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ORIGINAL RESEARCH

Characterisation of a cell-free supernatant obtained from cultures of *Enterococcus faecalis* DBFIQ E24 with antagonistic activity against bacteria, yeasts and moulds

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Enterococcus faecalis *DBFIQ E24 strain produces antimicrobial substances that inhibit the growth of food-borne pathogenic bacteria, yeasts and moulds. This broad antimicrobial spectrum was determined by obtaining the cell-free supernatant (CFS) and employing the agar-well-diffusion method. Its antibacterial activity was completely inactivated by the action of proteinase K and not affected by catalase action. Besides, it was stable to thermal treatments, showing maximum antibacterial activity at pH 7.0. Moreover, CFS showed a bactericidal effect against* Escherichia coli *DBFIQ Ec9 strain. All these interesting physicochemical properties allow that either CFS or* E. faecalis *DBFIQ E24 strain to be considered as potential food biopreservatives.*

Keywords Bacteriocin, *Enterococcus faecalis* strain, Cell-free supernatant, Characterisation, Food biopreservative.

INTRODUCTION

It has been shown that lactic acid bacteria (LAB) have the ability to produce, besides its final fermentation products, other inhibitory-growth substances: bacteriocins and bacteriocin-like substances (Tagg *et al.* 1976; Klaenhammer 1988).

Bacteriocins are ribosomally synthesised proteins or polypeptides that exert their action in an extra cellular manner, inhibiting the growth of taxonomically closed related bacteria. However, more recent studies have proved the existence of bacteriocins that prevent the proliferation of other Gram +ve bacteria not closely related to the producing strain from a taxonomic point of view (Raccach and Geshell 1995; Susani *et al.* 1995). The so-called bacteriocin-like substances are, in fact, bacteriocins that differ from those belonging to the classic definition in possessing a broader antibacterial spectrum than bacteriocins, also reaching Gram (−) species (Piard and Desmazeaud 1992; Ray and Sandine 1992; Vignolo *et al.* 1993).

Simultaneously, it has been demonstrated that LAB produce antifungal compounds that inhibit food-borne pathogenic and spoilage mould and yeast species belonging to *Aspergillus*, *Fusarium*, *Penicillium*, *Rhodotorula*, *Pichia* and *Kluyveromyces* genera (Magnusson *et al.* 2003; Savadogo *et al.* 2006).

Particularly, enterococci species are found in fermented vegetable microbiota and in most of cheese types, not only in those farmhouse-produced but also in those manufactured with pasteurised milk. Their ability to produce bacteriocins (enterocins) is a very important characteristic for their application in food technology. These enterocins have acquired a major relevance over the last decade, owing to the fact that producing strains can be easily isolated from several fermented foods (Vaughan *et al.* 1994). These bacteriocins are also very relevant because most of them are usually active against food-borne pathogens like *Listeria* sp. and *Clostridium* sp. (Gálvez *et al.* 1998; Ohmomo *et al.* 2000) and against Gram (−) bacteria such as *Vibrio cholerae* (Simonetta *et al.* 1997).

For the exposed reasons, the aim of this research work was to study and characterise a cell-free supernatant (CFS) with broad spectrum of

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antimicrobial activity obtained from cultures of *Enterococcus faecalis* DBFIQ E24 strain, isolated from Argentinian raw milk.

MATERIALS AND METHODS

Strains and cultures preservation

The chosen bacteriocin producer strain was *E. faecalis* DBFIQ E24, isolated from raw milk produced in Santa Fe region (Argentine). To test its antagonistic activity, 17 bacterial strains and 28 fungi and yeast strains from our culture collection were used as target, as indicated in Table 1.

All bacterial strains were lyophilised to maintain a reference stock and frozen at −80 °C in MRS or M17 Broth (Difco Laboratories, Detroit, MI, USA), for LAB, or Nutrient Broth (Merck KGaA, Darmstadt, Germany), for nonlactic bacteria (non-LAB), with 15% (v/v) glycerol. Yeast strains were frozen at −80 °C in YM Broth (Difco) with 15 % (v/v) glycerol. Moulds were preserved at 4 °C in Malt Extract Agar (MEA; Difco) slants previously incubated for 7 days at 25 °C, with the addition of sterile liquid vaseline (Heckly 1978).

Preparation of *E. faecalis* DBFIQ E24 CFS

From a stock culture, *E. faecalis* DBFIQ E24 was activated by two successive culturing procedures in M17 Broth (Difco) at 37 °C for 24 h. The final culture was obtained by adding 30 mL of the last propagation culture to 1000 mL of M17 Broth (Difco) and incubating at 37 °C for 24 h. The cell concentrations achieved were established between 1.0×10^9 and 7.4×10^9 CFU/mL. Then, cells were removed by centrifugation at 5200 *g* for 15 min at 4 °C. Afterwards, the CFS obtained was concentrated 10-fold at 70 °C in a Büchi RE 111 rotavapor and, subsequently, sterilised by filtration using 0.22 μm porous membranes (Sartourius Stedim, Goettingen, Germany) and immediately used or stored at −20 °C until needed. pH range of concentrated CFS was around 6.5–7.0.

Determination of antagonistic activity

The agar-diffusion assay described by Tagg and Mc Given (1971) was used to determine antimicrobial activity against moulds and yeasts. Moulds were grown on MEA (Difco) slants during 7 days at 25 °C or until sporulation, when required. Then, the spore suspensions were prepared by collecting spores after vigorously shaking of slants with 10 mL of 0.1 % (w/v) sterile peptone water. For assay, 0.3 mL of spore solution was inoculated on sterile MEA (Difco) plate and homogenously distributed. Immediately afterwards, 7-mm-diameter wells were made into the agar plates into which 60 μL of CFS was added. Mould growth inhibition zones were examined constantly during 7 days. Yeast cell inocula were prepared from liquid cultures grown in YM Broth (Difco) at 25 °C for 24–48 h. For assay, 0.25 mL of yeast cell suspension was added and homogenously distributed over the surface of a YM Agar (Difco) plate. Afterwards, this procedure was performed as in moulds, except that growing was done for 24–48 h at 25 °C.

Table 1 Target strains used in antimicrobial activity assays

Indicator strains
Yeasts
Candida albicans ATCC 10231[a]
Candida albicans ATCC 64548[a]
Candida parasilopsis ATCC 22019[a]
Candida tropicalis Ct8[b]
Candida tropicalis Ct9[b]
Kluyveromyces marxianus var. *marxianus* KM1[b]
Rhodotorula rubra R16[b]
Saccharomyces cerevisiae Sc5[b]
Schizosaccharomyces octosporus SO14[b]
Schizosaccharomyces pombe Sp4[b]
Moulds
Aspergillus clavatus AC23[b]
Aspergillus flavus AF14[b]
Aspergillus flavus AF25[b]
Aspergillus flavus AF11[b]
Aspergillus niger AN21[b]
Fusarium chlamydosporum F13[b]
Fusarium chlamydosporum F9[b]
Fusarium sp. F2[b]
Geotrichum candidum GC10[b]
Mucor circillenoides MC3[b]
Penicillium camemberti PC9[b]
Penicillium chrysogenum PC5[b]
Penicillium citrinum PC1[b]
Penicillium commune PC17[b]
Penicillium islandicum PI9[b]
Penicillium roquefortii PR1[b]
Rhizopus oryzae RO8[b]
Rhizopus stolonifer RS4[b]
Bacteria
Staphylococcus aureus subsp. a*ureus* Sa1[b]
Listeria monocytogenes Lm4[b]
Bacillus cereus B28[b]
Bacillus subtilis Bs23[b]
Pseudomonas sp. P36[b]
Vibrio cholerae O1 EL Tor serotype *Inaba* Vch5[b]
Escherichia coli Ec9[b]
Salmonella Enteritidis SE2[b]
Lactobacillus plantarum LP7[b]
Lactobacillus plantarum LP25[b]
Lactobacillus plantarum LP31[b]
Enterococcus faecium E2[b]
Enterococcus faecium E3[b]
Enterococcus faecalis E13[b]
Lactobacillus delbrueckii subsp. *bulgaricus* LB92[b]
Streptococcus thermophilus SF1-1[b]
Lactococcus lactis Ch3-3[b]
Lactococcus lactis subsp. *lactis* CRL 63[c]

[a]ATCC, American Type Culture Collection. [b]All these strains belong to the Food Engineering Department (Microbiology and Biotechnology Section), Facultad de Ingeniería Química (FIQ), Universidad Nacional del Litoral, Argentina, culture collection. [c]CRL, CERELA (CONICET, Argentina) culture collection.

Evaluation of CFS antagonistic activity against non-LAB was also developed by the agar-diffusion assay. Briefly, 0.1 mL of each indicator strain overnight culture was added to 15 mL of molten Nutritive Agar (Merck) and placed into a sterile Petri dish. After cooling and drying, 7-mm-diameter wells were filled with 60 μL of CFS. Plates were incubated at 37 °C for 24 h and the diameter of the inhibition zones was measured. To discard inhibition owing to the presence of lactic acid, 10-fold concentrated and sterilised M17 Broth (Difco) was acidified with lactic acid up to CFS pH value and used in every assay as negative control.

Lactic acid bacteria were screened for their antagonistic activity using a dual-culture overlay assay as described by Foulquié Moreno *et al.* (2003). *Enterococcus faecalis* DBFIQ E24 strain was inoculated by touching twice with a sterile loop on MRS Agar (Difco) plates and allowed to grow for 24 h at 37 °C. Subsequently, the plates were overlaid with 10 mL of 0.8 % (w/v) soft MRS Agar (Difco) containing 0.1 mL of the sensitive strain with a cell concentration of 1×10^6 CFU/mL. After 24 h of incubation at 37 °C, inhibition zones were measured. Each assay was performed in triplicate, and results were expressed as an average of the measured values.

Determination of CFS title against bacterial strains

Serial twofold dilutions were made to the CFS. The title, expressed in Arbitrary Units by ml (AU/mL), was defined as the reciprocal of the maximum dilution that generated an inhibition zone of 7.5 mm (employing the agar-diffusion method), divided by the sample volume tested (in mL). *Pseudomonas* sp. DBFIQ P36, *E. coli* DBFIQ Ec9, *B. cereus* DBFIQ B28 and *B. subtilis* DBFIQ Bs23 were employed as target bacterial strains.

Enzymatic characterisation of antibacterial components of CFS

To test the enzymatic susceptibility of the antibacterial substances produced by *E. faecalis* DBFIQ E24 strain, CFS was treated with catalase, lipase, lysozyme, papain, pepsin, pronase E, proteinase K and trypsin (all from Sigma) at a final concentration of 0.5 mg/mL. The reaction was incubated for 4 h at 37 °C and carried out in sterilised conditions. Afterwards, remaining inhibitory activity was evaluated by the agar-diffusion assay (Strasser de Saad and Manca de Nadra 1993). Controls were made assaying 10-fold concentrated M17 Broth (Difco) with enzymes at specified concentrations. All non-LAB strains were used as target bacteria. Each assay was performed in triplicate, and results were expressed as an average of the measured values.

Determination of physicochemical parameters of antibacterial components of CFS

Thermal stability

Cell-free supernatant was heated at 100 °C for 10 and 30 min and at 121 °C during 15 min, assaying afterwards the residual inhibitory activity by the agar-diffusion assay. All non-LAB strains were employed as target bacteria. Each assay was performed in triplicate, and results were expressed as an average of the measured values.

Influence of acidity on antibacterial activity

The pH of CFS was adjusted to different values, between 4.5 and 9.0. To reduce pH, concentrated lactic acid was used, and to increase it, a 1 M NaOH solution was added. Afterwards, inhibitory activity was evaluated by the agar-diffusion assay (Schillinger and Lücke 1989; Park *et al.* 2003). Tenfold concentrated M17 Broth (Difco) adjusted to the different pH values was used as controls. All non-LAB strains were used as target bacteria. Each assay was done in triplicate, and results were expressed as an average of the measured values.

Molecular weight estimation

The technique was performed by ultrafiltration using a Centriprep-3 device (MWCO 3 kDa; Amicon, EMD Millipore, Billerica, MA, USA). For assay, 2 mL of CFS was diluted five times by adding 8 mL of Milli-Q water and centrifuged at 3000 *g* at 25 °C for 65 min. The filtrate and retentate were obtained, and the title and antagonistic activity were evaluated against *B. cereus* DBFIQ B28, *E. coli* DBFIQ Ec9, *Pseudomonas* sp. DBFIQ P36 and *B. subtilis* DBFIQ Bs23 strains.

Effect of storage time and temperature on CFS antibacterial activity

Cell-free supernatant aliquots were stored for 5 months at the following temperatures: −20, 4, 25 and 37 °C. At appropriate time intervals, inhibitory activity, as title, was determined by the agar-diffusion assay, employing *E. coli* DBFIQ Ec9 and *B. cereus* DBFIQ B28 strains as target micro-organisms. Each assay was done in triplicate, and results were expressed as an average of the measured values.

Effect of the surfactants on CFS antibacterial activity

Liquid detergents or saturated solutions from solid surfactants were added to CFS aliquots, to obtain a 1% (v/v) final concentration. Cell-free supernatant without the addition of detergents and 1% (v/v) solutions of each surfactant in 10-fold concentrated M17 Broth (Difco) were used as controls (Muriana and Klaenhammer 1991). Samples and controls were incubated at 37 °C for 6 h. Finally, serial twofold dilutions were carried out to samples and controls, and antagonistic activity was determined using the agar-diffusion assay and expressed as title (AU/mL). *Bacillus cereus* DBFIQ B28 and *E. coli* DBFIQ Ec9 were used as target strains.

The nonionic surfactants used in this assay were Triton X-100, Tween 20 and Tween 80 (all from Sigma); the anionic detergents employed were sodium dodecyl sulphate (SDS, J.T. Baker, Covidien, Mansfield, MA, USA), glycocholic acid (Fluka, Sigma, St. Louis, MO, USA), cholic acid (BDH, Merck KGaA, Darmstadt, Germany), deoxycholic acid (Fluka),

taurocholic acid (Biochem, Buenos Aires, Argentina); and the cationic detergent used was cetyltrimethylammonium bromide (BDH). Each assay was done in triplicate, and results were expressed as an average of the measured values.

Mode of action of antibacterial components of CFS

To evaluate bactericidal or bacteriostatic effect of CFS on Gram +ve and Gram (−) bacteria, 2 mL of CFS was added to 18 mL of an exponential-growth-phase culture of indicator bacteria developed in Nutrient Broth (Merck). *Escherichia coli* DBFIQ Ec9 and *B. cereus* DBFIQ B28 were employed as indicator bacteria. At appropriate time intervals (0, 3, 6, 9, 12 and 24 h), the number of viable cells was determined.

For evaluation of CFS lytic ability, absorbances at 525 nm were measured in a Metrolab 1700 spectrophotometer to a sample of the CFS-added cultures at different time intervals using sterile Nutrient Broth (Merck) as negative control. Each assay was done in triplicate, and results were expressed as an average of the measured values.

RESULTS

The inhibition assay carried out with different moulds strains showed that all *Penicillium* strains except *P. citrinum* DBFIQ PC1 and *P. commune* DBFIQ PC17 were inhibited by CFS. Besides, an inhibitory effect was observed against three *Aspergillus* strains, one *Fusarium* strain and one strain belonging to *Mucor* genus. Results are summarised in Table 2.

All yeasts strains were inhibited by CFS with the exception of three strains belonging to *Candida* and *Schizosaccharomyces* genera. In general, CFS inhibitory action was weak except against *Schizosaccharomyces pombe* DBFIQ Sp4 and *Rhodotorula rubra* DBFIQ R16, which were deeply antagonised by CFS action.

Table 3 shows that all assayed bacterial strains were susceptible to CFS antagonistic effect, except for *S. aureus* subsp. *aureus* DBFIQ Sa1. Sensitive Gram (−) and Gram +ve bacterial strains behaved similarly between each other against CFS antagonistic properties with the exception of *V. cholerae* DBFIQ Vch5, which resulted in the strain most inhibited. Otherwise, *L. monocytogenes* DBFIQ Lm4 proved to be the least antagonised.

Cell-free supernatant title (expressed in AU/mL) against *Pseudomonas* sp. DBFIQ P36, *B. cereus* DBFIQ B28, *E. coli* DBFIQ Ec9 and *B. subtilis* DBFIQ Bs23 resulted in 267 AU/mL.

After treatment of CFS with different enzymes, total inactivation of antibacterial activity using trypsin was observed, except against *E. coli* DBFIQ Ec9; however, a deep decrease in the inhibition zone corresponding to this target strain was evidenced. Treatments with pronase E as well as proteinase K inactivated almost all CFS inhibitory activity against *E. coli* DBFIQ Ec9 and *B. cereus* DBFIQ B28 strains. Remaining proteolytic enzymes deactivated, totally or partially, CFS antagonistic activity against all studied bacterial strains. Furthermore, catalase did not affect CFS antibacterial activity. On the other hand, after lysozyme treatment, partial inactivation of CFS antagonistic activity against three target bacterial strains was observed. CFS treated with lipase caused a considerable decline of antagonistic activity against *E. coli* DBFIQ Ec9 and *B. cereus* DBFIQ B28, the only target strains assayed.

Thermal treatments only partially modified CFS antibacterial activity, which demonstrates its elevated thermal resistance. Cell-free supernatant bacterial antagonistic activity assays at different pH showed that between 4.5 and 5.0, inhibitory

Table 2 Antagonistic activity of cell-free supernatant obtained from *E. faecalis* E24 cultures against different yeasts and moulds

Indicator strains	*Antimicrobial activity*	*Diameter of inhibition zone (mm)*[a]
Yeasts		
C. albicans ATCC 10231[b]	−	−
C. albicans ATCC 64548[b]	+	9
C. parasilopsis ATCC 22019[b]	−	−
C. tropicalis DBFIQ Ct8[c]	+	7.5
C. tropicalis DBFIQ Ct9[c]	+	9
K. marxianus var. *marxianus* DBFIQ KM1[c]	+	7.5
R. rubra DBFIQ R16[c]	+	15
S. cerevisiae DBFIQ Sc5[c]	+	8
Sch. octosporus DBFIQ SO14[c]	−	−
Sch. pombe DBFIQ Sp4[c]	+	11
Moulds		
A. clavatus DBFIQ AC23[c]	+	10
A. flavus DBFIQ AF14[c]	+	7.5
A. flavus DBFIQ AF25[c]	−	−
A. flavus DBFIQ AF11[c]	+	9
A. niger DBFIQ AN21[c]	−	−
F. chlamydosporum DBFIQ F13[c]	−	−
F. chlamydosporum DBFIQ F9[c]	+	8
Fusarium sp. DBFIQ F2[c]	−	−
G. candidum DBFIQ GC10[c]	−	−
M. circillenoides DBFIQ MC3[c]	+	8
P. camemberti DBFIQ PC9[c]	+	8
P. chrysogenum DBFIQ [c] PC5	+	7.5
P. citrinum DBFIQ PC1[c]	−	−
P. commune DBFIQ PC17[c]	−	−
P. islandicum DBFIQ PI9[c]	+	10
P. roquefortii DBFIQ PR1[c]	+	9
Rh. oryzae DBFIQ RO8[c]	−	−
Rh. stolonifer DBFIQ RS4[c]	−	−

Diameter of wells: 7 mm. [a]Diameter of wells was subtracted from the inhibited zone. [b]ATCC: American Type Culture Collection. [c]DBFIQ: collection of the Food Engineering Department (Microbiology and Biotechnology Section), Facultad de Ingeniería Química, Universidad Nacional del Litoral, Argentina. – No inhibition zone.

Table 3 Antibacterial activity, enzymatic characterisation, influence of acidity and thermal stability of the cell-free supernatant (CFS) obtained from *E. faecalis* DBFIQ E24 cultures

	Diameter of inhibition zone (mm)[a]							
Treatment	*L. m.*[b]	*V. ch.*[b]	*B. c.*[b]	*B. s.*[b]	*Ps. sp.*[b]	*E. c.*[b]	*Staph. a.*[b]	*Salm. E.*[b]
Untreated CFS	10	23	16	15	15	16	–	16
Catalase	10	23	16	15	15	16	–	16
Lipase	NA	NA	13	NA	NA	11	NA	NA
Lysozyme	8	18	16	12	14	15	–	11
Papain	NA	NA	10	NA	NA	10	NA	NA
Pepsin	9	15	11	10	–	14	–	12
Pronase E	NA	NA	10	NA	NA	11	NA	NA
Proteinase K	NA	NA	8	NA	NA	10	NA	NA
Trypsin	–	–	–	–	–	9	–	–
pH = 4.5	15	25	25	19	18	25	14	25
pH = 5.0	9	25	20	16	13	25	–	20
pH = 6.0	9	23	18	14	14	20	–	19
pH = 7.0	10	23	16	15	15	16	–	16
pH = 8.0	8	21	14	13	13	14	–	14
pH = 9.0	8	21	14	13	13	14	–	14
M17 control, pH = 4.5	10	21	15	14	14	16	14	16
M17 control, pH = 5.0	8	17	11	12	11	12	12	14
M17 control, pH = 6.0 to 9.0	–	–	–	–	–	–	–	–
10 min, 100 °C	9	20	12	14	13	14	–	16
30 min, 100 °C	8	19	11	12	11	12	–	13
15 min, 121 °C	8	18	10	12	11	12	–	12

Diameter of wells: 7 mm. [a]Diameter of wells was subtracted from the inhibited zone. [b]DBFIQ: collection of the Food Engineering Department (Microbiology and Biotechnology Section), Facultad de Ingeniería Química, Universidad Nacional del Litoral, Argentina. *Ps.* sp.: *Pseudomonas* sp. DBFIQ P36 – *Staph. a.*: *Staphylococcus aureus* subsp. *aureus* DBFIQ Sa1 – *L. m.*: *Listeria monocytogenes* DBFIQ Lm4 – *B.c.*: *Bacillus cereus* DBFIQ B28 – *B. s.*: *Bacillus subtilis* DBFIQ Bs23 – *E. c.*: *Escherichia coli* DBFIQ Ec9 – *Salm. E.*: *Salmonella Enteritidis* DBFIQ SE2 – *V. ch.*: *Vibrio cholerae* O1 EL Tor serotype Inaba DBFIQ Vch5. NA Not assayed. –, No inhibition zone.

activity increased owing to lactic acidity; however, at pH values between 6.0 and 7.0, antagonistic activity proved to be maximum and slightly decreased between pH 7.0 and 9.0.

Enterococcus faecalis DBFIQ E24 possess the ability to hinder the growth of closely related LAB except for *L. plantarum* DBFIQ LP7 and *L. delbrueckii* subsp. *bulgaricus* DBFIQ LB92. Employing the dual-layer assay, inhibition zones with a diameter between 9 and 30 mm were observed as shown in Table 4.

On the other hand, when *E. faecalis* DBFIQ E24 was treated as a target strain against all others LAB, its growth was mainly inhibited by *L. delbrueckii* subsp. *bulgaricus* LB92 and *L. plantarum* LP31 with diameters of inhibition zones of 20 and 19 mm, respectively.

Molecular weight (MW) estimation of active compounds present in CFS was performed by ultrafiltration with Amicon membranes (cut-off value, 3 kDa). As a result, it was feasible to estimate the presence of two fractions with antibacterial activity: one active fraction retained (MW >3000 Da), called retentate, with scarce activity, and other active fraction not retained by the membrane (MW lower than 3000 Da), called filtrate, with a marked inhibitory activity against *B. cereus* DBFIQ B28, *B. subtilis* DBFIQ Bs23, *E. coli* DBFIQ Ec9 and *Pseudomonas* sp. DBFIQ P36. Results are shown in Table 5.

The effect of time and storage temperature on CFS inhibitory activity assayed against *E. coli* DBFIQ Ec9 and *B. cereus* DBFIQ B28 can be appreciated in Figure 1, in which both bacteria showed the same curve shapes. It was observed that CFS can be stored at 4, 25 or −20 °C for at least 1 month without losing any antimicrobial activity. However, after this time, a significant decline in antimicrobial activity at all temperatures was verified. Cell-free supernatant incubation at 37 °C caused a pronounced decrease in antagonistic activity, and it was evidenced even before a month of incubation time. This was probably due to the fact that this temperature is optimum for the action of many proteolytic enzymes that could be naturally present in CFS. Moreover, after 90 days of storage time, the remaining antibacterial activity did not decrease anymore until the end of the assay, except for 25 °C, which continued to decline up to the title found at 37 °C. This final decrease in antibacterial activity could be explained by a slower action of proteolytic enzymes at 25 °C.

Table 4 Crossed dual-layer inhibition assay employing different lactic acid bacteria

	E. faecalis DBFIQ E24	
Target bacteria	*As inhibitory strain*[a]	*As target strain*[a]
E. faecium DBFIQ E2	15	13
E. faecalis DBFIQ E13	14	11
E. faecium DBFIQ E3	14	15
L. lactis DBFIQ Ch3-3	14	14
L. lactis subsp. *lactis* CRL 63	13	14
S. thermophilus DBFIQ SF1-1	19	18
L. plantarum DBFIQ LP7	–	18
L. plantarum DBFIQ LP25	30	–
L. plantarum DBFIQ LP31	9	19
L. delbrueckii subsp. *bulgaricus* DBFIQ LB92	–	20

[a]Diameter of inhibition zone (mm).

Table 5 Antibacterial activity of the different fractions obtained after cell-free supernatant (CFS) ultrafiltration

	Diameter of inhibition zone (mm)[a]			
	B. cereus[b]	*B. subtilis*[b]	*E. coli*[b]	*Pseudomonas sp.*[b]
Untreated CFS	11	18	20	18
CFS Filtrate	10	17	18	16
CFS Retentate	–	14	14	15

Diameter of wells: 7 mm. [a]Diameter of wells was subtracted from the inhibited zone. [b]DBFIQ: collection of the Food Engineering Department (Microbiology and Biotechnology Section), Facultad de Ingeniería Química, Universidad Nacional del Litoral, Argentina. –, No inhibition zone.

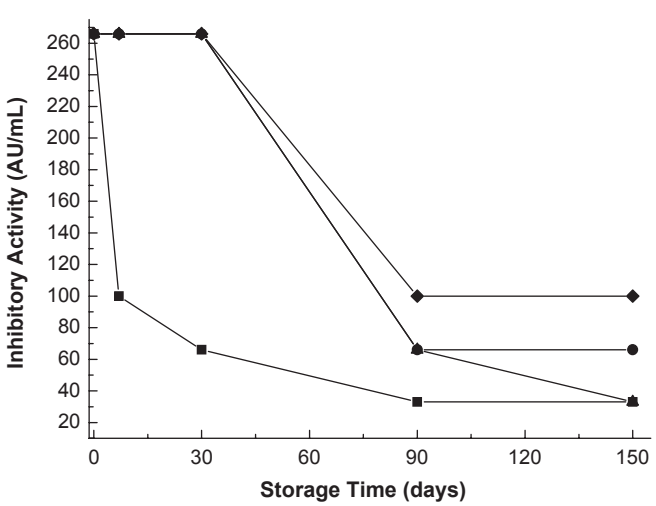

Figure 1 Effect of time and storage temperature on cell-free supernatant bacterial inhibitory activity. Storage temperatures: −20 °C (♦), 4 °C (•), 25 °C (▲) and 37 °C (■). *Escherichia coli* DBFIQ Ec9 and *B. cereus* DBFIQ B28 were used as target strains for antimicrobial activity determination.

Table 6 Effect of surfactants addition on cell-free supernatant (CFS) activity

	CFS title (AU/mL)[ab]		*Surfactant title control (AU/mL)*[ab]	
	E. coli[c]	*B. cereus*[d]	*E. coli*[c]	*B. cereus*[d]
Nontreated CFS	267	267		
Triton X-100	533	533	–	133
Tween 20	267	267	–	133
Tween 80	267	267	–	–
SDS	267	133	–	33
Glycocholic acid	533	267	67	67
Cholic acid	267	33	–	–
Deoxycholic acid	533	267	–	–
Taurocholic acid	267	67	–	133
Cetyltrimethylammonium bromide	533	533	133	133

Diameter of wells: 7 mm. [a]Diameter of wells was subtracted from the inhibited zone. [b]Title, in AU/mL, was defined as the reciprocal of the maximum serial twofold dilution that generated an inhibition zone of 7.5 mm (employing the agar-diffusion method), divided by the sample volume tested (in mL).[c]*E. coli* DBFIQ Ec9. [d]*B. cereus* DBFIQ B28. –, No CFS title.

The activity of the antimicrobial substances that antagonise *E. coli* DBFIQ Ec9 was not affected by the addition of most of the surfactants, as shown in Table 6. However, Triton X-100 and deoxycholic acid duplicated CFS title specifically, which means without showing positive results when using the detergents alone. On the other hand, the addition of four surfactants did not change CFS antagonistic activity against *B. cereus* DBFIQ B28, but Triton X-100 and cetyltrimethylammonium bromide duplicated the mentioned inhibitory activity, although causing an unspecific inhibition effect. The remaining detergents strongly decreased the antimicrobial activity and induced an unspecific action.

Disparity in results allowed to observe that Gram (−) bacteria are more resistant to surfactants than Gram +ve bacteria, attributing this difference to the different cell wall structures that both micro-organisms possess. Finally, nonionic surfactants Tween 20 and Tween 80 did not produce changes in inhibitory activity against bacterial strains assayed.

To evaluate whether the mode of action of the antibacterial substance was bactericidal or bacteriostatic, CFS was added at a 10 % (v/v) concentration to *E. coli* DBFIQ Ec9 and *B. cereus* DBFIQ B28 cultures grown in Nutritive Broth (Merck), and viability was monitored during 24 h. In relation to Gram (−) strain, addition of CFS to the microbial culture produced a marked decrease in viable cells, dropping off up to three logarithmic cycles after 24 h. Furthermore, as shown in Figure 2,

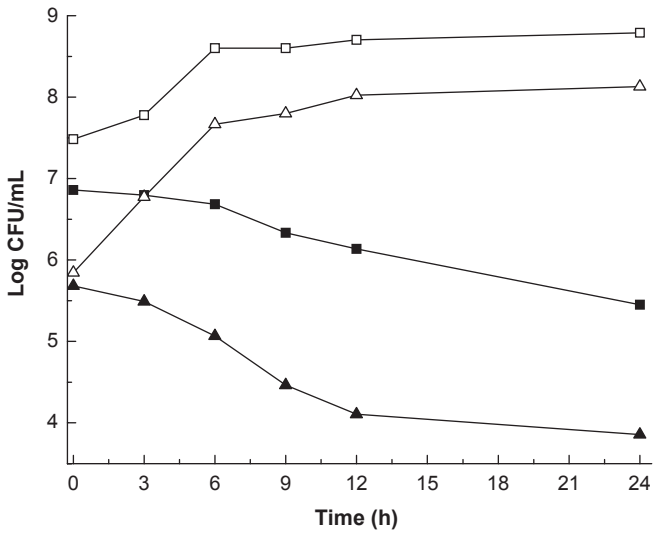

Figure 2 Effect of cell-free supernatant from *E. faecalis* DBFIQ E24 on growing cultures of *Escherichia coli* DBFIQ Ec9 and *Bacillus cereus* DBFIQ B28 in Nutrient Broth. In the absence of concentrated cell-free supernatant (CFS): (–□–) *E. coli* DBFIQ Ec9; (–Δ–) *B. cereus* DBFIQ B28; in the presence of CFS: (–■–) *E. coli* DBFIQ Ec9; (–▲–) *B. cereus* DBFIQ B28.

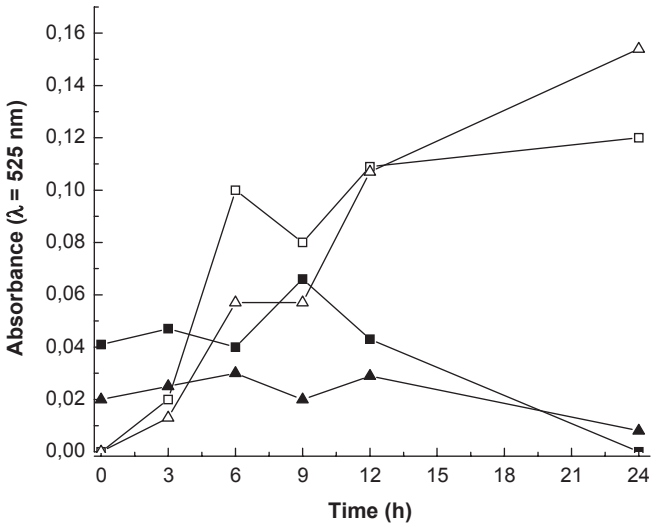

Figure 3 *E. faecalis* DBFIQ E24 cell-free supernatant (CFS) lytic action on growing cultures of *Escherichia coli* DBFIQ Ec9 and *Bacillus cereus* DBFIQ B28 in Nutrient Broth. In the absence of concentrated CFS: (–□–) *E. coli* DBFIQ Ec9; (–Δ–) *B. cereus* DBFIQ B28; in the presence of CFS: (–■–) *E. coli* DBFIQ Ec9; (–▲–) *B. cereus* DBFIQ B28.

CFS addition induced a decline in viable *E. coli* cells from 7.9×10^6 CFU/mL to bellow 5.6×10^5 CFU/mL after monitoring for 24 h; while, in the absence of CFS, viable cells raised from 3.2×10^7 to 6.3×10^8 CFU/mL after 24 h of incubation.

With regard to Gram +ve strain, addition of CFS produced a four logarithmic cycle decline in cell concentration after 24 h of incubation. In that way, cell counting dropped off from 6.0×10^5 to 8.3×10^3 CFU/mL after CFS addition and incubating for 24 h. On the other hand, the absence of CFS allowed that cell counting increased from 8.0×10^5 to 1.1×10^8 CFU/mL. This analysis would indicate that the inclusion of CFS to Gram (−) and Gram +ve cultures caused a bactericidal effect.

Figure 3 shows lytic action of CFS on both Gram (−) and Gram +ve bacteria. It can be observed that CFS possess not only a bactericidal but also a lytic effect, being higher on *E. coli* DBFIQ Ec9 cells than *B. cereus* DBFIQ B28 cells. Besides, lytic action was evidenced only after 12 h of incubation for both micro-organisms, which would indicate a stepped action.

DISCUSSION

This investigation has allowed the study and characterisation of a wide inhibitory spectrum CFS produced by *E. faecalis* DBFIQ E24 isolated from regional raw milk, which resulted in being active against bacteria, yeast and moulds. Thus, CFS arises as a promising and effective antimicrobial substance against these organisms.

Many food-borne pathogenic and spoilage processes are produced by yeasts and moulds that are present in many raw and fermented foods. In that way, a yeast included in this work, *K. marxianus* var. *marxianus*, arises as one of the most important spoilage organisms in dairy products. In the same sense, moulds such as *P. roquefortii* and *P. commune* have usually been found in spoilt hard cheese, and *Fusarium* species have been found in different types of grains being well known as mycotoxins-producing moulds. Therefore, it is necessary to find safe and efficient ways to prevent fungal growth in raw material and food products. In that way, LAB and derived products are adequate preservative agents because they are naturally present in many foods and are considered as GRAS organisms.

According to the results, different food-borne pathogenic and spoilage organisms were antagonised by the studied CFS. For instance, *P. roquefortii* PR1 was inhibited by CFS action, but other *Enterococcus* strains evaluated by other authors in a dual-layer inhibition assay could not antagonise this mould (Magnusson *et al.* 2003). Besides, *P. commune* PC17 was not antagonised by CFS action, coinciding with Magnusson *et al.*'s (2003) report. Several *Aspergillus* species found to be inhibited by CFS action, while its activity against *Penicillium* and *Fusarium* species resulted in being strain dependent. Moreover, yeasts such as *K. marxianus* var. *marxianus* DBFIQ KM1 were barely inhibited by CFS, in contrast to Magnusson *et al.*'s (2003) report, where *K. marxianus* J186 was inhibited by any *Enterococcus* strain. *Rhodotorula rubra* R16 strain employed in this work was deeply antagonised by the CFS, whereas a specific action against *Candida* species was not evidenced because a random behaviour in antagonistic activity was observed. Finally, CFS was not effective against *S. aureus* subsp. *aureus* Sa1 and *L. monocytogenes* Lm4 strains, coinciding with the performance reported by Diop *et al.* (2007). *Bacillus cereus* B28 and *B. subtilis* Bs23 strains were hindered by CFS

differing with Diop's report (Diop *et al.* 2007). However, similar results were achieved by Foulquié Moreno *et al.* (2003).

The dual-layer inhibition assay revealed the great bacteriocinogenic potential of *E. faecalis* DBFIQ E24 and its CFS as it inhibited the growth of almost all LAB strains tested, with the exception of *L. delbrueckii* subsp. *bulgaricus* DBFIQ LB92, *L. plantarum* DBFIQ LP7 and *L. plantarum* DBFIQ LP31. The inability of *E. faecalis* DBFIQ E24 to antagonise these LAB could be partially explained by the bacteriocinogenic behaviour of this strain. In fact, a bacteriocin with a broad inhibitory spectrum was previously characterised and purified from *L. plantarum* DBFIQ LP31 (Müller *et al.* 2009). In addition, Foulquié Moreno *et al.* (2003) reported that different *E. faecium* and *E. faecalis* strains were assayed as antimicrobial substance producers against 50 Gram +ve strains belonging to *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus* and *Staphylococcus* genera. Results have shown that *E. faecium* SF 68 and *E. faecalis* Y were not inhibitory towards any of the indicator strains tested. However, *E. faecium* RZS C5 and RZS C13 strains were the most inhibitory strains towards target bacteria assayed, demonstrating a great disparity in the inhibitory responses, which mainly depends on both producer and indicator strains.

Characterisation tests were performed and assayed primarily against bacteria as described previously. Stability to heat was demonstrated after heat treatments. In general, most of bacteriocins and bacteriocin-like substances of low MW show this behaviour (Muriana and Klaenhammer 1991; Park *et al.* 2003; Martín-Platero *et al.* 2006). Furthermore, the best storage temperatures for CFS resulted to be −20 and 4 °C. However, activity rapidly decayed at 25 and 37 °C. These results are similar to those obtained by Aktypis *et al.* (1998).

Antibacterial activity was slightly influenced by pH medium, being inversely proportional to pH. Unlike these results, Martín-Platero *et al.* (2006) and Sparo *et al.* (2006) did not observe modifications in antimicrobial activity in the pH range from 4.0 to 8.0.

Activity of CFS was completely inactivated by trypsin action, whereas other proteases, such as proteinase K, partially neutralised antibacterial activity. Besides, antagonistic activity was not attributed to hydrogen peroxide because the diameter of the inhibition zones remained unaltered. Furthermore, activity was partially affected by lysozyme and lipase action. These results indicate that CFS antibacterial activity is not exclusively attributed to proteic compounds. The nonproteic substances could exert their inhibitory action independently or in association with peptides and proteins and complementing their action. Foulquié Moreno *et al.* (2003) obtained similar behaviours from several CFS from *Enterococcus* strains using the same enzymes, except for lipase and lysozyme, for which inhibitory activity of CFSs did not result affected.

After CFS ultrafiltration, two active fractions were obtained. Major activity was achieved in the filtrate fraction. The residual activity found in the retentate fraction may be caused by aggregation of the peptides that prevents them from crossing the membrane. This phenomenon has been described by many authors and is particularly common in low-molecular-weight bacteriocins. For example, Gálvez *et al.* (1998) isolated a bacteriocin named enterocin EJ97 whose structure has hydrophobic zones that play an important role in the biological activity of the molecule and in its trend to form aggregates in aqueous solutions. Besides, Aktypis *et al.* (1998) found a decrease in the activity of termophilin T after each purification step and attributed this finding to the ability to form aggregates, either by self-association or by interaction with other components. Martín-Platero *et al.* (2006) and Yamamoto *et al.* (2003) reported similar results. According to the above, we estimate that the MW of CFS antibacterial peptidic compound is lower than 3000 Da.

The results have shown that CFS action is bactericidal and lytic. This is in agreement with several previous reports (Lauková *et al.* 1993; Susani *et al.* 1995). Sparo *et al.* (2006) also found similar bactericidal and lytic effects from an *Enterococcus* strain CFS against a strain of *L. monocytogenes*, while our CFS barely inhibited the assayed *L. monocytogenes* strain.

Moreover, CFS antagonistic effect was stronger against Gram +ve bacterial cells than against Gram (−) bacteria. However, according to our bibliographic knowledge, CFS is the only reported one, which exerts a lytic and bactericidal action towards *E. coli*. Furthermore, only enterocin AS-48 has been described as a bacteriocin with inhibitory activity against *E. coli*, but its mode of action was not assayed (Gálvez *et al.* 1989).

After addition of several surfactants to CFS, inhibitory activity increased twofold relative to nontreated CFS. Other detergents caused inhibition by themselves in such a way that the increase or decrease in antagonistic activity was a consequence of combined CFS specific and surfactant unspecific effects. Muriana and Klaenhammer (1991) observed an increase in the antimicrobial activity after treatment with different surfactants, and Sparo *et al.* (2006) confirmed an inverse effect on the activity generated after treatment with the same detergents. This could be supported in the way that surfactant addition to CFS promotes the disaggregation of bacteriocins complexes, so a larger number of bacteriocin units are available, which indeed increase the CFS antimicrobial title.

CONCLUSIONS

Enzymatic treatments revealed that inhibitory substances in CFS obtained from *E. faecalis* DBFIQ E24 cultures are integrated mainly by peptides or proteins. Cell-free supernatant has a broad and heterogeneous inhibitory range, particularly against food-borne pathogenic bacteria, yeasts and moulds, suggesting that the addition of CFS or the strain itself to certain foods may be advantageous for their biopreservation. Besides, CFS has a bactericide and bacteriolytic action against Gram +ve and Gram (−) bacteria.

It has been proved that the addition of certain surfactants improves CFS antimicrobial activity, probably favouring the disaggregation of the bacteriocin complexes. Furthermore, it is worth saying that the behaviour of each bacteriocin against detergents is unique and strain dependent, explaining the great difference in the results observed in the bibliography.

The inhibitory activity of CFS against *E. coli* Ec9 strain would convert into one of the few CFS produced by LAB strains, which expresses antibacterial activity against these Gram (−) bacteria, increasing its technological interest.

Studies related to purification of the different antimicrobial compounds are being accomplished to fully characterise the inhibitory substances, according to their own antagonistic activity.

Bearing in mind that each food is a real and particular ecosystem where life of microbial colonies is ruled out by great complexity phenomena, *E. faecalis* DBFIQ E24 strain would be very adequate as a starter playing its technological role in the maturation of certain fermented foods. Therefore, its use as a 'natural' food biopreservative shows a promissory future.

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