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ORIGINAL ARTICLE

Yeasts from autochthonal cheese starters: technological and functional properties

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Abstract

Aims: The aim of this work was to identify 20 yeasts isolated from autochthonal cheese starters and evaluate their technological and functional properties.
Methods and Results: The capacities of the yeasts to grow at different temperatures, pH, NaCl and lactic acid concentrations as well as the proteolytic and lipolytic activities were studied. Moreover, survival to simulated gastrointestinal digestion, hydrophobicity, antimicrobial activity against pathogens and auto- and co-aggregation abilities were evaluated. The sequentiation of a fragment from the 26S rDNA gene indicated that *Kluyveromyces marxianus* was the predominant species, followed by *Saccharomyces cerevisiae*, *Clavispora lusitaniae*, *Kluyveromyces lactis* and *Galactomyces geotrichum*. RAPD with primer M13 allowed a good differentiation among strains from the same species. All strains normally grew at pH 4·7–5·5 and temperatures between 15 and 35°C. Most of them tolerated 10% NaCl and 3% lactic acid. Some strains showed proteolytic (eight isolates) and/or lipolytic (four isolates) capacities. All strains evidenced high gastrointestinal resistance, moderate hydrophobicity, intermediate auto-aggregation and variable co-aggregation abilities. No strains inhibited the growth of the pathogens assayed.
Conclusions: Some strains from dairy sources showed interesting functional and technological properties.
Significance and Impact of the Study: This study has been the first contribution to the identification and characterization of yeasts isolated from autochthonal cheese starters in Argentina. Many strains could be proposed as potential candidates to be used as probiotics and/or as co-starters in cheese productions.

Introduction

Probiotics are defined as live micro-organisms which, when administered in adequate amounts, confer a benefit on the host (FAO/WHO 2002). Numerous micro-organisms are currently used as probiotics; among them *Lactobacillus* and *Bifidobacterium* constitute the most frequently utilized genera, mainly included in the formulation of dairy functional products. However, certain strains of *Bacillus* and *Pediococcus* have also been found as effective probiotics (Soccol *et al.* 2010). More recently, this increasing interest in the use of functional foods containing probiotic micro-organisms for health promotion and disease prevention has also reached the field of yeasts (Zanello *et al.* 2011). Their use as active dietary supplements, mainly for farm animals, is still limited despite their demonstrated occurrence as an integral part of the predominant or characteristic microflora of several fermented food products (Fleet and Mian 1987; Cocolin *et al.* 2006; Fleet 2007).

Saccharomyces boulardii is practically the only yeast commercialized as a probiotic for humans (Martins *et al.*

2005). This species is closely related to *Saccharomyces cerevisiae* from a genetic point of view, but both species are metabolically very different (Generoso *et al.* 2010). This leads to the question whether other yeasts possess biotherapeutic properties as well. Some authors have suggested the use of other yeast species or genera based essentially on *in vitro* assays and very few clinical trials (Kovacs and Berk 2000; Psomas *et al.* 2001; Kumura *et al.* 2004; Van der Aa Kühle *et al.* 2005; Pennacchia *et al.* 2008).

Yeasts play important roles in the dairy industry, mainly in cheese production, where they are commonly present. On the positive side, there is an increasing interest in their beneficial properties as novel probiotic and biocontrol agents; on the negative side, dairy-associated yeasts could be an underestimated source of defects (Jakobsen and Narvhus 1996). This fact emphasizes the need to develop and evaluate reliable tools for the characterization and identification of yeasts from dairy products. Following isolation, the first step is to identify the strains to the genus and species level using internationally accepted methodologies. Several studies have shown that traditional methods, based on the phenotypic properties of micro-organisms (morphological, biochemical and physiological tests) are laborious and lack discriminatory power, and misidentification occurs frequently (Lopandic *et al.* 2006). In addition, some tests show a high degree of variability (e.g. fermentation of glucose for *Debaryomyces hansenii*) and hence cannot be relied upon for identification purposes (Prillinger *et al.* 1999). The progress in molecular techniques in the last two decades has opened up numerous possibilities to identify and characterize yeasts at the genomic level, as it was recommended by the guidelines for the evaluation of probiotics in food (FAO/WHO 2002). In this sense, pulsed field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and, mainly, sequencing of the genes coding for 18S and 26S ribosomal RNA (rRNA) and ITS regions represent a step forward in the identification and classification of yeasts (Lopandic *et al.* 2006).

The aim of this work was to characterize, from a technological and functional point of view, yeasts isolated from Argentinean autochthonal cheese starters.

Materials and methods

Strains: origin and culture conditions

Twenty yeast strains isolated from autochthonal milk and whey starters used in cheese productions were studied (Table 1). Yeasts from nine factories of the Tandil (Argentina) area were isolated, as was described by Bruschi *et al.*

Table 1 Origin, source and identification of yeasts isolated from autochthonal cheese starters

Strain name	Origin (factory)	Source	Closest known species (% similarity)*	GenBank accession N° of closest known species
L1	Q5	Whey starter	*Saccharomyces cerevisiae* (99)	JN083825.1
L2	Q6	Milk starter	*Saccharomyces cerevisiae* (99)	JF757233.1
L3	Q7	Whey starter	*Saccharomyces cerevisiae* (100)	JF757233.1
L4	Q3	Whey starter	*Clavispora lusitaniae* (100)	FJ627986.1
L5	Q4	Whey starter	*Kluyveromyces marxianus* (100)	HQ262326.1
L6	Q1	Whey starter	*Kluyveromyces marxianus* (100)	HQ262358.1
L7	Q1	Milk starter	*Kluyveromyces marxianus* (100)	HQ262358.1
L8	Q9	Whey starter	*Kluyveromyces marxianus* (99)	AF543841.1
L9	Q4	Whey starter	*Kluyveromyces marxianus* (99)	HQ262358.1
L10	Q9	Whey starter	*Kluyveromyces marxianus* (100)	HQ262317.1
L11	Q2	Whey starter	*Saccharomyces cerevisiae* (100)	JN083825.1
L12	Q8	Whey starter	*Kluyveromyces lactis* (100)	GU225748.1
L13	Q3	Whey starter	*Kluyveromyces lactis* (99)	GQ121572.1
L14	Q7	Whey starter	*Galactomyces geotrichum* (100)	DQ907938.1
L15	Q4	Milk starter	*Galactomyces geotrichum* (99)	DQ907938.1
L16	Q2	Milk starter	*Kluyveromyces marxianus* (100)	HQ262326.1
L17	Q1	Whey starter	*Kluyveromyces marxianus* (100)	HQ262358.1
L18	Q2	Milk starter	*Pichia kudriavzevii* (99)	JN004188.1
L19	Q3	Milk starter	*Clavispora lusitaniae* (100)	FJ627986.1
L20	Q3	Milk starter	*Clavispora lusitaniae* (100)	FJ627986.1

*Accession numbers were determined by searching the GenBank database using the local BLAST program. We selected those sequences showing maximum identity among those displaying maximum coverage when aligned with sequences from PCR amplicons as closest relatives.

(2009). These factories are small establishments that produce only artisanal cheeses using these natural whey and milk starters. Strains were cultivated in YM (0·5% w/v peptone, 0·3% w/v yeast extract, 0·3% w/v malt extract, 1·0% w/v dextrose; pH: 6·2 ± 0·2) broth at 30°C and were maintained as frozen stocks at −80°C in the presence of 15% (v/v) glycerol as cryoprotective agent.

Identification and RAPD analysis

Total DNA of isolates was obtained from overnight cultures using the DNeasy® Blood and Tissue kit (QIAGEN, Venlo, the Netherlands) according to the manufacturer's instructions. Purified DNA samples were stored at −20°C until use. The identity of isolates was analysed by amplifying, sequencing and comparing a 607-bp fragment (D1/D2 region) from the 26S rRNA encoding gene, obtained using the primers NL1 (5′-GCATATCAATA AGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTC AAGACGG-3′) (White *et al.* 1990). All PCRs were carried out using 1 μl of DNA as template, 2·5 U *Taq* DNA polymerase (GE Healthcare, Little Chalfont, UK), 200 μmol l^{-1} dNTPs mix (GE Healthcare) and 400 nmol l^{-1} each primer (Sigma-Genosys, The Woodlands, TX, USA) in a final volume of 50 μl. Amplifications were performed in a GeneAmp PCR System (Applied Biosystems, Foster City, CA USA) under the following conditions: an initial step of 94°C for 5 min, 40 cycles of: 94°C for 40 s, 55°C for 40 s and 72°C for 30 s, and a final step of 72°C for 10 min. The PCR products were separated on 1·5% (w/v) agarose gels in TBE buffer, stained with GelRed (Biotium, Hayward, CA, USA) and visualized under UV light (Sambrook and Russell 2001). Amplicons were purified with MicroSpin Columns (GE Healthcare), and their nucleotide sequences were determined by primer extension at the DNA Sequencing Service of Macrogen (Seoul, Korea). The identity of strains was checked by nucleotide–nucleotide BLAST of the NCBI database (www.ncbi.nlm.nih.gov/BLAST).

Genotypic diversity among strains was analysed by RAPD-PCR, using the single arbitrary primers RF2 (5′-CGGCCCCTGT-3′, Paffetti *et al.* 1995; Andrighetto *et al.* 2000) and M13 (5′-GAGGGTGGCGGTTCT- 3′, Huey and Hall 1989), in independent reactions. Amplifications were performed under the following conditions: an initial step of 94°C for 3 min, 35 cycles of 94°C for 30 s (RF2) or 2 min (M13), 36°C for 1 min (RF2) or 45°C for 20 s (M13), 72°C for 1 min (RF2) or 2 min (M13), and a final step at 72°C for 7 min. PCRs were performed in a total volume of 25 μl with 1 μl template DNA, 1 μmol l^{-1} primer (Sigma-Genosys), 2·5 U *Taq* DNA polymerase (GE Healthcare) and 200 μmol l^{-1} dNTPs mix (GE Healthcare). A negative control (without template) was included in all reactions. Amplification products were analysed by electrophoresis on 1·8% (w/v) agarose gels in TBE buffer, following standard protocols.

The amplification pattern images of the gels were captured using the Gel Logic 112 Imaging System (Carestream Health Inc., New York, NY, USA) equipped with the Kodak MI Image Analysis Software (V 4.0). Images were saved as TIFF files and analysed with the pattern analysis software package BioNumerics™ (version 5.0; Applied Maths BVBA, Saint-Martens-Latem, Belgium). Calculation of similarity of band profiles was based on the Pearson's correlation coefficient *r*, and strains were grouped using the unweighted pair group method with arithmetic averages (UPGMA) (Vauterin and Vauterin 1992).

Technological characterization

The yeast strains were grown in YNB medium (0·67% w/v) (Sigma-Aldrich, St. Louis, MO, USA) added with 0·5% w/v glucose (Vivier *et al.* 1994) under the following conditions: at pH 4·7, 5·15 and 5·5 and incubation at 27°C for 3 weeks; at 5, 10, 18, 35 and 45°C and incubation for 3 weeks; in the presence of different concentrations (2·5, 5, 10, 15 and 20% w/v) of NaCl (Besançon *et al.* 1992) and incubation at 27°C for 3 weeks; and different concentrations (1, 2 and 3% v/v) of lactic acid and incubation at 27°C for 3 days (Besançon *et al.* 1992). The extracellular proteolytic activity was tested in milk agar (Merck, Darmstadt, Germany), under incubation at 27°C for 3 weeks. After this time, the zone of clearance was measured (Hansen and Jakobsen 2001). The lipolytic activity was tested in tributyrin agar (Merck), under incubation at 27°C for 5 days. Then the zone of clearance was measured (Hansen and Jakobsen 2001). The assays were performed in triplicate.

Functional characterization

Survival to simulated gastrointestinal (GI) digestion

This assay was performed according to Burns *et al.* (2010) with modifications as follows: 40 ml of overnight cultures grown in YM broth was harvested by centrifugation (5000 ***g***, 10 min). Pellets were washed twice with phosphate buffer (PB) 0·1 mol l^{-1} (pH 7·0) (0·5382% w/v NaH_2PO4, 1·6363% w/v $Na_2HPO_4.7H_2O$), and cells were resuspended in 40 ml of saline solution (125 mmol l^{-1} NaCl, 7 mmol l^{-1} KCl, 45 mmol l^{-1} $NaHCO_3$ and 3 g l^{-1} pepsin, pH 2·1 adjusted with HCl). Yeast suspensions were then incubated for 90 min at 37°C, and afterwards, 5 ml of culture was harvested (5000 ***g***, 10 min), resuspended in the simulated ileal juice (0·3% w/v bovine bile and 0·1% w/v pancreatin in PB, pH 8·0) and incubated for 60 min at 37°C. Viable counts (YM agar,

3 days, 30°C) were obtained from the initial cultures and after simulation of each condition tested. The assays were performed in triplicate.

Hydrophobicity

To evaluate surface hydrophobicity, the ability of cells to adhere to hydrocarbons was determined according to Burns *et al.* (2008). Overnight cultures of the strains were harvested by centrifugation at 5000 ***g*** for 10 min at 5°C, washed twice in PB 0·1 mol l^{-1} (pH 7·0) and resuspended in the same buffer. The cell suspension was adjusted to an OD_{560nm} of approximately 1·0 in the same buffer, and 3 ml of these suspensions was added to 0·6 ml of *n*-hexadecane (Merck) and vortexed for 120 s. The two phases were allowed to separate for 1 h at 37°C. The aqueous phase was carefully removed and its OD_{560nm} was measured. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%), which was calculated with the formula $H\% = [(OD_0 - OD)/OD_0] \times 100$, where OD_0 and OD are the optical density before and after extraction with *n*-hexadecane, respectively. This assay was performed in triplicate.

Antimicrobial activity

Escherichia coli V517, *Salmonella enteritidis* OMS-Ca and *Staphylococcus aureus* 76 (INLAIN Collection) were included as pathogens in inhibition assays. An aliquot (5 ml) of each yeast strain culture was spot-inoculated on YM agar. After incubation at 30°C for 48 h, the cells were killed by exposure to chloroform for 20 min. Residual chloroform was allowed to evaporate and the Petri dish was overlaid with 3·5 ml of TS (Tryptone Soya; Britania, Buenos Aires, Argentina) soft agar (0·6%) that had been inoculated with 10^6 CFU of the indicator strain. After 24 h of incubation at 37°C, the dish was evaluated for the presence of antagonism. A positive result was registered when an inhibition halo was observed (Nardi *et al.* 1999). This assay was performed in triplicate.

Auto-aggregation ability

Overnight yeast strain cultures in YM broth were centrifuged for 10 min at 5000 ***g*** and washed twice with PB 0·2 mol l^{-1} (pH 7·2). The pellets were resuspended in the same buffer (2 ml), vortexed for 30 s and incubated for 2 h at 37°C. An aliquot (1 ml) of these suspensions was carefully removed from the upper zone, and the OD_{560nm} was measured (Kos *et al.* 2003). Auto-aggregation ability was calculated using the formula $Au\% = 1 - (OD_t/OD_0) \times 100$, where OD_0 and OD_t are the optical density before and after incubation, respectively. Percentage values (Au%) <30 were considered low, between 30 and 60 intermediate, and >60 high. This assay was performed in triplicate.

Co-aggregation with pathogens

Escherichia coli V517 and *Salm. enteritidis* OMS-Ca were included as pathogens in co-aggregation assays. They were grown and counted in TS broth and agar (Britania), respectively (37°C, 24 h). Yeast cell suspensions from overnight cultures were prepared as described above in PB 0·2 mol l^{-1} (pH 7·2). Yeasts and pathogens were mixed in a CFU–CFU relation of 1 : 1 (approx. 10^8 CFU ml^{-1}) (Burns *et al.* 2011). The cell suspensions were allowed to stand at 37°C for 3 h and then pathogenic cells were counted. Yeast suspensions of pure cultures after incubation were used as controls. The assay was performed in triplicate. Co-aggregation ability was calculated using the formula $Co\% = 1 - (CFU_c/CFU_t) \times 100$, where CFU_t and CFU_c are pathogenic cell counts at the end of incubation in the mixtures and the control, respectively. Percentage values (Co%) <30 were considered low, between 30 and 60 intermediate, and >60 high. This assay was performed in triplicate.

Results

Identification of strains

The identification of the 20 isolated yeast strains is showed in Table 1. Taking into account the methodology selected for the analysis, 50% (10 isolates) of the strains belonged to the genus *Kluyveromyces*, and of these, 80% (eight isolates) belonged to the species *Kl. marxianus* and 20% (two isolates) to the species *Kl. lactis*. *S. cerevisiae* accounted for 20% (four isolates) of the isolates, followed by *Clavispora lusitaneae* (15%, three isolates), *Galactomyces geotrichum* (10%, two isolates) and *Pichia kudriavzevii* (one isolate).

When the genetic diversity was analysed by RAPD with primer M13, two clusters were clearly visualized (Fig. 1), being named Cl I and Cl II (similarity coefficient < 50·0%). Cluster I comprised 12 strains (nine of *Kluyveromyces* and the three strains of *C. lusitaniae*). This cluster was divided into three subclusters (similarity coefficients > 78·0%), named Scl I-1 (five strains), Scl I-2 (four strains) and Scl I-3 (three strains). Cluster II included *G. geotrichum*, *S. cerevisiae, P. kudriavzevii* and *Kl. marxianus* strains and was also divided into three subclusters (similarity coefficients > 78·0%), named Scl II-1 (two strains), Scl II-2 (four strains) and Scl II-3 (two strains). Surprisingly, eight *Kl. marxianus* strains showed a different profile from those of the other strains belonging to the same species, and it was grouped with *P. kudriavzevii*. This methodology was very useful to discriminate among strains from the same origin. For example, the strains L6, L7 and L17 (*Kl. marxianus*) came from the factory Q1. L6 and L7 (Scl I-2) could be the same strain, since their

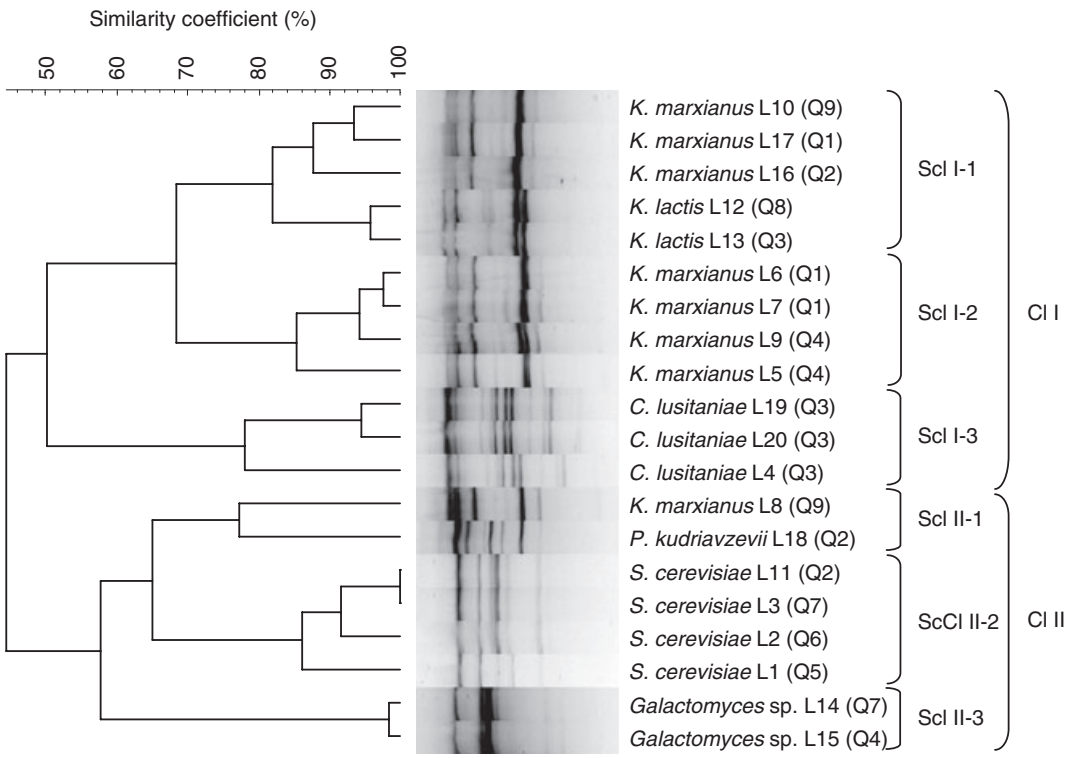

Figure 1 Dendrogram obtained by comparison (Gel Compar Software, Applied Maths, Sint-Martens-Latem, Belgium) and clustering (UPGMA method: unweighted pair group method using arithmetic averages) of RAPD profiles of yeast strains with primer M13. Clusters (Cl, similarity coefficients < 50%) and subclusters (Scl, similarity coefficients > 78%) are indicated. Source of yeasts is also indicated.

similarity coefficient was 97·5%, while these strains and L17 (Scl I-1) were discriminated by a similarity coefficient of 68·0%. Similar results were obtained for strains L5 and L9 (*Kl. marxianus*, from Q4, Scl I-2), which showed a similarity coefficient of 82·9%, L8 and L10 (*Kl. marxianus,* from Q9, Scl II-1 and Scl I-1 respectively), with a similarity coefficient of 68·9%, and L4 and L19 (*C. lusitaniae*, from Q3, Scl I-3), which shared a similarity coefficient of 78·0%. Moreover, the dendrogram showed that the strains L12 and L13 (*Kl. lactis,* from Q8 and Q3, respectively, Scl I-1) had a high similarity coefficient (96·0%), but, obviously, they could not be the same strain. The same analysis could be made for strains L3 and L11 (*S. cerevisiae,* from Q7 and Q2, respectively, Scl II-2), for which the M13 profiles were identical. RAPD-PCR amplification using primer RF2 showed characteristic profiles for each species; however, since few bands were obtained (data not shown), no discrimination among strains from the same species could be made.

Technological characterization

All yeast strains were capable of growing at the various pH values tested (4·7, 5·15 and 5·5). At 5°C, *C. lusitaniae* strains (L4, L19 and L20) and *P. kudriavzevii* L18 did not grow, while at 10 and 45°C, only *C. lusitaniae* strains did. In YBN medium with NaCl, the growth of all strains was normal up to a concentration of 10% (w/v). *Galactomyces geotrichum* (L14 and L15) grew even at 15% (w/v) and *Kl. lactis* (L12 and L13) tolerated up to 20% (w/v). All strains were capable of tolerating up to 3% (v/v) lactic acid, except *P. kudriavzevii* that only grew up to 2% (v/v).

Of the 20 strains studied, eight isolates (40%) showed proteolytic activity with mean values ranging between 6·8 and 8·7 mm of halo diameter, including only three species: *Kl. marxianus*, *C. lusitaniae* and *G. geotrichum*. All *C. lusitaniae* strains (L4, L19 and L20) showed proteolytic activity, their values being the highest ones obtained (Table 2). Among *Kl. marxianus* strains, only four strains (50%) showed proteolysis halos, and their values were lower than those from *C. lusitaniae.* The only proteolytic strain of *G. geotrichum* (L15) presented a high mean value, similar to that achieved by *C. lusitaniae*. The other species (*S. cerevisiae, Kl. lactis* and *P. kudriavzevii*) did not show this ability (absence of proteolysis halos).

When lipolytic activity was evaluated, only three isolates (15%) of all strains were able to hydrolyse the tributyrin, and these belonged to the species *Kl. marxianus, Kl. lactis* and *G. geotrichum* (Table 2).

Table 2 Proteolytic and lipolytic abilities of the yeast strains and percentages of positive responses for each property, considering their taxonomic classification

Species (N° of total strains)	Proteolytic activity		Lipolytic activity	
	Halo diameter (mm) (mean value ± SD)*	% of positive strains	Halo diameter (mm) (mean value ± SD)*	% of positive strains
Kluyveromyces marxianus (8)	6·8 ± 1·3	50	11·7 ± 1·5	12·5
Kl. lactis (2)	-	0	8·0 ± 0·1	50
Saccharomyces cerevisiae (4)	-	0	-	0
Clavispora lusitaniae (3)	8·67 ± 0·6	100	-	0
Galactomyces geotrichum (2)	8·33 ± 0·6	50	7·7 ± 0·6	50
Pichia kudriavsevii (1)	-	0	-	0

-, Non detectable.
*Mean value and standard deviation (SD) of three determinations.

Table 3 Survival of yeast strains to simulated gastrointestinal digestion

Strain	Counts (log CFU ml^{-1}) at time (min)*			% diminution of counts (at 150 min)
	0	90	150	
L1	8·36 ± 0·11	6·03 ± 0·13	6·32 ± 0·12	99·0
L2	8·62 ± 0·21	6·90 ± 0·12	6·20 ± 0·15	99·6
L3	8·69 ± 0·19	7·50 ± 0·15	6·34 ± 0·14	99·5
L5	8·62 ± 0·15	8·00 ± 0·17	7·98 ± 0·20	77·0
L6	8·00 ± 0·12	7·03 ± 0·18	7·00 ± 0·25	90·0
L7	8·08 ± 0·11	7·79 ± 0·19	7·59 ± 0·13	67·5
L8	8·38 ± 0·20	7·90 ± 0·20	7·70 ± 0·18	79·2
L9	8·59 ± 0·18	8·20 ± 0·23	7·00 ± 0·21	97·4
L10	7·38 ± 0·18	7·32 ± 0·15	7·18 ± 0·23	37·5
L11	7·43 ± 0·14	7·32 ± 0·14	7·23 ± 0·14	37·0
L12	6·32 ± 0·15	6·11 ± 0·13	5·60 ± 0·25	81·0
L13	7·34 ± 0·13	7·25 ± 0·16	7·32 ± 0·19	4·5
L14	8·60 ± 0·12	8·78 ± 0·17	8·90 ± 0·19	0·0
L15	8·45 ± 0·16	8·18 ± 0·2	7·75 ± 0·14	80·0
L16	7·72 ± 0·15	7·65 ± 0·21	6·20 ± 0·20	97·0
L17	7·48 ± 0·13	7·38 ± 0·13	6·04 ± 0·18	96·3
L18	8·49 ± 0·18	7·97 ± 0·12	7·00 ± 0·16	96·8

*Mean and standard deviation (SD) of three determinations.

Functional characterization

Survival to simulated gastrointestinal digestion

The gastrointestinal resistance of the yeast strains is showed in Table 3. Different performances were observed for strains of same species, indicating a clear strain dependence. In fact, L14 and L15 strains (both identified as *G. geotrichum*) showed a percentage of count diminution (at 150 min) of 0 and 80%, respectively. A similar behaviour was detected for *Kl. lactis* L12 and L13 (reductions of 81 and 4·5%, respectively). Anyway, for all yeasts, the highest reduction in viable counts after GI simulation was two logarithmic orders. Some strains (*Kl. marxianus* L10, *S. cerevisiae* L11, *Kl. lactis* L13 and *G. geotrichum* L14) showed significant (lower than two log orders) GI resistance.

Hydrophobicity

Figure 2 shows the hydrophobicity values obtained for the studied strains, which ranged from 45·3% to 85·3%. The highest value corresponded to *S. cerevisiae* L1 (80·1%). For *Kl. marxianus* (predominant species), the highest value was obtained for the strain L9 (72·5%). L14 and L15 strains were not included in this test because they showed variable results due to their agglutinant condition inherent to the genus.

Antimicrobial activity

The products of the yeast strain metabolism used in this study were unable to inhibit the growth of pathogenic micro-organisms used as controls. Inhibition halos were not observed in any case.

Auto-aggregation and co-aggregation with pathogens

Table 4 shows the results of auto-aggregation and co-aggregation abilities of yeast strains on pathogenic micro-organisms. Most of the strains showed intermediate auto-aggregation ability (mean values between 31·0 and 57·0% Au), and *G. geotrichum* L15 was the only strain highly (70·1% Au) auto-aggregating.

When co-aggregation ability was evaluated, a clear dependence on the pathogenic micro-organism was observed. For the two micro-organisms assayed (*E. coli* V517 and *Salm. enteritidis* OMS-Ca), low, intermediate and high co-aggregating strains were recognized. In general, the strains showed higher co-aggregation

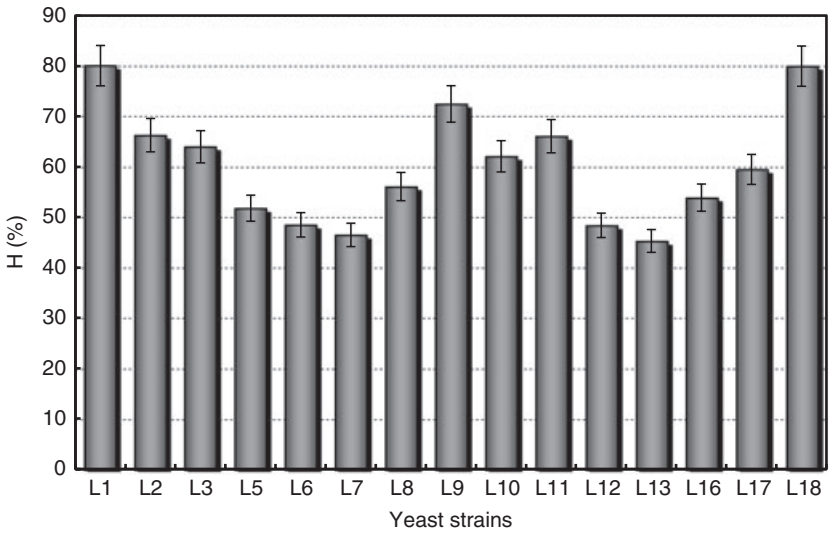

Figure 2 Hydrophobicity (H%) obtained for yeast strains isolated from autochthonal cheese starters. The values represent the mean and standard deviation (bars) of three determinations.

Table 4 Auto- and co-aggregation with intestinal pathogenic micro-organisms of yeast strains isolated from autochthonal cheese starters

		Co-aggregation ability (% Co)* against	
Strain	Auto-aggregation capacity (% Au)*	*Escherichia coli* V517	*Salmonella enteritidis* OMS-Ca
L1	48·6 ± 17·5	2·4 ± 0·1	55·2 ± 0·8
L2	44·1 ± 2·4	24·8 ± 0·8	57·3 ± 0·7
L3	37·9 ± 6·7	29·1 ± 0·9	79·0 ± 6·0
L5	28·6 ± 1·3	47·1 ± 1·1	25·7 ± 0·7
L6	44·4 ± 5·5	34·6 ± 0·8	70·1 ± 0·3
L7	57·0 ± 2·7	61·2 ± 1·3	92·4 ± 0·8
L8	31·0 ± 2·4	66·7 ± 1·5	62·1 ± 0·4
L9	16·0 ± 0·1	61·3 ± 2·2	81·4 ± 1·4
L10	25·5 ± 1·1	55·5 ± 1·0	73·1 ± 2·8
L11	45·3 ± 1·0	40·9 ± 0·0	63·7 ± 1·2
L12	41·3 ± 7·0	91·8 ± 1·9	74·1 ± 2·9
L13	29·3 ± 7·9	94·2 ± 1·4	47·5 ± 2·5
L14	53·3 ± 3·2	94·4 ± 0·9	nd
L15	70·1 ± 2·5	93·7 ± 2·1	16·0 ± 1·0
L16	48·6 ± 9·4	14·4 ± 1·4	67·9 ± 1·4
L17	52·9 ± 9·6	35·7 ± 0·7	61·8 ± 1·1
L18	nd	14·0 ± 0·5	55·7 ± 2·4

nd, not determined.
*Mean value and standard deviation (SD) of three determinations.

activity values on *Salm. enteritidis* than on *E. coli*. In fact, the percentage of strains with high (>60% Co) co-aggregation ability increased from 41·2% (for *E. coli*) to 64·7% (for *Salm. enteritidis*). Some strains of *Kluyveromyces* genus (*Kl. marxianus* L7, L8 and L9, and *Kl. lactis* L12) showed high co-aggregation activity values on both pathogenic micro-organisms (between 61·3 and 92·4%).

Discussion

Cheese ripening is a complex process where, besides the role of the starter lactic acid bacteria (LAB), an important contribution is recognized to the secondary microbiota, mainly constituted by enterococci, micrococci, nonstarter LAB (NSLAB) and yeasts (Beresford *et al.* 2001). Yeasts, in particular, are associated with the secondary microflora of a wide variety of cheeses; however, in most cases, their contribution to cheese ripening remains unclear. Many reasons could explain the occurrence of yeasts in cheese, the more important being tolerance to low pH, reduced water activity and high salt concentrations, as well as yeast ability to grow at the low storage temperature typical of the ripening environment (Ferreira and Viljoen 2003). Other important features that could explain their occurrence in cheeses are the assimilation/fermentation of lactose and galactose and the assimilation of succinic, lactic and citric acids. In addition, they are widely distributed in the dairy environments and appear as natural contaminants in raw milk, air, dairy utensils, brine and smear water (Wyder and Puhan 1999).

In our study, the predominant species found in autochthonal whey and milk starters used in artisanal cheese production was *Kl. marxianus* (eight of 20 isolates), followed by *S. cerevisiae* (four isolates), *C. lusitaniae* (three isolates), *Kl. lactis* (two isolates), *G. geotrichum* (two isolates) and *P. kudiavsevii* (one isolate). Many preliminary studies report that the common microflora of yeasts isolated from cheeses and dairy environments belong to the species *Debaryomyces hansenii*, *Kl. marxianus* and *lactis*, and *Yarrowia lipolytica*, *Geotrichum candidum* and *S. cerevisiae* (Prillinger *et al.* 1999; Andrighetto *et al.* 2000; Suzzi *et al.* 2000; Romano *et al.* 2001;

Vasdinyei and Deák 2003; Gardini *et al.* 2006; Lopandic *et al.* 2006; Aponte *et al.* 2010). *Debaryomyces hansenii* and *Y. lipolytica* were not found in this study. This could be attributed to the fact that, due to their ability to grow under stringent conditions, these species are predominant at later stages in the cheese ripening (Gardini *et al.* 2006).

A combination of physiological and morphological characteristics has traditionally been used in taxonomic studies of cheese yeasts (Kreger-van Rij 1984; Barnett *et al.* 2000). However, there is a lot of evidence that such tests are not satisfactory for the delineation and identification of yeast species (Prillinger *et al.* 1999). In recent times, different molecular methods relying on the comparative DNA studies have been introduced ensuring accurate and reliable characterization and identification of yeasts (Lopandic *et al.* 2006). In this work, the sequencing of the amplified variable fragment (D1/D2 region) of 26S rRNA encoding gene was used to identify the isolates. This technique was combined with random amplified polymorphic DNA (RAPD-PCR) analysis with two arbitrary primers in order to evaluate the genetic diversity among strains. These methodologies were reported by several authors to identify and establish the biodiversity within dairy yeasts (Prillinger *et al.* 1999; Andrighetto *et al.* 2000; Suzzi *et al.* 2000; Lopandic *et al.* 2006; Aponte *et al.* 2010). The results obtained in our study showed that RAPD-PCR analysis was an adequate methodology to differentiate among strains from the same species, although not so good to identify strains. This conclusion was also reported by Vasdinyei and Deák (2003). This analysis allowed us to discriminate strains from the same origin, mainly when using the primer M13.

Another objective of this work was to study the technological properties of the isolates, which could contribute to the desirable sensory characteristics of fermented products. Results concerning the enzymatic activity of yeasts isolated from dairy products have been reported in several previous works (Jakobsen and Narvhus 1996; Roostita and Fleet 1996; Welthagen and Viljoen 1998; Fadda *et al.* 2004; Gardini *et al.* 2006; Aponte *et al.* 2010). These results were positively evaluated for their contribution to understanding the ripening process of various types of cheeses. Yeasts can play a significant role in sensory and functional properties of cheese ripening based on the interactions between yeasts and starter cultures, proteolytic and lipolytic activities, aroma compound formation and other metabolic activities (such as fermentation of lactose and/or galactose). Our strains showed low lipolytic and variable proteolytic ability, depending on the species studied.

The study of functional properties of these yeast strains was another purpose of this work, with the intention to propose their use as potential probiotics. From this viewpoint, it is well known that *C. lusitaniae* is considered an emergent nosocomial pathogen (Cantón *et al.* 2001; Chabasse *et al.* 2009; Gómez *et al.* 2010). This fact determined that the functional assays on the strains belonging to this species have not been performed.

The ability of a strain to survive at low pH values and bile presence is an important criterion to select a micro-organism to be used as potential probiotic, since the stomach acidity and the concentration of bile salts in the intestine are the first biological barriers to overcome after digestion (Gueimonde and Salminen 2006). Pennacchia *et al.* (2008) adopted a count diminution of 30% as maximum value to select yeast strains as potential probiotic micro-organisms. Based on this criterion, only two of 20 strains (*Kl. lactis* L13 and *G. geotrichum* L14) were able to resist the GI-simulated digestion at this level. Anyway, all strains decreased their counts of only two logarithmic orders; hence, they could survive the GI-simulated passage and could reach the intestine in high numbers. The resistance to acidity is not surprising since the strains come from an acid environment where they coexist with lactic acid bacteria. Psomas *et al.* (2001) demonstrated that, among yeast strains isolated from Feta cheese, all isolates showed a high acidity resistance. Kumura *et al.* (2004) also reported a good GI resistance for all *Kl. lactis* (30 isolates) and *S. cerevisiae* (10 isolates) strains from blue-veined cheeses.

The hydrophobic nature of the outermost surface of micro-organisms has been shown to be involved in the attachment of bacteria to host tissues (Rosenberg *et al.* 1980). This property could confer a competitive advantage, important for bacterial maintenance in the human GI tract (Naidu *et al.* 1999). In our work, this property was variable (between 45 and 80%) and strain dependent. A high hydrophobicity of the cell surface has been frequently suggested to explain why certain microbial strains show slower elimination kinetics from the digestive tract than others after cessation of their administration and to have different health effects (Martins *et al.* 2009). However, a good correlation between hydrophobicity (*in vitro* assays) and intestinal adhesion (*in vivo* assays) was not always found, as was previously reported (Martins *et al.* 2009; Kotzamanidis *et al.* 2010).

Another important property of probiotic strains is their antagonistic activity against pathogenic bacteria. LAB can produce antimicrobial substances capable of inhibiting the growth of pathogenic and spoilage micro-organisms. Organic acids, hydrogen peroxide, diacetyl and bacteriocins are included among these compounds (Servin 2004). Nevertheless, yeast antimicrobial features, viz., the ability to secrete mycocins and other metabolic activities against pathogenic micro-organisms, would increase their importance in probiotic formulations or as a secondary

microflora in dairy starters. Our isolates did not inhibit the growth of the pathogenic micro-organisms selected (i.e. *Salm. enteritidis*, *E. coli* and *S. aureus*). These results are in agreement with those reported by other authors which, in general, demonstrated that only few yeast strains belonging to the species commonly found in dairy products exhibit inhibitory activity against pathogenic micro-organisms (Goerges *et al.* 2006, 2011; Psani and Kotzekidou 2006; Martins *et al.* 2009; Silva *et al.* 2011).

Auto-aggregation ability, as well as hydrophobicity, has been related to the adhesion ability of the strain to intestinal epithelial cells (Kotzamanidis *et al.* 2010; Burns *et al.* 2011), although they seem to act by independent traits (Del Re *et al.* 2000). Also, co-aggregation ability could prevent the host from enteric infections, removing the intestinal pathogenic micro-organisms from the lumen or by direct adhesion to the gut epithelium interfering with attachment of pathogens (Yuksekdag and Aslim 2010; Burns *et al.* 2011). Our strains showed low and intermediate auto-aggregation abilities. A clear correlation between hydrophobicity and auto-aggregation ability was not found. These results would be consistent with those found by Del Re *et al.* (2000), who reported that both properties would be independent, even though both would contribute to the adhesion.

This study has been the first contribution to the identification and characterization of yeasts isolated from autochthonal cheese starters in Argentina. Some strains (*Kl. marxianus* L10, *S. cerevisiae* L11 and *Kl. lactis* L13) showed interesting functional properties, which suggest that these strains could be potential candidates to be used as probiotic micro-organisms in food or pharmaceutical preparations. Following the recommendations of FAO/WHO Guidelines, further *in vivo* research (including safety and selection of a functional dose, modulation of immune response or the capacity of preventing enteric infections) must be carried out with these strains. Moreover, some strains (*Kl. marxianus* L5, L8 and L9, and *Kl. lactis* L12) showed interesting technological properties, considering their proteolytic/lipolytic activities and tolerance to NaCl. These strains could be proposed as co-starters in cheese making to improve the sensory properties and reduce the ripening time (Ferreira and Viljoen 2003; Boutrou and Guéguen 2005; Gardini *et al.* 2006). However, a careful selection of strains must be made to avoid possible defects in the final product, such as blowing or excessive lipolysis.

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