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RESEARCH ARTICLE

GH136-encoding gene (*per*B) is involved in gut colonization and persistence by *Bifidobacterium bifidum* PRL2010

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Abstract

Bifidobacteria are commensal microorganisms that typically inhabit the mammalian gut, including that of humans. As they may be vertically transmitted, they commonly colonize the human intestine from the very first day following birth and may persist until adulthood and old age, although generally at a reduced relative abundance and prevalence compared to infancy. The ability of bifidobacteria to persist in the human intestinal environment has been attributed to genes involved in adhesion to epithelial cells and the encoding of complex carbohydrate-degrading enzymes. Recently, a putative mucin-degrading glycosyl hydrolase belonging to the GH136 family and encoded by the *per*B gene has been implicated in gut persistence of certain bifidobacterial strains. In the current study, to better characterize the function of this gene, a comparative genomic analysis was performed, revealing the presence of *per*B homologues in just eight bifidobacterial species known to colonize the human gut, including *Bifidobacterium bifidum* and *Bifidobacterium longum* subsp. *longum* strains, or in non-human primates. Mucin-mediated growth and adhesion to human intestinal cells, in addition to a rodent model colonization assay, were performed using *B. bifidum* PRL2010 as a *per*B prototype and its isogenic *per*B-insertion mutant. These results demonstrate that *per*B inactivation reduces the ability of *B. bifidum* PRL2010 to grow on and adhere to mucin, as well as to persist in the rodent gut niche. These results corroborate the notion that the *per*B gene is one of the genetic determinants involved in the persistence of *B. bifidum* PRL2010 in the human gut.

Sonia Mirjam Rizzo and Laura Maria Vergna contributed equally to this work.

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INTRODUCTION

In recent decades, scientific interest has been focused on the characterization of members of the genus *Bifidobacterium* since they are not only recognized as dominant and symbiotic inhabitants of the human gut, particularly in the early stages of life, but also as microorganisms purported to exert various health-promoting effects upon their host (Alessandri et al., 2021; Bottacini et al., 2017; Hidalgo-Cantabrana et al., 2017). In this context, convincing scientific evidence has accumulated, highlighting that the presence of bifidobacteria in the human intestine supports host immune system development, promotes intestinal barrier integrity, and contributes to maintain intestinal eubiosis, limiting pathogen proliferation, and preventing the onset of inflammatory bowel disease, ulcerative colitis, and celiac disease (Duranti et al., 2016; Fujimura et al., 2016; Longhi et al., 2020; Luck et al., 2020; Milani, Duranti, et al., 2017; Milani, Mangifesta, et al., 2017; Shang et al., 2022). At the same time, bifidobacteria can produce bioactive metabolites, including vitamins, polyphenols, and short-chain fatty acids, which may elicit beneficial effects on both intestinal epithelial host cells and may also affect growth and abundance of other gut commensals (Bottacini et al., 2014; Bunesova et al., 2018; Khromova et al., 2022).

Being vertically transmitted and, therefore, among the first colonizers of the human gut, bifidobacteria are believed to elicit their health-promoting effects soon after birth (Duranti et al., 2017; Henrick et al., 2021; Kumar et al., 2020; Milani, Duranti, et al., 2017; Milani, Mangifesta, et al., 2017). Indeed, during the first months of life, the highest relative abundance and prevalence of bifidobacteria have been recorded in the human intestinal tract (Milani, Duranti, et al., 2017; Milani, Mangifesta, et al., 2017). However, despite a decline in their levels when the gut microbiota evolves from an “infant-” to an “adult-like” gut microbial ecosystem, bifidobacteria remain relatively stable and persist over time until old age (Alessandri et al., 2019; Arboleya et al., 2016; Milani, Duranti, et al., 2017; Milani, Mangifesta, et al., 2017).

The ability of members of the genus *Bifidobacterium* to persist in the host throughout life may be attributed to specific genomic traits. Indeed, bifidobacterial genomes contain genes involved in the production of certain extracellular structures such as exopolysaccharides, teichoic acids, and pili, which contribute to bifidobacterial persistence in the human gut by promoting their interaction with the host and other commensal microorganisms (Alessandri et al., 2021; Fanning et al., 2012; Milani, Duranti, et al., 2017; Milani, Mangifesta, et al., 2017; Turroni et al., 2013). In parallel, bifidobacteria possess a set of genes, which encodes a large enzyme arsenal required for carbohydrate metabolism, providing bifidobacteria with a selective advantage to colonize and persist in the competitive human intestinal environment, possibly throughout host life (Arzamasov & Osterman, 2022; Turroni et al., 2018). In this context, bifidobacteria are not only able to degrade complex, diet-derived carbohydrates but may also be able to access host-associated complex glycans, including Human Milk Oligosaccharides (HMOs) and mucin (Arzamasov & Osterman, 2022; Egan et al., 2014; Nishiyama et al., 2020; Sakanaka et al., 2019). In particular, since the ability to degrade HMOs and mucin is restricted to a limited number of intestinal microbes, this feature has been proposed to represent a crucial advantage in ensuring colonization and persistence of bifidobacteria in the human intestine (Alessandri et al., 2021; Katoh et al., 2017; Turroni et al., 2010, 2011). Indeed, it has been demonstrated that bifidobacterial strains possessing a genetic repertoire for mucin metabolism, including *Bifidobacterium bifidum* and *Bifidobacterium longum* subsp. *longum*, show higher resilience and long-term colonization in the human gut than strains lacking such genetically predicted ability (Katoh et al., 2023; Tarracchini et al., 2023). Specifically, the more efficient persistence of strains of *B. bifidum* and *B. longum* subsp. *longum* was attributed to *per*B, a gene encoding an enzyme belonging to the glycosyl hydrolase family 136 (GH136), which is proposed to facilitate mucin degradation (Tarracchini et al., 2023).

Based on these observations, the aim of this study was to investigate and validate the involvement of PerB in mucin breakdown and enhancement of gut persistence. A comparative genomic survey was performed, revealing that the presence of a *per*B homologue is a specific genetic trait harboured by just eight bifidobacterial species, all originating from the intestinal tract of primates. Furthermore, in vitro and in vivo experiments confirmed the involvement of *per*B in mucin degradation and enhancement of bifidobacterial cell persistence in the gut environment.

RESULTS AND DISCUSSION

Assessing the prevalence of *per*B homologues among members of the genus *Bifidobacterium*

A previous study had identified *per*B (for Persistence of Bifidobacteria), predicted to encode a glycosyl hydrolase (GH), i.e., GH136 (Yamada et al., 2017), as a key genetic determinant involved in bifidobacterial gut persistence across human life (Tarracchini et al., 2023). A very similar enzyme, designated LnbX, originally identified in a *B. longum* subsp. *longum* strain had been shown to act as an extracellular lacto-N-biosidase

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(Gotoh et al., 2015; Sakurama et al., 2013; Yamada et al., 2022). Transcription of *lnbX* is co-regulated by the global regulator NagR, which controls transcription of all genes involved in the utilization of *N*-acetylglucosamine-containing host glycans (Arzamasov et al., 2022). However, despite the role of *per*B/*lnb*X in host-derived glycan utilization, an analysis of all complete and well-annotated publicly available genome sequences of bifidobacterial species commonly found in the infant gut microbiota, that is, *B. bifidum*, *Bifidobacterium breve*, *B. longum* subsp. *longum*, and *Bifidobacterium pseudocatenulatum*, highlighted the non-ubiquitous nature of *per*B/*lnb*X in bifidobacterial genomes (Tarracchini et al., 2023). While all examined *B. bifidum* and certain *B. longum* subsp. *longum* strains were shown to possess a *per*B homologue, none of the *B. breve* and *B. pseudocatenulatum* genomes appeared to contain a homologue of this sequence (Tarracchini et al., 2023). Based on these results, to further investigate the presence of *per*B among all currently recognized species of the genus *Bifidobacterium*, a BlastP analysis was performed between a custom database containing the amino acid sequences deduced from *per*B homologues previously identified in *B. longum* subsp. *longum* and *B. bifidum* (Tarracchini et al., 2023) and the derived amino acid sequences from genes identified in publicly available bifidobacterial genomes. Genomes exhibiting an average nucleotide identity (ANI) of ≥99% were excluded from the BlastP analysis to reduce genetic redundancy, generating a custom-database containing a total of 877 non-redundant bifidobacterial genomes (Table S1). Interestingly, only 201 of these 877 genomes, covering 27 different bifidobacterial (sub)species, harboured a *per*B homologue (Figure 1 and Table S2). However, in-depth insights into the amino acid length match coupled with a protein domain check for all genes with a sequence homology with *per*B revealed that the genomes of only eight bifidobacterial species, i.e., *Bifidobacterium aerophilum*, *B. bifidum*, *Bifidobacterium colobi*, *Bifidobacterium imperatoris*, *Bifidobacterium leontopitheci*, *Bifidobacterium saguini*, and *Bifidobacterium samirii*, together with a portion (111 out of the 312 tested genomes) of the *B. longum* subsp. *longum* strains (Tables S1 and S2) encompassed the GH136 catalytic region corresponding to the FIVAR domain (Tarracchini et al., 2023) and a full-length *per*B homologue in their genomes. Conversely, all other genomes with a *per*B homologue did not encode the FIVAR domain or the other domain identified in the *per*B gene, i.e., a secretion signal peptide, and various beta-helix and Rib domains (Tarracchini et al., 2023), except for the perB homologue of *Bifidobacterium jacchi* which encodes multiple Rib domains (Figure 2 and Figure S1). The *per*B gene is predicted to be involved in mucin degradation, a metabolic feature exclusive to the species *B. bifidum* of the genus *Bifidobacterium* with certain *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, and *B. breve* strains that, despite lacking the complete genetic arsenal dedicated to mucin breakdown, can take advantage of the extracellular release of mucin components through cross-feeding events (Alessandri et al., 2021; Egan et al., 2016; Katoh et al., 2017, 2020). Furthermore, a small number of other intestinal bacterial members are also capable of mucin degradation, i.e., *Akkermansia muciniphila*, *Ruminococcus gnavus*, *Ruminococcus torques*, *Phocaeicola vulgatus*, and certain members of the genus *Bacteroides*, including *Bacteroides caccae*, *Bacteroides fragilis*, and *Bacteroides thetaiotaomicron*. Such bacteria require various genes encoding for the degradation of mucin-associated glycans, such as neuraminidases/sialidases (GH33), fucosidases (GH29 and GH95), exo- and endo-β-*N*-acetylglucosaminidases (GH84 and GH85), β-galactosidases (GH2, GH20, and GH42), α-*N*-acetylglucosaminidases (GH89), and α-*N*-acetylgalactosaminidases (GH101 and GH129), sulfatase, and GlcNAc phosphate-deacetylase (Hayase et al., 2022; Kim et al., 2021, 2023; Tailford et al., 2015). Interestingly, the abovementioned species corresponding to bifidobacterial taxa were exclusively isolated from the faeces of either humans or non-human primates (Bottacini et al., 2017; Duranti et al., 2019, 2020; Endo et al., 2012; Lugli et al., 2018, 2021; Michelini et al., 2016; Modesto et al., 2019). These results suggest that species of bifidobacteria adapted to colonize the intestinal niche of humans or non-human primates have specifically acquired or evolved *per*B to support their colonization and persistence in the associated competitive intestinal environment.

In addition, since it has been demonstrated that the expression of the *lnb*X gene in *B. longum* subsp. *longum* requires the chaperone molecule LnbY (Sakurama et al., 2013), the presence of conserved *lnb*Y homologues in the other bifidobacterial genomes with a *per*B gene was investigated through a BlastP analysis. Interestingly, only *B. aerophilum*, *B. imperatoris*, and *B. saguini* showed an *lnb*Y homologue in their genomes (Table S3). This suggests that this chaperone-encoding gene is not conserved in all *per*B-containing bifidobacterial genomes, leading to suggest that *per*B expression and activation may be subject to a different control.

Furthermore, to evaluate whether *per*B is an exclusive genetic feature of some members of the genus *Bifidobacterium*, BlastP analysis was performed against the deduced amino acid sequences corresponding to all bacterial genomes contained in the National Center for Biotechnology Information, NCBI by excluding bifidobacteria. Interestingly, this analysis highlighted the presence of *per*B homologues in bacterial species other than bifidobacteria (Table S4). However, evaluation of the protein domains present in the deduced protein sequences of such *per*B homologues indicated that only a small proportion of the *Bifidobacteriaceae* family,

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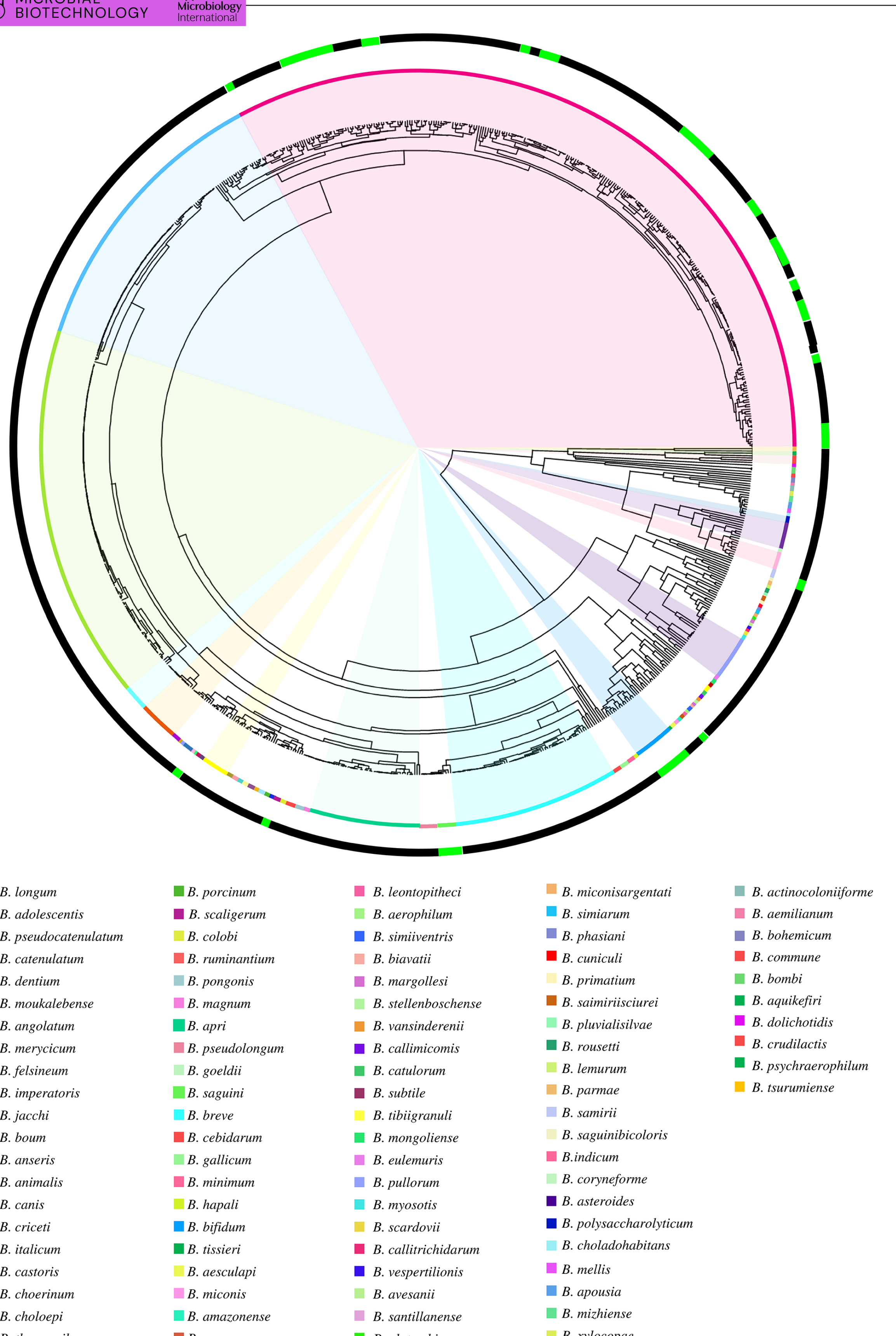

FIGURE 1 Prevalence of *per*B among the genus *Bifidobacterium*. The panel reports a circular cladogram generated through a comparative genomic analysis based on the average nucleotide identity (ANI) of all publicly available genome sequences of the genus *Bifidobacterium*. The inner circle provides a division of bifidobacteria into species. The outer circle around the cladogram depicts the presence, indicated in green, and the absence marked in black, of *per*B among bifidobacterial strains.

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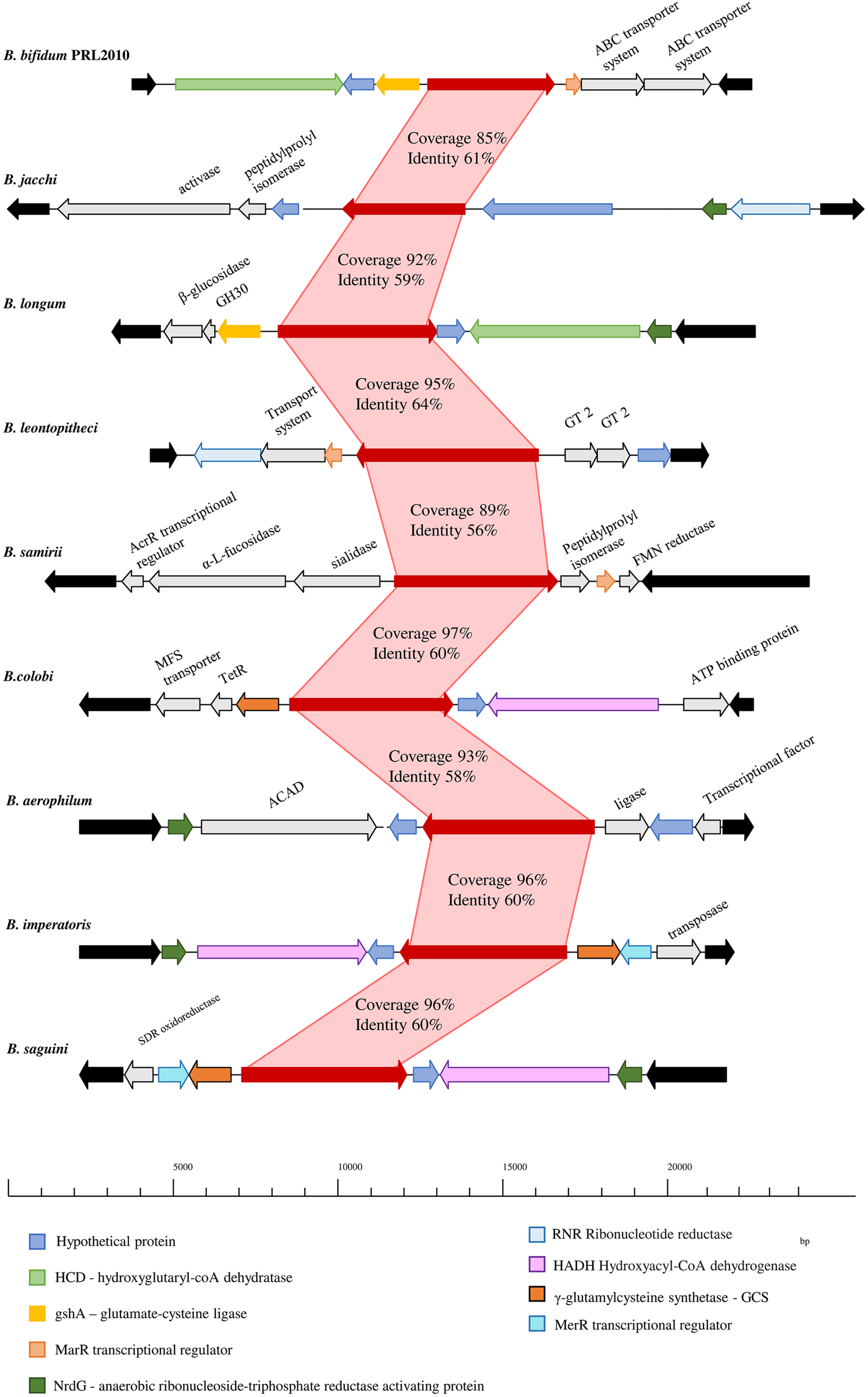

FIGURE 2 Locus map of the nine species of *Bifidobacterium* containing a full-length *per*B gene. The image shows the genomic region belonging to *Bifidobacterium aerophilum*, *Bifidobacterium bifidum*, *Bifidobacterium colobi*, *Bifidobacterium imperatoris*, *Bifidobacterium leontopitheci*, *Bifidobacterium saguini*, *Bifidobacterium samirii*, *Bifidobacterium longum* subsp. *longum*, and *Bifidobacterium jacchi*, in which a *per*B homologue has been identified. The amino acid sequence coverage and identity percentage between *per*B of *B. bifidum* PRL2010 and the other strains are reported.

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excluding *Gardnerella vaginalis*, shared all identified PerB domains, while putative PerB homologues found in other species lack the functional domain or possess a fibronectin type III domain (Table S4). Assuming that the fibronectin type III domain is involved in adhesion to and interaction with human intestinal epithelial cells (Alessandri et al., 2023), these results suggest that only certain members of the *Bifidobacteriaceae* family, represented by particular bifidobacterial species/strains, possess the *per*B gene and that *per*B provides them with a selective and competitive advantage to colonize and persist in the human gut environment.

Impact of *perB* inactivation on *B. bifidum* PRL2010 ability to grow on and adhere to mucin

Based on the transcriptome results of a recent study in which *B. longum* PRL2022 exposed to human intestinal cells revealed an up-regulation of the *per*B gene compared to the control (Tarracchini et al., 2023), other mucin-related experiments were carried out. To validate the involvement of *per*B in enhancing the gut persistence of bifidobacterial strains, the ability of a *B. bifidum* prototype, that is, *B. bifidum* PRL2010, to grow on and adhere to mucin and to persist in a rodent-model gut environment was compared to that of the isogenic *B. bifidum* PRL2010 *per*B mutant.

Since bifidobacteria, especially *B. bifidum*, have been reported to be recalcitrant to transformation due to the cell wall composition and thickness and the action of restriction-modification (R/M) systems (Sakaguchi, et al., 2012, O'Connell Motherway et al., 2009), an optimized protocol for high-efficiency transformation was developed. First, the R/M recognition sites of *B. bifidum* PRL2010 were identified based on methylome sequencing (Pacbio) and methylated site prediction from REBASE. The methylome analysis was performed to identify sequence motifs recognized by the strain endonucleases which cut any exogenous DNA that contains such motifs if they are non-methylated, thereby decreasing the efficiency of transformation. Analysis of PacBio sequencing data allowed identification of two Type I (CGAYNNNNNGGT, CAAYNNNNNCTC) and one Type II (CTGCAG) R/M recognition motifs, while the bioinformatics prediction based on REBASE highlighted the presence of different Type II sites (GTCGAC, GGCGCC, GCSGC, and GAATTC), allowing the delineation of the *B. bifidum* PRL2010 methylome. Subsequently, these recognition motifs, when present, were removed from the plasmids of interest, pNZ003 and pFREM30, to increase the transformation efficiency of the strain (O'Callaghan et al., 2015). The first plasmid, which is a derivative of pNZ44 (McGrath et al., 2001), was used to optimize the transformation protocol until an efficiency of 10E+06 CFU (colony-forming unit)/μg of pNZ003 DNA was reached. Optimization was achieved by assessing various critical transformation parameters, such as electroporation voltage and growth/washing/recovery media to be used for growth, transformation, and plating of the strain, as based on a previous study (Serafini, et al., 2012). Following optimization, the integration vector pFREM30, which is a derivative of plasmid pFREM28 (Hoedt et al., 2021), was employed to target and successfully disrupt *per*B in *B. bifidum* PRL2010 to generate *B. bifidum per*B::pFREM30.

*B. bifidum per*B::pFREM30 was then tested for its ability to use mucin. For this purpose, two in vitro growth assays were performed using mucin as the sole carbon source. Firstly, *B. bifidum* PRL2010 wild-type (wt) and *B. bifidum per*B::pFREM30 were grown in mMRS without lactose and supplemented with 0.5% mucin to evaluate possible differences in growth performance between the mutant and wt strains (using mMRS plus lactose as control). Additionally, to assess if the presence of chloramphenicol influences the ability of *per*B::pFREM30 to grow on mucin, the mutant strain was cultivated both in the presence and absence of this antibiotic. Subsequently, growth of each strain was monitored over time (0, 5, 10, 24, 36 and 48 h) by plating on mMRS agar and in parallel by a viability assay and total bacterial cell count through flow cytometry (Figure 3A,B and Figure S2A).

Interestingly, no statistically significant difference was observed of the mutant when grown in the presence or absence of the antibiotic neither in the medium supplemented with mucin nor in mMRS (Bonferroni Post Hoc test p-value >0.05), except for the 24-h time point when grown in mMRS where the mutant strain in chloramphenicol showed a significantly higher growth performance compared to the condition without the antibiotic (Figure 3A,B). Therefore, since the presence of chloramphenicol did not appear to affect mutant growth

FIGURE 3 Evaluation of *Bifidobacterium bifidum* PRL2010 and *B. bifidum per*B::pFREM30 ability to grow on mucin. Panels (A) and (B) depict growth performances of *B. bifidum* PRL2010 and *B. bifidum per*B::pFREM30 (grown with or without chloramphenicol) on mucin as the sole carbon source at different time points (5, 10, 24, 36 and 48 h) and on mMRS, respectively. The *x*-axis reports the examined time points, while the *y*-axis displays the logarithmic total bacterial count obtained by plating an aliquot for each time point and expressed as CFU/mL. The vertical bars indicate standard deviations. The experiments were carried out in triplicates, and the Bonferroni post-hoc test was used to statistically analysed data. Panel (C) shows light microscope images of HT29-MTX cells with *B. bifidum* PRL2010 and *B. bifidum per*B::pFREM30 and coloured with the Giemsa staining. Bar, 10 μm. Panel (D) displays the quantification of the ability of *B. bifidum per*B::pFREM30 and *B. bifidum* PRL2010 to adhere to HT29-MTX secreting mucin cells. The horizontal bars indicate standard deviations; the three asterisks indicate a *t*-test p-value <0.001.

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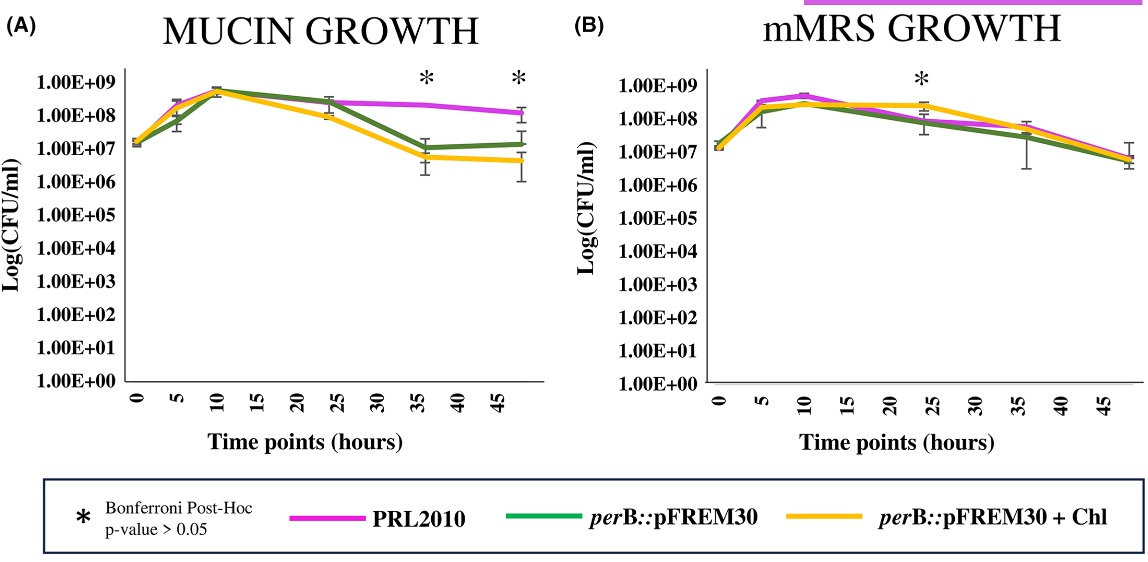
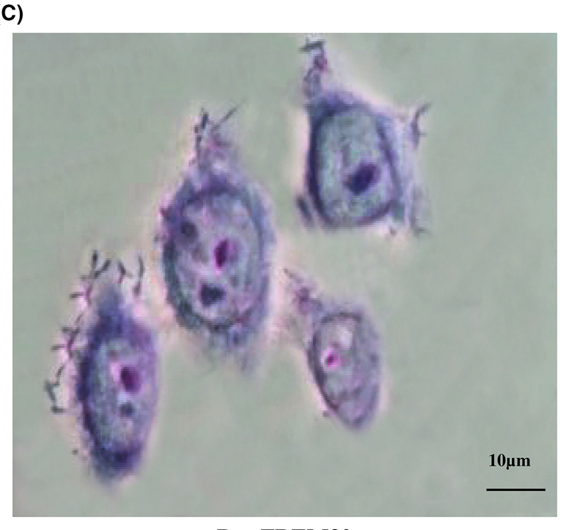
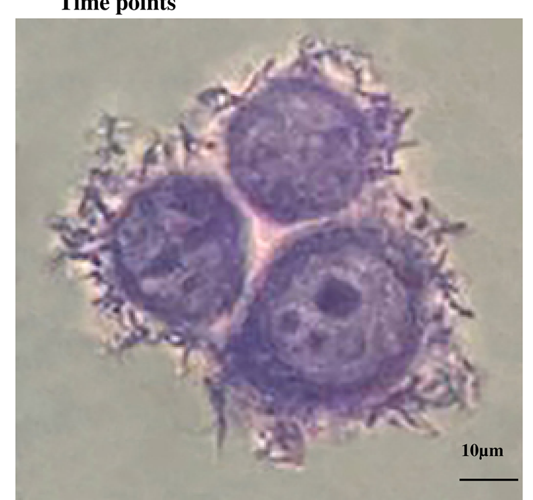


(C)

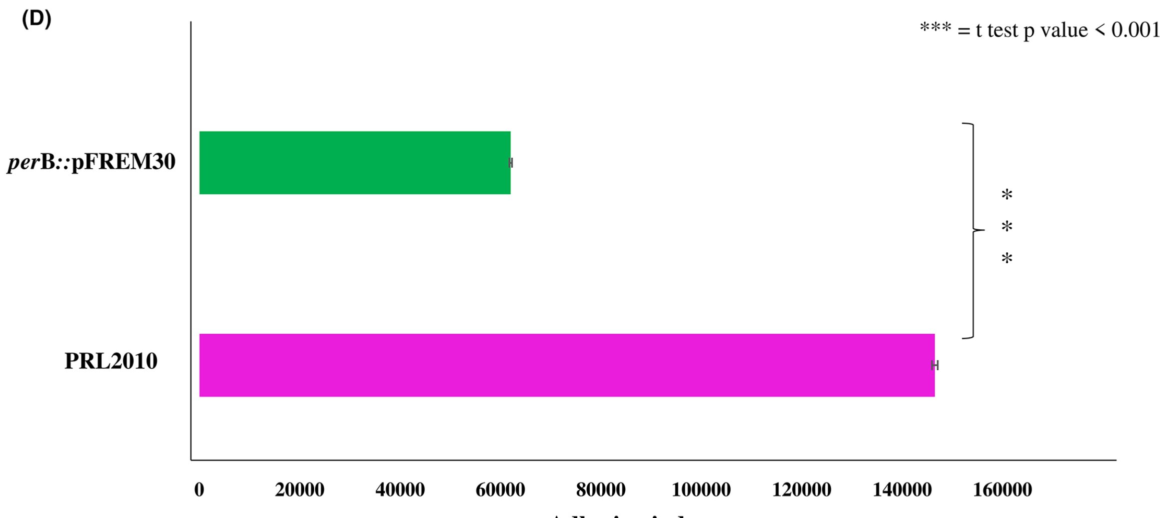
Time points



perB::pFREM30



PRL2010



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performance, to maintain the selective pressure of the integrated plasmid, all subsequent experiments involving growth and adhesion to mucin were performed by growing the mutant in the presence of the antibiotic.

Furthermore, in-depth insight into the mucin growth assay data obtained through the plating method revealed no significant differences between *B. bifidum* PRL2010 wt and perB::pFREM30 (Bonferroni Post Hoc test *p*-value >0.05) for the 5, 10 and 24 h timepoints (Figure 3A). Conversely, at 36 and 48 h, a significantly higher CFU value was observed for *B. bifidum* PRL2010 wt growing on mucin as the sole carbon source (2.08E+08 CFU/mL ± 1.83E+07 and 1.19E+08 ± 5.74E+07, respectively) when compared to the mutant (5.69E+06 CFU/mL ± 1.79E+06 and 4.42E+06 ± 3.39E+06, respectively) (Bonferroni Post Hoc *p*-value <0.001 and *p*-value = 0.008 for the 36 and 48 h, respectively). Therefore, despite an initial equal ability of the mutant to grow when compared to the wt strain, probably due to other genetic determinants involved in mucin degradation that are present in both strains (Egan et al., 2014; Turroni et al., 2010, 2011) and/or to the presence of contaminating carbohydrates in the partially purified mucin; these data suggest that the presence of the *per*B gene enhances and supports growth/persistence of *B. bifidum* PRL2010 wt on mucin at 36 and 48 h.

In addition, the data obtained by plating *B. bifidum* PRL2010 wt and perB::pFREM30 strains grown on mucin as the unique carbon source were further confirmed by analysing the normalized number of viable cells obtained by combining the viability assay and total bacterial cell count via flow cytometry. Indeed, although the mutant displayed a slight yet significantly higher growth performance at 5 h when compared to the wt strain with a total number of viable cells of 1.71E+07 and 1.13E+07, respectively, for all other time points except for 10 h, a significant higher number of viable cells was recorded for the *B. bifidum* PRL2010 wt strain when compared to the mutant (Figure S2). These observations support the notion that *per*B plays a role in improving the growth performance of *B. bifidum* PRL2010 in the presence of mucin.

Therefore, since the ability to access complex carbohydrates, such as mucin, is limited to a small number of intestinal microbial players, especially bifidobacterial species, and is considered to be a crucial feature for the survival and successful colonization of the intestinal environment, where high levels of competition for nutrients occur (Alessandri et al., 2021; Paone and Cani, 2020), these results support the hypothesis that *per*B plays a role in *B. bifidum* PRL2010 colonization in the human gut.

Furthermore, to corroborate the impact of the *per*B gene in promoting *B. bifidum* PRL2010 persistence in the human intestine via mucin anchoring, the adhesion abilities of *B. bifidum* PRL2010 wt and perB::pFREM30 to human mucin-secreting HT29-MTX cells were assessed. Interestingly, a statistically significant (Student's *t*-test *p*-value of <0.001) reduction in the adhesion index, calculated as the average number of bacterial cells/100*HT29-MTX cells, to HT29-MTX was observed for *B. bifidum* perB::pFREM30 (adhesion index 62,001 ± 260) when compared to *B. bifidum* PRL2010 wt (adhesion index 146,499 ± 577) (Figure 3C,D). These results demonstrate that *per*B gene inactivation not only appears to affect the ability of *per*B::pFREM30 to utilize mucin, but that it also diminishes its ability to adhere to human intestinal cells.

A rodent model-based assessment of *per*B involvement in intestinal colonization

To assess whether a functional *per*B gene contributes to a successful gut colonization and persistence in a competitive environment, the ability of *B. bifidum* PRL2010 wt and *B. bifidum per*B::pFREM30 to colonize the intestinal tract was tested in a rodent model. We first assessed the stability of the integrated pFREM30 plasmid used to create *B. bifidum* PRL2010 *per*B::pFREM30, as it is not possible to select for chloramphenicol resistance in the context of a murine model. For this purpose, *B. bifidum* PRL2010 *per*B::pFREM30 was grown in the presence or absence of 5 μg/mL chloramphenicol and sub-cultivated daily for a total of 2 weeks. Furthermore, every 2 days of sub-culturing, cells were spotted on mMRS plates with or without antibiotics, and after 48 h of incubation, the presence of the integrated plasmid was confirmed by colony PCR. This analysis not only confirmed the stability of the integrated plasmid over time in the absence of antibiotic selection but also that the number of colony-forming units (CFUs) in both tested conditions remained stable (10E+08 CFU/mL) for the full duration of assessment. After plasmid stability confirmation, a murine model-based in vivo trial was performed. The latter consisted of two groups of animals: one receiving a 1 mL daily inoculum of approximately 10E+09 cells/ml of *B. bifidum* PRL2010 *per*B::pFREM30 (Group 1), while the second group received the same amount of *B. bifidum* PRL2010 wt (Group 2) (Figure 4A). The abundance of *B. bifidum* PRL2010 in the faecal samples of the animals enroled in this study was evaluated using qPCR (Figure 4B). Interestingly, analysis of data collected from qPCR revealed no significant differences in the abundance of *B. bifidum* PRL2010 between the two groups after the first week of strain administration (T1) (Mann–Whitney test *p*-value = 0.694), even if a slight increment in the genome copy number (GCN) of *B. bifidum per*B::pFREM30 was observed when compared to the wild-type. In contrast, a statistically significant increase in the GCN (Mann–Whitney test *p*-value < 0.01) of *B. bifidum* PRL2010 *wt* (7.23E+04 GCN/g ± 1.52E+05) was observed after

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FIGURE 4 Timeline and experimental design of the in vivo study. Panel (A) shows the timeline of the experimental procedures in rats. Panel (B) reports qPCR data associated with the load of *Bifidobacterium bifidum per*B::pFREM30 and *B. bifidum* PRL2010 in the faecal samples of rats at T1, T2, and T3. The *x*-axis represents the different time points for each group, while the *y*-axis indicates the genome copy numbers of *B. bifidum* PRL2010 per gram of faeces. The boxes represent the 25th and 75th percentiles, and the whiskers represent the standard deviations. The lines in the boxes represent the medians, while the squares represent the average. The rhombi indicate outliers. * = Mann–Whitney test *p*-value <0.05.

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2 weeks of strain administration (T2) compared to *B. bifidum per*B::pFREM30 (7.11E+03 GCN/g ± 8.34E+03) (Figure 4B). These data suggest that, despite the initial similar ability of the two strains to colonize the intestinal environment, over a prolonged intervention period, the wild-type strain showed a more efficient gut colonization and a higher persistence performance when compared to *B. bifidum per*B::pFREM30, with a 10-fold higher average load in the rodent faecal samples. This finding was further confirmed by the observation that after the washout period (T3), the wild-type strain showed a significantly higher abundance (Mann–Whitney test *p*-value < 0.05) than *B. bifidum per*B::pFREM30 (7.56E+03 ± 5.31E+03 GCN/g and 4.41E+03 ± 3.01E+03 GCN/g for wt and *per*B::pFREM30 strains, respectively) (Figure 4B). In addition, we previously demonstrated that *perB* is involved in favouring bifidobacterial persistence in the human intestinal tract throughout life, yet also that this gene appears to contribute to a more efficient colonization of bifidobacteria in females compared to males (Tarracchini et al., 2023). We therefore also assessed possible differences in the ability of *B. bifidum* PRL2010 wt and *per*B::pFREM30 strains to colonize the intestine of female and male rats. No statistically significant differences were observed in *B. bifidum* GCN between female and male rats for either of the strains at T1 and T2 (Bonferroni post hoc *p*-value >0.05) (Figure S2), even if a slight increase of the average *B. bifidum* GCN/g was observed at T2 for the female group administered with *B. bifidum* PRL2010 wt (average GCN/g of 5.98E+04) (Figure S2), thus suggesting that, after 2 weeks of treatment, *per*B presence may play a role in favouring *B. bifidum* PRL2010 colonization performance in female hosts as previously observed in the human host (Tarracchini et al., 2023). The limit of detection is 95% (based on the Ct of the NTC). In contrast, at T3, a significantly higher average of *B. bifidum* GCN/g was recorded for the faecal samples of male rats when compared to the female counterpart for both the wt and *per*B::pFREM30 strains (Bonferroni post-hoc *p*-value >0.05) (Figure S2). These seemingly conflicting results should be contextualized knowing that there are differences in intestinal mucous composition between humans and rodents (Hugenholtz & de Vos, 2018; Robinson et al., 2019) and that, therefore, PerB is specialized to mucin present in human females as opposed to that of rodents.

Overall, these results suggest that *per*B support *B. bifidum* PRL2010 host colonization, allowing enhanced persistence of the strain in the intestinal environment.

CONCLUSIONS

To investigate the contribution of the previously identified mucin-degrading glycosyl hydrolase PerB to bifidobacterial gut persistence, we first investigated the distribution of *per*B homologues in all currently recognized bifidobacterial species, demonstrating that only eight bifidobacterial taxa possessed in their genomes a full-length *per*B homologue, which encodes the FIVAR domain, corresponding to the GH136 catalytic domain. These eight species correspond to bifidobacterial taxa that typically colonize the human or non-human primate intestinal environment, suggesting that only these bifidobacterial species have specifically acquired or evolved *per*B to support their colonization and persistence in the associated competitive intestinal environment.

This notion was confirmed by in vitro experiments with human mucin-producing cells, involving a strain with a *per*B gene in its genome, that is, *B. bifidum* PRL2010, and its isogenic *per*B negative variant, emphasizing the role of this gene in enhancing growth on and adherence to mucin. In addition, our in vivo rodent experiment highlighted how *per*B inactivation results in a reduced ability of *B. bifidum* PRL2010 cells to persist in the gut environment with a consequent decrease in its colonization capacity when compared to the wild-type strain. Our results, therefore, suggest that, beyond previously characterized bifidobacterial genetic determinants involved in bifidobacterial colonization of and persistence in the human gut environment, also *per*B is implicated in these specific functions (Alessandri et al., 2019, 2021; Turroni et al., 2022). Nonetheless, biochemical assays and mechanistic studies involving PerB are required to better characterize and confirm the involvement of this enzyme in mucin degradation as well as its multifaceted nature since this enzyme not only seems to degrade mucin but also HMOs, as previously reported (Sakurama et al., 2013; Tarracchini et al., 2023). A limitation of the present study is that a murine model was used to evaluate *per*B involvement in *B. bifidum* PRL2010 gut colonization, although this gene is exclusively present in bifidobacterial species that typically colonize the human or non-human primate gut. However, the murine model has been and continues to be the gold standard model organism for human-related studies to avoid in vivo trials that do not allow the administration of in vitro-obtained mutants. In addition, the application of the protocols used in this study on other *B. bifidum* strains and on the other bifidobacterial species that possess the *per*B gene would be necessary to confirm the role of the GH136 across bifidobacteria.

EXPERIMENTAL PROCEDURES

Bifidobacterial genome sequences

Publicly available genomes (complete and draft genome sequences) encompassing all currently characterized bifidobacterial species were retrieved in

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July 2023 from the NCBI public database. Duplicate bacterial genomes (ANI value >99%) were removed to avoid genetic redundancy, resulting in a final dataset consisting of 813 bifidobacterial genome sequences.

Presence of *per*B homologues in bifidobacterial species

The deduced proteome of each publicly available bifidobacterial genome was screened for the presence of *per*B homologues, based on sequence similarity to a custom reference database. The latter was obtained by considering all PerB protein sequences previously identified in *B. bifidum* and *B. longum* subsp. *longum* strains. The latter comprised four non-redundant protein sequences (sequence similarity <90%) of the *per*B gene, previously identified in *B. bifidum* and *B. longum* subsp. *longum* strains (Tarracchini et al., 2023). Subsequently, all redundant sequences (sequence similarity >90%) were removed, resulting in a custom database of four (three from *B. bifidum* strains and one from a *B. longum* subsp. *longum* strain) non-redundant *per*B protein sequences, covering the genetic variability of this gene in these species. BlastP analysis was performed employing DIAMOND software (Buchfink et al., 2015). In addition, Pfam v34.0 (https://pfam.xfam.org/), InterPro 86.0, (https://www.ebi.ac.uk/interpro/), and HMMER (http://hmmer.org/) were employed to identify protein domains. The deduced proteome of each publicly available bifidobacterial genome was also screened for the presence of *lnb*X and *lnb*Y homologues.

Bacterial strains, plasmids, and cultivation conditions

Plasmid pNZ003, which is a derivative of the pNZ44 plasmid (McGrath et al., 2001), was used as a positive control, whereas plasmid pFREM30 is a derivative of pFREM28 (Hoedt et al., 2021), a suicide vector used to target *per*B for mutagenesis by gene disruption. Construction of these two plasmids and their derivatives are described below. *Escherichia coli* EC101 (Law et al., 1995) was used as a host strain for the propagation of the aforementioned plasmids and was cultivated at 37°C in LB medium (Luria Bertani, Scharlab, Spain) supplemented with chloramphenicol at a final concentration of 25 μg/mL. *B. bifidum* PRL2010 was cultivated in modified de Man-Rogosa-Sharpe (MRS) medium without glucose supplemented with 0.05% cysteine–HCl and 2% lactose (mMRS) in an anaerobic chamber (Davidson and Hardy; Belfast; United Kingdom) at 37°C for 24 h when in broth and 48 h on agar plates. For the cultivation of bifidobacterial transformants or mutants, the mMRS medium was supplemented with 5 μg/mL chloramphenicol. The *B. bifidum* PRL2010 strain used in this study is a bifidobacterial strain isolated from the faecal sample of a breast-fed infant (Turroni et al., 2010).

Prediction of the methylome of *B. bifidum* PRL2010

To remove the restriction and modification (R/M) sites present on certain plasmids and targeted by endogenous R/M systems encoded by *B. bifidum* PRL2010, the methylome of *B. bifidum* PRL2010 was predicted by assessing the *B. bifidum* PRL2010 genome using the REBASE database (http://rebase.neb.com/rebase/rebase.html) and sequencing its genome using the Pacific Biosciences (PacBio) sequencing platform. For PacBio sequencing, *B. bifidum* PRL2010 was grown in MRS broth supplemented with 0.05% cysteine–HCl until it reached an optical density at 600 nm (OD_{600}) of approximately 0.6. Genomic DNA was extracted from harvested PRL2010 cells using the GenElute Bacterial Genomic DNA kit and then subjected to sequencing using PacBio Sequel I technology and SMRT cells (Macrogen service). The PacBio sequencing reads were processed and mapped to the *B. bifidum* PRL2010 sequence. The interpulse durations were measured as previously described (Murray et al., 2012). To identify methylated positions, the PacBio SMRTPortal analysis platform was adopted, employing an in silico kinetic reference and a *t*-test based kinetic score detection of modified base positions.

Plasmid manipulation and construction

Plasmid pNZ003 was obtained by removing three different R/M sites present in its predecessor, pNZ44 (McGrath et al., 2001), by PCR using the primers listed in Table 1. Primers pNEW_002, pNEW_003, pNEW_001, and pNEW_004 were used to amplify the pNZ44 plasmid with Q5 polymerase (New England Biolabs). After purification (GeneJet Gel Extraction Kit—Thermo Fisher Scientific), the amplicons obtained were digested with ApaLI and Esp3I (New England Biolabs), ligated (T4 DNA ligase, New England Biolabs), and used to transform *E. coli* EC101 prepared using a modified protocol for chemically competent cells (Hanahan et al., 1991). Transformants were selected on LB supplemented with 25 μg/mL chloramphenicol, and individual transformants were screened for the presence of the expected plasmid by colony PCR. Plasmid pFREM30 was obtained by amplifying the chloramphenicol gene (Cm^R) with primers CLMC_207 and CLMC_208 and the backbone of pFREM28 (Hoedt et al., 2021) with primers CLMC_009 and CLMC_010. Following DNA fragment purification (GeneJet Gel Extraction Kit, Thermo Fisher Scientific),

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TABLE 1 List of all primers used.

Target	Primer	Primer sequence 5′–3′
pNZ003	PNEW_001	AACAATGTGCACGACGCGGATTATGCGACGCGTGCATGCGGTACCACTAGTTC
	PNEW_002	CGCGTCGTGCACATTGTTAGATCTGGAGCTGTAATATAAAAACCTTCTTC
	PNEW_003	GATCGACGTCTCAGCTGCGTTAGCTATAGAAGAATATGCAAGAAAG
	PNEW_004	GATCGACGTCTCACAGCAACCGCAGATTTTGAAAAACC
pFREM30	CLMC_009	GATCGCTCTTCTCCCCACCAAAACCGAAATCCAC
	CLMC_010	GATCGCTCTTCTAAGGTGTGCTCCTTTCCCTCAC
	CLMC_207	GATCGCTCTTCGGGGATTATAAAAGCCAGTCATTAGGCCTATCTGAC
	CLMC_208	GATCGCTCTTCACTTATGAACTTTAATAAAATTGATTTAGACAATTGGAAGAG
	PFREM30 MCST	ATATATCCATGGTACCCGGGGTGCACGAAGCTTACGCGTAGACGTCATATGG ATATCGCCGTGCTCGAGT
	PFREM30 MCSB	ATATATTCTAGACTCGAGCACGGCGATATCCATATGACGTCTACGCGTAAG CTTCGTGCACCCCGGGTAC
Colony PCR-screening	LMV_015 LMV_016 CLMC_022	CGCAGCAGCGGTCGCTATG GAAGTTGAGGTACGCGGTGTAGC GCCAACGTTTTCGCCAACG
*per*B gene	LMV_011 LMV_012	TATTGGGTGCACGGCGAACGGCGTCAAGG GGTATGTCTAGACCGCCGTTGGCCATGTGC
qPCR	BBPR_0282_FW BBPR_0282_RV	GCGAACAATGATGGCACCTA GTCGAACACCACGACGATGT

the generated amplicons were used to obtain plasmid pFREM30 using the Golden Gate Cloning approach (Engler & Marillonnet, 2014), utilizing the Type IIS enzyme SapI (New England Biolabs). The product of the reaction was introduced into *E. coli* EC101, following the protocol for chemically competent cells. Subsequently, the colonies grown on LB supplemented with 25 μg/mL chloramphenicol were screened by colony PCR for the presence of the expected recombinant plasmid pFREM30, the genetic integrity of which was then validated by sequencing (Genewiz, Leipzig, Germany).

For the construction of plasmid pFREM30-*per*B, the to-be-targeted, internal region of the gene was amplified by PCR from chromosomal *B. bifidum* PRL2010 DNA (GenElute Bacterial Genomic DNA kit, Sigma, Germany) using the Q5 polymerase and the primers LMV_011 and LMV_012. Plasmid DNA was isolated from *E. coli* using the GeneJET Plasmid Maxiprep Kit (Thermo Fisher Scientific, USA). The amplicon and plasmid were digested with ApaLI and XbaI, ligated, and introduced into *E. coli* EC101 as previously reported (Hanahan et al., 1991). To select for transformants, the manipulated cells were plated on LB supplemented with 25 μg/mL chloramphenicol, and the colonies were screened for the presence of the expected plasmid construct by colony PCR.

Preparation of bifidobacterial cells for DNA electroporation

An overnight culture of PRL2010 was inoculated into fresh mMRS broth supplemented with 7% (v/w) sucrose (sMRS) and cultivated at 37°C until the exponential growth phase was reached, i.e., an OD_{600} between 0.5 and 0.6. Subsequent steps of the protocol were performed by maintaining the cells refrigerated on ice. Specifically, cells were harvested by centrifugation (4500*g* for 10 min at 4°C), washed twice with an ice-cold citrate-sucrose buffer (0.5 M sucrose, pH 5.8), and resuspended in 250 μL of the same buffer before electroporation.

Electroporation and selection of PRL2010 mutants

100 μL of concentrated bacterial cells resuspended in citrate–sucrose buffer (pH 5.8) was mixed with 1.5 μg of the plasmid in a precooled disposable electroporation cuvette with an interelectrode distance of 0.2 cm (Cell project, Kent, United Kingdom). A resistance of 200 Ω, capacity of 25 μF, and voltage of 2.5 kV were applied using a Gene Pulser apparatus (BioRad, UK). After cell electroporation, bacteria were resuspended in 950 μL of sMRS and incubated for 3 h at 37°C in an anaerobic cabinet. Following this, cells were plated on sMRS agar supplemented with 5 μg/mL of chloramphenicol and incubated anaerobically at 37°C for 48 h. Potential mutants were screened by colony PCR using the primers LMV_015 and LMV_016, which annealed to the chromosomal gene outside of the gene target region, and CLMC_02, which annealed to the (integrated) pFREM30 plasmid. The expected amplicon sizes are approximately 2000 bp and 800 bp, respectively. The amplicons obtained were sent

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for sequencing (Sanger) to further confirm the expected sequence following the expected homologous recombination-mediated integration event. All primer sequences are listed in Table 1.

Mutant stability

To assess the stability of the integrated pFREM plasmid, a spot assay was performed, and the presence of the plasmid was checked by colony PCR. For this assay, *B. bifidum per*B::pFREM30 was cultivated in mMRS broth, with or without 5 µg/mL chloramphenicol. Every day, for a total of 14 days, an aliquot of the culture was sub-cultured in fresh medium, and every second day, the overnight inoculum of the two growth conditions was serially diluted (1.00E-04, 1.00E-05, 1.00E-06) and spotted on mMRS plates with or without 5 µg/mL of chloramphenicol. After the anaerobic incubation of the plates at 37°C for 48 h, a colony PCR was performed on one colony per replicate with primers LMV_015 and LMV_016. The experiments were performed in triplicate.

Mucin growth assay

To evaluate possible differences in mucin utilization, *B. bifidum* PRL2010 wild-type (wt) and *B. bifidum per*B::pFREM30 were grown overnight until the exponential growth phase was reached in mMRS, with and without the addition of 5 µg/mL of chloramphenicol to the mutant. Subsequently, strains were washed twice with phosphate-buffered saline (PBS) (Sigma, Germany), and inoculated in a final volume of 20 mL of both mMRS and mMRS with 0.5% mucin (from porcine stomach—Type III, Sigma, Germany) as the sole carbon source (with and without 5 µg/mL of chloramphenicol for the mutant for both culture media) to reach a final inoculum with an OD_{600} of ~0.1. Cells were grown in biologically independent triplicates. Cells were incubated under anaerobic conditions at 37°C for 48 h. Aliquots of the bacterial cultures were collected at different time points (0, 5, 10, 24, 36, and 48 h) and subjected to a viability assay and total bacterial cell count using flow cytometry, as described below. Each aliquot was in parallel plated on mMRS agar. Plates were incubated under anaerobic conditions at 37°C for 48 h. Bacterial growth was assessed by colony-forming unit counting.

Evaluation of bacterial cell density and viability assay by flow cytometry

For total cell counts, each culture replicate was 100,000-fold diluted in physiological solution (PBS). Subsequently, 1 mL of the obtained bacterial cell suspension was stained with 1 µL of SYBR®Green I (ThermoFisher Scientific, USA) (1:100 dilution in dimethylsulfoxide; Sigma, Germany), vortexed, and incubated at 37°C in the dark for at least 15 min before measurement, as previously described (Alessandri et al., 2022; Vandeputte et al., 2017). For the bacterial cell viability assay, the fluorescent dyes SYTO9 (3.34 mM) and propidium iodide (PI; 20 mM) of the LIVE/DEAD BacLight Bacterial Viability Kit (ThermoFisher Scientific, USA) were used, following the manufacturer's instructions. Specifically, five different tubes of the diluted cells (final volume of 1 mL each) were obtained per sample. One of the tubes was subjected to centrifugation at 2515 g for 8 min, the supernatant was discarded, and the microbial cell pellet was resuspended in 70% isopropyl alcohol for 1 h to allow the permeabilization of the microbial cell membrane to induce cell death. The treated cells were then centrifuged, resuspended in PBS, and dyed with the addition of 1.5 µL of PI. Other two tubes were stained with 1.5 µL of one of the two dyes, while a fourth tube was not stained. Finally, the last tube was stained with both SYTO9 and PI. Immediately following staining, samples were vortex-mixed and incubated in the dark for 15 min at room temperature. For instrument parameter adjustment, single-dyed samples and the sample exposed to isopropyl alcohol were used as controls, while the non-stained cells were used as a background control.

Both count and viability experiments were performed with an Attune NxT flow cytometry (ThermoFisher Scientific, USA) equipped with a blue laser set at 50 mW and tuned to an excitation wavelength of 488 nm. Multiparametric analyses were performed on both scattering signals, that is, forward scatter (FSC) and side scatter (SSC), while fluorescence was detected on a BL1 530/30 nm optical detector. Cell debris were excluded from the acquisition analysis by setting the BL1 threshold. Furthermore, gated fluorescence events were evaluated on the forward–sideways density plot to exclude remaining background events and to obtain an accurate microbial cell count, as previously described. All data were analysed with the Attune NxT flow cytometry software.

Adhesion of *B. bifidum* PRL2010 to HT29-MTX cells

Bifidobacterial adhesion to HT29-MTX cells was assessed by slightly modifying a previously described protocol (Guglielmetti et al., 2008; Rizzo et al., 2023; Serafini et al., 2013). Briefly, human colon carcinoma-derived mucin-secreting goblet HT29-MTX cells (kindly provided by Prof. Antonietta Baldi, University of Milan) were cultured in Minimum Essential Medium (MEM) with

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high glucose (4.5 g/L) as previously described (Bianchi et al., 2019). The medium was supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, and 10 mM HEPES. For the experiment, HT29-MTX cells were seeded on microscope cover glasses previously settled in 10 cm^2 Petri dishes. The cells were carefully washed twice with PBS before the addition of bacterial cells. *B. bifidum* PRL2010 wt and *B. bifidum per*B::pFREM30 were grown as previously described until a concentration of 1.00E+08 cells mL^{-1} was reached. The two strains were then centrifuged at 5000*g* for 8 min, resuspended in PBS, and incubated with HT29-MTX cells. After 1-h incubation at 37°C, cells were washed twice with 2 mL of PBS to remove unbound bacteria. The cells were then fixed with 1 mL of methanol and incubated for 8 min at room temperature. Finally, the cells were stained with 1.5 mL of Giemsa stain solution (1:20 in PBS) (Sigma Aldrich, Milan, Italy) and incubated in the dark for 30 min at room temperature. After two washes with 2 mL of PBS, cover glasses were removed from the Petri plate, mounted on a glass slide, and examined using a Leica DM 1000 phase contrast microscope (objective: 100X/1.4 oil). The adherent bacteria in 20 randomly selected microscopic fields were counted and averaged, as previously described (Rizzo et al., 2023). The proportion of bacterial cells that remained attached to HT29-MTX was determined to reflect the extent of specific host–microbe interactions. The adhesion index is calculated as the average number of bacterial cells counted on 20 random spots/(100 the number of HT29-MTX cells) (Guglielmetti et al., 2008).

Ethics statement

All experimental procedures and protocols involving animals were approved by the Italian legislation on animal experimentation (D.L. 04/04/2014, n. 26, authorization n° 370/2018-PR) and conducted in accordance with the European Community Council Directives dated September 22, 2010 (2010/63/UE).

Animal housing and design of the in vivo experiment

The in vivo rodent experiments involved 10 male and 10 female 5-week-old Wistar rats bred at the University of Parma. After weaning, rats were individually housed in polymethyl methacrylate cages in a room under controlled humidity (50% ± 10%) and temperature (22 ± 2°C) conditions, maintained on a 12-h light–dark cycle (lights on at 7 a.m.), and with food and water available ad libitum. The first week of the in vivo trial corresponded to the acclimatization period, during which rats were fed a standard chow diet supplemented with an oral administration of 500 μL of sucrose solution (2%) through a syringe to adapt them to this form of administration (Figure 4). For the following 2 weeks (intervention period), rats were randomly and equally divided into a control (administration of *B. bifidum* PRL2010 wt) and treatment (administration *B. bifidum per*B::pFREM30) group, both orally administered once a day with 1.00E+09 cells/mL of the specific strain (Figure 4). For each day of the intervention period, bifidobacterial cells were harvested by centrifugation, washed in PBS, and resuspended in 1 mL of sucrose solution (2%) for oral administration to rats (10E+09 cells/mL).

Five females and five males were included in each group (Kiss et al., 2019). Finally, the last week corresponded to the washout period (Figure 4).

During the in vivo experiment, fresh faecal samples were collected at three different time points. Faecal samples were collected after the first week of bifidobacterial strain administration (T1), after the second week of treatment, and at the end of the washout (T2 and T3, respectively) (Figure 4). The cage litter was changed 1 h prior to each faecal collection. Afterward, fresh faecal samples were collected in the morning and stored at −20°C until analysis.

DNA extraction and qPCR analysis

0.2 g of the collected rat faecal samples was individually subjected to DNA extraction using the QIAmp DNA Stool Mini kit, following the manufacturer's instructions (Qiagen, Germany). Subsequently, quantitative PCR (qPCR) was performed using *B. bifidum* PRL2010-specific primers: BBPR_0282_FW and BBPR_0282_RV (Table 1). These primers had previously been designed and tested to specifically identify *B. bifidum* PRL2010 cells under in vitro and/or in vivo conditions (Duranti et al., 2016; Turroni et al., 2013, 2015, 2016). qPCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, USA) on a CFX96 system (BioRad, CA, USA) following previously described protocols (Milani et al., 2015). PCR products were detected using SYBR green fluorescent dye and amplified according to the following protocol: one cycle at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 1 min. Negative controls (no DNA) were included for each run. A standard curve was generated through CFX96 software (BioRad) using *B. bifidum* PRL2010 chromosomal DNA extracted with the GenElute bacterial genomic DNA kit (Sigma-Aldrich) following the manufacturer's guide.

Statistical analyses

SPSS software (www.ibm.com/software/it/analytics/spss) was employed to compute statistical analyses,

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including ANOVA with Bonferroni post-hoc multiple comparison, Student's *t*-test, and the non-parametric Mann–Whitney test.

AUTHOR CONTRIBUTIONS

Sonia Mirjam Rizzo: Data curation (equal); formal analysis (equal); investigation (equal); validation (equal); visualization (equal); writing – original draft (equal). **Laura Maria Vergna:** Data curation (equal); formal analysis (equal); investigation (equal); validation (equal); visualization (equal); writing – original draft (equal). **Giulia Alessandri:** Data curation (supporting); formal analysis (supporting); investigation (supporting); validation (equal); visualization (equal); writing – original draft (supporting). **Ciaran Lee:** Conceptualization (equal); funding acquisition (equal); supervision (equal). **Federico Fontana:** Data curation (equal); formal analysis (equal). **Gabriele Andrea Lugli:** Data curation (equal); formal analysis (equal). **Luca Carnevali:** Conceptualization (equal); supervision (equal); validation (equal); visualization (equal). **Massimiliano G. Bianchi:** Conceptualization (equal); investigation (equal); supervision (equal); validation (equal); visualization (equal). **Margherita Barbetti:** Investigation (equal); validation (equal); visualization (equal). **Giuseppe Taurino:** Validation (supporting); visualization (supporting). **Andrea Sgoifo:** Conceptualization (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Ovidio Bussolati:** Conceptualization (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Francesca Turroni:** Conceptualization (equal); funding acquisition (equal); project administration (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Douwe van Sinderen:** Conceptualization (equal); funding acquisition (equal); project administration (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Marco Ventura:** Conceptualization (equal); project administration (equal); resources (equal); supervision (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Raw sequences of PacBio sequencing data are available in the SRA database with accession number PRJNA999613.

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