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Short communication

# Bacteriocin-producing *Enterococcus casseliflavus* IM 416K1, a natural antagonist for control of *Listeria monocytogenes* in Italian sausages (“cacciatore”)

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**Abstract**

The bacteriocinogenic *Enterococcus casseliflavus* IM 416K1 (Bac+) isolated from Italian sausages or its bacteriocin Enterocin 416K1, with strong anti-listerial activity, were used in trials to evaluate the effect on *Listeria monocytogenes* NCTC 10888 in artificially inoculated Italian sausages (“cacciatore”).

In trials with Enterocin 416K1 added, *L. monocytogenes* showed a significant reduction as compared to the control inoculated with *L. monocytogenes* alone. The elimination of *L. monocytogenes* was only obtained in sausages added with *E. casseliflavus* IM 416K1 Bac+.


*Keywords:* Sausages; Enterocin; *Listeria monocytogenes*; Lactic acid bacteria

## 1. Introduction

*Listeria monocytogenes* is an important food-borne pathogen isolated from many types of foods that represents a serious health hazard to immunocompromised patients and pregnant women (McLauchlin, 1997; Rocourt and Cossart, 1997). Recently, particular attention has been directed towards meat products, considering that *L. monocytogenes* is frequently found in raw materials where it survives and proliferates at typical refrigeration temperatures (Farber et al., 1989; Farber and Peterkin, 1991). According to Fenlon et al. (1996), meat contamination occurs during slaughter and in the subsequent processing.

Because lactic acid bacteria (LAB) are generally recognised as safe (GRAS) in food production (Schillinger et al., 1996), in the last years, the use of either their bacteriocins or the bacteriocin-producing LAB starter cultures has received a special attention as a new preservation method to control pathogenic bacteria (Klaenhammer, 1993; Holzapfel et al., 1995; Jack et al., 1995; Ennahar et al., 1999). Preservation of meat with bacteriocin-producing *Pediococcus* spp. and *Lactobacillus* spp. has already been described (Berry et al., 1990; Foegeding et al., 1992; Campanini et al., 1993; Winkowski et al., 1993; McMullen and Stiles, 1996; Muriana, 1996; Stiles, 1996). With regard to enterococci, their bacteriocins (enterocins), generally belong to class II and have the potential to

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inhibit the growth of a narrow range of strains closely related to the producer microorganism (Tagg et al., 1976; Klaenhammer, 1993), Gram-positive food-borne pathogens and spoilage bacteria (Klaenhammer, 1988; Cintas et al., 1997; Ennahar et al., 1998; Ennahar and Deschamps, 2000; Ohmomo et al., 2000), and Gram-negative species (Lewus et al., 1991; Jennes et al., 2000). Bacteriocin-producing *Enterococcus* strains with strong anti-*Listeria* activity have been isolated from dairy products (Nuñez et al., 1997; Ennahar et al., 1998), fermented sausages (Aymerich et al., 1996; Cintas et al., 1997; Cintas et al., 1998), fish (Ben Embarek et al., 1994), vegetables (Bennik et al., 1998; Floriano et al., 1998), fermented olives (Franz et al., 1996) and silages (Kato et al., 1994). The ability of enterococci to inhibit *L. monocytogenes* (Arihara et al., 1993; Vlaemynck et al., 1994; Giraffa et al., 1995; Maisnier-Patin et al., 1996; Sabia et al., 2002) may be explained by the fact that enterococci and listeria are phylogenetically closely related (Stackebrandt and Teuber, 1988; Devriese and Pot, 1995). Since enterococci are common in various food systems and their technological and probiotic benefits are widely recognised (Giraffa et al., 1997), these microorganisms might be good candidates for potential application of bacteriocin-mediated antagonism against *L. monocytogenes* in foods (Muriana, 1996). An enterocin-producing *E. faecium* was recently found suitable for use as a meat starter culture in dry fermented sausage (Lauková and Czikková, 1999; Callewaert et al., 2000).

In a previous work (Sabia et al., 2002), Enterocin 416K1, a bacteriocin with strong anti-listerial antagonistic activity was studied. The aim of this study was to investigate the possible use of this enterocin or the bacteriocinogenic *Enterococcus casseliflavus* 416K1 IM Bac+ to control the growth of *L. monocytogenes* in Italian sausages (“cacciatore”).

## 2. Materials and methods

### 2.1. Bacterial strains and media

The following organisms were used for direct inoculation in the “cacciatore” standard mixture described below: *E. casseliflavus* IM 416K1 Bac+ isolated from Italian sausages and previously selected for anti-*listeria* activity, *E. casseliflavus* IM 416K1 (Bac − ), a derivative of the parental strain obtained by curing with ethidium bromide (Sabia et al., 2002) and *L. monocytogenes* NCTC 10888, also used as indicator strain in the bacteriocin activity assay. Enterococci were cultured in MRS broth or agar (Oxoid, Milan, Italy) and *L. monocytogenes* in Tryptic Soy broth or agar (TSB or TSA, Difco Laboratories, Detroit, MI) supplemented with 0.6% yeast extract (Oxoid). All strains were maintained at − 80 °C in the appropriate cultivation broth containing 20% (v/v) glycerol (Merck, Darmstadt, Germany).

### 2.2. Inocula

Overnight cultures at 30 °C in TSB of *L. monocytogenes* NCTC 10888 and in MRS broth of Bac+ and Bac − *E. casseliflavus* IM 416K1 were enumerated on TSA and MRS agar plates respectively, and expressed as colony forming units per milliliter (cfu $ml^{-1}$). After centrifugation at 10,000 × *g* for 10 min at 4 °C, pellets were resuspended in peptone saline (PSS, Oxoid) to a final bacterial count of about $10^5$ cfu $g^{-1}$ for enterococci and $10^4$ cfu $g^{-1}$ for *L. monocytogenes*.

### 2.3. Antimicrobial activity of concentrated Enterocin 416K1

Crude filtrate supernatant fluid (CFSF) from a culture of *E. casseliflavus* IM 416K1 in MRS broth grown at 30 °C for 24 h was collected by centrifugation as described above, dialyzed against 30 $mmol^{-1}$ sodium acetate buffer (pH 5.3) and filter sterilized (0.45-μm-pore-size filter; Millipore, Bedfort, MA). Dialyzed CFSF was concentrated two-fold by ultrafiltration through 3 kDa mol exclusion membranes (Diaflo Ultrafiltration Membranes, Amicon, Beverly, MA, USA). The inhibitory activity of the concentrate was determined by agar well diffusion assay (Rogers and Montville, 1991) against *L. monocytogenes* NCTC 10888. The concentrate of Enterocin 416K1 was added to the standard mixture giving a final concentration of 10 AU $g^{-1}$.

### 2.4. Preparation and inoculation of “cacciatore”

The standard mixture used to produce “cacciatore” consists of ground pork meat added NaCl 2.8% (w/w),

black pepper in wheat 0.2% (w/w), milk powder 3% (w/w) (containing up to 60% (w/w) lactose), 0.015% sodium nitrite and sodium ascorbate. To study the ability of *E. casseliflavus* IM 416K1 Bac+ or Enterocin 416K1 to control *L. monocytogenes* growth in ''cacciatore'', six independent trials (A, B, C, D, E, F) were conducted including three samples for each stage of ripening. The trial A comprised the untreated standard mixture and in trials B, C, D, E, F, the standard mixture was inoculated as shown in Table 1.

The inoculated and non-inoculated standard mixtures in sterile bags were homogenized by hand and stuffed into 5-cm-diameter natural casing using a mechanical stuffer. The sausages were about of 25 cm long and 300 g in weight. They were dried in the pilot plant at 25 °C and 80–85% relative humidity for 3 days, matured at 18 °C and 70–75% relative humidity for up to 10 days, and subsequently stored at 4 °C for 10 days.

### 2.5. Bacterial count, pH and $a_w$ measurement

Listeria and LAB were counted in samples immediately after preparation (time 0), after drying (3rd day), at half ripening (7th day), at final ripening (10th day) and after storage at 4 °C (20th day). The sausages were sampled aseptically, removing three 10-g portions from the centre, the top and bottom. The samples, diluted with 90 ml peptone saline, were homogenized for 1 min in a stomacher (Lab Blender, Seward, London, UK).

*Listeria* spp. were counted by spread plating five 0.2-ml portions of the initial suspension on Palcam agar (Oxoid) and incubating at 30 °C for 48 h. In addition, 25-g portions of each sausage were subjected to enrichment according to the U.S. Food and Drug Administration (US/FDA) modified method, followed by isolation on Palcam agar (Lovett and Hitchins, 1988). Enterococci and lactobacilli were counted by spread plating on Kanamycin aesculin azide agar (Oxoid) and on MRS agar, respectively, with incubation at 30 °C for 48 h.

Differences in counts between total enterococci and *E. casseliflavus* IM 416K1 Bac+ were evaluated by the deferred antagonism method using *L. monocytogenes* NCTC 10888 as indicator strain. The pH and the $a_w$ was monitored using a Beckman pHmeter 690 (Beckman Instruments, Inc. Fullerton, U.S.A.) and an Aqualab CX-2 (Decagon Devices, Pullman, WA, USA), respectively.

Table 1
Inoculation trials for standard mixture of "cacciatore"

| |
|---|
| A—standard mixture, no innoculation |
| B—standard mixture with *E. casseliflavus* IM 416K1 Bac+ added |
| C—standard mixture with *L. monocytogenes* added (control) |
| D—standard mixture with *L. monocytogenes* and Enterocin 416K1 added |
| E—standard mixture with *L. monocytogenes* and *E. casseliflavus* IM 416K1 Bac+ added |
| F—standard mixture with *L. monocytogenes* and *E. casseliflavus* IM 416K1 Bac− added |

### 2.6. Statistical tests

Bacterial counts were carried out in triplicate and the standard deviation was calculated on the average of the values obtained. The differences of decline rates of *L. monocytogenes* NCTC 10888 were analyzed with a *t*-test for paired data. Statistical probability equal to or less than 0.05 was considered significant.

## 3. Results and discussion

The antimicrobial titre of two-fold concentrated Enterocin 416K1, exhibited against *L. monocytogenes* NCTC 10888, was 2400 arbitrary units (AU $ml^{-1}$).

There was no significant variation in pH, $a_w$ values among the six trials. At time 0 the pH was 5.8 for all sausages, at the end of ripening, it decreased to about 5.1 and increased slightly up to 5.4 during further storage (results not shown). At the same time, the $a_w$ values decreased from 0.96 to 0.90 (data not shown).

Natural and added enterococci (Fig. 1a) increased initially by about 2–3 log cfu $g^{-1}$ up to the end of the drying period (3rd day) and further until half ripening (7th day), while a small reduction occurred in the subsequent determinations (10th and 20th days). In particular, the *E. casseliflavus* IM 416K1 Bac+ counts increased by about 1 log cfu $g^{-1}$ from the beginning to the end of the experiment (data not shown). Lactobacilli (Fig. 1b) increased by about 4 log cfu $g^{-1}$ up to the end of the drying period (3rd day), and this value was maintained during the whole experiment.

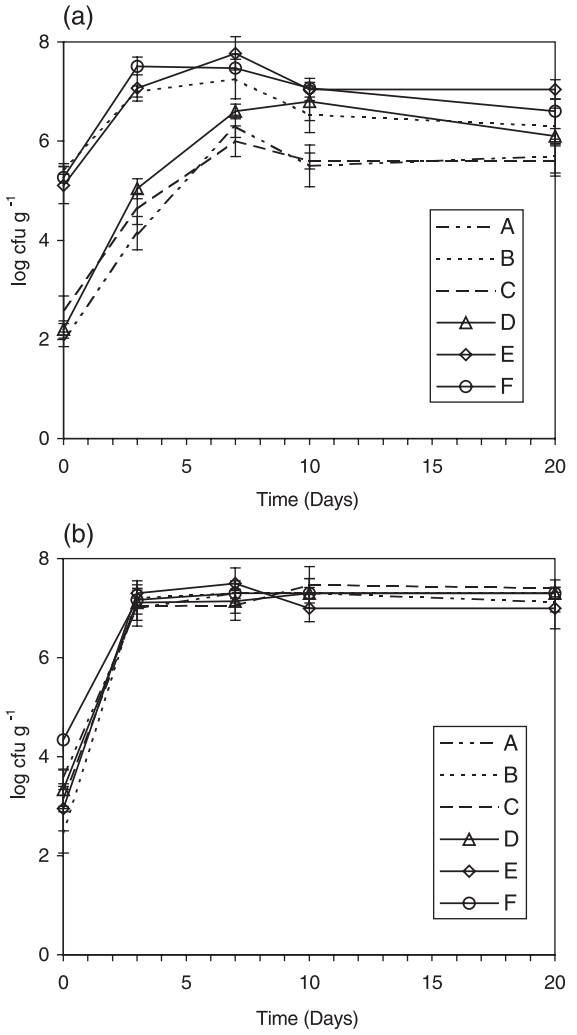


Fig. 1. Model "cacciatore" ripening: (a) number of enterococci and (b) number of lactobacilli during ripening of "cacciatore" at 25 °C for 3 days followed by 10 days at 18 °C and storage at 4 °C for 10 days. Trial A: standard mixture (– - - –); trial B: standard mixture with *E. casseliflavus* IM 416K1 Bac+ added (-----); trial C: standard mixture with *L. monocytogenes* added (control) (– – –); trial D: standard mixture with *L. monocytogenes* and Enterocin 416K1 added (△); trial E: standard mixture with *L. monocytogenes* and *E. casseliflavus* IM 416K1 Bac+ added (◇); trial F: standard mixture with *L. monocytogenes* and *E. casseliflavus* IM 416K1 Bac – added (○). Vertical bars show standard deviation.

In the "cacciatore" standard mixture (trial A), *Listeria* spp. were not detected, even when using the enrichment US/FDA modified method. Fig. 2 shows the number of *L. monocytogenes* in trials C, D, E, F, artificially inoculated with this microorganism. In trials C and F, *L. monocytogenes* declined to about 2 log at the end of the experiment. This could be related to the pH and $a_w$ decrease and to the competition with LAB.

In trial D shortly after Enterocin 416K1 was added (time 0), *L. monocytogenes* was reduced by 2.09 log. Although in the following sampling (3rd day), *L. monocytogenes* count increased slightly (from 1.91 to 2.49 log cfu $g^{-1}$), a significant difference was maintained during the whole experiment in comparison with trials C and F ($p<0.017$ and $p<0.009$, respectively). This is in agreement with results published by Lauková and Czikková (1999).

In trial E, *L. monocytogenes* decreased initially by about 2.5 log up to the end of the drying period (3rd day), and a further smaller reduction (from 1.51 to 1.30 log cfu $g^{-1}$) occurred after 1 week of ripening. In the subsequent determinations (10th and 20th days), *L. monocytogenes* was not detectable. The disappearance of *L. monocytogenes* could be due to an uninterrupted production of Enterocin 416K1 by *E. casseliflavus* IM 416K1 Bac+.

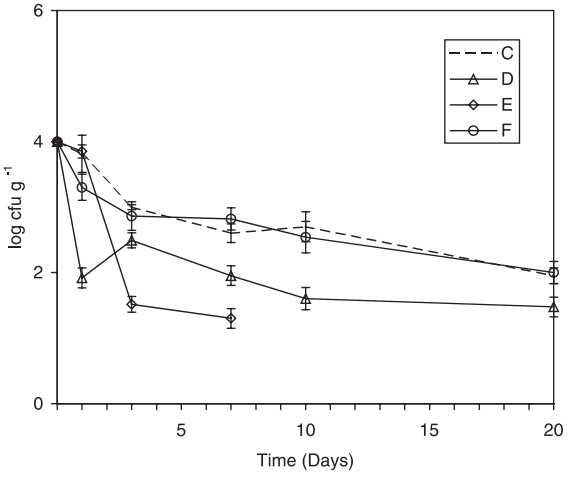


Fig. 2. Decrease of *L. monocytogenes* NCTC 10888 in trials of artificially contaminated "cacciatore". Trial C: standard mixture with *L. monocytogenes* added (positive control) (– – –); trial D: standard mixture with *L. monocytogenes* and Enterocin 416K1 added (△); trial E: standard mixture with *L. monocytogenes* and *E. casseliflavus* IM 416K1 Bac+ added (◇); trial F: standard mixture with *L. monocytogenes* and *E. casseliflavus* IM 416K1 Bac – added (○). Vertical bars show standard deviation.

As during the slaughter and processing contamination of beef occurs, food spoilage microorganisms or pathogens can easily contaminate the meat. In fact the detection of listeria in final products such as medium-acid salami is rather frequent even if the contamination level is generally low. Consequently, fast growth of highly competitive starter cultures and acidification of the meat are essential for the prevention of spoilage and pathogenic bacterial growth, and the use of strains that produce antimicrobial substances could be a further advantage (Callewaert et al., 2000). The results of the present work also indicate that the pH and $a_w$ decrease, in addition to the natural competition of LAB, leads to a decline in *L. monocytogenes* count by about 2 log at the end of ripening, showing that these factors in combination are favourable for preservation of sausages (Stecchini et al., 1992; Leistner, 1995, Scanell et al., 1977; Luecke, 1998). Unlike in other food matrixes, in this product, *Listeria* spp. do not find a favourable environment for growth (Guyer and Jemmi, 1991; Rorvik et al., 1991). However, only by adding *E. casseliflavus* IM 416K1 Bac+, we observed a reduction of *L. monocytogenes* NCTC 10888 to non-detectable levels, suggesting (Callewaert et al., 2000) the need for a continuous presence and activity of enterocin throughout. This result is in contrast with the study by Aymerich et al. (2000), where the addition of enterocin to dry fermented sausages significantly reduced listerial counts, whereas the same result was not obtained adding the producer *Enterococcus faecium* CTC492 as starter culture. The rapid multiplication of *E. casseliflavus* IM 416K1 Bac+ and the bacteriocin production in "cacciatore" is not surprising since this strain was isolated from the same type of food and therefore adapted to this environment (De Martinis and Franco, 1998). The smaller anti-listerial activity of Enterocin 416K1 when added alone could be due to the limited diffusion of the compound into the meat mixture, and there might also be a loss of activity over time (Hugas et al., 1996; Lauková and Czikková, 1999).

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