J Ind Microbiol Biotechnol (2010) 37:65–69
DOI 10.1007/s10295-009-0648-2

ORIGINAL PAPER

Pediocin production in milk by *Pediococcus acidilactici* in co-culture with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*

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Received: 27 May 2009 / Accepted: 28 September 2009 / Published online: 21 October 2009

Abstract The production of pediocin in milk by *Pediococcus acidilactici* was evaluated in co-culture with the dairy fermentation cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. The cultures were tested singly and in different combinations in milk (0 or 2% fat content) during incubation at 40°C for up to 10 h. Cell-free milk samples taken every 60 min were tested for bacteriocin activity against *Listeria monocytogenes*. Pediocin activity was not detectable when *P. acidilactici* was inoculated into milk as a monoculture. When *P. acidilactici* was grown in combination with the yogurt starter cultures *S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*, pediocin concentration reached 3,200–6,400 units ml^{-1} after 8 h of incubation. The results showed that pediocin producing pediococci may be useful adjunct components in mixed cultures of *S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* to amplify the bioprotective properties of fermented dairy foods against Listeria contamination.

Introduction

Pediococci are homofermentative lactic acid bacteria (LAB) with important applications as starter cultures in the fermentation of meats and vegetables [27]. In several strains of *Pediococcus acidilactici,* a species widely used in fermented sausages, the production of pediocins (PA-1, AcH, SJ-1, F and PO_2) is plasmid-linked [22, 23, 26]. Pediocins are broad-range bacteriocins with activity against several food-borne pathogens, including Listeria, and have the potential for applications as natural biopreservatives to improve the safety of food products [9, 14].

Two main characteristics of *P. acidilactici* account for the continued interest in the application of this species in dairy food fermentations. First, pediococci are fast acid producers and resistant to bacteriophages that infect *Streptococcus thermophilus* and could replace the latter as starters in the manufacture of Italian-style cheeses [8]. Second, as producers of the natural antimicrobial peptide pediocin, they may contribute to an increased level of food safety by preventing the growth of *Listeria monocytogenes*, which is the causative agent of listeriosis in cheeses [20]. As bacteriocin-producing adjunct cultures in dairy fermentations, pediococci may also be effective in protecting low-pH fermented products, such as yogurt, in which *L. monocytogenes* may actually survive [8, 15, 24, 25].

The direct application of *P. acidilactici* in fermented dairy foods either as starter culture or bacteriocin-producing adjunct culture is prevented or severely limited by its lack of or slow lactose fermentation [3]. Approaches to circumvent this problem have included the use of pediococci genetically modified to ferment lactose [6] and the transfer of the pediocin gene complex from pediococci to cheese starter cultures [10, 28].

In yogurt fermentation, the disaccharide lactose in milk is hydrolyzed to glucose and galactose by the β-galactosidase enzyme present in both *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* that are traditionally used together in starter culture mixes. However, it has been shown that many *S. thermophilus* strains metabolize only glucose, but not galactose [12, 13, 21, 30]. During growth of *L. delbrueckii* ssp. *bulgaricus* in milk, the rate of lactose hydrolysis may outpace the rate of glucose metabolism, resulting in an initial excess of glucose in milk fermentation [5]. Therefore,

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based on the available data, it is reasonable to anticipate that in yogurt fermentation the hydrolytic breakdown of lactose may result in a transient surplus of glucose and an overall accumulation of galactose that is fermentable by pediococci and is known to support pediocin synthesis [4].

In this study, we evaluated pediocin production by *P. acidilactici* in milk co-fermented with the commonly used yogurt starter cultures *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, and tested the effect of bacteriocin produced on the survival of *L. monocytogenes* during a 16-day storage of the fermented product at 4°C.

Materials and methods

Bacteria and media

All bacterial strains used were from an in-house collection. The lactic acid bacteria (LAB) *Streptococcus thermophilus* strains ST114 (NRRL B-59391) and ST129 (NRRL B-59392), and the *Lactobacillus delbrueckii* ssp. *bulgaricus* strains LDB11 (NRRL B-59388) and LDB12 (NRRL B-59389) were deposited in the Agricultural Research Service Culture Collection, NCAUR-USDA, Peoria, IL (http://nrrl.ncaur.usda.gov). The pediocin-producing *Pediococcus acidilactici* strain F (PAF) was a gift from B. Ray (University of Wyoming, Laramie). Cultures of PAF, LDB11 and LDB12 were maintained in deMan, Rogosa and Sharpe medium (MRS; Becton–Dickinson) at 37°C, while the strains ST114 and ST129 were grown in tryptone-yeast extract-lactose medium (TYL) at 37°C [29]. *Listeria monocytogenes* Scott A (LMS, ATCC49594) was incubated in brain heart infusion medium (BHI, Becton–Dickinson) at 37°C.

Pediocin production in microbiological media and bioassays

To test the capacity of *P. acidilactici* PAF for pediocin production in various media, MRS was formulated with glucose (MRSGlu), lactose (MRSLac) and galactose (MRSGal), using each carbohydrate at 1% (w/v) concentration. Inocula were prepared by centrifuging 1 ml of a PAF culture grown overnight in MRSGlu (37°C) at 12,500×*g* and resuspending the pellet in 1 ml of sterile peptone (0.05%, w/v) water. After inoculating 10 ml of each medium with 100 µl of PAF cell suspension, tubes were incubated for 8 h at 37°C. Pediocin production by PAF was tested by the spot-on-the-lawn antimicrobial assay [16]. Cell-free supernatants were prepared by centrifugation, and pediocin activity was measured after a two-fold serial dilution of cell-free supernatants with sterile distilled H_20, and depositing 5 µl samples on the surface of 2-mm-deep BHI agar plates inoculated with 0.5% (v/v) of a 16-h culture of *L. monocytogenes* Scott A. After storage at 6°C for 4 h the plates were incubated at 30°C for 16 h. The reciprocal of the highest dilution showing a visible zone of inhibition was defined as one pediocin activity unit (AU). The amount of pediocin produced (AU ml^{-1}) was calculated by multiplying the reciprocal of the highest active dilution by a factor of 200. Each sample was assayed in triplicate.

Pediocin production in fermented milk

Pediocin production by *P. acidilactici* strain PAF was tested in commercial samples of 0%-fat (skim) and 2%-fat milk each supplemented with 0.2% yeast extract and sterilized by autoclaving. Milk samples (10 ml) were inoculated with PAF in various combinations with LDB and ST strains that were grown overnight at 37°C. The inoculum size for each culture was 1% (v/v) and corresponded to an initial of 10^7 cfu ml^{-1}. Milk fermentations were carried out at 40°C in a water bath for 8–10 h to simulate conditions of yogurt production. Starting after 4 h of incubation, samples were withdrawn every 60 m, and after pH measurement, milk samples were centrifuged at 12,500×*g* at 4°C for 15 min, adjusted to pH 6.5 and twofold serially diluted to check pediocin titers in agar plates inoculated with *Listeria monocytogenes* Scott A.

Enzyme assays

Galactose was determined enzymatically according to the method described by Hutkins et al. [17]. Galactose dehydrogenase and other components of the assay mixture were purchased from Sigma–Aldrich Corporation (St. Louis, MO). The concentration of galactose in triplicate samples was calculated using a standard curve.

Pediocin production and survival of *L. monocytogenes* Scott in fermented milk

Milk samples were inoculated with PAF and lactic acid starter cultures in different combinations and preincubated at 30°C for 4 h before inoculating each sample with 100 µl of a 16-h LMS culture adjusted to yield an initial bacterial load of 10^3 cfu ml^{-1}. This was followed by an additional 5 h of incubation at 40°C. Samples from each tube were withdrawn at the time of inoculation with LMS and again 5 h later, and serially diluted to determine the LMS colony-forming units (cfu ml^{-1}) on Modified Oxford Listeria selective agar plates (MOX; Becton, Dickinson and Co.) after incubation at 37°C for 24 h. At the end of the fermentation, the products were stored at 4°C for 16 days, and samples were tested daily to determine the survival of LMS (cfu ml^{-1}).

Results and discussion

Pediocin production in microbiological media

The production of pediocin by *P. acidilactici* PAF in different media is summarized in Table 1. The results clearly indicated that lactose-based media (MRSLac and TYL) were not suitable for growth, and only marginal amounts of pediocin were produced, which confirmed the lack of or limited lactose-hydrolyzing capacity in *P. acidilactici* [3]. At the same time, media formulated with glucose (MRSGlu) and galactose (MRSGal) supported excellent growth by PAF, and pediocin production in these media reached 12,800 AU ml^{-1}.

Pediocin production in fermented milk

The capacity of PAF to produce pediocin during milk fermentation was also tested under conditions that simulated yogurt production (40°C). Changes in acidity (pH) and the amount of pediocin produced were tested every 60 m, starting after 4 h of incubation. The results obtained with skim milk are shown in Table 2. Milk samples inoculated with PAF alone showed only traces of pediocin by the assay technique used even after 8 h of incubation (data not shown). On the other hand, antilisterial activity was clearly measurable when either skim or 2%-fat milk were co-cultured by PAF with either ST or LDB strains, or in combination of both LAB cultures. This was attributed to the assimilation of fermentable carbohydrate generated by the traditional starter culture components (ST or LDB), which permitted growth and pediocin production by PAF. While low levels of pediocin titer (300–400 AU ml^{-1}) were detectable after 8 h in milk co-fermented with LDB12 and PAF, co-culturing PAF with strain ST129 alone or in combination with ST129 and LDB12 resulted in significantly greater pediocin production, with pediocin titers reaching levels of 3,200 AU ml^{-1}. In fact, galactose assays showed that in samples taken during milk fermentation by ST114 or ST129, galactose concentration increased from 0.07 to 1.9 mg ml^{-1} within the first 4 h of incubation. Similar results were obtained when milk was co-fermented with ST114 and LDB11 or LDB129.

These results demonstrated that the ST rather than the LDB component in the culture mixes played the key role in creating favorable conditions for the growth and pediocin synthesis by PAF, and this was apparently due to the generation of galactose that is fermentable by the pediococci. While the augmentation of the apparently critical PAF–ST culture combination with the LDB11 and LDB12 strains did not result in further improvement of pediocin yields, the presence of the LDB component is essential in yogurt fermentation for the development of flavor and aroma properties that are considered characteristic of this product. The results of trials with 2%-fat milk were essentially the same as that observed with using skim milk (data not shown).

Table 1 Pediocin production by *P. acidilatici* PAF in microbiological media

Medium	A_{660}	pH	Pediocin AU ml^{-1}
MRSLac	0.08	6.24	800
TYL	0.09	6.33	800
MRSGlu	1.12	4.35	12,800
MRSGal	1.15	4.25	12,800

Incubation time: 8 h at 37°C

Table 2 Pediocin production in skim milk fermented with *Pediococcus acidilactici* and LAB starter cultures

Time h	LB/PAF		ST/PAF		LB/ST/PAF	
	pH	AU/ml	pH	AU/ml	pH	AU/ml
4	6.54	TR[a]	5.35	TR	5.21	TR
5	6.46	TR	4.90	TR	4.74	400
6	6.26	200	4.64	400	4.50	800
7	5.96	200	4.50	800	4.33	1,600
8	5.75	300	4.36	1,600	4.24	3,200
9	5.38	400	4.27	3,200	4.19	3,200
10	5.32	400	4.20	3,200	4.14	3,200

LAB cultures: LB, *L. delbrueckii* subsp. *bulgaricus* LDB12; ST, *S. thermophilus* ST129; PAF: *P. acidilactici*

Survival of *Listeria monocytogenes* Scott A in milk co-fermented with PAF and LAB

Since *Listeria monocytogenes* has been reported to survive in acidic environments [15, 24], it was of interest to examine

Table 3 Survival of *L. monocytogenes* Scott A in skim milk fermented with LAB in co-culture with *P. acidilactici*

Time day	Plate counts of *Listeria monocytogenes* (cfu/ml)			
	Skim milk		2% Milk	
	ST/LB	PAF/ST/LB	ST/LB	PAF/ST/LB
0	1.3×10^3	1.2×10^3	1.6×10^3	1.5×10^3
0.25	10^3	10^2	1.6×10^3	10^2
2	1.9×10^3	10^2	2.8×10^3	2×10^2
4	2×10^3	10^2	2.8×10^3	2×10^2
6	2×10^3	10^2	3.8×10^3	2.8×10^2
8	2×10^3	10^2	4×10^3	3×10^2
10	1.6×10^3	0.8×10^2	4×10^3	2×10^2
14	10^3	0.6×10^2	3×10^3	2×10^2
16	10^3	0.1×10^2	3×10^3	2×10^2

LAB cultures: ST: *S. thermophilus* ST129; LB: *L. delbrueckii* subsp. *bulgaricus* LDB12; PAF: *P. acidilactici*

whether the amount of pediocin produced by PAF in co-culture with yogurt starter bacteria would be sufficient to inhibit the growth of this pathogen in yogurt-type dairy foods. At the start of the simulated yogurt production, both skim and 2%-fat milk samples were inoculated with LMS at 10^3 cfu ml^{-1}. The fermentation was stopped after 5 h, followed by storage of milk samples at 4°C. The results of plate counts for LMS on MOX agar plates over a period of 16 days are shown in Table 3. It was apparent that the inclusion of PAF in the starter mix caused a decrease in LMS cfu ml^{-1} in both skim and 2%-fat milk samples. During the first few days of storage, there was a slight increase in LMS plate counts in skim milk fermented without PAF, but on continued storage, the cfu ml^{-1} values returned to the initial levels and remained essentially unchanged during the rest of the storage period. In 2%-fat milk, there was a slight increase in LMS plate counts that persisted throughout the storage period. The results indicated that following the initial impact on the LMS population present in the yogurt mixes, the amount of pediocin produced was not adequate to destroy all LMS cells present in the coagulum. More work is needed on the selection of LB and ST companion cultures and also on production conditions that would promote pediocin output by PAF leading to a higher level titer that in turn would provide for greater efficiency in controlling LMS in fermented dairy foods.

In developed countries *L. monocytogenes* is responsible for over 20% of outbreaks of food-borne illnesses caused by dairy foods [11]. Listeria not only occurs in cheeses [20], but may survive the acidified conditions in fermented dairy products [8, 15, 19, 24, 25]. Therefore, continuing research on controlling this pathogen in all types of dairy foods is needed.

Various approaches have been suggested to control *L. monocytogenes* in fermented dairy products, including the application of the lantibiotic nisin produced by selected *Lactococcus lactis* strains, which is approved by the FDA for food uses [1, 7]. However, this broad-spectrum bacteriocin, which is highly active against Listeria [2], may negatively impact the performance of yogurt starter cultures depending on their level of sensitivity [2, 18]. An alternative solution suggested by Benkerroum et al. [2] involved the selection of bacteriocin-producing *S. thermophilus* strains for use in yogurt production. These authors reported that a bacteriocin-producing ST strain prevented the proliferation of LMS and extended shelf life of yogurt.

Pediocin production in milk or fermented milk products is not expected since pediococci are either slow- or non-lactose-fermenting microorganisms. Nevertheless, the results of this demonstrated that in dairy products fermented by yogurt starter cultures (LDB, ST), there may be a sufficient amount of monosachharides (glucose, galactose) generated from lactose hydrolysis that allows growth and pediocin production by PAF. The amount of pediocin produced under the experimental conditions was insufficient for the total elimination of Listeria from the yogurt samples during the storage period. Nevertheless, the results demonstrated the value of pediocin as a natural biopreservative in designing hurdle technologies to enhance the safety of yogurt and other fermented milk products [7]. Further work is required to establish the optimum ratio of starter culture components on pediocin production during yogurt fermentation and the impact of pediococci as adjunct cultures on the survival of *L. monocytogenes* during product storage.

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