Acta Alimentaria, Vol. 32 (1), pp. 103–111 (2003)

STUDIES ON THE GROWTH OF *LISTERIA MONOCYTOGENES* AND *LACTOBACILLUS CASEI* IN MIXED CULTURES

M. PÁLMAI[a]* and G. KISKÓ[b]

[a] Department of Food Microbiology and Food Hygiene, National Institute of Food Hygiene and Nutrition, H-1476 Budapest, P.O. Box 52. Hungary
[b] Department of Microbiology and Biotechnology, Faculty of Food Science, Szent István University, H-1118 Budapest, Somlói út 14–16. Hungary

(Received 21 March 2001; revision received: 24 June 2002; accepted: 16 August 2002)

The effect of lactic acid production of *Lactobacillus casei* on the growth characteristics of *Listeria monocytogenes* was examined in milk. The effect of temperature (7, 13, 20 °C) and the co-ratios with *Lb. casei* at different cell count ratios (10:1, 100:1, 10 000:1) on the growth of *L. monocytogenes* were studied. At a 10 000:1 ratio induction of an early stationary phase of *L. monocytogenes* occurred. An exponentially decreasing correlation was observed between the logarithmic maximum population of *L. monocytogenes* and the initial log counts of lactobacilli. The Baranyi model was fitted to the obtained growth curves. The Baranyi equation provided good fit for the lag and exponential phase of *L. monocytogenes*. According to our observations, pH decrease does not seem to be the main factor of early stationary phase induction of *L. monocytogenes* in milk.

Keywords: *Listeria*, lactobacilli, competition, curve-fitting

There is an increasing interest in the food industry for new biological preservation methods. The preservative effect of the different metabolites of lactic acid bacteria (LAB) has been known for a long time. Numerous researchers studied the preventive effect of LAB metabolites to increase the safety of foods by suppressing the growth of food-borne pathogenic bacteria.

Since the 1980s *Listeria monocytogenes* has been considered as a significant human food-borne pathogen and has received considerable attention in the food industry. *Listeria monocytogenes* caused outbreaks of food poisoning in many countries (ROCOURT & BILLE, 1997; JACQUET et al., 1999). Recently, an outbreak occurred in France due to consumption of rillettes (DE VALK et al., 2000). This bacterium is an environmental contaminant and has been isolated from different food industrial sources such as meat, milk, vegetables and raw agricultural products (RYSER & MARTH, 1991; RODRIGUES et al., 1994; WANG & MURIANA, 1994). *Listeria monocytogenes* can survive food processes including cheese-, yoghurt- and sausage production (SCHAACK, 1988; BRUNCIC et al., 1991; GENIGEORGIS et al., 1991; RYSER & MARTH, 1991). The

* To whom correspondence should be addressed.
Phone: 36-1 215 4130/121; fax: 36-1 215 1545; e-mail: mpalmai@axelero.hu

U.S. Food and Drug Administration and Food Safety Inspection Service specified a zero tolerance for *L. monocytogenes* in e.g. ready-to-eat foods and dairy products.

The lactic acid bacteria are widely distributed in nature, including humans and animals. They are also used as starters in several dairy products and other fermented foods. Therapeutic application of lactic acid bacteria preparations have also found use (FULLER, 1989). They are also used to inhibit the growth and activity of several micro-organisms (GONZALES, 1993; VESCOVO et al., 1996).

As lactobacilli inhibit certain pathogens and growth of *L. monocytogenes* can occur during cheese production, the aim of the present work was to study the inhibitory effect of the inoculum level of *Lactobacillus casei* and incubation temperature on the growth of *Listeria monocytogenes* in milk.

1. Materials and methods

1.1. Organisms

Listeria monocytogenes 4ab No. 10 was obtained from the Hungarian Meat Research Institute (Budapest, Hungary). *Lactobacillus casei* 20012 pSB168 was obtained from the University of Nottingham (Loughborough, U.K.). Bacterial strains were maintained on BHI agar (MERCK) and stored at 6 °C. Cultures transferred into BHI broth incubated for 24 h at 30 °C were used for the experiments.

1.2. Inoculum and culture conditions

UHT skim milk was purchased from a retail store. It was considered to be practically sterile media for the experiments. Fat content of the milk was 2.8%. Two hundred ml of skim milk was transferred to screw cap bottles under aseptic conditions and inoculated with 2–2 ml of the diluted strains. Strains were diluted in peptone-saline solution (8.5 g l^{-1} NaCl, 1 g l^{-1} peptone).

The initial cell count of *Lb. casei* varied from approx. 10^4 CFU ml^{-1}, to 10^6–10^7 CFU ml^{-1} to result 10:1, 100:1 and 10 000:1 ratio compared to the number of *L. monocytogenes* (10^3 CFU ml^{-1}). A mono-culture of *L. monocytogenes* was also inoculated (10^3 CFU ml^{-1}) as control. The growth of the mono-culture and the interaction of the two microorganisms in the co-culture were examined at three different incubation temperatures (7, 13, 20 °C).

During incubation 4-ml samples were taken at regular time intervals of which 1 ml was appropriately diluted in peptone-saline solution, and viable cell numbers were determined by surface plating using a semi-automatic spiral platter (Model DS – Interscience, France). The rest of the sample was used for measuring the pH.

Selective enumeration of *Listeria monocytogenes* was carried out using OXFORD Listeria selective media (OXOID) with selective supplement. Plates were incubated at 30 °C for 24 h. For selective counting of lactobacilli MRS (MERCK) media was used.

BHI agar was used to check any contamination in the mixed culture during growth period. Plates were incubated at 30 °C for 24–48 h.

2. Results and discussion

Figures 1–3 present growth curves and pH decrease of the mixed cultures in skim milk as affected by temperature and time of incubation. When *Listeria monocytogenes* grew in pure culture, its maximum population density (MPD) reached 10^9 CFU ml^{-1} independently of the incubation temperatures. Growth rate and lag phase were affected by incubation temperature (figures not shown). Its exponential phase lasted between 34–36 h at 20 °C, 106–110 h at 13 °C and between 180–220 h at 7 °C. When it was cultivated in co-culture with lactobacilli the exponential phase was slightly shortened.

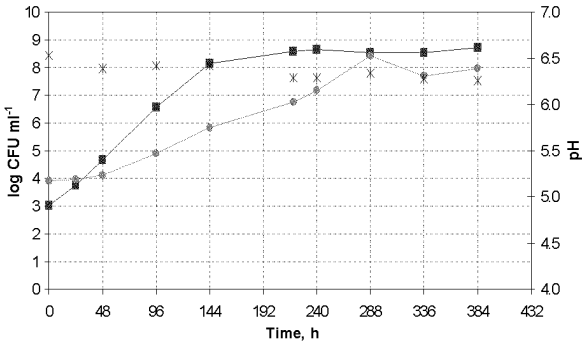
When co-inoculated in a 10:1 ratio (Figs. 1A–3A), the growth of the two organisms was neither inhibited nor stimulated. *Lactobacillus casei* did not effect the growth of *L. monocytogenes*. In this case of close-to-equal (10:1) initial level *L. monocytogenes* even grew to higher population level than *Lb. casei* at all temperatures. When *Lb. casei* was inoculated at a level hundredfold (100:1) or more (10 000:1) above that of *L. monocytogenes* the maximum population density of the *Listeria* strain decreased as the proportion of lactobacilli was increased in the mixed culture. Induction of an early stationary phase of *L. monocytogenes* occurred. A linear correlation was observed between the log MPD of *L. monocytogenes* and the initial log counts of lactobacilli (Fig. 4). This observation is in accordance with the findings of SZIGETI (2001) who examined the inhibition of *L. monocytogenes* in mixed culture with other lactic strains, among others, a bacteriocin-negative *L. lactis*.

Reaching stationary phase of a cell population is commonly explained by accumulation of toxic metabolic products, the exhaustion of essential nutrients required for growth or a change in pH. BAIL (1929) suggested that the maximum number in the stationary phase was set by an inherent attribute of the organisms and the necessity of a certain amount of physical or biological space. Multiplication may depend also on the maintenance of a critical minimal concentration of food per unit of surface or volume (LAMMANA et al., 1973).

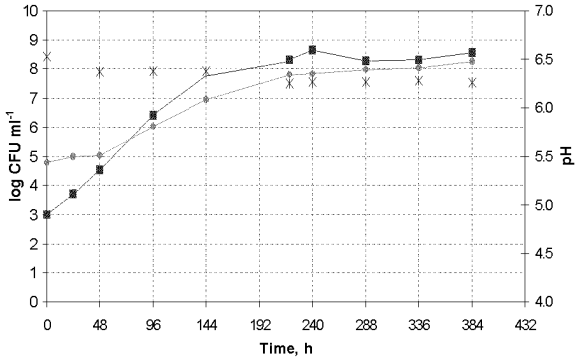
The antibacterial action of *Lb. casei* is probably due to a combination of factors including the release of lactic acid and hydrogen peroxide. It was shown that the inhibitory substances are normal products of the metabolic activity of lactobacilli. In the study of GONZALES and co-workers (1993) the antibiotic compound by *Lb. casei* has been released after a long incubation period (48 h).

According to our observations pH decrease did not seem to be the main factor of early stationary phase induction of *L. monocytogenes* in this set of experiments, since pH decreased only slightly and never dropped below 6.0. Our results concerning the effect of pH drop are similar to that of GONZALES and co-workers (1993).

A



B



C

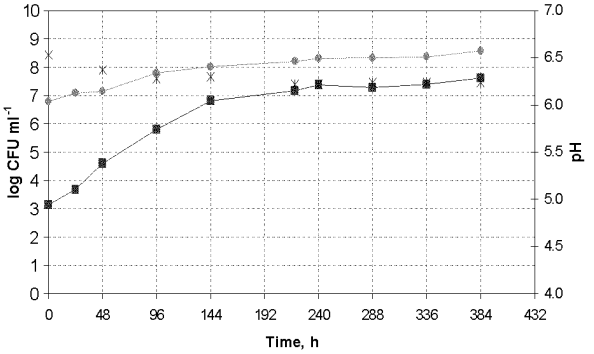


Fig. 1. Growth of mixed culture of *Listeria monocytogenes* and *Lactobacillus casei* in skim milk at 7 °C. ●: *Lb. casei*; ■: *L. monocytogenes*; ✳: pH. Approx. *L. monocytogenes* – *Lb. casei* ratio: **A:** 1:10; **B:** 1:100; **C:** 1:10 000

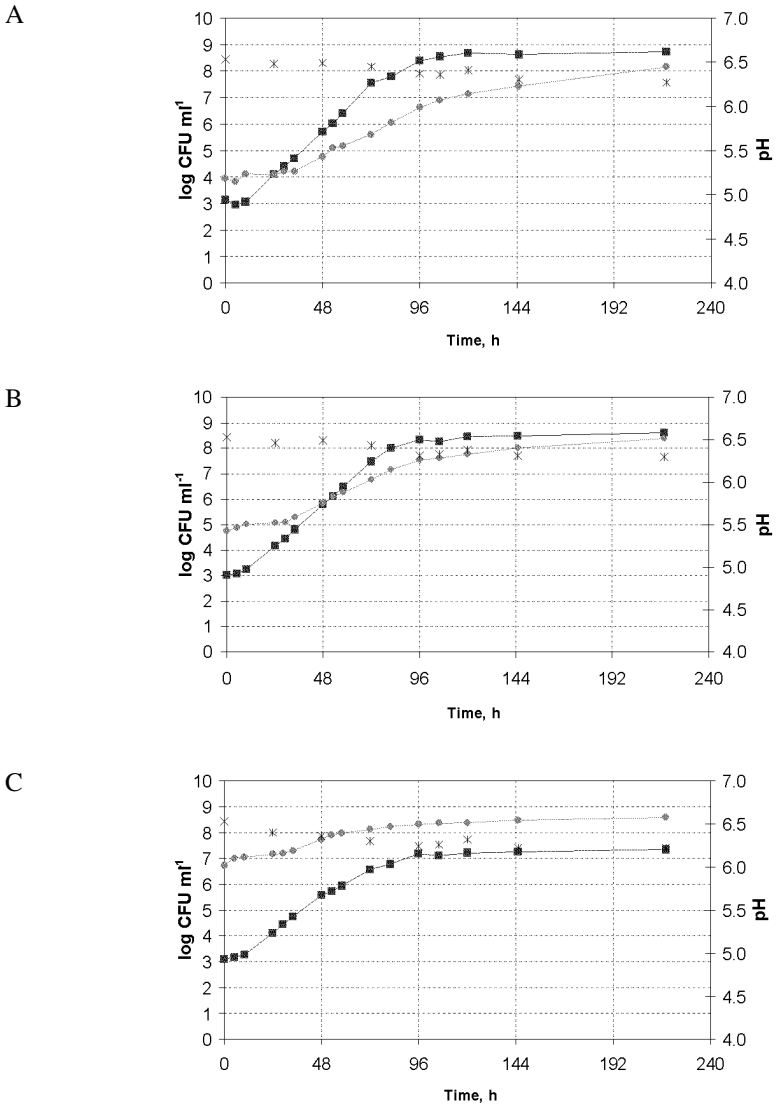


Fig. 2. Growth of mixed culture of *Listeria monocytogenes* and *Lactobacillus casei* in skim milk at 13 °C. ●: *Lb. casei*; ■: *L. monocytogenes*; ⚹: pH. Approx. *L. monocytogenes* – *Lb. casei* ratio: **A:** 1:10; **B:** 1:100; **C:** 1:10 000

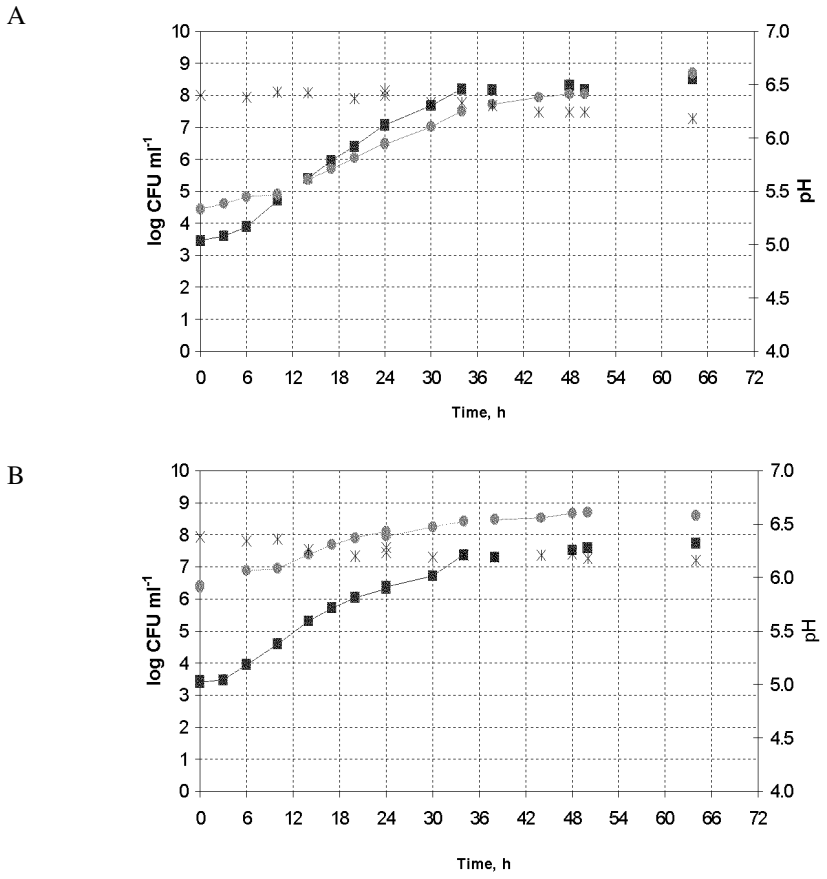


Fig. 3. Growth of mixed culture of *Listeria monocytogenes* and *Lactobacillus casei* in skim milk at 20 °C. ●: *Lb. casei*; ■: *L. monocytogenes*; ⚹: pH. Approx. *L. monocytogenes – Lb. casei* ratio: **A:** 1:10; **B:** 1:1 000

They investigated lactobacilli isolated from faeces. Inhibition of pathogenic strains by low pH only could not be considered because all of them were isolated from faeces between pH 4.6–8.4. The addition of active catalase to the associative cultures did not significantly modify the rate of inhibition. This suggested that hydrogen peroxide could be only slightly contributing to the inhibitory effect. We concluded that there must have been more factors besides pH that caused the inhibition of *L. monocytogenes* in the presence of high numbers of lactobacilli. Our investigations are in accordance with the observations of SCHIEMANN and OLSON (1984) which support a theory of a certain critical space for continued cell multiplication, effects of metabolic crowding.

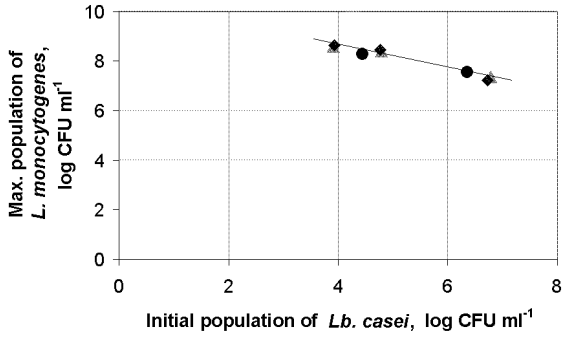


Fig. 4. Relationship between maximum population level of *L. monocytogenes* and initial cell count of *Lactobacillus casei* in logarithmic values. Incubation ▲: at 7 °C; ♦: at 13 °C; ●: at 20 °C

Among the models already available for modelling listerial growth the Baranyi equation was used to calculate growth parameters (BARANYI & ROBERTS, 1994). Lag and exponential phase, μ_{max} and doubling time of growth of *L. monocytogenes* were satisfactorily predicted by this model (Table 1). A fitted curve by the Baranyi model can be seen in Fig. 5.

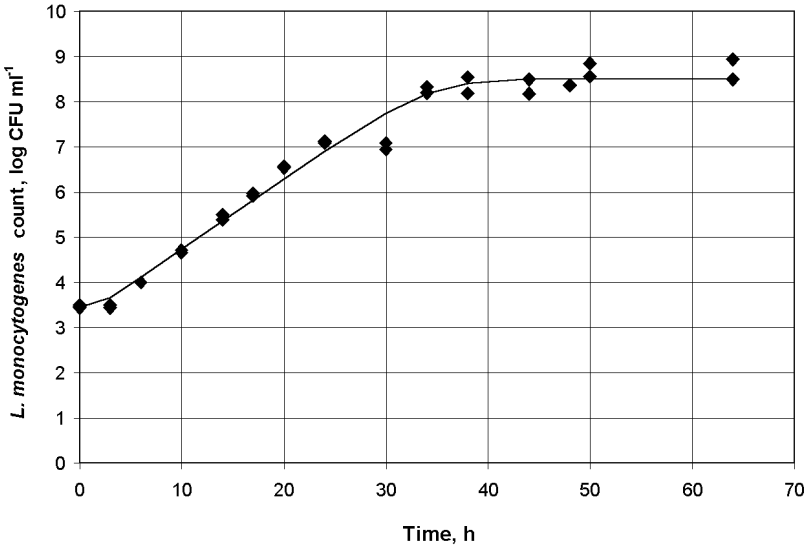


Fig. 5. Observed data and fitted values for *L. monocytogenes* by the Baranyi model in the case of incubation at 20 °C. ♦: Measured values; —: fitted values

Table 1. Growth characteristics of *Listeria monocytogenes* 4ab in monoculture and in co-culture with *Lactobacillus casei* at 7 °C (A), at 13 °C (B) and at 20 °C (C)

A

Microbes	Maximum population (log CFU ml^{-1})		μ_{max} ($1\ h^{-1}$)		Lag phase (h)		R^2
L.m.:L.c.	estimated	std. error	estimated	std. error	estimated	std. error	
Monoculture	8.63	0.03	0.094	0.003	8.79	3.02	0.9978
1:10	8.61	0.02	0.089	0.002	4.70	1.71	0.9990
1:100	8.42	0.05	0.086	0.005	5.04	3.93	0.9954
1:10 000	7.36	0.05	0.063	0.003	–	–	0.9913

B

Microbes	Maximum population (log CFU ml^{-1})		μ_{max} ($1\ h^{-1}$)		Lag phase (h)		R^2
L.m.:L.c.	estimated	std. error	estimated	std. error	estimated	std. error	
Monoculture	8.75	0.04	0.162	0.003	7.86	1.12	0.9980
1:10	8.63	0.04	0.160	0.003	7.93	1.20	0.9977
1:100	8.46	0.03	0.160	0.003	7.28	1.07	0.9983
1:10 000	7.22	0.03	0.124	0.003	3.96	1.81	0.9961

C

Microbes	Maximum population (log CFU ml^{-1})		μ_{max} ($1\ h^{-1}$)		Lag phase (h)		R^2
L.m.:L.c.	estimated	std. error	estimated	std. error	estimated	std. error	
Monoculture	8.50	0.05	0.404	0.014	2.55	0.65	0.9945
1:10	8.28	0.04	0.389	0.010	2.36	0.49	0.9965
1:1 000	7.55	0.06	0.302	0.011	0.99	0.80	0.9894

3. Conclusions

The aim of our investigation was to study microbial antagonism in mixed cultures of *Lactobacillus casei* and *Listeria monocytogenes*. In this set of preliminary experiments we demonstrated the effect of inoculum level of *Lb. casei* on the prevention of *L. monocytogenes* growth. The outcome of the experiments was similar and independent of temperature. Induction of an early stationary phase of *L. monocytogenes* occurred at *Lb. casei* ratios of 100:1 and 10 000:1. A linear correlation was observed between the total log counts of *L. monocytogenes* and the initial log counts of lactobacilli. From an industrial point of view it seems interesting to know the risk of *L. monocytogenes* proliferation, even at low temperature, and to be able to develop hurdle technology to avoid it. The growth data obtained in these mixed cultures can be used for microbial modelling of competitive interactions. Our results also confirmed the importance of prevention of listerial contamination and application of active and high inoculum of lactobacilli starter cultures to ensure production of microbiologically safe fermented products.

*

This work was supported by the Hungarian Scientific Research Fund: No.: OTKA F-030124.

References

BAIL, O. (1929): Ergebnisse experimenteller Populationsforschung. *Zitatsf. Immun. Forsch*., *60*, 1–22.

BARANYI, J. & ROBERTS, T.A. (1994): A dynamic approach to predicting bacterial growth in food. *Int. J. Fd Microbiol*., *23*, 277–294.

BRUNCIC, S., PAUNOVIC, L. & RADISIC, D. (1991): The fate of *Listeria monocytogenes* in fermented sausages and in vacuum packaged frankfurters. *J. Fd Prot*., *54*, 413–417.

DE VALK, H., ROCOURT, J., LEQUERREC, F., JACQUET, CH., VAILLANT, V., PORTAL, H., PIERRE, O., PIERRE, V., STAINER, F., SALVAT, G. & GOULET, V. (2000): Bouffée épidémique de listériose liée à la consommation de rillettes, France, octobre-décembre 1999. *Bull. Epidemiol. Hebdom*., *4*, 15–17.

JACQUET, CH., BROUILLE, SAINT-CLOMENT, C., CATIMEL, B. & ROCOURT, J. (1999): La listériose humaine en France en 1998. *Bull. Epidemiol. Hebdom*., *37*, 153–155.

FULLER, A. (1989): Probiotics in man and animals. Review. *J. appl. Bacteriol*., *66*, 365–378.

GENIGEORGIS, C.A., CARNICIU, M. & DUTULESCU, D. (1991): Growth and survival of *Listeria monocytogenes* in market cheese stored at 4 °C to 30 °C. *J. Fd Prot.*, *54*, 1–7.

GONZALES, S.N., APELLA, M.C., ROMERO, N.C., NADER DE MACIAS, M.E. & OLIVER, G. (1993): Inhibition of enteropathogens by lactobacilli strains used in fermented milk. *J. Fd Prot*., *56*, 773–776.

LAMMANA, C., MALLETTE, M.F. & ZIMMERMAN, L. (1973): *Basic bacteriology: its biological and chemical background.* 4th ed. The Williems & Wilkins Co., Baltimore.

ROCOURT, J. & BILLE, J. (1997): Foodborne listeriosis. *World Health Statistics Q*., *50*, 67–73.

RODRIGUES, J.L., MEDINA, M.P. & NUNEZ, M. (1994): Incidence of *Listeria monocytogenes* and other *Listeria* spp. in ewes' raw milk. *J. Fd Prot*., *57*, 571–575.

RYSER, E.P. & MARTH, E.H. (1991): *Listeria, listeriosis and food safety*. Marcel Dekker, New York.

SCHAACK, M.M. (1988): Behavior of *Listeria monocytogenes* in skim milk and yoghurt mix during fermentation by thermophilic lactic acid bacteria. *J. Fd Prot*., *51*, 607–614.

SCHIEMANN, D.A. & OLSON, S. (1984): Antagonism by Gram-negative bacteria to growth of *Yersinia enterocolitica* in mixed cultures. *Appl. environ. Microbiol*., *48*, 539–544.

SZIGETI, E. (2001): Interaction between *Lactococcus lactis* strains and *Listeria monocytogenes* in mixed cultures. *Acta Alimentaria*, *30*, 247–265.

VESCOVO, M., TORRIANI, S., ORSI, C., MACCHIAROLO, F. & SCOLARI, G. (1996): Application of antimicrobial-producing lactic acid bacteria to control pathogens in ready-to-use vegetables. *J. appl. Bacteriol*., *81*, 113–119.

WANG, C. & MURIANA, P.M. (1994): Inactivation and injury of *Listeria monocytogenes* in a minimal medium as affected by benzoic acid and incubation temperature. *J. Fd Sci*., *54*, 650–652.