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

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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.*

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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

Table 1
General features of the isolated enterococci.

		IQ23	GM75	IQ110	GM70
Phenotype		Cit^+ Agg^+	Cit^+ Agg^-	Cit^- Agg^-	Cit^+ Agg^-
Genomic size		3,123,992	2,848,961	2,757,341	2,696,915
%GC		37.7	38.1	37.9	38.0
CDS		3069	2921	2714	2741
ANI		99.9[b]	94.7[a]	98.9[a]	98.6[a]
% isDDH		96[b]	70[a]	88[a]	84[a]
IS		5	5	7	10
Prophages		6[c]	3[c]	3[c]	3[c]
Virulence factors		1	5	4	4
Sugar utilization	D-Ribose	+	+	+	+
	D-Galactose	+	+	+	+
	D-Fructose	+	+	+	+
	D-Maltose	+	+	+	+
	D-lactose	+	+	+	+
	D-Trehalose	+	I	+	+
	L-Rhamnose	−	−	I	−
	L-Arabinose	−	I	+	+
	D-Arabinose	−	−	−	−
Tyrosine decarboxylase activity		+	−	+	−

[a] Relative to *E. faecium* NRRL B-2354.
[b] Relative to *E. durans* IPLA 655.
[c] Including incomplete prophages.

and Holzapfel, 1999). The composition of the medium remained the same, except for the Tween 20, $MgSO_4$, $MnSO_4$, $FeSO_4$, ammonium citrate, thiamine and pyridoxal phosphate which were removed from such medium. Tyrosine was added to the plates at 1% w/v. Purple color development was considered a positive reaction.

3. Results

3.1. General features of the Enterococcus genomes and phylogenomic analyses

The four *Enterococcus* strains evaluated in the present study were isolated from Argentinean cheeses and selected according to their citrate fermentation and aggregation properties summarized in Table 1 (Martino et al., 2016; Suarez et al., 2012). Complete genomic sequences of GM70, GM75, IQ23 and IQ110 strains have been recently determined, assembled, and ordered in a single sequence for each strain (Martino et al., 2016).

The general genomic features and RAST's CDS classification in subsystems for each strain are presented in Table 1. No differences were found in the basic aspects of the predicted general metabolic pathways such as DNA and RNA metabolism, protein metabolism, cell cycle and division and stress response. However, major differences were observed in the predicted prophages, transposable elements or plasmid sequences and carbohydrates transport and utilization routes (see details below).

The phylogenomics of enterococci strains was analyzed through two approaches. First, a Gegenees-generated tree comparing whole genome sequences with 33 representative strains from *Enterococcus* genus (Fig. 1A); secondly, a MLSA carried out with all core genes of those strains, as described in Material and Methods (Fig. 1B). As shown in Fig. 1, GM70 (red dot), GM75 (yellow dot) and IQ110 (blue dot) strains clustered with *E. faecium* DO and NRRL-B2354 strains, whereas IQ23 (green dot) strain grouped with *E. durans* IPLA655 and ATCC6056 strains. To further analyze the taxonomic identity of the strains, *is*DDH and ANI values were calculated for each strain under study and its closest relative (according to phylogenomic data) (Table 1). As expected, ANI values for IQ110, GM70 and GM75 compared to *E. faecium* strain were higher than the 94% accepted as a threshold for species designation (Konstantinidis and Tiedje, 2005), while values over 99% were obtained for IQ23 against *E. durans* type strains. *is*DDH values reflected the same relationships between strains (Goris et al., 2007). Therefore, our phylogenomic analysis indicated that the strain IQ23 previously described as a member of *E. faecium* species (Martino et al., 2016; Martino et al., 2016) actually belongs to the *E. durans* species. The discrepancy between the taxonomic identity assignation in the RefSeq and GenBank available genomes and the one established with whole genome analyses was previously reported for other Firmicutes (Espariz et al., 2016).

Synteny at genomic level among GM70, GM75, IQ23 and IQ110 strains and reference enterococci is shown in Fig. 2. The genomic arrangement of *E. durans* IQ23 and *E. durans* KLDS 6.0930 (a Chinese cream-isolated strain (Guo et al., 2016)) showed a high degree of conservation. This can be noticed by the few locally collinear blocks (LCBs; 6) revealed by Mauve alignments (Fig. 2A). On the other hand, a higher number of LCBs (29) were found for *E. faecium* GM70, GM75, and IQ110 strains against the non-pathogenic *E. faecium* strain NRRL-B2354 (Fig. 2B; Kopit et al., 2014). This correlates with the structural genomic diversity present in the *E. faecium* species (Martino et al., 2016).

3.2. Industrial and probiotic features

Although the use of enterococci in food industry is still controversial, they are naturally present in dairy products and have positive characteristics, which make them potentially applicable in fermentative processes and as probiotics. In a detailed inspection of the genomic annotation, several functions of interest were found. A full list of such functions is presented in Supplementary Table 1S.

3.2.1. Sugar utilization

Lactose uptake and metabolism are essential in LAB intended to be used in milk fermentation (Table 1). API test indicates that all the strains are capable of using D-lactose. Accordingly, RAST annotates several genes related to lactose and galactose consumption. Lactose phosphotransferase system repressor LacR, operon *lacABCD* and the three components of lactose uptake PTS system are annotated for IQ23, IQ110, and GM70 strains (Fig. 2, black arrow and Supplementary Table 1S). While RAST also identified these functions for GM75 strain, a detailed inspection of the cluster reveals partial sequences or a truncated version of the genes; thus, the PTS uptake system, disruption of the molecule and subsequent conversion to tagatose (common feature among LAB) are probably not functional. However, all of the strains have a permease (*lacS* in *E. faecium* strains and *lacY* in *E. durans* IQ23). Besides, *lacZ* encoding beta galactosidase activity is also present in all four strains. All of these features could account for GM75 positive reaction in API test. D-Galactose is also consumed by all of the strains and *galM*, *galA*, *galE*, *galT*, *galK* and *galR* genes were annotated in all cases (Table 1 and Supplementary Table 1S).

Maltose and maltodextrins are common sugars in the GT, the utilization of these as carbon sources is an important property in commensal bacteria, especially in probiotic ones. Maltose gave a positive reaction in API test, which could be due to the presence of *malT*, *malB*, *malM*, *malP* and *malR* (Joyet et al., 2017; Mokhtari et al., 2013; Sauvageot et al., 2017) (Table 1 and Supplementary Table 1S). Maltodextrine uptake and utilization through *malEFG*, *malA* and neopullulanase gene *aglB* could be possible in the enterococci isolates studied. Additionally, all three *E. faecium* strains have YcjW regulon, which is involved in maltodextrine use (Fig. 2, yellow arrow).

Furthermore, *ara* genes for arabinose uptake and utilization (*araK*, *araD*, *araA*, *araT* and *araR*) were found in IQ110, GM70 and GM75; however, all of them are absent in IQ23, strain supporting the observed negative reaction with L-arabinose in API test (Table 1 and Supplementary Table 1S).

Besides these common carbohydrates, only IQ110 and GM70 strains are predicted to use trehalose since the PTS uptake system and hydrolase genes are present in their genomes. Nevertheless, all the strains are

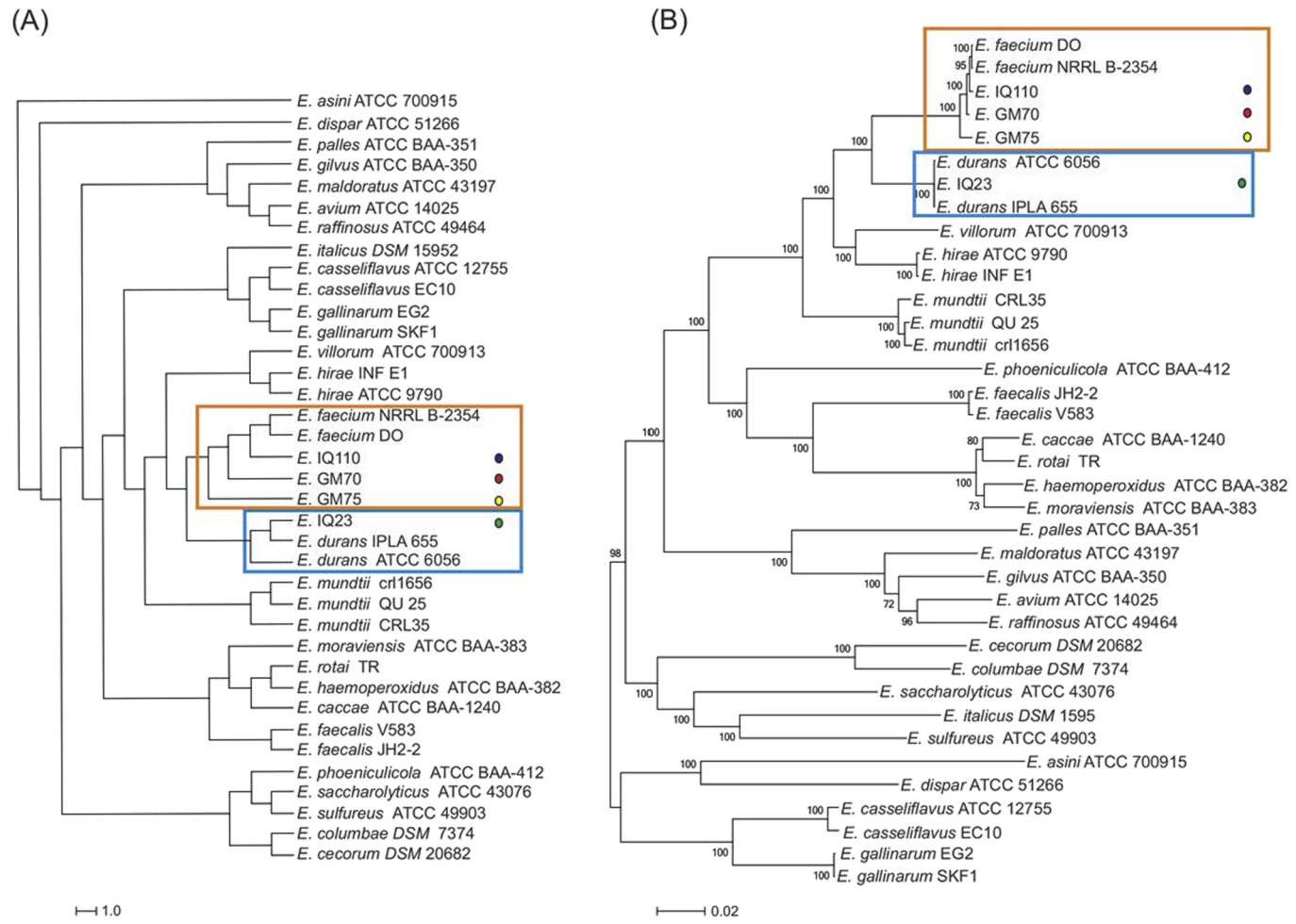

Fig. 1. Phylogenomic analysis of IQ23, GM70, GM75, and IQ110. (A) Gegeenes generated tree using complete genome sequences of the indicated strains. (B) 33 BLAST core genes were individually aligned, concatenated and trimmed, resulting in a final alignment. The evolutionary history of the indicated strains was inferred with Mega5. Reliability of the inferred tree was tested by bootstrapping with 1000 replicates. The orange rectangle highlights GM70 (red dot), IQ110 (blue dot) and GM75 strain (yellow dot) while blue rectangles highlights IQ23 strain (green dot). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

somehow capable of using these sugars (Table 1). The detected fructose and ribose utilization could be associated with the presence of fructose uptake and phosphorylation system and with a ribose ABC transporter and kinase in all the strains. IQ110 and GM75 have *rha* genes, but only IQ110 gave an intermediate reaction when tested *in vivo* (Table 1 and Supplementary Table 1S).

3.2.2. Casein utilization

Protein degradation is one of the most important activities in fermentative LAB. Casein molecule breakdown to form small peptides is one of the main processes involving flavor and texture development (Savijoki et al., 2006). Therefore, genes for proteinases, transporters, permeases and peptidases involved in casein degradation were searched in the four genomes (Fig. 2, purple and light blue arrow). The complete list of the searched genes involved in protein and peptide disruption can be found in Supplementary Table 1S. Curiously, GM70 is the only strain which has a proteinase similar to the cell-envelope proteinase PrtR from *Lactobacillus rhamnosus* necessary for casein disruption (Fig. 2, white arrow). The hydrolysis of casein was tested by using LB agar containing 1.5% of skimmed milk for caseinolytic activity but no clear halos were detected in the four strains (not shown).

3.2.3. Lipid metabolism

Esterase and lipase activities are also important for flavor development in cheese not only *via* milk fat hydrolysis, but also for ester synthesis *via* alcoholysis (Holland et al., 2005). Free fatty acids can provide typical cheesy flavor, while esters give fruity flavor notes which help mask the undesirable effects of short-chain fatty acids. RAST annotates four, three, three, and two esterases in IQ23, IQ110, GM75, and GM70, respectively. All the strains have one of these genes annotated as a tributyrin esterase (Fig. 2, grey arrow), which has high identity percentages with *estA* gene from *E. faecium* DO (PRJNA55353). Also, four lipases for the *E. faecium* strains and three for *E. durans* IQ23 were annotated by RAST.

3.2.4. Citrate metabolism and diacetyl formation pathway

Certain specific features present in IQ23, GM75, GM70, and IQ110 are essential in cheese manufacturing. Citrate metabolism is a catabolic pathway that leads to flavor production through pyruvate and posterior diacetyl, acetoin and 2,3-butanediol formation. Except for IQ110 strain, all the analyzed enterococci have the *cit* cluster and *als* operon (Fig. 2, green and orange arrow respectively, and Supplementary Table 1S), both necessary for C4 compound production. IQ110 lacks *cit* genes, but is capable of producing aroma *via* ALS and ALD proteins (Fig. 2; (Martino et al., 2016)). Aroma producing capacity was tested with the Voges Proskauer reaction. Except for the IQ110, all of the strains were capable of producing diacetyl and acetoin when citrate was available in the growth medium. This reinforces the existing connection between

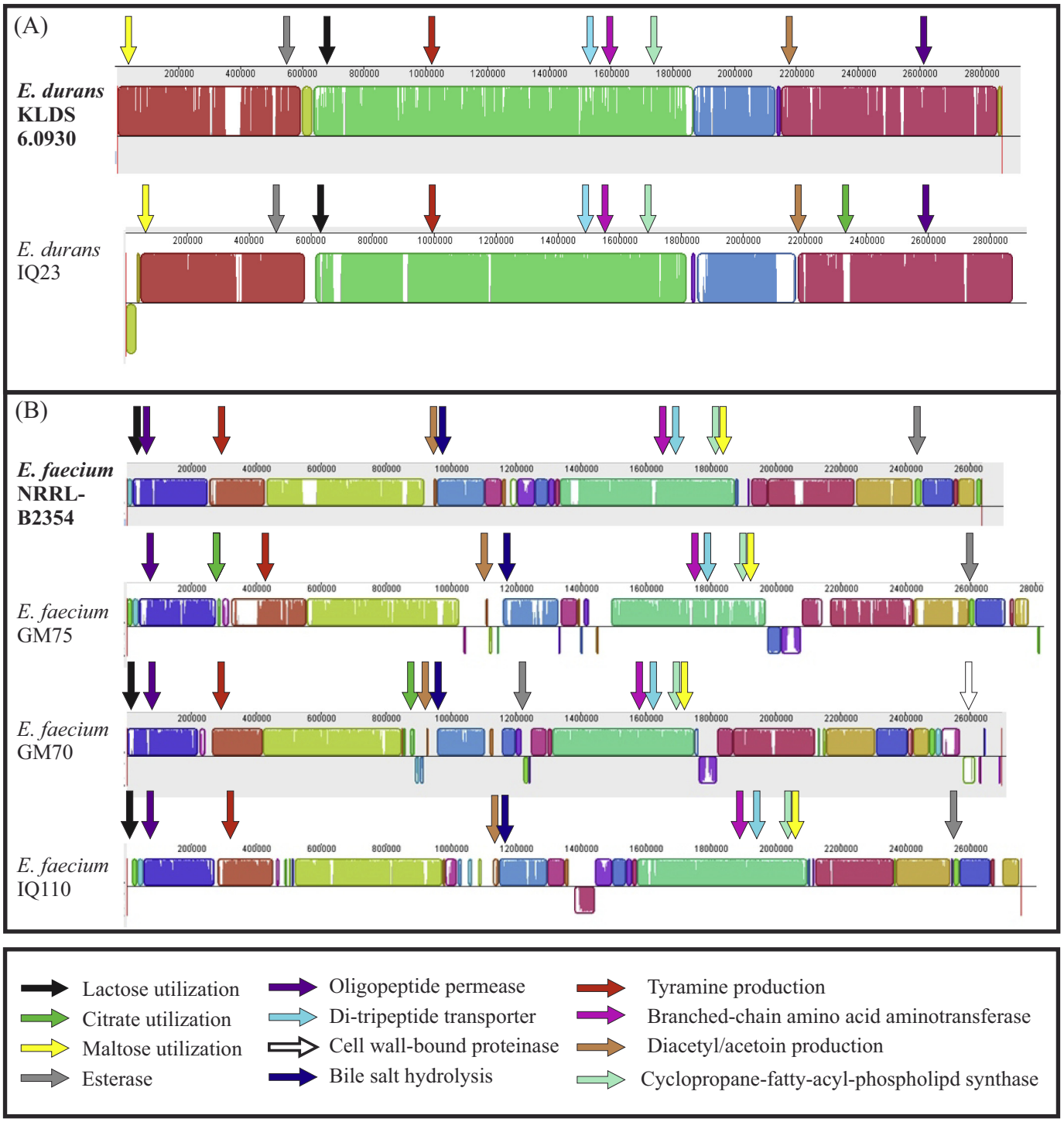

Fig. 2. Full genomic alignments and synteny of IQ23, GM70, GM75, and IQ110 against reference strains. Each colored block represents a conserved genomic region. A different position of the same block reflects its rearrangement. Blocks under the center line represent inverted regions. Blank spaces denote unique sequences. Features of special interest are indicated with colored arrows: black for *lacABCD* operon, green for *citMPCDEFXG* and *citI* genes, yellow for *malEFGA* operon y *aglB* gene, grey for *estA* gene, purple for *oppDFBCA* operon, light blue for *dtpT* gene, white for *prtR* gene, blue for the choloyl glycil hydrolase gene, red for *tyrPDC* operon, orange for *alsSD* operon, pink for *bcaT* gene and light green for the cyclopropane-fatty-acyl-phospholipid synthase gene. (A) *E. durans* KLDS 6.0930 was chosen as a reference for IQ23. (B) *E. faecium* NRRL-B2354 was chosen as a reference for IQ110, GM70 and GM75. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

citrate and C4 pathways.

3.2.5. Probiotic properties

GM75, GM70, and IQ110 strains have the gene sequence for the hydrolytic activity of bile salt hydrolase enzyme (BSH EC 3.5.1.24 also named choloyl glycil hydrolase) which is not only related to survival in GT, but also associated to host cholesterol diminution (Fig. 2, blue arrow). IQ23 does not encode BSH; however, to cope with bile salt stress, both IQ23 and *E. faecium* strains have a cyclopropane-fatty-acyl-phospholipid synthase (EC 2.1.1.79; Fig. 2, light green arrow), which

Table 2
In silico predictions and antibiotic susceptibility testing.[d]

Tested antibiotic	Strain							
	IQ23		GM75		IQ110		GM70	
	MIC/ phenotype	Encoded features	MIC/ phenotype	Encoded features	MIC/ phenotype	Encoded features	MIC/ phenotype	Encoded features
Ampicillin	< 2/S	*pbp5* hybrid form	< 2/S	*pbp5* hybrid form	< 2/S	*pbp5* hybrid form	< 2/S	*pbp5* hybrid form
Ciprofloxacin	< 0.5/S	*pmrA* or *lde* type efflux pumps	< 0.5/S	*pmrA* or *lde* type efflux pumps	< 0.5/S	*pmrA* or *lde* type efflux pumps	1/I	*pmrA* or *lde* type efflux pumps
Nitrofurantoin	< 16/S	–	64/I	–	64/I	–	64/I	–
Tetracycline	< 1/S	Efflux pump	< 1/S	Efflux pump	< 1/S	Efflux pump	< 1/S	Efflux pump
Vancomycin	< 0.5/S	ABC transporter, *vanR*, and *vanS*	< 0.5/S	ABC transporter, *vanR*, and *vanS*	< 0.5/S	ABC transporter, *vanR*, and *vanS*	< 0.5/S	ABC transporter, *vanR*, and *vanS*
Gentamicin (HL)[a]	SYN-S/S	AAC(6′)-Iih	SYN-S/S	AAC(6′)-Ii	SYN-S/S	AAC(6′)-Ii	SYN-S/S	AAC(6′)-Ii
Streptomycin (HL)[a]	SYN-S/S	AAC(6′)-Iih	SYN-S/S	AAC(6′)-Ii	SYN-S/S	AAC(6′)-Ii	SYN-S/S	AAC(6′)-Ii
Levofloxacin	< 0.12/S	*pmrA* or *lde* type efflux pumps	0.5/S	*pmrA* or *lde* type efflux pumps	2/S	*pmrA* or *lde* type efflux pumps	2/S	*pmrA* or *lde* type efflux pumps
Erythromycin	< 0.25/S	–	> 8/R	–	2/R[b]	–	2/R[b]	–
Quinupristin/dalfopristin	< 0.25/S	–	1/S	–	1/S	–	1/S	–
Linezolid	2/S	–	2/S	–	2/S	–	2/S	–
Teicoplanin	< 0.5/S	–	< 0.5/S	–	< 0.5/S	–	< 0.5/S	–
Minocycline	< 0.5/S	–	< 0.5/S	–	< 0.5/S	–	< 0.5/S	–
Trimethoprim/ sulfamethoxazole	< 10 R[c]	*dfrE*	< 10 R[c]	*dfrE*	< 10 R[c]	*dfrE*	< 10 R[c]	*dfrE*

[a] HL: high level resistance was tested because of enterococci natural resistance to low levels of these antibiotic.
[b] Changed S and I results with R for IQ110 and GM70, respectively. Vitek's Advanced Expert System (AES) modification.
[c] Changed S with R, Vitek's Advanced Expert System (AES) modification.
[d] CLSI M100-S16 MIC interpretation guideline and phenotypic interpretation (2014) using Vitek 2 system. Strains were classified as sensitive (S) or with intermediate (I) or full resistance phenotype (R); no MIC value but SYN-S results for antibiotics used in high concentrations and synergistically.

could give these bacteria the ability to survive in the gut medium. Also, the production of bacteriocins in probiotic strains is considered important, since these small proteins can inhibit pathogen growth or modulate the composition of the gut microbiota, and, consequently, modulate the host immune system (Dobson et al., 2012). RAST annotates several bacteriocin genes in the analyzed strains. In fact, one enterocin P precursor, one colicin V and one enterocin similar to a carnobacteriocin BM1 in all the strains were found. One more non-identified bacteriocin in the *E. faecium* strains was also detected, while two more were found in IQ23.

3.2.6. Negative features

As biogenic amines have negative effects on human health, their formation should be controlled in cheese manufacturing. *Enterococcus* strains are generally capable of producing mainly histamine, tyramine, cadaverine and putrescine (decarboxylation products of histidine, tyrosine, lysine and ornithine, respectively). Only tyramine formation was predicted to be feasible by the strains under study since they have the entire TDC gene cluster (Fig. 2, red arrow) whereas genes for histamine, putrescine and cadaverine production were not found (Supplementary Table 1S). Tyrosine decarboxylation activity was tested in the Bover-Cid and Holzapfel decarboxylase screening medium (Bover-Cid and Holzapfel, 1999). IQ110 and IQ23 strains gave positive results while GM70 had an intermediate reaction and GM75 a negative one. Thus, this pathway seems not to be active in all of the *E. faecium* group-strains, although they all have the complete TDC cluster (Table 1 and Supplementary Table 1S).

3.3. Mobile genetic elements and CRISPR – Cas systems

Insertion sequences (ISs), transposons, plasmids, prophages and other mobile genetic elements (genomic islands, integrative and conjugative elements) provide variability and play a crucial role in horizontal gene transfer and genomic reorganization. Five ISs were detected for IQ23, 6 for GM75, 7 for IQ110 and ten IS were found for GM70 strain using the ISfinder tool (Siguier et al., 2006). All of the IS belonged to families with origins in *Lactococcus*, *Enterococcus* and *Lactobacillus* species (Supplementary Table 2S A). IS16, an enterococci marker for clinically associated strains (Werner et al., 2011), was not found in IQ23, IQ110, GM70 or in GM75.

On the other hand, three putative prophages for GM70, IQ110 and GM75 strains were identified using Phaster tool (Arndt et al., 2016). All of them are considered intact by the software for GM70 strain, but only two of the three predicted regions are considered intact for the GM75 strain and one for IQ110 (Supplementary Fig. 1S and Table 2S). In *E. durans* IQ23 strain a total of 6 putative prophages have been identified, half of which are intact. The complete description of the predicted regions can be found in Supplementary Table 2S B.

CRISPR-*cas* loci not only provide the carrying strain with the ability to defend itself from strange DNA molecules, but also their presence is suggested as a marker for antibiotic-sensitive enterococci strains (Palmer and Gilmore, 2010), since most of the resistance genes are acquired from mobile elements. In GM70, GM75 and IQ23 this service found one putative complete CRISPR region composed of three directed repeats (24 bp long) and 3 spacers. These sequences were always found inside putative Rep protein coding gene. Since no Cas-encoding genes were found near these regions, these repeats are probably not related to CRISPR sequences.

Conversely, for IQ23, RAST annotates five putative CRISPR-associated genes with an organization similar to that of Nmeni sub-type CRISPR-cas clusters (Haft et al., 2005). Palindromic sequences next to these genes could not be located. Moreover, alignments of this region with homologous ones in other *E. durans* strains revealed a 94 pb deletion downstream this locus in IQ23 that may indicate that the putative *cas* genes found are probably not functional.

Genomic islands were predicted using IslandViewer web tool (Dhillon et al., 2015). Neither antibiotic resistance related genes nor virulence factors were found in the predicted regions (data not shown).

3.4. Antibiotic resistances

Antibiotic resistances were tested with Vitek®2 analyzer. The four

strains were found sensitive to the majority of the clinically used antimicrobial compounds (Table 2). All of the strains were resistant to trimethoprim/sulfamethoxazole. Furthermore, an intermediate resistance was observed for GM70, GM75 and IQ110 strains for nitrofurantoin. GM70 also showed intermediate resistance to ciprofloxacin. Interestingly, *E. durans* IQ23 strain presented the lowest MICs for nitrofurantoin (for which it was sensitive), levofloxacin, erythromycin and quimuspristin/dalfopristin (Table 2). Following the recommendations for phenotypic characterizations, for the *E. faecium* species, intermediate results for erythromycin were changed to full resistance.

Additionally, a bioinformatic search of relevant antibiotic resistance related features was performed. Features associated with the antibiotics tested were found using ResFinder service (Kleinheinz et al., 2014), Rast, BLAST and CARD (Jia et al., 2017); the results are listed in Table 2.

3.5. Pathogenicity and virulence factors

In order to use enterococci strains in food production, their pathogenicity should be evaluated. Hence, as a first approach, PathogenFinder was used to determine a probability value "of being pathogen". Very low probabilities were calculated and consequently "non-human pathogen" results were obtained with this tool. Secondly, VirulenceFinder was used to search known virulence factors in the genomes under study. Only *efaAfm* virulence factor was found in the three *E. faecium* strains, with identity percentages higher than 94%. On the other hand, no virulence factors were detected with this service in IQ23 strain (Table 3). Finally, BLAST searches were performed in the genomes using ten known virulence factors as query. Only three of them were identified in GM70, GM75 and IQ110 strains and one in IQ23. Complete results are listed in Table 3.

To further investigate their pathogenic potential, the four strains were compared in *G. mellonella* model host. Insect larvae have emerged as an alternative to mammalian models since their immune system has similar features to the innate immune system of mammals (Bergin et al., 2005; Wojda, 2017). Also, a correlation in microbial virulence between this and the mammalian models was demonstrated for several microorganisms (Aperis et al., 2007; Jander et al., 2000; Peleg et al., 2009) including *E. faecium* (Chibebe Junior et al., 2013). Survival of larvae groups was registered for 72 h after inoculation with PBS suspensions of 1×10^7 and 9×10^6 CFU/larva. Kaplan-Meier curves were constructed with the collected data (Fig. 3). GM70 strain differentiates itself ($P < 0.05$) from the rest since it did not produce any insect death with both CFU tested; the same result was observed for innocuous starter strain *L. lactis* CRL264 at 1×10^7 and 9×10^6 CFU/larva (not shown). As observed in Fig. 3, survival curves of IQ110, IQ23 and GM75 inoculated larvae did not differentiate from *E. faecalis* JH2-2, a non-pathogenic laboratory model strain.

Indeed, the virulence of our isolated strains and *E. faecalis* JH2-2 is remarkably lower than the virulence exhibited by clinical enterococci. As it had been previously reported, inoculations with *E. faecalis* V583 produced almost 100% larvae killing and OG1RF approximately 72% at 48 h when inoculating with 2×10^6 CFU (Gaspar et al., 2009), a much lower CFU value, than the lowest CFU tested in this paper. As shown in Fig. 3, when inoculating 9×10^6 CFU, IQ23 produces 50% larvae death at 50 h, while IQ110 and GM75 only 20% and GM70 0% death in the same lapse of time.

4. Discussion

The selection of bacteria with remarkable properties and safe for human consumption has been a crucial activity since the origin of fermented food. To date, most of the strains used are usually selected by traditional methodologies, such as the screening of specific activities or properties. Nowadays, this selection strategy is supported by the ability to obtain whole genomic sequences and by the huge amount of available data to compare these with. In an attempt to obtain and characterize enterococci bacteria in order to improve the quality and safety of cheeses, our group had previously isolated 75 enterococci strains from Argentinean regional cheeses (Martino et al., 2016; Suarez et al., 2012). These strains were genetically and biochemically studied. IQ23, IQ110, GM70, and GM75 strains were further selected, based on their citrate utilization and aroma compound production profile (Martino et al., 2016). Enterococci have been consumed by different cultures for centuries. However, in the last few years they have emerged as opportunistic pathogens in hospital environments. Consequently, the *Enterococcus* genus has not yet been classified as safe for human consumption (GRAS denomination by the FDA or Qualified Presumption of Safety, QPS by the EFSA), which is one of the main concerns in public health, since it has the capacity to mediate gene transfer with different genetic elements, including plasmids, phages and conjugative transposons (Clewell et al., 1975; Coburn et al., 2007; Davis et al., 2005; Flannagan and Clewell, 1991). Thus, for a future application in food industry, a thorough identification of the putative elements capable of this transfer, and of the genes involved in antibiotic resistances, is crucial. The transfer of these markers to and from pathogenic microorganisms, must be controlled or minimized (Wozniak and Waldor, 2010).

Accordingly, we performed a detailed inspection of the industrial potential and safety of the selected enterococci cheese isolates. In the present study, a combination of *in silico* tools and *in vivo* assays were used. Remarkably, the phylogenomic analysis determined that IQ23 strain belongs to *E. durans*, while GM70, GM75, IQ110 to *E. faecium* species. This fact refutes the initial taxonomic assignation of IQ23, based on the 16S RNAr sequence, and highlights the need for more

Table 3
Predicted virulence factors.

Virulence factor	Role	Gene/s	Presence			
			IQ23	IQ110	GM75	GM70
Aggregation substance	Adhesion	*asa1*(1)	No	No	No	No
Cytolysin	Cell lysis	*cylM*, *cylL*, *cylS*(1)	No	No	No	No
Gelatinase	Translocation	*gelE*(1)	No	No	No	No
Hyaluronidase	Translocation	*hyl*(2)	No	No	No	No
Enterococcal surface protein	Adhesion	*esp* (3)	No	No	No	No
Endocarditis and biofilm-associated pili proteins	Biofilm formation	*ebpR*, *ebpA*, *ebpB*, *ebpC*(2)	Yes	Yes	No	No
Biofilm enhancer in *Enterococcus*	Biofilm formation	*bee1*, *bee2*, *bee3* and sortase genes (4)	No	No	Yes	Yes
Second collagen adhesion protein	Adhesion	*scm*(2)	No	Yes	No	Yes
Adhesion to collagen protein	Adhesion	*acm*(2)	No	No	Yes	No
Secreted antigen	Adhesion	*sagA*(2)	No	Yes	Yes	Yes
Number of predicted virulence factors	1	3	3	3		

Gene sequences from *E. faecalis* V583 (1), *E. faecium* DO (2), *E. faecium* Aus0004 (3) and *E. faecalis* E99 (4) were used as query for BLAST searches.

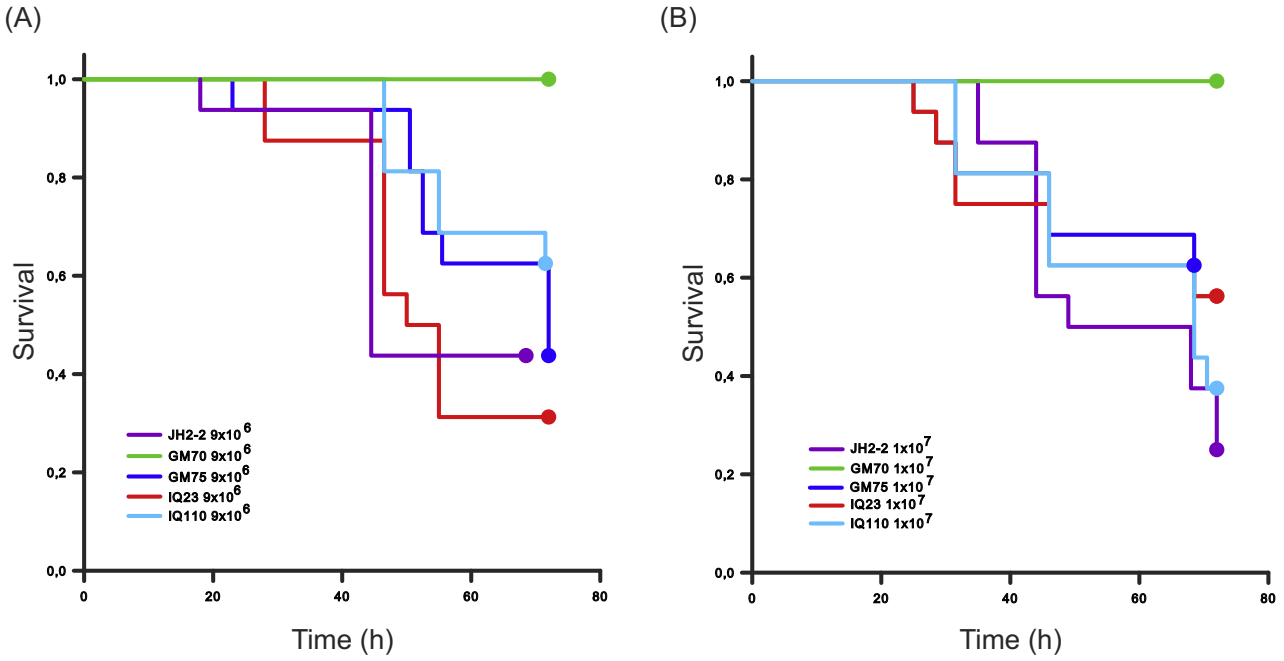

Fig. 3. Survival curves of *G. mellonella* larvae inoculated with IQ23, GM70, GM75 or IQ110. Survival of *G. mellonella* after inoculation with 9×10^6 CFU/larva (A) or 1×10^7 CFU/larva (B). Experiments were repeated twice and a representative example was used for each group. Comparison was carried out using Log-rank test.

accurate methodologies to resolve the identity of the *E. faecium* group-strains. The proper species assignation of these and other highly phylogenetically related strains is a *sine qua non* condition to perform any comparative bioinformatic analysis (Espariz et al., 2016).

The profile of specific features related to cheese production was explored, and common positive elements were found, such as proteinases, peptide permeases, citrate catabolic genes, lactose and maltose utilization operons, bile salt tolerance genes, diacetyl and acetoin pathway, as well as negative ones, such as tyramine production genes. Despite finding a putative Prt protein in GM70 strain, hydrolysis of caseins, in many cheese varieties, is mainly caused by the coagulant which results in the formation of large and intermediate-sized peptides (Sousa et al., 2001).Then, during cheese maturation, enterococci adjunct cultures could profit from the remaining casein degradation products. Nevertheless, *L. lactis* requires a PrtM maturation protein (Vos et al., 1989) for the activation of the proteinase. We could not find any homologous to the *prtM* gene in GM70 strain, thus explaining the observed phenotype. As noted, the sole presence of certain genes such as protein degradation genes, which could confer highly desirable properties, does not guarantee an active metabolism. So, in order to obtain reliable technological data, every function of interest must be experimentally analyzed in cheese matrix. On the other hand, the factors involved in the biogenic amines production, which constitute a health risk, should be inactivated in the strains selected to be cheese adjuncts.

Also, regarding the strains safety, putative genes involved in fluoroquinolones, lincosamides, tetracycline and vancomycin resistance were found, as well as genes associated with pathogenicity and virulence, such as those involved in biofilm formation and adhesion. Nevertheless, the presence of resistance genes did not correlate with the observed phenotype (Table 2). In fact, all the strains turned out to be susceptible to the antibiotics commonly used in clinic.

Furthermore, there were no obvious pathogenicity or virulence genes found. This correlates with the observed phenotype in *G. mellonella* model, due to the low mortality rates that were obtained (Table 3 and Fig. 3). Moreover, 100% of GM70 inoculated larvae survived in the tested conditions, similar behavior to the larvae inoculated with innocuous *L. lactis* strains.

The present study shows the advantages of using a combined approach of whole genome sequence analysis and phenotypic testing to pursue a rational selection of microorganisms intended to be used in cheese production. Additionally, these results revealed that *E. faecium* GM70 strain presents no virulence in *G. mellonella* while having a plethora of positive features, which makes it a good candidate for food production. In particular, this microorganism could be used as adjuvant in a starter culture to prevent the development of undesirable microbiota during the cheese ripening process. This hypothesis is currently under investigation in our lab.

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