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Short communication

Combination of bioprotective cultures with EDTA to reduce *Escherichia coli* O157:H7 in frozen ground-beef patties

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ABSTRACT

The effectiveness of bacteriocin-producing *Lactobacillus curvatus* CRL705 and *Lactococcus lactis* CRL1109 in combination with Na_2EDTA on frozen ground-beef patties contaminated with *Escherichia coli* O157:H7, was investigated under temperature abuse conditions (5 °C during 9 days). The presence of the bioprotective cultures (ca. 10^7 CFU/g) and chelator (48 mM) resulted in one log CFU/g reduction for *E. coli* strain, compared to the control on day 0. Similarly, a significant decline for indigenous coliforms in ground-beef patties was also observed in the presence of bacteriocinogenic strains and chelator. However, in the absence of Na_2EDTA, neither *E. coli* nor coliforms were inhibited by the bioprotective cultures, the pathogen reaching similar counts than control samples (5.22 and 3.60 log CFU/g, respectively) at 9 days. When the growth of bacteriocinogenic strains on patties was evaluated, they were able to increase their population producing bacteriocins after 48 h up to the end of incubation period while a near neutral pH in the presence of Na_2EDTA was detected. Non substantial effect on of ground-beef patties color was produced in the presence of bioprotective cultures, while a darker color developed in those added with the chelator. The simultaneous treatment with bioprotective cultures and Na_2EDTA may be of value for the control of *E. coli* O157:H7 in temperature abused ground-beef patties.

1. Introduction

Foodborne illness outbreaks and ground-beef recalls caused by *Escherichia coli* O157:H7 contamination continues to be a major concern to consumers, regulatory authorities, and food industry. In Argentina, the hemolytic-uremic syndrome (HUS) caused by this microorganism is endemic and approximately 400 new cases are reported each year by hospital nephrology units. The high incidence rate of HUS in Argentina (Padola et al., 2004) is closely related with the high consumption of bovine meat per inhabitant (>69 kg/year) (<http://www.inta.gov.ar/balcarce/Carnes/sitgan.htm>; accessed 7/03/2010). A quantitative risk assessment for verocytotoxigenic *E. coli* in ground-beef patties in Argentina indicated that the most important factors associated with infection and HUS were the pathogen concentration in the hides of cattle destined to slaughter, the temperature during meat processing and thermal abuse during storage and food preparation (Signorini & Tarabla, 2009).

Methods to substantially reduce or inhibit Gram-negative bacteria by foodgrade compounds are of most interest to the food industry. Biocontrol based on bacteriocinogenic lactic acid bacteria (LAB) has been proposed as a promising tool to ensure the hygienic quality of foods. LAB have a major potential for use in biopreservation because they are safe to consume (GRAS status) and during storage they naturally dominate the microbiota of many foods. During the last 30 years knowledge of LAB antimicrobial peptides has dramatically improved; however their application is limited mainly due to restrictive legislation concerning food additives. Since nisin is the only LAB bacteriocin approved to be used in foods, an alternative to introduce bacteriocins in food is the use of live LAB that produce bacteriocins *in situ* in the food. Bioprotective cultures offer a wide spectrum of potential applications against pathogenic and spoilage bacteria in foods (Chen & Hoover, 2003; Gálvez, Abriouel, Lucas López, & Ben Omar, 2007). Selected LAB protective cultures demonstrated the ability to control *Listeria monocytogenes* in different meat systems, in which this pathogen constitutes a frequent post-processing contaminating agent (Castellano, Belfiore, Fadda, & Vignolo, 2008; Castellano, González, Carduza, & Vignolo, 2010). LAB can also be applied for the inactivation of Gram-negative pathogens on foods in combination with other hurdles or treatments to induce cell damage and partial disorganization of the outer membrane protective layer (Gálvez, Abriouel, Lucas López, Valdivia, & Ben Omar, 2008). Treatments with chelating agents as ethylene-diaminetetraacetate (EDTA)

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generally results in removal by chelation of divalent cations from the lipopolysaccharide layer in the protective outer membrane of Gram-negative cells, making them sensitive to hydrophobic peptides such as bacteriocins (Alakomi, Saarela, & Helander, 2003; Hancock & Rozek, 2002). The enhanced effect of chelators such as EDTA and LAB bacteriocins against Gram-negative bacteria has been reported for nisin, both under laboratory conditions and in foods (Belfiore, Castellano, & Vignolo, 2007; Boziaris & Adams, 1999; Cutter & Siragusa, 1995; Fang & Tsai, 2003). On the other hand, EDTA has been approved by FDA as a food additive preventing oxidation of meat products that is generally recognized as safe (US Code of Federal Regulations, http://cfr.vlex.com/vid/172-135-disodium-edta-1970780521). In a previous work, the inhibitory activity of the bacteriocins produced by *Lactobacillus curvatus* CRL705 and *Lactococcus lactis* CRL1109 in combination with chelating agents against *E. coli* strains at low temperature in TSB medium was demonstrated (Belfiore et al., 2007). This study was undertaken to determine whether both bacteriocinogenic strains in conbination with EDTA would be effective to protect frozen ground-beef patties from *E. coli* O157:H7 growth during temperature abuse conditions.

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. curvatus CRL705 and *Lc. lactis* CRL1109 from CERELA culture collection were propagated (24 h at 30 °C) in MRS and LAPTg broth (Raibaud, Galpin, Duclezeau, Mocquot, & Oliver, 1963), respectively. *E. coli* NCTC12900 (ATCC700728), a non-toxigenic O157:H7 strain that has the shiga-like toxin genes deleted was cultivated in BHI broth at 37 °C. *Listeria innocua* 7 (Unité de Recherches Laitieres et Genetique Appliqué, INRA, France) used as indicator organism was grown at 30 °C in TSB with 0.5% added yeast extract. All the microbiological media used were supplied by Britania (Argentina), unless otherwise stated.

2.2. Preparation of ground-beef patties and experimental design

Six loin-ball (*quadriceps femoris* muscles; right and left selected at random) were obtained from a local retail supermarket. To minimize the initial numbers of resident microbiota, 3 mm of the beef surface were aseptically trimmed off. The lean beef was aseptically ground in a manual meat grinder with a plate having 100-mm diameter multiple perforations (model N°22, Bs As, Argentina) and mixed with NaCl (1.7% w/w), sodium erythorbate (0.02% w/w), monosodium glutamate (0.07% w/w) and sodium tripolyphosphate (0.01% w/w). All ingredients were purchased from Sigma Chemical CO (St Louis, MO, USA). *E. coli* O157:H7 and bioprotective cultures were appropriately diluted in sterile peptone water (0.1% w/v), and added according to a complete factorial experimental design (Table 1) to obtain a final concentration of ca. 10^2 and 10^7 CFU/g of ground-beef, respectively. Disodium EDTA solution was added to ground-beef mass at 18 g/kg (ca. 48 mM). The

Table 1
Experimental design[a] used for *E. coli* O157:H7 challenge by bioprotective cultures with or without Na_2EDTA[b] in frozen ground-beef patties (24 h at −12 °C) subjected to temperature abuse conditions (5 °C during 9 days).

Treatments	*E. coli* O157:H7 (~10^3 CFU/g)	Bioprotective cultures (~10^7 CFU/g)	Na_2EDTA
1	−	−	−
2	−	+	−
3	+	−	−
4	+	+	−
5	−	−	+
6	−	+	+
7	+	−	+
8	+	+	+

[a] Controls (1, uninoculated; 3,inoculated) and treatments (2, 4, 5, 6, 7 and 8) were performed in duplicate.
[b] Na_2EDTA (18 g/kg of beef).

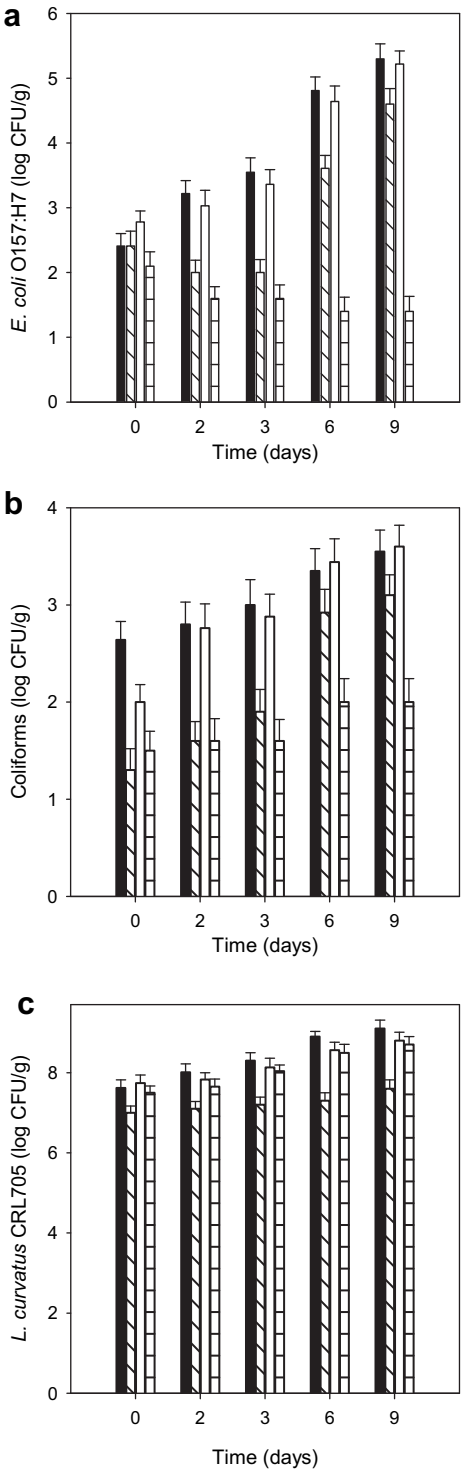

Fig. 1. Growth of *E. coli* O157:H7 (a), coliforms (b) and *L. curvatus* CRL705 (c) in ground-beef patties stored at 5 °C for 9 days. (■) controls; (▨) + Na_2EDTA; (□) + bioprotective cultures (a,b)/*E. coli* O157:H7 (c); (▤) + Na_2EDTA + bioprotective cultures (a,b)/*E. coli* O157:H7+ Na_2EDTA (c).

Table 2
pH values and antimicrobial activity of frozen ground-beef patties (24 h at −12 °C) subjected to temperature abuse conditions (5 °C during 9 days).

Treatment/Day	pH[a]					Antimicrobial activity[b]				
	0	2	3	6	9	0	2	3	6	9
1	5.59 ± 0.15a	5.56 ± 0.19a	5.34 ± 0.17a	5.43 ± 0.18a	5.48 ± 0.19a	−	−	−	−	−
2	5.51 ± 0.18a	5.47 ± 0.20a	5.41 ± 0.22a	5.52 ± 0.19a	5.53 ± 0.21a	−	+	++	++	++
3	5.44 ± 0.14a	5.57 ± 0.17a	5.53 ± 0.19a	5.51 ± 0.20a	5.55 ± 0.20a	−	−	−	−	−
4	5.59 ± 0.14a	5.58 ± 0.16a	5.49 ± 0.18a	5.52 ± 0.21a	5.54 ± 0.22a	−	+	++	++	++
5	6.20 ± 0.19bc	6.22 ± 0.21bc	6.10 ± 0.14bc	6.16 ± 0.15bc	6.21 ± 0.17bc	−	−	−	−	−
6	6.00 ± 0.20bc	5.96 ± 0.19b	6.12 ± 0.18bc	6.14 ± 0.18bc	6.18 ± 0.18bc	−	+	+	+	+
7	6.25 ± 0.18c	6.26 ± 0.17c	6.15 ± 0.17bc	6.14 ± 0.17bc	6.20 ± 0.19bc	−	−	−	−	−
8	6.28 ± 0.17c	6.15 ± 0.20bc	6.14 ± 0.18bc	6.13 ± 0.16bc	6.22 ± 0.24bc	−	+	+	+	+

[a] Mean ± standard deviation; means with the same letters (a, b, c) are not significantly different ($P < 0.05$).
[b] against *Listeria innocua* 7; - no inhibition, + and ++ inhibition halos <5 and >5 mm, respectively.

ground-beef patties (100 g) were aseptically hand formed, placed into individual packing films (Polyvinyl chloride 20 m of thickness) and stored at −12 °C for 24 h followed by a storage at 5 °C for 9 days.

2.3. Microbiological analysis

A 25 g sample from each experimental case was aseptically weighed into a sterile stomacher bag, diluted with 225 ml of sterile saline solution (NaCl 0.85%) and homogenized for 1 min with a Stomacher 400 system (Seward Laboratory, London, UK). Decimal dilutions were prepared in the same diluent, and the following analyses were carried out: *E. coli* O157:H7 on Sorbitol MacConkey agar (48 h at 37 °C); coliforms on MacConkey agar (48 h at 37 °C); *L. curvatus* CRL705 on MRS agar + 200 μg/ml of spectinomycin (48 h at 30 °C) and *Lc. lactis* CRL1109 on LAPTg agar. All the microbiological media used were supplied by Britania (Argentina). The antibiotic spectinomycin was purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.4. Antimicrobial activity, pH and color measurements

A semiquantitative diffusion assay was used to determine *L. curvatus* CRL705 and *Lc. lactis* CRL1109 bacteriocins activity (Castellano et al., 2010). Five μl of each homogenate obtained as described above was spot placed in a semi-solid TSB plates overlay inoculated with *L. innocua* 7 and positive bacteriocin activity was evidenced as an inhibition zone on the indicator organism lawn. For pH measurement a Metrohn 692 pH/ion meter was applied on the meat homogenates. The surface color (L [lightnes], *a*, *b*) of ground-beef patties was measured using a Minolta colorimeter (Chroma meter CR-300). The L, *a* and *b*-values are given as the average value of six determinations and three measurements on each surface.

2.5. Statistical analysis

Two separate replications of each experiment were carried out. The microbial counts and pH values were analyzed using Tukey post-test for multiple comparisons. Principal component analysis (PCA) was used for samples differentiation based on the color changes produced by treatments. PCA was done by means of multivariate data analysis software (Infostat Versión 2010; Universidad Nacional de Córdoba, Argentina).

3. Results and discussion

The combined effect of bioprotective cultures and EDTA on the control of *E. coli* O157:H7 in frozen ground-beef patties under temperature abuse is shown in Fig. 1a. Pre-incubation at −12 °C for 24 h prior to storage at 5 °C did not produce significant changes in the analyzed bacterial counts (data not shown). The enumeration of the pathogen showed the population to increase from 2.41 on day 0 to 5.30 log CFU/g on day 9 at 5 °C. Growth of *E. coli* O157:H7 during the storage of beef patties showed to be higher compared to those previously reported for ground-beef (Mann & Brashears, 2006; Ruby & Ingham, 2009; Smith, Mann, Harris, Miller, & Brashears, 2005). Differences in pathogen cell counts may be assigned to the slight selectivity of the enumeration media used, the previous decontamination of beef used for patties production as well as to the adaptation of pathogen strain during pre-incubation of inoculated ground-beef patties before refrigerated storage. In presence of Na_2EDTA, although a significant reduction was observed until day 3, on day 9 an increase of 2.19 log CFU/g occurred as compared with the inoculated samples on day 0. On the contrary, when Na_2EDTA was combined with the bioprotective cultures significant population reductions, 1.62, 1.95, 3.41 and 3.90 log cycles compared to the control ($P \leq 0.05$), were observed from day 2, 3, 6 and 9, respectively. When the effect of the different treatments against the naturally present coliforms population was analyzed, no significant difference between the uninoculated control and bioprotective cultures-treated ground-beef patties were observed during the 9 days of storage (Fig. 1b). Similarly to *E. coli* O157:H7, when compared to the uninoculated control, a reduction of 1.55 log cycles on indigenous coliforms on day 9 was produced when Na_2EDTA was combined with the bioprotective cultures. These results are in agreement with those reported in a previous study, in which EDTA in combination with the bacteriocins produced by *L. curvatus* CRL705 and *Lc. lactis* CRL1109, demonstrated to be an effective *in vitro* strategy for *E. coli* O157:H7 inhibition (Belfiore et al., 2007). In addition, Branen and Davidson (2004) reported various combinations of nisin, lysozyme and monolaurin with EDTA to be bactericidal against Gram-negative bacteria in TSB medium, whereas none of the antimicrobials alone showed inhibitory activity. In coincidence with our results, *in situ* produced nisin combined with EDTA was reported to effectively inactivate a cocktail of *E. coli*, *Pseudomonas aeruginosa* and salmonellae in a model substrate (Boziaris & Adams, 1999). When the growth of *L. curvatus* CRL705 was evaluated in the ground-beef patties, it showed to increase its population from 7.62 to 9.10 log CFU/g during the storage period at 5 °C (Fig. 1c). In presence of Na_2EDTA, a significant *L. curvatus* CRL705 population reduction occurred, while this effect was not observed in presence of *E. coli* O157:H7. Similarly, *Lc. lactis* CRL1109 grew by 1.90 CFU/g during storage, its population also being affected by the presence of EDTA (data not shown). This inhibitory effect may be assigned to the chelating activity of Na_2EDTA that binds trace metals valuable for fastidious LAB growth while in presence of *E. coli*; the chelator exhibits a higher affinity for outer membrane ions of Gram-negative strains than for substrate metallic trace elements (Martell & Smith. 1986).

When the pH of the ground-beef patties was measured, no significant differences were observed at 9 days of storage for

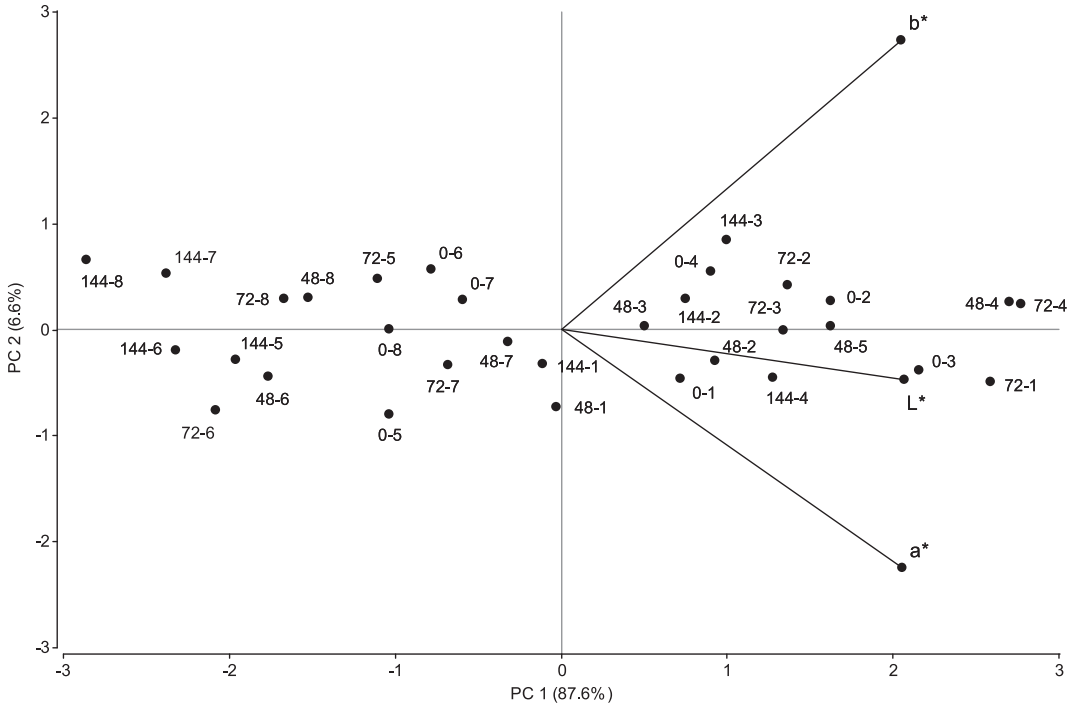

Fig. 2. Factorial biplot defined by the principal components 1 and 2 (PC1: 87.6%; PC2: 6.6%) resulting from the PCA performed on color variables (CIE a*, b* and L*) for ground-beef patties different treatments at 0, 48, 72 and 144 h.

control samples as well as for those inoculated with bioprotective cultures (Table 2). However, when Na_2EDTA or its combination with bioprotective cultures was added to patties, pH values resulted significantly higher than those from samples without chelator, these results being in coincidence with those reported on vacuum-packaged fresh beef treated with EDTA or nisin combined with EDTA during storage at 4 °C for 30 days (Tu & Mustapha, 2002; Zhang & Mustapha, 1999). The higher pH in presence of Na_2EDTA may be due to the suppression of acid-producing bacteria and the chemical dissociation of the chelator. When antimicrobial activity was evaluated (Table 2), bacteriocin activity against the indicator organism in ground-beef patties was detected after 48 h of cold storage in presence of bioprotective cultures alone or combined with Na_2EDTA; this remained stable throughout the storage period. This result is in agreement with previous studies (Castellano & Vignolo. 2006; Castellano, Holzapfel, & Vignolo. 2004), in which the biopreservative culture *L. curvatus* CRL705 inoculated in a meat slurry and chilled meat discs was able to produce bacteriocins during 21 and 36 days, respectively.

In order to visualize the influence of treatments on ground-beef patties color, a multivariate analysis technique such as PCA was applied (Fig. 2). PCA allowed to clearly differentiate Na_2EDTA treated samples which presented a darker color when compared to the control, while no significant difference in color (CIE a*, b* and L* values) among treatments was found in the presence of bioprotective cultures during 6 days of storage at 5 °C. There was no difference between 6 and 9 days of storage in the color developed for all samples (data not shown). Conversely, when the effect of this chelator was assayed to inhibit the pink color development in cooked and uncured turkey breast, no significant changes occurred in color units, particularly in CIE a* values that measure redness in meat (Schwarz et al., 1998). Nevertheless, in this study a role in color changes may be assigned to the high EDTA amount used, which was near 20 times higher.

In conclusion, the results obtained confirm that bacteriocinogenic *L. curvatus* CRL705 and *Lc. lactis* CRL1109 combined with Na_2EDTA will provide a significant reduction of *E. coli* O157:H7 growth in ground-beef patties submitted to temperature abuse during storage. Even when further studies must be done to evaluate sensory acceptance due to color changes, the development of a strategy preventing the growth of this pathogen in this massively consumed meat product will strongly impact on food safety.

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