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Growth inhibition of *Listeria monocytogenes* by a nonbacteriocinogenic *Carnobacterium piscicola*

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ABSTRACT

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Aims: This study elucidates the mechanisms by which a nonbacteriocinogenic *Carnobacterium piscicola* inhibits growth of *Listeria monocytogenes*.

Methods and Results: *Listeria monocytogenes* was exposed to live cultures of a bacteriocin-negative variant of *C. piscicola* A9b in co-culture, in a diffusion chamber system, and to a cell-free supernatant. Suppression of maximum cell density (0–3·5 log units) of *L. monocytogenes* was proportional to initial levels of *C. pisciola* (10^3–10^7 CFU ml^{-1}). Cell-to-cell contact was not required to cause inhibition. The cell-free *C. piscicola* supernatant caused a decrease in *L. monocytogenes* maximum cell density, which was abolished by glucose addition but not by amino acid, vitamin or mineral addition. The fermentate also gave rise to a longer lag phase and a reduction in growth rate. These effects were independent of glucose and may have been caused by acetate production by *C. piscicola*. 2D gel-electrophoretic patterns of *L. monocytogenes* exposed to *C. piscicola* or to *L. monocytogenes* fermentate did not differ. Treatment with *C. piscicola* fermentate resulted in down-regulation (twofold) of genes involved in purine- or pyrimidine metabolism, and up-regulation (twofold) of genes from the regulon for vitamin B_{12} biosynthesis and propanediol and ethanolamine utilization.

Conclusions: A nonbacteriocinogenic *C. piscicola* reduced growth of *L. monocytogenes* partly by glucose depletion.

Significance and Impact of the Study: Understanding the mechanism of microbial interaction enhances prediction of growth in mixed communities as well as use of bioprotective principles for food preservation.

Keywords: 2-chamber system, 2D PAGE, carnobacteriocin B2, *Carnobacterium piscicola*, DNA micro-arrays, glucose competition, *Listeria monocytogenes*, microbial interaction.

INTRODUCTION

Growth of the food-borne human pathogen, *Listeria monocytogenes*, to high numbers in ready-to-eat food products dramatically increases the disease risk of susceptible consumers (Chen *et al.* 2003). As it is virtually impossible to avoid cross-contamination during processing or after opening of food packages, a large number of studies have been devoted to developing principles that can inhibit growth of the pathogen in foods (reviewed by Nilsson and Gram 2002). Amongst the principles studied are combinations of lactate and diacetate (Mbandi and Shelef 2001), bacteriocins (Cleveland *et al.* 2001), and live bacterial (bioprotective) cultures (O'Sullivan *et al.* 2002).

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Concerns have been raised with respect to possible resistance development as a consequence of widespread use of pure bacteriocins or bacteriocinogenic lactic acid bacteria (Ennahar *et al.* 2000; Gravesen *et al.* 2002). Several studies have demonstrated that also lactic acid bacteria that do not produce bacteriocins are capable of controlling growth of *L. monocytogenes* in food products (Buchanan and Bagi 1997; Nilsson *et al.* 1999) although the precise mechanism of inhibition is not known.

Carnobacterium piscicola A9b which has been isolated from cold-smoked salmon inhibits the growth of *L. monocytogenes* by production of carnobacteriocin B2 (Nilsson *et al.* 2002). However, a plasmid-cured derivative of *C. piscicola* A9b (bac$^-$ mutant) that did not produce any extracellular antimicrobial compound also had a marked antilisterial effect, demonstrating the presence of a significant nonbacteriocin-dependent inhibition (Nilsson *et al.* 2004). It was suggested that the antilisterial effect of *C. piscicola* A9b bac$^-$ was because of nutrient competition as *L. monocytogenes* entered stationary phase earlier in co-cultures with *C. piscicola* bac$^-$ when compared with monocultures of *L. monocytogenes* (Nilsson *et al.* 2004).

Competition for nutrients is considered to be one of the major evolutionary driving forces in the bacterial world (Christensen *et al.* 2002). In food microbiology, several studies have shown that the population of an indigenous microbial flora suppresses the growth of pathogens in foods (Mattila-Sandholm and Skytta 1991; Carlin *et al.* 1996; Babic *et al.* 1997; Samelis *et al.* 1998; Vold *et al.* 2000). Although microbial competition is an important quality and safety issue in food manufacturing, only limited work has been conducted on mechanisms of action that do not include antimicrobial compounds. An interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts was mediated by cell-to-cell contact presumably as a result of competition for space (Nissen and Arneborg 2003).

Nonbacteriocin-producing lactic acid bacteria may hold great potential for bioprotection against *L. monocytogenes*, however, to control the activity and ensure a reliable and reproducible growth inhibition, knowledge of the mechanisms of inhibition and factors influencing them must be obtained. The purpose of this study was therefore to obtain insight into the mechanisms by which a nonbacteriocinogenic *C. piscicola* inhibits *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacteriocin-negative mutant of *C. piscicola* A9b, strain A9b bac$^-$ (Nilsson *et al.* 2004), and *L. monocytogenes* O57 (Ben Embarek and Huss 1993) were used throughout this study.

Media and nutrients

Carnobacterium piscicola A9B bac$^-$ and *L. monocytogenes* O57 were propagated in brain–heart infusion (BHI) broth (code CM225; Oxoid) at 30°C. When required, BHI broth and peptone saline (0·1% peptone) were buffered with 0·1 mol l^{-1} phosphate buffer (K_2HPO_4/KH_2PO_4) to pH of 6·2, giving B-BHI and B-PS respectively.

Stock solutions of glucose, vitamins, minerals and amino acids were prepared as a modification of the procedure described by Phan-Thanh and Gormon (1997). A 50% glucose solution (250-fold) was sterilized at 121°C for 15 min and stored at room temperature. For each amino acid and vitamin, a 100-fold concentrated stock solution was made with (g l^{-1}): L-leucine (1·0), DL-isoleucine (1·0), DL-valine (1·0), DL-methionine (1·0), L-arginine HCl (1·0), L-tryptophan (1·0), L-phenylalanine (1·0), L-cysteine (1·0), L-glutamine (6·0), biotin (0·05), thiamine HCl (0·1), pyridoxal HCl (0·1), calcium pantothenate (0·1), nicotinamide (0·1), para-aminobenzoiz acid (0·1), thioctic acid (alpha-lipoic acid) (0·0005), and riboflavin (0·5). The stock solutions of minerals contained 65·6 g l^{-1} KH_2PO_4 (10-fold concentrated), 16·32 g l^{-1} Na_2HPO_4 (10-fold concentrated), 4·1 g l^{-1} $MgSO_4$ (100-fold concentrated) and 4·4 g l^{-1} ferric citrate (50-fold concentrated). All amino acid, vitamin and mineral stock solutions were adjusted to pH 7·2, sterile filtered and stored at −20°C, with the exception of L-glutamine and L-cysteine, which were stored at room temperature, and biotin, thioctic acid and riboflavin, which were stored at 5°C.

Inoculum for culture competition and fermentate challenge experiments

Listeria monocytogenes O57 and *C. piscicola* A9b bac$^-$ were propagated twice at 30°C overnight in B-BHI broth, transferred to fresh B-BHI broth and grown until mid-exponential phase (O.D.$_{600}$ *ca* 0·4; Novaspec. II; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), and subsequently used as inoculum for batch culture competition, diffusion chamber competition or fermentate challenge experiments. Cultures were diluted in fresh media to appropriate cell numbers.

Batch culture competition between *L. monocytogenes* O57 and *C. piscicola* A9b bac$^-$

Listeria monocytogenes at an initial concentration of 10^3 CFU ml^{-1} was co-inoculated with 10^3, 10^4, 10^5, 10^6 or 10^7 CFU ml^{-1} *C. piscicola* A9b bac$^-$ and growth was followed by colony counts on selective media. Growth of monocultures of all cultures was followed in parallel. *Listeria monocytogenes* was enumerated by surface plating on *Listeria*-selective agar base (CM856; Oxoid) with *Listeria* selective

supplement (SR140; Oxoid). Plates were incubated at 25°C for 48 h. Cell numbers of *C. piscicola* were estimated by plating on nitrate polymyxin agar [NP, composed as nitrite actidion polymyxin (NAP) agar without actidion] based on APT agar (0654–17; Difco), pH 6·7 (Davidson and Cronin 1973). Plates were incubated at 25°C for 48 h. Pinpoint (<1 mm) colonies were not counted as these were *L. monocytogenes* capable of weak growth on NP agar.

The diffusion chamber growth system

A chamber with membrane-filter (0·2 μm pore size) side walls (Technical Services, College of Engineering, Montana State University, Bozeman, MT, USA) was used to study the interaction between live cells of *L. monocytogenes* and *C. piscicola* without mixing the two cultures (McFeters and Stuart 1972; Roper and Marshall 1978). The system consisted of three Plexiglas rings; the two membrane side walls were placed between either of the two outer rings and the central ring, which was 0·9 cm thick and constituted the peripheral wall of the inner chamber. The central ring was mounted with inlets allowing aseptical sampling. The inner chamber had a volume of 20 ml, and the membrane side walls were circular with a diameter of 5·7 cm. The unassembled chamber was sterilized at 121°C for 15 min, assembled aseptically and placed in a glass beaker. Sterilized B-BHI broth was added to the beaker (outer chamber) covering the inner chamber with fluid. Broth was loaded in the inner chamber using a syringe. The chamber system was covered with silver foil and heat-treated at 105°C for 30 min. The broth in the outer chamber was inoculated with the appropriate culture solution and the broth in the inner chamber was replaced with appropriately inoculated medium.

Diffusion chamber competition between *L. monocytogenes* O57 and *C. piscicola* A9b bac$^-$

Listeria monocytogenes O57 was inoculated at an initial level of 10^3 CFU ml^{-1} in the inner chamber and either *C. piscicola* A9b bac$^-$ (10^6 CFU ml^{-1}) or *L. monocytogenes* (10^3 CFU ml^{-1}) was added to the outer chamber. The beakers were incubated at 30°C with continuous stirring. Samples were removed aseptically every second hour for determination of viable cell count and pH. In control batch cultures with the same inoculum, *L. monocytogenes* O57 was grown as monoculture and as co-culture with *C. piscicola* A9b bac$^-$.

Challenge of *L. monocytogenes* O57 with fermentate from *C. piscicola* A9b bac$^-$ and effect of nutrient supplementation

Culture fermentate was used in challenge experiments with *L. monocytogenes*. *Carnobacterium piscicola* A9b bac$^-$ or *L. monocytogenes* O57 were grown overnight in B-BHI broth at 30°C and centrifuged (10 000 *g*, 30 min). The supernatant was filter sterilized (0·45 μm pore-size nylon membrane filter; Sartorius, Göttingen, Germany) and stored at −80°C. The glucose content of the two fermentates was determined using an enzymatic kit (R-Biopharm; Boehringer Mannheim GmbH, Darmstadt, Germany). *Listeria monocytogenes* O57 exponential cells were inoculated at *ca* 10^3 cells ml^{-1} into microtitre plate wells containing B-BHI broth with *C. piscicola* A9b bac$^-$ fermentate at 0, 10, 20, 30, 40, 50, 60, 70 or 80% v/v. As control, B-PS was added at the same concentrations. Bacterial growth at 30°C in each media was determined in triplicate by measuring O.D.$_{600}$ at 10-min intervals using a temperature-controlled automatic microtitre plate reader (Bioscreen C Unit; Transgalactic Ltd., Helsinki, Finland).

For the nutrient supplementation experiment, fermentate of *C. piscicola* A9b bac$^-$ and B-PS was added to the growth medium of *L. monocytogenes* at 60% v/v. Ten sets of nutrient supplementation combinations were performed, where bac$^-$ fermentate or B-PS were supplemented with stock solutions (see above for media preparation) of: (i): glucose, (ii) vitamins, (iii) minerals, (iv) amino acids, (v) glucose and vitamins (vi) glucose, vitamins and amino acids, (vii) glucose and minerals, (viii) glucose, minerals, and amino acids, (ix) glucose, vitamins, and minerals, (x) glucose, vitamins, minerals, and amino acids. Nutrient components were added at final concentrations corresponding to those in a chemically defined minimal medium for *Listeria* spp. (Phan-Thanh and Gormon 1997).

Analysis of growth data

O.D.$_{600}$ as a function of time was fitted to a logistic growth model (Dalgaard and Koutsoumanis 2001). The O.D.$_{max}$ parameter of the model was kept fixed as the max. O.D.$_{600}$ observed within 20 h of incubation. Fitting was carried out using the method of least squares resulting in estimates of the maximum specific growth rate, μ_{max}. In addition, ΔO.D.$_{600}$ was calculated as the difference between the observed O.D.$_{max}$ and observed O.D.$_{min}$ and the time to reach an increase in O.D.$_{600}$ of 0·1, $T_{0·1}$, was also determined. For nonautomatically generated growth data, ΔO.D.$_{600}$ was expressed as the initial O.D.$_{600}$ subtracted from the O.D.$_{600}$ obtained after a 3-h incubation period.

To analyse the effect of bac$^-$ fermentate on the growth of *L. monocytogenes*, ΔO.D.$_{600}$,μ_{max}, and $T_{0·1}$ were used as response variables in analyses of variance with fixed effects of the dilution media (bac$^-$ fermentate and B-PS) and of the dilution factors (0, 10, 20, 30, 40, 50, 60, 70 and 80%). In addition, ΔO.D.$_{600}$ was tested in a covariate analysis with dilution factor as covariate and dilution medium as a fixed effect. The ΔO.D.$_{600}$ for the nonautomatically generated

growth data was tested in an analysis of variance with fixed effect of the growth media B-BHI, 50% B-PS, 50% bac$^-$ fermentate and 50% *L. monocytogenes* O57 fermentate.

For the nutrient supplements with glucose, amino acids, vitamins, minerals and all four components, ΔO.D.$_{600}$ and $T_{0{\cdot}1}$ were tested pairwise for the following five growth media: B-BHI, 60% B-PS, 60% B-PS with nutrient supplement, 60% bac$^-$ fermentate and 60% bac$^-$ fermentate with nutrient supplement. Additionally, μ_{max} was tested in the case of glucose supplement.

The procedure GLM in the SAS system for Windows, release 8.02 TS level 02MO Windows version 5.1.2600 (SAS Institute Inc., Cary, NC, USA), was used for all tests and when pairwise tests were conducted the statement LSMEANS and the option PDIFF was used.

Challenge of *L. monocytogenes* O57 with fermentate from *C. piscicola* A9b bac$^-$ for molecular analyses

To harvest cells for protein and RNA extraction, the challenge of *L. monocytogenes* with culture fermentates was performed in batch cultures. Overnight cultures of *L. monocytogenes* O57 in B-BHI broth at 30°C were inoculated into fresh media and maintained in exponential growth phase for several generations (*ca* 24 h; O.D.$_{600}$ kept below 0·2 by recurring dilution). A sample of exponential *Listeria* cells was taken for protein extraction prior to additions. Subsequently, exponentially growing *L. monocytogenes* cells were exposed to four different treatments (added at 50% v/v): (i) fermentate of *L. monocytogenes* O57, (ii) fermentate of *C. piscicola* A9b bac$^-$, (iii) fresh B-BHI and (iv) B-PS. Cultures were incubated at 30°C and samples for plate counting on BHI agar and O.D.$_{600}$ were taken every 30 min. At transition to stationary phase ($t = 80$ min), cells from the two fermentate-treated cultures (treatments i and ii) were harvested for RNA isolation. Two hours after the entry into stationary phase ($t = 3{\cdot}5$ h for i, ii and iv; $t = 4{\cdot}5$ h for iii), cells were collected for protein extraction.

Proteome analysis of fermentate-treated *L. monocytogenes* O57

Total protein extraction from *L. monocytogenes* cells and two-dimensional gel electrophoresis (2D PAGE) were performed as described by Ramnath *et al.* (2003). Briefly, chloramphenicol was added at a final concentration of 20 μg ml^{-1} to halt protein synthesis. Protein concentrations were determined with a PlusOne 2-D Quant kit (Amersham Biosciences). Isoelectrofocusing for the first dimension was performed using 11 cm precast Immobiline DryStrip with a linear gradient at pH 4–7 (Amersham Biosciences); *ca* 200 μg protein were cup loaded at the anodic end. The second dimension separation was performed with precast ExcelGel XL SDS 12–14 gels using a Multiphore II apparatus (Amersham Biosciences). Gels were stained with Coomassie colloidal blue G250 (Neuhoff *et al.* 1988). For each growth condition, proteins were extracted from cells harvested in two independent experiments, and two to four gels were run of each protein sample (i.e. four to eight gels in total per growth condition). Representative gels from each growth condition were compared, and observed differences in spot intensity were verified in all gels from both experiments. Only differences that were apparent in all gels from each growth condition were considered.

Transcriptome analysis of fermentate-treated *L. monocytogenes* O57

RNA was isolated from cells harvested in two independent experiments essentially as described previously (Gravesen *et al.* 2000, 2002) but using the RNeasy midi kit (Qiagen) with in-column DNase digestion. The RNA samples were used for DNA array and Northern blot hybridizations.

Whole-genome arrays of *ca* 500 bp internal gene PCR products spotted onto nylon membranes were hybridized with ^{33}P-labelled single-stranded cDNA as described recently (Milohanic *et al.* 2003). Two membranes were hybridized with the labelled cDNA of bac$^-$ fermentate and O57 fermentate-treated cultures of *L. monocytogenes* O57 harvested at the onset of stationary phase. Spot intensities were quantified and visualized using the ARRAYVISION software (Imaging Research, St Catherines, ON, Canada). After visualization of the signal, the membranes were stripped and the probes were swapped, but using cDNA from an independent experiment. The ratios of expression in the bac$^-$ fermentate-treated culture to expression in the O57 fermentate-treated culture were calculated, and genes that in both experiments had at least 2·0-fold difference in expression were considered. The average ratios and confidence interval at the 99% significance level were calculated.

Northern blotting employing 3 μg RNA was performed as previously described (Gravesen *et al.* 2000, 2002), but using the Megaprime DNA labelling system (Amersham Biosciences) to ^{33}P-label the probes. For the *pyrR* gene, the probe was a 506-bp PCR product made with primers pyrR-F1, 5′-ATGGACGAGGCGGCAATCAAAC-3′, and pyrR-R1, 5′-GCATCTTCTGCATGATCCACATCTG-3′. For *lmo1885*, a 455-bp PCR product made with 1885-F1, 5′-GGTTTTAATGCAAGCGATGG-3′, and 1885-R1, 5′-CGTTTTCAAGTGAGGCAATTC-3′, was used. For *cbiP*, a 597-bp PCR product made with primers cbiP-F1, 5′-AAGCCCAGCAGAAATCAAC-3′, and cbiP-R1, 5′-CCACCACAAATCCCGATTAC-3′, was used. The signal intensities were quantified using the ImageQuant

software version 5.0 (Molecular Dynamics; Amersham Biosciences).

RESULTS

Batch culture competition between *L. monocytogenes* O57 and *C. piscicola* A9b bac⁻

In batch co-cultures of *L. monocytogenes* O57 and *C. piscicola* A9b bac⁻, *L. monocytogenes* reached maximum cell numbers of 8×10^5, 2×10^7, 2×10^8, 10^9 and 2×10^9 CFU ml^{-1} in the presence of initial levels of 10^7, 10^6, 10^5, 10^4 and 10^3 CFU ml^{-1} of *C. piscicola* A9b bac⁻ respectively (Fig. 1a). The corresponding maximum cell numbers of *C. piscicola* A9b bac⁻ were 10^9, 10^9, 10^9, 5×10^8 and 3×10^8 CFU ml^{-1} respectively.

Diffusion chamber culture competition between *L. monocytogenes* O57 and *C. piscicola* A9b bac⁻

As the cultures in the diffusion chamber system were physically separated by membranes, interaction could only happen through the diffusion of extracellular compounds. Growth of *L. monocytogenes* in monoculture reached a maximum cell number of 10^9 CFU ml^{-1} after 17 h of incubation at 30°C (Fig. 1b). No substantial difference was observed between growth of *L. monocytogenes* in the inner and outer chamber or as monoculture. However, the maximum cell number of *L. monocytogenes* in the inner chamber was reduced by *ca* 3 log units when *C. piscicola* A9b bac⁻ was growing in the outer chamber, while the initial growth rate was unaffected by the presence of *C. piscicola* A9b bac⁻ (Fig. 1b). The growth suppression of *L. monocytogenes* was similar to the inhibitory effect obtained in co-culture with *C. piscicola* A9b bac⁻.

Challenge of *L. monocytogenes* O57 with fermentate from *C. piscicola* A9b bac⁻

To investigate if the addition of *C. piscicola* A9b bac⁻ fermentate could provide a simplified model for the inhibition of *L. monocytogenes* O57, exponential cultures were treated with increasing amounts of *C. piscicola* A9b bac⁻ fermentate or B-PS. The maximum cell density of *L. monocytogenes* decreased with increasing concentrations of bac⁻ fermentate or B-PS added to the growth medium (Fig. 2), similar to the effect of increasing start inocula of *C. piscicola* A9b bac⁻ in the co-culture experiment (Fig. 1a). Furthermore, addition of increasing concentrations of bac⁻ fermentate extended the lag phase of *L. monocytogenes* and also reduced growth rate.

ΔO.D.$_{600}$ was inversely linearly correlated to the added amount of bac⁻ fermentate or B-PS (Fig. 3a) with regression

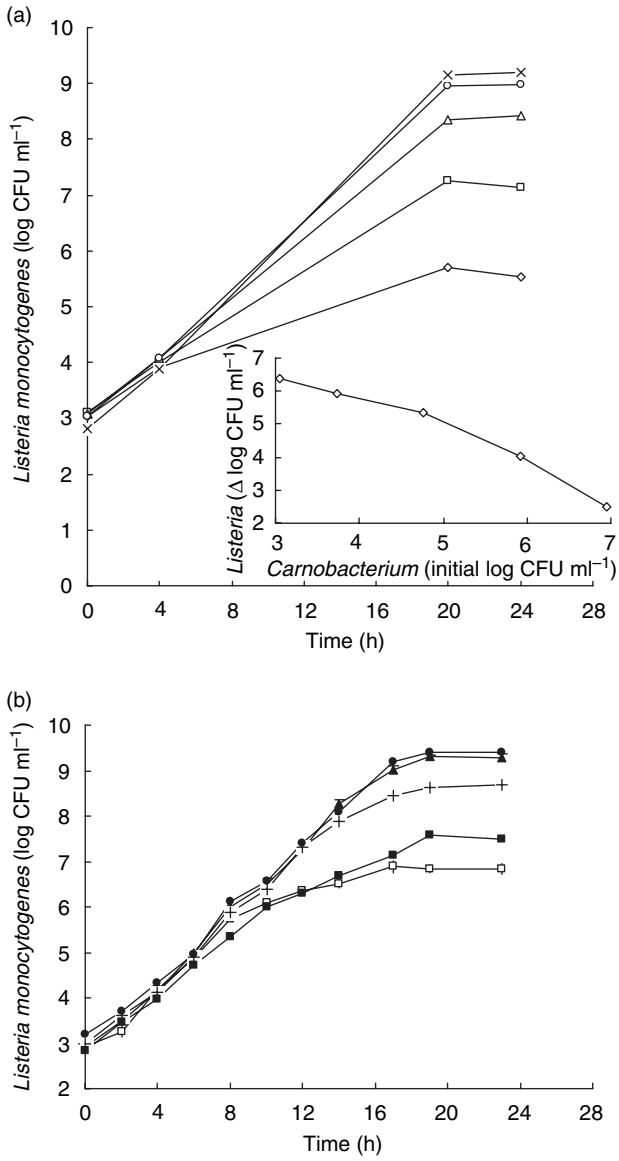

Fig. 1 Growth of *Listeria monocytogenes* O57 in buffered brain–heart infusion broth at 30°C (start inoculum *ca* 10^3 CFU ml^{-1}) in a mixed batch co-culture (a) with *Carnobacterium piscicola* A9b bac- (start inoculum *ca* 10^3 (×), 10^4 (○), 10^5 (△), 10^6 (□) or 10^7 (◇) CFU ml^{-1}). The insert depicts maximum growth of *L. monocytogenes* O57 as a function of *C. piscicola* A9b bac⁻ start inoculum. Growth of *L. monocytogenes* in diffusion chamber (b) as monoculture in the inner diffusion chamber (+), as monoculture in the outer diffusion chamber (●) or as co-culture (inner chamber) (■) with *C. piscicola* (outer chamber, start inoculum 10^6 CFU ml^{-1}). Growth in batch system as monoculture (▲) or as co-culture with *Carnobacterium piscicola* (□)

coefficients of 0·971 and 0·959 respectively. The decrease in ΔO.D.$_{600}$ when compared with the untreated culture (0% addition to B-BHI) was statistically significant for all levels of bac⁻ fermentate ($P \leq 0{\cdot}017$) and for 20% or higher B-PS

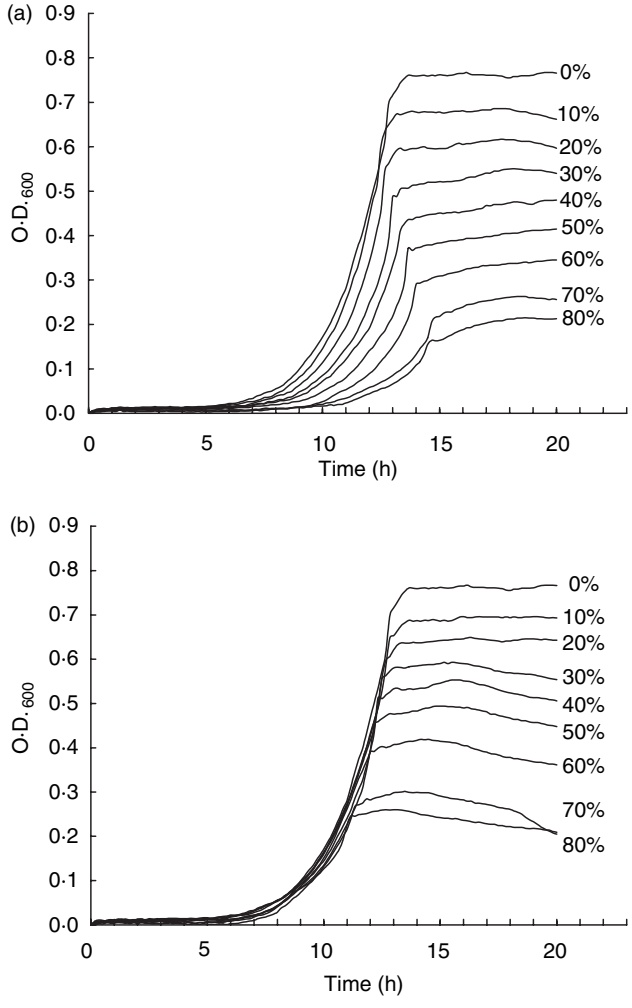

Fig. 2 Growth of *Listeria monocytogenes* O57 in buffered brain–heart infusion (B-BHI) broth at 30°C treated with 0 to 80% cell-free supernatant from *Carnobacterium piscicola* A9b bac- (bac- fermentate) or buffered peptone saline (B-PS). The optical density ($O.D._{600}$) was measured at 10 min intervals with an automatic microplate reader; triplicate determinations of each culture were made and representative growth curves are shown

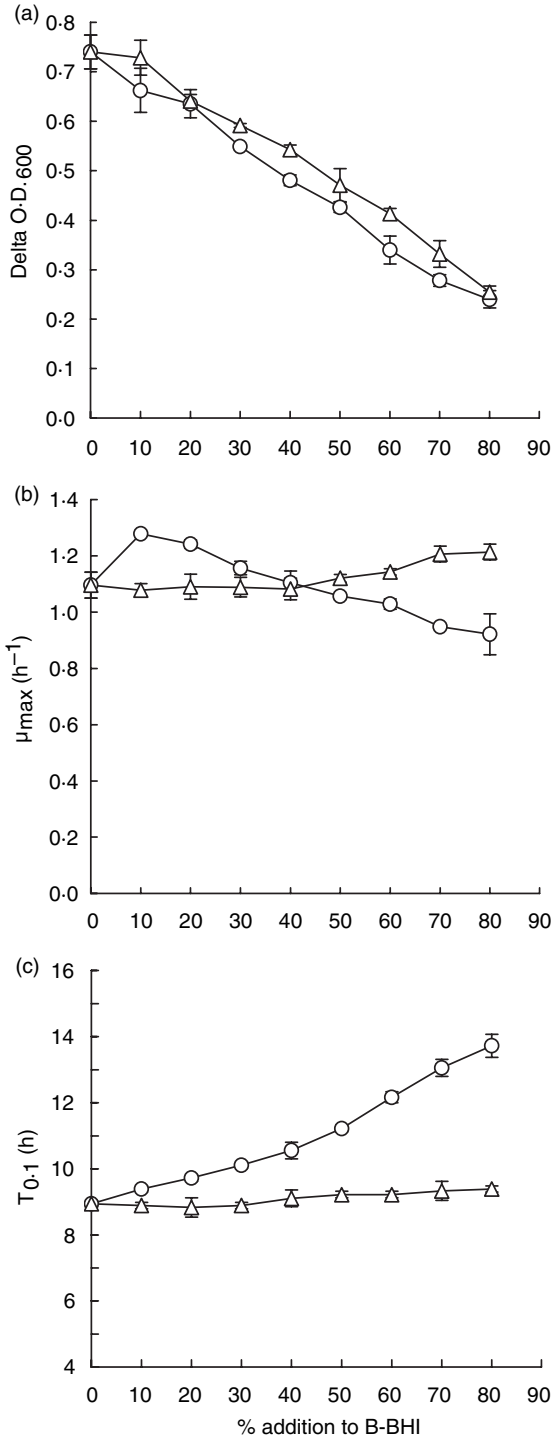

Fig. 3 Increase in optical density (delta $O.D._{600}$) (a), maximum specific growth rate (μ_{max}) (b), and time to reach delta $O.D._{600}$ of 0·1 ($T_{0\cdot1}$) (c) for cultures of *Listeria monocytogenes* O57 treated with 0 to 80% cell-free supernatant from *Carnobacterium piscicola* A9b bac^- (bac- fermentate, ○) or buffered peptone saline (B-PS, △). The parameters were calculated as averages from triplicate determinations, as described in Materials and methods. Representative growth curves are shown in Fig. 2

($P \leq 0{\cdot}002$). The bac^- fermentate resulted in a significantly larger reduction in $\Delta O.D._{600}$ than B-PS ($P < 0{\cdot}001$).

The relationship between μ_{max} and content of bac^- fermentate was nonlinear (Fig. 3b). Low levels of bac^- fermentate increased μ_{max} significantly ($P \leq 0{\cdot}043$) whereas high levels of bac^- fermentate resulted in a significant decrease in μ_{max} ($P \leq 0{\cdot}034$). μ_{max} of the B-PS control was not significantly different from the untreated culture except for 70 and 80%, where μ_{max} was increased ($P < 0{\cdot}001$). It was observed that the μ_{max} estimate was not very robust; the values increased for shorter growth periods. Comparison of the growth curves (Fig. 2) with the μ_{max} values (Fig. 3b)

indicated that the increase in μ_{max} for 70 and 80% B-PS could be an artefact as a result of overestimation by the model. However, the changes in μ_{max} calculated for the bac$^-$ fermentate-treated cultures were reflected in the growth curves.

As a measure of lag phase, the last panel in Fig. 3c depicts the time to reach an O.D.$_{600}$ of 0·1, $T_{0·1}$. All concentrations of bac$^-$ fermentate gave a statistically significant ($P \leq 0·010$) increase in $T_{0·1}$ compared with the untreated culture. $T_{0·1}$ of the B-PS treatment was not significantly different from the untreated culture except for 70 and 80%, where there was a marginal increase ($P \leq 0·016$).

Supplementation of bac$^-$ fermentate with various nutrients

Growth of *L. monocytogenes* O57 cultures was monitored in 60% bac$^-$ fermentate or B-PS supplemented with glucose, amino acids, vitamins and minerals, or all four components (Fig. 4). Addition of glucose (Fig. 4a) increased ΔO.D.$_{600}$ significantly ($P < 0·001$) when compared with the cultures with 60% bac$^-$ fermentate or B-PS, but $T_{0·1}$ was not affected ($P \geq 0·205$). For both cultures with glucose, O.D.$_{600}$ reached a higher level than in an untreated B-BHI culture, but declined relatively quickly after entry into stationary phase. Furthermore, glucose treatment caused a slight reduction in the growth rate. Estimation of μ_{max} showed a significant reduction in the glucose-supplemented cultures compared with the cultures with 60% fermentate or B-PS ($P \leq 0·007$) and compared with the untreated B-BHI culture ($P \leq 0·001$).

The addition of amino acids (Fig. 4b) had no significant effect on ΔO.D.$_{600}$ or $T_{0·1}$ in either culture ($P \geq 0·388$). The vitamin mix (Fig. 4c) significantly increased $T_{0·1}$ in the two cultures ($P \leq 0·038$) and there is a tendency that ΔO.D.$_{600}$ is reduced (not significant). Supplementation with minerals (Fig. 4d) in the culture with 60% bac$^-$ fermentate caused a reduction of ΔO.D.$_{600}$ as well as $T_{0·1}$ ($P < 0·001$). In the B-PS culture, the minerals similarly reduced ΔO.D.$_{600}$ ($P < 0·001$).

Addition of glucose, amino acids, vitamins and minerals simultaneously (Fig. 4e) increased ΔO.D.$_{600}$ significantly ($P < 0·001$) in the bac$^-$ fermentate and the B-PS cultures, although not up to the level of the untreated B-BHI culture. In the fermentate-treated culture, the four components together gave a reduction in $T_{0·1}$ ($P < 0·001$), however, $T_{0·1}$ was increased in the culture with B-PS ($P = 0·008$).

Combinations of glucose and other nutrients were added to the culture with 60% bac$^-$ fermentate (results not shown). All combinations with glucose gave an increased ΔO.D.$_{600}$ ($P < 0·001$). $T_{0·1}$ was significantly increased ($P < 0·001$) by combinations containing vitamins (glucose plus vitamins; glucose plus vitamins and amino acids), and reduced ($P < 0·001$) by combinations containing minerals (glucose plus minerals; glucose plus minerals and amino acids). The combination of glucose, vitamins and minerals had no significant affect on $T_{0·1}$ ($P = 0·388$).

Molecular analyses of bac$^-$ fermentate challenge of *L. monocytogenes* O57

Batch cultures of *L. monocytogenes* O57 were treated with 50% *C. piscicola* A9b bac$^-$ fermentate, and used for proteome and transcriptome analyses of the inhibitory effect. Addition of bac$^-$ fermentate, B-PS or O57 fermentate reduced the maximum cell density of *L. monocytogenes* in four independent growth experiments (Fig. 5), as seen in the microtitre experiments with bac$^-$ fermentate and B-PS (see above). Additionally, ΔO.D.$_{600}$ of the bac$^-$ fermentate-treated cultures were consistently lower ($P = 0·011$) than that of the B-PS-treated cultures. Both the bac$^-$ and the O57 fermentate were depleted of glucose, i.e. the concentrations were below the detection limit of the enzymatic kit.

To understand the growth inhibitory effect of bac$^-$ fermentate, the protein profile (proteome) of *L. monocytogenes* in early stationary phase was studied (Fig. 5). No consistent differences were observed between the protein profiles of *L. monocytogenes* treated with the bac$^-$ fermentate, O57 fermentate, B-PS or B-BHI. However, substantial differences between *L. monocytogenes* cells collected from the exponential and early stationary phase of growth were observed. The most pronounced differences were up-regulation of 10 spots and decreased intensity of two spots in early stationary phase compared with exponential growth (Fig. 6). The protein profile of *L. monocytogenes* O57 was compared with the location of proteins from strain EGDe that were identified in previous work (Ramnath *et al.* 2003; Folio *et al.* in press). Spot 1, which had the largest increase in intensity in early stationary phase, could possibly correspond to Fri, a nonhaeme iron-binding ferritin encoded by *lmo0943*. Spots 2 and 8, which both had a lesser increase, corresponded to GroES (*lmo2069*) and succinyldiaminopimelate desuccinylase (*lmo2511*), respectively, and the down-regulated spot 11 to CspB (*lmo2016*). Both the early stationary and the exponential cultures in this study had pronounced increased expression of one spot compared with the exponential culture of strain O57 grown at 37°C in a previous study (Ramnath *et al.* 2003). This spot was located at a similar position as the flagellin protein, FlaA (Folio *et al.* in press). The increased flagellin expression at 30°C is in accordance with the temperature-dependent production of flagella in *Listeria* (Peel *et al.* 1988).

To screen for a possible specific inhibitory mechanism conferred by the bac$^-$ fermentate, gene expression in a bac$^-$ fermentate-treated culture was compared with that of an O57 fermentate-treated culture by total genome DNA

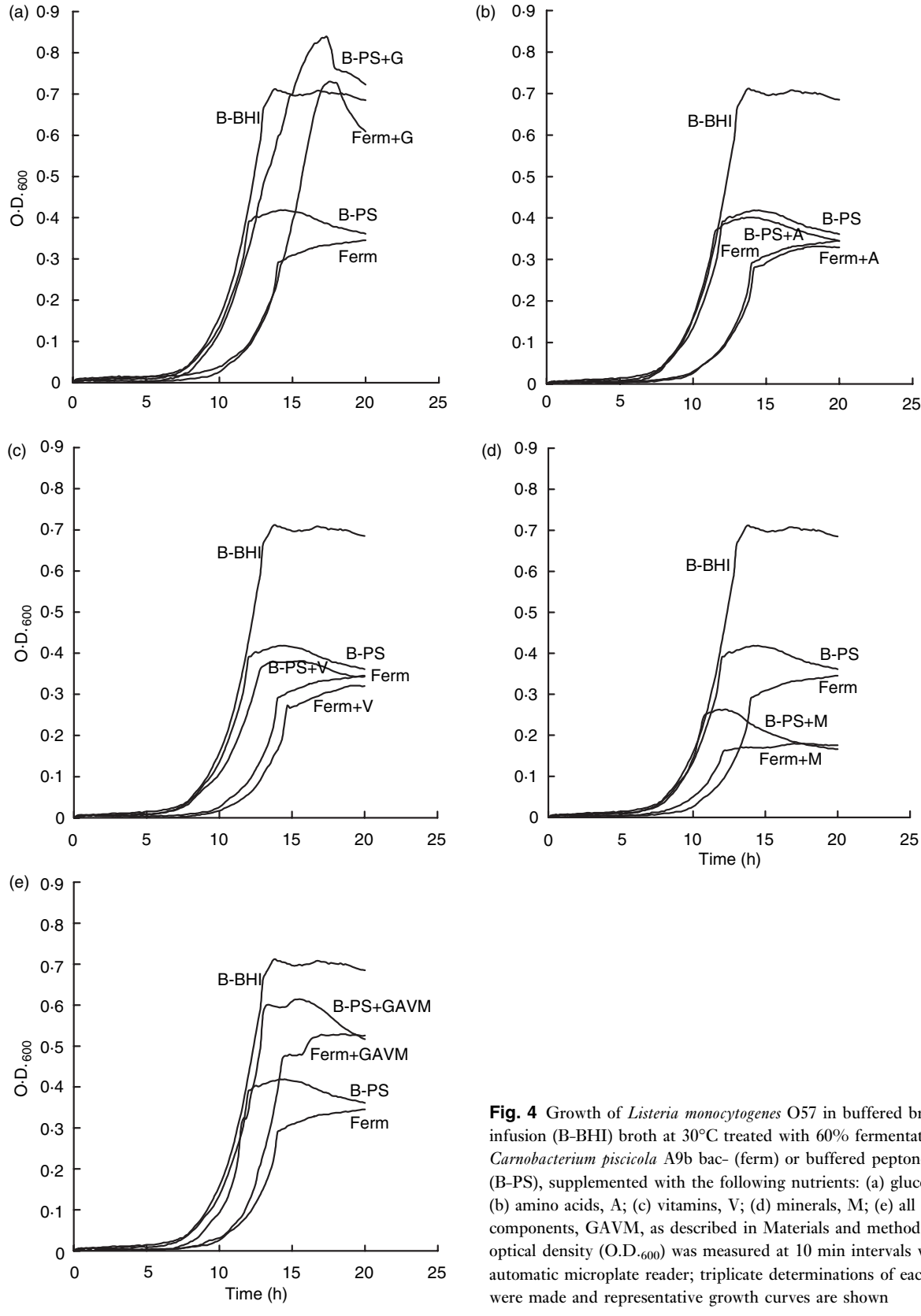

Fig. 4 Growth of *Listeria monocytogenes* O57 in buffered brain–heart infusion (B-BHI) broth at 30°C treated with 60% fermentate from *Carnobacterium piscicola* A9b bac- (ferm) or buffered peptone saline (B-PS), supplemented with the following nutrients: (a) glucose, G; (b) amino acids, A; (c) vitamins, V; (d) minerals, M; (e) all four components, GAVM, as described in Materials and methods. The optical density ($O.D._{600}$) was measured at 10 min intervals with an automatic microplate reader; triplicate determinations of each culture were made and representative growth curves are shown

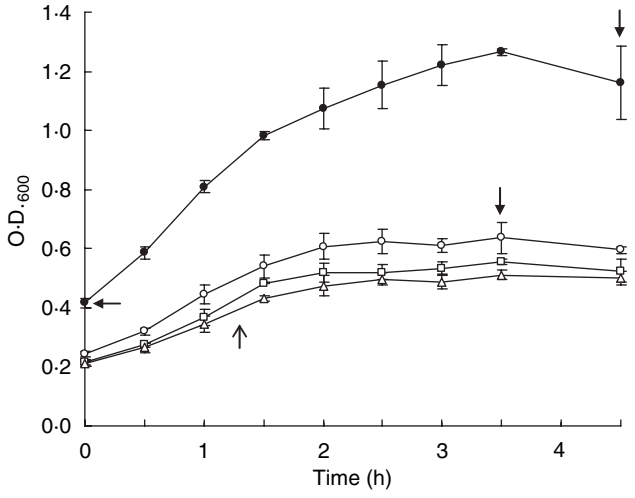

Fig. 5 Growth of *Listeria monocytogenes* O57 in buffered brain–heart infusion broth at 30°C. At time zero, the exponential culture of *L. monocytogenes* was treated with 50% v/v of *Carnobacterium piscicola* A9b bac⁻ fermentate (△), *L. monocytogenes* O57 fermentate (□), buffered peptone saline (○). The untreated exponential culture of *L. monocytogenes* O57 (●) is also shown. The error bars indicate standard deviations of two to four independent experiments. The arrows indicate harvest of cells for protein analyses (closed arrows; at 0, 3·5, and 4·5 h for the untreated exponential control, the three fermentate and saline-treated cultures, and the untreated stationary control respectively) or for RNA analyses (open arrow; at 1·3 h for the two fermentate-treated cultures)

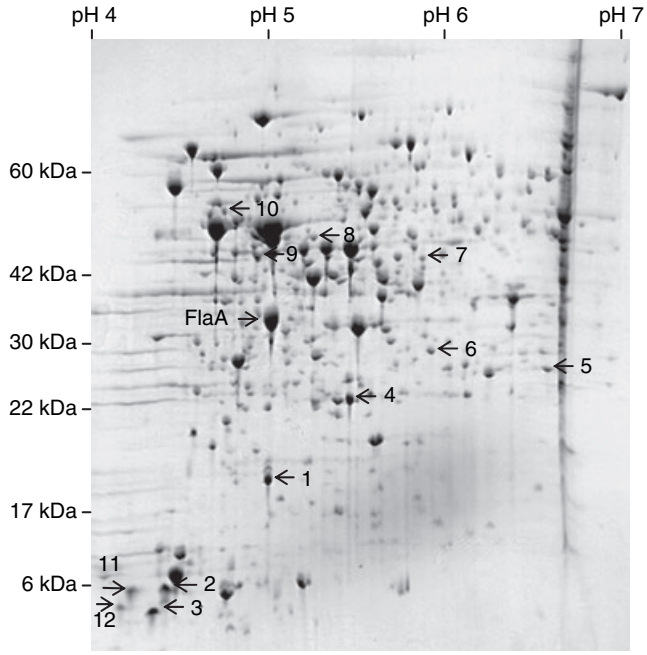

Fig. 6 Two-dimensional gel electrophoresis of proteins extracted from an early stationary culture of *Listeria monocytogenes* O57. The indicated spots had distinct increase (spots 1–10) or decrease (spots 11 and 12) in intensity compared to an exponential culture

macroarray analysis. Cells were harvested at the onset of stationary phase (Fig. 5), i.e. at the time that the inhibition of the cultures was apparent, in two independent growth experiments. Nine genes consistently had at least 2·0-fold difference in expression between the two treatments in both of the growth experiments (Table 1). The significance of these changes was supported by the observation that a further number of genes from the same regulons or pathways had differential expression in one of the two experiments.

There was about twofold lower expression following bac⁻ fermentate treatment in *lmo1885* (encoding a putative protein similar to xanthine phosphoribosyltransferase) and *groES* (heat-shock protein) (Table 1), where the former is involved in purine metabolism. The *lmo1884* (similar to xanthine permease) and *groEL* genes, which are transcriptionally linked to *lmo1885* and *groES*, respectively, similarly had over twofold lower expression following bac⁻ fermentate treatment in one of the two growth experiments (average ratios of 0·53 and 0·55 respectively). Additionally, three genes involved in pyrimidine metabolism, *pyrR*, *pyrAB*, and *pyrAa*, had at least 2·0-fold lower expression in one of the experiments (average ratios of 0·47, 0·62 and 0·69 respectively). To further test the changes in the pyrimidine and purine metabolism, the *pyrR* and *lmo1885* gene expression was analysed by Northern blotting. Quantification of the signals gave ratios of 0·44 and 0·54, respectively, for bac⁻ vs

Table 1 Consistent differences in gene expression of at least 2·0-fold between bac- fermentate vs O57 fermentate-treated *Listeria monocytogenes* O57 determined by macroarray analysis

Gene	Function from homology*	Average ratio†
lmo1885	Xanthine phosphoribosyltransferase	0·48 ± 0·02
groES	Class I heat-shock protein (chaperonin)	0·47 ± 0·09
lmo0866	ATP-dependent RNA helicase	3·23 ± 0·63
lmo1155	Diol dehydrase gamma subunit (PduE‡)	2·81 ± 1·46
lmo1157	Diol dehydratase-reactivating factor small chain (PduH‡)	3·24 ± 3·05
lmo1158	Propanediol utilization protein, PduK	4·75 ± 0·09
lmo1159	Carboxysome structural protein (PduJ‡)	2·51 ± 0·26
lmo1161	Ethanolamine utilization protein, EutJ	2·87 ± 2·07
cbiL	S-adenosyl-methionine:precorrin-2-methyltransferase	2·82 ± 1·06

*According to http://genolist.pasteur.fr/ListiList/.
†Average of one determination from each of two independent growth experiments, ±CI at the 99% significance level.
‡Buchrieser *et al.* (2003).

O57 fermentate-treated cultures, verifying the results of the array analysis.

Of the seven genes with higher expression following bac$^-$ fermentate treatment (Table 1), six were from the vitamin B_{12} biosynthesis (*cbi*), propanediol (*pdu*) and ethanolamine (*eut*) utilization regulon (Buchrieser *et al.* 2003). A further 17 genes of the *cbi-pdu-eut* regulon had at least twofold higher expression in the bac$^-$ fermentate-treated culture in one of the experiments (average ratios from 1·58 to 2·24). Northern blot verification proved to be difficult because of low expression of the genes. However, the increased expression of *cbiP*, which is the last gene of the regulon, was detectable. This gene had an average ratio of 1·72 in the array hybridization, and quantification of the Northern blot signals gave a ratio of 1·3.

DISCUSSION

This study presents, to our knowledge, the first mechanistic investigation of nonbacteriocinogenic inhibition of a food-borne pathogenic bacterium by lactic acid bacteria. Non-bacteriocinogenic *C. piscicola* were observed to repress the maximum cell density of *L. monocytogenes*, and nutrient depletion was indicated to be involved (Buchanan and Bagi 1997; Nilsson *et al.* 2004). In the present work, we note that the degree of depression was proportional to the initial cell density of the inhibitory organism. This observation indicates that the inhibition of *L. monocytogenes* by *C. piscicola* could be due to a ratio-dependent competition. In general, competition between micro-organisms arises when they compete for the same ecological niche (Boddy and Wimpenny 1992), and several observations indicate that this is the case for *C. piscicola* and *L. monocytogenes*. The two organisms can be found on the same food products, e.g. cold-smoked salmon, and they utilize some of the same nutrients, e.g. both deplete laboratory media of glucose (Nilsson *et al.* 2002; Jydegaard-Axelsen *et al.* in press). Glucose depletion was confirmed for the conditions and the two strains used in this study. Furthermore, our data showed that growth of *C. piscicola* was also slightly restricted by the presence of *L. monocytogenes* at similar initial levels of the two organisms.

The diffusion chamber, which has been designed to study survival and persistence of pathogenic agents in situ in natural aquatic environments (Roper and Marshall 1978; Kreader 1998), has, to our knowledge, not been used before to study bacterial interactions. Using this technique, we were able to demonstrate that the inhibitory activity of *C. piscicola* strain A9b bac$^-$ did not require cell-to-cell contact but was diffusible through a 0·2-μm pore, which precludes competition for space.

The inhibitory activity of a crude fermentate from *C. piscicola* A9b bac$^-$ was similar to the co-culture systems, and was used as a model to study changes in the target organism. The inhibitory activity of bac$^-$ fermentate could partly be explained by glucose limitation: first, the depression of maximum cell density was similar to that obtained when exposing the *Listeria* culture to an equal volume of diluent and secondly, the depression was abolished when supplementing the system with glucose. However, the addition of fermentate also influenced lag phase and had a slight impact on growth rate indicating that additional 'factors' contribute to the interaction. As opposed to the *Lactobacillus*, *Carnobacterium* spp. are sensitive to acetate (Baya *et al.* 1991; Holley *et al.* 2002), however, *Carnobacterium* can under some conditions produce acetate (Hansen 1995; Leisner 2002) although this is not always the case (Joffraud *et al.* 2001). Acetate acts as an inducing factor for bacteriocin production in *C. piscicola* A9b (Nilsson *et al.* 2002). *Listeria monocytogenes* is also sensitive to acetate (Kouassi and Shelef 1996) and we hypothesize that the additional inhibitory factor present in *Carnobacterium* fermentate could be acetate. Indeed, HPLC analysis of the bac- fermentate revealed that *C. piscicola* A9B bac$^-$ had produced *ca* 14 mM acetate under the used growth conditions (data not shown). This suggests that acetate could give rise to the increase in lag phase and reduction of growth rate of *L. monocytogenes* exposed to bac- fermentate. Thus, the observed effects on maximum cell density, lag phase, and growth rate are presumably a combination of glucose deficiency and toxicity of acetate, and possibly also other as yet unidentified factors.

Analysis of gene transcription and protein expression may uncover the mechanisms that enable micro-organisms to grow under sub-optimal conditions. The response of *L. monocytogenes* to adverse conditions such as low temperature or a high osmotic environment includes expression of a range of stress proteins as revealed by 2D gel electrophoresis (Duche *et al.* 2002; Liu *et al.* 2002). The protein profiles of *L. monocytogenes* in early stationary phase were similar for cultures exposed to bac$^-$ fermentate, to its own fermentate, or to buffered saline. In contrast, the proteomic profile revealed major differences in protein expression between exponential and stationary phase cultures, as also demonstrated recently for *Bacillus subtilis* (Bernhardt *et al.* 2003). Thus, it was not possible to further characterize the *C. piscicola* A9b bac$^-$ inhibition mechanism by proteomic analyses, however, the 2D gel electrophoresis methodology is known to under-represent e.g. membrane proteins, as discussed previously (Ramnath *et al.* 2003).

DNA-microarray analysis has been used to identify the genes involved in bacterial response to stressful conditions (Tucker *et al.* 2002) or to elucidate the mechanisms of antibacterial systems (Hong *et al.* 2003; Utaida *et al.* 2003; Hansen *et al.* 2004). This prompted us to use whole-genome DNA microarrays to evaluate if any particular

defence systems were used by *L. monocytogenes* when exposed to *Carnobacterium* when compared with exposure to itself. A limited number of genes were differentially affected and only to a limited degree. However, the array data supported the findings that glucose competition was an important mechanism of interaction: the increase in *pdu–eut* regulon transcription suggests that *Listeria* is 'searching' for an alternative carbohydrate source. The decrease in expression of pyrimidine biosynthetic genes, on the other hand, could indicate that *C. piscicola* A9b bac$^-$ does not deplete pyrimidine precursors as efficiently as *L. monocytogenes* O57.

As disease is primarily associated with consumption of high numbers of the organism (Chen *et al.* 2003), restricting *L. monocytogenes* growth is indeed a viable risk management option if initial contamination levels are kept low. This study corroborates earlier observations (Buchanan and Bagi 1997; Nilsson *et al.* 2002, 2004) that nonbacteriocinogenic lactic acid bacteria can control growth of *L. monocytogenes*, and demonstrates that competition for nutrients, in this case glucose, is an important mechanism. Concerns have been expressed that biopreservation using bacteriocinogenic lactic acid bacteria (or pure bacteriocins) may be hampered by bacteriocin resistance. Nutrient competition is unlikely to cause development of resistance and thus represents an effective supplement to biopreservation with bacteriocins. The gained insight helps our understanding of microbial interactions in food, thereby enhancing the options for future use of biocontrolling cultures.

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