International Dairy Journal 39 (2014) 193–200

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

# Safety and potential beneficial properties of *Enterococcus* strains isolated from kefir

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ARTICLE INFO

*Article history:*
Received 9 May 2014
Received in revised form
22 June 2014
Accepted 23 June 2014
Available online 5 July 2014

ABSTRACT

Kefir is a fermented milk containing microorganisms showing several properties beneficial to health. In the present work the safety aspects and beneficial properties of kefir-isolated *Enterococcus* strains were investigated for the first time. *Enterococcus* strains were identified as *Enterococcus durans* using phenotypical and molecular characterisation methods. Neither resistance to clinically important antibiotics, nor genes encoding for eight different virulence factors were found. Interestingly, selected *E. durans* strains were able to inhibit different Gram+ and Gram− pathogens. These strains were able to survive simulated gastrointestinal conditions and showed a similar power of adhesion to mucins. Of note, the *E. durans* strains possessed anti-inflammatory properties as demonstrated by a significant inhibition of flagellin-induced response on Caco-2 cells. Our results indicate that the presence of *E. durans* in kefir does not represent a threat to consumers' health, and shows their potential both functionality and as probiotics.

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## 1. Introduction

Kefir is a viscous, acidic, and mildly alcoholic product produced through fermentation of milk with mixed microbial population confined to "kefir grains" as a starter culture (FAO/WHO, 2011). Several health-promoting properties such as inhibitory activity against foodborne bacterial pathogens, antitumoral, immunological, hypocholesterolaemic effects (Güven & Gülmez, 2003; Guzel-Seydim, Kok-Tas, Greene, & Seydim, 2011; Thoreux & Schmucker, 2001; Vinderola et al., 2005, 2006) and changes in human intestinal microbiota (Forejt & Simuunek, 2007) have been associated with kefir-consumption. It has been also demonstrated that kefir-isolated strains of lactic acid bacteria (lactobacilli and lactococci) and yeasts have potentially beneficial properties in vitro (Bolla, Carasi, Bolla, De Antoni, & Serradell, 2013; Carasi, Trejo, Pérez, De Antoni, & Serradell, 2012; Golowczyc, Mobili, Garrote, Abraham, & De Antoni, 2007; Romanin et al., 2010) or in vivo (Bolla et al., 2013; Romanin et al., 2010).

The members of the genus *Enterococcus* belong to the lactic acid bacteria (LAB). Enterococci are ubiquitous; they can be found in a variety of foods and dairy products, water surfaces and plants, and are natural inhabitants of the gastrointestinal tract of man and animals (Ahmed, Sidhu, & Toze, 2012; Gelsomino, Vancanneyt, Cogan, Condon, & Swings, 2002; Klein, 2003). The genus *Enterococcus* is the most controversial group of LAB, even though many enterococcal strains are used probiotics or are relevant for technological properties of foods such as ripening, shelf life improvement and aroma development (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006; Morandi, Silvetti, & Brasca, 2013) not all the strains are recognised as safe. Indeed, *Enterococcus faecalis* and *Enterococcus faecium* are among the leading causes of nosocomial infections such as endocarditis, urinary tract infections and bacteraemia (Fernández-Guerrero et al., 2002; Lee, 2013; Peel, Cheng, Spelman, Huysmans, & Spelman, 2012; Tan et al., 2010). On the other hand, cases of infection with other enterococcal species, including *Enterococcus durans* and *Enterococcus hirae*, are very

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http://dx.doi.org/10.1016/j.idairyj.2014.06.009
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rare (Kenzaka, Takamura, Kumabe, & Takeda, 2013; Lee et al., 2013; Stepanovic, 2004).

Although enterococci isolated from food have not been conclusively identified as direct causes of clinical infections, the consumption of food carrying antibiotic-resistant bacteria is a possible route of transfer of this trait to host-adapted strains (Hayes et al., 2003). The presence of antibiotic-resistant enterococci in food of animal origin has been reported by several authors (Hayes & English, 2004; Hayes et al., 2003; Koluman, Akan, & Çakiroğlu, 2009; Novais et al., 2005; Sparo et al., 2012) and constitutes a potential risk of transmission to humans.

The association of enterococci with human disease has raised concern regarding their use as probiotics (Franz, Huch, Abriouel, Holzapfel, & Gálvez, 2011; Giraffa, 2003). Some enterococal strains were able to restore the microbiota balance in antibiotic-induced dysbiosis (Tarasova et al., 2010), to have antiviral activity (Wang et al., 2013), or beneficial effects in anti-tumoral protective responses (Castro et al., 2010; Thirabunyanon & Hongwittayakorn, 2013). Additionally, *E. faecium* is one of the most common probiotics used in animal feed to improve growth, to prevent diarrhoea and for immune stimulation (Lodemann, Hübener, Jansen, & Martens, 2006; Taras, Vahjen, Macha, & Simon, 2006). In relation to this, the European Food Safety Authority (EFSA), through its Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) edited a new guidance document (EFSA, 2012a) to differentiate between safe strains and the potentially harmful clinical strains of *E. faecium*, based on their susceptibility to the ampicillin and the absence of three genetic markers associated with virulence (*esp*, *hylEfm*, IS*16*).

Moreover, it has described that enterococci produce antimicrobial substances such as lactic acid, hydrogen peroxide and bacteriocins (Franz, van Belkum, Holzapfel, Abriouel, & Gálvez, 2007; Sparo et al., 2006), which could explain their activity against several food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*.

In this context, the purpose of this work was to isolate and identify new strains belonging to genus *Enterococcus* from kefir-fermented milk, and then to investigate both their safety aspects and beneficial properties in vitro.

## 2. Materials and methods

### 2.1. Bacterial strains

Pure cultures used in this study comprised *E. faecium* ATCC 6569, *E. faecium* DSMZ 25390, *E. hirae* CIP 5348 lot 15409, *E. faecalis* ATCC 29212, *E. faecalis* ATCC 51299, clinical isolates *E. faecium* CHU 5088, CHU 4812 y CHU 3091 (CHU, Centre Hospitalo-Universitaire de Bordeaux, France), *S. aureus* ATCC 6538, *Shigella flexneri* ATCC 9199, *Pseudomonas aeruginosa* ATCC 15442, clinical isolate *Salmonella enterica* serovar Enteritidis CIDCA 101 (Hospital de Pediatría Prof. Juan P. Garrahan, Buenos Aires, Argentina), enterohaemorragic *Escherichia coli* EDL 933, and *L. monocytogenes* ATCC 7644. All strains were grown using Brain Heart Infusion (BHI) broth (BIOKAR) in aerobic conditions at 37 °C for 16 h.

### 2.2. Isolation of microorganisms

In this study, six different kefir grains belonging to the Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA) collection were used: CIDCA AGK1, AGK2, AGK3, AGK4, AGK5 y AGK11 (Garrote, Abraham, & De Antoni, 2001). Such grains have no common "history" previous to their arrival to the institute, and frozen stocks of them were maintained at −80 °C in cow skim milk. Kefir grains were sub-cultured in sterilised commercial cow milk before use.

Kefir grains were disrupted in a mortar and resuspended in 0.1% (w/v) tryptone. For the direct isolation Bile Esculin Azide Agar (Isenberg, Goldberg, & Sampson, 1970) or MRS agar supplemented with 6.5% (w/v) NaCl (MRS-NaCl) was used. An enrichment step was performed in different media: either azide dextrose broth (in w/v: 1% meat peptone, 1% casein peptone, 0.5% dextrose, 0.5% sodium chloride, 0.3% dipotassium hydrogen phosphate, 0.3% potassium dihydrogen phosphate and 0.02% sodium azide, final pH 7.0) or Kenner Fecal (KF) Streptococcus with bromocresol purple (Kenner, Clark, & Kabler, 1961), and incubated for 24 h at 37 °C in aerobic conditions. After enrichment, 20 μL aliquots were isolated in Bile Esculin Azide Agar plates and MRS-NaCl. After least five passages by isolated colony, pure bacteria isolates were obtained.

The same procedure was used to isolate strains from sterilised milk fermented with kefir grains (10%, w/w) for 24 h at 32 °C.

### 2.3. Enumeration of microorganisms

Enumeration was performed in disrupted kefir grains resuspended in 0.1% (w/v) tryptone and in samples of sterilised milk fermented with kefir grains (10%, w/w) during 24 h at 32 °C. Differential count was performed by plating on MRS agar supplemented with 6.5% (w/v) NaCl to selectively allow the growth of enterococci.

### 2.4. Bacterial identification

#### 2.4.1. Morphological and biochemical analysis

For presumptive identification of the isolates the criteria described by Facklam and Elliott (1995) was used. Cellular morphology, Gram staining, motility, catalase reaction, growth in MRS, growth at 45 °C and at pH 9.6, growth in presence of 6.5% (w/v) NaCl, 2:3:5-triphenyltetrazolium chloride reduction (Barnes, 1956), haemolysin production on blood agar using 5% (v/v) human blood, and acid production from the following carbohydrates 1% (w/v): L-arabinose, mannitol, D-raffinose, and sorbitol, were analysed.

#### 2.4.2. Molecular characterisation and identification

Species identification was confirmed by 16S rDNA gene sequencing (Weisburg, Barns, Pelletier, & Lane, 1991) and species-specific primers based upon the superoxide dismutase (sodA) gene (Jackson, Fedorka-Cray, & Barrett, 2004). Positive controls (*E. faecalis* ATCC 29212, *E. faecium* ATCC 6569, *E. hirae* CIP 5348 lot 15409) were used in all reactions. Total DNA of enterococcal strains was extracted using the Genomic DNA Purification Kit (Fermentas, France) according to manufacturer's specifications. Finally, all strains were characterised by random amplified polymorphic DNA-PCR (RAPD-PCR) using two selected 10-nucleotide single-strand primers OPA3 (5′-AGTCAGCCAC-3′), and OPL12 (5′-GGGCGGTACT) (Bioprobe, Montrevils-sous-Bois, France).

### 2.5. Strain characterisation

#### 2.5.1. Antimicrobial susceptibility

Susceptibility to antibiotics was evaluated by using the disk diffusion method in Mueller-Hinton agar (Société Française de Microbiologie, 2008). The following antibiotics were tested: ampicillin, ciprofloxacin, erythromycin, linezolid and vancomycin. *E. faecalis* ATCC 29212 was used as quality control strain. The minimum inhibitory concentration (MIC) for ampicillin was also determined by broth microdilution according to ISO 20776-1 (ECS, 2006).

*2.5.2. PCR screening for virulence genes*

The presence of virulence genes was screened in DNA extracts from all the *Enterococcus* strains isolated: enterococcal surface protein (*esp*) (Ahmed et al., 2012), sex pheromones (*ccf*), gelatinase (*gelE*), cytolysin (*cylA*), aggregation substance (*agg*) (Eaton & Gasson, 2001), cell-wall anchored collagen adhesin (*acm*) (Nallapareddy, Singh, Okhuysen, & Murray, 2008), IS*16* (Werner et al., 2011), $hyl_{Efm}$ (Rice et al., 2003) and *van A*, *van B*, *van C2* (Satake et al., 1997).

Positive controls were used in all PCR reactions (*E. faecalis* ATCC 29212, *E. faecalis* ATCC 51299, *E. faecium* DSMZ 25390), clinical isolates *E. faecium* CHU 5088, CHU 4812 y CHU 3091 (CHU, Centre Hospitalo-Universitaire de Bordeaux, France).

*2.5.3. Resistance to simulated gastric and intestinal compartments*

Viability of bacterial suspensions incubated sequentially in solutions simulating the gastric and intestinal compartments was assessed following the protocol described by Grimoud et al. (2010). Briefly, bacteria in stationary phase were washed twice with phosphate-buffered saline (PBS; in w/v: 0.014% $KH_2PO_4$, 0.9% NaCl, 0.08% $Na_2HPO_4$, pH 7.2) and resuspended in simulated gastric fluid (in w/v: 0.73% NaCl, 0.05% KCl, 0.4% $NaHCO_3$, and 0.3% pepsin) at pH 2.5 (adjusted with 18.5%, w/v, HCl) and a final bacteria concentration of $10^8$ cfu $mL^{-1}$. Suspensions were incubated at 37 °C with stirring at 200 rpm for 90 min. After incubation, cells were washed with PBS and the pellets were resuspended in simulated intestinal fluid (comprising 0.1%, w/v, pancreatin and 0.15%, w/v, bovine bile salts) at a pH 8.0 (adjusted with 20%, w/v, NaOH). Suspensions were incubated at 37 °C with stirring at 200 rpm for 3 h. Cell viability was assessed by plate counting after incubation in simulated gastric fluid and after incubation in simulated intestinal fluid. Independent experiments were performed at least three times.

*2.5.4. Bacterial binding assay to mucin*

Binding assays to commercial type III porcine gastric mucin (Sigma-Aldrich) were performed as described by Carasi et al. (2014), with an initial bacterial count of $9 \times 10^7$ cfu $cm^{-2}$. Bacterial adhesion was quantified by plate counting and at least four replicates were used to estimate the adhesion of a given strain. Bacterial adhesion to the plate without substrate was lower than 30 cfu $cm^{-2}$.

*2.5.5. Growth inhibition of bacterial pathogens*

An agar spot test, performed as described by Schillinger and Lücke (1989), was used to evaluate growth inhibition of bacterial pathogens. Briefly, overnight cultures ($10^6$ cfu) of each isolate to be tested were spotted onto the surface of MRS agar (MRS containing 1.5%, w/v, agar) and incubated for 24 h at 37 °C. The pathogenic bacteria were inoculated into 8 mL of soft BHI agar (containing 0.7%, w/v, agar) at a final concentration of approximately $5 \cdot 10^6$ cfu $mL^{-1}$ and poured over the plate on which the potentially antimicrobial producer strain was grown. After incubation for 16 h at 37 °C, plates were checked for inhibition zones. Inhibition was scored negative (−) if the width of the clear zone around the colonies was less than 2 mm, a low inhibition capacity (+) was considered if the width of the clear zone ranged between 2 and 5 mm, finally, a high inhibition capacity (++) was considered if the width was 6 mm or larger. Three independent experiments were performed.

*2.5.6. Stimulation assay with Caco-2 ccl20:luc reporter system*

Caco-2 cells stably transfected with a luciferase reporter construction under the control of CCL20 promoter (Caco-2 ccl20:luc) (Nempont et al., 2008) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL Life Technologies, Rockville, MD, USA) supplemented with 15% (v/v) inactivated (30 min 60 °C) foetal bovine serum (FBS, Gibco BRL Life Technologies, Rockville, MD, USA), 0.2% (w/v) $NaHCO_3$, 10 mg $L^{-1}$ streptomycin and 10 IU $mL^{-1}$ penicillin G and incubated at 37 °C in a 5% (v/v) $CO_2$ 95% (v/v) air atmosphere. The culture medium was changed every 2 days. Fully differentiated cells (14 days in culture) were used throughout. The experiments were performed as described by Romanin et al. (2010). Briefly, confluent Caco-2 ccl20:luc cells were co-cultured 2 h with a suspension of the *Enterococcus* ($10^7$ cfu per well) strains to be tested (multiplicity of incubation = 100). Then, cells were stimulated using flagellin from *S. enterica* ser. Typhimurium (FliC) (1 μg $mL^{-1}$) for 6 h. Luciferase activity was measured in a Labsystems Luminoskan TL Plus luminometer (Thermo Scientific, USA) using a luciferase assay system (Promega, Madison WI, USA). Luminescence was normalised and expressed as the percentage of the mean of stimulated control (NAL).

*2.6. Statistical analysis*

One-way ANOVA with Bonferroni post-test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

## 3. Results

*3.1. Enumeration of microorganisms at high salt concentration*

The bacterial counts on fermented milk with kefir grains 10% (w/v) or disrupted kefir grains were performed using MRS supplemented with 6.5% (w/v) NaCl, since enterococci are the only LAB capable of growing in these conditions. In fermented milk, the quantity of Gram positive cocci ranged from 3.34 to 4.87 $\log_{10}$ cfu $mL^{-1}$, while in disrupted grains the count was lower than 30 cfu $g^{-1}$.

*3.2. Bacterial identification*

A total of 36 Gram positive coccoid bacteria (24 isolated from kefir-fermented milk and 12 from kefir grains) were obtained and the initial identification was performed on the basis of physiological and biochemical tests according to the criteria established by Facklam and Elliott (1995). All the isolates were identified phenotypically as "potentially" *E. durans*. These findings were confirmed by partial sequencing of the 16S RNA gene (Weisburg et al., 1991) and using species specific primers for *E. durans*, *E. hirae* and *E. faecium* (Jackson et al., 2004).

The isolates were characterised by RAPD-PCR, and a total of 29 different patterns were obtained. Fig. 1 shows the amplification patterns obtained using the primer OPA3 for 16 selected strains isolated from milk fermented with different kefir grains, as example. One representative strain of each 29 RAPD group was selected for further studies.

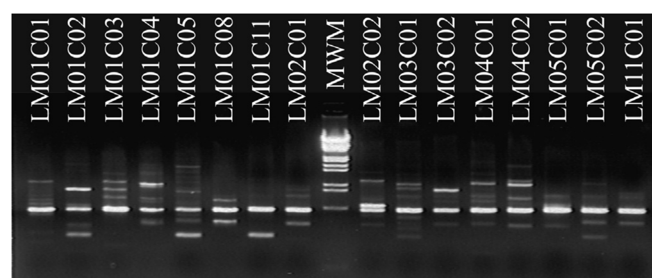


**Fig. 1.** RAPD-PCR patterns of kefir-isolated *E. durans* strains obtained with OPA3 primer. MWM: molecular weight marker Lambda DNA/*Hin*dIII Marker 2.

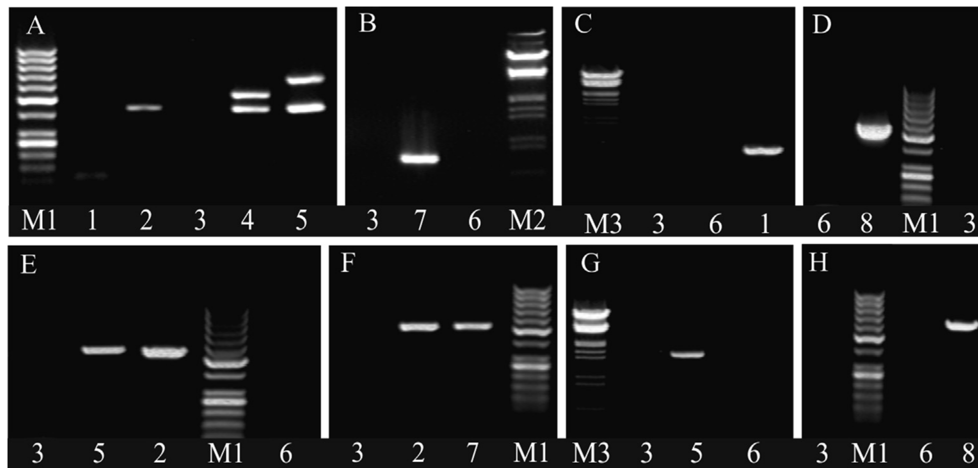


**Fig. 2.** PCR screening for virulence genes in *E. durans* strains. Panel A, multiplex PCR for the genes *acm* (104 bp), *gelE* (440 bp), *cylA* (517 bp), *van B* (643 bp); panel B, *van A* (732 bp); panel C, *van C2* (484 bp); panel D, *IS16* (547 bp); panel E, *ccf* (543 bp); panel F: *esp* (510 bp); panel G, *agg* (1553 bp), panel H, $hyl_{Efm}$ (661 bp). M1 and M2, molecular weight marker Thermo Scientific GeneRuler 50 bp and 100 bp, respectively; M3, lambda DNA/*Hind*III Marker 2; Lane designations are: 1, *E. faecium* CHU 3091; 2, CHU 5088; 3, *E. durans* isolated from kefir LM01C01; 4, *E. faecalis* ATCC 29212; 5, ATCC 51299; 6, LM01C02; 7, CHU 4812; 8, DSMZ 25390.

### 3.3. Strain characterisation

#### 3.3.1. Antiobiotic resistance

All the 29 strains tested were susceptible to vancomycin, ciprofloxacin, erythromycin or linezolid by disk diffusion method. However, three strains showed resistance to ampicillin (LM01C04, LM01C08 and LM01C11) and they were no included in subsequent assays. Additionally, the other 26 strains exhibited MIC values for ampicillin lower than the recommended breakpoint (2 mg mL$^{-1}$).

#### 3.3.2. Virulence genes

The presence of virulence genes was assessed by PCR. All the 26 strains were negative for *ccf*, *esp*, *gelE*, *cylA*, *agg*, collagen (*acm*), IS*16*, $hyl_{Efm}$, *van A*, *van B*, *van C2* (Fig. 2). In the case of the last three genes, it is important to note that these results correlate with the lack of resistance to vancomycin observed using the disk diffusion method.

#### 3.3.3. Inhibition of pathogens in vitro

Based on antibiotic resistance, absence of virulence genes and RAPD patterns, we selected 13 strains isolated from different kefir grains or different kefir-fermented milk, and the inhibition of different pathogenic bacteria was evaluated in vitro by agar spot test. The results obtained are shown in the Table 1. Twelve strains showed inhibition of at least four different pathogens, meanwhile LM11C01 was able to inhibit only *S. aureus* and *E. faecalis*. Noteworthy, the strains LM01C02 and LM04C02 inhibited all the pathogens tested here. On the other hand, strains named as LM01C01, LM01C05, LM03C01 and LM03C02 showed the same antimicrobial profile and failed only to inhibit the growth of *L. monocytogenes*.

#### 3.3.4. Resistance to simulated gastrointestinal conditions

The ability to survive to the simulated gastrointestinal conditions was assessed. In all cases, the critical step was the gastric passage which lowered viability significantly around one logarithmic unit. Results obtained for six representative strains are shown in Fig. 3. After complete simulation, the viability ranged from 6.44 to 7.03 $\log_{10}$ cfu mL$^{-1}$ for all tested strains.

#### 3.3.5. Bacterial binding assay to mucin

The capacity of adherence to mucus is a desirable property for potentially probiotic bacteria since it could allow the competitive exclusion of pathogens and also the interaction with epithelial and immune cells in the gut. For the 13 different *E. durans* strains, adhesion to mucins ranged from 5.42 to 6.31 $\log_{10}$ cfu cm$^{-2}$ and no significant differences were observed among them. Fig. 4 shows the results obtained for six representative strains. Similar results were obtained for *Lactobacillus plantarum* 299v, already described as

**Table 1**
Growth inhibition of bacterial pathogens by kefir-isolated *E. durans* strains.[a]

| Strain | *Salmonella enterica* | *Pseudomonas aeruginosa* | *Shigella flexneri* | EHEC | *Listeria monocytogenes* | *Staphylococcus aureus* | *Enterococcus faecalis* |
|---|---|---|---|---|---|---|---|
| LM01C01 | + | + | + | + | − | + | + |
| LM01C02 | + | + | + | + | ++ | + | + |
| LM01C03 | − | − | + | + | + | + | + |
| LM01C05 | + | + | + | + | − | ++ | + |
| LM02C01 | + | + | + | + | − | + | + |
| LM02C02 | + | − | + | + | + | + | + |
| LM03C01 | + | ++ | + | + | − | + | + |
| LM03C02 | + | ++ | + | + | − | + | ++ |
| LM04C01 | + | − | + | + | + | + | + |
| LM04C02 | ++ | + | + | + | + | + | + |
| LM05C01 | + | ++ | − | + | + | + | + |
| LM05C02 | + | + | + | + | − | − | − |
| LM11C01 | − | − | − | − | − | + | + |

[a] EHEC, enterohaemorragic *Escherichia coli*. −, no inhibition (width of the clear zone around colony < 2 mm); +, low inhibition capacity (width of the clear zone around colony between 2 and 5 mm); ++, high inhibition capacity (width of clear zone around colony > 6 mm).

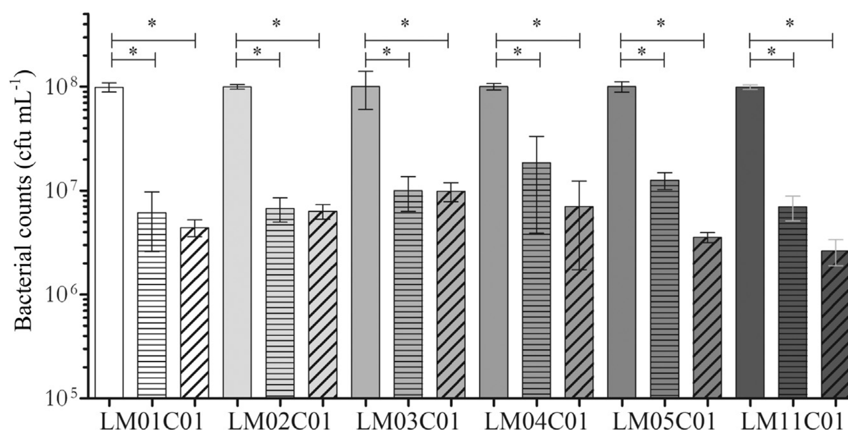


**Fig. 3.** Resistance of *E. durans* (6 representative strains) to simulated gastrointestinal conditions. Counts of viable microorganisms (cfu mL$^{-1}$) without any treatment (□), after incubation in simulated gastric fluid (▤), and sequential incubation in simulated gastric and intestinal fluid (▨); results are expressed as mean ± standard deviation and are representative of at least three independent experiments (*$P < 0.05$).

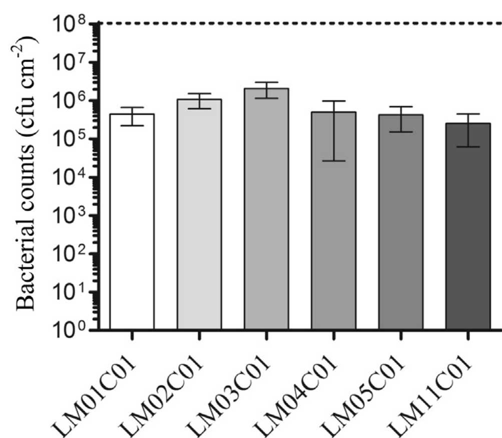


**Fig. 4.** Adhesion of *E. durans* (6 representative strains) to gastric mucin. Counts of viable microorganisms per area (cfu cm$^{-2}$); results are expressed as mean ± standard deviation and are representative of at least three independent experiments.

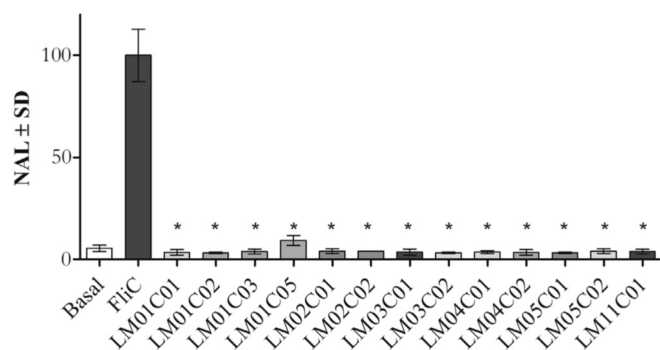


**Fig. 5.** Modulation of pro-inflammatory response in Caco-2 ccl20:luc reporter system by kefir-isolated *E. durans* (13 representative strains). NAL: normalised average luminiscense expressed as percentage of activity induced with flagellin stimulation; FliC: *Salmonella*-isolated flagellin; basal: without any stimulation. Results are expressed as mean ± standard deviation and are representative of at least three independent experiments. *$P < 0.001$.

adherent strain for other authors (Tallon, Arias, Bressollier, & Urdaci, 2007).

*3.3.6. Stimulation assay with Caco-2 ccl20:luc reporter system*

The ccl20 promoter activity is contains NF-kB, AP1 and ESE-1 transcription factors binding sites and is a sensitive indicator of activation of innate response and is highly inducible in Caco-2 cells using TLR agonists such as flagellin (Nempont et al., 2008). All the *E. durans* strains tested were capable of reducing the cell activation induced by FliC to almost basal level since a very strong inhibition of luciferase activity was observed (Fig. 5).

## 4. Discussion

Several species of bacteria and yeasts have been isolated and identified from kefir microbiota by using selective growth media, morphological and biochemical characteristics, and molecular biology based-techniques (Chen, Wang, & Chen, 2008; Garrote et al., 2001; Hamet et al., 2013; Kesmen & Kacmaz, 2011; Simova et al., 2002). To our knowledge, the presence of enterococci in kefir has been reported only by few authors (Hsieh, Wang, Chen, Huang, & Chen, 2012; Rea et al., 1996; Ünsal, 2008; Yüksekdağ, Beyatli, & Aslim, 2004), but no subsequent studies regarding their safety were described. In this sense, this work constitutes the first report about isolation, safety evaluation and characterisation of beneficial properties of different strains belonging to genus *Enterococcus* from kefir-fermented milk. Noteworthy, all the 36 initial isolates phenotypically and genotypically characterised as *Enterococcus* were lately identified as *E. durans*, in concordance with results previously described by Yüksekdağ et al. (2004) who worked with samples of Turkish kefir.

It has been reported that *E. faecium* are the most frequently occurring enterococcal species in dairy products (Morandi, Brasca, Andrighetto, Lombardi, & Lodi, 2006), fermented vegetable and sausages (Kim et al., 2009; Landeta, Curiel, Carrascosa, Muñoz, & de las Rivas, 2013). Indeed, the isolation of *E. faecalis* and *E. faecium* from kefir grains was described by Hsieh et al. (2012) and Ünsal (2008) respectively, however these species were not found in our kefir samples. It is important to note that in our case the highest level of enterococci was found in fermented milk instead in kefir grains, where counting of these bacteria was very low. In contrast, other authors have failed to find enterococci in fermented milk (Ünsal, 2008) or have reported a higher counting in the grains than in fermented milk (Hsieh et al., 2012).

In this regard, several studies have revealed that microbial composition of the final product may not be equivalent to that found in kefir grains, mainly because of fermentation conditions and location of the microorganisms in the grain matrix which could affect the release of them to the milk (Chen et al., 2008; Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011). Besides, it is known that grains from different origins showed differences in their microbiota

(Garrote et al., 2001; Kesmen & Kacmaz, 2011), which could explain the apparent disagreements between our results and those from other authors. Also, our findings are in concordance with the results obtained by Rea et al. (1996) who were able to detect a maximum of $5 \times 10^5$ cfu $mL^{-1}$ of enterococci in kefir milk after 30 h of fermentation.

The Qualified Presumption of Safety (QPS) list updated by the EFSA Panel on Biological Hazards (BIOHAZ) (EFSA, 2012b), no longer includes *Enterococcus* species even though in 2009 the EFSA had concluded that a strain specific evaluation was necessary to assess the risk associated to the intentional use of enterococci in the food chain (EFSA, 2012a).

The resistance against different antibiotics and the production of virulence factors are two important factors for the evaluation of safety in enterococci. In particular, the prevalence of vancomycin-resistant enterococcal strains is a relevant issue to be aware of, since it is the last choice antibiotic for treatment of severe nosocomial infections by enterococci in hospitalised or immunocompromised patients in case penicillins were proved not to be effective (Klein, 2003). Several studies showed the occurrence of vancomycin-resistant enterococci in food of animal origin, mainly in *E. faecalis* and *E. faecium* species, although the isolation frequency seems to be lower than in clinical samples (Klein, 2003).

In our case, all the tested strains (29 in total) were found to be susceptible to vancomycin, and this lack of resistance correlated with the absence of *van A*, *van B* and *van C2* genes. Besides, only 3 strains showed resistance to ampicillin, meanwhile the other 26 strains were susceptible to this antibiotic (MIC $< 2$ mg $mL^{-1}$) and did not amplified for *esp*, $hyl_{Efm}$ or *IS*16 genes. According to the EFSA, if one or more these genetic elements were detected, the strain is considered as unsafe and should not be used as a feed additive (EFSA, 2012a). Moreover, no resistance to other clinically important antibiotics such as ciprofloxacin, erythromycin or linezolid were found in these kefir-isolated enterococci.

The potential pathogenicity of these kefir-isolated strains was evaluated also by investigating the presence of genes encoding for other virulence factors, as determined by studies in different enterococcal species (Ben Omar et al., 2004; Morandi et al., 2013). Genes encoding for aggregation substance (*agg*), cell-wall anchored collagen adhesin (*acm*), gelatinase (*gelE*) and cytolysin (*cylA*) were not found in our isolated strains. Besides, the amplification for a sex pheromones genes (*ccf*), that could be involved in initiating conjugation processes (Clewell, 2011), was negative in all tested strains, diminishing the possibility that apparently safe cultures might acquire virulence factors by conjugation (Eaton & Gasson, 2001).

Taking into account most of the *E. durans* strains tested may be considered as safe, we decided to test some characteristics that are recommended as desirable for probiotic microorganisms. It is known that the adverse conditions that microorganisms will find in the stomach (low pH and pepsin activity) constitute an effective barrier against the entry of bacteria and their survival in the intestine. Our results indicate that all the *E. durans* strains tested were able to survive to simulated gastrointestinal conditions, being the gastric transit the critical step (with a loss of viability of around one logarithm), meanwhile the presence of pancreatin and bile salts in the intestinal environment did not greatly affected bacterial viability. These findings are in agreement with results reported by other authors for *Enterococcus lactis* (Morandi et al., 2013) and *E. faecium* (Hosseini et al., 2009) isolated from food, and suggest that these *E. durans* strains isolated from kefir could have the ability to reach the intestinal lumen and keep alive in that environment. Moreover, adhesion to gastrointestinal mucus could be considered as a prerequisite for bacterial colonisation, at least transiently, of the gut (Tallon et al., 2007). All the *E. durans* strains tested here were able to adhere to mucins, which could allow them to interfere with pathogen binding and also interact with the mucosal immune system, as reported for other kefir-isolated lactic acid bacteria (Carasi et al., 2014).

Numerous strains of enterococci associated with food systems show antimicrobial activity against pathogenic bacteria, which is of special interest for the application of these bacteria as starter or protective cultures in food industry. In our case, the ability to inhibit growth of pathogens was strain-dependent, as expected. In this sense, all tested strains were able to inhibit at least two different pathogenic bacteria, and only two strains isolated from different kefir grains (LM01C02, LM04C01) inhibited all the pathogenic bacteria assessed. Activity against Gram negative and Gram positive bacteria were observed in the same extent, being enterohaemorragic *E. coli*, *S. aureus* and *E. faecalis* inhibited by the greatest number of kefir-isolated *E. durans* strains. In particular, the activity against some Gram negative bacteria, which are usually considered to be resistant to inhibitory power of lactic acid bacteria, has been described in *E. durans* (Batdorj et al., 2006) and other enterococci strains (Kang & Lee, 2005; Line et al., 2008) and could be an important issue to be aware of because of the prevalence of Gram negative pathogenic bacteria as causal agents of gastrointestinal infections. Besides, 6 out of 13 tested strains showed an inhibitory activity against *L. monocytogenes*. Taking into account that milk is one of the sources of food-borne listeriosis (Arqués, Rodríguez, Gaya, Medina, & Nuñez, 2005), the antilisterial activity is of the great interest in food and dairy product industries. As reported by other authors, a common feature of enterococci is their ability to produce bacteriocins with a broad spectrum of activity (Cocolin, Foschino, Comi, & Grazia Fortina, 2007; Giraffa, 2003; Morandi et al., 2013), which is in concordance with our observations although we did not focus the present study in the identification of this kind of substances by kefir-isolated *E. durans*.

On the other hand, the ability of many different bacterial species (mostly *Lactobacillus* and *Bifidobacterium*) to modulate the immune response has been of special interest in the last years (Corthésy, Gaskins, & Mercenier, 2007). Romanin et al. (2010) reported the use of the Caco-2 ccl20:luc reporter system to study the capability of kefir-isolated yeasts of modulate the mucosal innate immune response. Yeasts from different genera showed a strong down-regulation of the pro-inflammatory response induced by flagellin, but only some lactobacilli strains showed a modest anti-inflammatory activity. Noteworthy, all the *E. durans* strains assessed here showed an inhibition of flagellin-induced activation of epithelial cells to basal level, similarly to kefir yeasts. These findings encourage to further study of the anti-inflammatory effects of these enterococcal strains in the future.

## 5. Conclusions

Altogether, the results presented in this work indicate that the presence of the strains of *E. durans* in our kefir grains does not represent a threat to the health of consumers, but instead might explain some of the beneficial properties traditionally associated with kefir consumption. In turn, the isolation of safe enterococcal strains able to resist to gastrointestinal conditions, showing antimicrobial activity and capable of down-regulating inflammatory response in intestinal epithelial cells proves that kefir fermented milk constitutes a very valuable source of bacterial strains with great potential for functional food industry.

## Acknowledgements

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (PICT 00479/06), CONICET (PIP 0598),

Universidad Nacional de La Plata (Project 11/X548), and Bordeaux Science Agro, Ministère de l'Agriculture Français. P Carasi is a fellow of CONICET; G De Antoni is a researcher of the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC-PBA); M Serradell is a member of the Carrera de Investigador Científico y Tecnológico of CONICET. MC Urdaci is researcher of Bordeaux Science Agro, Université de Bordeaux.

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