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Research Article
Genetic of Microorganisms

Genomic analysis of *Enterococcus durans* LAB18S, a potential probiotic strain isolated from cheese

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Abstract

Gut microbiota exerts a fundamental role in human health and increased evidence supports the beneficial role of probiotic microorganisms in the maintenance of intestinal health. *Enterococcus durans* LAB18S was previously isolated from soft cheese and showed some desirable *in vitro* probiotic properties, for that reason its genome was sequenced and evaluated for genes that can be relevant for probiotic activity and are involved in selenium metabolism. Genome sequencing was performed using the Illumina MiSeq System. A variety of genes potentially associated with probiotic properties, including adhesion capability, viability at low pH, bile salt resistance, antimicrobial activity, and utilization of prebiotic fructooligosaccharides (FOS) were identified. The strain showed tolerance to acid pH and bile salts, exhibited antimicrobial activity and thrived on prebiotic oligosaccharides. Six genes involved in selenium metabolism were predicted. Analysis of the SECIS element showed twelve known selenoprotein candidates. *E. durans* LAB18S was the only food isolate showing absence of plasmids, virulence and antimicrobial resistance genes, when compared with other 30 *E. durans* genomes. The results of this study provide evidence supporting the potential of *E. durans* LAB18S as alternative for probiotic formulations.

Keywords: *Enterococcus*, probiotc, prebiotic, genome, selenoproteins.

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Introduction

Enterococcus genus belongs to the group of lactic acid bacteria (LAB) of the phylum Firmicutes, showing the ability to survive under various environmental conditions (Byappanahalli *et al*., 2012). This genus is an important component of the intestinal microbiota of humans and other animals and is found in commercial products, such as Cernivet® and FortiFlora® (containing *Enterococcus faecium* SF68®, Cerbios -Pharma SA, Switzerland) and Symbioflor® 1 with *Enterococcus faecalis* (Symbiopharm, Herborn, Germany) (Hanchi *et al*., 2018). Many enterococci isolated from fermented dairy products proven to be natural probiotics and have been considered beneficial and safe to the host (Franz *et al*., 2011).

Currently, the role of probiotic bacteria in gut health and functionality of human, livestock animals and pets has been greatly emphasized. The intestinal microbiome has a great importance in human health, promoting intestinal homeostasis, development of the immune system, protection against pathogens and stimulating the production of micronutrients and energy (Clemente *et al*., 2012; Martín and Langella, 2019).

Some *in vitro* assays are recommended to characterize a microorganism with probiotic potential, including adherence to human and/or mucosal epithelial cells, antimicrobial activity against pathogens, ability to decrease the adhesion of pathogens and stimulate the hydrolysis of bile salts (Hill *et al*., 2014). These assays have become the dogma for probiotic characterization, but phenotypic characterization is not enough to provide a full description of probiotic microorganisms. Thus, the study of genomic data obtained by high-throughput DNA sequencing tools may provide novel useful information, expanding the current knowledge on probiotic strains. Genomic analysis may be useful to identify genes related to probiotic properties and to find additional molecules and metabolic routes that contribute to the specific activity of a probiotic strain (Li *et al*., 2018). These genes can codify proteins associated with survival to gastrointestinal tract transit, such as bile salt hydrolases, production of antimicrobial substances like bacteriocins, and beneficial enzymes, such as β-galactosidase (BGL) and inulinase (Ladero *et al*., 2013; Bonacina *et al*., 2017).

In addition to these probiotic characteristics, antioxidant properties play an important role and can be associated to the

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ability of a probiotic to produce selenoproteins. Selenium (Se) is a trace element known primarily for its functions in redox homeostasis as a promising chemo-preventive agent for cancer (Hartfield *et al*., 2006) and because it has beneficial effects associated with probiotic bacteria (Galano *et al*., 2013). The major biological form of Se is selenocysteine (Sec, the 21st amino acid), which is co-translationally inserted into selenoproteins by recoding the UGA codon (Hatfield and Gladyshev, 2002). In bacteria, the mechanism of Sec biosynthesis and its insertion into proteins requires an in-frame UGA codon, a Sec insertion sequence element (SECIS). SECIS is a hairpin structure within the selenoprotein mRNA immediately downstream of the Sec codon encoding the UGA codon (Zhang and Gladyshev, 2005).

Although genome sequences of *Enterococcus* species like *E. faecalis* and *E. faecium* have been largely described (Bonacina *et al*., 2017; Zhong *et al*., 2017), minor information is available for *E. durans* (Li *et al*., 2018). The *E. durans* LAB18S was previously isolated from a typical Brazilian soft cheese and exhibited some desirable probiotic properties *in vitro* (Pieniz *et al*., 2015). In addition, this strain thrives in selenium enriched medium, accumulating this element in the biomass (Pieniz *et al*., 2017). Further research is needed to prove its potential health benefits and application as a probiotic lineage in the industry. Thus, the aim of this study was to characterize the genome of *E. durans* LAB18S strain, searching for relevant genes associated with probiotic properties and selenoproteins, in addition to performing comparative analyzes with *E. durans* genomes from different isolation sites.

Material and Methods

Genomic DNA preparation and high-throughput sequencing

E. durans LAB18S was isolated from soft cheese, was retrieved from the collection of Laboratory of Applied Microbiology and Biochemistry (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil). The strain was maintained as frozen stock cultures in Brain Heart Infusion (BHI, Oxoid) containing 20% (v/v) glycerol. The bacterium was grown in MRS broth (de Man *et al*., 1960) at 37 °C at mid log phase (8 h).

E. durans LAB18S total DNA was extracted with phenol-chloroform following usual procedures and purified using a Genomic DNA Clean & Concentrator (Zymo Research). The quality and quantity of the DNA were assessed by spectrophotometry analysis using NanoDrop™ (Thermo Scientific) and fluorometry (Qubit™; Invitrogen), respectively. DNA fragment libraries were further prepared with 50 ng of DNA using a Nextera™ XT DNA sample preparation kit and sequenced using an Illumina™ MiSeq System (2x250 paired-end reads with the Illumina™ v2 reagent kit), manufacturer's instructions.

After quality checking with FastQC software, reads were trimmed with Geneious software (version 10.2.3) (https://www.geneious.com). The paired-end sequence reads were then assembled by *de novo* assembly using SPAdes 3.9.0 (Bankevich *et al*., 2012), and Geneious software version 10.2.3 followed by template-assisted assembly to the reference *E. durans* KLDS6.0933 (NZ_CP012366).

Gene prediction and bioinformatics analysis

Annotation NCBI Prokaryotic Genome Annotation Pipeline (PGAAP) was employed to identify coding sequences (CDS) based on the best-placed reference protein set. Similarly, to aid the gene prediction and annotation, *E. durans* genome were performed by RAST (Rapid Annotation Subsystem Technology) webservice (https://rast.nmpdr.org). Genes of interest had their annotation refined manually. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession NCVP00000000. The version described in this paper is version NCVP01000000.

Genes involved in the biosynthesis of secondary metabolites were analyzed *in silico* using the antiSMASH algorithm (Medema *et al*., 2011). We then used bSECISearch to predict candidates for bacterial SECIS elements and their putative coding genes with weight scores greater than the cutoff (> 30) in order to analyze the genome of *E. durans* LAB18S for full complement of selenoprotein genes (Zhang and Gladyshev, 2005). BLAST search (tblastn + blastx) was performed at NCBI to filter out false positive elements involved with selenium.

Comparative analysis

Antimicrobial resistance genes were identified using ResFinder 3.2 (Zankari *et al*., 2012) following the thresholds 60% identity over a length of 60% coverage, respectively. VirulenceFinder (Joensen *et al*., 2014) and PlasmidFinder (Caratoli *et al*., 2014) were used to predict potential virulence genes and plasmids, respectively. Identification thresholds were set at 60% identity over a minimum length of 60% for PlasmidFinder, and 85% identity over a length of 60% for VirulenceFinder.

Core genome Single Nucleotide Polymorphism (SNP) tree were performed using Parsnp v1.2 program included in Harvest (Treangen *et al*., 2014). A total of 31 *E. durans* genomes, one draft genome from this study and 30 genomes from previous studies obtained from the NCBI database were used (Table 1). Core genome SNPs of *E. durans* were identified, the reference genome was randomly selected using the parameter '-r!' and recombination regions were used (Treangen *et al*., 2014). An approximately maximum likelihood tree was constructed from concatenated SNPs using FastTree2 (Price *et al*., 2010), and interactive Tree Of Life (iTOL) v4 software (Letunik and Bork, 2019) were used for visualization and edition of the phylogenomic tree.

Phenotypical characteristics

E. durans LAB18S was evaluated for tolerance to acid pH and bile salts, β-galactosidase activity and growth on prebiotic oligosaccharides.

Acid tolerance

The resistance under acid conditions was investigated according to Erkkila and Petaja (2000) with some modifications. *E. durans* LAB18S cells were grown in BHI (Brain Heat Infusion broth; Oxoid) without shaking at 37 °C for 24 h. Then, the culture was standardized at an optical density (OD_{600}) = 1.0 ± 0.05. One milliliter of standardized culture was added into tubes containing 10 mL of sterile BHI broth with the following pH values: 2.0, 3.0, 4.0 and 7.0 (adjusted

with HCl), in which pH 7.0 was used as a control. Viable cell counts were determined after exposure to acidic condition for 0, 1, 2, 3 and 4 h at 37 °C. The experiment was performed in triplicate. Survival cell counts were expressed as log values of colony-forming units per ml (CFU/mL).

Bile tolerance test

Growth in the presence of 0.3% (w/v) oxbile was analyzed as described by Gilliland *et al*. (1984). Overnight grown (16 ± 2 h at 37 °C) assay cultures were centrifuged at 8,000 x g for 15 min at 4 °C and the pellet collected was resuspended in same volume of saline (0.85% NaCl). Fresh BHI broth (5 ml), without ox bile with pH 7 (for control), and BHI broth (5 ml) containing 2.5, 5, 10 and 15 mg/mL of ox bile was inoculated with 250 µl (5%) of cell suspension. The growth was monitored hourly by measuring the OD at 600 nm using spectrophotometer. The survival percentage was calculated as follows: % survival = final (OD) / control (OD) x 100.

β-Galactosidase (BGL) activity

BGL activity was assayed by a modified procedure, based on the method of Hang and Woodams (1994). The source of BGL was a cell-free supernatant of *E. durans* LAB18S culture in BHI broth (Brain Heart Infusion) and sonicated LAB18S cells. Besides, this isolate was grown in BHI broth supplemented with 10 g/L lactose and the same assay was performed. The reaction mixture (200 µL) contained 90 µL of citrate buffer (250 mM, pH 4.5), 10 µL of *p*-nitrophenyl-β-D-galactopyranoside (pNPGal; 4 mg/mL), and 100 µL of the enzyme source. After incubation at 37 °C for 30 min, the reaction was stopped by adding 1 mL of cold sodium carbonate buffer (500 mM, pH 10). The activity of β-galactosidase was estimated spectrophotometrically by reading the absorbance of the liberated *p*-nitrophenol at 405 nm (ε = 18,700). One unit (U) of β-galactosidase activity was defined as the amount of enzyme required for the hydrolysis of 1 µmol of substrate pNPGal per min, under the assay conditions.

Table 1 – Complementary information of *Enterococcus durans* genomes from NCBI.

Species	Strain	GenBank assembly	Genome size (bp)	Contigs	n50
E. durans	18S	GCF_003945985.1	2760363	61	210893
E. durans	4928STDY7071618	GCA_902162045.1	3173223	140	69586
E. durans	4928STDY7071587	GCA_902161685.1	3129748	140	53780
E. durans	4928STDY7071465	GCA_902160745.1	2952049	115	66799
E. durans	4928STDY7071468	GCA_902160735.1	3049809	33	266845
E. durans	4928STDY7071461	GCA_902160695.1	2843396	99	68904
E. durans	4928STDY7071424	GCA_902160425.1	2993992	134	66221
E. durans	4928STDY7071423	GCA_902160385.1	2987662	135	66543
E. durans	4928STDY7071358	GCA_902159875.1	2937777	113	70649
E. durans	4928STDY7071318	GCA_902159725.1	3070184	143	59610
E. durans	4928STDY7071647	GCA_902159525.1	2965835	49	151918
E. durans	4928STDY7071469	GCA_902159215.1	3065464	56	207797
E. durans	4928STDY7071427	GCA_902159205.1	2986615	134	63841
E. durans	4928STDY7071462	GCA_902159195.1	3052558	42	228325
E. durans	4928STDY7071385	GCA_902159095.1	3126814	142	53818
E. durans	NCTC8129	GCF_900447815.1	3259358	6	3126530
E. durans	NCTC8130	GCF_900447695.1	3357395	8	3078716
E. durans	OSY-EGY	GCF_004330425.1	3230625	227	52003
E. durans	am_0171	GCF_004167095.1	3002381	120	55320
E. durans	C11	GCF_004102865.1	2988164	115	54843
E. durans	P16CLA28	GCF_003796805.1	2886365	28	215484
E. durans	AF1132H	GCF_003465125.1	3083830	215	34708
E. durans	FDAARGOS_396	GCF_002554315.1	3395970	4	3104428
E. durans	BDGP3	GCA_002277935.1	2988928	2	2983334
E. durans	F0321E104	GCF_002077535.1	2931215	43	147275
E. durans	NBRC100479	GCF_001544215.1	3017302	122	53575
E. durans	IQ23	GCF_001455455.1	3125512	127	70907
E. durans	KLDS6.0930	GCF_001267865.1	3071879	3	2867090
E. durans	KLDS6.0933	GCF_001267395.1	3071804	3	2867028
E. durans	ATCC6056	GCF_000406985.1	3153755	19	411581
E. durans	IPLA655	GCF_000350465.1	3059052	145	73480

Growth on prebiotic oligosaccharides

E. durans LAB18S cells were grown in BHI without shaking at 37 °C for 24 h. Then, the culture was inoculated (1%, v/v) in individual sterile vials containing M9 medium (5 g/L NH_4Cl, 33.9 g/L Na_2HPO_4, 15 g/L KH_2PO_4, and 2.5 g/L NaCl), added with 10 g/L of either glucose, lactose, FOS or GOS and incubated at 37 °C. The growth was monitored by measuring the OD at 600 nm using spectrophotometer.

Results

Structure and general features of E. durans LAB18S genome

The genome sequence of *E. durans* LAB18S was obtained using the Illumina® MiSeq system, and compared with the complete genome sequence of *E. durans* KLDS6.0933 (GenBank accession number CP012366.1). The complete genome of *E. durans* LAB18S is composed of a chromosome with 2,867,357 bp, GC content of 38%, 2,579 CDSs, 108 RNAs and 180 pseudogenes (Table S1). By assembling the genome, a total of 82 contigs were obtained and a mean coverage of 31.7 x giving reliability to the results. Comparatively, the reference strain (*E. durans* KLDS6.0933) has 2,867,028 bp and the *E. durans* LAB18S genome is slightly larger with additional 329 bp.

The genes were grouped into subsystems through the RAST webservice (Figure S1). In brief, there are 126 genes for cell wall and capsule; 342 genes for carbohydrate transport and metabolism, which contains 17 genes related with fructooligosaccharides (FOS) and raffinose utilization; 63 genes for virulence, disease and defense, which contains adhesion, bacteriocins, resistance to antibiotics and toxic compounds, invasion and intracellular resistance genes; 2 genes for phages and prophages; 58 for membrane transport; 219 for protein metabolism; 6 for dormancy and sporulation, and 69 for stress response.

Genes associated with probiotics properties

The *E. durans* LAB18S genome showed several genes that may be related with probiotic activity (Table 2). It encodes an S-layer protein (LIU RS11695), and two fibronectin-binding proteins (LIURS07910 and LIU RS10480), which may contribute to bacterial adherence. Besides, this genome carries an exopolysaccharide (EPS) cluster that could be related with improved adhesion properties and persistence in the gut. In addition, it also contains genes that can be associated to viability at lower pH (Na^+/H^+ antiporters) and bile salt tolerance (Table 2). In this regard, *E. durans* LAB18S demonstrated ability to survive at pH 3.0 and higher, and up to 15 mg/mL bile salts (Figure 1).

The potential for carbohydrate utilization was also analyzed and genes for fructooligosaccharide (FOS) and disaccharides utilization were found. Besides, the β-galactosidase (BGL) gene was identified in the genome (Table 2). These properties were confirmed by phenotypical assays showing the *E. durans* LAB18S has ability to growth on probiotic oligosaccharides FOS and GOS and produce BGL activity (Figure 2).

Table 2 – Genes associated with potential probiotic properties of *E. durans* LAB18S.

Protein	Gene	Function
Maintenance in the gastrointestinal tract		
S-layer protein	*lbs*	Improves adhesion properties and persistence in the gut
Fibronectin-binding protein	*prtF*	Improves adhesion properties and persistence in the gut
Heat-shock protein 33	*hsp33*	Improves persistence in the gut
EPS cluster	*epsABCDE*	Improves adhesion properties and persistence in the gut
Na^+/H^+ antiporter	*nhaC*	Improves viability at low pH
Cyclopropane-fatty-acyl-phospholipid synthase	*Cfa*	Key protein in bile salt tolerance
Bacteriocins and toxin-antitoxins		
Microcin cluster	*micJ25*	Low molecular mass bacteriocins produced under stress conditions
Enterocin A immunity protein	*entI*	Putative protection against the effect of bacteriocin enterocin A
Colicin V precursor	*cvaC*	Kills sensitive cells by disrupting their membrane potential
Zeta-toxin	*pSM19035*	Inhibits cell wall biosynthesis
Toxin RelE	*relE*	Cleaves translating mRNA in the ribossomal A-site upon aminoacid starvation
Resistance to heavy metals		
Multi-copper oxidase	*cueO*	Provides copper tolerance
Copper-transporting efflux system	*cusCFBA*	Mediates resistance to copper and silver
Cation efflux system protein CzcA	*czcA*	Provides resistance to cobalt, zinc and cadmium
Mercuric reductase	*merA*	Provides resistance to mercury
Carbohydrate utilization		
Raffinose operon regulatory protein	*rafR*	Metabolism of fructooligosaccharides (FOS) and raffinose
Lactose operon	*lacZYA*	Metabolism of lactose and galactose
Maltodextrin phosphorylase	*malP*	Metabolism of maltodextrin and α-1,4-glucans
4-alpha-glucanotransferase	*malQ*	Starch metabolism

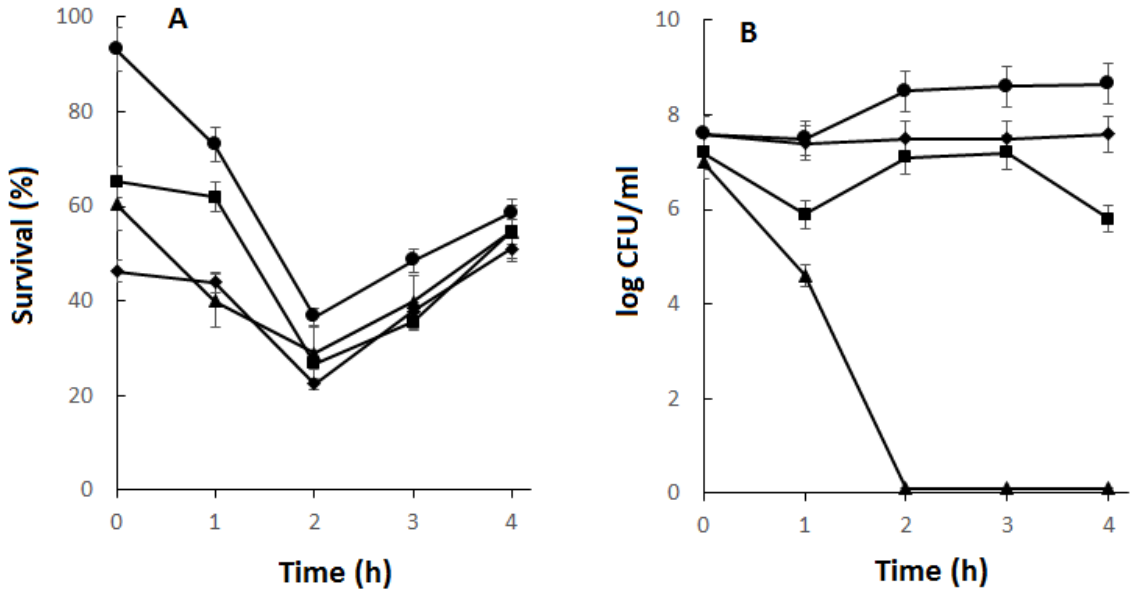

Figure 1 – Tolerance of *E. durans* LAB18S to bile salts and acid pH. (A) The strain was incubated for up to 4 h at 37 °C in the presence of bile salts at concentrations 2.5 mg/mL (●), 5.0 mg/mL (■), 10 mg/mL (▲) or 15 mg/mL (♦). Results are expressed as percentage of surviving cells in comparison to incubation without bile salts used as a control. (B) The strain was incubated for up to 4 h at 37 °C in pH 2 (▲), 3 (■), 4 (♦) or 7 (●). Viable cell counts were monitored at each 1 h interval. Values are the means ± standard deviations of three independent experiments.

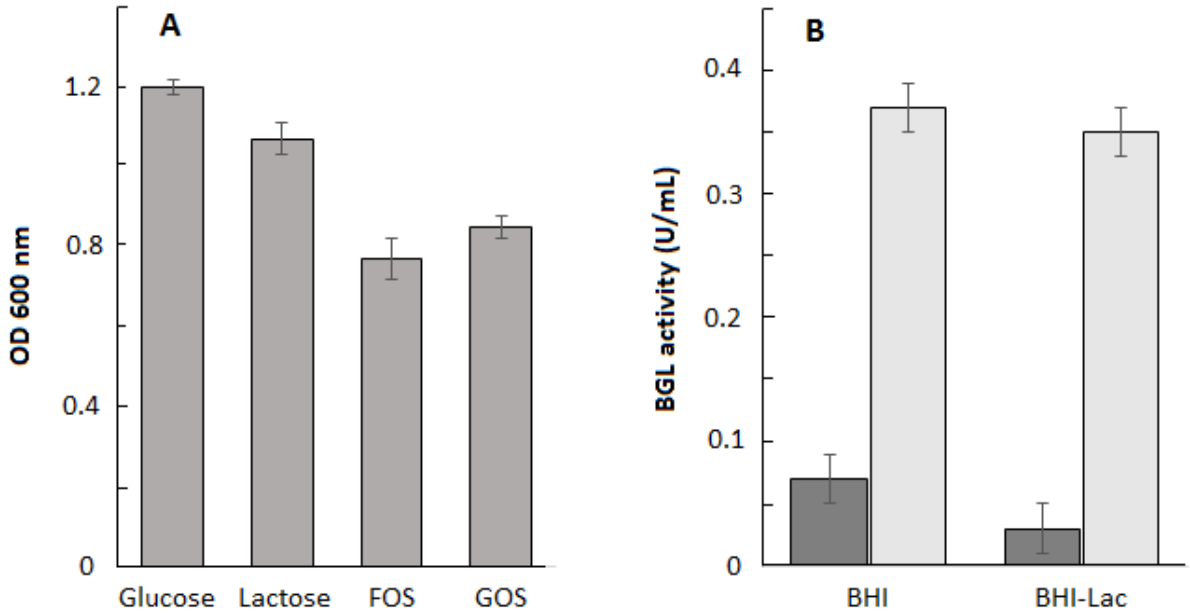

Figure 2 – Phenotypical characteristics of *E. durans* LAB18S. (A) Growth on different carbon sources. The strain was cultivated in medium containing 10 g/L glucose, lactose, FOS or GOS and the cell density was estimated by OD_{600} after 24 h at 37°C. (B) The β-galactosidase (BGL) activity was measured in the cell culture supernatant (dark gray bars) and cell lysates (pale gray bars) after growth in BHI or BHI supplemented with 10 g/L lactose (BHI-Lac). Values are the means ± standard deviations of three independent experiments.

Secondary metabolite analysis revealed the presence of genes associated with colicin V, enterocin A, and the small bacteriocin microcin J25 (Table 2). In agreement, the culture supernatant of *E. durans* LAB18S showed inhibitory haloes ranging 9-10 mm against strains of *Listeria* spp.. Furthermore, two genes of toxin-antitoxin proteins, namely RelE and Zeta-toxin, were also identified. The BLAST algorithm was used to align the deduced colicin V sequence of *E. durans* LAB18S with colicin V and colicin V production protein CvpA from other genera and species. This sequence is quite conserved among different species of *Enterococcus*, *Bacillus* and *Carnobacterium* and strain *E. durans* LAB18S (Figure S2).

Genes related to selenoproteins

The *E. durans* LAB18S genome contains seven genes involved in selenium metabolism (Table 3). Five genes encode typical selenoproteins, namely glutathione peroxidase (*gpx*), thioredoxin reductase (*trxB1, trxB2*), glycine reductase complex selenoprotein B (*grdB*), and peroxiredoxin (*prX*). Another two genes are related with selenium metabolism:

Table 3 – Selenoprotein related genes predicted in *E. durans* LAB18S genome.

Protein	Gene	Function
Selenoproteins		
Glutathione peroxidase	*gpx*	Catalyzes the reduction of H_2O_2; protection against oxidative stress
Thioredoxin reductase	*trxB1*, *trxB2*	NADPH-depended oxidoreductase activity
Glycine reductase complex	*grdB*	Active protein in the peroxidase reaction
Peroxiredoxin	*prX*	Antioxidant enzyme that uses thioredoxin (Trx) to recharge after reducing H_2O_2
Other selenium-related proteins		
L-seryl-tRNA selenium transferase	*selA*	Converts seryl-tRNA(Sec) to selenocysteinyl-tRNA (Sec) required for selenoprotein biosynthesis
Selenocysteine-specific elongation factor	*selB*	Translation factor necessary for the incorporation of selenocysteine into proteins
YggS family pyridoxal phosphate	*yggS*	Decomposes selenocysteine to alanine and elemental Se or H_2Se during selenium metabolism

L-seryl-tRNA selenium transferase (*selA*) and YggS family pyridoxal phosphate (*yggS*).

In the analysis of the selenocysteine insertion sequence (SECIS) element, 1,274 hits were identified as candidates of bacterial SECIS (bSECIS)-like elements. These hits were divided into homologs of previously known selenoproteins (40 sequences) and candidates of selenoproteins (1,234 sequences). Then, optimal bSECIS elements and their predicted putative coding genes were identified as 26 known selenoproteins and 765 unknown bSECIS elements were detected. After the BLAST search was performed to filter out false positives, 12 bSECIS elements involved with selenium were identified. This indicates the ability of the *E. durans* LAB18S to produce selenoproteins.

After the BLAST search to filter out false positives, 12 bSECIS elements involved with selenium were identified. This indicates the ability of *E. durans* LAB18S to produce selenoproteins.

Comparative analysis

Virulence genes were not found in the *E. durans* genomes compared in this study, such as aggregation substance (*agg*), surface adhesins (*esp*, *ace*), sex pheromones (*cob*, *cpd*, *ccf*), D-alanylation of lipoteichoic acid (*dlt*), the lytic enzymes gelatinase (*gelE*) and hyaluronidase (*hyl*), and the toxin cytolysin (*cylA*). Antimicrobial resistance was checked against the ResFinder database and genes associated with tetracycline resistance, namely *tet*(M) and *tet*(O)-like were found in seven genomes of *E. durans*, mostly from fecal origin (Table 4). Only three enterococci under study did not present any plasmids, including *E. durans* LAB18S. Besides the absence of plasmids, these three strains also showed no virulence and antimicrobial resistance genes. The 31 genomes of *E. durans* were clusterized into a phylogenetic tree (Figure 3). *E. durans* LAB18S has been clusterized with isolates NCTC8130, FDAARGOS_396 and ATCC 6056, which are of fecal origin, and NRBC10079, which lacks source information. None of these isolates showed antimicrobial resistance or virulence genes. The presence of plasmids was found in these isolates, excepting for *E. durans* LAB18S (Table 4).

Discussion

Complete knowledge of genome sequences may allow a precise genetic analysis of probiotic bacteria. This includes the genetic features that can be associated with beneficial effects and those potentially associated with undesirable characteristics. The genus *Enterococcus* contains strains associated with severe infections, while other strains form part of the commensal human microbiome of the mouth, skin, and gut. Some strains have probiotic properties, including *E. durans* (Liaskovs'kyĭ *et al*., 2008; Li *et al*., 2018). Interestingly, virulence is very different among enterococci derived from community or hospital environments, which appears to be associated to some strain-specific genetic features (Douillard and de Vos, 2014). Thus, the study of whole genomes is relevant to acquire information on the potential benefits and drawbacks. In this work, the genome of *E. durans* LAB18S isolated from Minas Frescal cheese showed some desirable characteristics for a probiotic strain.

The survival of probiotic bacteria under gastrointestinal tract conditions has been extensively studied. Probiotics, after ingestion, are exposed to the acidic conditions and the activity of digestive enzymes of the stomach. *E. durans* LAB18S is equipped with a gene coding for Na^+/H^+ antiporter, contributing to regulate intracellular pH (Guo *et al*., 2015). The reduction of bacterial survival in the gut may be due to secretion of bile that breaks the microbial cell membrane, and tolerance to bile salt concentrations between 0.15 and 0.5% has been recommended for probiotics (Lavermicocca *et al*., 2008). The gene encoding cyclopropane-fatty-acyl-phospholipid synthase (HUO 05315), present in the genome of *E. durans* LAB18S, might be associated with bile salt tolerance. Comparative proteomic studies on *Lactobacillus plantarum* identified this enzyme as a key protein in bile tolerance (Hamon *et al*., 2011). Bile salt hydrolase identified in the genome of *E. durans* KLDS6.0933, has been also associated with cholesterol removal ability (Li *et al*., 2018).

Adhesive properties can prolong the contact between bacteria and the host and therefore enhance the desired probiotic effect (Botta *et al*., 2014). Although mucus-binding proteins and adhesion genes are absent, *E. durans* LAB18S

Table 4 – Comparative analysis of the presence of virulence genes, antimicrobial resistance genes and plasmids of 31 *E. durans* genomes.

Species	Strain	Origin	Resistance		Virulence	Plasmids						
			tet(M)	*tet*(O)		*rep*1	*rep*2	*rep*4	*rep*11	*rep*18	*rep*US1	*rep*US15
Enterococcus durans	NCTC8129	Unknown										
Enterococcus durans	NCTC8130	Unknown										
Enterococcus durans	P16CLA28	Cloaca (*Gallus gallus*)										
Enterococcus durans	F0321E104	Feces (*Bos taurus*)										
Enterococcus durans	**LAB18S**	Frescal cheese										
Enterococcus durans	KLDS6.0930	Water										
Enterococcus durans	KLDS6.0933	Water										
Enterococcus durans	IQ23	Cheese										
Enterococcus durans	AF1132H	Feces (*Homo sapiens*)										
Enterococcus durans	ATCC6056	Feces (*Homo sapiens*)										
Enterococcus durans	IPLA655	Cheese										
Enterococcus durans	C11	Kimchi										
Enterococcus durans	OSY-EGY	Egyptian hard Cheese										
Enterococcus durans	am_0171	Feces (*Homo sapiens*)										
Enterococcus durans	**BDGP3**	Feces (*Drosophila melanogaster*)										
Enterococcus durans	4928STDY7071618	Feces (*Homo sapiens*)										
Enterococcus durans	4928STDY7071587	Feces (*Homo sapiens*)										
Enterococcus durans	4928STDY7071465	Feces (*Homo sapiens*)										
Enterococcus durans	4928STDY7071468	Feces (*Homo sapiens*)										
Enterococcus durans	**4928STDY7071461**	Feces (*Homo sapiens*)										
Enterococcus durans	4928STDY7071424	Feces (*Homo sapiens*)										
Enterococcus durans	4928STDY7071423	Feces (*Homo sapiens*)										
Enterococcus durans	4928STDY7071358	Feces (*Homo sapiens*)										
Enterococcus durans	4928STDY7071318	Feces (*Homo sapiens*)										
Enterococcus durans	4928STDY7071647	Feces (*Homo sapiens*)										
Enterococcus durans	4928STDY7071469	Feces (*Homo sapiens*)										
Enterococcus durans	4928STDY7071427	Feces (*Homo sapiens*)										

Table 4 – Cont.

Species	Strain	Origin	Resistance		Virulence	Plasmids						
			tet(M)	*tet*(O)		*rep*1	*rep*2	*rep*4	*rep*11	*rep*18	*rep*US1	*rep*US15
Enterococcus durans	4928STDY7071462	Feces (*Homo sapiens*)										
Enterococcus durans	4928STDY7071385	Feces (*Homo sapiens*)										
Enterococcus durans	FDAARGOS_396	Feces (*Homo sapiens*)										
Enterococcus durans	NBRC 100479	Unkwnown										

Black boxes indicate the presence of resistance genes, dark gray boxes indicate the presence of plasmids, light gray lines indicate the strains that were negative for virulence genes, antimicrobial resistance genes and plasmids.

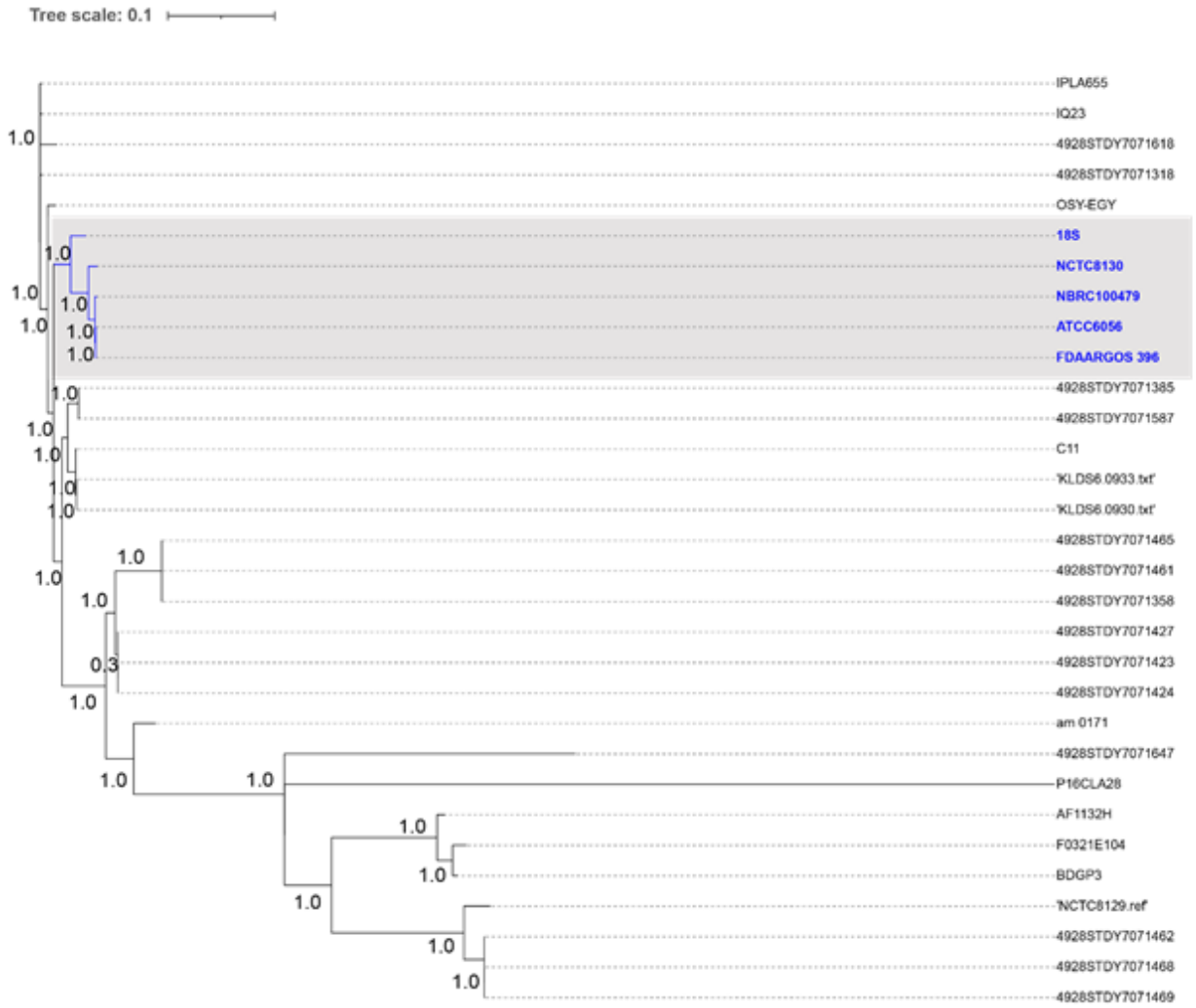

Figure 3 – Core genome SNP tree of 31 *E. durans*. The phylogenomic reconstruction was built using Parsnp and Fast tree 2. The percentage of the reference that is covered by core alignments was above 60%. Strains related with *E. durans* LAB18S are highlighted in the grey box.

genome presents an S-layer protein (LIURS 11695), and fibronectin-binding proteins (LIURS 07910 and LIURS 10480), which may contribute to bacterial adherence. In addition, a gene encoded aggregation-promoting factor (LIURS 03835) was also identified, suggesting that this strain can bind to receptors in the gut environment (Senan *et al*., 2015). Some EPS produced by probiotics can improve its adhesion properties and its persistence in the gut (Ruas-Madiedo *et al*., 2006), and the *E. durans* LAB18S genome carries an EPS cluster. All these genetic elements corroborate to the potential adhesive characteristics of *E. durans* LAB18S.

The production of bacteriocins by probiotic strains has been recognized as a desirable feature (Hegarty *et al*., 2016). Analysis for secondary metabolite clusters of *E. durans*

LAB18S genome revealed the presence of genes associated with the synthesis of microcin J25, colicin V and enterocin A, which may endow competitive advantages to combat pathogenic bacteria. The inhibitory activity of *E. durans* LAB18S against *Listeria* spp. agrees with the typical antilisterial activity of *Enterococcus* bacteriocins (Rocha *et al*., 2019). Colicin V is produced by many strains of *Escherichia coli* and its precursor peptide is similar to some bacteriocins of the *Enterobacteriaceae* family, which fits the definition of class II bacteriocins from Gram-positive bacteria (Håvarstein *et al*., 1994). The transfer of genes encoding bacteriocins from Gram-negative bacteria, such as colicin V, to food-grade lactic acid bacteria (LAB) host has been described (Langa *et al*., 2017). In this regard, Horn *et al*. (2004) were the first to show the coproduction of nisin and colicin V in *Lactococcus lactis* as a host enhancing the antimicrobial activity against both Gram-positive and Gram-negative bacteria.

The *E. durans* LAB18S genome contains genes of toxin-antitoxin systems, which have been associated with survival under stress conditions (Fernández-García *et al*., 2016). Zeta-toxin is bactericidal for *Bacillus subtilis* and bacteriostatic for *E. coli*, while the toxin RelE degrades mRNA at specific sequences when it is bound to the ribosomal A site (Pedersen *et al*., 2003). As a concern, the presence of omega/epsilon/zeta toxin-antitoxin system seems to stabilize plasmids carrying *vanA* in *E. faecium* and *E. faecalis* resistant to vancomycin (Fernández-Gracía *et al*., 2016).

Genes related to the metabolism of molecules associated with prebiotic properties were also identified. The strain LAB18S presented genes related to the use of frutooligosaccharides (FOS), a non-digestible dietary component that undergo selective colonic fermentation. FOS cause significant changes in the composition of the gut microbiota, increasing the numbers of potentially health-promoting bacteria and reducing potentially harmful species, respectively (Slavin, 2013). Cultivation of *E. durans* LAB18S on FOS revealed an increased number of overexpressed proteins, including L-asparaginase and arginine deiminase, two enzymes of clinical importance for the treatment of cancer (Comerlato *et al*., 2020). The BGL gene was also detected in the genome. This enzyme is produced by several LAB with both hydrolase and transglycosylase activities, beneficial from technological and health point of views for applications as probiotic cultures in dairy industry or synthesis of prebiotic GOS (Meira *et al*., 2012). Because they are not digested by humans, GOS represents a rich source of substrate for probiotic organisms, including *Enterococcus* (Park and Oh, 2010).

Selenium is an essential metalloid required for the expression of selenoproteins. It was previously observed that *E. durans* LAB18S bioaccumulates selenium when grown in medium containing Na_2SeO_3 (Pieniz *et al*., 2017). Selenium was mainly found as selenoproteins, reaching 2.6 mg/g biomass. Selenoprotein genes, to insert SEC into UGA codons, have developed a stem-loop shaped RNA structure, called SECIS. These SECIS elements are located downstream of the Sec UGA codons in bacteria. Through a computer program we were able to identify conserved structural characteristics of these structures. Bacterial SECISearch recognize a bacterial consensus SECIS element in sequence databases and the results indicate the ability of the *E. durans* LAB18S to produce selenoproteins. Selenium antioxidant properties stimulates the activity of some antioxidant enzymes, such as glutathione peroxidase, thioredixin reductase, and iodothyronine deiodinase, which contain selenocysteine (Lin *et al*., 2015). One biological form of Se has been identified as selenocysteine (Sec) (Hatfield and Gladyshev, 2002), but selenium could form selenomethionine (SeMet) by replacing sulfur in methionine and thus could be incorporated into proteins instead of methionine (Schrauzer, 2000). Although some microorganisms are capable of transforming high concentrations of selenium into selenate and selenite, only few studies on selenite uptake and biotransformation have been conducted with probiotic microorganisms (Zhang *et al*., 2009; Pieniz *et al*., 2017). Comparative genomic analyses were performed in order to identify new genes associated to Se utilization in *Enterococcus faecalis*. Seven candidate genes for selenoproteins were identified (Zhang *et al*., 2008), the same number found in this study.

Enterococci may have resistance to various antibiotics, due to their innate resistance to widely used antibiotics such as penicillin or to their ability to easily acquire antimicrobial resistance, especially by horizontal gene transfer. Horizontal transfer of antimicrobial resistance in enterococci has been associated with mobile genetic elements, such as plasmids and transposons (Palmer *et al*., 2012; Beukers *et al*., 2015). Resistance to tetracycline in *Enterococcus* spp. is frequently associated with the resistance genes *tet*(M) and *tet*(O) (Roberts, 2005; Anderson *et al*., 2016). Recently, a PCR-based plasmid classification system has been established by targeting specific replicon initiation genes (*rep*) of plasmid DNA. Rep-family, already found in the genus *Enterococcus*, may confer multiple antibiotic resistance as well as the mechanism of stabilization of toxin-antitoxin plasmids (Zankari *et al*., 2012; Bonacina *et al*., 2017). The absence of such genetic elements in *E. durans* LAB18S reinforce its promising as probiotic strain. Another recent study concludes that a cheese isolate *E. faecalis* does not represent a substantial reservoir of antimicrobial resistance and virulence when compared to clinical strains (Silvetti *et al*., 2019). The *E. durans* LAB18S genome was more closely to human feces genomes, which can be explained in part because enterococci are enteric bacteria commonly associated with the gastrointestinal tract of animals. In this regard, many probiotic lineages have been identified from animals or human feces (Hanchi *et al*., 2018; Nagpal *et al*., 2018; Bazireh *et al*., 2020).

In summary, the genome of *E. durans* LAB18S presents a variety of genes that can be associated with probiotic properties, such as adhesion properties, viability at lower pH, bile salt tolerance, production of bacteriocins, and utilization of prebiotic molecules. Besides, this strain presents genes encoding for known selenoproteins, which should contribute to the antioxidant properties. In comparison with other *E. durans* genomes, *E. durans* LAB18S was the only food isolate with absence of plasmids, virulence and antimicrobial resistance genes. *E. durans* LAB18S exhibited a probiotic potential and its potential health benefit and application as probiotic strain in the feed industry merits future investigation. This work significantly improved the knowledge on the genetic characteristics of this promising strain.

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

CBC performed the experiments and data analysis and wrote the manuscript, JP and FMS performed data analysis and writing, ACR, AMPV and FQM, performed experiments and data analysis, AB conceived the idea, participated in design and coordination, and secured funding. All authors read and approved the final manuscript.

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Supplementary material

The following online material is available for this article:

Table S1 – General genome features of *E. durans* LAB18S compared with *E. durans* KLDS6.0933.

Figure S1 – *E. durans* LAB18S genes grouped into subsystems by RAST.

Figure S2 – Multiple sequence alignment of colicin V gene from *E. durans* LAB18S.

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