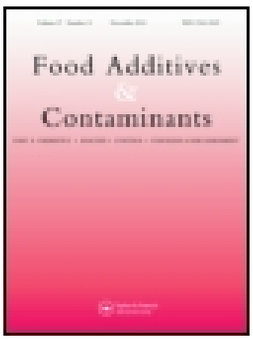

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Encapsulated whey–native yeast *Kluyveromyces marxianus* as a feed additive for animal production

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ABSTRACT

Whey is the main byproduct of the cheese industry. While the composition is variable, it retains up to 55% of milk nutrients. The beneficial features of whey indicates a promising source of new potentially probiotic strains for the development of food additives destined for animal production. The aim of this study was to identify *Kluyveromyces* spp. isolated from whey, to study some probiotic properties and to select the best strain to be encapsulated using derivatised chitosan. *Kluyveromyces marxianus* strains (VM003, VM004 and VM005) were isolated from whey and identified by phenotypic and molecular techniques. These three yeast strains were able to survive under gastrointestinal conditions. Moreover, they exhibited weak auto-aggregation and co-aggregation with pathogenic bacteria (*Salmonella* sp., *Serratia* sp., *Escherichia coli* and *Salmonella typhimurium*). In general the *K. marxianus* strains had a strong antimicrobial activity against pathogenic bacteria. The potential probiotic *K. marxianus* VM004 strain was selected for derivatised-chitosan encapsulation. Material treated with native chitosan exhibited a strong antimicrobial activity of *K. marxianus*, showing a total growth inhibition at 10 min exposure. However, derivatised-chitosan encapsulation showed a reduced antimicrobial activity. This is the first study to show some probiotic properties of whey–native *K. marxianus, in vitro*. An encapsulation strategy was applied using derivatised chitosan.

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Kluyveromyces marxianus; probiotics; encapsulation; whey

Introduction

Argentina annually produces 450,000 tons of whey, one of the main products of the dairy industry. About 33% goes to lactose and protein derivatives production, and 4–5% is transformed into whey powder. The remaining 60% is discarded as effluent or utilised at a low technological level in feed for pigs and cattle, generating important environmental pollution problems. While the composition is variable, it retains up to 55% of the milk nutrients such as lactose, soluble proteins, lipids and minerals, making it a source of microorganisms and a potential low-cost substrate for cellular biomass production (Panesar et al. 2007).

In recent years, much attention has been paid to the design of functional foods that contain probiotic microbial strains responsible for health benefits in the host (Kumura et al. 2004). Probiotics are defined as 'live microorganisms' which, when administered in adequate amounts, exert a beneficial effect on the health of the consumer (FAO/WHO 2002). Prevention of gastrointestinal tract (GIT) colonization by a variety of microbial pathogens is the primary mechanism mediated by probiotics. Above all, probiotics stimulate the immune system, suppress pathogens through competitive exclusion and/or synthesise inhibitory compounds (Papadimitriou et al. 2015). It is established that probiotics must be able to resist gastrointestinal conditions and adhere to gut epithelial tissue (Morelli 2000). Although lactic acid bacteria and bifidobacteria are the microorganisms most widely studied for probiotic properties, the use of yeast as a probiotic food supplement is gaining relevance in the last years (Fleet & Balia 2006).

Kluyveromyces, *Debaryomyces*, *Issatchenkia* and *Yarrowia* yeast genera have been mainly isolated from whey (Spencer & Spencer 1997; Spreer & Mixa 1998). *K. lactis* has become a 'non-conventional' model

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for studying the molecular physiology, especially in comparative studies with *Saccharomyces cerevisiae* strains (Breunig et al. 2000).

In recent years, *K. marxianus* has received attention due to the biotechnological potential. It can grow on a variety of substrates at high temperature and is the major producer of industrial enzymes of β-galactosidase interest, β-glucosidase, inulinase, endopolygalacturonase, carboxypeptidases and aminopeptidases. Since 1994 this yeast has been classified as 'generally regarded as safe' (GRAS) by the USFDA, and since 2005 it has been listed as a microorganism with 'qualified presumption of safety' (QPS) by the EFSA (Hensing et al. 1994; EFSA, 2005, 2013). Moreover, it is used as a source of oligonucleotides, used as flavour enhancers in food products; oligosaccharides, used as prebiotics, oligopeptides and immuno-stimulators (Fonseca et al. 2008).

Vehiculisation in biodegradable polymers matrices is a widely used technique. There are different methods of cell immobilisation, which can be divided into four major categories based on the physical mechanism employed: attachment or adsorption on solid carrier surfaces, entrapment within a porous matrix, self-aggregation by flocculation or with cross-linking agents, and cell containment behind barriers (Kourkoutas et al. 2004). Chitosan (Ch) is a polysaccharide derived from the alkaline hydrolysis of chitin. It is a natural, biocompatible, biodegradable, antioxidant, antimicrobial, non-toxic, immunostimulant, mucoadhesive, antitumor compound (Singla & Chawla 2001). Recently the use of Ch as growth promoter to reduce the use of antibiotic in animal feed has been proposed (Swiatkiewicz et al. 2015). The antimicrobial activity of Ch can be attributed to its polycationic nature resulting from protonation of NH_2 groups when the polymer is dissolved in acidic solutions (Aranaz et al. 2009) or its oligopolymer action which blocks transcription of RNA by adsorption of DNA (Benhabiles et al. 2012). To take advantage of the functional properties of Ch and to use it as a carrier of probiotics, derivatisation is necessary in order to reduce its polycationic character and consequently antimicrobial activity, without losing other potent biological properties with application in the formulation of functional foods.

The beneficial feature of whey indicates this substrate as a promising source of potential probiotic yeasts strains for the development of feed additives destined for animal production. Thus, the aim of this study was to isolate and identify yeasts from whey, and to study some of their probiotic capacities. Moreover, an encapsulation strategy was applied using derivatised Ch.

Materials and methods

Sampling and yeast strain isolation

Whey samples were obtained from the Cooperative 'Cuatro Esquina' located 8.6 km north of Villa Maria city, Córdoba. Samples from different stages of cheese production were collected and transported in refrigerated boxes containing cooling gel to the laboratory for immediate analysis.

Yeast strains were isolated from whey according to Massera et al. (2013). A total of 10 µl of chloramphenicol solution (50 mg ml^{-1}) were added to 10 ml of whey to inhibit bacterial growth and incubated at 150 rpm for 12 h at 28°C. Serial dilutions in PBS solution pH 7.4 were made and 0.1 ml aliquots were inoculated onto lactose–ML culture medium (4.5 g yeast extract, 7.5 g peptone, 20 g lactose, 20 g agar, 1000 ml distilled water and 1 mg ml^{-1} of phenol red) and incubated for 48 h at 30°C. Yeast colonies that showed colour change in the medium were selected.

Phenotypic identification of yeasts

For phenotypic identification, the methodology proposed by Kutzman et al. (2011) was followed.

The ability to ferment sugars such as lactose, glucose, sucrose, maltose and raffinose was evaluated in test tubes (Durham tubes) using 2% sugar solutions (w/v), except raffinose (4%). The basal medium (BM) for fermentation was constituted using 4.5 g of yeast extract, 7.5 g peptone and 20 g of the studied sugar (40 g raffinose) per litre and 4 ml of stock solution of bromothymol blue (50 mg in 75 ml distilled water) per 100 ml of BM. Final pH was 7–8. Tubes were inoculated with strains and incubated for 28 days at 28°C and inspected daily. The presence of gas in the Durham tube and the colour change of the indicator from dark green to yellow were considered positive.

The ability to assimilate lactose, glucose, sucrose, maltose and raffinose in solid media was evaluated by replica plate technique. The plates were incubated for 24–48 h at 30°C. An impression of the incubated plate was performed and a set of plates were inoculated with 2% sugar to study in agarised BM (5 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 2 g yeast extract, 20 g agar and 1000 ml distilled water) and autoclaved at 121°C for 15 min. Assimilation was considered positive when yeast growth was observed on the plate.

Petri plates with YPD agar in duplicate were inoculated to determine the growth at 37°C, and incubated for up to 2 weeks. The plates were inspected daily and the growth was considered positive.

Molecular identification of yeasts

Yeasts culture and DNA extraction

A pure colony of each isolate grown on a solid medium was transferred to 3 ml of YPD medium and incubated at 28°C for 24 h. After that, 1 ml was centrifuged (12,000 rpm 15 min) and the obtained pellet frozen in liquid nitrogen for 5 min. Fungal DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) procedure following the methodology proposed by Leslie and Summerell (2006).

Microsatellite-primed PCR

The one-step PCR-fingerprinting method was performed using the microsatellite primer $(GTG)_5$. PCR reactions were made with 20–30 ng of fungal DNA in a total volume of 25 µl of 1× reaction buffer containing 2 mM $MgCl_2$, 1.25 U Taq DNA polymerase (5 U μl^{-1}, Invitrogen by Life Technologies, Buenos Aires, Argentina), 0.2 mM of each dNTP and 0.6 µM of GTG_5 primer. A negative control, containing all reagents without fungal DNA, was included in every set of reactions. PCR was conducted according to the following cyclic conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles consisting of 94°C for 45 s, 54°C for 45 s and 72°C for 1 min, and a final extension step of 72°C for 10 min, and then held at 4°C indefinitely. DNA band patterns were visualised after electrophoresis using 1.5% agarose gels stained with 0.5 µg ml^{-1} ethidium bromide. Gels were photographed using a MiniBIS Pro, DNR Bioimaging systems analyser. The fragment sizes were measured by comparison with DNA 100-bp ladder (Invitrogen by Life Technologies) whose reference bands vary between 100 and 2072 bp.

Sequencing of ITS (5.8S) regions

The microsatellite-primed PCR results were confirmed by chosen strains for its sequencing at ITS region with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR was set up in a 50-µl reaction mixture containing 5 µl of genomic DNA (10 ng μl^{-1}), 1 × reaction buffer containing 2 mM MgCl2, 1.25 U Taq DNA polymerase (5 U μl^{-1}, Invitrogen by Life Technologies), 0.2 mM of each dNTP and 0.6 µM of each primer. A negative control, containing all reagents without fungal DNA, was included in every set of reactions. Amplification was performed in a MJ Research PTC-200 thermocycler (GMI Inc., Minnesota, MN, USA) programmed for 5 min at 94°C followed by 35 cycles of 1-min denaturation at 94°C followed by primer annealing 1 min at 55°C and primer extension 1 min at 72°C and a final 5-min elongation step at 72°C. PCR products were visualised after electrophoretic run on 1.5% agarose gel stained with ethidium bromide (0.5 µg ml^{-1}). For DNA sequencing of both strands, template DNA was send to Macrogen Inc. (Korea). After that each sequence was aligned with Clustal W (Thompson et al. 1994) as implemented in the program BioEdit version 7.0.9.0 (Hall 1999).

Tests for potential probiotic properties

Tolerance to gastric and intestinal solutions

Gastric stomach tolerance solution was as described by van der Aa Kühle et al. (2005), with modifications. A suspension in PBS of 10^7 cell ml^{-1} from each strain grown on YPD agar was performed by counting in a Neubauer chamber with trypan blue solution. A total of 1 ml of this suspension was added to 9 ml of YPD broth pH 2 and 1–9 ml of YPD broth supplemented with ox bile 0.5%, pH 6. The cultures were incubated at 37°C under agitation at 150 rpm for 3 h. After 1, 2 and 3 h from the time of inoculation, aliquots of 1000 µl of each broth was taken at each of these test times and placed in a sterile microtube.

The count was made by the droplet technique as follows: 100 µl of each microtube were taken, six serial dilutions were done and aliquots (0.01 ml) were inoculated into surface YPD agar and incubated for 24 h at 37°C. The control was performed in YPD medium without the addition of hydrochloric acid and the same determinations were made in for YPD pH 2 treatment.

Aggregation assay

Aggregation assay was performed according to Kos et al. (2003). Yeasts were grown for 24 h at 37°C in YPD broth. Cells were harvested by centrifugation and suspended in PBS to one optical density (OD) unit at 600 nm (T_0). A total of 2 ml yeast suspensions were placed in each tube and centrifuged. Cells were then resuspended in PBS. After incubation for 2 h at 37°C, 1 ml of the upper suspension was transferred to another tube and the OD was measured. Aggregation was expressed as 1 – (OD upper suspension/OD total yeast suspension) × 100.

Co-aggregation assay

Yeasts strains were tested for their capacity to co-aggregate with animal pathogens: clinical isolates of *Salmonella* sp. (LM001, LM006), *Serratia* sp. (LM007), *Escherichia coli* and *Salmonella typhimurium*. The assay was performed according to Collado et al. (2008) with some modifications. The inoculum of each pathogen strain was prepared at 37°C for 18 h in nutritive broth and harvested by centrifugation (5000 rpm for 20 min). Then cells were suspended in PBS (pH 7). The bacterial suspension was adjusted to absorbance (600 nm) 0.05 ± 0.25 to standardise the number of bacteria (10^7–10^8 CFU ml^{-1}) and yeast (10^5–10^6 CFU ml^{-1}). The suspensions of probiotic and pathogenic strains (500 µl) were mixed and incubated at 37°C for 4 h. Controls of probiotic and pathogenic strains were performed. After the incubation period the absorbance was measured. To determine the co-aggregation the following equation was used:

$$\frac{\left[\left(A_{pat}+A_{prob}\right)/2-\left(A_{mix}\right)\right]}{\left[\left(A_{pat}+A_{prob}\right)/2\right]}\times 100 \qquad (1)$$

where A_{pat} and A_{prob} represent the absorbance at 600 nm of the suspensions of pathogenic and probiotic strain, respectively; and A_{mix} represents the absorbance at 600 nm of the mixture of suspensions.

Antimicrobial activity to animal pathogens

Yeast strains were grown in YPD broth and incubated for 24 h at 37°C. Similarly, pathogenic strains *Salmonella* sp. (LM001, LM006), *Serratia* sp. (LM007), *E. coli* and *S. typhimurium* (10^5–10^6 CFU ml^{-1}) were grown in nutritive broth and incubated for 12 h at 37°C. Filter paper discs in the overnight culture were imbibed and deposited on Petri plates containing YPD agar and incubated for 24 h at 37°C. A layer of nutrient agar in each of the plates was added and allowed to solidify. A moistened swab tip with the pathogenic strain was plated on nutrient agar to obtain confluent growth and was incubated for 24 h at 37°C. After 24-h incubation, an antagonistic effect was determined by the appearance of clear zones, which indicated the inhibitory effect of one organism on the other.

Encapsulation of yeast strain

Ch was derivatised according to the method proposed by Abdelaal et al. (2013) with some modifications. Ch (1.5 g) (583 kDa; Sigma-Aldrich) was dissolved in 100 ml of 2% acetic acid and 1.5 g glucose was added under agitation for 4 h at 35°C.

Viability tests for *K. marxianus* VM004 strains in native chitosan (ChN) and derivatised chitosan (ChD) were performed. A total of 1 ml inoculum (10^7 cells ml^{-1}) was added to 9 ml of ChN or ChD, homogenised for 10 min and serial decimal dilutions were made until 10^{-6}. Using the micro-drop technique, aliquots of 0.01 ml in triplicate on YPD agar were plated and incubated for 24 h at 37°C.

The ionotropic gelation method for extrusion was used to form capsules of Ch with different yeast strains according to de Vos et al. (2010). An inoculum of grown yeast at 37°C for 12 h was added to a solution of ChD in a proportion 1:5. The suspension was dropped into the solution of ionic cross-linking of TPP 1% (w/v) using a syringe. The capsules formed were maintained in the solution of TPP 1% for 15 min, and then filtered using a sterile Whatman No. 5 paper and washed with sterile PBS (pH 7.4). The capsules were stored in sterile PBS at 4°C.

Determination of the viability of yeast in capsules

Viability of capsules was determined by taking the preservative solution with a flamed sterile spatula, placed in a sterile bag with 10 ml of PBS and macerated in a stomacher for 10 min at 650 rpm.

Serial decimal dilutions were performed to 10^{-4} dilution and seeded in Petri dishes on agar YPD using the microdrop technique. They were incubated at 37°C for 24 h. To determine the encapsulation efficiency (EE) the following equation was used and was informed as colony forming unit per bead (CFU ml^{-1}):

$$\mathrm{EE}: \frac{Count\ x\ aliquot\ of\ dilution}{n^{\circ}\ of\ capsules} \times 143 beads/ml \quad (2)$$

The average bead production per millilitre of bead-forming solution is 143 beads.

Determination of bead dry weight

A known number of beads was placed in a Petri dish, previously weighed, and heated in an oven at 80°C until constant weight.

Scanning electron microscopy (SEM)

Cell culture of *K. marxianus* VM004 strains, free and encapsulated, were used for electron microscopy. Samples were homogenised for 30 min and centrifuged at 10,000 rpm for 10 min. The dry pellet was processed for SEM according to Bozzola and Russell (1999). All samples were pre-fixed in 3% (w/v) glutaraldehyde in 0.1 M sodium phosphate buffers, pH 7.2 for 3 h at RT followed by thorough washing with phosphate buffer. Fixed materials were then post-fixed in 1% aqueous osmium tetroxide for 3 h at RT. Dehydration of samples was achieved by transferring to vials containing a graded water–acetone series (10% steps for 30–90% each of 60 min, 100% for 180 min and finally 100% overnight). Dehydrated specimens were embedded with EMbed 812 and acetone 100% by 24 h, then were embedded with EMbed 812 with 1.5% hardening agent, DMP-30 at 60°C by 24 h. Ultra-thin sections (60 nm) were cut and placed on copper grids, counterstained with saturated uranyl acetate and aqueous lead citrate. The sections were examined in transmission electron microscope Elmiskop 101 (Siemens, Germany).

Statistical analysis

The CFU data obtained from GIT condition analyses and the number of yeast cells adhesion were transformed using a logarithmical function $\log_{10}(x + 1)$ before applying the analysis of variance (ANOVA). Means were compared using Fisher's protected LSD test (Quinn & Keough 2002).

Results

Sources and isolated yeast strains are shown in Table 1. A total of 16 yeast strains were isolated from different samples. Strains with the ability to use lactose and grow at 37°C were selected and studied for the selection of potential probiotic characteristics. Phenotype identification revealed that three strains assimilated and fermented the tested sugars and grew at 37°C. They were identified as *Saccharomyces* spp. and *Kluyveromyces* spp.

Table 1. Yeast species isolated from different whey sources.

Source	Strain	Lactose	Growth at 37°C	Phenotypic identification	Molecular identification
Whey cold	VM001	–	+	*Saccharomyces* spp.	n.i.
	VM002	+	–	*Saccharomyces* spp.	n.i.
	VM003	+	+	*Kluyveromyces* spp.	*Kluyveromyces marxianus*
	VM004	+	+	*Kluyveromyces* spp.	*K. marxianus*
	VM005	+	+	*Kluyveromyces* spp.	*K. marxianus*
	VM006	–	+	*Saccharomyces* spp.	n.i.
	VM007	–	+	*Saccharomyces* spp.	n.i.
Whey tank	VM008	+	–	*Saccharomyces* spp.	n.i.
	VM009	–	+	*Saccharomyces* spp.	n.i.
Whey calorific	VM010	–	+	*Saccharomyces* spp.	n.i.
	VM011	–	+	*Saccharomyces* spp.	n.i.
	VM012	+	–	*Saccharomyces* spp.	n.i.
	VM013	–	+	*Saccharomyces* spp.	*S. cerevisiae*
	VM014	–	+	*Saccharomyces* spp.	*S. cerevisiae*
Whey brine	VM015	–	+	*Saccharomyces* spp.	n.i.
Whey fat	VM016	–	+	*Saccharomyces* spp.	n.i.

Note: n.i., not identified.

Table 2. Effect of gastrointestinal solution on viability of *Kluyveromyces marxianus* strains isolated from whey.

K. marxianus strain	Viable count during simulated transit tolerance (log_{10} CFU ml^{-1})							
	Gastric solution				Intestinal solution			
	0 h	1 h	2 h	3 h	0 h	1 h	2 h	3 h
VM003	6.72 ± 0.14^{a}	7.74 ± 0.34^{c}	7.16 ± 0.10^{b}	7.83 ± 0.14^{c}	6.73 ± 0.06^{a}	7.08 ± 0.15^{ab}	7.26 ± 0.01^{b}	7.74 ± 0.34^{c}
VM004	6.61 ± 0.08^{a}	6.34 ± 0.52^{ab}	7.10 ± 0.19^{bc}	7.52 ± 0.07^{c}	6.30 ± 0.26^{a}	6.11 ± 0.06^{a}	6.70 ± 0.11^{b}	7.02 ± 0.21^{b}
VM005	6.66 ± 0.05^{a}	6.64 ± 0.14^{bc}	6.50 ± 0.02^{ab}	6.80 ± 0.00^{c}	6.56 ± 0.07^{a}	6.58 ± 0.46^{a}	6.56 ± 0.07^{a}	6.66 ± 0.16^{a}

Results are shown as mean ± SD (standard deviation), n = 2. Values with the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.0001$). Statistical analysis compared means obtained from each yeast strain separately (different letters indicate statistical differences within each row).

Molecular identification determined that yeasts from whey belonged to the *Kluyveromyces* genus. Three strains were molecularly identified as *K. marxianus* (VM003, VM004 and VM005) by conducting BLAST searches of GenBank database with ITS sequences as the query. Other strains that did not comply with the conditions were not assigned to species level. The obtained ITS sequences were submitted to GenBank, while the other strains that did not comply with the conditions were not identified. After conducting BLAST searches of GenBank, they had high match with published *K. marxianus* sequences in GenBank showing maximum identities of 99–100% (January 2017, see http://blast.ncbi.nlm. nih.gov/Blast.cgi). The obtained sequences have been deposited in GenBank under accession numbers KY421189, KY421190 and KY421191 (see http://www.ncbi.nlm.nih.gov/nucleotide).

The effect of simulated GIT on viability of strains is presented in Table 2. In general, the strains retained viability through GIT conditions. Strains VM003 and VM004 showed an increase in viability during transit to gastric and intestinal conditions, whereas VM005 yeast strain did not increase the cell density but maintained the number of viable cells throughout the GIT.

Table 3 shows the auto-aggregation ability of the tested yeast strains. Results showed that *K. marxianus* VM003, VM004 and VM005 strains exhibited a weak auto-aggregation. The maximum aggregation was shown with *K. marxianus* VM004 with 48.18%.

Table 3. Auto-aggregation ability of *Kluyveromyces marxianus* strains isolated from whey.

K. marxianus strains	OD_{600nm} (t_0)	OD_{600nm} (t)	Auto-aggregation (%)	Aggregation score
VM003	1.4386	0.8394	41.65	+
VM004	1.5982	0.8283	48.18	+
VM005	1.6614	1.1129	33.02	+

Co-aggregation and antimicrobial activity among *K. marxianus* strains and pathogens are shown in Table 4. The results show that the ability of yeasts to bind a microorganism varies according to each yeast strain and the pathogen involved. The studied strains showed a co-aggregation capacity range from 5.3% to 66.2%; the three strains had variable capacity to bind *Serratia* sp. *K. marxianus* VM005 showed the lowest binding capacity, having no co-aggregation to *E. coli* 81,749 and poor binding to the other pathogenic bacteria studied, with the exception of *Serratia* sp. and *Salmonella* sp. LM006. *K. marxianus* VM004 showed the best co-aggregation capacity that ranged from 32.4% to 66.2%. The antimicrobial activity results showed that all strains had a good inhibition capacity against the pathogenic bacteria studied, with the exception of *K. marxianus* VM003 against *S. typhimurium* and *K. marxianus* VM004 against *E. coli* 81,749. However, *K. marxianus* VM004 showed a good inhibition capacity against the other studied *E. coli* strains. It was found that the antimicrobial activity was strain

Table 4. Antagonism activity among *Kluyveromyces marxianus* strains and pathogenic bacteria.

K. marxianus strains	*Escherichia coli* 81382	*E. coli* 81749	*Salmonella typhimurium*	*Serratia* sp.	*Salmonella* sp. LM001	*Salmonella* sp. LM006
Co-aggregation (%)						
VM003	27.5	21.1	26.1	50.3	31.8	33.3
VM004	39.9	32.4	36.9	66.2	34.6	47.5
VM005	5.3	n.d.	5.3	50.7	7.4	22.6
Antimicrobial activity[a]						
VM003	++	++	–	++	++	++
VM004	++	–	++	++	++	++
VM005	++	++	++	++	++	++

Note: [a]Diameters (mm): +++ ≥ 15; ++ ≥ 10 y < 15; + ≥ 5 y < 10; – < 5.

related. In general, *K. marxianus* VM004 showed the best antagonist capacity and was selected to be encapsulated with derivatised Ch.

When encapsulated with ChN, VM004 showed a strong antimicrobial activity; at 10 min of exposure, total inhibition of the yeast growth was observed. Therefore, Ch derivatised to obtain ChD was used to reduce its antimicrobial activity. The viability of the encapsulated strain at the start, and over time, is shown in Table 5. It can be observed that viability was maintained in the bead-forming solution and the beads until the fourth week in PBS at pH 7.4 and 4°C. The dry weight of ChD beads were 0.19 mg per bead giving an initial concentration of 2.26×10^8 CFU g^{-1} of dry weight of ChD bead.

Figure 1 shows the electron microscopy of the empty capsule and the encapsulated yeast. The capsules were spherical with an approximately 2 mm diameter. They had a smooth and compact surface outside and were porous inside. The porosity was due to cross-linking of the molecules of ChD with TPP, producing an open network (Figure 1(a,b)). Figures 1(c,d) show the encapsulated yeast, indicating that they were found inside the capsule and not on the surface. In addition, they were found viable and in a budding process.

Table 5. Viability over time of *Kluyveromyces marxianus* VM004-encapsulated in derivatised chitosan.

K. marxianus VM004 viable counts (log_{10} CFU ml^{-1}), media ± SD				
Initial[a]	Time 0[b]	First week[b]	Second week[b]	Fourth week[b]
6.45 ± 0.2	6.79 ± 0.08	6.05 ± 0.91	5.39 ± 0.25	< 3.48

Notes: [a]ChD bead-forming solution.
[b]ChD beads.

Discussion

The present work reports the isolation and selection of *K. marxianus* strains from whey with the ability to tolerate GIT conditions and with some beneficial probiotic properties *in vitro* for animal feed. In addition, the strain with the best features was selected to be encapsulated.

Whey is one of the main byproducts of the dairy industry that is produced during the manufacture of cheese and casein from milk during the coagulation process. It represents an important source of environmental pollution due its enormous global production rate and high organic matter content exhibiting a biological and chemical oxygen demand values of 50 and 80 g l^{-1}, respectively (Domingues et al. 1999).

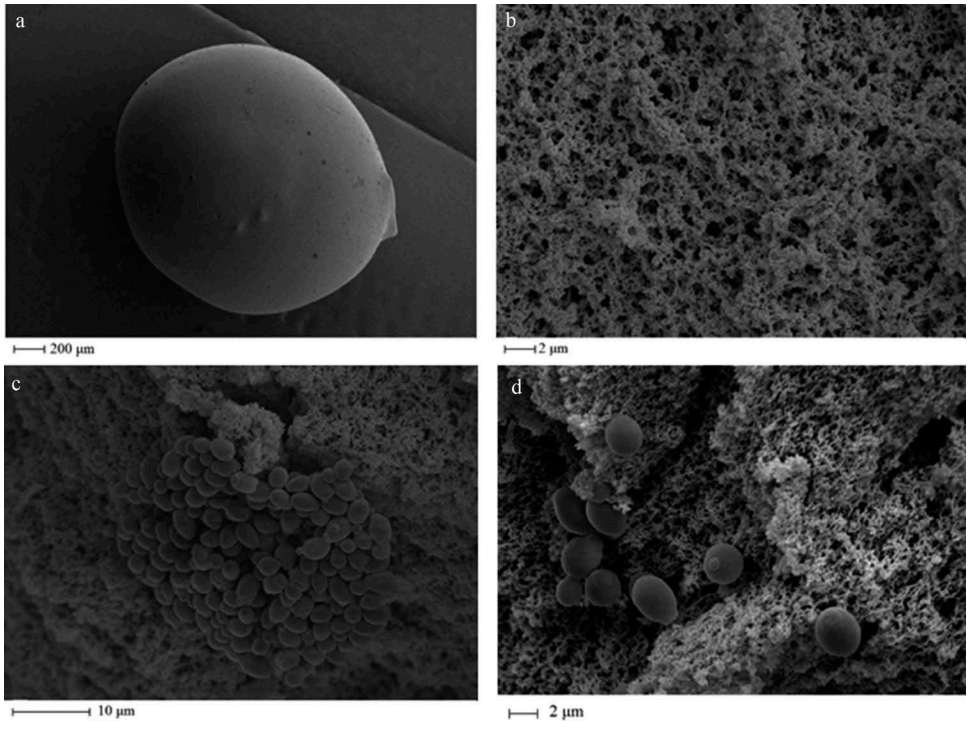

Figure 1. Electron microscopy of chitosan capsule by scanning electron microscopy (SEM): (a) empty capsule complete; (b) interior empty capsule; (c) *K. marxianus* VM004 on the inside of the capsules (10 µm); and (d) *K. marxianus* VM004 on the inside of the capsules (2 µm).

Moreover, the design of culture medium for isolation and growth of probiotic microorganisms is one of the most attractive options for reducing environmental pollution from whey. The presence of lactose as the only fermentable carbohydrate in whey confines its use to selective fermentations involving microorganisms which are capable of breaking down lactose with the enzyme galactosidase (Compagno et al. 1993; Grba et al. 2002). Apart from lactose, whey also contains vitamins and minerals which may improve the physiological activity of the cells.

In the present work, a total of 16 yeast strains were isolated from whey at different stages of cheese production. Three these strains were identified as *K. marxianus*. This microorganism is classified as GRAS by the USFDA and QPS by the EFSA, and fulfils one of the main characteristics necessary to classify it as probiotic (FAO/WHO 2002). Since 2005, *K. marxianus* has been listed as QPS by EFSA; this list was created based on taxonomic identity of the microorganism, body of knowledge, possible pathogenicity and end use, and is evaluated periodically to determinate the safety of the listed microorganisms (EFSA 2013).

Another required characteristic to be a probiotic is the ability to survive during passage through the GIT (Kumura et al. 2004; Saad et al. 2013). The *K. marxianus* strain studied in the present work demonstrated resistance to the GIT that must be highlighted. This feature and the ability to use lactose make this species an interesting option for use as a potential probiotic. Recent years have seen a great number of reports that describe different probiotic characteristics of this species (Anadón et al. 2006; Maccaferri et al. 2012).

The *K. marxianus* VM003, VM004 and VM005 strains showed a low percentage of auto-aggregation; however, when evaluating resistance to gastric and intestinal conditions, a high resistance was observed. In addition, an increase in the growth of *K. marxianus* VM003 and VM004 in these solutions was obtained. Other authors have shown similar results. Diosma et al. (2014) isolated *K. marxianus* strains from kefir with a high resistance to bile salts; however, they reported up to 70% of survival at acidic conditions. Psomas et al. (2001) showed growth of different *K. marxianus* strains isolated from faeces of infants and cheese in media with a pH between 3 and 5, and in the presence of bile.

At present, the reduction of levels of antibiotics for animal feed is hoped to reduce the resistance of pathogenic bacterial strains (Paphitou 2013). Yeast strains evaluated in this study showed a mild co-aggregation and very good antimicrobial activity against the pathogens tested. The antimicrobial activity of yeasts could be attributed to the production of 'killer' or 'mycocin' toxins. There is little information on the antimicrobial properties against bacteria. It has been reported that *S. boulardii* probiotic strains reduced *Clostridium difficile* infections when administered together with standard antibiotics (Surawicz et al. 2000). Bolla et al. (2013) used a mixed culture of microorganisms (*Lactobacillus plantarum*, *L. kefir*, *L. lactis*, *K. marxianus* and *S. cerevisiae*) isolated from kefir in a model of *C. difficile* infection in hamsters, showing that the mixed culture intake can prevent diarrhoea and enterocolitis triggered by this pathogen. Although the mode of action of yeasts as biocontrol agents is still largely unknown, the possible reasons for these interactions could be the production of killer toxins, predation, secretion of cell wall-degrading enzymes, the competition for nutrients etc.

The protection of microorganisms by microencapsulation is being developed and it is important to find a suitable, biocompatible carrier, stable over time. In this study, it was decided to encapsulate the strain with the best beneficial properties in Ch, since it is a natural, biocompatible, biodegradable, antioxidant, antimicrobial, antitumor and mucoadhesive polysaccharide. Recently, several studied informed the growth promoting capacity of Ch on animals, with a positive effect on the feed intake, body weight gain and feed-conversion ratio (Swiatkiewicz et al. 2015). The Ch used as a matrix for encapsulation of microorganisms presented difficulties because strong antimicrobial activity, a property mainly attributed to the amino groups present in the molecule (Goy et al. 2009).

ChN showed a strong antimicrobial activity against *K. marxianus* VM004; therefore, it was decided to use a ChD with blocked amino groups (Abdelaal et al. 2013). This technique significantly decreased the antimicrobial activity of Ch. The encapsulation efficiency shows no diminution of the CFU ml^{-1} of capsule-forming solution and the capsules (Table 5). When assessing the viability of encapsulated *K. marxianus* VM004 through time, a decrease was observed after 4 weeks, probably

because the preservation solution was not suitable for conservation for a long time.

The recommended concentration for most probiotics is approximately 10^9 CFU kg^{-1} of feed (Simon 2005). The concentration obtained in this work was 2.26×10^8 CFU dry g^{-1} of ChD bead, therefore it can achieve the recommended concentration with 5 g ChD beads kg^{-1} of feed, giving the final product the benefits of Ch and the probiotic yeast. Future studies should include other conservation solutions that enable preservation of viability over time and *in vivo* studies to confirm the probiotic and growth-promoting properties of the studied yeast, free and encapsulated in ChD.

Conclusions

The present work reports the isolation and selection of *K. marxianus* strains from whey with the ability to tolerate GIT conditions and with some proven beneficial probiotic properties *in vitro* for animal feed. In addition, the strain with the best features was selected to be encapsulated. This novel finding is important since the beneficial features of whey indicate a promising source of new, potentially probiotic strains for the development of feed additives destined for animal production.

Disclosure statement

No potential conflict of interest was reported by the authors.

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