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Safety assessment and functional properties of four enterococci strains isolated from regional Argentinean cheese

Gabriela P. Martino[a,b], Martín Espariz[a,b], Gabriel Gallina Nizo[a,b], Luis Esteban[c], Víctor S. Blancato[a,b,*], Christian Magni[a,b,*]

[a] Laboratorio de Biotecnología e Inocuidad de los Alimentos, Facultad de Ciencias Bioquímicas y Farmacéuticas (Universidad Nacional de Rosario) – Municipalidad de Granadero Baigorria, Sede Suipacha 590, Rosario, Argentina
[b] Laboratorio de Genética y Fisiología de Bacterias Lácticas, Instituto de Biología Molecular y Celular de Rosario - IBR, sede FCByF, CONICET – UNR, Argentina
[c] Facultad de Ciencias Médicas, Universidad Nacional de Rosario, Santa Fe 3000, Rosario, Argentina

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ABSTRACT

The members of the *Enterococcus* genus are widely distributed in nature. Its strains have been extensively reported to be present in plant surfaces, soil, water and food. In an attempt to assess their potential application in food industry, four *Enterococcus faecium* group-strains recently isolated from Argentinean regional cheese products were evaluated using a combination of whole genome analyses and *in vivo* assays. In order to identify these microorganisms at species level, *in silico* analyses using their newly reported sequences were conducted. The average nucleotide identity (ANI), *in silico* DNA-DNA hybridization, and phylogenomic trees constructed using core genome data allowed IQ110, GM70 and GM75 strains to be classified as *E. faecium* while IQ23 strain was identified as *E. durans*. Besides their common origin, the strains showed differences in their genetic structure and mobile genetic element content. Furthermore, it was possible to determine the absence or presence of specific features related to growth in milk, cheese ripening, probiotic capability and gut adaptation including sugar, amino acid, and peptides utilization, flavor compound production, bile salt tolerance as well as biogenic amine production. Remarkably, all strains encoded for peptide permeases, maltose utilization, bile salt tolerance, diacetyl and tyramine production genes. On the other hand, some variability was observed regarding citrate and lactose utilization, esterase, and cell wall-associated proteinase. In addition, while strains were predicted to be non-human pathogens by the *in silico* inspection of pathogenicity and virulence factors, only the GM70 strain proved to be non-virulent in *Galleria mellonella* model. In conclusion, we propose that, in order to improve the rational selection of strains for industrial applications, a holistic approach involving a comparative genomic analysis of positive and negative features as well as *in vivo* evaluation of virulence behavior should be performed.

1. Introduction

Enterococci are one of the most controversial microorganisms of the Lactic Acid Bacteria (LAB) group. They have remarkable ecological adaptability as they can be isolated from water, the gastrointestinal tract (GT) of mammals and insects, soil, plant surfaces, water and food products. This is probably due to their ability to grow in adverse conditions, such as high temperatures, low pH or high salinity (Lebreton et al., 2014). The genus was originally divided into subgroups according to the 16S rRNA sequence similarity; among these we can find the *E. faecium* group which gathers four closely related species: *E. faecium*, *E. hirae*, *E. mundtii* and *E. durans* (Dworkin and Falkow, 2006). Of the four species, only *E. faecium* has been largely studied because of the increasing number of hospital-acquired infection cases caused by this bacterium (Hidron et al., 2008). This may be due to the wide genomic diversity of strains with several antibiotic resistances and virulence factors. On the other hand, *E. hirae*, *E. mundtii* and *E. durans* rarely cause human infections (Agudelo Higuita and Huycke, 2014).

Nevertheless, *E. faecium* group-strains are also known for their beneficial characteristics regarding food fermentation and preservation and for their contribution to human health (Byappanahalli et al., 2012). In fact, *E. faecium* and *E. durans* strains have been proposed and used as probiotics (Franz et al., 2011; Guo et al., 2016; Natarajan and Parani, 2015) and as adjunct starters to improve sensorial properties of dairy products (Gardiner et al., 1999; Gotova and Dimitrov, 2015; Sarantinopoulos et al., 2002). Although *E. faecalis* and *E. faecium* have traditionally been the main species found in milk derivatives, *E. durans* has also been isolated from cheese (Ogier and Serror, 2008). Indeed, *E.*

* Corresponding authors at: Instituto de Biología Molecular y Celular de Rosario (IBR), Suipacha 590, Rosario, Argentina.
E-mail addresses: blancato@ibr-conicet.gov.ar (V.S. Blancato), magni@ibr-conicet.gov.ar (C. Magni).

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faecium group-strains are industrially relevant in cheese production because of their contribution to flavor through proteolytic and lipolytic activities and the generation of aroma compounds (C4 metabolites such as diacetyl acetoin or 2, 3-butanediol) (Giraffa, 2003; Martino et al., 2016). Although the use of enterococci in industrial production remains controversial, in many countries (especially in Greece, Italy, France, Spain and Portugal) they are considered essential for flavor development, as their natural presence in milk gives traditional cheese specific flavor notes (Foulquié Moreno et al., 2006; Martino et al., 2016).

Our laboratory isolated several enterococci strains from regional cheeses and analyzed their ability to produce C4 compounds (diacetyl, acetoin) from citrate, which is naturally present in milk. The capacity to take up and use this metabolite gave Cit^+ strains an advantage over Cit^- strains with regards to C4 production (Martino et al., 2016). Also, the genomic arrangements of *cit* gene clusters were studied and it was found that this metabolic pathway was not always present, nor did it have a constant organization (Martino et al., 2016). Remarkably, neither the genetic variability concerning citrate metabolism nor the relationship between citrate fermentation and aroma production have been previously described in *E. faecium*. In the present study, a comparative genomic analysis of four enterococci strains isolated from Argentinean regional cheeses was performed (Martino et al., 2016; Martino et al., 2016; Suarez et al., 2012). In traditional manufacturing, these naturally occurring strains can pose a threat to human health. Thus, a high throughput sequencing technology complemented with *in vivo* experiments regarding antibiotic resistances profiles and pathogenic behavior could be a powerful tool for the rational selection of strains meant to be used in cheese industry.

2. Material and methods

2.1. Nucleotide sequence data, assembly and annotation

All the genomes used in this study are available at GenBank database (Benson et al., 2013) or EZBioCloud genome database (www.ezbiocloud.net). The genome sequence of GM70, GM75, IQ23 and IQ110 had been previously obtained and preliminary characterized (Martino et al., 2016). A procedure to improve these sequences was performed essentially as described in Repizo et al. (2014). Briefly, BLASTn analyses (all *versus* all) were performed and those contigs shorter than 1000 bp, which were also contained in a longer contig (higher than 99% identity), were deleted. The remaining contigs were ordered and oriented with Mauve version 2.3.1 against a close relative strain (Darling et al., 2004). Genome annotation was performed using RAST (Overbeek et al., 2014).

2.2. Phylogenetic analyses

The evolutionary history of the strains was inferred using Gegenees (Ågren et al., 2012) and through a Multi Locus Sequence Analysis as previously described (Espariz et al., 2016). In order to do so, the genome sequences of GM70, GM75, IQ23 and IQ110 as well as of 33 enterococci type strains were selected. Gegenees comparison was carried out using fragmented all-all comparison, with 200 pb of fragment size and 100 pb of step size. The resulting data were analyzed with Splitstree software (Huson and Bryant, 2006). MLSA was performed using 33 core genes defined by bidirectional best-hit BLAST searches with an *E*-value of $1E^{-30}$. These were individually aligned with ClustalW, concatenated with Perl script catfasta2phyml.pl and trimmed using Gblock 0.91b (Castresana, 2000). The best substitution model and the higher log likelihood tree were selected with Mega5 (Tamura et al., 2011). The reliability of the inferred tree was tested by bootstrapping with 1000 replicates. ANI values were calculated as described by Repizo et al. (2014) using the JSpecies software with BLAST algorithm (Richter and Rossello-Mora, 2009). The estimates of *in silico* DNA-DNA Hybridization (*is*DDH) were made using the Genome Blast Distance Phylogeny (GBDP) 2.0 Web server (http://ggdc.dsmz.de/distcalc2.php) and whole sequence length formulae described by Meier-Kolthoff et al. (2013). Genomic synteny was analyzed with Mauve version 2.3.1 (Darling et al., 2004).

2.3. Insertion sequences (IS), prophages and genomic islands

IS were searched in genomes using ISfinder blast search tool (Siguier et al., 2006) and the database available at http://www-is.biotoul.fr. The IS whose e-values resulted 0.0 (all of them were under 9×10^{-165}) were selected and manually located in the genomes. Putative prophages were searched using Phaster application (Arndt et al., 2016). Genomic islands were predicted using IslandViewer web tool (Dhillon et al., 2015).

2.4. Resistances, virulence factors and CRISPR-Cas predictions

ResFinder tool (Zankari et al., 2012), RAST and the Comprehensive Antibiotic Resistance Database (CARD, (Jia et al., 2017)) were used for the fast identification of antibiotic resistance genes. VirulenceFinder tool (Kleinheinz et al., 2014) and PathogenFinder (Cosentino et al., 2013) were used to predict common Gram-positive virulence factors and pathogenicity, respectively. CRISPR sequences and Cas encoding genes were explored using CRISPRFinder tool (Grissa et al., 2007) and RAST. In order to improve the gene mining in the genomes under study, well-known antibiotic resistance genes, virulence factors, and other relevant genes from LAB (described in text) were used as query in BLAST searches.

2.5. Antibiotic susceptibility

Minimum inhibitory concentrations (MICs) were tested by using Vitek®2 (bioMérieux) antibiotic susceptibility analyzer according to the manufacturer's instructions (software version 5.01 and AST-GP2 card). A service provided by Laboratorio de Microbiología del Hospital Escuela Eva Perón (FBioyF-UNR), Granadero Baigorria, Santa Fe, Argentina.

2.6. G. mellonella killing assay

G. mellonella killing assays were performed using 16 larvae per group. Each group was inoculated with PBS suspensions of the enterococci or *L. lactis* CRL264 strains at 1×10^7 or 9×10^6 CFU/larva; 72 h post-inoculation, Kaplan-Meier survival curves were constructed using R Software. LogRank test and Holm-Sidak multiple comparison test were used to estimate differences (Rich et al., 2010). *P* value was set at 0.05.

2.7. Phenotypic determinations of active metabolic pathways

The pattern of carbohydrate fermentation was determined by using the API 50 CH kit (bioMérieux, France) according to the manufacturer's instructions.

Diacetyl and acetoin (C4 compounds) production was determined by Voges Proskauer (VP) qualitative test. Exponentially growing cultures were diluted (initial OD_{600} of 0.1) in LB broth or LB supplemented with glucose 30 mM, or citrate 15 mM, at an initial pH value of 7. After 5 h of incubation at 37 °C, the presence of diacetyl and acetoin in the supernatant (SN) of cultures was determined as follows: 1 ml of SN was mixed with 0.6 ml of α-naftol 5% w/v of ethanol 96% and 0.2 ml of potassium hydroxide 40% w/v. The acetoin present in each culture was oxidized in such conditions to form diacetyl. The diacetyl present in the culture reacted with peptone from LB to produce color. Red color development was considered a positive reaction.

Biogenic amine determination was performed with an adaptation of Bover-Cid and Holzapfel decarboxylase detection medium (Bover-Cid