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Use of Bacteriocinogenic *Pediococcus acidilactici* in Sausage Fermentation

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The bacteriocin produced by *Pediococcus acidilactici* KFRI 168 exhibited a wide antimicrobial spectrum including many strains of lactic acid bacteria, *Listeria monocytogenes, Staphylococcus aureus,* and *Enterococcus faecium* by both disk and deferred assay methods. Inhibition of *Lis. monocytogenes* and *Stph. aureus* were observed only from deferred assay. Gram-negative bacteria were not inhibited. Bacteriocin production was observed at 10 h, and was maximized at 16 h in MRS broth incubated at 37°C. In a beaker sausage fermented with *P. acidilactici* KFRI 168, viable counts of *Stph. aureus, Salmonella, Escherichia coli, Clostridium perfringens,* and *Lis. monocytogenes* were reduced by 2.8, 2.3, 2.4, 0.7, and 0.5 log CFU/g, respectively. Inoculated *P. acidilactici* KFRI 168 maintained its viable count of more than 10^8 CFU/g during the whole fermentation period, and it took less than 8 h to reduce sausage pH below 5.

As the consumption of fermented sausage is increasing, the demand for using suitable lactic starter culture is expanding. The sausage batter provides an excellent environment not only for the proliferation of desirable lactic acid bacteria (LAB) but also for food poisoning microorganisms. The latter cause serious health threatening problems. Sporadic outbreaks of staphylococcal food poisoning, listeriosis and salmonellosis have been reported from the consumption of unhygienically prepared fermented meat products (6). It is therefore prerequisite to use lactic starter culture(s) which possess antimicrobial effects in the manufacture of fermented sausage (2). The major contribution of lactic starter culture for the inhibition of undesirable bacteria is a production of lactic acid. Besides, many LAB have been found to produce bacteriocin or bacteriocin-like compounds that also contribute to the safety aspect of fermented sausage (8). From the previous studies, Kim *et al.* (9) have reported the antimicrobial spectrum and plasmid linkage of bacteriocin production phenotype (Bac^+) in *P. acidilactici* M (redesignated as KFRI 168). The *in situ* antagonistic effect of this strain on microorganisms of hygienic concern during sausage manufacture has not been studied yet. This article deals with further characterization of bacteriocin of *P. acidilactici* KFRI 168, and its feasibility as sausage starter was evaluated in respect of inhibition of undesirable microorganisms and pH development during the fermentation of beaker sausage.

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Key words: Bacteriocin, *Pediococcus acidilactici* KFRI 168, starter, fermented sausage

MATERIALS AND METHODS

Test Microorganisms, Culture Condition and Media

All microorganisms used in this study were maintained in lyophilized vial. To activate before use, they were transferred twice in the suitable medium. All LAB including *P. acidilactici* KFRI 168 and *Ent. faecium* were maintained in MRS broth. Strains of *E. coli* and *Sal. typhimurium* were grown in Tryptic Soy Broth. *Stph. aureus* and *Lis. monocytogenes* were cultured in Brain Heart Infusion. *Cl. perfringens* was maintained in Reinforced Clostridial Agar in CO_2 generating anaerobic jar. To prepare agar plate, granular agar was added to broth at 1.5%. The incubation temperature for all microorganisms was 37°C. Table 1 shows the test microorganisms used in this study. The media used in this study were obtained from Difco.

Determination of Antimicrobial Spectrum

A large number of bacteria (25 strains) were tested

Table 1. Antimicrobial specrtrum of bacteriocin produced by *P. acidilactici* KFRI 168.

Test microorganisms	Detection method	
	Disk	Deferred
Leu. paramesenteroides NRRL B3471	+	+
Leu. mesenteroides subsp. *dextranicum* NRRL B1141	+	+
P. acidilactici NRRL B2039	+	+
P. pentosaceus NRRL B14009	+	+
P. halophilus NRRL B4243	−	−
Lb. delbrueckii KFRI 149	+	+
Lb. plantarum NRRL B4496	+	+
Lb. plantarum KFRI 144	−	−
Lb. fermentum KFRI 145	−	−
Lb. brevis KFRI 146	−	−
Lb. amylophilus KFRI 161	−	−
Lb. helveticus NRRL B4526	−	−
Lc. lactis KFRI 125	+	+
Lc. lactis KFRI 131	+	+
Lc. lactis KFRI 141	−	−
Lc. diacetylactis KFRI 139	+	+
Ent. faecium KFRI 134	+	+
Ent. faecium KFRI 132	+	+
Ent. faecium KFRI 130	+	+
Stph. aureus ATCC 14458	−	+
Stph. aureus ATCC 12692	−	+
Stph. aureus KFRI 188	−	+
Lis. monocytogenes KFRI 229	−	+
E. coli KFRI 174	−	−
Sal. typhimurium KFRI 191	−	−

+: presence of zone of inhibition, −: absence of zone of inhibition

by both disk (simultaneous) and deferred assays. In a disk assay, *P. acidilactici* KFRI 168 was grwon in MRS broth for 16 h at 37℃. The culture broth was obtained by centrifugation (13,000 rpm, 10 min) and pH was adjusted to 5.5 by adding 3N NaOH or HCl, and membrane sterilization (cellulose acetate, 0.45 μm) was followed. Forty *μl* of pH adjusted cell-free supernatant fluid was loaded on a sterile paper disk (6 mm) contacted on soft agar lawn of indicator bacteria which was prepared just before the experiment. Plates were incubated for 48 h at 37℃ and the presence or absence of clear zone of inhibition was observed. In a deferred assay method, which is a modification of Spelhaug and Harlander (14), overnight grown *P. acidilactici* KFRI 168 in MRS broth was stabbed on MRS agar and covered by dropping melted MRS soft agar (0.75%) on it. After incubation of the plate (16 h at 37℃), 5 *ml* of soft agar containing test bacterium was overlaid and incubated further for 24 h at 37℃. In both methods, the test bacterium was grown overnight in appropriate medium and was added at approximately 10^6 CFU per plate. The presence of clear zone of inhibition around the disk and/or stabbed colony was regarded as bacteriocin sensitive strain. Diffused zone of inhibition, which is mainly derived from the inhibitory effect of organic acids, was not considered as sensitive strain.

Production of Bacteriocin in MRS, TGE, and M 17-Glu

To select the most efficient medium for bacteriocin production, *P. acidilactici* KFRI 168 was grown (for 16 h at 37℃) in three different media, MRS, TGE (3), and M17-Glu. The production of bacteriocin in each medium was compared by measuring the diameter of the zone of inhibition in deferred and disk assay. *Ent. faecium* KFRI 134, being the most sensitive strain to bacteriocin, was used as an indicator.

Determination of Bacteriocin Titer

P. acidilactici KFRI 168 grown in MRS broth (37℃ for 16 h) was transferred (1% inoculum, V/V) to a cotton-plugged MRS broth held statically at 37℃. At every 2 h, 1 *ml* culture was aseptically taken, centrifuged (13, 000 rpm, 10 mn) in an Eppendorf tube, and followed by serial two fold dilution. Sterile water was used as a diluent. Forty *μl* from each diluted sample solution was disk assayed on the soft agar lawn of *Ent. faecium* KFRI 134. The bacteriocin titer was defined as the reciprocal of the highest dilution that showed clear zone of inhibition on the indicator lawn and was expressed as activity unit per *ml* (AU/*ml*). The growth curve, monitored by counting viable cell counts of KFRI 168, and pH development pattern were also obtained.

Enumeration of Microorganisms in Beaker Sausage

Overnight grown *P. acidilactici* KFRI 168 (MRS broth, 37℃) was harvested, resuspended in 0.1% sterile peptone water, and was added at approximately 10^8 CFU per gram of beakerk sausage batter (Table 2). Exactly 25 g of sausage was aseptically wrapped in sterile aluminum foil and fermentation was carried out at 37℃ for

Table 2. Composition of beaker sausage

Ground beef	72.0%
Ground beef fat	13.7%
Dextrose	0.86%
NaCl	2.06%
White pepper powder	0.26%
Mustard powder	0.26%
Liquid smoke	0.26%
Nutmeg powder	0.001%
Garlic powder	0.0008%
Sodium nitrate	70.0 ppm
Sodium ascorbate	450.0 ppm
Water	10.27%

24 h. At every 4 h, 100 ml of sterile 0.1% peptone water was added to a sample and blended (Stomacher Lab-Blender, UK) for 10 min. The pH was directly read from the sausage slurry, and the viable count of each target microorganism in sausage batter was enumerated. As a negative control, beaker sausage without the addition of *P. acidilactici* KFRI 168 was prepared. The selective media for the enumeration of *E. coli, Lis. monocytogenes, Salmonella, Stph. aureus, Cl. perfringens* and LAB were MacConkey Agar, PALCAM Listeria Selective Agar with supplement, Bismuth Sulfite Agar, Vogel Johnson Agar with 1% Chapman tellurite solution, Tryptose Sulfite Cycloserine Agar, and Rogosa SL Agar, respectively. All plates were incubated at 37℃ for 48 h.

RESULTS AND DISCUSSION

Antimicrobial Spectrum

P. acidilactici KFRI 168 produced bacteriocin which was inhibitory to a wide variety of Gram-positive bacteria (Table 1). Many investigators have indicated that most of the pediocins exhibit wide antimicrobial spectrum (8). A new finding in this experiment is the highest sensitivity of *Ent. faecium* KFRI 134 to bacteriocin, which enabled *Ent. faecium* KFRI 134 as an indicator in determination of bacteriocin titer. To the best of our knowledge, this is the first finding that *Ent. faecium* is inhibited by pediocin.

Disk assay of bacteriocin against *Stph. aureus* and *Lis. monocytogenes* did not show any antagonism, however, they were inhibited by deferred method. This was the same as a large number of similar reports indicating more bacteriocin production in agar than broth (7, 12) or no activity in broth (10). We could not find the reason, however, we could postulate in several ways: (a) a deplection of the nutrient by *P. acidilactici* KFRI 168; (b) continuous or more bacteriocin production in agar than in broth; (c) adaptation to bacteriocin (1) or the outgrowth of a mutant population (5); (d) adoption of inappropriate detection method (14); (e) anaerobiosis resulted from overlaying *P. acidilactici* KFRI 168 with soft agar in deferred assay may be favorable for bacteriocin production (11).

As it was indicated before (9), bacteriocin produced by *P. acidilactici* KFRI 168 seems to be strain specific, rather than species specific (i.e., *Lactococcus lactis* strain 131 was inhibited, while strain 141 was not).

Production of Bacteriocin and pH Change During Cell Growth

Bacteriocin production was observed as the cell entered late log phase of grwoth, and it was maximized during stationary phase (Fig. 1). This indicates that bacteriocin of *P. acidilactici* KFRI 168 is a secondary metabolite.

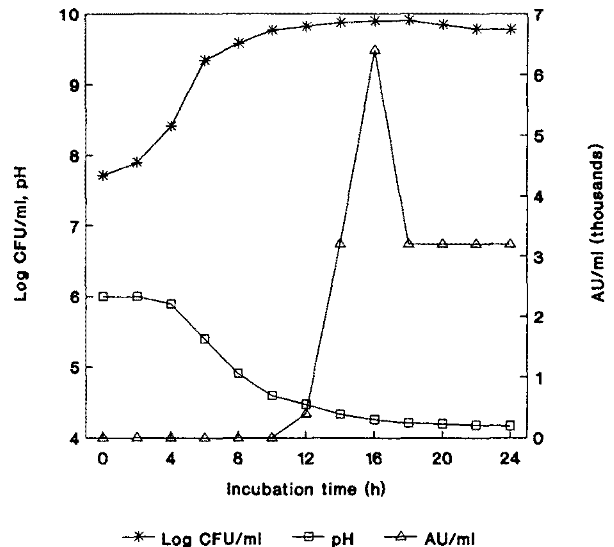

Fig. 1. Growth curve, bacteriocin production and pH development during static culture of *P. acidilatici* KFRI 168 in MRS broth at 37℃.

The maximum activity was observed at pH near 4.5 and it suddenly declined as cells entered the late stationary phase. The highest amount of bacteriocin, based upon the size of zone of inhibition, was produced in MRS agar, following TGE and M17-Glu agar (data not shown). Biswas *et al.* (3) have reported that *P. acidilactici* H, a closely related strain, produced maximum amount of bacteriocin in TGE broth, its titer was higher than ours, and it was gradually reduced. Such differences may be derived from using different indicator (*Lb. plantarum* NCDO 955 versus *Ent. faecium* KFRI 134). This report and previously publicized one (9) indicate both *P. acidilactici* strains H and KFRI 168 seem to share common as well as some different features. Further studies such as DNA-DNA homology and restriction mapping of the plasmids in these two closely related strains will be helpful to elucidate the differences.

Microbial Changes and pH Development in Beaker Sausage

The initial number of coagulase positive *Stph. aureus* in both groups (*P. acidilactici* KFRI 168 added and negative control) was quite high (ca. 3×10^5 CFU/g). Addition of *P. acidilactici* KFRI 168 resulted in approximately 2.8 log reduction in the viable cell count of *Stph. aureus*, while the value was only 0.6 log in negative control (Table 3). Insurgence or adaptation of *Stph. aureus* in late phase of fermentation (1) was not observed in this experiment. Daly *et al.* (4) have reported that when the initial number of *Stph. aureus* is high (ca. 1.9×10^5 CFU/g), the degree of inhibition by commercial starter cultures (Lactacel, Lactacel MC, and Lactacel DS) was

reduced even though they actively grow. Similarly, Raccach (13) reported addition of *P. acidilactici* NRRL B-5624 to beaker sausage in the range of 7.5 to 8.8 log CFU/g and manufacturing at 43℃ showed only 0.5 log reduction when *Stph. aureus* was added at 10^4 CFU/g. Throughout the whole fermentation time, the number of *Spth. aureus* was maintained below 10^6 CFU/g, the number necessary to cause staphylococcal food poisoning. In this respect, the antistaphylococcal effect of *P. acidilactici* KFRI 168 is rather prominent. At this point, it is not clear whether reduction of staphylococcal population is mediated by bacteriocin or organic acid. However, it has been reported that *Stph. aureus* is somewhat resistant to low pH (6), and we also could not observe zone of inhibition when *Stph. aureus* was challenged against plain MRS broth whose pH was adjusted at 4.5 by lactic acid. There was a slight increase in viable cell count of *Stph. aureus* (between 8 and 12 h) in *P. acidilactici* KFRI 168 added group, even though the pH of sausage batter was gradually declining (Fig. 2), and *Stph. aureus* was inhibited in deferred bacteriocin assay (Table 1). Furthermore, *P. acidilactici* KFRI 168 is catalase negative and H_2O_2 is unstable at room temperature. This results, at least, indicate H_2O_2 and acid may not be the sole answer to the reduction of *Stph. aureus*. In other words, there seems to be an additional factor. Manufacturing of sausage with isogenic strain with Bac^- trait will clearly elucidate whether bacteriocin was really involved in antistaphylococcal effect or not. However, we may carefully anticipate bacteriocin is also participating to some extent.

Both *Salmonella* and *E. coli* were insensitive to bacteriocin by deferred and disk assay (Table 1), but their population was greatly reduced in sausage made with starter, while there was slight increase in negative control (Table 3 and Fig. 3). Acid might play a role because their population was rapidly decreased as the pH of the sausage was also lowered (Fig. 2).

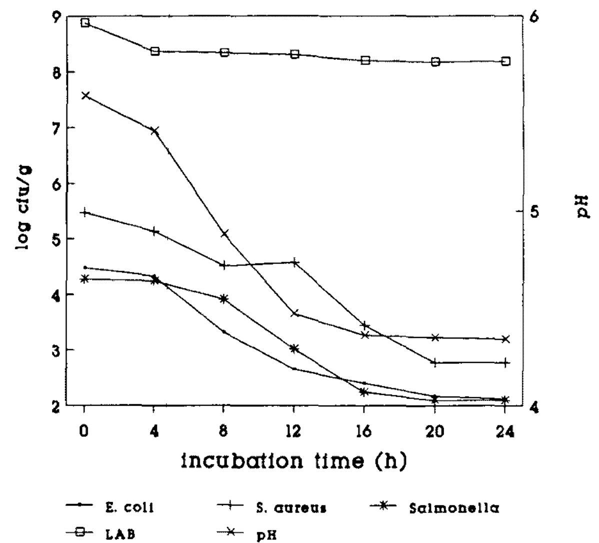

Fig. 2. Changes in viable cell counts of *Stph. aureus*, *E. coli*, *Salmonella* and LAB, and pH development in beaker sausages fermented with the addition of *P. acidilacitici* KFRI 168.

The population of LAB in starter added sausage was slightly decreased for the first 4 h, however, it regained its population at 10^8 CFU/g for the remaining fermentation time (Fig. 2). This indicates *P. acidilactici* KFRI 168 can survive well, and therefore actively produce lactic acid in sausage environment.

To enumerate listeria population in samples, three selective agars including PALCAM Listeria Selective Agar were tried. Both *Lis. monocytogenes and Stph. aureus* grew on McBride Listeria Agar and Tryptic Soy Agar, however, only *Lis. monocytogenes* grew on PALCAM Listeria Selective Agar. Therefore PALCAM was chosen. The initial population of *Lis. monocytogenes* in sausage

Table 3. Changes in viable cell counts (log CFU/g) and pH during fermentation of beaker sausages made with and without *P. acidilactici* KFRI 168

Microorganisms	With KFRI 168			Without KFRI 168		
	Initial	Final	Difference[a]	Initial	Final	Difference[a]
Stph. aureus	5.5	2.7	2.8	5.5	4.9	0.6
Salmonella	4.3	2.0	2.3	4.2	4.3	−0.1
E. coli	4.5	2.1	2.4	4.6	4.3	−0.3
Lis. monocytogenes	2.7	2.2	0.5	2.7	2.8	−0.1
Cl. perfringes	2.8	2.1	0.7	2.8	2.7	0.2
LAB	8.9	8.2	0.2	5.4	7.5	−2.1
pH	5.6	4.4	1.2	5.6	4.8	0.8

The fermentation was continued for 24 h at 37℃.
[a] (initial value) − (final value)
All figures represent mean value after triplicated experiments

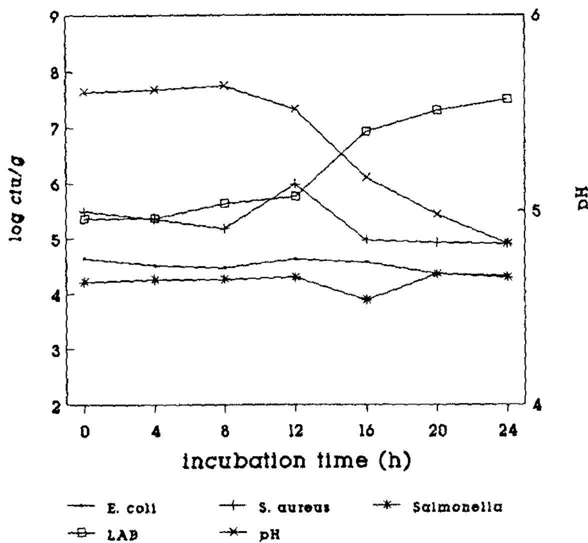

Fig. 3. Changes in viable cell counts of *Stph. aureus, E. coli, Salmonella* and LAB, and pH development in beaker sausages made without the addition of *P. acidilactici* KFRI 168.

batter was low. Even though the decrease in listeria population was minor, at least no increase was observed (Table 3).

The population of *Cl. perfringens* was slightly reduced (0.7 log CFU/g) in starter added, while it was only 0.2 log CFU/g in negative control.

The time to reach pH below 5 for starter added and negative control were 8 and 20 h, respectively (Fig. 2 and 3). This indicates addition of *P. acidilactici* KFRI 168 could save time and energy in the commercial preparation of fermented sausage.

In microbiological view point, *P. acidilactici* KFRI 168 is an excellent starter culture for sausage fermentation. Being the homofermentative, it rapidly lowered the meat pH, strongly reduce the population of *Stph. aureus, Salmonella, E. coli*, and survive well in sausage batter at high number, and additionally, it produces a novel bacteriocin with broad antimicrobial spectrum. Additional studies such as sensory evaluation, pilot scale manufacture of regular sausage, and detection of enterotoxin and biogenic amines will be necessary to ascertain its candidacy as sausage starter.

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