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ORIGINAL ARTICLE

Controlling *Listeria monocytogenes* in Cottage cheese through heterologous production of enterocin A by *Lactococcus lactis*

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Abstract

Aims: Enterocin A is an example of a class IIa bacteriocin with potent anti-listerial activity. This study was initiated with a view to harnessing this activity, through heterologous production by a lactococcal starter strain, to limit levels of *Listeria monocytogenes* in a food (Cottage cheese).

Methods and Results: Plasmid pEnt02 (containing *entA*, *I*, *T* and *D* genes under the control of a constitutive promoter) was introduced into a *Lactococcus lactis* strain capable of fermenting lactose. When this bacteriocin-producing starter was used in combination with a non-enterocin A producer, thereby compensating for an associated reduction in acid production, during a Cottage cheese fermentation, a decrease in *L. monocytogenes* (tagged with *lux* genes for convenience) levels was evident.

Conclusions: Enterocin A, heterologously produced by a food grade lactic acid bacteria (LAB), was therefore shown to have potential for use as a biocontrol agent in food.

Significance and Impact of the Study: Many of the most active anti-listerial compounds identified to date are enterocins. However, because of *Enterococcus*-associated concerns, the use of these antimicrobials in a food setting has been curtailed. Although enterocins have been heterologously produced in LAB to overcome this problem, this study represents the first occasion upon which the benefits of such heterologous production have been demonstrated in a food context.

Introduction

Listeriosis is a food-borne disease that presents an acute hazard to susceptible groups; in particular, to the elderly, new born, pregnant women and those who are immunocompromised (McLaughlin 1987). Although the causative agent, *Listeria monocytogenes*, has been isolated from a wide range of food sources (Gravani 1999), a number of foods, including soft cheese, pose a particularly high risk (Ryser 1999; Rudolf and Scherer 2001). As a consequence of an increased demand from consumers for foods that contain lower concentrations of chemical preservatives, there has been an ever greater focus on the identification of natural antimicrobials, such as bacteriocins (Twomey *et al.* 2002; Cotter *et al.* 2005), that can be employed to control the growth of pathogens in food. Bacteriocins have been shown to be able to affect population dynamics in a cheese matrix and thus offer a potential solution to control undesirable flora or to cause the release of enzymes from adjunct flora, contributing to cheese flavour (Morgan *et al.* 1995; Ryan *et al.* 1996; Fenelon *et al.* 1999). A large number of bacteriocins possess anti-listerial activity and many of them have been applied to the control of *L. monocytogenes* in or on cheese (Maisnier-Patin *et al.* 1992; Davies *et al.* 1997; Nuñez *et al.* 1997; McAuliffe *et al.* 1999; Morgan *et al.* 2001; O'Sullivan

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et al. 2006). A number of the most active of these are enterocins produced by enterococci. The potential benefits of applying enterocins to control *Listeria* in food have been well documented, particularly in dairy products (Giraffa 1995; Giraffa and Carminati 1997). Unfortunately, the commercial application of these micro-organisms as live producing cultures in food has been limited by their association with certain human infections (Foulquié Moreno *et al.* 2006). One approach to overcoming this issue is the heterologous production of the *Enterococcus*-associated bacteriocins (enterocins) (Rodríguez *et al.* 2003).

Enterocin A, produced by *Enterococcus faecium*, is a class IIa bacteriocin and is one of the most potent antilisterial bacteriocin known (Eijsink *et al.* 1998; Ennahar *et al.* 1999; Ennahar and Deschamps 2000; Drider *et al.* 2006) and, in a semi-purified form, has been utilized in dry fermented sausage, ham, minced pork meat, deboned chicken breasts, pate and fermented sausage (Aymerich *et al.* 2000a,b). However, although it has been established that enterocin A can be heterologously expressed (O'Keeffe *et al.* 1999; Martínez *et al.* 2000; Klocke *et al.* 2005), such heterologous producers have not been applied for food protection purposes. Here, the enterocin A-associated genes were introduced into a *Lactococcus lactis* starter strain. After confirming an enterocin A^+ phenotype, this strain was successfully employed to control *L. monocytogenes* levels during Cottage cheese fermentation.

Materials and methods

Bacterial strains and culture conditions

Lactococcus lactis IL1403 pENT02 (O'Keeffe *et al.* 1999) was cultured in M17 with supplement of 0·5% (w/v) glucose (GM17) at 30°C. *Lactococcus lactis* MG1614 pLP712 (O'Sullivan *et al.* 1998) was propagated in M17 supplied with 0·5% (w/v) lactose (LM17) at 30°C. *Listeria monocytogenes* EGD*elux* (Riedel *et al.* 2007) and enterococci (*E. faecium* DPC1146; O'Keeffe *et al.* 1999 and *E. faecium* UCC127, a non-enterocin A producing *Enterococcus*) were routinely grown in brain heart infusion (BHI) medium at 37°C and 30°C, respectively. Erythromycin and rifampicin (Sigma Chemical Co., Dorset, UK) were used at a concentration of 5 μg ml^{-1} (*Lactococcus*) and 50 μg ml^{-1} (*Listeria*), respectively.

Plasmid DNA preparation and electroporation

After treatment with lysis buffer (Anderson and McKay 1983), plasmid DNA was isolated from *L. lactis* IL1403 pENT02 using the QIAprep Spin Miniprep kit (Qiagen Gmbh, Hilden, Germany). Electrocompetent *L. lactis* MG1614 pLP712 were prepared by the method of Holo and Nes (1989) and transformed with a Gene Pulser (Bio-Rad Laboratories, Richmond, CA, USA) at 2·5 kV, 25 μF and 200 ΩE. DNA was dialysed on filters with a 0·025-μm pore size (Millipore Corp., Bedford, MA, USA) for 20 min prior to the electroporation to reduce the ionic strength of the solution.

Bacteriocin activity assay

To assay for enterocin A production on solid medium, 10 μl of an overnight culture of the putative bacteriocin producer was spotted onto agar and was overlaid with soft BHI agar seeded with the indicator of choice (*L. monocytogenes* EGD*elux* and *E. faecium* UCC127) (0·5% v/v). Bacteriocin activity (AUml^{-1}) of the transformant in broth was quantified by an agar well diffusion assay (Ryan *et al.* 1996).

Bacteriocin purification and mass spectrometry

Lactococcus lactis MG1614 pLP712 containing pENT02 was subcultured twice in GM17 broth at 1% (v/v) at 30°C before use. Two litres of GM17 were inoculated with the culture at 0·5% and incubated at 30°C overnight. The culture was then centrifuged at 7000 rev min^{-1} for 15 min and both the cell pellet and supernatant were retained. The culture supernatant was passed through 60 g beads (Amberlite XAD16; Sigma-Aldrich Chemie, Steinheim, Germany), prewashed with 1 l of distilled H_2O. The column was washed with 500 ml of 30% ethanol, 500 ml of 30% isopropanol and the bacteriocin was eluted in 500 ml of 70% isopropanol 0·1% trifluoroacetic acid (TFA) and retained (S1). The cell pellet were resuspended in 300 ml of 70% isopropanol, 0·1% TFA and stirred at room temperature for approx. 3 h. It was then centrifuged at 7000 rev min^{-1} for 15 min and the supernatant (S2) retained. S1 and S2 were combined and the isopropanol was evaporated using a rotary evaporator (Büchi, Postfach, Switzerland) before applying to a 10 g (60 ml) Phenomenex SPE C-18 column pre-equilibrated with methanol (60 ml) and then water (60 ml). For mass spectrometric analysis, semi-purified peptide was loaded into an Axima CFR plus MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK).

Growth comparison assays

Growth of cultures was measured in both LM17 and milk. Optical density of LM17 and acid production of LM17 and milk were assessed on an hourly basis for 8 h and at 24 h, using pH meter (AGB Scientific Ltd, Schott Instruments, Woburn, MA) and spectrophotometer (DU 730;

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Beckman Coulter Inc., Fullerton, CA, USA), respectively. Milk was prepared by dissolving skimmed milk powder in water (10% w/v) and autoclaving at 110°C for 10 min. *L. lactis* MG1614 pLP712 and its pEnt02-containing counterpart were inoculated (2%) into milk separately or in combination and grown at 30°C.

Preparation of rifampicin-resistant strain of *L. monocytogenes* EGD*elux*

A rifampicin-resistant derivative of *L. monocytogenes* EGD*elux* was prepared as described previously (Begley *et al.* 2005). Briefly, cell pellets from a 10 ml overnight culture of *L. monocytogenes* EGD*elux* were resuspended in 200 μl of ¼ strength Ringer's solution. Of this resuspension, 100 μl was spread onto BHI agar containing rifampicin (50 μg ml^{-1}) and grown at 37°C.

Manufacture of Cottage cheese

Starter cultures were grown overnight in skimmed milk solution at 30°C. Fresh milk was standardized and pasteurized (73°C × 15 s), cooled to 30–31°C and inoculated with the starter cultures (2%). Cheese rennet (1 ml/100 l; CHR Hansen, Hørsholm, Denmark) was added according to the manufacturer's instruction and following stirring (5 min) the milk was covered and incubated at 30–31°C for 4–5 h. Once the coagulum was sufficiently firm, it was cut into 1–2 cm cubes and after a healing period, the curd particles were stirred for 30 min during which the temperature was increased from 31°C to 55°C, followed by continuous stirring at 55°C for 15 min. The curd particles were drained using cheese cloth, rinsed with warm water (25°C) and immersed repeatedly in cold water (<10°C/ice water) for at least 3 min. The curd particles were drained again, at which stage *L. monocytogenes* EGD*elux* (overnight culture) was added to the cheese and mixed evenly (through stirring) at a concentration of 10^4, 10^5, 10^6 or 10^7 CFU g^{-1}, and placed at 4°C. *L. monocytogenes* levels were assessed daily for 5 days and thereafter on days 10 and 15.

Enumeration of rifampicin-resistant *L. monocytogenes* EGD*elux*

To enumerate *L. monocytoegenes* levels in the cheeses, 5 g samples were taken and diluted in 45 ml of 2% tri-sodium citrate solution. After stomaching, 10 ml of the mixture was serially diluted in ¼ strength Ringers solution, plated onto BHI agar containing rifampicin and incubated at 37°C for 24 h. The identity of the *L. monocytogenes* EGD*elux* colonies was confirmed by imaging with a Xenogen 100 (Xenogen Corporation, Alandeda, CA, USA).

pH determination of cheese

Ten grams of cheese was sampled and mixed with 10ml distilled water. The mixture was then homogenized and pH of the homogenate was measured with a pH meter (AGB Scientific Ltd).

Statistical analysis

One-way analysis of variance using SPSS software (version 13.0; SPSS Inc., Chicago, IL, USA) was performed on all experimental data sets. *Post hoc* multiple comparisons were determined by least significant difference. All comparisons were made at a 5% level of significance. Mean values from each trial were determined from triplicate data. Each cheese trial was repeated three times and the final results represent the average of each set of experiments.

Results

Heterologous production of enterocin A by an *L. lactis* starter culture

It has previously been established that the introduction of pEnt02, containing *entA*, *I*, *T* and *D* genes (encoding the enterocin structural, immunity, transporter and accessory protein, respectively) under the control of the constitutive P32 promoter, into *L. lactis* IL1403 brings about the heterologous production of enterocin A (O'Keeffe *et al.* 1999). However, IL1403 is not suitable for cheese fermentations because of its Lac$^-$ phenotype and thus for this study, the pENT02 was introduced into *L. lactis* MG1614 containing pLP712, a *L. lactis* MG1614 transconjugate capable of fermenting lactose. In contrast to the MG1614 pLP712 host, the resulting bacteriocin-producing transformants were capable of inhibiting the growth of *L. monocytogenes* and non-enterocin A-producing enterococci, but not enterocin A-producing enterococci (data not shown). One such transformant was selected for further analysis and following partial purification and mass analysis, it was established that a peptide corresponding to the mass of enterocin A (i.e. 4·8 kDa) was indeed produced (data not shown). This strain is hereafter designated as *L. lactis*$_{\text{Ent+}}$. Despite being an efficient enterocin A producer, it was apparent from agar well diffusion assays that the activity of cell-free supernatant from *L. lactis*$_{\text{Ent+}}$ was four-fold less than that of the original enterocin producer *E. faecium* DPC1146 i.e. 160 *vs* 640 AU ml^{-1}, but equal to that of IL1403 pENT02 (data not shown). However, given the replacement of the original *entA* promoter which is strongly induced in enterococci, such a reduction was

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not unexpected and should be acceptable, given the strong anti-listerial activity of enterocin A.

Growth and bacteriocin activity of transconjugant in LM17 and milk

With a view to the potential use of *L. lactis*$_{\text{Ent+}}$ as a starter culture in Cottage cheese fermentation, its growth and that of the parental nonproducer was assessed in LM17 and skimmed milk. In LM17, it was noted that *L. lactis*$_{\text{Ent+}}$ grew at a slower rate during the first 8h post-inoculation (2%), although the final optical density of the two cultures was similar after 24h (Fig. 1a). Unsurprisingly, this reduced initial growth also manifested as a reduced ability to acidify the medium (Fig. 1b). The use of a higher initial inoculum (6%) did not compensate for this poorer growth/acid production (data not shown). This phenomenon was also apparent when the strain was grown in milk (10% solid; Fig. 1c). On the basis of its reduced ability to acidify milk, it was apparent that use of *L. lactis*$_{\text{Ent+}}$ as a sole starter culture in cheese fermentation would not be successful. As an alternative, the *L. lactis*$_{\text{Ent+}}$ was combined with its parental strain, *L. lactis* MG1614 (pLP712), in both a 1 : 1 and 1 : 2 ratio (2 : 2% and 1 : 2%). In both cases, acidification was rapid and proceeded in a manner corresponding to that observed when the parental strain alone was utilized (Fig. 1d).

To ensure that bacteriocin production also occurred in milk, overlay assays were carried out with all the cultures discussed above. Deferred antagonism assays demonstrated that the transformed culture in LM17 gave the largest clear zone of inhibition of *L. monocytogenes* and was followed by the same strain in milk. Mixtures of producing and nonproducing cultures with different ratios in milk also formed clear but smaller zones. No zone was observed from the untransformed culture in both LM17 and milk. It was apparent that enterocin A was produced by the transformed strain and was active in both LM17 medium and milk, while the activity in milk was lower, but remained effective (data not shown).

Inhibition of *L. monocytogenes* in cheeses containing *L. lactis*$_{\text{Ent+}}$

To assess the effectiveness of the mixed culture against *L. monocytogenes* in a model food, cottage cheese was

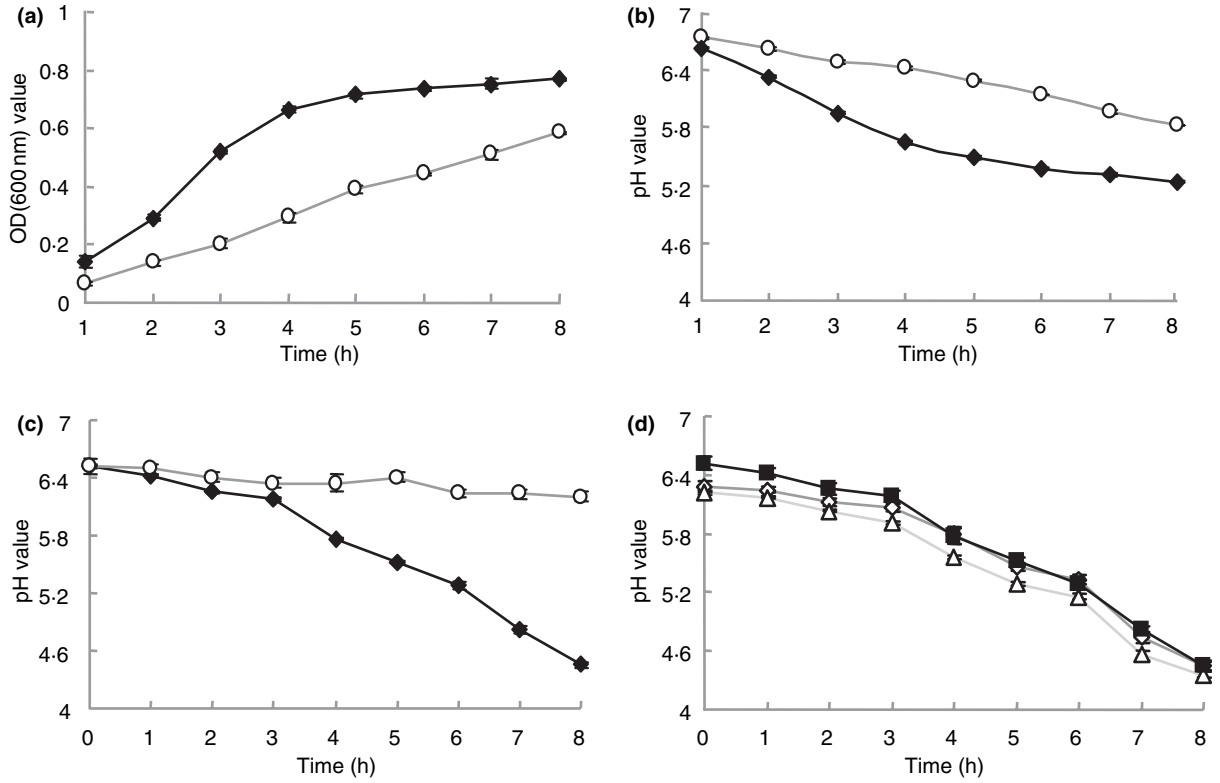

Figure 1 (a) Optical density at 600nm of LM17 with *Lactococcus lactis*$_{Ent+}$ (2%) and *L. lactis*$_{Ent-}$ (2%). (b) pH of LM17 with *L. lactis*$_{Ent+}$ (2%) and *L. lactis*$_{Ent-}$ (2%). (c) pH of milk with *L. lactis*$_{Ent+}$ (2%) and *L. lactis*$_{Ent-}$ (2%) [*L. lactis*$_{Ent+}$ (○); *L. lactis*$_{Ent-}$ (◆)]. (d) pH of milk containing *L. lactis*$_{Ent-}$ only (■) or containing a combination of *L. lactis*$_{Ent+}$ and *L. lactis*$_{Ent-}$ (◇) at a ratio of 1:2% or (△) 2:2%.

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prepared using either this combination (test cheese) or *L. lactis* MG1614 (pLP712) (control cheese). It was established that the pH of the test and control cheeses did not vary (data not shown). *L. monocytogenes* EGDe*lux* was introduced in both cheeses at concentrations of 10^4, 10^5, 10^6 or 10^7 CFU g^{-1}. Regular sampling of the cheeses established that, with respect to the cheeses initially containing 1×10^4 CFU g^{-1} *L. monocytogenes* EGDe*lux*, the presence of *L. lactis*$_{Ent+}$ resulted in a dramatic decrease in pathogen numbers which dropped below detectable levels (1×10^3 CFU g^{-1}) within 2 days. In contrast, *L. monocytogenes* EGDelux could still be detected in the control cheese after 10days (Fig. 2a).

When the initial level of *L. monocytogenes* EGDe*lux* in cheese was $1{\cdot}6 \times 10^5$ CFU g^{-1}, there was a significant beneficial impact ($P < 0{\cdot}01$ on each day after commencement of the trial) accruing from the presence of *L. lactis*$_{Ent+}$ in the cheese. This was most apparent on the final day of the trial (day 15) when the pathogen was present at a concentration of $3{\cdot}63 \times 10^3$ CFU g^{-1} in

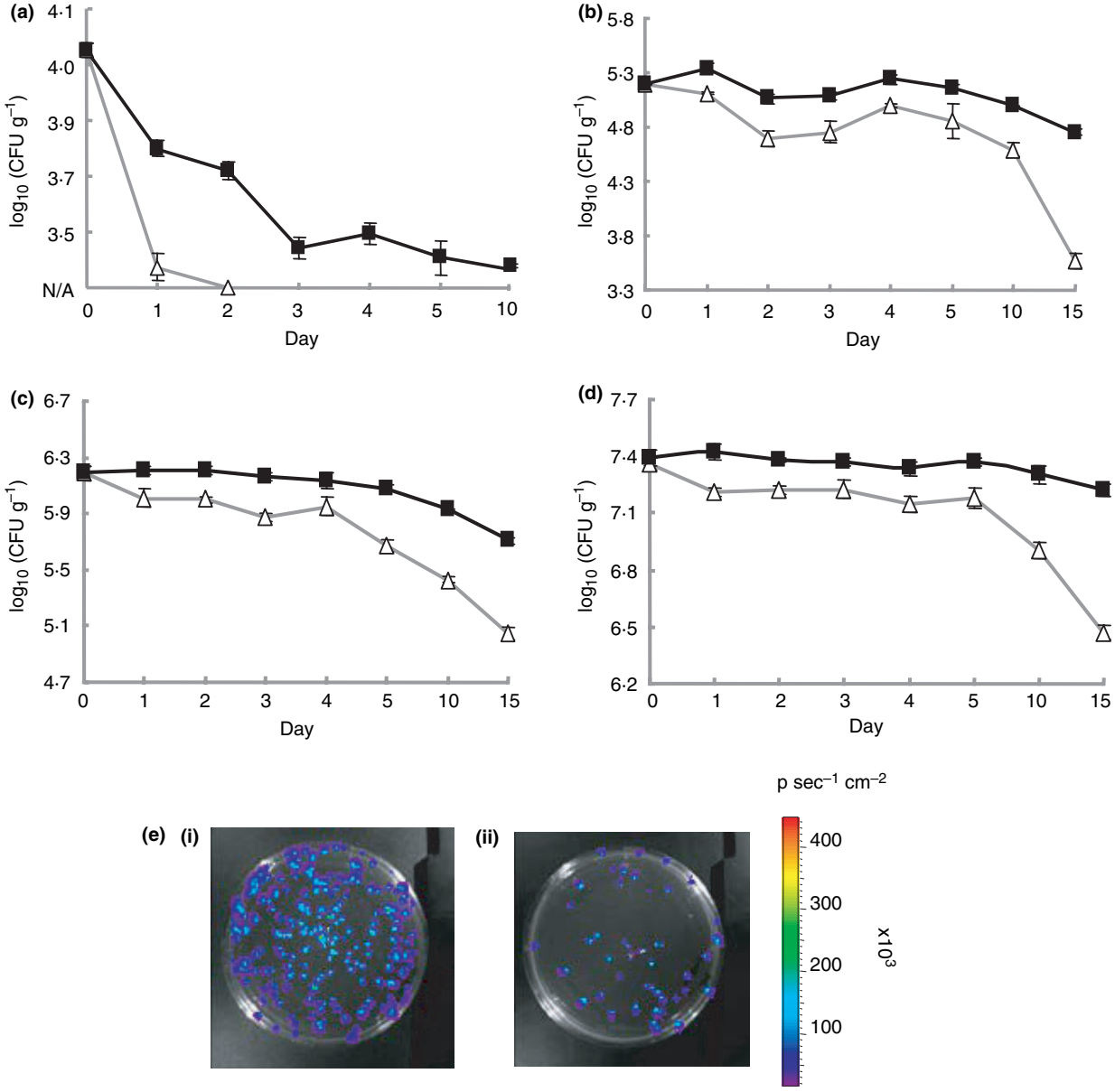

Figure 2 (a–d) Survival of *Listeria monocytogenes* EGDe*lux* in Cottage cheeses stored at 4°C in order of increasing initial inoculum. (△) Cheese containing *Lactococcus lactis*$_{Ent+}$ and *L. lactis*$_{Ent-}$, (■) cheese containing *L. lactis*$_{Ent-}$ only. (e) Image of *L. monocytogenes* EGDe*lux* in (i) cheese containing *L. lactis*$_{Ent-}$ only and (ii) cheese containing *L. lactis*$_{Ent+}$ and *L. lactis*$_{Ent-}$. Units are pixels s^{-1} cm^{-2}.

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the test cheese, whereas levels in the control cheese were at $1{\cdot}07 \times 10^5$ CFU g^{-1}; i.e. almost unchanged from the start of the trial (Fig. 2b).

A similar, although slightly less dramatic, trend was observed when the cheeses contained an initial level of $1{\cdot}5 \times 10^6$ and $2{\cdot}3 \times 10^7$ CFU g^{-1} *L. monocytogenes* with the final reductions in the test cheese of 1·1 and 1 log units, respectively. This again represented a significant difference ($P < 0{\cdot}01$ on all days) from the levels in the control cheese, where marginal reductions in *L. monocytogenes* EGD*elux* numbers were apparent (Fig. 2c,d).

The use of luciferase-marked strains of *L. monocytogenes* (EGD*elux*) as an indicator and bioluminescent imaging for monitoring pathogen levels was shown to be an efficient approach to assess the anti-listerial function of *L. lactis*$_{Ent+}$ in cheese (Fig. 2e). In addition to the advantage of being strain specific, it was also capable of distinguishing between the target and other colonies appearing on the selective agar and thus gave a more accurate estimation of *Listeria* numbers.

Discussion

Many bacteriocins of potential commercial value are produced by enterococci (Foulquié Moreno *et al.* 2006). As the debate surrounding the application of these micro-organisms in food continues, a number of researchers have instead focused on producing these bacteriocins heterologously. Lactic acid bacteria (LAB), and lactococci in particular, are a common choice as host by virtue of their generally regarded as safe status and the close evolutionary relationship between enterococci and lactococci. Although not successful in all cases (Fernandez *et al.* 2007), heterologous expression of enterocins in LAB has been achieved on a number of occasions (Rodríguez *et al.* 2003), but had not been investigated in a food context prior to this study. The successful implementation of this strategy to limit *L. monocytogenes* in Cottage cheese is greatly encouraging, and has an additional significance in which although the benefits of introducing purified enterocin A into foods (and meat in particular) with a view to controlling *L. monocytogenes* have previously been established (Aymerich *et al.* 2000a,b), no beneficial impacts were apparent when a natural enterocin A-producing strain was introduced into these foods as a source of the bacteriocin (Aymerich *et al.* 2000a). This phenomenon may be attributable to the micro-environments of the respective foods, the use of a lactococcal rather than enterococcal producer or the involvement of constitutive rather than natural, inducible promoter.

From the Cottage cheese data, it is apparent that the extent to which *L. monocytogenes* levels are reduced in the *L. lactis*$_{EntA+}$ containing cheese is inversely proportional to the number of the pathogens present, i.e. 0·99 (10^7), 1·14 (10^6) and 1·64 (10^5) log unit reductions over 15 days, or a reduction to below detectable levels after 2 days (10^4). These reductions are significant and would be expected to be further enhanced if *L. lactis*$_{EntA+}$ incorporation were to be employed as one of a number of preservative hurdles. Following on from this success, a number of approaches could be taken with a view to the further optimization of this approach. With respect to production levels, adjustments such as the utilization of promoters other than P32 merit investigation and from a stability perspective, the introduction of the enterocin A-associated genes into the lactococcal genome *via* double crossover homologous recombination would be beneficial, with an added bonus being that such an approach would reduce the level of nonlactococcal DNA present in the producing strain. It may also be possible to further extend the anti-listerial activity of this and other lactococcal strains through the production of enterocin A in conjunction with other anti-listerial bacteriocins. Thus, in addition to the first successful application of a heterologously produced enterocin in a food, there exist a number of additional adjustments which could make the application of such lactococcal producers a commercial reality.

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