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# Dissecting the Evolutionary Development of the Species *Bifidobacterium animalis* through Comparative Genomics Analyses

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**ABSTRACT** Bifidobacteria are members of the gut microbiota of animals, including mammals, birds, and social insects. In this study, we analyzed and determined the pangenome of *Bifidobacterium animalis* species, encompassing *B. animalis* subsp. *animalis* and the *B. animalis* subsp. *lactis* taxon, which is one of the most intensely exploited probiotic bifidobacterial species. In order to reveal differences within the *B. animalis* species, detailed comparative genomics and phylogenomics analyses were performed, indicating that these two subspecies recently arose through divergent evolutionary events. A subspecies-specific core genome was identified for both *B. animalis* subspecies, revealing the existence of subspecies-defining genes involved in carbohydrate metabolism. Notably, these *in silico* analyses coupled with carbohydrate profiling assays suggest genetic adaptations toward a distinct glycan milieu for each member of the *B. animalis* subspecies, resulting in a divergent evolutionary development of the two subspecies.

**IMPORTANCE** The majority of characterized *B. animalis* strains have been isolated from human fecal samples. In order to explore genome variability within this species, we isolated 15 novel strains from the gastrointestinal tracts of different animals, including mammals and birds. The present study allowed us to reconstruct the pangenome of this taxon, including the genome contents of 56 *B. animalis* strains. Through careful assessment of subspecies-specific core genes of the *B. animalis* subsp. *animalis/lactis* taxon, we identified genes encoding enzymes involved in carbohydrate transport and metabolism, while unveiling specific gene acquisition and loss events that caused the evolutionary emergence of these two subspecies.

**KEYWORDS** *Bifidobacterium*, bifidobacteria, pangenome, phylogeny, probiotic

Bifidobacteria are Gram-positive, anaerobic, nonmotile, and non-spore-forming bacteria, which are commonly found in the gastrointestinal tracts (GITs) of various animals, the human oral cavity, and sewage (1). Bifidobacterial species residing in the human GIT are believed to support host health in providing energy and nutrients, modulating the immune system and adjusting the gut physiology of the host (2–5). Currently, 72 different species of bifidobacteria have been identified and, depending on the species, more or less characterized (6). Among this large number of bifidobacterial taxa, just a few species, including *Bifidobacterium animalis* (7, 8), *Bifidobacterium bifidum* (9, 10), *Bifidobacterium breve* (11), and *Bifidobacterium longum* (12, 13), have been exploited as health-promoting bacteria. In particular, *B. animalis* strains have been

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extensively used as active ingredients in a variety of functional foods (14, 15). The *B. animalis* species consists of two subspecies, *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* (16). Of these two taxa, only members of *B. animalis* subsp. *lactis* have been utilized for their health-promoting purposes (17). To date, a number of scientific publications have investigated the purported probiotic features of a number of *B. animalis* subsp. *lactis* strains, such as their protective behavior against periodontitis (18), their ability to improve GIT health in abdominal discomfort and obesity disorder states (19, 20), and the inhibition of pathogenic bacteria (21).

Before the advent of next-generation sequencing (NGS) methods, the classification criteria to discriminate (what were then called) *B. lactis* and *B. animalis* were based on phenotypic characteristics, such as morphology and carbohydrate fermentation abilities. However, 16S rRNA gene sequence comparison, combined with DNA-DNA hybridization of the type strains of these two taxa, led to the proposal to consider *B. lactis* as a junior, synonymous taxon of the *B. animalis* species (22). Subsequently, using a polyphasic approach, *B. animalis* and *B. lactis* were reclassified as *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis*, respectively (16). Various genomic studies have revealed the existence of a high level of genome synteny between the two *B. animalis* subspecies (23, 24), as well as similar levels of acid, heat, and oxygen tolerance (25, 26).

To date, comprehensive comparative genomic analyses of bifidobacterial taxa have been performed (23, 27–29). In this context, members of the *B. bifidum*, *B. breve*, and *B. longum* species have been shown to exhibit a closed pangenome structure, revealing the presence of specific genetic strategies to establish and persist in the human gut, such as through the production of various types of pili (30, 31) or metabolic capabilities toward particular host glycans (32, 33). In the same fashion, members of the *B. animalis* subsp. *lactis* taxon have been investigated through genomic decoding. Notably, such analyses involved *B. animalis* subsp. *lactis* strains, which were isolated from the human GIT and dairy products. Overall, their genetic characterization highlighted the presence of a very modest number of genomic differences (23). Conversely, genotypic and phenotypic analyses of *B. animalis* subsp. *lactis* strains from commercial products and animals revealed some distinct differences in fermentation profiles and peptide mass fingerprints (34). In contrast to these investigations involving *B. animalis* subsp. *lactis*, very little investigative work has been done on members of the *B. animalis* subsp. *animalis* taxon.

The aim of this study was to investigate the genetic biodiversity of the *B. animalis* species by decoding genome sequences of isolates collected from the GITs of various animals, including mammals and birds. The identification of the genomic makeup of members belonging to either of the two subspecies is considered crucial in order to provide information regarding the subspecies-specific repertoire of genes that may have caused their evolutionary differentiation. Furthermore, such genomic analyses, combined with carbohydrate profiling experiments, support the hypothesis that the two *B. animalis* subspecies have been subject to genetic adaptations to environments that had a distinct glycan content.

## RESULTS AND DISCUSSION

**Isolation and genetic characterization of the *B. animalis* species.** To investigate the occurrence of *B. animalis* in the gut of animals, we screened the internally transcribed spacer (ITS) sequence profiling data derived from fecal samples of four mammalian and bird species, together with the bifidobacterial community data previously determined by Milani et al. (35) (Fig. 1). In this context, *B. animalis* was detected in 55% of such fecal samples, with a higher occurrence in the fecal samples of dogs (*Canis lupus*), onagers (*Equus hemionus kulan*), monkeys (*Chlorocebus pygerytrhus*, *Macaca fuscata*, *Macaca sylvanus*, and *Pan troglodytes*), and mice (*Mus musculus*) (Fig. 1). These data revealed a cosmopolitan lifestyle of this taxon, underlining the potential high genetic adaptation of *B. animalis* strains to different (host) environments.

In order to investigate the genetic contents of the *B. animalis* species, including representatives of both *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* taxa, we

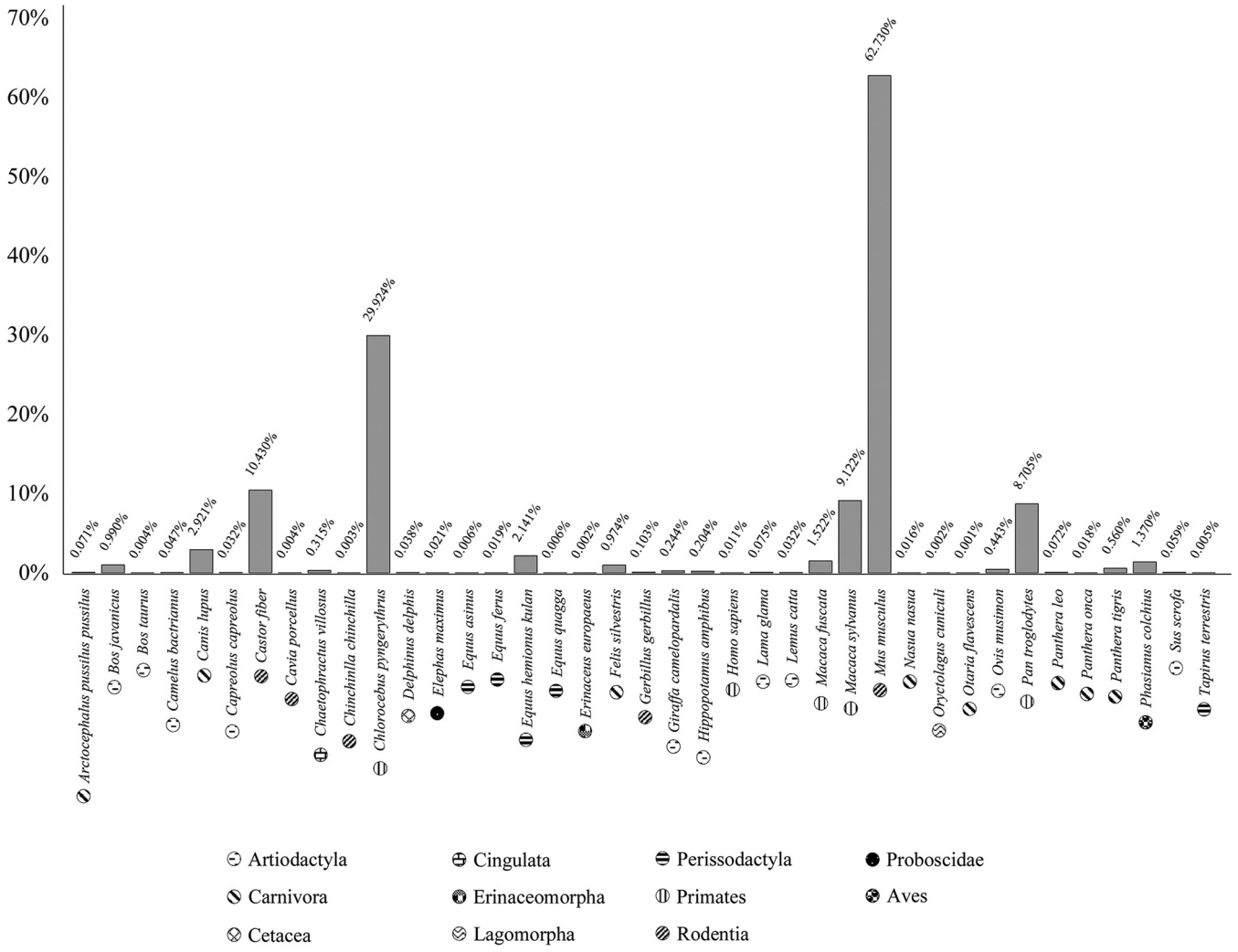


**FIG 1** *B. animalis* profiling data obtained from fecal samples of different animals. In this bar plot, the *x* axis represents the animals tested for the presence of *B. animalis*, while the *y* axis represents the percentage of *B. animalis* compared to other *Bifidobacterium* species present in the samples. Each pattern represents an animal order, as indicated in the key.

applied a bifidobacterial isolation protocol on fecal samples of animal species displaying a high abundance of these taxa. The above-mentioned analyses (see Materials and Methods) (36, 37) allowed the isolation of 15 novel *B. animalis* strains from birds (*Phasianus colchicus*) and various Mammalia, such as canine breeds, i.e., German shepherd, Pomeranian, Alaskan malamute, and flat-coated retriever, and three different nonhuman primates, i.e., *Pan troglodytes*, *Chlorocebus pygerythrus*, and *Macaca sylvanus*. Moreover, *B. animalis* subsp. *lactis/animalis* strains were isolated from fecal samples of rabbits (*Oryctolagus cuniculus*), beavers (*Castor fiber*), and pigs (*Sus scrofa domesticus*) (Table 1 and Fig. 1). Interestingly, we were also able to isolate different *B. animalis* strains from stool samples of animals in which the ITS bifidobacterial profiling analysis indicated a low relative abundance of this species, i.e., *Sus scrofa* (0.06%) and *Oryctolagus cuniculus* (0.002%). This may be due to the better growth performance (e.g., high tolerance to environmental stresses) of members of the *B. animalis* species compared to other bifidobacteria (38–40). A comparative genomic analysis between newly isolated strains was complemented with the inclusion of publicly available genomic repertoire of 41 *B. animalis* strains, thereby exemplifying a broad ecological representation, including the GITs of human and other animals (e.g., rats and chickens) (41), as well as different food matrices (e.g., milk and yogurt) and human vaginal swabs (23, 42) (Table 1). This information further validates the notion that *B. animalis* seems to be

**TABLE 1** *B. animalis* strains/genomes used in this study[a]

| Species and strain | Ecological origin | Genome size (Mb) | No. of ORFs | GC content (%) | No. of tRNAs | rRNA locus | Coverage depth (fold) | No. of contigs | Source or reference |
|---|---|---|---|---|---|---|---|---|---|
| *B. animalis* subsp. *animalis* | | | | | | | | | |
| 2022B | *Castor fiber* feces | 2.4 | 1,935 | 61.08 | 65 | 2 | 142 | 17 | RSDC00000000 |
| 2006B | *Canis lupus familiaris* (German shepherd) feces | 2.16 | 1,747 | 61.24 | 56 | 3 | 195 | 47 | RSDB00000000 |
| ATCC 25527 | Human feces | 1.93 | 1,622 | 61.35 | 52 | 2 | | | 24 |
| ATCC 27672 | Rat feces | 1.99 | 1,611 | 60.97 | 52 | 1 | | | NCBI database |
| IM386 | Human feces | 1.93 | 1,623 | 61.35 | 52 | 1 | | | NCBI database |
| LMG10508 | Rat feces | 1.92 | 1,619 | 60.53 | 52 | 2 | | | NCBI database |
| MCC0483 | Rat feces | 2.18 | 1,922 | 60.97 | 53 | 1 | | | NCBI database |
| MCC0499 | Rat feces | 2.13 | 1,870 | 61.05 | 62 | 1 | | | NCBI database |
| MCC1489 | Guinea pig feces | 1.91 | 1,619 | 61.35 | 52 | 2 | | | NCBI database |
| YL2 | Rat feces | 2.02 | 1,705 | 61.1 | 52 | 3 | | | 75 |
| *B. animalis* subsp. *lactis* | | | | | | | | | |
| 646 | Human feces | 1.92 | 1,673 | 61.4 | 52 | 4 | | | NCBI database |
| 1316B | *Phasianus colchicus* feces | 1.92 | 1,556 | 60.47 | 52 | 3 | 195 | 14 | RSDA00000000 |
| 1395B | *Oryctolagus cuniculus* feces | 1.92 | 1,557 | 60.47 | 52 | 2 | 99 | 12 | RSCZ00000000 |
| 1528B | *Sus scrofa domesticus* feces | 1.95 | 1,600 | 61.47 | 55 | 2 | 97 | 12 | RSCY00000000 |
| 1802B | *Macaca sylvanus* feces | 1.92 | 1,557 | 61.36 | 52 | 2 | 132 | 15 | RSCX00000000 |
| 1808B | *Chlorocebus pygerytrhus* feces | 1.92 | 1,556 | 61.35 | 52 | 2 | 87 | 15 | RSCW00000000 |
| 1811B | *Chlorocebus pygerytrhus* feces | 1.68 | 1,560 | 61.37 | 52 | 2 | 156 | 16 | RSCV00000000 |
| 1813B | *Pan troglodytes* feces | 1.68 | 1,557 | 61.36 | 52 | 2 | 247 | 12 | RSCU00000000 |
| 1821B | *Pan troglodytes* feces | 1.75 | 1,636 | 60.71 | 53 | 2 | 278 | 40 | RSCT00000000 |
| 1843B | *Pan troglodytes* feces | 1.68 | 1,557 | 61.36 | 52 | 2 | 85 | 14 | RSCS00000000 |
| 1869B | *Pan troglodytes* feces | 1.92 | 1,557 | 61.36 | 52 | 2 | 229 | 14 | RSCR00000000 |
| 2007B | *Canis lupus familiaris* (Pomeranian) feces | 1.97 | 1,599 | 61.28 | 52 | 1 | 194 | 25 | RSCQ00000000 |
| 2010B | *Canis lupus familiaris* (Alaskan malamute) feces | 1.98 | 1,607 | 61.21 | 52 | 2 | 121 | 29 | RSCP00000000 |
| 2011B | *Canis lupus familiaris* (Flat coated retriever) feces | 2.08 | 1,700 | 61.32 | 54 | 1 | 181 | 44 | RSCO00000000 |
| A6 | Human feces | 1.96 | 1,651 | 61.38 | 52 | 5 | | | NCBI database |
| AD011 | Infant feces | 1.93 | 1,642 | 61.38 | 52 | 2 | | | 76 |
| ATCC 27536 | Chicken feces | 1.91 | 1,632 | 61.35 | 52 | 1 | | | NCBI database |
| ATCC 27673 | Fermented milk sample | 1.95 | 1,685 | 61.52 | 52 | 3 | | | 77 |
| ATCC 27674 | Rabbit feces | 1.91 | 1,629 | 61.35 | 52 | 1 | | | NCBI database |
| B420 | Human feces | 1.94 | 1,633 | 61.37 | 52 | 3 | | | 78 |
| BB-12 | Food matrices | 1.97 | 1,639 | 61.38 | 52 | 3 | | | 79 |
| BF052 | Feces of breast-fed infant | 1.94 | 1,632 | 61.38 | 52 | 3 | | | NCBI database |
| Bi-07 | Human feces | 1.94 | 1,831 | 61.38 | 52 | 3 | | | 78 |
| Bifido_08 | Human feces | 1.95 | 1,757 | 61.32 | 52 | 4 | | | NCBI database |
| Bifido_11 | Human feces | 1.94 | 1,702 | 61.32 | 52 | 4 | | | NCBI database |
| BI_04 | Human feces | 1.94 | 1,633 | 61.38 | 52 | 3 | | | 5 |
| BI12 | Human colonoscopic sample | 1.94 | 1,633 | 61.37 | 52 | 3 | | | NCBI database |
| BL3 | Human feces | 1.94 | 1,639 | 61.38 | 52 | 3 | | | 80 |
| BLC1 | Human feces | 1.94 | 1,630 | 61.37 | 52 | 3 | | | 81 |
| BS01 | Human feces | 1.93 | 1,632 | 61.37 | 52 | 1 | | | NCBI database |
| CECT8145 | Infant feces | 1.96 | 1,766 | 61.38 | 52 | 1 | | | NCBI database |

(Continued on next page)

**TABLE 1** (Continued)

| Species and strain | Ecological origin | Genome size (Mb) | No. of ORFs | GC content (%) | No. of tRNAs | rRNA locus | Coverage depth (fold) | No. of contigs | Source or reference |
|---|---|---|---|---|---|---|---|---|---|
| CNCMI-2494 | Human feces | 1.94 | 1,635 | 61.38 | 52 | 3 | | | 82 |
| DS1_2 | Human feces | 1.92 | 1,636 | 61.36 | 52 | 2 | | | NCBI database |
| DS11_2 | Human feces | 1.92 | 1,637 | 61.36 | 52 | 2 | | | NCBI database |
| DS15_2 | Human feces | 1.92 | 1,635 | 61.37 | 52 | 3 | | | NCBI database |
| DS2_2 | Human feces | 1.92 | 1,634 | 61.35 | 52 | 2 | | | NCBI database |
| DS24_2 | Human feces | 1.92 | 1,670 | 61.35 | 52 | 1 | | | NCBI database |
| DS27_2 | Human feces | 1.92 | 1,642 | 61.35 | 52 | 1 | | | NCBI database |
| DS28_2 | Human feces | 1.92 | 1,633 | 61.35 | 52 | 2 | | | NCBI database |
| DSM10140 | Human feces | 1.94 | 1,635 | 61.37 | 51 | 3 | | | 42 |
| HN019 | Human feces | 1.92 | 1,645 | 61.35 | 52 | 1 | | | NCBI database |
| KLDS2.0603 | Human feces | 1.95 | 1,646 | 61.37 | 52 | 2 | | | NCBI database |
| LMG P-17502_1 | Food sample | 1.92 | 1,628 | 61.36 | 52 | 2 | | | NCBI database |
| LMG P-17502_2 | Food sample | 1.92 | 1,628 | 61.36 | 52 | 1 | | | NCBI database |
| RH | Human feces | 1.93 | 1,629 | 61.37 | 52 | 2 | | | NCBI database |
| V9 | Human feces | 1.94 | 1,633 | 61.38 | 52 | 3 | | | NCBI database |

[a]The references are based on the decoding genomes project according to the NCBI database.

genetically adapted to a large number of habitats. Notably, the ORFeome of *B. animalis* subsp. *animalis* strains, defined as the complete set of open reading frames (ORFs) in genomes of the same species, was shown to be substantially larger compared to that of *B. animalis* subsp. *lactis* strains, suggesting that members of the *B. animalis* subsp. *animalis* taxon exhibit a more extensive level of genetic diversity.

**Pangenome and core genome analyses of *B. animalis* species.** The reconstructed genomic data sets of the *B. animalis* species, encompassing a total of 56 chromosomal sequences, represents the genetic catalogue for this bifidobacterial species. The genetic makeup of the whole taxon was employed to predict the pangenome of the *B. animalis* species, i.e., the available collection of genes from strains of a given species (43). Moreover, these data were used to predict also the core genome, i.e., the collection of gene families shared between organisms of a given species, i.e., the *B. animalis* taxon, as based on the clusters of orthologous groups (COGs) (44). The pangenome size, consisting of 4,486 COGs, when plotted on a log-log scale as a function of the number of analyzed genomes, suggests that the power trend line has almost reached a plateau (Fig. 2). The average number of new genes discovered by sequential addition of genome sequences decreased from 130 COGs upon the addition of another genome, to 30 COGs in the final addition (Fig. 2). Thus, these findings indicate that genome sequencing of additional (novel) *B. animalis* strains are expected to increase the pangenome size by $<$0.7% (Fig. 2). Furthermore, the 56 *B. animalis* genomes were screened to identify shared orthologous genes, as well as unique genes. *In silico* analyses reveals that 1,098 ORFs were shared between the assessed strains, representing the core genome of this species. The functional examination of the core genome, based on the eggNOG database (45), reveals that 26.1% of the identified core genes are predicted to encode housekeeping functions and enzymatic activities related to amino acid and carbohydrate metabolism and their corresponding transport.

When we separately analyzed the core-genome of strains belonging to a specific *B. animalis* subspecies, subspecies-specific core genes could be identified (Fig. 2). In this context, 142 subspecies-specific genes were retrieved in the genomes of the *B. animalis* subsp. *animalis* subspecies, while just 82 were detected in the chromosome sequences of *B. animalis* subsp. *lactis* members. The existence of specific conserved genes among the two subspecies is suggestive of an evolutionary separation between these bifidobacterial taxa. Specifically, genes that have driven this differentiation are expected to be among the subspecies-specific core and include genes that are predicted to encode transporters and carbohydrate active proteins, i.e., 51 in the *B. animalis* subsp. *animalis*-specific and 31 in the *B. animalis* subsp. *lactis*-specific core genomes, respectively (see Table S2 in the supplemental material). Interestingly, the higher number of the above-mentioned genes in the *B. animalis* subsp. *animalis*-specific core genome compared to the corresponding number in the *B. animalis* subsp. *lactis*-specific core genome suggests that *B. animalis* subsp. *animalis* strains are able to metabolize a larger number of glycan substrates compared to *B. animalis* subsp. *lactis* strains (Table S2). Furthermore, the subspecies-specific core genomes include various DNA binding proteins, with a distinctly higher abundance in *B. animalis* subsp. *animalis* (13 genes) compared to *B. animalis* subsp. *lactis* (three genes), five of which belong to the MarR family of transcriptional regulators (Table S2). Altogether, the observed differences in the number of subspecies-specific core genes between the *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* were shown to be statistically significant ($P < 0.05$).

An *in silico* approach was used to calculate the average nucleotide identity (ANI) values, defined as a measure of nucleotide-level genomic similarity between the coding regions of two genomes, between *B. animalis* genomes (46), showing a highly syntenic genome structure among members of this species, with associated ANI values ranging from 95.81 to 99.99%. Moreover, different ranges of ANI values were identified between strains belonging to *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis*. Interestingly, the lowest ANI value between *B. animalis* subsp. *lactis* genomes was 98.7%, while for *B. animalis* subsp. *animalis* genomes this number was 96.1%. These data reflect the

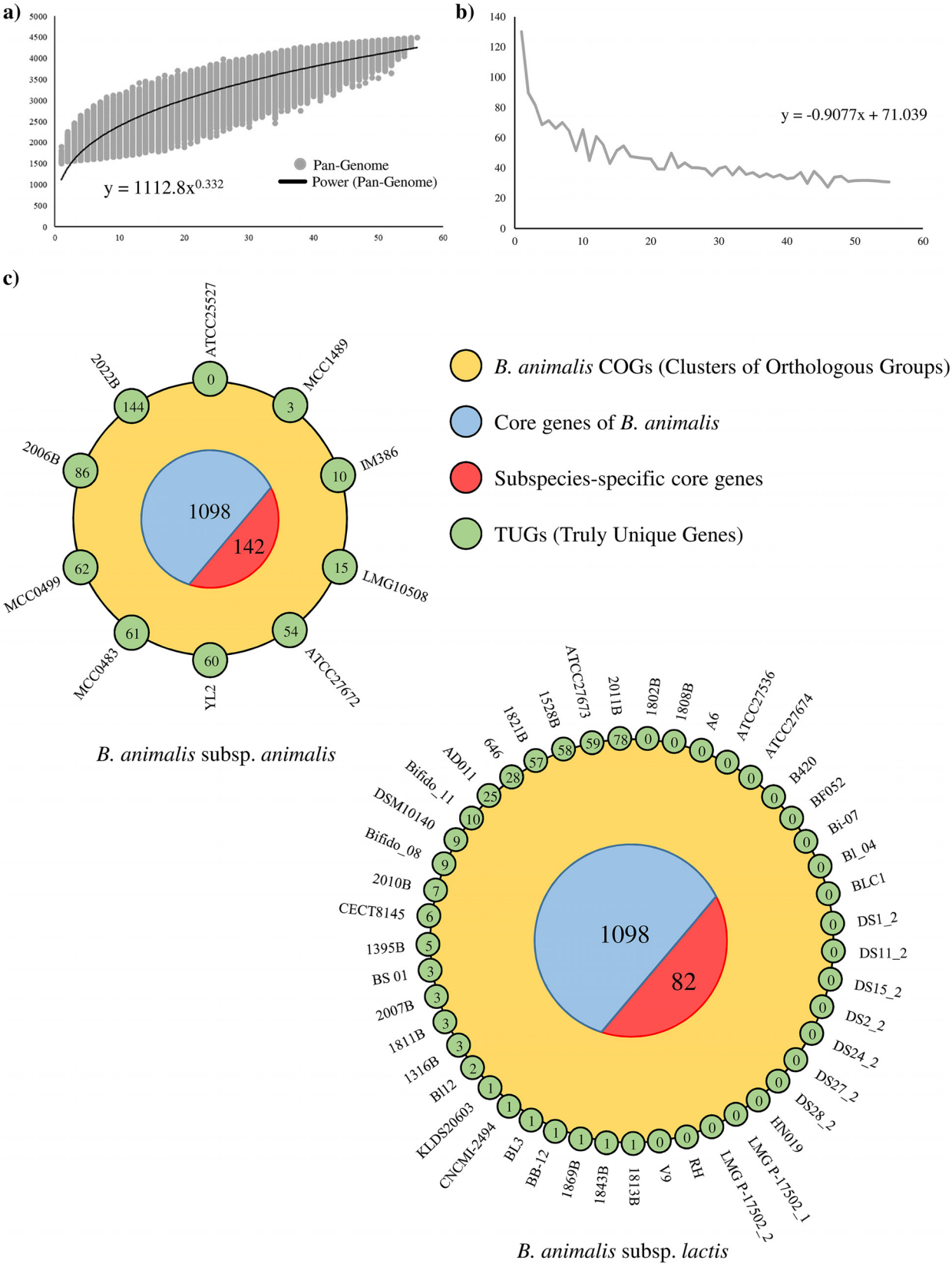


**FIG 2** Pangenome and core genome of the *B. animalis* species. (a) Pangenome of the *B. animalis* species. (b) Average of new genes upon sequential addition of the *B. animalis* genomes. (c) Two Venn diagrams representing shared orthologous, as well as unique, genes among the 56 *B. animalis* genomes. Numbers in blue circular segments represent the core genes of the *B. animalis* taxon, while numbers in red circular segments symbolize the subspecies-specific core genes. Moreover, the numbers of unique genes are highlighted in small green circles.

differences between these two subspecies, highlighting a highly syntenic genome structure among members of the *B. animalis* subsp. *lactis* subspecies. This statement was further validated by the pangenome analysis mentioned above that allowed us to highlight truly unique genes (TUGs) of each *B. animalis* strain (Fig. 2). In this context, a variable number of TUGs, ranging from 0 genes for 23 *B. animalis* subsp. *lactis* strains to 144 genes for *B. animalis* subsp. *animalis* 2022B, were detected (Fig. 2). Thus, the absence of TUGs within the majority of *B. animalis* subsp. *lactis* strains supports the previously noted high isogenic nature of members of this taxon (23). Furthermore, the ANI analysis highlights that genomes of two *B. animalis* subsp. *lactis* strains, i.e., ATCC 27674 and CNCM I-2494, displayed a genetic identity of 99.9% compared to that of the prototypical probiotic bifidobacterial strain, i.e., *B. animalis* subsp. *lactis* BB-12 (17). Thus, we can speculate that the latter strains exhibit similar probiotic characteristics (47). Nevertheless, additional functional genomics analyses coupled with *in vivo* studies should be performed in order to confirm this notion.

**Phylogenetic analyses of the *B. animalis* species.** Recently, a phylogenomic assessment of members of the genus *Bifidobacterium* allowed the identification of nine phylogenetic groups (6). Notably, *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis* taxa are members of the *Bifidobacterium pseudolongum* group, which also includes *Bifidobacterium choerinum*, *Bifidobacterium cuniculi*, *Bifidobacterium gallicum*, *Bifidobacterium magnum*, *Bifidobacterium pseudolongum* subsp. *globosum*, and *Bifidobacterium pseudolongum* subsp. *pseudolongum* (48). Accordingly, we reevaluated the evolutionary development of the 56 *B. animalis* strains analyzed here using a phylogenomic approach, which also included the genome sequences of the bifidobacterial type strains belonging to the *B. pseudolongum* phylogenetic group.

*In silico* analyses identified 667 orthologous genes, which were shared among sequenced genomes of the *B. pseudolongum* group, which were then used to build a so-called supertree (Fig. 3). This supertree showed that all 15 *B. animalis* strains isolated in this study cocluster with other publicly available *B. animalis* genomes. Furthermore, a clear division was identified between genomes belonging to the *B. animalis* subsp. *animalis* subspecies and those encompassing the *B. animalis* subsp. *lactis* subspecies (Fig. 3). As previously observed through molecular typing approaches, *B. animalis* subsp. *lactis* ATCC 27672 clusters together with members of the *B. animalis* subsp. *animalis* group, suggesting a misclassification of this strain (39). Interestingly, *B. animalis* subsp. *lactis* 2011B clusters on a separate branch with respect to other *B. animalis* subsp. *lactis* strains, suggesting that this isolate may have followed a different evolutionary pathway compared to the other members of *B. animalis* subsp. *lactis* taxon.

In order to assess the level of genetic differences between each *B. animalis* subspecies, we analyzed single nucleotide polymorphisms (SNPs) among genomes of this taxon, using the software Mauve (49). The number of identified SNPs was higher in *B. animalis* subsp. *animalis* genomes (123,338 SNPs) compared to those detected in the *B. animalis* subsp. *lactis* chromosomes (52,162 SNPs). In this context, 59.5% of the *B. animalis* subsp. *animalis* SNPs were identified only in two strains, i.e., *B. animalis* subsp. *animalis* 2006B and *B. animalis* subsp. *animalis* 2022B, while 54.8% of the *B. animalis* subsp. *lactis* SNPs were detected in only three strains, i.e., *B. animalis* subsp. *lactis* 2010B, *B. animalis* subsp. *lactis* 2011B, and *B. animalis* subsp. *lactis* 2007B. It should be noted that some of these differences may be correlated with the quality of the deposited genome sequences, which may have been affected by a low sequencing fold coverage. Nonetheless, strains that display the highest number of SNPs in their genomes also reflect their apparent phylogenetic distinctiveness in the supertree of the *B. pseudolongum* group (Fig. 3), perhaps reflecting divergent evolution compared to other members of their subspecies. Furthermore, the performed phylogenetic analysis may assist in the selection of novel probiotic strains. In this context, 18 *B. animalis* subsp. *lactis* strains cluster in the BB-12 branch (Fig. 3). Their genomic relatedness was also highlighted in the pangenome analysis, where half of the *B. animalis* subsp. *lactis* strains does not show any TUGs (Fig. 2).

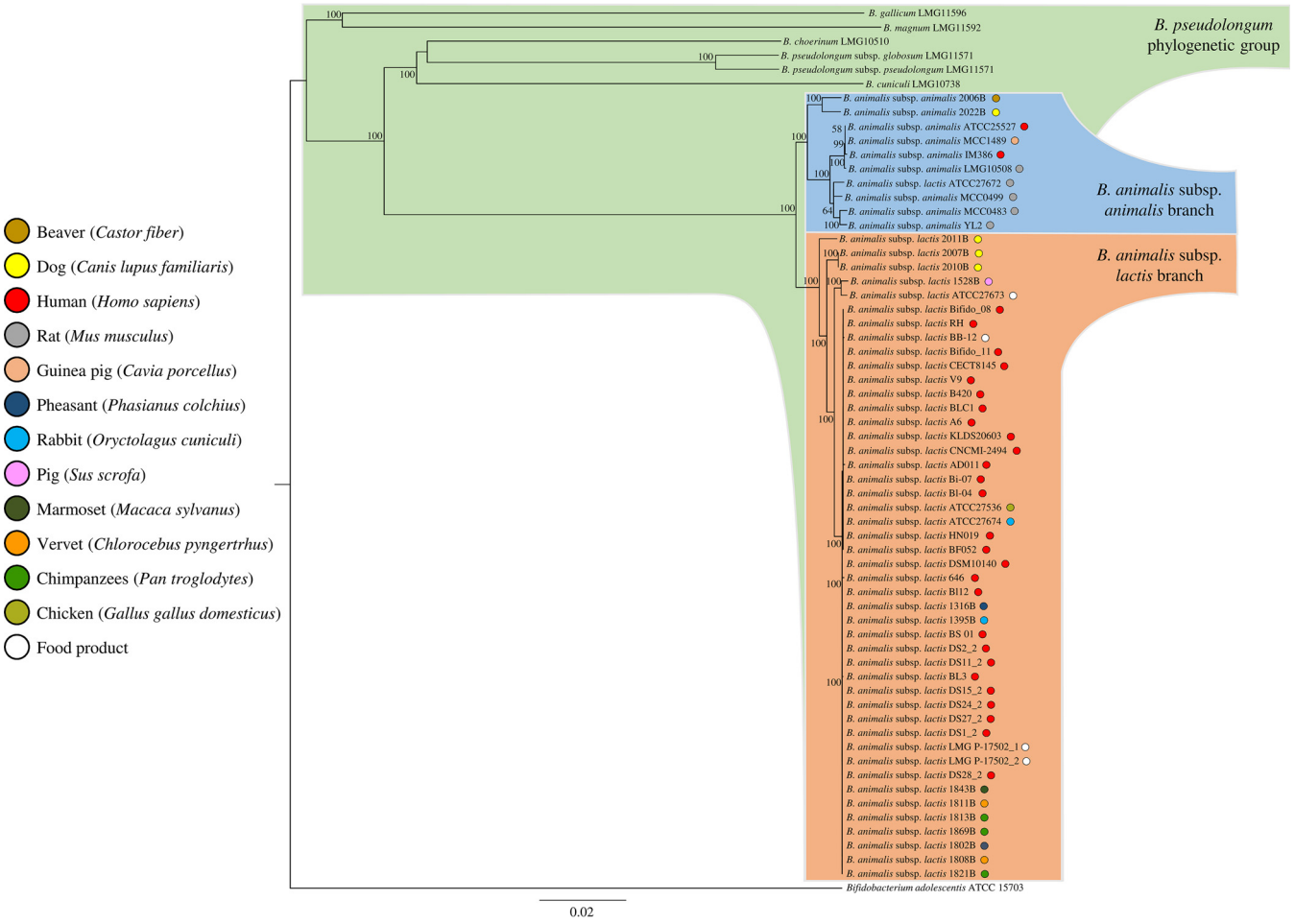


**FIG 3** Phylogenomic tree of the *B. animalis* taxa. A proteomic tree was developed based on the concatenation of 667 *B. animalis* core genes identified in the *B. pseudolongum* group phylogenomic analysis. This tree was constructed by the neighbor-joining method, and the genome sequence of *Bifidobacterium adolescentis* ATCC 15703 was used as outgroup. Bootstrap percentages of >50 are shown at node points, based on 1,000 replicates. Colored small circles indicate the ecological origins of each bacteria.

**Glycobiome of the *B. animalis* species.** Bifidobacteria are known to metabolize a wide range of carbohydrates as a carbon and energy source, ranging from dietary to host-derived glycans (50–53). In order to assess carbohydrate fermentation capabilities of the two *B. animalis* subspecies, we performed growth experiments involving 19 *B. animalis* species cultivated on semisynthetic medium with different carbohydrates as the sole carbon source. In order to obtain a complete overview of such carbohydrate metabolic abilities, we included both plant- and host-derived glycans (Fig. 4). As displayed in Fig. 4, all *B. animalis* subsp. *lactis* strains were able to grow on a common set of sugars, such as lactose, maltose, raffinose, and sucrose. In contrast, *B. animalis* subsp. *animalis* strains was shown to metabolize a broader array of sugars, with a high growth performance in medium containing arabinose, galactose, glucose, maltose, melibiose, sucrose, or xylose (54). Furthermore, *B. animalis* subsp. *lactis* 646, *B. animalis* subsp. *lactis* 1316B, and *B. animalis* subsp. *lactis* 1395B, in contrast to other members of this subspecies, exhibited appreciable growth on xylose (Fig. 4).

Statistical analyses were performed to corroborate the observed growth differences between *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis* strains on different sugars. As shown in Fig. 4, a significant growth difference ($P < 0.05$) for 14 carbohydrates was observed, with the highest growth performances of *B. animalis* subsp. *animalis* strains (compared to *B. animalis* subsp. *lactis* strains) in medium

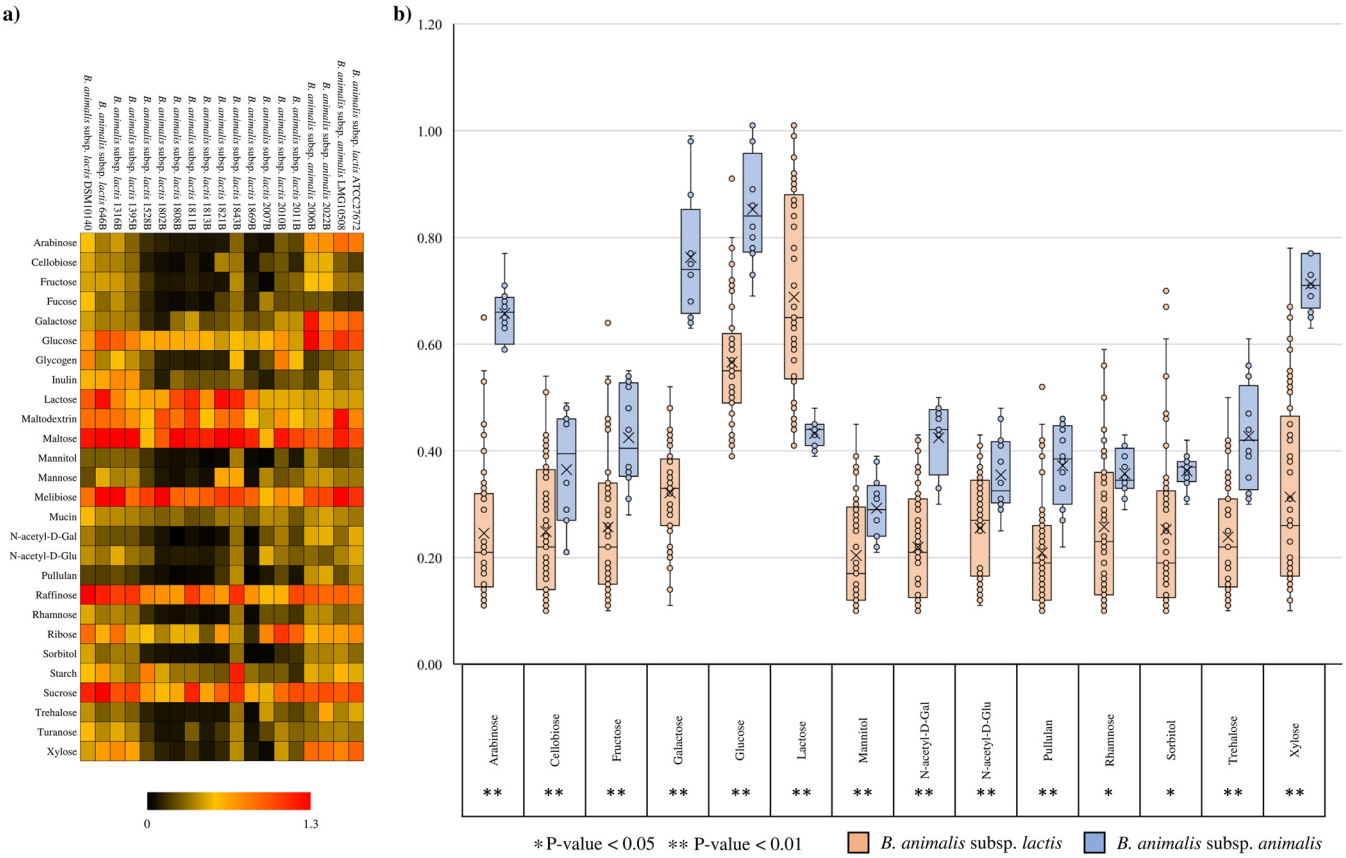


**FIG 4** Evaluation of carbohydrate utilization by *B. animalis* strains. (a) Heat map representing the growth performances of *B. animalis* strains on different sugars. Cultures were grown in biologically independent triplicates. Different shadings represent the optical densities reached by the assessed cultures. (b) Whiskers plot based on optical density values of sugars with a *P* value of $<0.05$ between subspecies (Student *t* test). The *x* axis represents the sole carbon source used for the growth experiments, while the *y* axis shows the optical density values obtained for *B. animalis* subsp. *animalis* strains (blue) and *B. animalis* subs. *lactis* strains (orange). Points reflect the distribution of a data set, while the boxes represent 50% of the data set, distributed between the first and third quartiles. The median divides the boxes into the interquartile range, while the "X" represents the mean. The lines extending vertically outside the boxes show the outlier range.

containing arabinose, fructose, galactose, glucose, pullulan, trehalose, or xylose (Table S1). On the other hand, *B. animalis* subsp. *lactis* strains were shown to grow significantly better (compared to *B. animalis* subsp. *animalis* strains) in MRS medium supplemented with lactose (Fig. 4). Moreover, in five cases, the obtained growth performances were shown to be highly significantly different, with *P* values of $<0.001$ (Fig. 4). Notably, none of *B. animalis* subsp. *lactis* strains was able to utilize mucin, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine, which indicates that the tested strains possess limited metabolic capabilities with regard to host-derived glycans (Fig. 4).

In order to validate the observed metabolic differences of the *B. animalis* subspecies, we predicted the glycosyl hydrolase (GH) enzymes involved in carbohydrate breakdown and belonging to the subspecies-specific core genes, as mentioned above. The *in silico* analyses were performed using the carbohydrate-active enzymes (CAZy) database (55) involving the 56 *B. animalis* genomes mentioned above. Interestingly, 13 subspecies-specific core genes of *B. animalis* subsp. *animalis* genomes are predicted to be involved in sugar metabolism, while 5 genes indicated as carbohydrate-active enzymes are present in the subspecies-specific core genes of *B. animalis* subsp. *lactis* genomes. Among these subspecies-specific carbohydrate-active enzymes, we retrieved seven GH-encoding genes in *B. animalis* subsp. *animalis* genomes and four within *B. animalis* subsp. *lactis* strains. Interestingly, one of the seven *B. animalis* subsp. *animalis*-specific GH belongs to the GH2 family, which typically represent $\beta$-galactosidase (56)

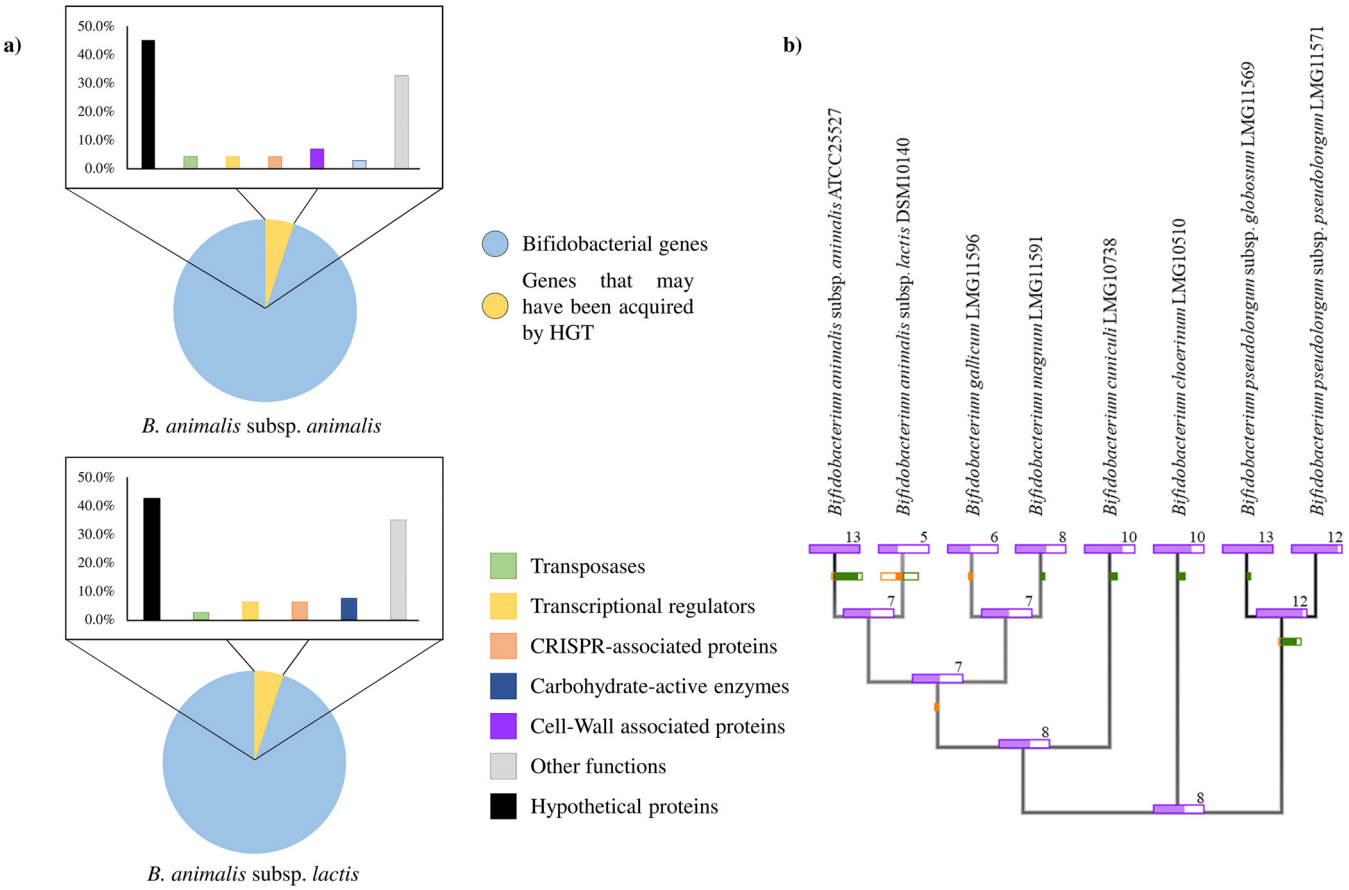


**FIG 5** Evolutionary gene gain and gene loss analysis within the *B. pseudolongum* phylogenetic group, as based on predicted subspecies-specific GHs. (a) Genes predicted to have been acquired by HGT events in type strains of the *B. animalis* species. Bar plots represent in different colors the functional annotations of the predicted genes. (b) Tree based on the core genome of the *B. pseudolongum* phylogenetic group. The different sticks represent the predicted subspecies-specific GHs within *B. pseudolongum* phylogenetic group. Each node reports the number of predicted GHs identified in the type strains tested. Green and orange bars on the edge leading to each node indicate gains and losses.

and exo-$\beta$-glucosaminidase (57) activities, confirming the observed high metabolic capabilities of this taxon toward galactose- and glucose-containing sugars (Fig. 4). Furthermore, two *B. animalis* subsp. *animalis* GH-specific genes belong to the GH3 family, representing $\beta$-glucosidases and xylosidases (58), and the GH43 family, representing xylosidases (58) and arabinosidases (59), which are involved in the metabolism of xylose- and arabinose-containing glycans. Therefore, *in silico* analyses showed a larger number of GH-encoding genes among the *B. animalis* subsp. *animalis* subspecies-specific core genes compared to the *B. animalis* subsp. *lactis* subspecies-specific core genes, confirming the observed broader carbohydrate-dependent growth performances displayed by this taxon.

**Evolutionary gain gene and loss gene analysis.** In order to identify genes that may have been acquired by horizontal genes transfer (HGT), the genomes of the type strains of *B. animalis* subsp. *animalis/lactis* were analyzed with the software suite COLOMBO v4.0 (60). Interestingly, 80 genes, representing 5.1% of the *B. animalis* subsp. *lactis* genes, seem to have alien origins of which 42.5% encode hypothetical proteins. Moreover, 7.5% of the genes that may have been acquired by HGT are predicted to be enzymes involved in carbohydrate metabolism, while 12.6% represent genes encoding transcriptional regulators, and genes involved in CRISPR-Cas systems (Fig. 5). In the case of *B. animalis* subsp. *animalis*, 4.6% of the genes seem to have been acquired by HGT, of which 45.1% represent hypothetical proteins. Moreover, 4.2% of these genes encode transposase and 8.4% are predicted to be involved in CRISPR-Cas and in transcriptional

regulation. These data suggest that HGT events represent a minor force in the evolution of genomes of *B. animalis* species.

To evaluate the acquisition and loss of the subspecies-specific GH genes through the *B. pseudolongum* phylogenetic group, we analyzed the predicted subspecies-specific carbohydrate-active enzymes using Count software (61). This evolutionary development analysis is based on the core-gene sequences retrieved from the type strains of the *B. pseudolongum* phylogenetic group. As indicated in Fig. 5, the *B. animalis* subsp. *animalis* taxon seems to have acquired five carbohydrate-active enzymes during evolution compared to the common ancestor of the phylogenetic group. Furthermore, the *B. animalis* subsp. *lactis* taxon was shown to be the subspecies with the higher prevalence of subspecies-specific GH gene loss, encompassing five specific GHs (Fig. 5). These findings suggest that the *B. animalis* subspecies has followed a different evolutionary path, confirming our observed differences between these two taxa identified in the phylogenomic analyses.

**Conclusions.** Isolation of 15 *B. animalis* strains from the GITs of different animals and representing the *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* taxa revealed the cosmopolitan lifestyle of this species. Genome sequencing of the collected strains allowed us to reconstruct the genomic data set of the *B. animalis* species, including 41 publicly available *B. animalis* genomes, unveiling that further genome sequencing of novel *B. animalis* strains will only slightly contribute to increase the pan-genome size. Nonetheless, phylogenetic analysis based on core genome sequences, among the 56 bifidobacterial genomes, showed a clear differentiation between the *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* branch. In fact, genome comparison of each strain showed the presence of a subspecies-specific core genome, representing the genetic differences between these two subspecies. Furthermore, the performed phylogenetic analysis highlights a cluster composed of 18 *B. animalis* subsp. *lactis* isolates that represent potential novel probiotic strains. Interestingly, a large proportion of the subspecies-specific genes of either *B. animalis* subspecies seems to be involved in sugar transport and metabolism. In this context, a larger number of such subspecies-specific transporter and GH activities was found in *B. animalis* subsp. *animalis* genomes. Growth performances on various sugars as their sole carbon source confirmed the ability of *B. animalis* subsp. *animalis* taxon to metabolize a broader set of sugars, e.g., arabinose, galactose, glucose, maltose, melibiose, sucrose, and xylose, whereas *B. animalis* subsp. *lactis* strains seems to be more specialized using a smaller number of sugars, such as lactose, maltose, raffinose, and sucrose. Altogether, these results seem to highlight a better ecological fitness of *B. animalis* subsp. *animalis* taxon compared to *B. animalis* subsp. *lactis* taxon. Moreover, a gene acquisition and loss analysis based on subspecies-specific glycosyl hydrolase genes revealed that *B. animalis* subsp. *animalis* taxon seems to have acquired several GHs through HGT, whereas *B. animalis* subsp. *lactis* species appears to have suffered loss of GH-encoding genes. Thus, these findings confirm the evolutionary differentiation between these two subspecies as highlighted in both phylogenetic and genomic analyses.

## MATERIALS AND METHODS

**Bifidobacterial selection.** In order to explore genome variability of the *B. animalis* species, 15 novel strains were isolated from fecal samples collected from different animals. Samples were composed of 10 g of fresh fecal material, which is a sufficient quantity to represent the overall biodiversity of the fecal microbiota as reported in a previously published study (62). One gram of fecal sample from each collected animal was mixed with 9 ml of phosphate-buffered saline (pH 6.5). Serial dilution and subsequent plating were performed using de Man–Rogosa–Sharpe (MRS) agar, supplemented with 50 $\mu$g/ml mupirocin (Delchimica, Italy) and 0.05% (wt/vol) L-cysteine hydrochloride. Agar plates were incubated for 48 h at 37°C in a chamber (Concept 400; Ruskin) with an anaerobic atmosphere (2.99% $H_2$, 17.01% $CO_2$, and 80% $N_2$). Morphologically different colonies that developed on MRS plates were randomly picked and restreaked in order to isolate purified bacterial strains. All isolates were subjected to DNA isolation and characterized as previously described by Turroni et al. (63). The *B. animalis* strains isolated in this study are listed in Table 1, together with other strains used for *in silico* analyses.

**Bifidobacterial ITS profiling.** Partial ITS sequences were amplified from extracted DNA using the primer pair Probio-bif_Uni/Probio-bif_Rev (64). Resulting reads were analyzed by means of an updated bifidobacterial ITS database encompassing all publicly available bifidobacterial genomes and a custom

bioinformatics script as previously described (64). ITS bifidobacterial profiling of mammalian species and birds were coupled with data of mammalian bifidobacterial communities as previously determined by Milani et al. (35).

**Genome sequencing and assemblies.** DNA extracted from bifidobacterial isolates was subjected to whole-genome sequencing using MiSeq (Illumina, UK) at GenProbio srl (Parma, Italy) according to the supplier's protocol (Illumina, UK). Fastq files of the paired-end reads obtained from targeted genome sequencing of isolated strains were utilized as input for genome assemblies through the MEGAnnotator pipeline (65). SPAdes software was used for *de novo* assembly of each bifidobacterial genome sequence (66, 67), while protein-encoding ORFs were predicted using Prodigal (68). The coverage depth of these newly isolated 15 *B. animalis* chromosomes ranged from 85- to 278-fold, which upon assembly generated 47 to 12 contigs (Table 1). The number of predicted ORFs ranged from 1,556 of *B. animalis* subsp. *lactis* 1808B to 1,935 of *B. animalis* subsp. *animalis* 2022B (Table 1). In order to ensure data consistency, *B. animalis* chromosomes retrieved from public databases were reannotated using the same bioinformatics pipeline applied for the 15 *B. animalis* strains isolated in the present study.

**Comparative genomics.** A pangenome calculation was performed using the pan-genome analysis pipeline PGAP (69), including each *B. animalis* genome collected from this study (Table 1). Each predicted proteome of a given *B. animalis* strain was screened for orthologues against the proteome of every collected *B. animalis* strain by means of BLAST analysis (70) (cutoff, E value of $<1 \times 10^{-4}$ and 50% identity over at least 80% of both protein sequences). The resulting output was then clustered into protein families by means of MCL (graph theory-based Markov clustering algorithm) (71), using the gene family method. A pangenome profile was built using all possible BLAST combinations for each genome being sequentially added. Using this approach, unique protein families encoded by the analyzed *B. animalis* genomes were also identified. Protein families shared between analyzed genomes allowed us to identify the core genome of the *B. animalis* species. Each set of orthologous proteins, belonging to the core genome, was aligned using Mafft software (72), and phylogenetic trees were constructed using ClustalW (73). Based on these comparative analyses, a *B. animalis* supertree was constructed and visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

**Carbohydrate growth assays.** Bifidobacterial strains were cultivated on semisynthetic MRS medium supplemented with a 1% (wt/vol) concentration of a particular sugar, and the optical densities (measured at a wavelength of 600 nm) were recorded using a plate reader (BioTek, Winooski, VT). The plate reader was read in intermittent mode, with absorbance readings performed at 3-min intervals for three times after 48 h of growth, where each reading was ahead of 30 s of shaking at medium speed. Cultures were grown in biologically independent triplicates, and the resulting growth data were expressed as the means of these replicates. Carbohydrates were purchased from Sigma and Carbosynth (Berkshire, UK). Carbohydrate-active enzymes were identified based on similarity to the Carbohydrate-Active enZYmes (CAZy) database entries.

**SNP identification.** Multiple alignment of conserved genomic sequence with rearrangements (Mauve) software (74) was employed to perform whole-genome sequence alignments between bifidobacterial genome sequences. SNPs reported by Mauve were manually evaluated to identify polymorphisms between subspecies.

**Gene gain or loss through evolutionary reconstruction.** Identification of genes that are predicted to be acquired by an HGT event was performed using COLOMBO v4.0 (60). Evolution-driven acquisition and loss of GH-encoding genes among members of the *B. pseudolongum* phylogenetic group was performed with Count (61) software using Wagner's parsimony.

**Statistical analyses.** SPSS software (IBM, Italy) was used to perform statistical analysis between *B. animalis* subsp. *animalis* strains group and *B. animalis* subsp. *lactis* group by Student *t* test. Furthermore, *t* test assumption was verified using the unequal variances Welch *t* test analysis to validate samples that exhibit unequal variance in the sample size (Table S1).

**Data availability.** Newly isolated *B. animalis* genomes were sequenced and deposited at DDBJ/ENA/GenBank under the accession numbers reported in Table 1 (BioProject PRJNA506409).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02806-18.

**SUPPLEMENTAL FILE 1**, XLSX file, 0.02 MB.

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