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Article in African journal of microbiology research · February 2012
DOI: 10.5897/AJMR10.175

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Inhibition of *Listeria monocytogenes* and *Escherichia coli* by bacteriocin-producing *Lactobacillus plantarum* EC52 in a meat sausage model system

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Accepted 14 December, 2011

The plantaricin-producing *Lactobacillus plantarum* strain EC52 isolated from *poto-poto*, an ethnic maize fermented food, showed a good capacity to grow in a meat sausage model system. In meat mixtures, this strain caused strong acidification both in single culture and in coculture with inoculated pathogenic bacteria: *Listeria monocytogenes* and *Escherichia coli* O157:H7. *L. plantarum* EC52 had a strong inhibitory effect on *L. monocytogenes* in meat. The observed antagonism depended on the inoculum concentration of the pathogen, resulting in logarithmic reductions of up to 2.4 log cycles after 15 days of storage at 22°C. Strain EC52 was also able to inhibit growth of *Escherichia coli* O157:H7 for at least 6 days. These results suggest that strain EC52 could be added in meat mixtures such as those used in the manufacture of fermented sausages as a protective culture to reduce the levels of *L. monocytogenes* and to inhibit proliferation of *E. coli*.

**Key words:** Biocontrol, sausage model system, *Lactobacillus, Listeria, Escherichia.*

INTRODUCTION

Meat and meat products can act as vehicles for transmission of food borne pathogens (EFSA, 2007; Mataragas et al., 2008). According to the Community Summary Reports on Trends and Sources of Zoonoses in the EU (EFSA, 2007) the main foods contaminated with *Listeria monocytogenes* were meat products. *L. monocytogenes* can be found along the entire pork-processing chain (Nesbakken et al., 1996; Müller et al., 2009), and also able to survive sausage fermentation (Thevenot et al., 2005), and has frequently been recovered from the end product (Duffy et al., 2001; Gianfranceschi et al., 2006). In dry-fermented sausages, several other pathogens like *Escherichia coli* O157:H7, *Salmonella enterica*, or *Staphylococcus aureus* may also prevail (Leroy et al., 2006). Outbreaks of *E. coli* O157:H7 have been linked to raw meat contamination and survival of the bacterium through the fermentation and drying steps (Castano et al., 2002; Duffy et al., 2001).

The lactic acid bacteria are well-known for the capacity to produce a variety of inhibitors including metabolic end products such as organic acids, reuterin, reutericyclin, antifungal peptides and bacteriocins. The directed application of LAB strains and/or their antimicrobial products to inhibit unwanted bacteria in food was introduced in the concept of biopreservation (Stiles, 1996), and many recent reviews have addressed this issue extensively (Cleveland et al., 2001; Deegan et al., 2006; O’Sullivan et al., 2002; Ross et al., 2002).

Several bacteriocinogenic strains have been tested to control pathogenic and spoilage bacteria in meats (Gálvez et al., 2008; Hugas, 1998; Lücke, 2000). In fermented sausages, bacteriocinogenic LAB may be added as functional starter cultures to improve the product safety and quality (Leroy et al., 2006). Most
studies on application of bacteriocin producing strains as protective cultures in meat fermentation have focused on *L. sakei* and *L. curvatus* strains. Addition of *Lactobacillus sakei* (Drosinos et al., 2006; Leroy et al., 2005a, b, 2006; Ravýts et al., 2008) or *Lactobacillus curvatus* (Leroy et al., 2006) starter cultures may reduce *Listeria* levels in fermented sausages. *Lactobacillus plantarum* was useful as a bacteriocinogenic sausage starter culture (Campanini et al., 1993; Dicks et al., 2004). *Enterococcus faecium* PCD71 and *Lactobacillus fermentum* ACA-DC179 applied as protective cultures in chicken meat significantly reduced growth of *L. monocytogenes* and *Salmonella* respectively (Maragkoudakis et al., 2009). However, *L. plantarum* BFE 5092 had no inhibitory effect on *L. monocytogenes* when tested as a protective culture in turkey meat in spite of its capacity to express plantaricin genes in the meat system (Cho et al., 2010). Strains *L. sakei* CWBI-B1365 (sakacin P producer) and *L. curvatus* CWBI-B28 (sakacin G producer) inhibited the growth of *L. monocytogenes* in raw beef individually, and also in chicken meat when inoculated together (Dortu et al., 2008). Lamb meat inoculated with the Sakacin-A producer *L. sakei* Lb706 had lower *L. monocytogenes* counts (Jones et al., 2009). Although *L. plantarum* bacteriocin producers have also been isolated from meat products (Enan et al., 1996; Messi et al., 2001; Müller et al., 2009; Phumkhachorn et al., 2007; Rekhif et al., 1995), only a few studies have addressed their inhibitory effects in meat systems. *L. plantarum* strains producing an uncharacterised bacteriocin (Campanini et al., 1993), bacteriocin 423 (Dicks et al., 2004) or bacteriocin UG1 (Enan et al., 1996) showed strong inhibitory effects against *L. monocytogenes* in salami or meats.

Strain *L. plantarum* EC52 was isolated from poto poto, a Congolese fermented maize (Ben Omar et al., 2008). This strain shows a broad spectrum of antibacterial activity (including *Bacillus cereus*, *S. aureus*, *L. monocytogenes*, *S. enterica*, *E. coli*, and *Enterobacter aerogenes*) and carries several plantaricin genes (Ben Omar et al., 2008) of the *pln* locus described initially in *L. plantarum* strain C11 and widely disseminated among other *L. plantarum* strains (Diep et al., 2009). Most of those strains were isolated from vegetable foods, but none has been tested so far in meat systems. Strain *L. plantarum* EC52 is interesting as a starter or protective culture for fermented foods. The purpose of the present study was to determine the antagonistic capacity of this strain against two foodborne pathogenic bacteria (*L. monocytogenes* and *E. coli* O157:H7) in a meat sausage model system.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

*L. plantarum* EC52 previously isolated from congolese poto-poto, a maize fermented food (Ben Omar et al., 2008), was used as starter culture. Strain EC52 carried several plantaricin genes (*plnABDEFIGKN*) and displayed broad antimicrobial activity (Ben Omar et al., 2008). The non-bacteriocinogenic strain *L. plantarum* 1.6 (Sánchez et al., 2008) was used as negative control. The bacteria used as challenge in meat were *L. monocytogenes* CECT 4032 and *Escherichia coli* CECT 4972 (serotype O157:H7) from the Colección Española de Cultivos Tipo (CECT, Burjasot, Valencia, Spain). *L. plantarum* EC52 was grown in MRS medium (Scharlab, Barcelona, Spain) at 30°C. *L. monocytogenes* CECT 4032 and *E. coli* CECT 4972 were grown in Brain Heart Infusion (BHI) (Scharlab, Barcelona, Spain) at 37°C; all strains were stored at 4°C or frozen as stocks at -20°C. MRS and BHI solid media were prepared by adding 1.5% agar (Scharlab) to the broths.

**Inoculum preparation**

To prepare inoculum, strain *L. plantarum* EC52 was grown 24 h at 30°C in MRS broth. This culture was diluted 1:10 in sterile saline solution (0.85%) to a final cell density of approximately 8 log cfu/ml. This dilution was used as the *L. plantarum* EC52-inoculum.

Cultures of *L. monocytogenes* CECT 4032 and *E. coli* CECT 4972 grown overnight in brain heart infusion broth at 37°C were diluted 1:100 in sterile saline solution (0.85%) to a final cell density of approximately 7 log cfu/ml. This dilution was used as the *Listeria* and *E. coli*-inoculum solution.

**Meat food system and microbial analysis**

A model sausage meat system was prepared with chopped meat obtained from a local supermarket (Mercadona, Jaen, Spain). The meat food system contained pork and beef (1:1) and water (50 ml/Kg), with the following ingredients (in g/Kg): sodium chloride 25; sodium nitrite 0.1; potassium nitrate 0.3; sodium ascorbate 0.5; monosodium phosphate 1.5; dextrose 7; lactose 10; skimmed milk powder 10; sodium caseinate 10; Ponceau 4R 0.02; black pepper 3.

The bacterial cultures, added in the water of the formulation, were applied to the meat system when indicated. Meat mixtures (200 g) were placed in Stomacher bags. The incubation was performed at 22°C for 15 days in a laboratory incubator (Memmert, Schwabach, Germany) and the meat samples were evaluated each 3 days. The pH of the samples was determined at room temperature using a Basic 20 pH-meter (Crison, Barcelona).

**Trials**

The following trials (in duplicate) were set up:

Trial A was carried out to investigate the biocontrol of *L. monocytogenes* by *L. plantarum* EC52. *L. monocytogenes* was tested at two different concentrations (4.0 and 5.2 log cfu/g), and *L. plantarum* EC52 was inoculated at 7.4-8.0 log cfu/g. Each set of experiments consisted of a control batch without strains, a control batch contaminated with *L. monocytogenes* (either low or high inoculum concentration), a control batch inoculated with *L. plantarum* EC52 and one batch contaminated with *L. monocytogenes* and inoculated with *L. plantarum* EC52.

Trial B was carried out to investigate the biocontrol of *E. coli* (5 log cfu/g) by *L. plantarum* EC52 (8.0 log cfu/g). Each set of experiments consisted of a control batch without strains, a control batch contaminated with *E. coli*, a control batch inoculated with *L. plantarum* EC52 and one batch contaminated with *E. coli* and inoculated with *L. plantarum* EC52.

**Meat sampling and microbial analysis**

At each step, samples (5 g) were mixed with 45 ml of sterile saline solution and homogenised (Stomacher). A 0.1 ml aliquot was removed from each sample and used for colonies determination by counting on the following media: MRS agar (Scharlab, Barcelona, Spain) for *L. monocytogenes*, BHI agar (Scharlab, Barcelona, Spain) for *E. coli* and MRS (Scharlab, Barcelona, Spain) for *L. plantarum*. Samples were incubated at 30°C for *L. monocytogenes*, 37°C for *E. coli* and 35°C for *L. plantarum*.
solution (0.85% NaCl) and pummeled for 2 min, normal speed, in a Stomacher 80 (Biomaster, Seward, UK) before they were serially diluted in sterile saline solution and plated on the respective selective media: MRS agar (Scharlab) for *L. plantarum* EC52 incubated at 30°C for 72 h; VRBA (Scharlab) for *E. coli*, incubated at 37°C for 48 h and PALCAM agar with added Listeria supplement (Merok, Madrid) for *L. monocytogenes* incubated at 37°C for 48 h. Counts of colonies showing features typical of *L. plantarum*, *E. coli* or *L. monocytogenes* were determined in order to calculate viable cell counts.

Confirmation of *L. monocytogenes* was done by PCR amplification of the *hlyA* gene with primers DG69 (GTGCCGCCAAGAAAAAGTTA) and DG74 (CAGCACATTAGGATAT) as described elsewhere (Choi and Hong, 2003). The expected 636-bp amplicon was visualized after agarose gel electrophoresis.

Confirmation of *L. plantarum* EC52 was done by RAPD-PCR analysis as described previously (Ben Omar et al., 2004). DNA was amplified with primer M13 (5′-GAG GTC GGC GGT TCT 3′) in 35 cycles of 94°C for 1 min, 40°C for 20 s, ramp to 72°C at 0.6°C/s for 2 min, and 72°C for 2 min, and products were separated electrophoretically (Ben Omar et al., 2004).

### Statistical analysis

The average data from duplicate trials ± standard deviations were determined with Excel programme (Microsoft Corp., USA). In order to determine the statistical significance of data, a t-test was performed at the 95% confidence interval with Statgraphics Plus version 5.1 (Statistical Graphics Corp., USA).

### RESULTS AND DISCUSSION

The *L. plantarum* EC52 strain multiplied efficiently in meat mixtures (both single and in cocultures), reaching final cell concentrations up to 11.5 log cfu/g (Figures 1A, B and C). The pH of cocultures decreased from 6.0 to 4.2-4.4 after 15 days. Growth of lactobacilli and pH parameters did not differ significantly (P > 0.1) between single cultures of EC52 strain and cocultures.

In meat mixtures inoculated with *L. monocytogenes* at initial cell concentrations of 5.2 log cfu/g, viable counts of *listeria* in cocultures with strain EC52 were significantly lower (P < 0.05) compared to single cultures for all incubation times (Figure 1A). The number of viable *listeria* in co-cultures decreased to 3.6 log cfu/g at day 12 of incubation, although it increased slightly at day 15 (Figure 1A). In contrast, the single *L. monocytogenes* control increased to up to 9.6 log cfu/g (day 3). For a lower starting *Listeria* concentration of 4.0 log cfu/g, the viable *listeria* decline was significantly (P < 0.05) greater: 1.6 to 1.8 log cfu/g at days 9 to 12 of incubation (Figure 1B). This is equivalent to a reduction of ca. 2.2-2.4 log cycles in the viable population compared to initial values, and of up to 5.4 log cycles compared to the untreated controls at the end of incubation. These results clearly indicate that the bactericidal effects of strain EC52 towards *L. monocytogenes* are dependent on the cell concentration of the target bacterium. Typically, this dependency is associated to the production of antimicrobial substances (such as bacteriocins), whereby a given concentration of bacteriocin can only kill a certain number of bacteria.

The negative control strain *L. plantarum* 1.6 was also able to reach high cell densities in meat mixtures and showed a similar acidification capacity as strain 5.2 (Figure 1D). Nevertheless, this strain was unable to inactivate *L. monocytogenes* in cocultures, and only caused a late inhibition of growth due to acidification (Figure 1D). The viable counts obtained for *L. monocytogenes* in cocultures with the negative control strain always were significantly higher (P < 0.05) compared to cocultures with the bacteriocin-producing strain EC52. A distinction should be made at this point between the inhibitory effects due to acid production (as in *L. plantarum* 1.6) and the bactericidal effects due to plantaricin production in strain *L. plantarum* EC52. Furthermore, the bactericidal activity of most bacteriocins increases in the acidic pH range. Therefore, a synergistic effect of bacteriocin production and acidification would be expected in the microbial inactivation observed for cocultures of *L. monocytogenes* in combination with the plantaricin-producing strain EC52.

Growth of *E. coli* in cocultures with bacteriocin-producing strain EC52 was significantly inhibited (P < 0.05) for the first 6 days of incubation (Figure 1C). After that, the concentrations of the pathogen increased up to 8 log cfu/g at day 15. In single cultures, viable counts reached much higher concentrations (up to 9.3 log cfu/g at day 9). Highest differences between cocultures and single cultures were observed at days 3 to 9, being significantly lower (P < 0.05) by 1.4 to 3.7 log cycles in cocultures. Cocultivation with the negative control strain *L. plantarum* 1.6 only caused some growth inhibition of *E. coli* (Figure 1D). *E. coli* viable counts obtained for such cocultures were significantly higher (P < 0.05) compared to cocultures with the bacteriocin-producing strain EC52 (except for day 15 of incubation).

In those cocultures where a strong inhibitory effect was observed, the identity of bacteria after co-cultivation was confirmed by molecular techniques. The identity of *L. monocytogenes* in meat samples after co-culture experiments with *L. plantarum* EC52 was confirmed by PCR amplification of the *hlyA* gene. Typically, PCR amplification of colonies isolated at random from PALCAM agar plates yielded a DNA band with the expected size of 635 bp (Figure 2A). Since the meat mixture used was not a sterile product, it was important to corroborate that viable determinations obtained on MRS agar plates corresponded to the inoculated LAB bacteriocin-producing strain. Confirmation of *L. plantarum* EC52 in meat samples after co-culture experiments was done by RAPD-PCR analysis. Typically, RAPD-PCR amplification from colonies isolated at random from MRS agar plates yielded a DNA band profile as the expected for pure cultures of EC52 strain (Figure 2B).

Most studies on application of bacteriocin producing...
strains as starter or protective cultures in meat fermentation have focused on *L. sakei* and *L. curvatus* strains, but none has focused on *L. plantarum* strains carrying the widely disseminated *pln* locus. Such strains are of great interest for food preservation since they may produce an array of antimicrobial peptides (such as plantaricins A, EF, JK, NC8 and J51) (Diep et al., 2009). The present study explores additional possibilities for application of such plantaricin-producing strains, exemplified by strain EC52 isolated from an ethnic fermented food.

Results from the present study indicate that the plantaricin-producing *L. plantarum* strain EC52 shows a good capacity to grow in a meat sausage model system, to reduce the levels of *L. monocytogenes* and to inhibit proliferation of *E. coli*. This strain was shown previously to carry several genes of the *pln* locus and exhibit antibacterial activity against foodborne pathogens in vitro (Ben Omar et al., 2008). Plantaricin-producing lactobacilli from traditional fermented gruels have been shown to inhibit foodborne pathogens in malted millet flour (Sánchez et al., 2008), but this is the first report

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**Figure 1.** Inhibition of *L. monocytogenes* CECT 4032 (A, B) and *E. coli* CECT 4972 (serotype O157:H7) (C) by *L. plantarum* EC52 in meat sausage model system. *L. monocytogenes* was inoculated at 5.2 log c.f.u./g (A) or 4.0 log c.f.u./g (B). *L. plantarum* EC52 in co-culture (■); pathogens (*L. monocytogenes* or *E. coli*) in co-culture with strain EC52 (▲); *L. plantarum* EC52 control single culture (□); controls of pathogens (*L. monocytogenes* or *E. coli*) in single cultures (Δ); pH values of cocultures (○). In (D), the negative control strain *L. plantarum* 1.6 was tested in cocultures: growth of *L. monocytogenes* CECT4032 in coculture with strain 1.6 (▲); growth of *E. coli* CECT 4972 in coculture with strain 1.6 (■). Open symbols in (D) indicate growth of strain 1.6 in coculture with *L. monocytogenes* CECT4032 (Δ) or with *E. coli* CECT 4972 (○), and pH (○).
on their activity in meats. The fast growth capacity shown by strain EC52 in the meat model system of present study and the high cell densities reached shortly during incubation at an abusive temperature of 22°C suggest that this strain is a potential candidate for preservation of meat mixtures, not only against L. monocytogenes but also against the Gram-negative pathogen E. coli O157:H7. This bacterium is a food borne pathogen of special concern in cow meats and meat products, which are increasingly being consumed by particular ethnic groups compared to pork meats. Ethnic fermented foods are being considered as potential sources of bacterial strains with useful technological properties such as bacteriocin production. Furthermore, application of LAB strains from ethnic fermented foods could be highly beneficial to the industries and economies of developing countries, while at the same time promote the exploitation of their natural microbial resources.

ACKNOWLEDGEMENTS

This work was partially supported by CEREFER project (Contract ICA4-CT-2002-10047) of the INCO-DC programme. Gloria Díaz received a grant for young visiting scientists (MEC, Ref. SB2004-0121). We also acknowledge the Junta de Andalucía Research Support Programme (PAI research group AGR230) and the University of Jaén research support plan.

REFERENCES


Díaz Ruiz et al. 1107


Figure 2. Confirmation of L. monocytogenes by PCR and L. plantarum EC52 by RAPD-PCR in meat samples. A) Verification of L. monocytogenes in 15th day samples. Lanes 1 - 6: co-cultures; L, L. monocytogenes control. B) Verification of L. plantarum EC52 in 9th day samples. Lanes 1 – 6: co-cultures. M, markers.


