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Research Paper

Bio-preservation of ground beef meat by *Enterococcus faecalis* CECT7121

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Abstract

Meat and particularly ground beef is frequently associated with Food Poisoning episodes and breeches in Food Safety. The main goal of this research was to evaluate the bactericide effect of the probiotic *Enterococcus faecalis* CECT7121, against different pathogens as: *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Clostridium perfringens* and *Listeria monocytogenes,* inoculated in ground beef meat. Three studies were performed to evaluate the inhibition of *E. faecalis* CECT7121 on ground beef meat samples inoculated with pathogens: *Study I:* Samples (100 g meat) were inoculated with pathogens (10^3 CFU/g)) and *E. faecalis* CECT7121 (10^4 CFU/g) simultaneously. *Study II:* Samples were inoculated with *E. faecalis* CECT7121 24 h before the pathogens. *Study III: E. faecalis* CECT7121were inoculated 24 h after pathogens. The viable counts were performed at 0, 24, 48 and 72 h post-inoculation. The simultaneous inoculation of *E. faecalis* CECT7121 with *E. coli* O157:H7 strains resulted in the absence of viable counts of bacteria at 72 h post-treatment. However, when the probiotic was added 24 h before and 24 h after the pathogen *E. coli* O157:H7, viable cells were not detected at 24 h and 48 h post-treatment, respectively. Consistently, neither *S. aureus* nor *Cl. perfringens* viable bacteria were detected at 48 h in whole assays when inoculated with *E. faecalis* CECT7121. The same trend than described before was obtained after applying the 3 models assayed for *L. monocytogenes*. The current assays demonstrated the bactericide activity of *E. faecalis* CECT7121 strain on bacterial pathogens in ground beef meat.

Key words: bio-preservation, probiotic, ground beef meat, *Enterococcus faecalis* CECT7121.

Introduction

Meat and particularly ground beef is frequently associated with Food Poisoning episodes and breeches in Food Safety. A recent Food Safety review by Newell *et al.* (2010) indicates that the microbiological safety of food remains a dynamic situation heavily influenced by multiple factors along the food chain from farm to fork. In addition, the pathogen populations relevant to food safety are not static, and meat is an excellent vehicle by which many pathogens (bacteria, viruses/prions and parasites) can reach an appropriate colonization site in a new host.

Among the emerging foodborne pathogens of the last 20 years, Shiga toxin-producing *Escherichia coli* (STEC) has found in Argentina a favourable and permissive ecosystem to express it virulence. Although *E. coli* is considered a food transmitted pathogen of the industrialized nations, this STEC O157:H7 serotype is the main responsible to cause the Haemolytic Uremic Syndrome (HUS) in young children and also responsible for the majority of HUS caused by STEC in Argentina (Rivas *et al.*, 2006).

The clinical syndrome caused by STEC can go from hemorrhagic colitis, to Thrombotic Thrombocytopenic Purpura (TTP) and Haemolytic Uremic Syndrome (HUS). The HUS is an endemic disease in Argentina with an increased number of reported cases during the spring and summer months (Mercado, 2007). Argentina reports 300

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HUS cases per year with an incidence of 12.5 positive cases per 100.000 children under the age of five years (Gianantonio *et al.*, 1973; López *et al.*, 1997; Rivas *et al.*, 1996). The virulence profile of STEC isolated from burgers and ground beef coincides with it prevalence in cattle (Parma *et al.*, 2000).

Other pathogenic bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Clostridium perfringens* are recognised as the main responsible agents for severe infections and toxicity to humans.

S. aureus is a leading cause of gastroenteritis resulting from the consumption of contaminated food. *S. aureus* is indeed found in the nostrils, and on the skin and-hair of warm-blooded animals (von Eiff, 2001). *S. aureus* is able to grow in a wide range of temperatures, pH and sodium chloride concentrations. These characteristics enable *S. aureus* to grow in a wide variety of foods (Bergdoll, 1989; Schmitt *et al.*, 1990, Le Loir *et al.*, 2003).

Cl. perfringens is the second most common causal agent of foodborne diseases in the US, after *Salmonella* spp. The ingestion of large numbers of vegetative cells can lead to concomitant sporulation, enterotoxin release in the gastrointestinal tract, and diarrhoea-like illness (Brynestad and Granum, 2002; Le Loir *et al.*, 2003).

L. monocytogenes is a ubiquitous, intracellular pathogen which has been implicated as the causative organism in several outbreaks of foodborne disease. The genus *Listeria* has been described as an important contaminant in raw ground meat and the pathogenic specie *L. monocytogenes* is capable of surviving for long periods at 4 °C (Farber and Daley, 1994; Yücel *et al.*, 2005).

Among some of the specific interventions applied to ground meat, artificial chemical preservatives have been traditionally used to inhibit those microorganisms capable of growing within foods. The increasing consumer concerns of potential health risks associated with some of these substances has led researchers to examine the opportunity of using natural bacteriocin producer Lactic Acid Bacteria (LAB) as bio-preservatives (Amezquita and Brashears, 2002; Lui *et al.*, 2010).

Recently, Sparo *et al.* (2008) documented, in craft dry-fermented sausages, the inhibitory activity of the environmental strain *Enterococcus faecalis* CECT7121 against *S. aureus* and gram negative bacilli included in the *Enterobacteriaceae* family. Molecular evaluations of *E. faecalis* CECT7121 have proven it is safety, absence of virulence genes, antibiotic multiresistance, gut translocation and compatibility with food quality (Castro *et al.*, 2007; Sparo *et al.*, 2008). *E. faecalis* CECT7121 strain produces a new bacteriocin (enterocin), the peptide AP-CECT7121, with proven inhibitory activity *in vitro* on Gram positive multiresistent and Gram negative bacteria (Sparo *et al.*, 2008, 2009a, 2009b).

The main goal of the current study was to evaluate the inhibitory effect of *E. faecalis* CECT7121, on ground beef samples inoculated with pathogenic wild and collection strains across different experimental laboratory models.

Materials and Methods

Experimental strains

The Collection strains used in this work were: *Escherichia coli* O157 ATCC 700728, *Staphylococcus aureus* ATCC 25923, *Clostridium perfringens* ATCC 13124, *Listeria monocytogenes* ATCC 19115, *Enterococcus faecalis* ATCC 29212 and *E. faecalis* CECT7121 (biological preserver, BP).

The Wild strains isolated at our laboratory were: *E. coli* CEB1146, *E. coli* CEB1320, *E. coli* CEB1192, *E. coli* CEB1123, *S. aureus* CEB1412, *S. aureus* CEB153, *S. aureus* CEB1308, *Cl. perfringens* CEB1041, *Cl. perfringens* CEB1012, *Cl. perfringens* CEB1074, *L. monocytogenes* CEB123, *L. monocytogenes* CEB218 and *L. monocytogenes* CEB247; from ground beef meat acquired from different butchers. However, *E. faecalis* MR99 and *E. faecalis* MR1024, were isolated from corn silage.

Selection criteria of bovine meat cuts

Meat samples were obtained from the meat cut known as Round roll / Eye of round and from a meat shop of known high hygiene standards. In order to minimize any potential external contamination of the samples, a superficial two centimetres deep layer of tissue was cut and removed under sterile laboratory conditions. The remaining central part of each sample was cut in cubes of 0.5 cm per side. The indigenous total viable cells count value for each sample was determined by the Most Probable Number (MPN) method. Ten grams of ground meat samples were placed in sterile plastic bags, and a solution of peptone in water was added until reaching a final 1/10 dilution. The sample was then homogenized in a Stomacher 400 (Seward *Laboratory Systems* Inc., US) by 3 min at 230 g. Sub-samples obtained from the meat homogenate were further diluted by triplicate and inoculated into test tubes containing a 2% of green lactose bile bright broth. These samples were incubated at 37 °C by 48 h. Those samples that showed gas formation where confirmed as coliforms following further culturing in violet red bile agar (Allaert and Escola, 2002).

E. faecalis CECT7121 culture preparation and strain identification

An 18 h culture of brain heart infusion broth (BHI, Lab. Britania, Argentina) was centrifuged at 3.000 g for 20 min; the bottom pellet was washed twice with phosphate buffer 74 mM at pH 7 and resuspended in 10 mL of the same buffer.

This 10 mL aliquot was kept for inoculation of meat samples. Each experimental sample was inoculated with a final concentration of 10^4 CFU/g of meat.

The BP strain count was performed using a bile esculin azide agar (Lab. Britania, Argentina) incubated at 35 °C for 48 h.

The specie phenotypic characterization was determined by sampling 10 colonies at random according Sparo and Mallo (2001) and Sparo *et al.* (2008). The BP identity was performed by RAPD-PCR (polymerase chain reaction using random amplified polymorphic DNA) analysing the differences between genomes of *E. faecalis* strains, following the protocols described by Suzzi *et al.* (2000) and Sparo *et al.* (2008). The control strains used in this assay were the *E. faecalis* CECT7121 used for ground beef meat samples inoculation (original strain deposited at the Colección Española de Cultivos Tipo (CECT), Universidad de Valencia, Spain), as well as the other strains mentioned above. Total DNA was extracted according to Persing *et al.* (1993). The primer used was D8635 (5' GAG CGG CCA AAG GGA GCA GAC 3'). The clonal relationship was assessed using the software RAPDistance 1.04 software package (Australian National University) and Mega 2.0 (Winer *et al.*, 1991; Zar, 1999).

Pathogenic bacteria culture preparation and phenotypic identification

Each strain of *E. coli* O157, *S. aureus* and *Listeria monocytogenes* was cultured for 18 h at 35 °C in BHI broth and *Cl. perfringens* was cultured in anaerobic conditions (Gas Pack System, Oxoid, UK) by 24 h at 35 °C in fluid thioglycolate medium with beef extract (Difco, US). Cultures were centrifuged for 20 min at 3.000 g. The sediments were washed twice with phosphate buffer 75 mM at pH 7 and resuspended. The assays were performed inoculating 100 g of ground beef meat with 10^5 CFU/ mL of each pathogen strain.

The viable *E. coli* and *L. monocytogenes*, counts were assessed using the CHROMagar™ O157 (BBL, US) and CHROMagar™ Listeria (BBL, US) respectively. For assessment of *S. aureus* viable counts the Agar Baird-Parker (Lab. Britania, Argentina) was used. Whole pathogens strains were incubated by 48 h, at 35 °C (*E. coli and S. aureus*) and at 30 °C (*L. monocytogenes*).

For determining *Cl. perfringens*, the meat samples were inoculated in test tubes adding Agar Sulphite Polymyxin Sulfadiazine (SPS) sealed with VAS-PAR and incubated at 35 °C for 48 h.

The phenotypic characterization of each strain was carried out by sampling 10 colonies randomly collected from the viability agar cultures following the conventional biochemist tests described by Winn *et al.* (2008).

Evaluation of *E. faecalis* CECT7121 pathogen inhibitory efficacy

Each strain of *E. coli* O157, *S. aureus* and *L. monocytogenes* was cultured for 18 h at 35 °C in BHI broth. However, *Cl. perfringens* was cultured in anaerobic conditions (Gas Pack System, Oxoid, UK) by 24 h at 35 °C in fluid thioglycolate medium with beef extract (Difco, US). Cultures were centrifuged for 20 min at 3.000 *g*. The sediments were washed twice with phosphate buffer 75 mM at pH 7 and resuspended. The assays were performed inoculating 100 g of ground beef meat with 10% v/p of the inoculums of each bacterium at a final concentration of 10^3 CFU.g^{-1}, allowing the spread and absorption of bacteria to the meat. Upon the excess of liquid was removed. The BP was added in the same way, at a concentration of 10^4 CFU.g^{-1}, following the three different experimental assays.

Study I: Samples were inoculated with pathogens and the BP simultaneously.

Study II: Samples were inoculated with the BP 24 h before the pathogenic bacteria addition.

Study III: Each pathogenic strain was inoculated 24 h before the BP addition.

Each and every group had a non inoculated sample as control. The bacterial counts were performed at 24, 48 and 72 h. Each study was performed twice by triplicate.

Results

The simultaneous inoculation of BP with *E. coli* O157 strains showed not detection of viable cells upon 72 h post-treatment. However, viable bacteria were not detected at 24 and 48 h when the BP, was added 24 h before and 24 h after *E. coli* strains, respectively (Figure 1).

The inoculation of BP simultaneously with *S. aureus* strains, 24 h before or 24 h after the incorporation of the strains resulted in the not detection of viable cells at 48 h in whole applied models (Table 1).

The same trend than described above was obtained for *Cl. perfringens* (see Table 2).

L. monocytogenes has shown to be the more sensitive to the inhibitory activity of the BP. Viable *Listeria* cells were not detected before 24 h after incubation on the three experimental models assayed. (Table 3).

Also it was observed a decrease at time zero on the implantation of the pathogen bacteria in ground beef meat (circa 1 Log) when the BP were inoculated 24 h before, compared with those sample where the BP was not used (Figure 1, Table 1, Table 2, Table 3). For example, in *E. coli* O157 at time zero the viable count without BP was 3.2 x 10^3 CFU/g and with BP previously inoculated was 2.4 x 10^2 CFU/g. The same trend occurred to other pathogens.

Detection of *E. faecalis* CECT7121

The incubation of *E. faecalis* CECT7121 in ground meat samples at 5 °C, have shown consistent and stable viable cell counts levels of 4 log up to 72 h after initial inoculation.

The identification and monitoring studies utilising the RAPD-PCR confirmed the presence of *E. faecalis* at each of the sampling times at 0, 24, 48 and 72 h from inoculation, while no BP was detected in the control samples.

The results obtained on the experiment when the ground beef meat was simultaneously inoculated with *E. coli* ATCC 700728 and the BP, illustrated Figure 2, shows the RAPD-PCR profiles of *E. faecalis* CECT7121 (BP) inoculated (Lane 3) compared with other *E. faecalis* strains isolated in the samples at different times. Lanes 3, 4 and 5 shows a group of profiles sharing a 90% similarity considered as identical profiles. Other non *E. faecalis* related isolates, used as controls, such as *E. faecalis* ATCC 29212, *E. faecalis* MR99 and *E. faecalis* MR1024 showed different RAPD-PCR profiles.

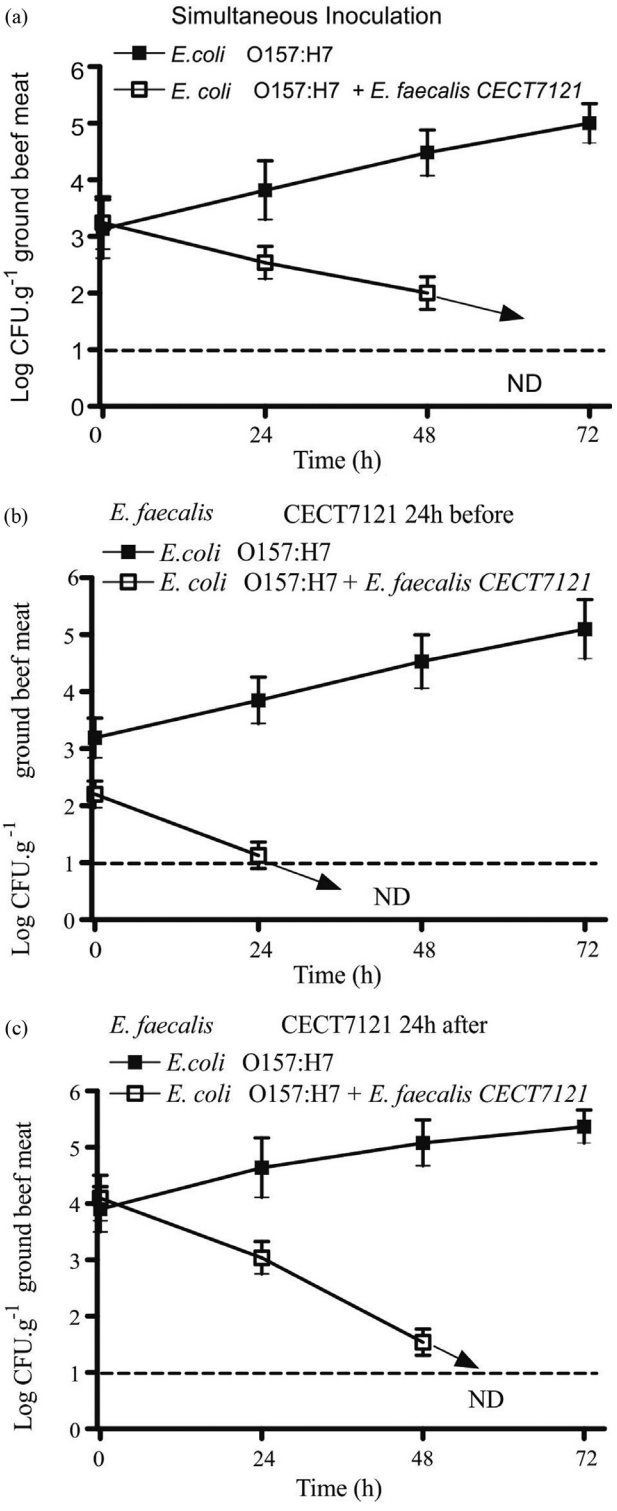

Figure 1 - Mean count of strains viable cells *vs.* time of *E. coli* O157:H7 when *E. faecalis* CECT7121 (10^4 CFU/g) was inoculated (a) simultaneously with the pathogen, (b) 24 h before the pathogen and (c) 24 h after the pathogen. Each curve represents the average of 5 analysed strains. The viable cells count for each strain is the average count of 3 experiments with 2 repetitions. ND = Not detected.

Discussion

It is widely accepted that *E. coli* 0157:H7 and non-O157 STEC represent the most serious foodborne Public Health risk and all possible pre and postharvest interventions must include these group of STEC pathogens. The utilization of different LAB probiotic strains on specific meat products is an interesting alternative in the prevention of ground meat contamination and pathogens development.

A number of successful experiences indicate the LAB inhibitory activity against *E. coli* 0157:H7 when incorporated directly to ground beef and various food products (Brashears *et al.*, 1996, 1997, 1998, 1999, 2003; Harris *et al.*, 2002). More recently, Smith *et al.* (2005) have reported the success of combining four LAB strains in the inhibition and postharvest control of *E. coli* 0157:H7 and *Salmonella* in ground beef kept at 5 °C for five days. This type of approach should allow the industry and health authorities to concentrate intervention efforts on specific high risk meat products under controlled conditions.

The current study has demonstrated that the presence of the probiotic strain *E. faecalis* CECT7121 in meat is able to inhibit substantially *E. coli* 0157:H7 to undetectable levels within 24 h of incubation (Figure 1). Similar inhibition results were obtained when *E. faecalis* CECT7121 was incorporated either simultaneously or 24 h after *E. coli* 0157:H7 inoculation, reaching non detectable values of viable counts at 72 and 48 h post inoculation respectively (Figure 1). However, when applied the same experimental models for *L. monocytogenes*, not viable cell counts were observed at 24h post-treatment, resulting in the total inhibition of the pathogens by *E. faecalis* CECT7121 (Table 3). The latter is considering pivotal in terms of foodborne disease prevention since *L. monocytogenes* is an emergent pathogen.

Interestingly, when the *E. faecalis* CECT7121 was inoculated 24 h before the implantation of *E. coli, Cl. perfringens and S. aureus,* the concentration of these bacteria was substantially lower in terms of implantation in ground beef meat samples at Time 0 (1 log). The later, would be relevant from the technological view, since the adherence or implantation of those pathogens may decrease when this probiotic strain is previously inoculated.

The current results from different model combinations show a broad spectrum inhibitory action over the pathogens assayed. Food poisoning from multiple origins is

Table 1 - Mean count of strains viable cells (CFU.g^{-1} ± Standard Deviation) *vs.* time of *S. aureus* when the probiotic *E. faecalis* CECT7121 (BP), was inoculated at 10^4 CFU/g, simultaneously with the pathogen , 24 h before the pathogen and 24 h after the pathogen. Each value represents the average of 5 analysed strains. The viable cells count for each strain is the average count of 3 experiments with 2 repetitions. ND = Not detected.

S.aureus						
	Simultaneous		24 h before		24 h after	
Time	Control	+ BP	Control	+ BP	Control	+ BP
0	2.37 ± 0.50	2.48 ± 0.40	3.31 ± 0.50	2.70 ± 0.50	4.17 ± 0.60	4.39 ± 0.70
24	3.12 ± 0.61	1.71 ± 0.40	3.42 ± 0.51	1.87 ± 0.40	4.64 ± 0.61	2.28 ± 0.50
48	3.73 ± 0.61	ND	3.67 ± 0.61	ND	5.19 ± 0.710	ND
72	4.15 ± 0.71	ND	3.98 ± 0.51	ND	5.63 ± 0.610	ND

Table 2 - Mean count of strains viable cells (CFU.g^{-1} ± Standard Deviation) *vs.* time of *Cl. perfringens* when the probiotic *E. faecalis* CECT7121 (BP) was inoculated at 10^4 CFU/g, simultaneously with the pathogen, 24 h before the pathogen and 24 h after the pathogen. Each value represents the average of 5 analysed strains. The viable cells count for each strain is the average count of 3 experiments with 2 repetitions. ND = Not detected.

Cl. perfringens						
	Simultaneous		24 h before		24 h after	
Time	Control	+ BP	Control	+ BP	Control	+ BP
0	3.51 ± 0.50	3.42 ± 0.60	3.69 ± 0.60	2.49 ± 0.40	4.16 ± 0.50	4.28 ± 0.60
24	4.39 ± 0.61	2.17 ± 0.400	4.32 ± 0.51	1.78 ± 0.40	4.59 ± 0.61	2.35 ± 0.40
48	4.87 ± 0.71	ND	4.86 ± 0.51	ND	5.07 ± 0.51	ND
72	5.14 ± 0.51	ND	5.31 ± 0.51	ND	5.49 ± 0.610	ND

Table 3 - Mean count of strains viable cells (CFU.g^{-1} ± Standard Deviation) *vs.* time of *L. monocytogenes* when the probiotic *E. faecalis* CECT7121 (BP) was inoculated at 10^4 CFU/g, simultaneously with the pathogen, 24 h before the pathogen and 24 h after the pathogen. Each value represents the average of 5 analysed strains. The viable cells count for each strain is the average count of 3 experiments with 2 repetitions. ND = Not detected.

L. monocytogenes						
	Simultaneous		24 h before		24 h after	
Time	Control	+ BP	Control	+ BP	Control	+ BP
0	3.74 ± 0.22	3.44 ± 0.22	3.64 ± 0.03	2.71 ± 0.10	3.64 ± 0.31	3.51 ± 0.10
24	4.35 ± 0.52	ND	4.05 ± 0.10	ND	4.05 ± 0.20	ND
48	4.64 ± 0.23	ND	4.51 ± 0.25	ND	4.51 ± 0.10	ND
72	4.73 ± 0.19	ND	4.86 ± 0.32	ND	4.86 ± 0.30	ND

a serious Public Health problem often underestimated due to lack of reporting.

The *E. faecalis* CECT7121 strain has shown to be capable of inhibiting the growth of *Cl. perfringens* and *S. aureus* in ground meat to undetectable levels at 24 h in all models assayed (Tables 1 and 2). This aspect of the study suggests the ability of *E. faecalis* CECT7121 to prevent secondary contaminations often associated to *Cl. perfringens* and *S. aureus* as described by Taormina and Dorsa (2004).

Among LAB experiences inhibiting *L. monocytogenes*, Skytta *et al.* (1991) have reported the results of three *Pediococcus* strains producing bacteriocin-like inhibitors in ground meat. The pathogens studied were *L. monocytogenes, Yersinia enterocolitica* and *Pseudomonas* spp. The inoculation levels of pediococci varied within a range of 10^3-10^8 CFU. g^{-1}, whereas the number of test organisms inoculated was approximately constant at 10^2 CFU/g. Aymerich *et al*. (2003) has also reported the use of enterococci as bio protective against *L. monocytogenes* in slightly fermented sausages, at concentrations of 10^4-10^5 CFU/g. Both above reports seem to have applied higher concentrations of LAB than the one used for *E. faecalis* CECT7121 at 10^4 CFU/g which resulted in the bactericidal effect of *L. monocytogenes* at 10^3 CFU CFU/g concentration (Table 3).

These current *E. faecalis* CECT7121 results are promising and may have a potential relevant impact in the safety and hygiene control of ground beef meat. These results correlate with previous studies reporting the ability of

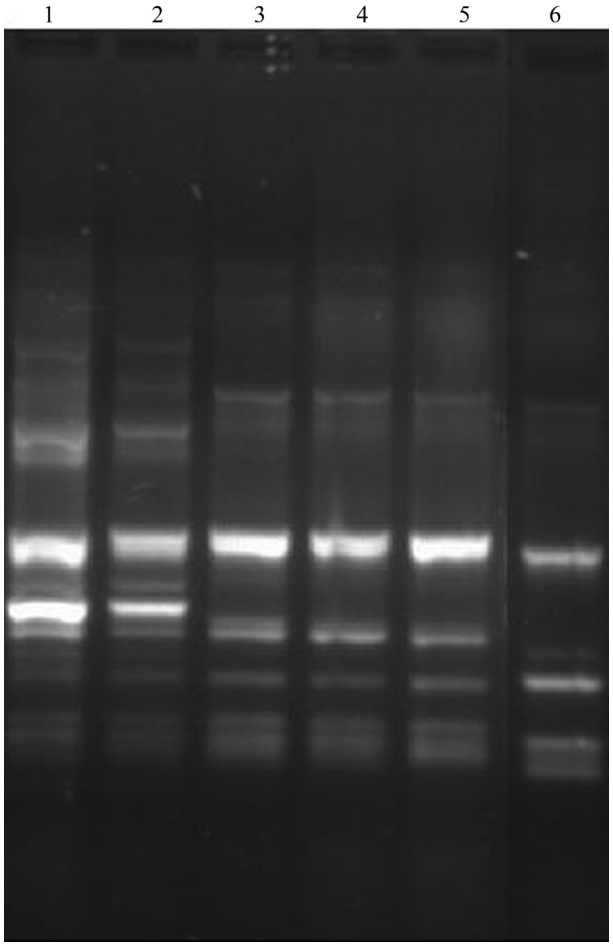

Figure 2 - Molecular typing of different isolations of *E. faecalis* from ground meat simultaneously inoculated with *E. coli* ATCC 700728 and *E. faecalis* CECT7121 by RAPD-PCR. Reference: Gel 1% agarose in buffer TBE 0.5X. Lane 1: *E. faecalis* MR99-1 (corn silage), Lane 2: *E. faecalis* MR1024-2 (corn silage), Lane 3: *E. faecalis* CECT 7121-3 (ground meat, T = 0), Lane 4: *E. faecalis* CECT 7121-4 (ground meat, T = 24 h), Lane 5: *E. faecalis* CECT 7121-5 (ground meat, T = 48 h), Lane 6: *E. faecalis* ATCC 29212-6.

E. faecalis CECT7121 to inhibit gram positive and gram negative strains in craft dry-fermented sausages; *in vitro* and *in vivo* mice *Salmonella* challenge studies (Castro *et al.*, 2007; Sparo *et al.*, 2008, 2009a, 2009b).

In conclusion, the current study has conclusively demonstrated the efficacy of the *E. faecalis* CECT7121 strain to inhibit the growth of pathogens in ground beef meat to undetectable levels achieving a significant bactericidal effect against *E. coli* O157:H7, *S. aureus, L. monocytogenes* and *Cl. perfringens in ground meat samples.*

The current biological model is capable to obtain the inhibition of the growth of pathogenic bacteria during incubation at 5 °C while maintaining *E. faecalis* CECT7121 at a consistent stable population. It must be noted that concentrations of bio-preserver strains in food over 10^4 CFU/g would not be allowed under existing food ingredients regulations.

Furthermore, the residual viability of *E. faecalis* CECT7121 in ground beef meat, allows the continuous protection of beef during storage preventing secondary contamination due to lack of hygiene or breakdown of the refrigeration chain. These results demonstrate that the *E. faecalis* CECT7121 strain is a viable and feasible post-harvest intervention for preserving the bio-security of ground beef meat.

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