Letters in Applied Microbiology 2002, **34**, 77–81

Bacteriocin production by spray-dried lactic acid bacteria

J. Silva, A.S. Carvalho, P. Teixeira and P.A. Gibbs

Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr António Bernardino de Almeida, Porto4200–072, Portugal

2001/196: received 2 July 2001, revised 4 October 2001 and accepted 15 October 2001

J. SILVA, A.S. CARVALHO, P. TEIXEIRA AND P.A. GIBBS. 2002.

Aims: Cell survival and antagonistic activity against *Listeria innocua*, *Listeria monocytogenes* and *Staphylococcus aureus* were investigated after spray-drying three bacteriocin-producing strains of lactic acid bacteria: *Carnobacterium divergens*, *Lactobacillus salivarius* and *Lactobacillus sakei*.

Methods and Results: Bacterial cell concentrates were spray-dried and stored at 4°C and 18°C and 0·3% ERH (equilibrium relative humidity). Enumeration and antagonistic activity were evaluated before and after spray-drying and at regular intervals during storage.

Conclusions: A higher survival rate was obtained when survival was performed at 4°C. With the exception of *Carnobacterium divergens* which lost the inhibitory activity against *Staph. aureus* after drying, antagonistic production was not affected by the process nor by the storage. Of the three species studied, *Lact. salivarius* showed the highest resistance to the spray-drying and storage processes.

Significance and Impact of Study: Spray-drying is a potentially useful process for large scale production of dried powders containing viable organisms with antagonistic activity against pathogens.

INTRODUCTION

Despite the recent progress in food biotechnology, with the introduction of modern technologies and safety concepts (e.g. HACCP), the problem of food safety and security remains to be solved. Protective cultures and associated antagonistic substances ('biopreservation') should be considered an additional factor with the potential for improving the microbiological safety of food. Their implementation should support good manufacturing practices, thereby reducing risks of growth and survival of pathogens and spoilage micro-organisms (Kim and Bhowmik 1990; Mauriello *et al*. 1999).

Lactic acid bacteria (LAB) have traditionally been used in food processing because of their ability to improve the organoleptic characteristics and healthiness of foodstuffs. Different antimicrobials, such as lactic acid and acetic acid, hydrogen peroxide, carbon dioxide and bacteriocins, produced by these bacteria, can inhibit pathogenic and spoilage micro-organisms, extending the shelf-life and enhancing the safety of food products (Aymerich *et al*. 2000).

Correspondence to: J. Silva, Escola Superior de Biotecnologia, Universidade Católica Portuguesa Rua Dr António Bernardino de Almeida, Porto 4200–072, Portugal (e-mail: joana@morango.esb.ucp.pt).

Increasing consumer demand for natural food additives has focused interest on bacteriocins. Bacteriocins of LAB are considered natural biopreservatives, as it is assumed that bacteriocins are degraded by the proteases of the gastrointestinal tract (Cintas 1995), and most of the LAB are considered as GRAS (Generally Recognized as Safe) micro-organisms (Holzapfel *et al*. 1995).

The application of bacteriocins as food preservatives could be achieved either by using the bacteriocinogenic strain as a starter culture and/or a protective culture, or by using the bacteriocin as a food additive. Therefore, from a commercial point of view, an inexpensive method for large-scale production of cultures containing high levels of viable bacteriocin producers, in a form suitable for product applications, is highly desirable (Gardiner *et al*. 2000). In previous studies researchers have investigated the production of spray-dried powders of *Lactobacillus* spp. (Kim and Bhowmik 1990; Teixeira *et al*. 1995). In addition to maintaining the viability of cultures, it is important that their bacteriocinogenic properties are maintained following the spray-drying process.

Spray-drying can be used to produce large amounts of dairy ingredients relatively inexpensively, the spray-dried powders can be transported at a low cost and can be stored in a stable form for prolonged periods (Gardiner *et al*. 2000).

Because of the advantages of spray-drying, in the present study we investigated the use of this method as a way to preserve bacteriocinogenic strains, e.g. *Lact. sakei*, *Lact. salivarius* and *Carn. divergens*. The purpose of this study was to evaluate the antimicrobial activity of these LAB strains, which are active against *L. monocytogenes*, *L. innocua* and *Staph. aureus* (Hugas *et al.* 1995; Pilet *et al.* 1995; Duffes *et al.* 1999; Dunne *et al.* 1999; Pascual *et al.* 1999), before and after spray-drying.

MATERIALS AND METHODS

Bacterial cultures and media

Lact. sakei CTC 494 and *Lact. salivarius* CTC 2197 were generously supplied by Dr Marta Hugas from Spain. *Carn. divergens* isolated from trout intestine by Pilet *et al.* 1995) was generously supplied by ENITIAA (France).

Lact. sakei and *Lact. salivarius* were grown in MRS broth (LAB M, Bury, UK) (DeMan *et al.* 1960) at 30°C and 37°C, respectively. *Carn. divergens* was grown in Elliker medium (Elliker *et al.* 1956) at 30°C for 24 h. Working cultures were stored as stab agar medium at 4°C. Fresh stab cultures were prepared from working cultures every month. *L. monocytogenes* T100 and *L. innocua* 2030 c (tetracycline resistant), previously isolated from cold smoked fish by Escola Superior de Biotecnologia, were grown in tryptic soy broth (TSB, LAB M) supplemented with 0·6% yeast extract (LAB M) at 37°C. *Staph. aureus* NCTC 08532 (supplied by Serviços de Microbiologia, ESB, Porto, Portugal) was grown in TSB at 37°C for 24 h.

Preparation of feed solution for spray-drying

Overnight cultures of each strain of lactic acid bacteria were inoculated at 1% v/v into MRS broth and Elliker broth and incubated at 30°C or 37°C for 24 h. After centrifugation at 7000 *g* for 15 min at 4°C, the cells were resuspended in an equal volume of sterile 11% wv^{-1} skim-milk powder (Oxoid). Each sample was then directly spray-dried.

Spray-drying and storage

Samples were spray-dried in a laboratory scale apparatus (Niro Atomizer, Gladsaxevej, Denmark). Moisture in spray droplets produced by the atomization of the feed liquid by a vaned wheel (rotary atomizer) rotating at high speed, was evaporated in a vertical co-current drying chamber, 0·8 m diameter and 0·6 m height. Spray-drier conditions were: outlet air temperature 70°C, inlet air temperature 200°C and atomizing air pressure 5 Bar. Powder was collected in a single cyclone separator. Samples of the spray-dried inoculum were stored at 4°C and 18°C in hermetically sealed glass bottles in which the ERH (equilibrium relative humidity) was controlled by equilibrium with silica.

Enumeration

Before drying, the organisms were enumerated on Elliker agar in the case of *Carn. divergens* and on MRS agar for the other two cultures in the study, by the drop count technique (Miles and Misra 1938). Each sample of spray-dried bacteria was rehydrated to the original volume with deionized water. The cells were allowed to rehydrate for 2 min under vigorous shaking and the suitable dilutions were plated as described above. Plates were incubated at 30°C (*Carn. divergens* and *Lact. sakei*) and 37°C (*Lact. salivarius*) for 24 h before enumeration.

Bacteriocin spectrum of activity

The inhibitory activity of the three bacteriocin producers against the *L. innocua*, *L. monocytogenes* and *Staph. aureus* strains was tested at various intervals during storage by the deferred antagonism test (Barefoot and Klaenhammer 1984). From each lactic acid bacteria, 10 μl were placed on the surface of a solid medium TSYE overlay seeded with 100 μl of an overnight target culture. The plates were examined after overnight incubation at 37°C.

Statistical analysis

Statistical analysis was done with the ANOVA methodology using Statview^{TM} Package (Abacus Concepts, Berkeley, CA, USA) using as independent variable the storage time.

The experiments were repeated at least three times. Viable counts on MRS agar were converted to log cfu ml^{-1}. Differences were considered significant at $P < 0{\cdot}05$. The error bars on the figures indicate the mean standard deviations for the data points.

RESULTS

In the present study, the use of spray-drying as a way to prepare dairy-based powders containing viable cultures of *Lact. sakei*, *Lact. salivarius* and *Carn. divergens* with antagonistic activity against selected pathogens was investigated. For each strain, three independent experiments were performed.

Survival kinetics of spray-dried cell concentrates during storage

The experimental results for the survival of *Lact. salivarius*, *Lact. sakei* and *Carn. divergens* during drying and subsequent storage at different temperatures (4°C and 18°C) are shown in Table 1 and Fig. 1, respectively. For all strains, no

significant differences were observed in the number of viable cells before and after drying. Survival of *Carn. divergens* and *Lact. sakei* decreased during the storage time at 4°C and 18°C being the rate of decrease higher at the higher temperature of storage ($P < 0{\cdot}05$). The survivor curve of *Lact. sakei* showed the greatest decrease during storage at both temperatures, and no survivors were observed at the end of the 2nd month of storage at 18°C.

Until the 3rd month of storage there was no significant survival decrease of *Lact. salivarius* at 4°C and 18°C.

Effect of spray-drying on bacteriocin production

The effect of spray-drying and storage in the dried state on the bacteriocinogenic activity of lactobacilli was investigated. The results obtained by deferred antagonism assay to detect the ability of bacteriocin-producing strains to inhibit *L. innocua*, *L. monocytogenes* and *Staph. aureus* are reported in Table 2.

In the case of *Carn. divergens* there was a loss of antagonistic activity against *Staph. aureus* immediately after spray-drying but not against the two *Listeria* spp. During spray-drying and during storage, *Lact. salivarius* maintained the bacteriocin activity against *L. innocua*, *L. monocytogenes* and *Staph. aureus* at least during the 3 months of storage. *Lact. sakei* maintained its bacteriocin activity while viable.

DISCUSSION

In this study it was found that the strains investigated, varied considerably in their ability to survive storage in dried state.

Results showed that the storage temperature was a critical parameter affecting the survival of the micro-organisms. In all experiments, survival rates were higher at the lower temperature of storage.

The bacteriocin produced by *Lact. sakei* CTC 494 (sakacin K), by *Lact. salivarius* CTC 2197 and by *Carn. divergens* (divercin V 41) was reported as having an antagonistic activity against both *Listeria* strains and *Staph. aureus* (*Lact. sakei* and *Lact. salivarius*) and against *Listeria* spp. (*Carnobacterium*) (Duffes *et al.* 1995; Hugas *et al.* 1995; Pascual *et al.* 1999; and Pilet *et al.* 1995). In this study, it was demonstrated that the spray-drying process did not affect this antagonistic activity of *Lact. sakei* and *Lact. salivarius*. Similar results were obtained by Gardiner *et al.* (2000) and Mauriello *et al.* (1999). *Carn. divergens*, however, lost its antagonistic activity against *Staph. aureus,*

Table 1 Survival of *Lact. sakei*, *Lact. salivarius* and *Carn. divergens* before and after spray-drying

	Lact. sakei		*Lact. salivarius*		*Carn. divergens*	
	4°C	18°C	4°C	18°C	4°C	18°C
Before spray-drying	8·73	8·75	8·84	8·82	8·53	8·45
After spray-drying	8·4	8·3	8·83	8·84	8·17	8·2

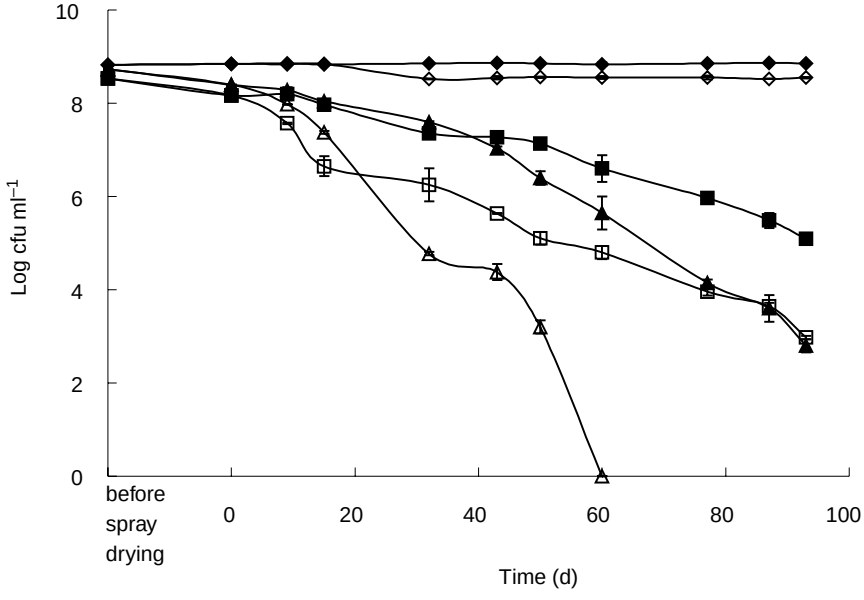

Fig. 1 Survival of spray dried *Lact. sakei, Lact. salivarius* and *Carn. divergens* during storage at 18°C and 4°C under controlled ERH ($P < 0{\cdot}05$). (□), (*Carn. divergens* 18°C; (■), *Carn. divergens* 4°C; (△), *Lact. sakei* 18°C; (▲), *Lact. sakei* 4°C; (◇), *Lact. salivarius* 18°C; (◆), *Lact. salivarius* 4°C)

Table 2 Sensitivity of three pathogenic micro-organisms to bacteriocins produced by *Lact. salivarius*, *Lact. sakei* and *Carn. divergens,* before and after drying and during storage, at 18°C, as determined by deferred antagonism assay

	Carn. divergens							*Lact. sakei*							*Lact. salivarius*						
Time (d)	BSD	0	9	32	50	77	93	BSD	0	9	32	50	77	93	BSD	0	9	32	50	77	93
L. innocua	+	+	+	+	+	+	+	+	+	+	+	+	*	*	+	+	+	+	+	+	+
L. monocytogenes	+	+	+	+	+	+	+	+	+	+	+	+	*	*	+	+	+	+	+	+	+
Staph. aureus	+	−	−	−	−	−	−	+	+	+	+	+	*	*	+	+	+	+	+	+	+

+, clearly defined zone of inhibition surrounding the producing bacterial colony
−, no inhibition
BSD = before spray drying
*no survivors

but not against *L. innocua* and *L. monocytogenes*. The production of more than one bacteriocin by a single organism has already been reported (Bhugaloo-vial *et al.* 1996) and Bhugaloo-vial reported the isolation and classification of two class II a bacteriocins produced by *Carn. piscicola* V1 which exhibit significant differences in their inhibitory activities against target Gram-positive bacteria. To our knowledge, this is the first time that loss of antagonistic activity during spray-drying is reported. This phenomenon might be explained by a possible loss of the plasmid responsible for that inhibitory activity. Bacteriocin production mediated by plasmids has already been reported (Kim and Bhowmik 1990; Hugas *et al.* 1995; Pilet *et al.* 1995).

In conclusion, spray-drying is potentially a useful process for large scale production of dried powders containing viable organisms with antagonistic activity against pathogens. However, this can not be generalized and for each individual organism, it must be investigated if these characteristics are maintained. Furthermore, given the numerous applications of skim milk powders, not only in dairy products but also in food such as instant desserts and mayonnaise, it is possible that the resulting culture containing powders could be used in a wide range of functional food applications, i.e. having antimicrobial properties in those foods.

ACKNOWLEDGEMENTS

Financial support for authors Silva and Carvalho was provided, respectively, by a PhD fellowship issued by PRAXIS XXI (JNICT–BD–19713–99 and JNICT–BD-18512–98, respectively).

REFERENCES

Aymerich, T., Artigas, M.G., Monfort, J.M. and Hugas, M. (2000) Effect of sausage ingredients and additives on the production of enterocins A and B by *Enterococcus faecium* CTC492. Optimization of *in vitro* production and anti-listerial effect in dry fermented sausages. *Journal of Applied Microbiology* **88**, 686–694.

Barefoot, S.F. and Klaenhammer, T.R. (1984) Purification and characterization of the *Lactobacillus acidophilus* bacteriocin lactacin B. *Antimicrobiological Agents Chemotherapy* **26**, 328–334.

Bhugaloo-vial, P., Dousset, X., Metiver, A., Sorokine, O., Anglade, P., Boyaval, P. and Marion, D. (1996) Purification and amino acid sequences of Piscicocins V1a and V1b, two class Iia bacteriocins secreted by *Carnobacterium piscicola* V1 that display significantly different levels of specific inhibitory activity. *Applied and Environmental Microbiology* **62**, 4410–4416.

Cintas, L. (1995) *Caracterización Bioquimica Y Genética Parcial de la Pediocina L50, Una Nueva Bacteriocia Producida Por Pediococcus Acidilactici*. L50 aislado de embutidos crudos curados. *Doctoral Thesis.* Madrid Autonomous University.

DeMan, J., Rogosa, C.M. and Sharpe, E. (1960) A medium for the cultivation of Lactobacilli. *Journal of Applied Bacteriology* **23**, 130–135.

Duffes, F., Leroi, F., Boyaval, P. and Dousset, X. (1999) Inhibition of *Listeria* strains in a simulated cold smoked fish system stored at 4°C. *International Journal of Food Microbiology* **47**, 33–42.

Dunne, C., Murphy, L., Flynn, S., O'Mahony, L., O'Halloran, S., Feeney, M., Morrissey, D., Thornton, G., Fitzerald, G., Daly, C., Kiely, B., Quigley, E.M.M., O'Sullivan, G.C., Shanahan, F. and Collins, J.K. (1999) Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antonie Leeuwenhoeck* **76**, 279–292.

Elliker, P.R., Anderson, A.W. and Hannesson, G. (1956) An agar culture medium for lactic acid streptococci and lactobacilli. *Journal of Dairy Science* **39**, 1611.

Gardiner, G.E., O'Sullivan, E., Kelly, Y., Auty, M.A.E., Fitzgerald, G.F., Collins, Y.K., Ross, R.P. and Stanton, C. (2001) Comparative survival rate of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray-drying. *Applied and Environmental Microbiology* **66**, 2605–2612.

Holzapfel, W.H., Geisen, R. and Schillinger, U. (1995) Biological preservation of foods reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology* **24**, 343–362.

Hugas, M., Garriga, M., Aymerich, M.T. and Monfort, J.M. (1995) Inhibition of *Listeria* in dry fermented sausages by the bacteriocinogenic *Lact. Sake* CTC 494. *Journal of Applied Bacteriology* **79**, 322–330.

Kim, S.S. and Bhowmik, S.R. (1990) Survival of lactic acid bacteria during spray-drying of plain yoghurt. *Journal of Food Science* **55**, 1008–1010.

Mauriello, G., Aponte, M., Andolfi, R. and Moschetti and Villani, F. (1999) Spray-drying of bacteriocin-producing lactic acid bacteria. *Journal of Food Protection 62* **7**, 773–777.

Miles, A.A. and Misra, S.S. (1938) The estimation of the bactericidal power of blood. *Journal of Hygiene (Cambridge)* **38**, 732–749.

Pascual, M., Hugas, M., Badiola, J.I., Monfort, J.M. and Garriga, M. (1999) *Lactob. saliv.* CTC 2197 prevents *Salmonella enteritidis* colonization in chickens. *Applied and Environmental Microbiology* **65**, 4981–4986.

Pilet, M., Dousset, X., Barré, R., Novel, G. and Desmazeaud and Piard, J. (1995) Evidence for two bacteriocins produced by *Carnobacterium piscicola* and *Carnobacterium divergens* isolated from fish and active against *Listeria monocytogenes*. *Journal of Food Protection* **58**, 256–262.

Teixeira, P., Castro, H. and Kirby, R. (1995) Spray-drying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus*. *Journal of Applied Microbiology* **78**, 456–462.