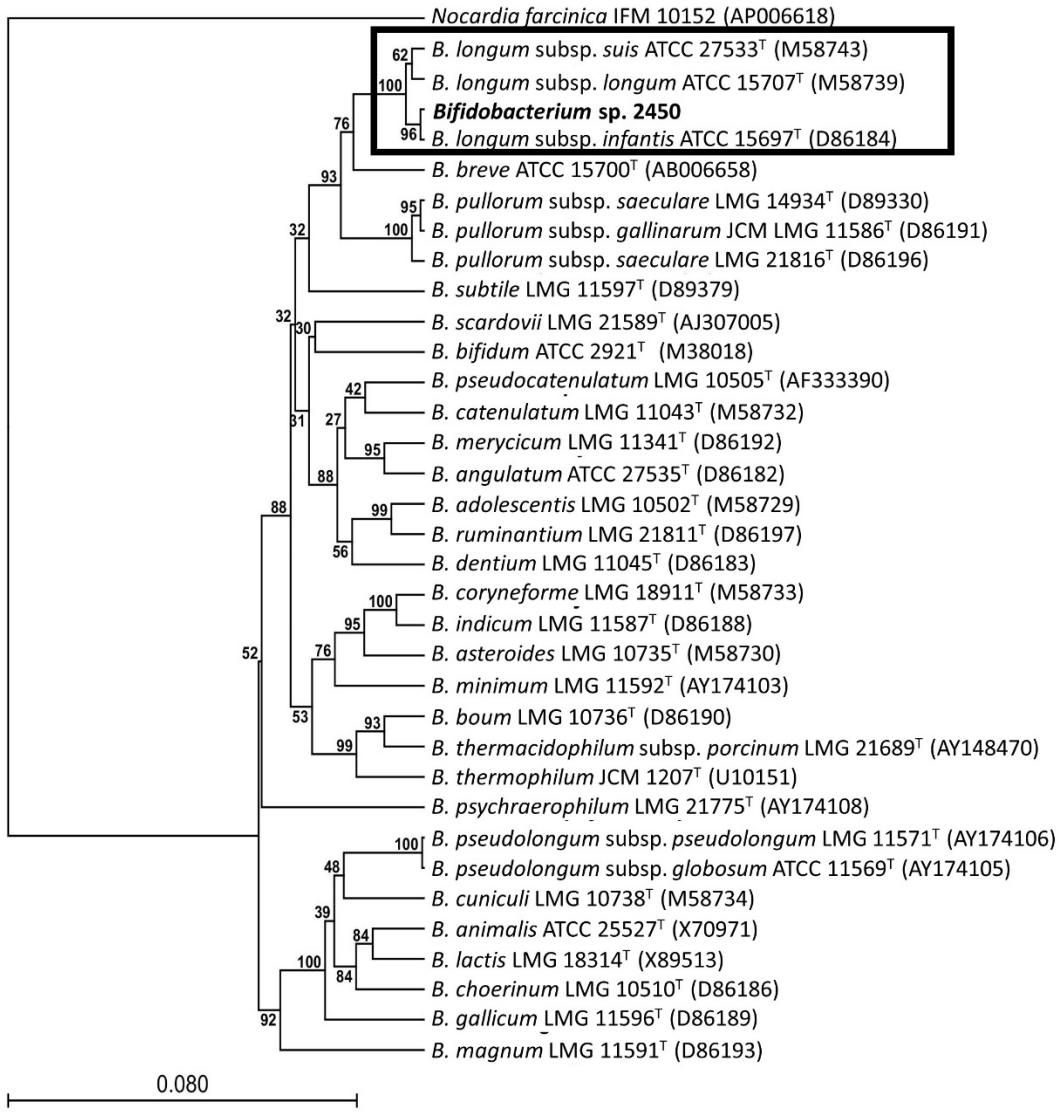

Figure 1. Clustering of *Bifidobacterium* sp. 2450 against different type strains within the genus *Bifidobacterium*. Clustering was based on a partial sequence of their 16S-rDNA gene (1285 nt). The tree was generated by the CLC Sequence Viewer software ver. 6.6.1 (www.clcbio.com, CLC bio A/S) by the UPGMA algorithm. Bootstrap values displayed at nodes were obtained based on 100 replicates. Sequences for type strains (GenBank accession numbers shown in parentheses) were selected according to Ventura et al. (2006).

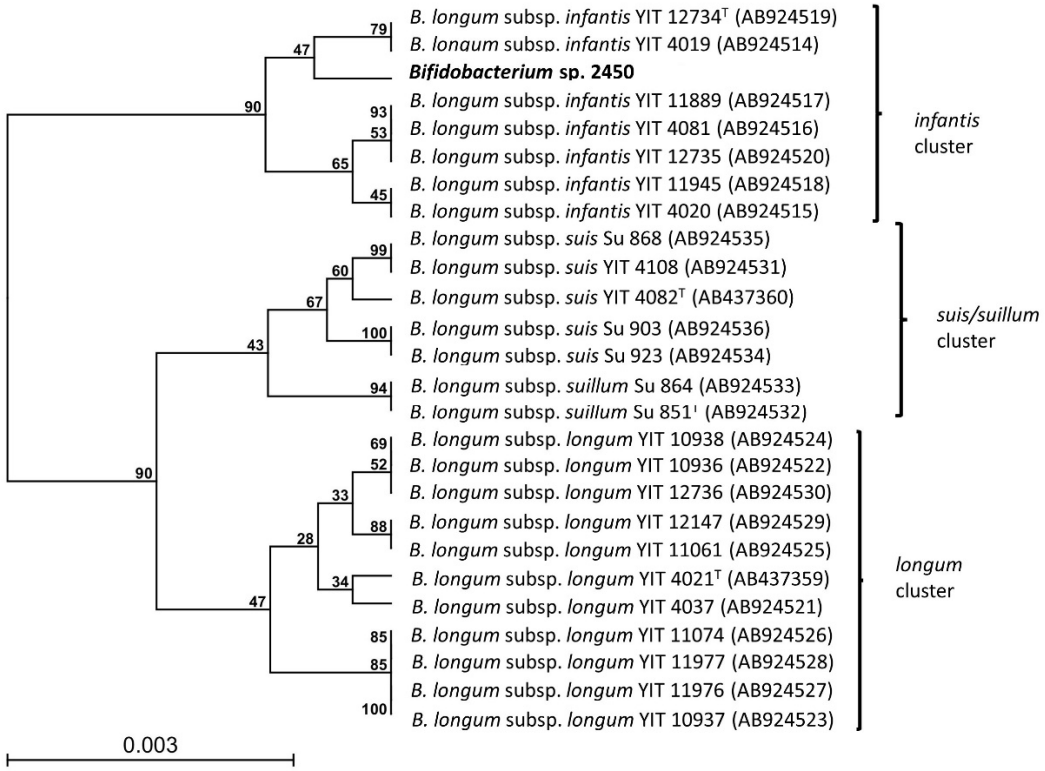

Figure 2. Clustering of *Bifidobacterium* sp. 2450 against a set of 25 *B. longum* strains. Clustering was based on a partial sequence of their 16S-rDNA gene (1490 nt). The tree was generated by the CLC Sequence Viewer software ver. 6.6.1 (www.clcbio.com, CLC bio A/S) by the UPGMA algorithm. Bootstrap values displayed at nodes were obtained based on 100 replicates. Sequences for the *B. longum* strains (GenBank accession numbers shown in parentheses) were selected according to Yanokura et al. (2015).

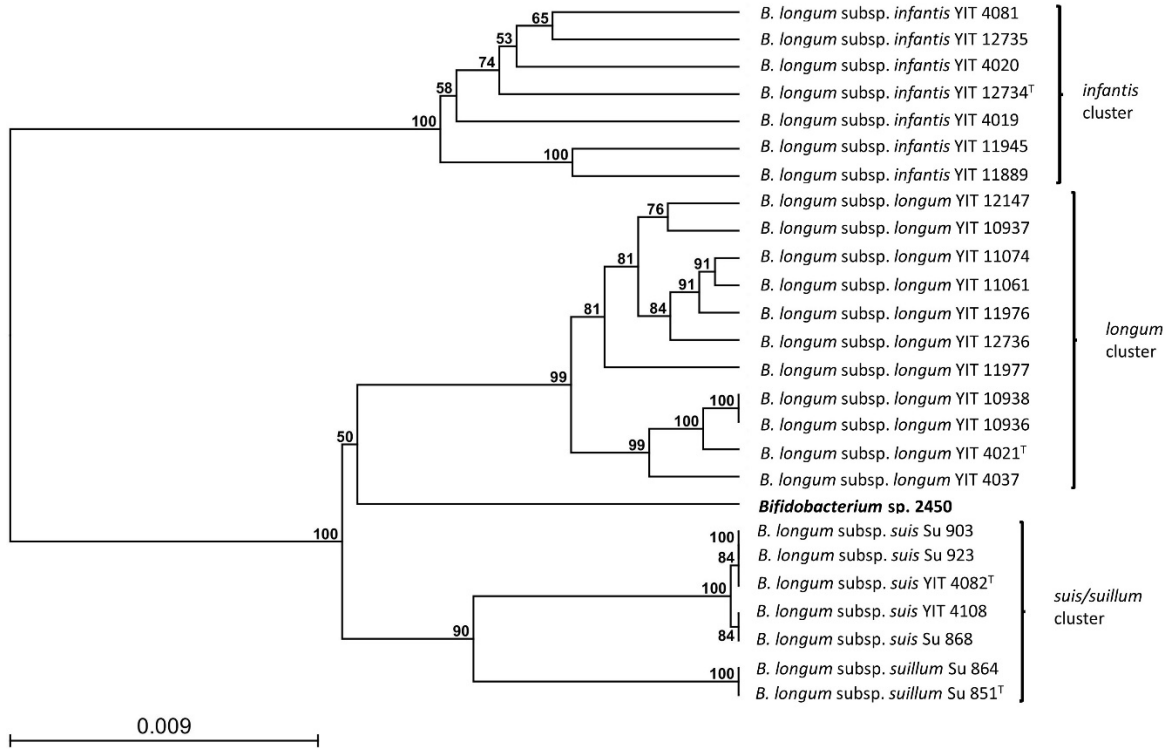

Figure 3. Clustering (MLST) of *Bifidobacterium sp.* 2450 against a set of 25 *B. longum* strains. Clustering was based on the concatenated partial sequences of seven house-keeping genes (4360 nt). The tree was generated by the CLC Sequence Viewer software ver. 6.6.1 (www.clcbio.com, CLC bio A/S) by the UPGMA algorithm. Bootstrap values displayed at nodes were obtained based on 100 replicates. Sequences for the *B. longum* strains (GenBank accession numbers shown in parentheses) derived from Yanokura et al. (2015).

Identification by Digital DNA:DNA hybridization

Next, after one- (16S-rDNA) and seven- gene (MLST) sequence analysis, dDDH permitted comparison of draft genome data of *Bifidobacterium* sp. 2450 to complete genomes of type strains. The hybridization values obtained for the draft genome of *Bifidobacterium* sp. 2450 with the type strain of *B. longum* ssp. *longum* DSM 20219^T were 75.8 against only 62.4 for *B. longum* ssp. *infantis* DSM 20088^T (Table 1). Moreover, the dDDH values of *Bifidobacterium* sp. 2450 against the type strains of *B. longum* ssp. *suis* and *B. longum* ssp. *suillum* were still higher (68.1-68.2) than those obtained for the type strain of *B. longum* ssp. *infantis* (62.4). Considering a species delineation threshold value for dDDH of 70, this method identified *Bifidobacterium* sp. 2450 strictly as *B. longum* ssp. *longum* supporting the identification results of MLST but not that of 16S-rDNA analysis. Furthermore, a phylogenetic tree, based on the distances, calculated from the sequence similarities in the genome sequences of *Bifidobacterium* sp. 2450 and *B. longum* ssp. *longum*, *B. longum* ssp. *infantis, B. longum* ssp. *suis* and *B. longum* ssp. *suillum* type strains clustered the new isolate at the least distance from *B. longum* ssp. *longum* away from the type strain of *B. longum* ssp. *infantis* (Figure 4). Clustering based on whole genome sequence similarities in the case of *Bifidobacterium* sp. 2450 produced very similar results to those, obtained by MLST (compare Figures 3 and 4), but contradicted the classical 16S-rDNA sequence analysis at intraspecies level.

Table 1. Pairwise comparison of *Bifidobacterium* sp. 2450 draft genome vs. type strain genomes

Type strain of	Strain	dDDH value	C.I.
B. longum ssp. *longum*	JCM 1217^T	75.8	72.7-78.5
	DSM 20219^T	75.8	72.8-78.6
	LMG 13197^T	75.7	72.7-78.4
	NCTC 11818^T	75.7	72.7-78.5
B. longum ssp. *suis*	DSM 20211^T	68.2	65.2-71.1
	LMG 21814^T	68.2	65.2-71.1
B. longum ssp. *suillum*	DSM 28597^T	68.1	65.2-71.0
B. longum ssp. *infantis*	DSM 20088^T	62.4	59.5-65.2
	JCM 1222^T	62.4	59.5-65.2
	NCTC 11817^T	62.4	59.5-65.2
	ATCC 15697^T	62.4	59.5-65.2

Note. dDDH values along with their confidence intervals (C.I.) are calculated following formula 2 of the Genome-to-Genome Distance Calculator (Meier-Kolthoff et al., 2013)

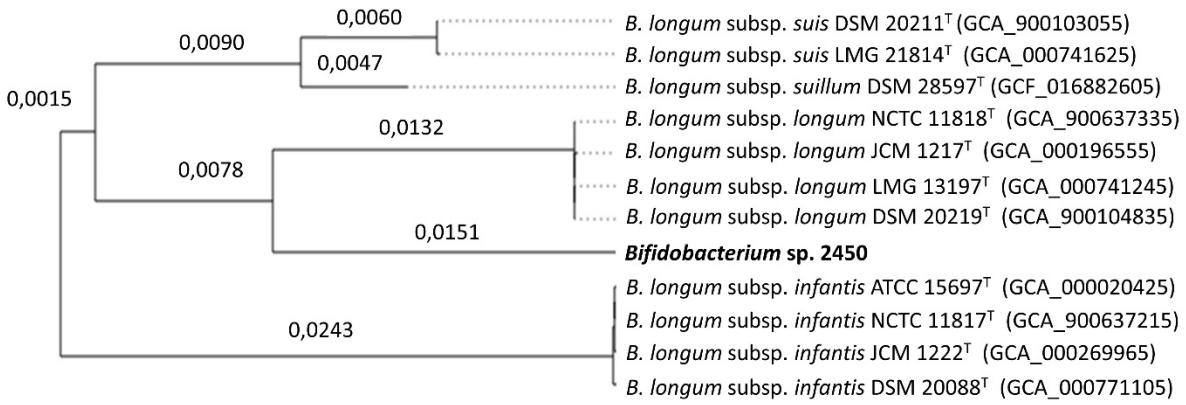

Figure 4. Phylogenetic tree, representing *Bifidobacterium* sp. 2450 and type strains of *B. longum* ssp. - *B. longum* ssp. *longum*, *B. longum* ssp. *infantis, B. longum* ssp. *suis* and *B. longum* ssp. *suillum.* Tree inferred with FastME 2.1.6.1 (Lefort et al., 2015) from the Genome BLAST Distance Phylogeny method (GBDP) distances, calculated from whole genome sequences. The numbers above branches are lengths scaled in terms of GBDP distance formula d_5. The average branch support was 33.3 %. The tree was rooted at the midpoint (Farris, 1972). GenBank assembly accession numbers are given in parentheses.

Discussion

The evolution of molecular methods and species delineation concepts have an especially good example with the taxonomy of *B. longum* and its related species/subspecies. Currently the *B. longum* species is divided into four subspecies *B. longum* ssp. *longum*, *B. longum* ssp. *infantis, B. longum* ssp. *suis* and *B. longum* ssp. *suillum*. The first three subspecies have been initially outlined as separate species based on phenotypic criteria (Scardovi et al., 1971). However, the high level of DNA-DNA relatedness between the *B. longum, B*. *infantis* and *B. suis* species was found to be in the range of 63-85%, what prompted Sakata et al. (2002) to propose the unification of these three species into one species – that of *B. longum*. On the opposite side, after extensive review of molecular typing methods such as amplified rDNA restriction analysis (ARDRA), randomly amplified polymorphic DNA (RAPD), BOX-PCR, comparison of the *recA*, *tuf* and *ldh* gene, etc., Mattarelli et al (2008) proposed to reclassify these three biotypes of *B. longum* into separate subspecies. The authors suggest that as these three taxonomic entities share greater than 97% 16S-rDNA sequence similarity (Sakata et al, 2006), the analysis of this sequence itself may not allow clear separation between them at species level (Mattarelli et al, 2008). A firm confirmation of such an observation is the fact that it was only after applying MLST of seven house-keeping genes that a new cluster, that of *B. longum* ssp. *suillum*, was introduced as a fourth subspecies in the *B. longum* species (Yanokura et al., 2015). With the wider availability of whole genome sequencing, digital DNA-DNA hybridization (dDDH) emerged as alternative and more precise method allowing the comparison of newly sequenced cultures to deposited type strains (Meier-Kolthoff &

Göker, 2019). An extensive study, reviewing the whole phylum of *Actinobacteria*, found that the intergenomic dDDH value of the type strain of *B. longum* ssp. *longum* DSM 20219^T against the type strain of *B. longum* ssp. *infantis* ATCC 15697^T was only 62.4 (Nouioui et al., 2018). With a species cut-off value of 70% (Wayne et al., 1987) and subspecies dDDH cut-off value of 79% (Meier-Kolthoff, 2014), Nouioui et al. (2018), proposed to reinstate the *B. infantis* species and separate it from *B. longum*.

In the present study, regardless of the applied molecular method for species identification, isolate *Bifidobacterium sp.* 2450 proved to belong to the *B. longum* species. From practical point of view, identification of bifidobacteria at this taxonomic level is sufficient to decide on further evaluation of the applicability of a new strain. Beyond species identification, WGS data is mandatory for testing and supporting any safety or probiotic claims, related to a strain. Once sequenced, genome data should undergo thorough search for the potential presence of antibiotic resistance and virulence factors genes (EFSA, 2018) as well as genetic content, related to biogenic amines synthesis (Elsanhoty & Ramadan, 2016). Furthermore, whole genome data may facilitate and confirm the selection of a bifidobacterial probiotic candidate by identifying genes, responsible for its probiotic properties or strain robustness in an industrial production process (Sundararaman et al., 2021).

Conclusion

The simultaneous existence of several species identification methods may result in different outcomes of the analysis. Comparison of the partial 16S-rDNA sequence of *Bifidobacterium* sp. 2450 clustered it with *B. longum* ssp. *infantis* while dDDH of its draft genome identified it as *B. longum* ssp. *longum*.

Analysis of the 16S-rDNA sequence can give satisfactory results for practical purposes at species level (here *B. longum*), however further intraspecies identification may require clustering based on multiple sequences (MLST) or whole genomes (dDDH).

REFERENCES

Arboleya, S., Ruas-Madiedo, P., Margolles, A., Solís, G., Salminen, S., De los Reyes-Gavilan, C. G., & Gueimonde, M. (2011). Characterization and in vitro properties of potentially probiotic Bifidobacterium strains isolated from breast-milk. *International journal of food microbiology*, *149*(1), 28-36. doi: 10.1016/j.ijfoodmicro.2010.10.036.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., ... & Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology*, *19*(5), 455-477. doi: 10.1089/cmb.2012.0021.

Besemer, J., Lomsadze, A., & Borodovsky, M. (2001). GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions. *Nucleic acids research*, *29*(12), 2607-2618. doi: 10.1093/nar/29.12.2607.

Dogra, S. K., Doré, J., & Damak, S. (2020). Gut microbiota resilience: definition, link to health and strategies for intervention. *Frontiers in microbiology*, *11*, 572921. doi: 10.3389/fmicb.2020.572921

Duranti, S., Lugli, G. A., Mancabelli, L., Armanini, F., Turroni, F., James, K., ... & Ventura, M. (2017). Maternal inheritance of bifidobacterial communities and bifidophages in infants through vertical transmission. *Microbiome*, *5*, 1-13. doi: 10.1186/s40168-017-0282-6

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). (2018). Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA Journal*, *16*, 5206. doi: 10.2903/j.efsa.2018.

Elsanhoty, R. M., & Ramadan, M. F. (2016). Genetic screening of biogenic amines production capacity from some lactic acid bacteria strains. Food Control, 68, 220-228. doi: 10.1016/j.foodcont.2016.04.002.

Farris, J. S. (1972). Estimating phylogenetic trees from distance matrices. *The American Naturalist*, *106*(951), 645-668. doi: 10.1086/282802

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. Journal of clinical microbiology, 45(9), 2761-2764. doi:10.1128/JCM.01228-07

Kordy, K., Gaufin, T., Mwangi, M., Li, F., Cerini, C., Lee, D. J., ... & Aldrovandi, G. M. (2020). Contributions to human breast milk microbiome and enteromammary transfer of Bifidobacterium breve. *PloS one*, *15*(1), e0219633. doi: 10.1371/journal.pone.0219633.

Lefort, V., Desper, R., & Gascuel, O. (2015). FastME 2.0: a comprehensive, accurate, and fast distance-based phylogeny inference program. *Molecular biology and evolution*, *32*(10), 2798-2800. doi: 10.1093/molbev/msv150

Lin, S. H., & Liao, Y. C. (2013). CISA: contig integrator for sequence assembly of bacterial genomes. *PloS one*, *8*(3), e60843. doi: 10.1371/journal.pone.0060843.

Li, R., Zhu, H., Ruan, J., Qian, W., Fang, X., Shi, Z., ... & Wang, J. (2010). De novo assembly of human genomes with massively parallel short read sequencing. *Genome research*, *20*(2), 265-272. doi: 10.1101/gr.097261.109.

Masco, L., Huys, G., Gevers, D., Verbrugghen, L., & Swings, J. (2003). Identification of Bifidobacterium species using rep-PCR fingerprinting. *Systematic and applied microbiology*, *26*(4), 557-563. doi: 10.1078/072320203770865864.

Mattarelli, P., Bonaparte, C., Pot, B., & Biavati, B. (2008). Proposal to reclassify the three biotypes of *Bifidobacterium longum* as three subspecies: *Bifidobacterium longum* subsp. *longum* subsp. nov., *Bifidobacterium longum* subsp. *infantis* comb. nov. and *Bifidobacterium longum* subsp. *suis* comb. nov. *International journal of systematic and evolutionary microbiology*, *58*(4), 767-772. doi: 10.1099/ijs.0.65319-0.

Meier-Kolthoff, J. P., & Göker, M. (2019). TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nature communications*, *10*(1), 2182. doi: 10.1038/s41467-019-10210-3

Meier-Kolthoff, J. P., Hahnke, R. L., Petersen, J., Scheuner, C., Michael, V., Fiebig, A., ... & Klenk, H. P. (2014). Complete genome sequence of DSM 30083 T, the type strain (U5/41 T) of Escherichia coli, and a proposal for delineating subspecies in microbial taxonomy. *Standards in genomic sciences*, *9*, 1-19. doi: 10.1186/1944-3277-9-2

Meier-Kolthoff, J. P., Auch, A. F., Klenk, H. P., & Göker, M. (2013). Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC bioinformatics, 14, 1-14. doi: 10.1186/1471-2105-14-60

Michaylova, M., Urshev, Z., Yungareva, T., Borisova, T., Gerogieva, R. (2022) Breast milk as a source of bifidobacteria and lactobacilli with potential application in breast milk enrichment and food for preterm infants (in Bulgarian). *Medinfo, 2022, 1,* 218-221.

Notarbartolo, V., Giuffrè, M., Montante, C., Corsello, G., & Carta, M. (2022). Composition of human breast milk microbiota and its role in children's health. *Pediatric gastroenterology, hepatology & nutrition*, *25*(3), 194. doi: 10.5223/pghn.2022.25.3.194

Nouioui, I., Carro, L., García-López, M., Meier-Kolthoff, J. P., Woyke, T., Kyrpides, N. C., ... & Göker, M. (2018). Genome-based taxonomic classification of the phylum Actinobacteria. *Frontiers in microbiology*, *9*, 2007. doi: 10.3389/fmicb.2018.02007

Oshiro, T., Nagata, S., Wang, C., Takahashi, T., Tsuji, H., Asahara, T., ... & Yamashiro, Y. (2020). Bifidobacterium supplementation of colostrum and breast milk enhances weight gain and metabolic responses associated with microbiota establishment in very-preterm infants. *Biomedicine hub*, *4*(3), 1-10. doi: 10.1159/000502935

Sakata, S., Kitahara, M., Sakamoto, M., Hayashi, H., Fukuyama, M., & Benno, Y. (2002). Unification of Bifidobacterium infantis and Bifidobacterium suis as Bifidobacterium longum. *International journal of systematic and evolutionary microbiology*, *52*(6), 1945-1951. doi: 10.1099/ijs.0.02221-0

Sakata, S., Ryu, C. S., Kitahara, M., Sakamoto, M., Hayashi, H., Fukuyama, M., & Benno, Y. (2006). Characterization of the genus *Bifidobacterium* by automated ribotyping and 16S rRNA gene sequences. *Microbiology and immunology*, *50*(1), 1-10. doi: 10.1111/j.1348-0421.2006.tb03762.x.

Scardovi, V., Casalicchio, F., & Vincenzi, N. (1979). Multiple electrophoretic forms of transaldolase and 6-phosphogluconic dehydrogenase and their relationships to the taxonomy and ecology of the bifidobacteria. *International Journal of Systematic and Evolutionary Microbiology*, *29*(4), 312-327. doi: 10.1099/00207713-29-4-312.

Selma-Royo, M., Lerma, J. C., Cortes-Macias, E., & Collado, M. C. (2021, October). Human milk microbiome: from actual knowledge to future perspective. In *Seminars in perinatology* (Vol. 45, No. 6, p. 151450). WB Saunders. 10.1016/j.semperi.2021.151450.

Simpson, J. T., Wong, K., Jackman, S. D., Schein, J. E., Jones, S. J., & Birol, I. (2009). ABySS: a parallel assembler for short read sequence data. *Genome research*, *19*(6), 1117-1123. doi: 10.1101/gr.089532.108.

Solís, G., de Los Reyes-Gavilan, C. G., Fernández, N., Margolles, A., & Gueimonde, M. (2010). Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. *Anaerobe*, *16*(3), 307-310. doi: 10.1016/j.anaerobe.2010.02.004.

Sundararaman, A., Bansal, K., Sidhic, J., Patil, P., & Halami, P. M. (2021). Genome of *Bifidobacterium longum* NCIM 5672 provides insights into its acid-tolerance mechanism and probiotic properties. *Archives of Microbiology, 203*, 6109-6118. doi: 10.1007/s00203-021-02573-3.

Ventura, M., Canchaya, C., Casale, A. D., Dellaglio, F., Neviani, E., Fitzgerald, G. F., & Van Sinderen, D. (2006). Analysis of bifidobacterial evolution using a multilocus approach. *International journal of systematic and evolutionary microbiology*, *56*(12), 2783-2792. doi: 10.1099/ijs.0.64233-0

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., ... & Truper, H. G. (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *International Journal of Systematic and Evolutionary Microbiology*, *37*(4), 463-464. doi: 10.1099/00207713-37-4-463.

Yanokura, E., Oki, K., Makino, H., Modesto, M., Pot, B., Mattarelli, P., ... & Watanabe, K. (2015). Subspeciation of *Bifidobacterium longum* by multilocus approaches and amplified fragment length polymorphism: description of *B. longum* subsp. *suillum* subsp. nov., isolated from the faeces of piglets. *Systematic and applied microbiology*, *38*(5), 305-314. doi: 10.1016/j.syapm.2015.05.001.

Zuo, F., Yu, R., Feng, X., Chen, L., Zeng, Z., Khaskheli, G. B., ... & Chen, S. (2016). Characterization and in vitro properties of potential probiotic Bifidobacterium strains isolated from breast-fed infant feces. *Annals of Microbiology*, *66*, 1027-1037. doi: 10.1007/s13213-015-1187-x.