Pediococcus acidilactici PO2 Bacteriocin Production in Whey Permeate and Inhibition of *Listeria monocytogenes* in Foods

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ABSTRACT

Growth of *Pediococcus acidilactici* PO2 and production of associated bacteriocin (pediocin PO2) were demonstrated in whey permeate (WP) supplemented with 0.5–4% yeast extract (YE). Initial pH 6.5 (without PH regulation during fermentation) was optimal for production of pediocin PO2 in WP containing 2% YE. A pediocin-rich dairy ingredient (PRDI) powder was made of the fermented WP and applied in heat-treated whole milk and pasteurized liquid whole egg. PRDI inhibited *Listeria monocytogenes* in milk. *L. monocytogenes* was inhibited by untreated liquid whole egg and PRDI appeared to contain a factor that offsets the inherent antilisterial action of the egg product.

Key Words: whey, pediococcus, bacteriocin, listeria

INTRODUCTION

WHEY PERMEATE (WP) is produced during ultrafiltration of cheese whey to manufacture whey protein concentrate. WP contains ca. 5.7% solids which are mainly lactose (Coton, 1980). In addition, WP contains the minerals and trace elements required for microbial growth (Moulin and Galzy, 1984). Possible uses of WP include the production of lactose, ethanol (through fermentation of lactose), ammonium lactate, galactose, baker's yeast, and single cell protein. Probably WP would support growth and bacteriocin production by bacteriocinogenic bacteria, but investigations to test this postulate are lacking.

Several newly discovered bacteriocins are produced by lactic acid bacteria (LAB) such as pediococci which appear to be potentially useful in food applications. Pediococci are homofermentative LAB used to produce several fermented foods (Franklin and Sharpe, 1963; Pederson, 1949; Smith and Palumbo, 1983). Hoover et al. (1988, 1989) found that *Pediococcus acidilactici* PO2 produce a bacteriocin (referred to as pediocin PO2) that inhibit several bacteria including *Listeria monocytogenes*.

L. monocytogenes, as established foodborne pathogen, has the potential for contaminating most types of foods (Brackett, 1988) and has become a major concern to the food industry and regulatory agencies. Listeriosis, caused by this organism, is estimated to cost much more per incident than any other common but less serious foodborne disease, because of the high fatality rate (Todd, 1989). Food-grade LAB such as *P. acidilactici* could be used to produce a bacteriocin in a low cost food ingredient such as WP and the product may be used as a natural biopreservative to control undesirable microorganisms (e.g. *L. monocytogenes*) in food. The objectives of our study were to produce the pediocin PO2 in WP by *P. acidilactici* PO2, and to demonstrate the inhibitory activity of the prepared pediocin-containing permeate against *L. monocytogenes* in selected food systems.

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MATERIALS & METHODS

Bacterial strains and media

Bacterial strains were obtained from the Food Research Institute, Madison, WI. *P. acidilactici* PO2 was used as pediocin-producing strain (Hoover et al., 1988, 1989) and *L. monocytogenes* Scott A and Ohio were used as pediocin-sensitive strains. Cultures were maintained as frozen stocks at −20°C in appropriate media containing 10% glycerol (Sigma Chemical Co., St. Louis, MO) and propagated twice before use. MRS broth (Oxoid LTD., Basingstoke, Hampshire, U.K.) and tryptose broth (Difco Laboratories, Detroit, MI) supplemented with 0.5% yeast extract (Difco) (TYE) were used as media for *P. acidilactici* and *L. monocytogenes*, respectively. Soft overlay agar was prepared to contain 0.75% agar. Whey permeate was prepared from fresh cheddar cheese whey by ultrafiltration using Hollow Fiber Cartridge PM 50 (Romicon Inc., Woburn, MA) and frozen at −20°C until used. Permeate was thawed and filtered with a 0.45-μm filter (Gelman Sciences, Ann Arbor, MI) before use.

Pediocin PO2 assay

Samples for detection of pediocin PO2 were centrifuged at 12,000xg for 20 min and then filtered through a 0.45-μm filter (Gelman). Assay for pediocin PO2 in the filtrate was as described by Barefoot and Klaenhammer (1983). Filtrate (5μL) of each sample was spotted onto a TYE soft overlay agar (5 mL) seeded with 10 μL of an overnight culture of the sensitive indicator strains i.e., *L. monocytogenes* Scott A or Ohio. Assay plates were incubated at 37°C for 18 hr and results were expressed in Arbitrary Units (AU) mL.

Production of pediocin PO2 in whey permeate

Whey permeate and MRS were used as basal media for testing the effect of different concentrations of yeast extract (YE) on production of pediocin by *P. acidilactici* PO2. The following liquid media were prepared: (a) MRS broth supplemented with 0, 1, 2 or 3% YE (in addition to a 0.5% YE originally present in MRS); (b) YE alone at concentrations of 0.5, 1, 2, 3 and 4% and (c) WP supplemented with 0, 0.5, 1, 2, 3 or 4% YE. pH value for all media was adjusted to 6.5. *P. acidilactici* PO2 (18-hr culture) was inoculated (0.01%) into duplicate flasks, each containing 25 mL of one of the former media, and cultures were incubated at 37°C. Samples were withdrawn at intervals during incubation periods to measure growth, pH and activity of pediocin PO2. Optical Density of cultures at 600 nm (OD_{600}) was measured in Spectronic 1001 spectrophotometer (Milton Roy, Rochester, NY) as an index of growth.

Initial pH and pH regulation during fermentation were studied in a 5-L bench-top fermenter (VirTis Co., Inc., Gardiner, NY) connected to an automatic pH controller (Cole Parmer, Chicago, IL). WP was supplemented with 2% YE, then pH was adjusted to 6.0, 6.5 or 7.0. *P. acidilactici* PO2 was inoculated at the 0.01% level into 2 L of medium in the fermenter, and the mixture was fermented with or without pH control. Controlling the pH was achieved by automatic addition of 10% NaOH. The fermenter vessel was held at 37°C, purged with N_2 and stirred at 200 rpm. Samples were continuously withdrawn and collected in a fraction collector under refrigeration (0°C) at a rate of 8mL/h by a peristaltic pump (Phamacia Fine Chemicals, Uppsala, Sweden). After ca. 45 hr of fermentation, all the collected samples (4 mL each) were analyzed for growth (OD_{600}), activity of pediocin

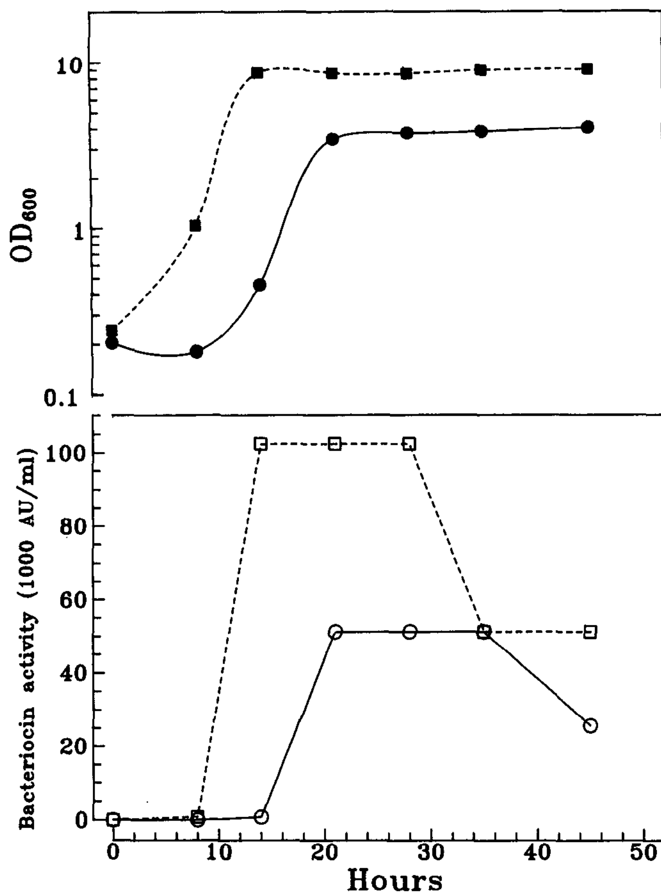

Fig. 1.—Growth of Pediococcus acidilactici *PO2 and production of the pediocin PO2 in MRS broth containing different concentrations of yeast extract (initial and final pH values were 6.2-6.3 and 4.1-4.3, respectively). Symbols: ■● = growth (OD_{600}), □○ = pediocin activity [Arbitrary units (A)/mL]. Concentrations of yeast extract in MRS: 0% (——) and 1% (--).*

Table 1—Growth[a] of Pediococcus acidilactici *PO2 and production of pediocin PO2 in whey permeate (WP) containing different concentrations of yeast extract (YE)*

	Incubation period (hr)						
	8	21	35	45	21	35	45
Media[b]	Growth (OD_{600})				Bacteriocin activity (AU/mL)[c]		
0.5% YE	0.18	0.25	0.27	0.30	0	0	0
WP + 0.5% YE	0.24	0.68	0.61	0.52	800	400	0
1.0% YE	0.25	0.50	0.47	0.44	0	0	0
WP + 1.0% YE	0.25	1.19	1.00	0.91	1600	800	200
2.0% YE	0.27	0.55	0.73	0.73	0	0	200
WP + 2.0% YE	0.38	1.60	1.00	0.91	3200	3200	800
3.0% YE	0.30	1.46	1.25	1.12	400	400	200
WP + 3.0% YE	0.43	2.01	1.81	1.52	6400	6400	3200
4.0% YE	0.44	2.10	1.73	1.48	800	1600	1600
WP + 4.0% YE	0.48	2.71	2.30	1.88	12800	12800	6400

[a] The initial and final pH values of all media were in the range 6.40–6.60 and 5.2–5.6, respectively.
[b] No growth or bacteriocin production occurred in WP.
[c] Production of pediocin PO2 was not detected after 8 hr of incubation. *Listeria monocytogenes* Scott A was used for the assay of pediocin PO2.

PO2 and pH value. The experiment was repeated and data represent the average of two trials.

Preparation of pediocin-rich dairy ingredient (PRDI) power

Whey permeate, supplemented with 2% YE and adjusted to pH 6.5 (without regulating the pH during fermentation), was used for pediocin production by *P. acidilactici* PO2. Fermented medium was centrifuged at 12,000 × *g* for 20 min and pH of the resulting supernatant was adjusted to 7.0 with 3N NaOH. Neutralized culture supernatant was filtered through a 0.45-µm filter, then mixed with nonfat dry milk (10% wt/vol) and the mixture was heated at 100°C for 5 min. The heated mixture was tested for pediocin activity and freeze dried (Consol 25SL; VirTis Co., Inc., Gardiner, NY) to produce the pediocin-rich dairy ingredient (PRDI) powder. A portion of the culture supernatant was neutralized, mixed with protease (type I, 200 µg/mL; Sigma Chemical Co., St. Louis, MO), incubated at 37°C for 1 hr and then mixed with nonfat dry milk and heated as indicated earlier. Protease-treated supernatant was checked for freedom of pediocin PO2, freeze dried to produce protease-treated PRDI powder, and used as a control.

Application of PRDI powder in listeria-contaminated foods

Commercial grade A whole milk was heated at 180°F for 60 min, cooled to 25°C and then used to test the inhibitory activity of the PRDI powder. A portion (1 or 2g) of PRDI powder was added in duplicate to 25 mL of heat-treated milk. Treated milk was inoculated with *L. monocytogenes* Scott A or Ohio (18-hr culture) at ca. 2×10^3 CFU/mL and incubated at 25°C. Samples were withdrawn after 4, 10, and 20 hr incubation. Samples or dilutions thereof were surface-plated onto tryptose phosphate agar (Difco) containing 0.5% each of ferric citrate and esculin (Sigma) (TPA-FE) (Foegeding and Leasor, 1990) and plates were incubated at 37°C for 48 hr. Blue-gray colonies (often with indented center) which caused blackening of the medium were enumerated as *L. monocytogenes*. The following treatments were included: (1) uninoculated, (2) inoculated, (3) inoculated plus PRDI powder, (4) inoculated plus protease-treated PRDI powder, (5) uninoculated plus PRDI powder, and (6) uninoculated plus protease-treated PRDI powder. Treatments 1, 5, and 6 were done to rule out the presence *L. monocytogenes* as a contaminant in the components of these treatments.

Commercial fresh eggs were disinfected by soaking whole eggs in fresh hypochlorite solution (500 ppm) for 15 min and then the contents were collected aseptically into a blender. After blending for 1 min, fresh liquid whole egg was heated at 65°C for 3.5 min. This pasteurized liquid whole egg was used for testing the inhibitory activity of PRDI powder against *L. monocytogenes*. Procedure and treatments were the same as those of heat-treated milk.

RESULTS

THIS STUDY was performed in two phases; (a) defining the optimum conditions for production of pediocin PO2 by *P. acidilactici* PO2 in WP, and (b) use of the resulting pediocin-containing WP to control *L. monocytogenes* in selected foods. In the first phase, factors such as YE supplementation, initial pH, and control of pH during fermentation were investigated.

Production of pediocin PO2 in whey permeate

The ability of *P. acidilactici* PO2 to produce large amounts of pediocin PO2 was tested in MRS broth, a nutritionally complex medium. Additionally, batches of MRS broth were supplemented with YE. Growth of *P. acidilactici* PO2 increased greatly by supplementing MRS broth with 1% YE (Fig 1). With 2 and 3% only a slight increase over 1% was observed (Data not shown). Pediocin activity also increased from 5.12×10^4 to 1.03×10^5AU/mL when MRS broth was supplemented with 1% YE (Fig. 1). Additional increase in the level of YE (2 or 3%) resulted in negligible change in the maximum activity of pediocin PO2. However, the maximum pediocin titer remained stable for longer periods when the high levels of YE were used (data not shown).

Growth of *P. acidilactici* PO2 in WP without supplementation was minimal, and no pediocin activity was detected during 45 hr incubation (data not shown). YE supported growth of *P. acidilactici* PO2, but limited amounts of pediocin PO2 were detected in the medium (Table 1). *P. acidilactici* PO2 grew and produced pediocin PO2 at greater rates in WP supplemented with YE than in WP or YE separately. For example, the medium made of 2% YE alone allowed growth of cells to 0.548 units of OD_{600} after 21 hr incubation, whereas cell growth increased to 1.599 OD_{600} during the same period using WP plus 2% YE as medium (Table 1). Pediocin production was not detected in 2% YE alone after 21 hr incubation, while it reached 3,200AU/mL in WP plus 2% YE during the same period (Table 1).

Production of pediocin PO2 in WP containing 2% YE by *P. acidilactici* PO2 was examined via fermentation with or without pH con-

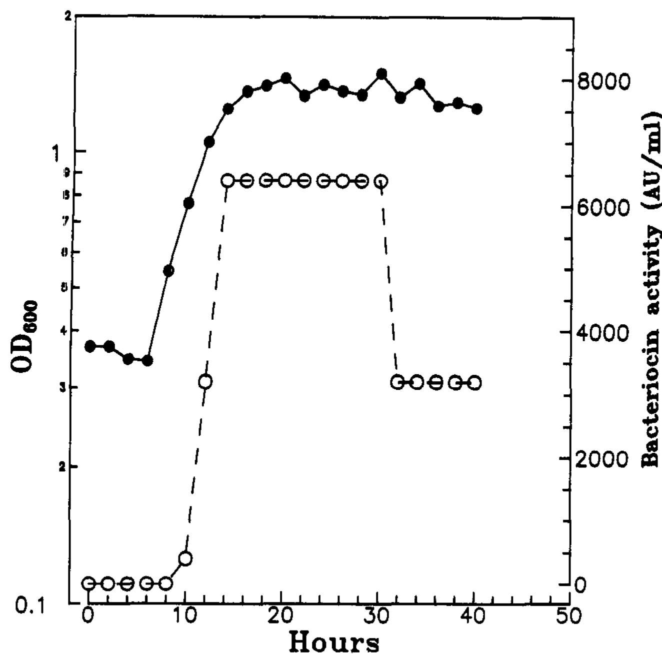

Fig. 2.—Growth (OD_{600}) of Pediococcus acidilactici *PO2 and production of pediocin PO2 (AU/mL) in whey permeated supplemented with 2% yeast extract during fermentation without controlling the pH (initial and final pH values were 6.5 and 5.1, respectively). OD_{600} (●—●), AU/mL (○--○).*

Table 2—Production of pediocin PO2 by Pediococcus acidilactici *PO2 in whey permeate supplemented with 2% yeast extract when fermented at different pH treatments in a 2-L fermenter*

pH[a]		Incubation period (hr)					
Initial	Final	12	20	28	12	20	28
		Growth (OD_{600})			Bacteriocin activity (AU/mL)[b]		
7.0	7.0	0.55	1.57	1.64	0	200	0
6.5	6.5	0.75	1.83	1.60	0	800	0
6.0	6.0	0.72	0.82	0.76	0	0	0
7.0	6.2	0.65	1.33	1.41	0	0	0
6.5	5.1	1.05	1.46	1.34	3200	6400	6400
6.0	5.2	0.51	0.83	0.95	1600	1600	1600

[a] Equal initial and final (after 28 hr of fermentation) pH values indicate that the fermentation was done under pH control.
[b] Bacteriocin activity was assayed against *Listeria monocytogenes* Scott A.

trol. *L. monocytegenes* Scott A was used as a sensitive indicator to monitor production of pediocin PO2. Among six different pH treatments (Table 2), the largest amount of pediocin PO2 was produced in media with initial pH 6.5 which was fermented without controlling pH. Under these conditions, pediocin titer reached 6,400 AU/mL after 14 hr and maintained activity until 30 hr (Fig. 2). By using an initial pH of 6.0 without control during fermentation, activity of pediocin PO2 was only 25% of that at pH 6.5 (Table 2). Appreciable growth was detected in the other four pH treatments, but production of pediocin PO2 was less than 800 AU/mL.

Inhibition of *L. monocytogenes* by PRDI powder in heat-treated milk

The pediocin-containing powder (PRDI) was added to heat-treated milk which was previously inoculated with *L. monocytogenes* Scott A or Ohio. After 20 hr incubation at 25°C, the cell population of *L. monocytogenes* Scott A in the inoculated control (without any PRDI powder) increased from 3.3×10^3 to 2.7×10^7 CFU/mL. Pediocin PO2 in PRDI powder delayed the growth of *L. monocytogenes* Scott A by ca. 10 hr (Fig. 3). At the end of incubation, count of *L. monocytogenes* Scott A was 5.0×10^7 CFU/mL in the control treatment (inoculated milk containing protease-treated PRDI powder), whereas it was 5.5×10^5 CFU/mL (99% decrease) in inoculated milk with added PRDI powder (20,480 AU/mL). *L. monocytogenes* was not detected in the uninoculated control during 20 hr incubation. *L. monocytogenes* strain

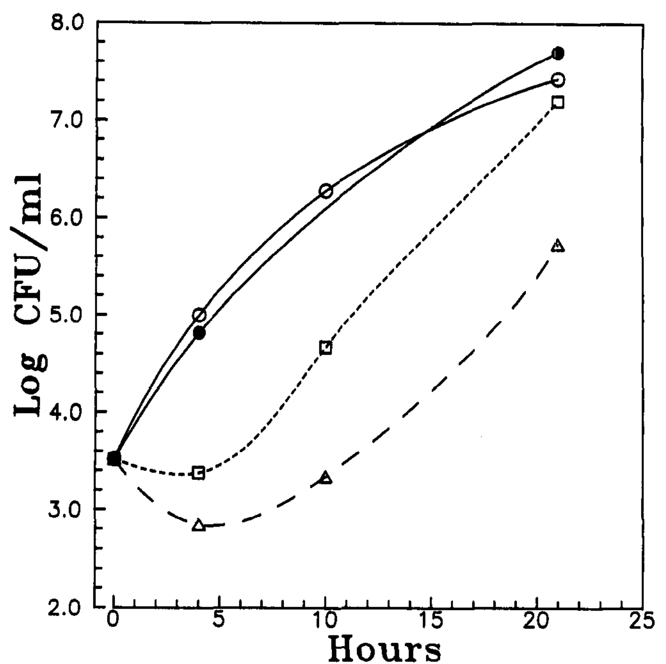

Fig. 3.—Growth of Listeria monocytogenes *Scott A in heat-treated milk in the presence of pediocin PO2 (added as PRDI powder). Concentrations of pediocin PO2: 0 [uninoculated control] (○—○), 0 [control containing protease treated PRDI powder] (●—●), 10,240 (□---□), and 20,480 (△--△) AU/mL.*

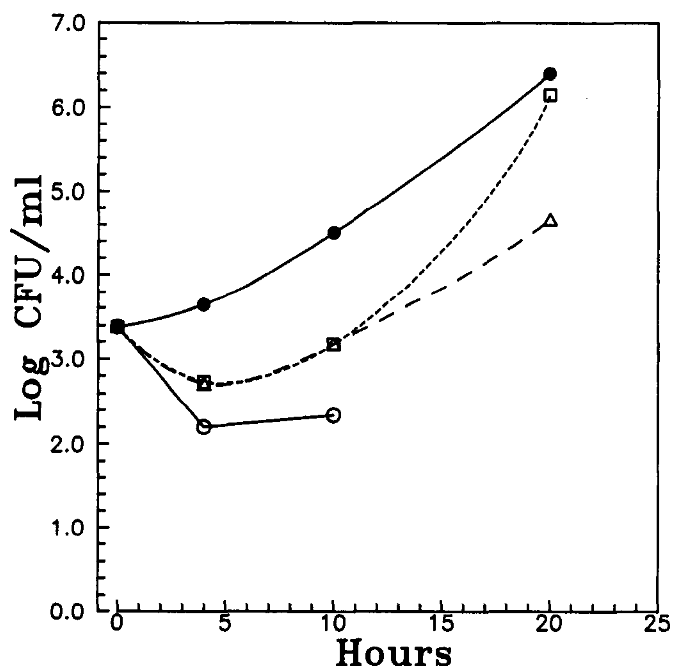

Fig. 4.—Growth of Listeria monocytogenes *Scott A in pasteurized liquid whole egg in the presence of pediocin PO2 (added as PRDI powder). Symbols are the same as in Fig. 3. Count of* L. monocytogenes in the control treatment after 10 hr of incubation was undetectable.

Ohio in heat-treated milk was also inhibited by PRDI powder. Counts of *L. monocytogenes* Ohio after 20 hr of incubation at 25°C were 87% lower in treated milk than in controls (data not shown).

Inhibition of *L. monocytogenes* by PRDI powder in pasteurized liquid whole egg

Pasteurized liquid whole egg was used to test the effect of PRDI powder on *L. monocytogenes*. Growth of *L. monocytogenes* Scott A

was delayed by ca. 10 hr in the pediocin-treated liquid egg, in comparison with the product that contained enzyme-treated PRDI powder (Fig. 4). After 20 hr incubation at 25°C, the cell population of *L. monocytogenes* Scott A was 2.5×10^6 CFU/mL in control containing enzyme-treated powder, and 4.6×10^4 CFU/mL (98% decrease) in product containing PRDI powder (20,480 A/mL). However, the number of *L. monocytogenes* Scott A in inoculated control (without PRDI powder) decreased from 2.4×10^3 CFU/mL initially to 2.4×10^2 CFU/mL after 10 hr and then cell count was below detection level after 20 hr incubation at 25°C (Fig. 4). *L. monocytogenes* was not detected in the uninoculated control during the same period. Count of *L. monocytogenes* strain Ohio in inoculated control also decreased from an initial count of 2.4×10^3 CFU/mL to 80 CFU/mL after 4 hr and count was nondetectable after 10 hr incubation at 25°C (data not shown).

DISCUSSION

PRODUCTION of pediocin PO2 by *P. acidilactici* PO2 in MRS or WP increased considerably in the presence of YE in these media. A former study showed that bacteriocin production by various strains of *Streptococcus mutans* was enhanced by adding 2% YE to a basal trypticase medium (Rogers, 1972). Addition of 0.6% YE increased the production of bacteriocin by *S. lactis* subsp. *diacetylactis* S1-67/C in YE-dextrose broth, but additional increase of YE concentration showed a negligible change in bacteriocin production (Reddy and Ranganathan, 1983).

Pediocin PO2 was produced in greater amounts in WP containing YE than in YE alone (Table 1). Media that contained WP and YE enhanced pediocin production by *P. acidilactici*, although WP alone did not provide sufficient nutrients for production of pediocin PO2. WP usually contains lactose (4.9%), protein (0.03%), non-protein nitrogen (0.1%), ash (0.5%), fat (<0.01%) and lactic acid (0.15%) (Coton, 1980). Lactose may be used as a carbon source by pediococci (Garvie, 1986). In addition, WP contains some trace elements, such as iron, copper, zinc and manganese, and vitamins including vitamin A, thiamin, pyridoxin, riboflavin, calcium pantothenate, biotin, cobalamin and vitamin C (Moulin and Galzy, 1984). Lactose, trace elements and vitamins in WP may have been utilized, thus supporting the growth and production of pediocin by *P. acidilactici* PO2.

Production of pediocin PO2 in the fermenter depended on the changes in pH during the course of fermentation. In our range of pH values, the highest pediocin titer in WP containing 2% YE was obtained when initial pH was 6.5, and final pH was 5.1 (Table 2). Other investigators (Tagg et al., 1973) reported that bacteriocin production by streptococci on Todd-Hewitt agar was increased by adjusting the initial pH of the medium to 6.5. Biswas et al. (1991) also found that the optimal initial pH for bacteriocin production by *P. acidilactici* H was 6.5, but when the final pH of broth was ≥ 5.0 negligible amounts of bacteriocin were produced. Our study also showed that production of pediocin PO2 in YE-fortified WP coincided with the late logarithmic phase of *P. acidilactici* PO2 and amounts produced remained stable for 16 hr during the stationary phase (Fig. 2).

Addition of pediocin-containing powder (PRDI) to milk delayed the growth of *L. monocytogenes* by 4–10 hr. Added pediocin PO2 decreased the initial load of *L. monocytogenes* in milk, but the remaining cells grew and attained high numbers (Fig. 3). Residual antilisterial activity in pediocin-treated milk was determined using a technique similar to that of pediocin assay. Pediocin PO2 was not detected in treated milk that was stored for 2 days at 25°C. Apparently, pediocin PO2 combined irreversibly with susceptible cells and thus it had lost activity during storage of milk. Bhunia et al. (1991) provided evidence for the presence of specific receptors for pediocin AcH on the cell walls of susceptible bacteria. Therefore, the amount of pediocin PO2 to be used in food is governed by the load of susceptible cells and probably by the type of food. In a previous study, bacteriocin from another strain, *P. acidilactici* PAC 1.0, was produced in MRS containing 2% YE and applied in creamed cottage cheese, half-and-half cream and cheese sauce at 4°C (Pucci et al., 1988). The bacteriocin preparation inhibited growth of *L. monocytogenes* in half-and-half cream and cheese sauce during refrigerated storage.

L. monocytogenes was inactivated in untreated liquid whole egg (Fig. 4). Addition of protease-treated PRDI powder protected *L. monocytogenes* against the intrinsic bacteriocidal action of liquid whole egg. Residual antilisterial activity in pediocin-treated liquid whole egg was determined after 2 days incubation at 25°C using methods similar to that of bacteriocin assay. The product contained residual antilisterial activity equivalent to 25,600 AU/mL; a value greater than the maximum activity (20,480 AU/mL) attributed to added pediocin PO2. These results suggested that nontreated liquid whole egg contained a factor that caused inhibition of *L. monocytogenes* in this product. It is also likely that PRDI (prepared with or without protease treatment) contained a substance that interacted with the egg antilisterial factor, thus eliminated the bactericidal action of this factor. After the inherent bactericidal activity in egg was eliminated (by added PRDI powder), increased concentrations of pediocin PO2 caused corresponding increases in antilisterial activity in the treated liquid egg (Fig. 4). Accordingly, application of pediocin PO2 to enhance the safety of liquid whole egg should be preceded by efforts to exclude from PRDI powder the substance that suppresses the egg intrinsic antilisterial factor.

Reports describing growth and survival of *L. monocytogenes* in eggs are not in agreement. Five strains of *L. monocytogenes* grew in liquid whole egg at 4 to 30°C. Scott A was an exception and did not grow at 4 or 10°C (Foegeding and Leasor, 1990). However, Sionkowski and Shelef (1990) indicated that cell population of *L. monocytogenes* declined in raw whole eggs and albumin but increased in the yolks at 20°C. At 5°C, the organism grew in raw whole egg and egg yolks but not in albumin. It was suggested that proteins such as lysozyme in egg albumin might be responsible for the death of *L. monocytogenes*. It was demonstrated that egg white lysozyme lysed *L. monocytogenes* cell suspended in phosphate buffer (Hughey and Johnson, 1987). Wang and Shelef (1991) also reported that the antilisterial effects of albumin were primarily due to lysozyme.

The pediocin-containing powder (PRDI) caused noticeable inhibitory activity against *L. monocytogenes* Scott A and Ohio in heat-treated milk. The fermented whey we developed may be applicable in other dairy products like cheese sauce and cold-pack cheese food. Although several bacteriocins produced by pediococci were previously reported, those were produced in high-cost and/or non-food grade media (Gonzales and Kunka, 1987; Hoover et al., 1988; Nielson et al., 1990; Pucci et al., 1988). Our study indicates that pediocin PO2 can be produced in a low-cost food-grade medium (WP plus YE) and can be prepared as a powder which may be useful in controlling *L. monocytogenes* in selected food systems.

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WATER VAPOR PERMEABILITY OF MODEL EDIBLE FILMS. . .From page 429

erential water vapor transfer through the methylcellulose matrix in the emulsified film was confirmed. In the laminated films, methylcellulose is only used as a support and has no effect on the transfer resistance of the film, compared to the paraffin wax resistance.

CONCLUSION

AN ACCURATE method aimed at continuously following the permeation process was perfected to reveal that barrier efficiency depends both on polarity of the film, and on its structure (homogeneity). A film of laminated paraffin wax on a methylcellulose support was an efficient barrier to transport water vapor. Its permeability was close to that of a plastic (polyethylene) film and 10 times smaller than the permeability of an emulsified film. SEM revealed that paraffin wax distribution within or on the surface of the film strongly depended on film preparation techniques. SEM made it possible to schematize the structure of the film: starting from the permeability or water vapor transfer resistance of each component, it was possible to estimate with a model the permeability of resistance of the entire film.

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