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, probiotic, 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®, Cebrios -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
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.

Materials 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 broth; Oxoid) without shaking at 37 °C for 24 h. The bacterium 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 broth 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 hSEECISearch 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 (Jøensen et al., 2014) and PlasmidFinder (Carattoli 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 ‘-t!’ 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 Heart Infusion broth; Oxoid) without shaking at 37 °C for 24 h. Then, the culture was standardized at an optical density (OD600) = 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 oxbile with pH 7 (for control), and BHI broth (5 ml) containing 2.5, 5, 10 and 15 mg/mL of oxbile 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_902160895.1  | 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_000447815.1  | 3259358          | 6       | 3126530 |
| *E. durans* | NCTC8130   | GCF_000447695.1  | 3557395          | 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      | GCF_002277935.1  | 2988928          | 2       | 283334  |
| *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* | KLD6S0.930 | GCF_001267865.1  | 3071879          | 3       | 2867090 |
| *E. durans* | KLD6S0.933 | 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 NH₄Cl, 33.9 g/L Na₂HPO₄, 15 g/L KH₂PO₄, 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.

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 | fnbF | Improves adhesion properties and persistence in the gut |
| Heat-shock protein 33 | hsp33 | Improves persistence in the gut |
| EPS cluster | epsABCD1E | Improves adhesion properties and persistence in the gut |
| Na⁺/H⁺ antiporter | nhaC | Improves viability at low pH |
| Cyclodextrin-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 | entl | 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 ribosomal 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 |

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 (LIU RS07910 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).
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:
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, cef), D-alanylation of lipoteichoic acid (dlt), the lytic enzymes gelatinase (gelE) and hyaluronidase (hyl), and the toxin cytolsyn (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 clustered into a phylogenetic tree (Figure 3). E. durans LAB18S has been clustered 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’kyi 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 (Lavernicocca 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 | Plasmids |
|------------------|---------------|---------------------------------|------------|----------|
| *Enterococcus durans* | NCTC8129     | Unknown                         | tet(M)     |          |
| *Enterococcus durans* | NCTC8130     | Unknown                         | tet(O)     |          |
| *Enterococcus durans* | P16CLA28     | Clouca (Gallus gallus)          | rep1       |          |
| *Enterococcus durans* | F0321E104    | Feces (Bos taurus)              | rep2       |          |
| *Enterococcus durans* | LAB18S       | Frescal cheese                  | rep4       |          |
| *Enterococcus durans* | KLDS6.0930   | Water                           | rep11      |          |
| *Enterococcus durans* | KLDS6.0933   | Water                           | rep18      |          |
| *Enterococcus durans* | IQ23         | Cheese                          | repUS1     |          |
| *Enterococcus durans* | AF1132H      | Feces (Homo sapiens)            | repUS15    |          |
| *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* | 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 |
|---------------|----------------|-------------------|------------|-----------|----------|
| Enterococcus  | 4928STDY7071462| Feces (Homo sapiens) | tet(M)     | rep1      | repUS1   |
| durans        | 4928STDY7071385| Feces (Homo sapiens) | tet(O)     | rep2      | repUS15  |
| Enterococcus  | FDAARGOS_396   | Feces (Homo sapiens) | rep4       | rep11     |          |
| durans        | NBRC 100479    | Unknown           | rep18      | rep15     |          |

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.

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 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.
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-Garcia 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-Gracia et al., 2016).

Genes related to the metabolism of molecules associated with probiotic properties were also identified. The strain LAB18S presented genes related to the use of fructooligosaccharides (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 culturites 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 Na₂SeO₃ (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, thioredoxin reductase, and thyroid hormone deiodinase, which contain selenocysteine (Lin et al., 2015). One biological form of Se has been identified as selenocysteine (Sec) (Hatfield and Gladyszew, 2002), but selenium could form selenomethionine (SeMet) by replacing sulfur in methionine and thus could be incorporated into proteins instead of methionine (Schrauer, 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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