Short communication: 3-Phenyllactic acid production in milk by *Pediococcus pentosaceus* SK25 during laboratory fermentation process

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ABSTRACT

3-Phenyllactic acid (PLA) is a broad-spectrum antimicrobial compound, produced by a wide range of lactic acid bacteria. A novel lactic acid bacteria strain with high PLA-producing ability, *Pediococcus pentosaceus* SK25, was isolated from traditional Chinese pickles. When grown in de Man, Rogosa, Sharpe broth at 30°C for 36 h, this strain produced 135.6 mg/L of PLA. Using this strain as starter for milk fermentation, 47.2 mg/L of PLA was produced after fermentation for 12 h. The PLA production was significantly improved by phenylalanine supplement, but was completely inhibited by tyrosine supplement.

Key words: 3-phenyllactic acid, lactic acid bacteria, fermentation, *Pediococcus pentosaceus*

Short Communication

3-Phenyllactic acid (PLA) is an organic acid with broad-spectrum antimicrobial activity (Mu et al., 2012b). It inhibits not only food-borne pathogenic bacteria, including *Listeria monocytogenes* (Dieuleveux and Gueguen, 1998), *Staphylococcus aureus*, and *Escherichia coli* O157:H7 (Ohhira et al., 2004), but also food-spoiling fungi, including yeasts such as *Candida pulcherrima*, *Candida parapsilosis*, and *Rhodotorula mucilaginosa* (Schwenninger et al., 2008), and a wide range of molds, such as *Aspergillus ochraceus*, *Penicillium roqueforti*, and *Penicillium citrinu* (Lavermicocca et al., 2003). Due to its broad inhibitory activity against a variety of food-borne microorganisms, PLA has interesting potential as an antimicrobial agent in the food industry.

3-Phenyllactic acid is a phenolic acid commonly found in honey, and its content is commonly much higher than that of other phenolic acids in honey. It has even been suggested as a chemical marker for some honeys (Tuberoso et al., 2011). Recently, PLA was proven to be a regular metabolite from lactic acid bac-

teria (LAB) and was produced by a wide range of LAB species (Valerio et al., 2004). Laboratory research has suggested that PLA could be produced in fermented foods using LAB as starter, such as sourdoughs (Van der Meulen, et al., 2007; Ryan et al., 2009). During the sourdough fermentation process, fungal growth was significantly delayed in the presence of PLA-producing LAB strain, *Lactobacillus plantarum* 21B, in coculture with *Saccharomyces cerevisiae* (Lavermicocca et al., 2000).

In our study, a novel LAB strain, *Pediococcus pentosaceus* SK25, was isolated from traditional Chinese pickles and its PLA-producing ability was measured in de Man, Rogosa, Sharpe (MRS) broth. We subsequently evaluated PLA production by this strain in milk through simulating the LAB fermentation manufacturing process, and we analyzed the effect of supplementation with AA precursors on PLA production during the fermentation process.

Pediococcus pentosaceus SK25 was isolated from traditional Chinese pickles and cultivated in MRS medium at 30°C without shaking. To obtain the LAB starter for milk fermentation, the overnight culture of *P. pentosaceus* SK25 was collected and centrifuged at $4,000 \times g$ for 15 min at 4°C. The pellet was then washed twice and resuspended in sterile 0.9% (wt/vol) NaCl to give a final concentration of approximately 5×10^9 cfu/mL for further inoculation of the milk fermentation process.

The PLA production by *P. pentosaceus* SK25 was tested in commercial homogenized milk. Commercial fresh milk (full-fat pasteurized milk, Mengniu Modern Meadow brand, product license No. QS10005010354; Huhehaote, Inner Mongolia, China) was purchased from a local supermarket. Every 100 mL of milk contained 3.4 g of protein, 4.0 g of fat, 5.1 g of carbohydrate, 66 mg of sodium, 118 mg of calcium, and 292 kJ of energy. Milk samples (100 mL) supplemented with 2% glucose were inoculated with *P. pentosaceus* SK25 with initial dose of 10^7 cfu/mL. Milk fermentations were conducted at 30°C in a water bath for 12 h to simulate conditions of yogurt production. Starting after 6 h of incubation, the samples were withdrawn every 1 h for PLA quantification analysis.

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The fermented milk samples were centrifuged at $4,000 \times g$ for 15 min at 4°C . The supernatant was treated with TCA to remove the proteins by precipitation, followed by another round of centrifugation at $20,000 \times g$ for 15 min at 4°C . The ultimate supernatant was filtered ($0.22\text{-}\mu\text{m}$ pore size membrane) for further PLA determination. The PLA was determined using an Agilent 1260 liquid chromatography system equipped with a 1260 Infinity Diode Array Detector (Santa Clara, CA) and an Agilent Zorbax SB-C₁₈ column ($4.6\text{ mm} \times 150\text{ mm}$, $5\text{ }\mu\text{m}$). Linear gradient elution was used with methanol/0.05% trifluoroacetic acid (solvent A) and water/0.05% trifluoroacetic acid (solvent B) at 1 mL/min and A:B ratios of 10:90, 100:0, 100:0, and 10:90, with run times of 0, 20, 23, and 25 min, respectively. The PLA was detected by UV detection at 210 nm.

The PLA-producing microorganisms were isolated from traditional Chinese pickles using MRS broth for cell culture and HPLC for detection. Forty-five strains were isolated that produced more than 0.1 mM PLA when grown in MRS broth at 30°C for 36 h, and the strain SK25 displayed the highest PLA-producing ability of 0.81 mM (135.6 mg/L; Figure 1). The 16S rDNA sequence of the strain SK25 displayed 100% identity with that of *P. pentosaceus* DSM 20336 (GenBank

accession No. NR_042058); thus, the screened strain was identified as *P. pentosaceus*. *Pediococcus* strains are widely distributed in fermented beverages and foods (Tanasupawat et al., 1993). In a previous study, *Pediococcus acidilactici* DSM 20284, which was isolated from barley (Tanasupawat et al., 1993), was reported to be able to produce 0.65 mM PLA in MRS broth after 36 h of fermentation (Mu et al., 2012a). The PLA is a possible metabolite of phenylalanine and could be produced by a wide range of LAB strains. But the PLA productivities of different LAB strains vary greatly. Shown in Table 1, most LAB strains produced less than 1 mM PLA when grown in MRS broth; however, *Lactobacillus plantarum* 1081 produced 5.2 mM PLA (Gerez et al., 2010).

3-Phenyllactic acid is commonly found in honey; however, few reports of PLA in other commercial food products exist. Plenty of reports have shown that PLA could be produced by a wide range of LAB during microbial fermentation, so theoretically PLA may also be produced in LAB-fermented foods. We first determined the PLA content in commercial LAB-fermented yogurts. Four brands of LAB-fermented yogurts were selected for PLA detection and 3 samples were tested for each brand. The results showed that 2 did not contain

Figure 1. Production of 3-phenyllactic acid (PLA) during *Pediococcus pentosaceus* SK25 fermentation at 30°C in de Man, Rogosa, Sharpe broth. Values are means of 3 replications $\pm$ SD.

Table 1. 3-Phenyllactic acid (PLA) production by selected LAB strains grown in de Man, Rogosa, Sharpe broth

Strain	Source	PLA production (mM)	Reference
<i>Pediococcus pentosaceus</i> SK25	Traditional Chinese pickles	0.81 ± 0.06	This study
<i>Pediococcus acidilactici</i> DSM 20284	Barley	0.65	Mu et al., 2012a
<i>Lactobacillus</i> spp. SK007	Traditional Chinese pickles	0.55	Li et al., 2007
<i>Lactobacillus plantarum</i> CECT-221	Spanish Collection of Type Cultures	1.38	Rodriguez et al., 2012
<i>L. plantarum</i> 1081	Culture Collection of the Centro de Referencia para Lactobacilos	5.2	Gerez et al., 2010
<i>L. plantarum</i> CRL 778	Wheat dough	0.1	Dallagnol et al., 2011
<i>Enterococcus faecium</i> ATCC 882	Cheese	0.09	Valerio et al., 2004
<i>Weissella confusa</i> ITM14A	Sourdough	0.06	Valerio et al., 2004
<i>Lactobacillus alimentarius</i> ATCC 29643	Fish products	0.37	Valerio et al., 2004
<i>L. plantarum</i> VE56	Fermented cassava	0.48	Ndagano et al., 2011
<i>Weissella cibaria</i> FMF4B16	Milk flour	0.1	Ndagano et al., 2011

PLA, but the other 2 contained 13.2 ± 0.2 and 21.8 ± 0.3 mg/L of PLA, respectively. The PLA content in the commercial LAB-fermented yogurts was similar to that in most honeys, which generally have less than 100 mg/kg of PLA (Dimitrova et al., 2007).

We also tested the capacity of *P. pentosaceus* SK25 to produce PLA during milk fermentation at 30°C. The PLA content was 17.0, 23.7, 29.1, 34.8, 39.3, 43.5, and 47.2 mg/L when *P. pentosaceus* SK25 was fermented in the milk for 6, 7, 8, 9, 10, 11, and 12 h, respectively, showing that the PLA content gradually increased with longer fermentation times (Table 2). The total PLA production of milk fermentation using *P. pentosaceus* SK25 as a starter was higher than that of the 2 selected commercial LAB-fermented yogurt products in which PLA was detected. In previous studies, wheat sourdough fermentation was simulated using several types of PLA-producing LAB strains as starter, and it was found that 5 to 35 mg/kg of PLA could be produced after fermentation for 48 h (Ryan et al., 2009). Van der Meulen et al. (2007) analyzed the metabolite production during laboratory fermentations of wheat and spelt sourdoughs and found PLA in 35 out of 40 sourdough samples at concentrations usually lower than 0.3 mM/kg (49.8 mg/kg). Fermentation of quinoa and wheat slurries by *L. plantarum* CRL 778 could produce 45.5

and 17.5 mg/L of PLA, respectively (Dallagnol et al., 2013).

Use of antimicrobial agent-producing LAB strains as starter improves the antimicrobial activity of LAB-fermented food products. Lavermicocca et al. (2000) reported that wheat sourdough fermented by the PLA-producing LAB strain (*L. plantarum* 21B) in coculture with *Saccharomyces cerevisiae* had much longer shelf life (>7 d) than that fermented by only *S. cerevisiae* (<2 d). Pediocin is another antimicrobial compound with activity against several food-borne pathogens. Somkuti and Steinberg (2010) reported that in simulated yogurt fermentation, the pediocin-producing strain *P. acidilactici* F in coculture with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* could effectively produce pediocin and showed inhibitory activity against *Listeria monocytogenes*. Our study showed that *P. pentosaceus* SK25 starter produced PLA when cultured in milk. The inhibitory activity of PLA-produced fermented milk against bacteria and fungi will be extensively investigated in the future.

Milk fermentation by *P. pentosaceus* SK25 was also studied under supplementation with 1 mg/mL of phenylalanine or tyrosine. The 2 AA supplements produced different results. The phenylalanine supplement significantly increased PLA production during the milk fer-

Table 2. 3-Phenyllactic acid (PLA) production (mg/L) in milk fermented with *Pediococcus pentosaceus* SK25 as starter¹

Time (h)	Milk + 2% glucose	Milk + 2% glucose + 1 mg/mL of phenylalanine	Milk + 2% glucose + 1 mg/mL of tyrosine
6	17.0 ± 3.2	36.1 ± 3.7	ND ²
7	23.7 ± 3.5	39.3 ± 4.2	ND
8	29.1 ± 3.8	47.7 ± 5.3	ND
9	34.8 ± 4.7	57.2 ± 4.3	ND
10	39.3 ± 4.2	69.7 ± 4.9	ND
11	43.5 ± 4.6	78.4 ± 3.8	ND
12	47.2 ± 5.3	82.5 ± 6.3	ND

¹Values are means of 3 replications ± SD.

²ND = none detected.

Figure 2. Possible pathway of 3-phenyllactic acid (PLA) production by *Pediococcus pentosaceus* SK25 in fermented milk.

mentation process, and the PLA concentration reached 82.5 mg/L after fermentation for 12 h; in contrast, PLA biosynthesis was almost completely inhibited by the tyrosine supplement (Table 2).

The PLA biosynthesis pathway in LAB is well characterized (Mu et al., 2012b). 3-Phenyllactic acid production is a side pathway of phenylalanine metabolism, in which phenylalanine is transaminated to phenylpyruvic acid by aminotransferase and phenylpyruvic acid is further reduced to PLA by a dehydrogenase (Figure 2). Phenylalanine supplementation may thus increase PLA biosynthesis. Conversely, the transamination reaction of phenylalanine is catalyzed by aromatic aminotransferase, which has a broad substrate spectrum, including tyrosine (Yvon et al., 1997). Therefore, tyrosine inhibits the transamination of phenylalanine and tyrosine supplementation thus decreases PLA production. Many studies have shown that tyrosine supplementation strongly inhibits PLA production, but it did not completely inhibit the PLA production of LAB strains in flask fermentation (Valerio et al., 2004; Mu et al., 2012a). In our experiment, PLA production was almost completely inhibited within 12 h by tyrosine supplementation during *P. pentosaceus* SK25 fermentation in milk.

In conclusion, PLA was produced in milk by *P. pentosaceus* SK25 during laboratory fermentation process, and the PLA production level was increased by supplementation with phenylalanine. Our study presents a possibility that using PLA-producing LAB strains as starters may produce PLA in LAB-fermented milk products to improve antimicrobial activity against food-borne pathogenic bacteria and food-spoiling fungi.

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