Current Microbiology (2023) 80:399
https://doi.org/10.1007/s00284-023-03515-6

SHORT COMMUNICATION

Draft Genome Sequence and Comparative Genome Analysis Reveal Potential Functional Properties in *Lacticaseibacillus paracasei* ItalPN16

Cristian Mauricio Barreto Pinilla[1] · Frank Guzman Escudero[2] · Adriana Torres Silva e Alves[1] · Leila Maria Spadoti[1] · Adriano Brandelli[3]

Received: 14 July 2023 / Accepted: 6 October 2023 / Published online: 1 November 2023

Abstract

Nowadays, there is a great interest on rapid and effective methods for initial identification of probiotic bacteria. In this work, potential probiotic features of the lactic acid bacteria strain ItalPN16 isolated from a traditional Brazilian cheese were studied using bioinformatic tools. The complete genome sequence was obtained, and in silico analyses were carried out to identify the strain and its potential probiotic properties. The sequenced genome (3.02 Mb) presented 3126 protein-coding sequences distributed on 244 SEED subsystems, classifying the strain as nomadic lactobacilli. Phylogenetic and ANI analyses allowed to locate the ItalPN16 strain as a member of the *Lacticaseibacillus paracasei* group, due to the highest number of orthologous genes in common with reference *L. paracasei* strains (>98%). In silico analyses revealed the presence of CDSs related to microbe–host interactions, such as adhesion proteins and exopolysaccharide biosynthesis genes. The comparative analysis reveals the presence of a strain-specific glycosyl transferases, compared with other three *L. paracasei* strains and a high level of protein expression (92%) with the probiotic *L. paracasei* BL29. The results obtained here indicated interesting probiotic features of the strain *L. paracasei* ItalPN16 that could favor a future application in the food industry.

Introduction

The search for new bacterial strains with probiotic properties and inherent technological attributes has increased due to the several benefits reported by scientific and medical communities. The continuous consumption of some lactic acid bacteria (LAB), in particular from the lactobacilli group, has proven to be beneficial for human health and consequently some strains are considered as probiotics [1]. The lactobacilli are found in a large group of Gram-positive, non-spore-forming bacteria with varied phylogenetic and ecological adaptability [2]. Several species of the *Lactobacillaceae* family have been used in food products to confer diverse health benefits to the consumers, beyond basic nutrition [3]. However, due to the probiotic properties that could vary according to the strain, the identification, safety confirmation, and complete characterization of each strain are of high importance prior to their utilization in foods.

Recently, a new classification of the *Lactobacillaceae* family has been released based on novel genetic approaches and markers [4], including changes in diverse species previously designated to the genus *Lactobacillus.* In fact, based on the new classification, some species of *Lactobacillus* largely used in foods have been renamed as *Limosilactobacillus* (e.g., *L. reuteri*), *Lactiplantibacillus* (e.g., *L. plantarum*), and *Lacticaseibacillus* (e.g., *L. casei*), among others. However, their status in the list of "Qualified Presumption of Safety" (QPS) at the species level by the European Food Safety Authority and the designation of "Generally Recognized as Safe" (GRAS), at the strain level by the U.S. Food & Drug Administration, was maintained [5].

Lacticaseibacillus paracasei (formerly *Lactobacillus paracasei*) belongs to the *L. casei* group, found in diverse niches, such as fermented foods, plants, and intestines of human and animals [6]. Moreover, *L. paracasei* has demonstrated beneficial properties against several diseases [7]. In

✉ Cristian Mauricio Barreto Pinilla
cristianmaobarreto@gmail.com

[1] Dairy Technology Center (TECNOLAT) of the Food Technology Institute (ITAL), Campinas, São Paulo 13070-178, Brazil

[2] Facultad de Medicina, Universidad Científica del Sur, 150142 Lima, Peru

[3] Laboratory of Applied Microbiology and Biochemistry, Institute of Food Science and Technology (ICTA), Federal University of Rio Grande Do Sul (UFRGS), Porto Alegre 91501-970, Brazil

a previous study, the strain ItalPN16, isolated from a traditional ripened cheese in Brazil and preliminarily characterized as *L. paracasei*, demonstrated in vitro probiotic and technological features including auto-aggregation and cell adhesion properties [8]. Here, we report the whole-genome sequence and phylogenetic analysis of ItalPN16, which allowed its precise taxonomic location inside the *Lacticasei* group and an in silico comparative study, providing important insights on industrial potential and possible probiotic properties of this strain.

Materials and Methods

Genome Sequencing, Assembly, and Annotation

The strain ItalPN16 was maintained in a frozen stock culture in MRS (Merck, Darmstadt, Germany) broth containing 20% (v/v) glycerol. For DNA extraction, the strain ItalPN16 was activated by incubation in MRS broth at 37 °C for 24 h under anaerobic conditions. For total genomic DNA extraction of ItalPN16, the DNeasy PowerSoil Microbial Kit (Qiagen, Valencia, CA, USA) was used according to the manufacturer's protocols and then a Spark 10 M spectrophotometer (Tecan Trading AG, Männedorf, Switzerland) was used to assess the quantity and quality of the obtained DNA. The genome sequencing of ItalPN16 was carried out using paired-end sequencing technology with a NextSeq500 Illumina sequencer in the Center of Biotechnology (CBiot, Porto Alegre, Brazil). The quality and nucleotide composition of the raw sequences were visualized by FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and adapter trimming was performed by Trim Galore (www.bioinformatics.babraham.ac.uk/projects/trim_galore/) using a cutoff of q30. The 8,022,957 of paired-end reads (read length 150 pb) obtained were used for De novo assembly of the cleaned reads, performed with IDBA assembler version 1.1.3 [9] using default parameters. The assembled scaffolds were used for gene prediction and annotation in the Rapid Annotation Subsystems Technology (RAST) server [10]. Finally, the draft genome data were deposited in GenBank (accession number JALGQW000000000).

Genomic Analysis and Bioinformatics

Genome annotation was performed using RAST; this software was used to obtain the main features of the genome and to search for genes related to adhesion and bacteriocins. The ResFinder 4.1 software [11] with default parameters was used to identify acquired antimicrobial resistance genes in *L. paracasei* ItalPN16. The presence of genes encoding virulence factors was determined with VirulenceFinder 2.0 [12]. MEGA11 software was used to compute the alignment and the phylogenetic tree using the Maximum Likelihood method [13]. In this analysis, we used the 16S ribosomal DNA sequences of *L. paracasei* ItalPN16 and the other *Lactobacillus* species obtained using EzBioCloud tools [14]. Average nucleotide identity (ANI) analysis between the ItalPN16 and others 18 representative *Lactobacillus paracasei* strains with available genomes in the NCBI database was performed to determine their relationship, and clustermap was generated using the ANI calculator available ANIclustermap (v1.2.0) [15].

Comparative Genomics Analysis

Specific features of this strain were searched performing a comparative analysis where the predicted proteins for *L. paracasei* ItalPN16 genome were compared against the proteins of another three *L. paracasei* genomes obtained from GenBank (NCBI), using the online platform OrthoVenn with default parameters [16]. These three *L. paracasei* strains ATCC 25302, BL23, and DTA95 were selected due to their probiotic properties (accessions numbers VTYT00000000, NZ_PUFK00000000, and NC_010999, respectively).

Nucleotide Sequence Accession Numbers

The strain ItalPN16 has been previously isolated from a commercial cheese and is part of the collection of the Dairy Technology Center (TECNOLAT) of the Food Technology Institute (ITAL), Campinas, Brazil. This Draft Genome Shotgun project has been deposited at EMBL/ENA/GenBank under the accession code JALGQW000000000. The version described in this paper is version JALGQW000000000.

Results and Discussion

Genome Features

Nowadays, genome sequence analysis has become an important tool to obtain useful information about safety issues and functional properties of novel potential probiotic strains [1]. In this context, the use of whole-genome sequencing is an important approach to determine the exact taxonomical location of a strain and to make an in-depth inspection of its industrial potential and possible health benefits.

The assembling of shotgun reads generated 70 contigs, resulting in a genome size of 3.020.335 bp with a G+C content of 46.3%. The main features of ItalPN16 genome are reported in Table 1. Several studies have shown that genome size of lactobacilli could vary from 1.28 to 4 Mb, depending on their ideal environmental niche [17]. The genome reduction of members of the *Lactobacillus* sensu lato is an evolutionary process, resulting from the transition

Table 1 Genome features of *Lacticaseibacillus paracasei* ItalPN16

Genome features	Values
Genome size (bp)	3,020,335
G+C content (%)	46.3
Contig N50	110,907
Contig L50	11
Number of contigs (with PEGs)	70
Number of protein-coding sequences (CDSs)	3126
Number of tRNAs	57
Number of CDSs related to host adhesion	7
Number of CDSs related to Exopolysaccharide Biosynthesis	6
Number of CDSs related to bacteriocins	7
Number of genes related to antimicrobial resistance	1
Number of genes related to virulence	0

of free-living to matrix-associated and nomadic bacteria [18]. Large genomes ranged 3–4 Mb are usually found in free-living and nomadic strains; this feature supports their survival in heterogenous matrixes, improving their ability to survive in dairy products and in association with the host [19]. Thus, according to the *L. paracasei* ItalPN16 genome metrics, we can classify it as a nomadic strain, different to host-associated strains, such as *Bifidobacterium,* which possess smaller genomes (1.28–3 Mb) resulting from extensive gene loss due to their specialization and habitat adaptation to more selective energy sources [18]. However, a recent comparative analysis of 1665 sequenced genomes belonging to 14 species of *Lactobacillus* and 5 species of *Bifidobacterium* revealed that nomadic lactobacilli could present more intestinal tissue-anchored surface structures compared with some host-adapted ones. The authors also reported that the occurrence of genetic elements in high colonization phenotypes are species specific and strain specific [20].

The draft genome prediction and annotation indicated a total number of 3126 predicted protein coding sequences (CDSs) and 57 structural RNAs within the *L. paracasei* ItalPN16 (Table 1). From the genome annotation data, seven CDSs related to bacteriocins, seven CDSs to bacterial adhesion (three enolases, two fibronectins, and two LPXTG motifs), and six CDSs related to EPS biosynthesis were found. In addition, the analysis using ResFinder detected the vancomycin resistance gene (*vanZ*) in the ItalPN16 genome and the bioinformatic analysis with VirulenceFinder showed that the strain does not harbor virulence factors that could be transported to bacteria of the gut microbiome.

Regarding the potential probiotic properties, bacteriocin production and adhesion ability are desirable, since they provide antagonistic effect against unwanted gut bacteria, an effective colonization of the gut environment, allowing the exclusion of pathogens [21]. Proteomic studies have related that bile and acid stress resulted in the overexpression of these CDSs in *L. paracasei* species, improving their adhesion ability [22]. In addition, surface (LPxTG) adhesins present in the bacterial surface, provide specific interactions with host receptors. Therefore, the presence of these structures on *L. paracasei* ItalPN16 indicates higher adhesion capacity of the strain.

Production of EPS is an interesting strain property since EPS with varied carbohydrate compositions retrieved from probiotic bacteria could present several beneficial properties to the host [23]. In fact, surface EPS in *L. paracasei* strains could play a key role in their interaction with epithelial intestinal cells, triggering the biological and health-promoting effects [24].

The probiotic character of a given strain also includes sensitivity to common antibiotics and lack of virulence genes [25]. In this work, the vancomycin-resistant gene (*vanZ*) was detected in the ItalPN16 genome. The mechanism of action of VanZ remains unknown; however, some evidence indicate increase in the minimum inhibitory concentration by the interaction with vancomycin and other lipoglycopeptide antibiotics in the cell wall. However, vancomycin resistance is a shared characteristic in lactobacilli and does not raise safety concerns [26].

Phylogenetic Analysis

The strain ItalPN16 isolated from a commercial cheese was identified based on draft genome sequencing. The phylogenetic tree developed by MEGA11 software confirmed that the strain ItalPN16 belongs to the *L. paracasei* species, according to its location in the dendrogram (Fig. 1). The highest similarity at nucleotide level was obtained with the *L. paracasei* strains JMC 1171 and ATCC 25302 both isolated from dairy products as well the ItalPN16. However, due to their nomadic character, *L. paracasei* strains usually form clades based on their metabolic and fermentation capabilities and do not form phylogenetic clusters based on their origin [17]. Overall, based on the phylogenetic analysis, it was possible to indicate that the strain ItalPN16 belongs to the *Lacticaseibacillus* group and is closely related to reference *L. paracasei* species.

After the initial phylogenetic location, the ANI analysis was performed to determine the relationship of species with other 18 genomes of *L. paracasei* available on NCBI. According to the ANI values (Fig. 2), the genome of ItalPN16 was most similar (99.05%) to the *L. paracasei.* Sub *tolerans* FX-6, which was isolated from Tibetan kefir [27], followed by the *L. paracasei* DTA93 was isolated from healthy infant feces with 98.59% [28] and the strain *L. paracasei* DSM 28675 (98.53%) was isolated from caecal content. Such dissimilarities may be due to host-specific strain diversity and niche adaptation. Overall, this genotypic

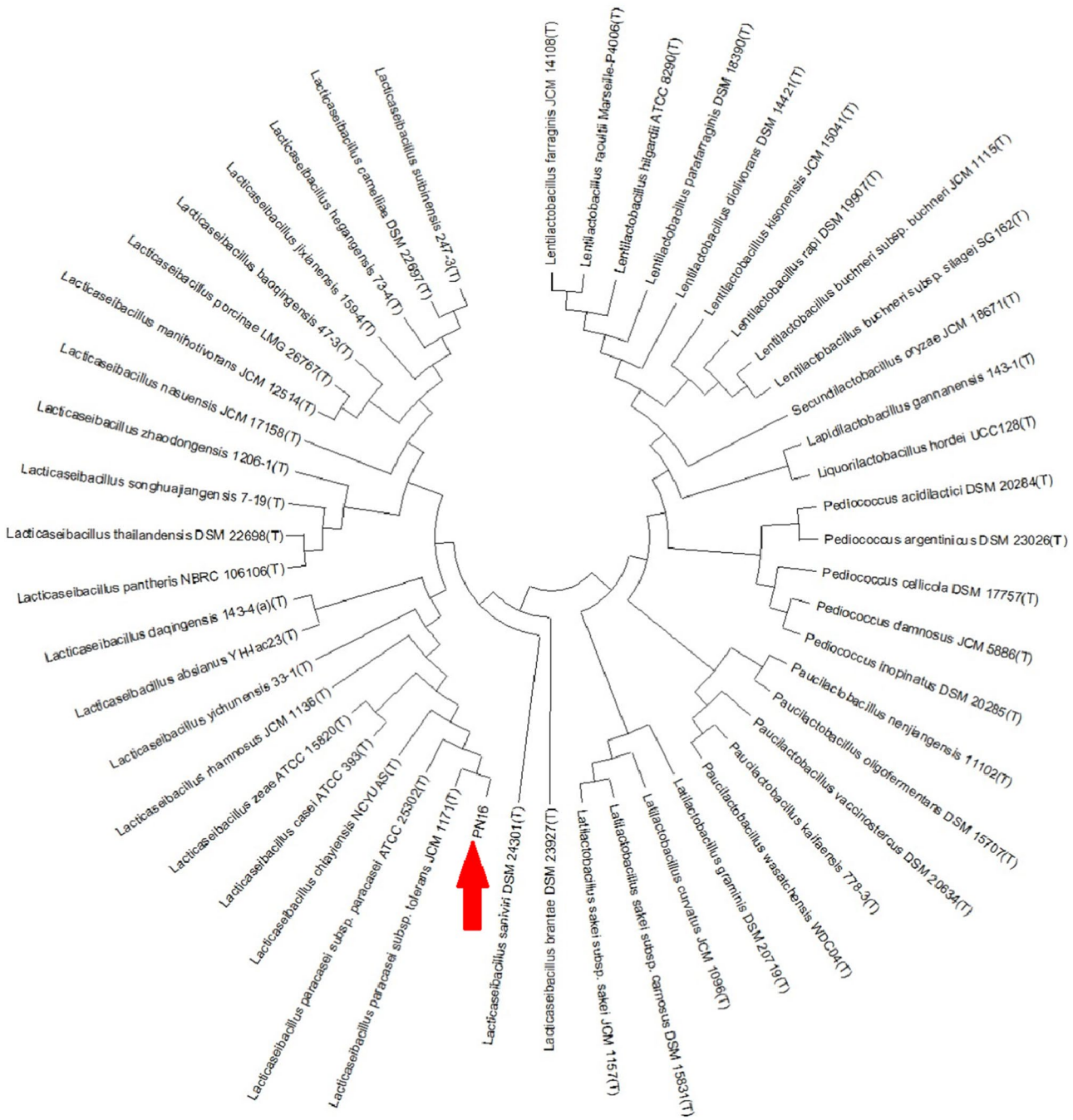

Fig. 1 Phylogenetic tree of *L. paracasei* ItalPN16 derived from whole-genome analysis

methodology is an important tool to determine the taxonomic status and nomenclature of NSLAB strains due to the phenotypically similarity of the *L. casei* group, which include the *L. casei*, *L. paracasei*, and *L. rhamnosus* [29].

Graphical Genome Annotation

Figure 3 depicts the protein subsystems found in the RAST annotation, which collectively perform some specific biological process or structural complex. The functional annotation (COG) of these core genes showed that most of them belong to carbohydrate metabolism with 21.47% of the total, followed by production of amino acid and derivates (13.35%), protein metabolism (11.32%), DNA metabolism (6.19%), cofactors, vitamins, prosthetic groups and pigments (5.32%), and cell wall and capsule with 4.84%. A previous report of our research group demonstrated that *L. paracasei* ItalPN16 can grow successfully in sweet and

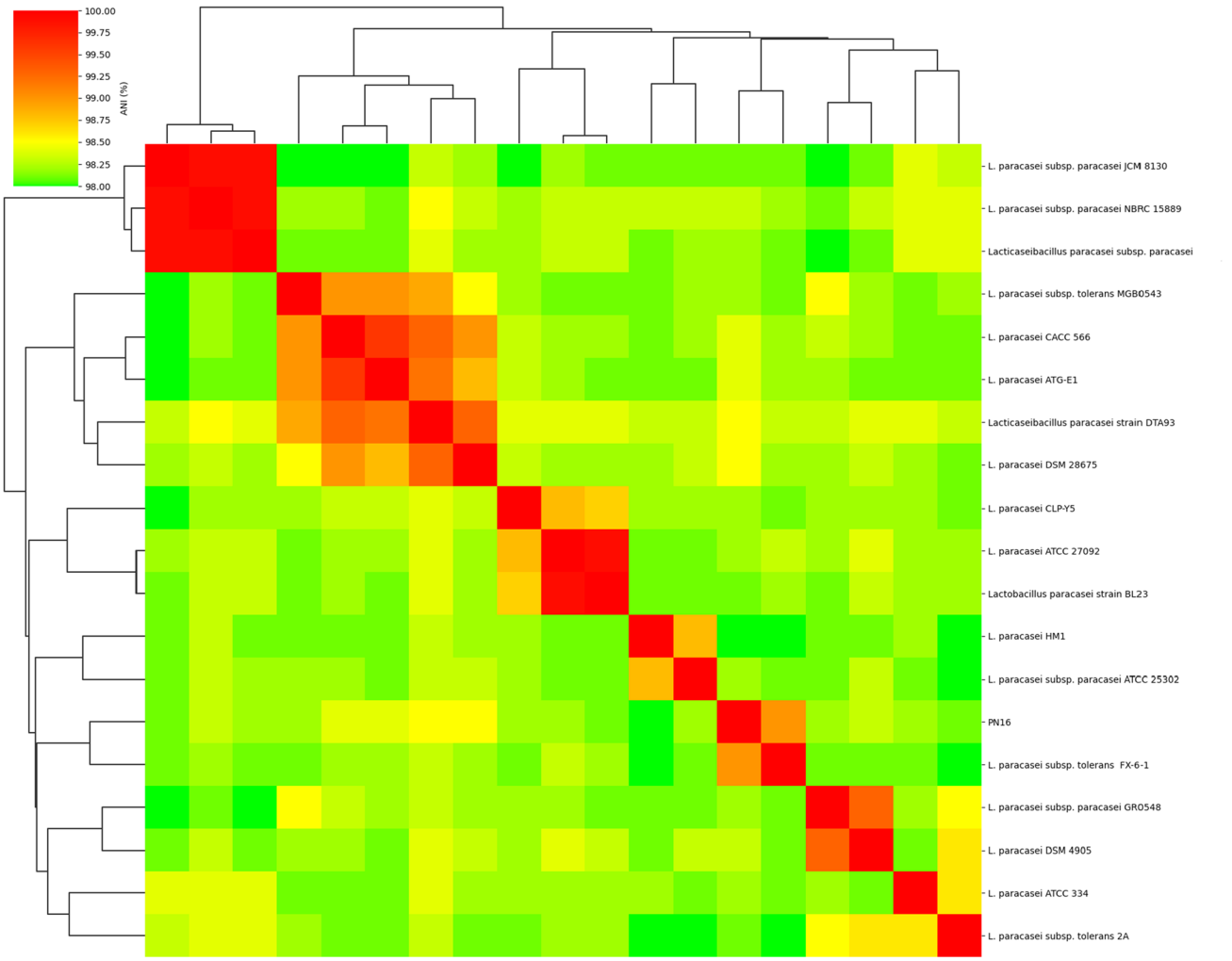

Fig. 2 Heatmap showing relative average nucleotide identity (ANI) between all-vs-all microbial genomes

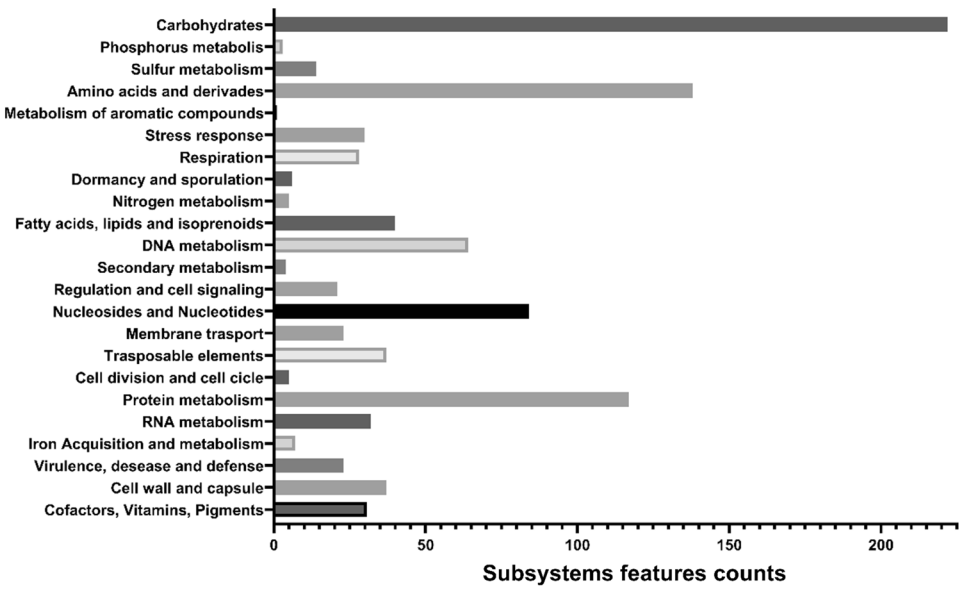

Fig. 3 An overview of the RAST annotation and subsystems of *L. paracasei* ItalPN16 genome

acid whey, despite nutrient limitation and excess lactose [9]. These results support the lack of niche specialization of the strain, revealing its ability to grow in a great variety of environments. However, further analysis must be carried out to determine the functional classification of genes and prediction of the metabolic potential of *L. paracasei* ItalPN16.

Comparative Genome Analysis

In this work, orthologous proteins from a specific group of *L. paracasei* strains were used in a comparative analysis to strengthen taxonomical identification and to understand and identify specific features of the ItalPN16 genome structure and function. OrthoVenn with default parameters was used to define an orthologous cluster structure within the *L. paracasei* genomes. The results revealed that the core genome consisted of 2237 orthologous proteins common to all the examined strains. The analysis indicated that the strain ItalPN16 has the highest number of orthologous proteins (2715) among the four strains evaluated, and from these, 2237 are present in all the four strains. Interestingly, the ItalPN16 share 2507 orthologous proteins with the *L. paracasei* BL23, around 92% of the total BL23 proteins (2711). With the other two strains, the ItalPN16 share 2480 and 2427 orthologous proteins for the DTA93 and the ATCC 25302, respectively. The *L. paracasei* BL23 is considered a probiotic lactobacilli and previous studies has conferred anti-inflammatory effect and protection against the development of colitis [30]. These findings indicate the potential of ItalPN16 as probiotic, although in vivo studies must be conducted to evaluate possible health effects in the host.

Based on the pan/core genome analysis (Fig. 4), the presence of 8 specific single-copy genes/proteins (singletons) was also observed in ItalPN16, indicating individual proteins that confer specific features compared to other *L. paracasei* strains. The specific ItalPN16 singletons (genes/proteins) and their functions are reported in Table 2. Interestingly, from the eight specific proteins predicted in the strain ItalPN16, four were identified as glycosyl transferases. Proteins of the glycosyl transferases group, including the fructosyl transferases, have an important role in the synthesis of extracellular polysaccharides (EPS) in LAB [31]. Biosynthesis of EPS by potential probiotic strains is desirable due to their biological activities, such as antioxidant, antiviral, immunomodulatory, and probiotic properties; EPS also contribute for the probiotic–host interaction, representing an important asset for probiotic candidates [32], besides exhibiting diverse technological applications, improving texture and rheological properties of fermented foods. In addition, two transposases belonging to the families IS5/IS1182 and IS30 were found as specific proteins in ItalPN16 (Table 2). Despite the lack of information about these two transposases families on lactobacilli species, Yi and collaborators [33]

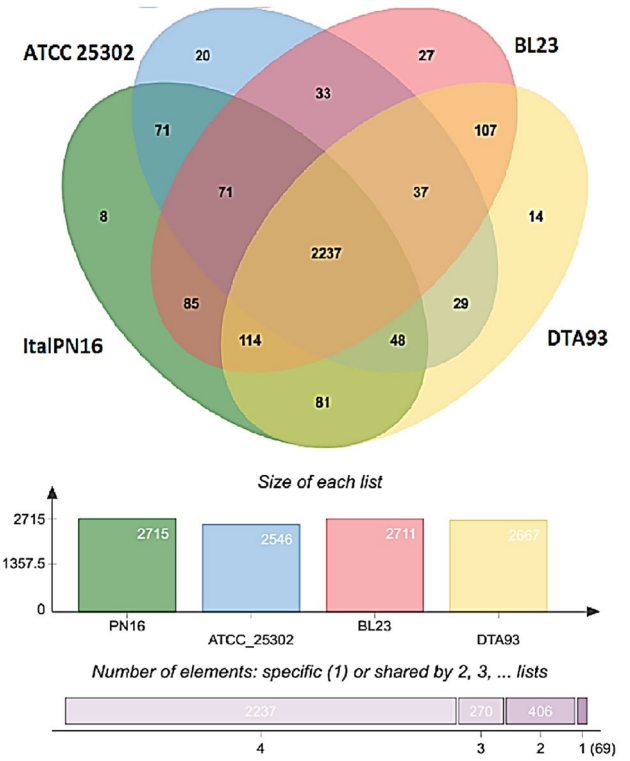

Fig. 4 Pan/core genome analysis generated with OrthoVenn software. Venn diagram indicates the distribution of common gene families (orthologous clusters) among *L. paracasei* ATCC 25302T, *L. paracasei* DTA93, *L. paracasei* BL23, and *L. paracasei* ItalPN16. The number of clusters for each strain is also reported in the histogram graph

reported the presence of proteins of these two families in a predicted gene cluster for bacteriocin biosynthesis in *L. crustorum* MN047. Thus, the possible production of EPS by the ItalPN16 strain is an interesting probiotic characteristic regarding its antimicrobial and gut colonization properties.

Conclusion

Considering the results of this study, the genome analysis of *L. paracasei* ItalPN16 allowed a precise taxonomic identification and its genome features supported its nomadic character. In addition, CDSs related to probiotic features such as bacteriocins synthesis, adhesion, and EPS production were identified. Concerning to the strain safety, the *L. paracasei* ItalPN16 does not harbor acquired antimicrobial resistance genes or virulence factors. The in silico comparative analysis indicated high genetic proximity with the probiotic strain *L. paracasei* BL23 and a specific set of glycosyl transferases that could improve antimicrobial and gut adhesive properties. Despite the role of probiotics is not determined by a single gene, but by a combination of genomic elements, this in silico analysis provided important insights for identification

Table 2 Specific single-copy genes/proteins (singletons) and their related functions in *L. paracasei* ItalPN16

Gene NCBI access code	Number of proteins	Protein	Description
WP_226897455.1	1	Glycosyl transferase	Glycosyl transferase family A (GT-A) includes diverse families of glycosyl transferases with a common GT-A-type structural fold
MBX4164975.1	1	IS5/IS1182 family transposase	Derived by protein homology
NLT82680.1	1	IS30 family transposase	Derived by protein homology
EPC62331.1	2	Putative antifreeze protein	No information
WP_076653741.1	1	Glycosyl transferase	Glycosyl transferase family A (GT-A) includes diverse families of glycosyltransferases with a common GT-A-type structural fold
WP_123018234.1	1	Glycosyl transferase family 2 protein	GT2_RfbF_like protein. RfbF is a putative dTDP-rhamnosyl transferase
NCU14874.1	1	Transcriptional regulator	Derived by protein homology
WP_076653763.1	1	Glycosyl transferase	Glyco_tranf_GTA_type protein

and underlying probiotic features, for a future application of *L. paracasei* ItalPN16 as probiotic strain in foods and for biotechnological innovation. Further studies, including proteomics and in vivo approaches, will provide a more complete view of its possible health-promoting properties.

Author Contributions CMBP and FGE conducted strain reactivation and isolation, formal bioinformatic analysis, data curation, and wrote the original draft. ATSA, LME, and AB were responsible for the project supervision, obtaining financial resources, and revision and edition of the manuscript. All authors read and approved the final version of the manuscript.

Funding This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [grant number 308880/2021-8] and São Paulo Research Foundation (FAPESP) [grant number 2021/04987-0].

Data Availability The main data used in the current study are available in EMBL/ENA/GenBank under the accessions code JALGQW000000000, VTYT00000000, NZ_PUFK00000000, and NC_010999. Other data generated and/or analyzed in this work are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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