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TECHNOLOGICAL AND PROBIOTIC PROPERTIES OF *Enterococcus faecium* STRAINS ISOLATED FROM TUNISIAN CAMEL MILK

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ABSTRACT

Sixty-two lactic acid bacteria (LAB) strains isolated from Tunisian raw camel milk were studied by focusing on their technological and probiotic potential. Strains were tested for their acidification activity, proteolytic and lipolytic activity, ability to use citrate, biomass yield, growth rate, and Exopolysaccharide (EPS) production. Probiotic tests used were pH and bile tolerance, antimicrobial activity, antimicrobial susceptibility, adhesion, cell surface hydrophobicity and aggregation abilities. Twenty-six strains showed survival at pH 2; only 10 strains tolerated 0.3 % of bile salt and will therefore further assessed for their probiotic properties. These strains were identified by partial 16S rRNA gene sequencing and were represented by *Enterococcus faecium* as the only group. Eight strains present ΔpH ≥0.3 U and then were considered as rapid acidifier strains. All strains have a significant proteolytic power. All strains produced EPS.

Survival to the simulated *in vitro* digestion was strain-dependent. Strains were tested for cell surface acid-base properties and adhesion to gastric mucin and STC-1 cells. Isolates showed good adhesion rate to gastric mucin and only two strains (SCC1-33 and SLch6) were able to colonise STC-1 cells with an adhesion value of 7.8×10^3 and 4.2×10^3, respectively. Auto- and co-aggregation abilities were interesting and values were ranged from 33.10 to 63.10 %. Results from the cytotoxicity test showed a negative effect on STC-1cells of all the studied strains. *Enterococcus faecium* isolated from camel milk was characterised by their technological and probiotic properties.

Key words: Camel milk, *Enterococcus faecium*, probiotics, technological properties, Lactic Acid Bacteria

Camel milk, alone or in combination with bacterial strains having probiotic properties and/or producing physiologically active metabolites, represents one of the technology options for manufacturing dairy functional beverages (Gomes *et al,* 1998). Probiotics are non-pathogenic microorganisms that, when ingested in adequate amounts, exert a positive influence on their host's health. The most studied probiotic strains are lactic acid bacteria (LAB) especially belong to the genera *Lactobacillus* and *Bifidobacterium*. However, other genera such as *Streptococcus, Lactococcus* and *Enterococcus*, are also considered as probiotics. *Enterococcus* identified as potential probiotics are available on the market known as *Enterococcus faecium* SF68® (NCIMB 10415, Cerbios-Pharma SA, Barbengo, Switzerland) and *Enterococcus faecalis* Symbioflor 1 (SymbioPharm, Herborn, Germany).

The nutritional health benefits (for consumers) attributed to probiotic bacteria include their role in enhancing the bio-availability of calcium, zinc, iron, manganese, copper and phosphorus, increasing the digestibility of protein and synthesis of vitamins (Sudha, 2014). Camel milk is also known as a source of LAB strains, mainly *Lactobacilli, Lactococci* and *Enterococci* (Abdou *et al,* 2018). *Lactobacillus* spp. was the major group which has been isolated from camel milk (Abushelaibi *et al,* 2017). According to Li *et al (*2020), lactococcus was used in the production and maturation of dairy fermented products. The *Lactobacillus* (Lb.) was added as fermenting agents for the manufacture of fermented milks and cheeses *Lb. plantarum, Lb. casei* and *Lb. acidophilus* involved in the development of the flavour and texture of cheese. Enterococci (*E. faecium* and *E. feacalis*) has an important role in the maturation of several varieties of cheese, probably because of their proteolytic activity, lipolytic, their capacity diacetyl and other volatile components contributing to flavouring, flavour and taste characteristic (Terzić-Vidojević *et al,* 2021). The isolation of the microflora in camel milk as a basis for possible development of suitable

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starter cultures for fermented camel milk products is therefore necessary.

The objective of this work was to isolate and characterise lactic acid bacteria from camel milk and to evaluate their technological and probiotic properties.

Materials and Methods

Bacterial strains and culture conditions

LAB strains were isolated from camel milk (*Camelus dromadarius*) belonging to the herd of Arid Lands Institute (IRA, Medenine, Tunisia). Milk samples were collected from camel flock from Arid Land Institute, Medenine, Tunisia. LAB were isolated on MRS agar (Pronadisa, Madrid, Spain) and incubated at 37°C for 24 to 48 h in order to apply the conventional tests for identification and screening of probiotics. The strains were tested for Gram staining, catalase and mobility. Gram-positive, catalase-negative and non-motile isolates were selected and stored in MRS broth (Pronadisa, Madrid, Spain) supplemented with 30% sterile glycerol and conserved at -80°C. At the time of analyses, the purified cultures were activated by sub-culturing twice in MRS broth before use.

16S rRNA gene identification

The genomic DNA of the strains was isolated using the DNA extraction and purification kit according to the manufacturer's instructions (Fermentas, Cambridge, UK). The PCR reaction mixture contained 0.5 µL of template DNA, 2.5 µL of reverse primer (10 mM), 2.5 µL of forward primer (10 mM), 2 µL of dNTP (25 mM), 4 µL of $MgCl_2$ (25 mM), 5 µL of PCR buffer (10X) and 1µL Taq polymerase, in a 50 µL final volume. The primers sequences used were S1 (5'AGAGTTTGATC (A,C) TGGCTCAG 3') and S2 (5' GG (A,C) TACCTTGTTACGA (T,C) TTC 3'). The cycling programme was 94ºC for 3 min, 29 cycles at 94ºC for 40 sec, 55ºC for 50 sec and 72ºC for 2 min. The PCR products were visualised on agarose gel electrophoresis and 1500 bp bands were purified. The resulted amplicons were cloned into the pGEM-T Easy Vector System (Promega Corp., Charbonnières-les-Bains, France), followed by plasmid extraction using Gene JET plasmid Mini prep (Thermo Fisher Scientific, Surrey, UK). Sequencing of the amplicons was performed by the sequencing facility offered by Eurofins (Ebersberg, Germany). The obtained nucleotide sequences, displayed by BioEdit software, were analysed using the blast tool of the NCBI site in order to find identity percentages with the sequences present in databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Probiotics screening tests

pH and bile tolerance

The tolerance of the isolated LAB to acidic pH was performed as described by Yu *et al* (2013) with some modifications. pH 2 was used as a representative gastric pH value. After 16 to 18 h of culture in aerobic conditions, cells were harvested by centrifugation for 10 min at 5000 rpm at 4°C. The pellets were washed once in phosphate buffered saline PBS (8 g/LNaCl, 0.2 g/LKCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, at pH 7.2) then resuspended in PBS (pH 2) and incubated at 37°C. After 4 h of incubation, colonies were counted and the number of LAB was calculated according to the standard ISO 15214 (1998). The survival rate was calculated as the percentage of LAB colonies number grown on MRS agar (Oxoid CM0361, Thermo Fisher Diagnostics, Dardilly, France) after 4 h of incubation compared to the initial LAB colonies number. Strains showing resistance to low pH were tested for bile tolerance. The experiment was applied at this concentration of bile 0.3% (w/v) for 4 h. MRS medium containing 0.3% bile (Oxoid, Thermo Fisher Diagnostics, Dardilly, France) was inoculated with active cultures (incubated for 16-18 h). During the incubation for 4 h, viable cells were enumerated for every hour with pour plate technique and also growth was monitored at OD600. A non-probiotic strain: *Lactococcus lactis* was used as a negative control.

Adhesion tests

Adhesion to organic solvents

The bacterial adhesion to hydrocarbons test (BATH) was performed according to Sepova *et al (*2017) with some modifications.

Cells were washed once with phosphate-buffered saline PBS (pH 7.2) and resuspended in the same buffer to an optical density (OD_{600}) of about 0.25 ± 0.05 (OD_0) in order to standardise the number of bacteria (10^7-10^8 CFU/mL). Then an equal volume of solvent was added. Both phases system was mixed by vigorous mixing for 5 min. The aqueous phase was removed after 1 h of incubation at room temperature, and its optical density at 600 nm (OD_1) was measured. The percentage of bacterial adhesion to the solvent was calculated as follows: $[(OD_0-OD_1)/OD_0)]*100$

Two different solvents were tested in this study: chloroform, a monopolar and acidic solvent; and

ethyl acetate, a monopolar and basic solvent. The values obtained with the two solvents, chloroform and ethyl acetate, were considered as a measure of electron donor (basic) and electron acceptor (acidic) characteristics of bacteria, respectively.

Adhesion to gastric mucin

The isolated LAB strains were evaluated for adhesion to immobilised porcine stomach mucin (Type III, Sigma-Aldrich, St Louis, Missouri, USA), in 96-well polystyrene microplates (NuncMaxisorp, Thermo Fisher Scientific, Roskilde, Denmark) (Sanchez *et al,* 2010).

Each well was covered by 100 µL of porcine gastric mucin type III solution at 10 mg/mL in sterile PBS (pH 7) and microplates were incubated at 4°C, overnight. Wells were washed twice with 200 µL of sterile PBS to remove unbound mucin. A bovine serum albumin solution (200 µL at 2% (w/v) in PBS) was added and the microplates were incubated for 2 h at 37°C.

The wells were then washed twice with 200 µL of sterile PBS, before adding the bacterial cells. One mL of culture was collected at three different stages of growth (9, 12 and 24 h) by centrifugation at 3500 g for 5 minutes at 4°C. The obtained pellet was washed twice with 1 mL of Tris-HCl 0.1 M, pH 7.5 then centrifuged. Cells were resuspended in PBS and diluted to achieve an OD_{600} of 0.1±0.02 (which corresponds to about 3.10^7 CFU/mL). Each bacterial suspension (100 µL) was added per well and the microplate was incubated for 1 h at 37°C then the liquid was removed by pipetting. Each well was washed five times with 200 µL of PBS. The desorption of adherent cells was carried out by adding 200 µL of Triton X-100 0.5% solution (v/v) for 20 min at room temperature, with orbital stirring at 150 g and bacteria were counted on MRS plates. Adhesion to gastric mucin was performed in triplicate. Percentage adhesion was calculated from the viable counts adherent to the mucin with respect to the initial counts (%) = (CFU/mL recovered bacteria/CFU/mL initial bacteria) × 100.

Adhesion to STC-1 cells

Adhesion to mouse intestinal endocrine tumor cell line (STC-1) was investigated (Borah *et al,* 2019). Cells were cultured in DMEM medium (Dulbecco Modified Eagle's Minimal Essential Medium, high glucose, HEPES, GlutaMAX™, Gibco, Life Technologies, Paisley, United Kingdom) supplemented with 10% (v/v) calf serum (South America origin, Gibco), 100 µg/mL of streptomycin and 100 UI/mL of penicillin(Gibco) at 37°C in 95% air/5% CO_2. The medium was changed every 2 days and the cells were used after 15 days when they had a full confluence. Cells were inoculated at 5.10^4 cells per well, grown on 24 wells plates (Nunclon™ Surface, Nunc, Thermo Fisher Scientific, Roskilde, Denmark) at 37°C until a confluent monolayer was obtained. Prior to adhesion, wells were washed with a pre-warmed medium to discard antibiotics. Bacteria were grown in MRS medium for 18 h at 37°C then washed twice in 100 mM phosphate buffer (pH 7.0). Bacteria were then diluted in DMEM to obtain 1.10^7 CFU/mL and 1 mL was added in each well. After 2 h of incubation, free bacteria were removed by 1 mL of pre-warmed phosphate buffer. Then, 1 mL of 1% triton X100 was added and after 10 min, serial dilutions were plated on MRS medium and incubated 48h at 37°C. This test was done in triplicate.

Auto-aggregation assays

Auto-aggregation assays were performed according to (Holst *et al,* 2019) with certain modifications. LAB strains were grown on MRS broth for 18 h at 37°C. The cells were harvested by centrifugation at 5000 g for 15 min, washed twice and resuspended in phosphate buffered saline (PBS) to give viable counts of approximately 10^8 CFU/mL. Cell suspensions (4 mL) were mixed by vortexing for 10s. After incubation at 37°C for 4 h, 0.1 mL of the upper suspension was transferred to another tube with 3.9 mL of PBS and the optical density (OD) was measured at 600 nm. Aggregation was expressed as:

$$1 - (OD_{uppersuspension} / OD_{total\ bacterial\ suspension}) * 100$$

Co-aggregation assays with Saccharomyces cerevisiae

The co-aggregation test was performed as described by Ribeiro *et al (*2020) with some modifications. Briefly, bacterial suspensions were prepared as described for auto-aggregation analysis. Equal volumes of cells (100 mL) of the different *Enterococcus* strains and *Saccharomyces cerevisiae*; grown in Sabouraud Dextrose Broth (Sigma-Aldrich, St Louis, Missouri, USA) for 24h, were mixed and incubated at 20 and 37°C without agitation. Pure bacterial suspensions (200 mL each) were incubated under conditions similar to the mixtures in order to check self-flocculation. The optical density of the mixtures and the pure bacterial suspensions was measured at 600 nm after 4 h of incubation. The co-aggregation (%) was calculated according to the equation:

$[(OD_{Sac}+OD_{Bact}t) - (OD_{Mix}) / (OD_{Sac}+OD_{Bact})] * 100$

Where: OD_{Sac} represent OD_{600} of *S. cerevisaeat* time T_0, OD_{Bact} represent OD_{600} of bacterial suspension at time T_0 and OD_{Mix} represents OD_{600} of the mixture after 4 h of incubation.

Survival to simulated in vitro digestion

Successive *in vitro* gastric and intestinal digestion was performed according to (Haghshenas *et al,* 2016). The strains were grown in skimmed milk for 8 h at 30°C. One gram of the resulting fermented milk was diluted to 1/10 in phosphate-buffered saline PBS. To simulate the gastric digestion, the sample was adjusted to pH 3.0 and Pepsin was added to a final concentration of 5% (w/v). The mixture was incubated at 37°C for 90 min with agitation at 110 rpm. To create intestinal digestion conditions samples were adjusted to pH 6.0 and solutions of pancreatin and bile salts at a final concentrations of 0.1% and 0.3% (w/v), respectively were added. Samples were incubated at 37°C for 150 min with agitation (110 rpm). After that, the number of cells was determined before and after both gastric and intestinal digestion. An aliquot was serially diluted and then plated on MRS agar in double. The plates were incubated under anaerobic condition for 48 h. The survival of LAB was expressed as log percentage of the final bacterial count (log_{10} CFU/mL) in comparison to their log of the initial count (log_{10} CFU/mL), which allowed the comparison of different isolates regardless of differences in initial counts.

Antagonistic Activity

The antibacterial activity of the selected isolates was determined by agar spot on-lawn test. The indicator bacteria used in this study were *Saccharomyces cerevisiaea, Listeria innocua, Micrococcus luteus,* and *Escherichia coli* (Lab collection).

One microlitre of each overnight culture of selected LAB was spotted on MRS plates (containing 0.2% glucose and 1.2% agar) and incubated under anaerobic conditions for 48 h to develop colonies. A portion of 0.25 mL of 1:10 dilution of an overnight culture of the indicator bacteria was inoculated in 9 mL of Brain Heart Infusion (Merck, Darmstadt, Germany) soft agar (0.7% agar). The medium was immediately poured over the MRS plate on which the tested *Enterococcus* was grown. The plates were incubated anaerobically at 37°C for 24 h. The antibacterial activity was related to the clear inhibition zone which calculated as the difference between the total of inhibition zone and the diameter of the growth spot of selected strains. Zones with diameters larger than 1 mm were determined to have antagonistic activity according to Nisin, used as a control, was prepared by dissolving the commercial preparation Nisaplin® (2.5% pure nisin, Sigma-Aldrich, St Louis, Missouri, USA) in 0.02 N HCl to assure complete solubilisation. Subsequently, sterile distilled water was added until a final concentration of 1mg/mL of nisin. The solution of nisin was prepared on the day of the experiment. The Antagonistic Activity was determined by replacing 1 µL of LAB strain culture by 1 µL of nisin solution.

Safety Evaluation of Enterococcus faecium strains

Antibiotic susceptibility

The antibiograms of the strains were determined using agar antibiotic diffusion discs. Strains were grown overnight in MRS broth at 37°C and 100 µL of the diluted culture (approximately 10^6 viable cells) were streaked on MRS agar. The antibiotics were used at the following concentrations: 30 µg tetracycline, 10 µg ampicillin, 1000 µg kanamycin, 15µg erythromycin, 30 µg rifampicin and 30 µg vancomycin. The plates were incubated at 37°C under anaerobic conditions for 18 h and the inhibition zones were measured. According to CLSI zone diameter interpretative standards, strains were considered resistant if the inhibition zone diameter was less than 17 mm for Vancomycin, Erythromycin and tetracycline; less than 16 mm for ampicillin and than 10 mm for Kanamycin CLSI (2017).

Cytotoxic assay on STC-1 cells

The cytotoxic assay was performed according to Espíndola *et al* (2022), where Caco-2 cell line was replaced by STC-1 cell line. Cells were seeded on a 96 well plate at 7000 cells per well in 150 µL of DMEM medium. A volume of 80 µL of minimum medium containing bacterial strains at 10^5 or 10^7 CFU/mL were added; sterile minimum medium was used as a control. Twenty microlitres of propidium iodide at 5 µg/mL were then added in each well. The emission was monitored during 60 minutes every 30 seconds using excitation/emission wavelengths of 575/615 nm and 5 nm slits for both on spectrofluorimeter (SAFAS, Monaco, France). Results where expressed in fluorescence emission "fold of control" observed with non-treated cells.

Technological characteristics of strains

Acidifying activity

Acidifying activity of strains was measured according to the International Dairy Federation (IDF)

standard 306 (1998), (Martínez-Cuesta *et al,* 2021) and (Ribeiro *et al,* 2021).

Acid production ability was assayed by inoculating 10% skim milk with 24 h old cultures at 1% level and incubation at 30°C. Acidity was determined during 24h of incubation.

Proteolytic activity

To determine the proteolytic activity of LAB, MRS agar supplemented with 10% skim milk was poured, solidified and then dried. Sterile Whatman paper discs were deposited on the surface of the agar. Each disk received a volume of 20µl of a young culture. After incubation at 37 ° C for 24 h, proteolysis is indicated by clear zones around discs (Artha *et al,* 2019).

Lipolytic activity

To determine the lipolytic activity, the strains were inoculated on agar spot in Tween 80 (1, 3, 5%). Incubation was carried out at 25°C for 72 hours. Strains with an opaque area due to the formation of esters with calcium liberated fatty acids were considered positive (Silva *et al,* 2019).

Biomass production

Strains were sub cultured on MRS broth; 100 ml of the medium were inoculated with 10% of the active culture. Bacterial growth was monitored by measuring the optical density at 600 nm (OD600) using a spectrophotometer (CECIL CE 2041/2000 Series) during 6 h. The difference between the initial OD and the OD at which cells were collected (ΔOD) was taken as an indication for the growth amount. The maximum growth rate was determined from the slope of the linear part of curve representing Log OD versus time. At the early stationary phase, 30 ml of culture were harvested by centrifugation (Sigma GmbH, Model 6K15, Gottingen, Germany) at 5000 g for 30 min at 4°C. The dry weight was determined after drying the pellet at 105°C for 24 h. The remaining 70 ml were used to study the separation of biomass by centrifugation and measurement of OD600 of supernatant (Berisvil *et al,* 2020).

Exopolysaccharides production

The cultures were streaked on modified MRS (m-MRS; glucose replaced with 100 g/l sucrose) (Prete *et al,* 2021) and incubated at the optimum growth temperature for 24 h, then tested for slime formation using the inoculated loop method (Mazlumi *et al,* 2022). Formed colonies were dragged up using a metal loop and the strains were considered positively slimy producer if the length of slime was above 1.5 mm (Berisvil *et al,* 2020).

Results and Discussion

Isolation of Lactic Acid Bacteria

A total of 62 strains were isolated from camel milk using MRS agar at 37°C under anaerobic conditions. All isolates were Gram positive, non-motile, cocci shaped and catalase negative as preliminary characteristics.

pH and bile resistance

The major selection criterion for probiotic strains is resistance to low pH because they have to pass through the stressful conditions of the stomach and reach the small intestine. In our case, only 26 isolates were tolerant to low pH, thereby tested for bile salt tolerance. At this stage, 10 strains were retained for further probiotic assessment and molecular identification. These strains showed a good resistance to bile salts: 8 strains (SSC1-2, SCC1-6, SCC1-8, SCC1-13, SCC1-15, SCC1-24, SCC1-33 and SLch14) were resistant after the incubation period with a survival rate higher than 0.5 unit of OD_{600} and only 2 strains (SCC1-7 and SLch6) had a survival rate greater than 1 unit.

Molecular identification of the selected isolate

At least a 1500 bp fragment of the 5′ region of the 16S rRNA gene was sequenced for the 10 retained strains. Comparison of sequences in the NCBI data base revealed that the 10 strains showed 99% of identity with *Enterococcus faecium* (Table 1). Sequences of the 16S rRNA genes have been deposited at the NCBI gene bank under the accession numbers: JN560903.1, KF149320.1, JX847611.1, JQ726533.1,

Table 1. Identification of LAB isolates from camel milk and their Genbank accession numbers.

Strains	Identification	% of similarity	NCBI accession number
SCC1-2,	*E. faecium*	99%	JN560903.1
SCC1-6	*E. faecium*	99%	KF149320.1
SCC1-7	*E. faecium*	99%	JX847611.1
SCC1-8	*E. faecium*	99%	JQ726533.1
SCC1-13	*E. faecium*	99%	EU878170.1
SCC1-15	*E. faecium*	99%	KC422716.1
SCC1-24	*E. faecium*	99%	JN560911.1
SCC1-33	*E. faecium*	99%	JN560898.1
SLch6	*E. faecium*	99%	HM162421.1
SLch14	*E. faecium*	99%	AY587799.1

EU878170.1, KC422716.1, JN560911.1, JN560898.1, HM162421.1 and AY587799.1 corresponding to the strains: SCC1-2, SCC1-6, SCC1-7, SCC1-8, SCC1-13, SCC1-15, SCC1-24, SCC1-33, SLch6 and SLch14 respectively. Ours results were according to previous reports finding that LAB isolates from Camel Milk were especially *Enterococcus* species (Abushelaibi *et al,* 2016).

Adhesion to organic solvents, gastric mucin and STC-1 cells

Adhesion to organic solvents

Bacterial adhesion to solvents is implicated in various interfacial phenomena such as microbial adhesion. Bacterial adhesion to chloroform and ethyl acetate was tested to assess the Lewis acid-base characteristics of the bacterial cell surfaces. Our findings showed that the strains have medium to low affinities to both solvents (Table 2). The greatest affinities with chloroform were observed in *E. faecium* SCC1-13 (30.4±7.58%), *E. faecium* SCC1-15 (28.6 ±5.05%) and *E. faecium* SLch6 (25.5±0.76%), while the least affinities were observed in *E. faecium* SCC1-2(12±5.66%). The bacterial affinities to ethyl acetate were relatively high when compared to chloroform and ranging from 0 to 34.56 ± 4.88%; the highest value was obtained for the strain *E. faecium* SCC1-8 and the lowest value was obtained for the strain *E. faecium* SCC1-13; indicating the acidic and electron acceptor property of most strains. However, the strain SCC1-13 showed higher affinity to chloroform than to the ethyl acetate (30.4% vs. 0.0%, respectively). As reported by [26] three strains of *Lactobacillus acidophilus* showed strong affinities to chloroform, which means they are strong electron donors. Unlike chloroform, the bacterial adhesion to ethyl acetate was low, ranging from 5.1 to 16.9%.

Table 2. Adhesion to organic solvents (%), coaggregation (%) with S. cerevisae at 20°C and 37°C and auto-aggregation of the different *E. faecium* strains.

Strains	Adhesion to ethyl acetate	Adhesion to chloroform	Coaggregation with *S. cerevisae*		Auto-aggregation
			20°C	37°C	
SCC1-2	16± 5.66	12± 5.66	61.77± 11.05	46.78± 2.16	41.03
SCC1-6	26.0± 2.83	16.7± 2.62	58.10± 13.38	45.07± 2.23	62.76
SCC1-7	20.7± 9.75	14.4± 4.08	48.74± 1.99	43.31± 12.29	59.66
SCC1-8	31.8± 10.71	18.8± 2.95	48.43± 6.93	44.94± 18.76	34.48
SCC1-13	00± 00	30.4± 7.58	62.59± 0.68	43.41± 0.1	60.69
SCC1-15	25.0± 8.16	28.6± 5.05	55.28± 4.13	43.98± 0.29	33.10
SCC1-24	34.5± 4.88	14.8± 5.24	44.40± 7.2	43.46± 2.08	62.76
SCC1-33	18± 2.83	18.5± 00	48.64± 2.6	41.03± 19.46	40.34
SLch6	22± 2.83	25.5± 0.76	50.00± 1.57	42.15± 8.19	63.10
SLch14	15.4± 5.44	18.8± 4.42	55.15± 14.34	41.18± 10.72	60

Adhesion to gastric mucin and STC-1 cells

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 (Dell'Anno *et al,* 2021). Strains were tested for their adhesion to gastric mucin at different stages of growth. The results are shown in Fig 1. All *E. faecium* strains showed good adhesion rate to gastric mucin (higher than 60% at all growth stages) which decreased at stationary phase due to a possible reduction in nutrients and an increase in metabolic. Strains able to adhere to mucins, could allow them to interfere with pathogen binding and also interact with the mucosal immune (Han *et al*, 2021). Furthermore, the strains were also examined for their ability to adhere to STC-1 cells *in vitro*. Our results showed that *E. faecium* strains had low adhesion to STC-1 cells and only two strains: SCC1-33 and SLch6 were able to colonise epithelial cells with an adhesion value of 7.8 10^3 and 4.2 10^3 CFU/mL respectively.

Auto-aggregation and co-aggregation

Auto-aggregation and co-aggregation are essential properties of probiotic organisms as they prevent their elimination from gastro-intestinal tract environment. Co-aggregation with *S. cerevisae* and auto-aggregation of the different probiotic strains are presented in Table 2. Results showed that among the ten *E. faecium* tested, six strains showed a high auto-aggregation rate (higher than 60%). All tested strains were well aggregated with *S. cerevisae* and co-aggregation was higher at 20°C. The process of adhesion appears to be multifactorial because adhesion cannot be attributed to one component and includes electrostatic interactions, hydrophobic interactions, and specific bacterial structures (Elbourne *et al,* 2019).

Survival in simulated in vitro digestion

The ability to survive in the GIT is one of the main crucial properties required for probiotic strains. Since, the therapeutic effect of probiotic bacteria is correlated with their concentration in the intestine lumen. Thus, the selected strains were tested for their viability in simulated stressful conditions of gastric and intestinal digestion. Results in Fig 2 indicated that two strains (SCC1-2 and SCC1-7) were able to multiply in simulated intestinal fluid. Two strains (SCC1-5 and SLCch6) had a good survival rate higher than 50%. Three strains had low survival rate (between 11 and 37%) whereas, two strains did not survive simulated gastric nor intestinal transit (less than 5% survival rate). Similar results were found by (Coimbra-Gomes *et al,* 2022) for the strains *E. faecium* SJRP20 and *E. faecium* SJRP65.

Antagonistic effect

Antagonistic activity to inhibit the pathogens in the GIT is considered an important probiotic trait. The ten selected strains were tested for their antagonistic effect (Fig 3). All the *E. faecium* strains showed high antagonistic effect towards all tested pathogen strains with different intensity between strains except for SCC1-13 strain that did not inhibit the growth of *M. luteus*. Several studies reported the antibacterial effect of the *Enterococcus* genus.

E. faecium LCW 44, isolated from camel milk exhibited a large antibacterial spectrum with inhibitory activity against several Gram-positive strains belonging to the genera *Clostridium, Listeria, Staphylococcus,* and *Lactobacillus* (Choeisoongnern *et al,* 2021).

Safety evaluation of E. faecium strains

The susceptibility of *E. faecium* strains to several antibiotics was determined (Table 3). All the strains were susceptible to Tetracycline, Vancomycin, Erythromycin, Ampicillin and Kanamycin and resistant to Rifampicin. The antibiotic susceptibility profile of *E. faecium* SLCch6 is in agreement with previous reports concerning *E. faecium* strains that are commonly found in foods and have safety criteria (Golob *et al,* 2019). Results from the cytotoxicity test showed a negative effect on STC1- cells.

Technological properties

Study of technological properties of LAB strains isolated from camel milk is an important criterion for selection of starter cultures to be used in the standardised production of dairy products.

Table 3. Antibiotic resistance and susceptibility of the studied *E. faecium* strains.

Strains	T30	R30	V30	E15	A10	K1000
SCC1-2	S	R	S	R	S	S
SCC 1-6	S	R	S	S	S	S
SCC 1-7	S	R	S	S	S	S
SCC 1-8	S	R	S	S	S	S
SCC 1-13	S	R	S	S	R	S
SCC 1-15	S	R	S	S	S	S
SCC 1-24	S	R	R	S	S	S
SCC 1-33	S	R	S	S	S	S
SLC ch6	S	R	S	S	S	S
SLC ch14	S	R	S	S	S	S

T30: Tetracycline; R30: Rifampicin; V30: Vancomycin; E15: Erythromycin; A10: Ampicillin; K1000: Kanamycin
(R): resistant, (S): susceptible

Acidifying activity

The strains were characterised on the basis of acid production ability. The acidity increased during the fermentation time and there was variability in acidification rate between the different strains used to inoculate milk (Fig 3). The strain is considered fast, medium and slow when ΔpH reached 0.4 U for 3, 3 to 5 and > 5 h respectively (Ayad *et al,* 2004). This is applicable using cow's milk as a substrate. In our case, only strains with ΔpH ≥ 0.3 U after 6 h were kept for the next steps considering the antimicrobial activity of camel milk. Thus, the strains selected are: SCC1-33, SCC1-8, SCC1-7, SCC1-15, SCC1-6, SCC1-24 and SLCch14.

A rapid decrease in pH during the initial step of cheese preparation is crucial importance in cheese manufacture, since it is essential for coagulation and prevention or reduction of the growth of adventitious microflora. The fast acidifying strains are good candidate in the dairy fermentation process as primary starter organisms, whereas, the poor acidifiers strains can be used as adjunct cultures depending on their other important properties, e. g., proteolytic and autolytic activity.

The difference observed from one lactic acid bacteria species to another were suitably explained (Abozead *et al,* 2022). In fact, the acidifying activity of each strain is related to its specific capacity to break down the substances in the medium and render the capability of assimilation. On occasion, differences are also due to the presence or absence of nutrient transport systems (Mercha *et al,* 2020).

Proteolytic activity

The results obtained during the implementation of this test are summarised in Table 4. The table

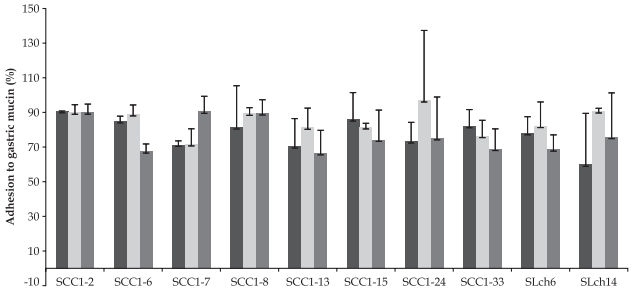

Fig 1. Adhesion to gastric mucin at three different stages of bacterial growth: exponential phase (■), early stationary phase (■) and stationary phase (■).

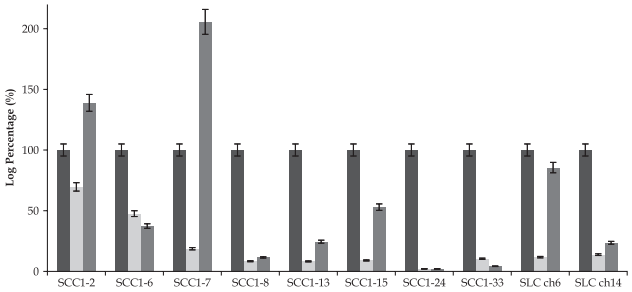

Fig 2. Resistance of *Enterococcus faecalis* strains to simulated *in vitro* digestion: viability at the beginning of the assay (■), viability after simulation of gastric conditions (■) and viability after intestinal digestion (■).

shows that all strains studied show growth with proteolytic activity resulted in the emergence of a clear halo around the discs. According to (Kieliszek *et al,* 2021), the strain is called proteolytic if it has a zone of lysis of diameter between 15 and 21 mm.

Table 4. Proteolytic activity of lactic isolates.

Strains	Diameter of inhibition zone en mm
SCC1-2	15±1.4
SCC1-6	15±0.0
SCC1-7	18±1.41
SCC1-8	16±0.0
SCC1-13	21±0.0
SCC1-15	16.5±3.53
SCC1-24	16.5±3.53
SCC1-33	16.5±3.53
SLch6	19±0.0
SLch14	18±0.0

The proteolytic activity of dairy lactic acid bacteria is essential for the bacterial growth in milk and involved in the development of organoleptic properties of different fermented milk products (Razzaq *et al,* 2019; Worsztynowicz *et al,* 2019). The production of high quality fermented dairy products is dependent on proteolytic systems of starter bacteria, since peptidase and amino acids formed have a direct impact on flavour or serve as flavour

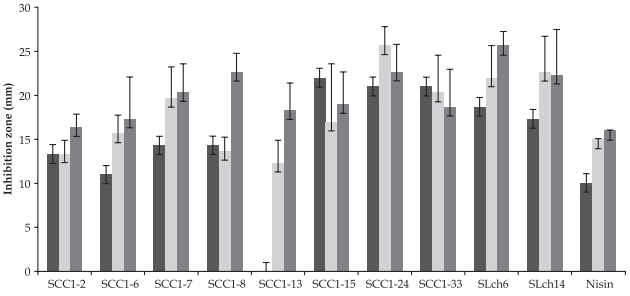

Fig 3. Antagonistic effect of *Enterococcus faecalis* strains against *Micrococcus luteus* (■), *Listeria innocua* (■) and *Escherichia coli* (■).

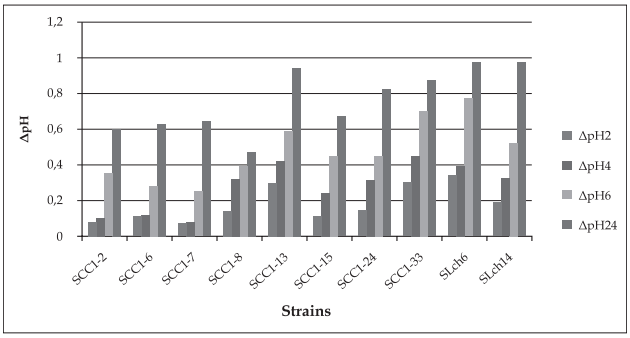

Fig 4. Evolution of ΔpH during the fermentation of camel milk after 2H , 4H , 6H and 24H inoculated with different lactic strains and incubated at 30°C.

precursors in these products. Several peptidases with different specificities have been identified in lactic acid bacteria; all peptidases have been found to be intracellular and liberated in fermented milk products after cell lysis (Sebastián-Nicolas *et al,* 2021; Razzaq *et al,* 2019).

Acidification and proteolytic activity are difficult to dissociate and the differences of acidifying power between the different strains were certainly related to a difference in the initiation of proteolytic activity (Raveschot *et al,* 2020).

Lipolytic activity

The results of the lipolytic activity of lactic strains are shown in Table 5. Lactic acid bacteria are considered weakly lipolytic (García-Cano *et al,* 2019) in comparison with other bacterial species such as *Pseudomonas, Acinetobacter* and *Flavobacterium* (Brennan *et al,* 2002).

Tavakoli *et al* (2019) stated that the addition of autochthonous LAB on dairy products contributes to the production of free fatty acids and linoleic acid from milk fat lipolysis, providing a hipolipidemic effect in the host. These bacteria are found in large amounts on lactic foods due to their adaptation capacity in this substrate rich in proteins, lipids and fatty acids. The wide distribution of these bacteria is attributed to their lipolytic and proteolytic properties,

capacity to ferment/assimilate lactose and to use fatty acids. The Free Fatty Acid contributes to the aroma and flavour of some foods, especially cheese (Gomes *et al,* 1998).

Table 5. Lipolytic activity of lactic acid bacteria.

Strains	Lipolytic diameter zone		
	1%tween80	3%tween80	5%tween 80
SCC1-2	11,375	9	9
SCC1-6	8,5	9	9,125
SCC1-7	9,5	10	9,875
SCC1-8	9,25	9	9,125
SCC1-13	13,5	9,75	9,5
SCC1-15	9,625	10,5	11,5
SCC1-24	8,875	9,5	10,25
SCC1-33	9,5	8,75	9,5
SLch6	8,625	9,5	11
SLch14	11,125	9	9,5

Biomass production and growth rate

The fermentation broth was centrifuged and the pellet was dried in order to determine biomass. The difference between the initial optical density (OD600) and the OD600 at which cells were collected (ΔOD600) as well as the dry weight of strains were used to reflect the growth amount (Table 6). Based on the biomass, cultures were divided into 3 groups: major yields when biomass ≥ 1.30 mg/L, an average yield when the formed biomass ranged from 0.6 to 1.29 mg/L, poor performance when the biomass was <0.6 mg/L (Berisvil *et al,* 2020). Strains SCC1-6, SCC1-15, SCC1-33 and SLCch14 were characterised by a high value of ΔDO600 and an important growth rate. The strains SCC1-24, SCC1-2, SCC1-13 and SCC1-13 presented a weak biomass and growth rate.

Table 6. Characteristics of starters growth.

Strains	ΔOD600*	Biomass (g/l)	µmax (h-1)	EPS	OD600 Supernatant
SCC 1-2	0.448	0.53	0.052	-	0.003
SCC1-6	1.136	0.81	0.132	-	0.008
SCC1-7	0.67	0.69	0.071	-	0.019
SCC1-8	0.864	0.20	0.080	+	0.106
SCC1-13	0.737	0.06	0.073	+	0.115
SCC1-15	1.322	0.79	0.123	+	0.024
SCC1-24	0,737	0,06	0,073	+	0.049
SCC1-33	1 ,322	0,79	0,123	+	0.056
SLch6	0.689	0.887	0.146	+	0.068
SLCch14	1.935	0.98	0.131	+	0.02

* ΔOD 600, difference between the initial optical density and optical density after 6 h of culture; +, EPS producing strains; -, non EPS producing strains.

Indeed, the production of small quantities of biomass could be an inconvenient for the industrial use of these strains. However, this low yield could be explained by the loss of biomass during centrifugation and this was due to the production of exopolysaccharides that prevent the separation of bacterial cells and culture medium. This was visualised in the OD values of supernatant (Table 6). According to Ren *et al* (2022), a good separation of biomass was represented by an OD600 ranging between 0 and 0.1. The majority of strains had an OD600 <0.1 reflecting a good separation of biomass. Only two strains SCCl-8 and SCCl-13 had values greater than 0.1. As mentioned earlier, this was due to the production of EPS which prevent separation during centrifugation.

Exopolysaccharide production

Many strains of LAB produce EPS that can be a capsule, closely attached to the bacterial cell, or loosely attached or excreted as slime (Angelin and Kavitha, 2020; Sørensen *et al,* 2022). The strains that showed poor and fair pellet separation after centrifugation (see above) were screened for EPS production.

Lactic acid bacteria have the ability to synthesise and excrete during their growth, extracellular sugar polymers called polysaccharides or exopolysaccharide (EPS), which can improve the texture and viscosity of the final product (Korcz and Varga, 2021). In general, the presence of polysaccharides in fermented products such as yogurt can increase the homogeneity of the product and make its presentation more enjoyable (Wa *et al,* 2021). The texture of fermented milk depends also on the interactions between bacteria and the different proteins (spatial conformation, interaction, pH, ionic strength) (Prete *et al,* 2021). Our results showed that seven strains (SCC1-8, SCC1-13, SCC1-15, SCC1-24, SCC1-33, SLch6 and SLch14) were able to produce EPS (Table 6).

A relatively high number of *Enterococci* strains were evaluated and the results indicated good production and technological performance of many strains of *E. faecium*. However, enterococci comprise a major part of the fresh cheese curd microflora and in some cases, they are the predominant microorganisms in the ripened cheese in other countries (Berisvil *et al,* 2020). The role of these strains in the manufacture of cheese has been investigated in the last few years (Dapkevicius *et al,* 2021; Rhoades *et al,* 2021; Sarkar *et al,* 2020).

Conclusion

Several LAB isolated from Tunisian camel milk and identified as *Enterococcus faecium* showing potentially important properties are valuable for practical application as starter and a potential probiotic. *E. faecium* SLCch6 strain had great potential to be used as an effective probiotic in both food industry or therapy purposes. This strain presented high tolerance to the passage through the GIT environment, high co-aggregation and auto-aggregation ability, high adhesion to gastric mucin and acceptable adhesion to epithelial cells suggesting high adhesiveness to the host tissues which favour the colonisation and survival in the GIT. Moreover, this strain showed strong antagonistic activity against pathogens. Furthermore, the susceptibility of *E. faecium* SLCch6 toward antibiotics suggested the absence of transferable antibiotic genes. However, careful safety investigations must be greatly emphasised before the application of *E. faecium* SLCch6 as probiotic.

Author Contributions

Imen Fguiri designed and performed the experiments. Imen Fguiri and Manel Ziadi analysed the data and wrote the manuscript. Samira Arroum contributed in the experimental analysis. Touhami Khorchani revised the paper

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Competing Interests

The authors declare that there is no Competing Interests regarding the publication of this paper.

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