Lactic acid bacteria isolated from raw and fermented pork products: Identification and characterization of catalase-producing *Pediococcus pentosaceus*

Suree Nanasombat*, Patcharee Treebavonkusol, Sunisa Kittisrisopit, Thitirut Jaichalad, Saranya Phunpruch, Achaporn Kootmas, and Imboon Nualsri

Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand

Received July 14, 2016
Revised October 26, 2016
Accepted November 2, 2016
Published online February 28, 2017

*Corresponding Author
Tel: +662-3298000 (ext. 6264)
Fax: +662-3298427
E-mail: snanasombat@gmail.com

pISSN 1226-7708
eISSN 2092-6456

© KoSFoST and Springer 2017

Abstract Lactic acid bacteria (LAB) from raw and fermented pork samples were screened for their inhibitory activity by an agar spot test in order to obtain a LAB strain with suitable property to be used as meat starter cultures. Among the 174 isolates, 73 were positive to inhibit at least one of the seven indicator bacteria, which were further characterized. The most suitable isolate was isolate P0805, identified as *Pediococcus pentosaceus*. This bacterium was catalase- and nitrate reductase-positive and amino acid decarboxylase-negative; moreover, it produced inhibitory substances against *Salmonella* Typhimurium with the activity of the partially purified inhibitory substances of 409,600 AU/mL. To further characterize the catalase-producing ability of *P. pentosaceus* P0805, the effect of hematin on its catalase activity in Sausage Model Broth (SMB) was evaluated, and it enhanced catalase production. The catalase activity was found in both SMB with and without hematin. It was concluded that catalase produced by this bacterium was heme-independent catalase.

Keywords: lactic acid bacteria, inhibitory substance, catalase, meat starter culture, *Pediococcus pentosaceus*

Introduction

Lactic acid bacteria (LAB), including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, and *Pediococcus*, are commonly found in raw meat. LAB play an important role in meat fermentation by rapidly decreasing the pH. They produce some inhibitory substances such as organic acids, bacteriocins, diacetyl, hydrogen peroxide, and other compounds, thereby inhibiting the growth of spoilage and pathogenic bacteria (1,2). LAB starter cultures should rapidly produce adequate amount of antimicrobial compounds to improve the safety of fermented meat products. LAB have been used as starter cultures, i.e., viable microorganisms added to meat for improving the keeping quality, safety, and consumer acceptability of the meat products (3).

LAB starter cultures are usually homofermentative since the formation of carbon dioxide and other fermentation products leads to undesirable off-flavors and other defects (3). The starter cultures should not cause accumulation of biogenic amines, such as histamine, tyramine, cadaverine, and putrescine, in fermented foods as excessive consumption of these amines can affect the nervous, gastric, and intestinal systems and blood pressure. Availability of precursors, including amino acids and microorganisms with amino

acid decarboxylase activity, can lead to accumulation of biogenic amines in foods at conditions favorable for growth and decarboxylation (4). In addition, starter cultures with probiotic properties should also be considered. Several species of LAB are proved to function as probiotics (5). If they are ingested in sufficient amount, they can provide health benefits to the consumers. For this purpose, they should be tolerant to hydrochloric acid (in the stomach) and bile salts (in the small intestine) in order to survive the passage through the gastrointestinal tract (6).

However, hydrogen peroxide produced by most LAB in fermented meat products can have serious consequences. Hydrogen peroxide activates metmyoglobin during lipid oxidation in meat and therefore it is the major cause of quality deterioration. This further leads to rancidity of fat and undesirable color components in fermented meat products. In general, hydrogen peroxide produced during meat fermentation could be removed by catalase of some micrococcal starter cultures such as *Staphylococcus xylosus* and *Micrococcus varians*. Catalase is not generally produced by most of LAB; however, some LAB strains can have true or pseudocatalase activity (7). They produce either heme-dependent catalase or heme-independent catalase (pseudocatalase). Heme-dependent catalase was induced by the addition of heme compounds, which is an iron-porphyrin

complex such as hematin (7,8). Moreover, LAB also participate in the spontaneous reduction of nitrites to nitric oxides, which induces a typical pink color. Some meat LAB have been reported to possess nitrate reductase, which reduces nitrates to nitrites (9). Thus, it will be useful to find a new LAB strain with catalase- and nitrate reductase-producing ability along with other important characteristics for future use as meat starter cultures.

Materials and Methods

Raw pork and fermented pork samples Ten samples of raw pork and 22 samples of Thai fermented pork products (16 samples of *nham* and 6 samples of *saingonyisan*) were purchased from the local markets in Bangkok, Thailand. The pH and water activity values of these samples were measured at 30°C.

Enumeration and isolation of lactic acid bacteria Total LAB counts in all the raw pork samples, *nham* and *saingonyisan*, were determined on de Man Rogosa and Sharpe agar (MRS; Difco Laboratories, Detroit, MI, USA) containing 0.5% calcium carbonate under microaerophilic condition at 37°C for 48 h. Colonies with a clear zone were counted and different morphology colonies were picked and purified on MRS agar.

Screening of lactic acid bacteria with antagonistic and some enzymatic activities

Antagonistic activity: The antagonistic activity of the selected LAB isolates was assessed by an agar spot test (10). A loopful of each LAB isolate on the MRS agar slope was transferred into 10 mL MRS broth and incubated at 37°C for 48 h. The cultures were inoculated on the surface of a bacteriocin-screening medium (11). The plates were incubated under microaerophilic condition at 37°C for 48 h and then covered with 5.0 mL semisoft Tryptic Soy Agar (TSA; Difco Laboratories, with 1.0% agar) or MRS agar containing 2.0% each of the seven indicator strains: *Lactobacillus plantarum* TISTR 050, *P. pentosaceus* TISTR 414, *Staphylococcus aureus* TISTR 118 (obtained from the Microbiological Resources Centre for Southeast Asian Region, Bangkok MIRCEN), *Salmonella* Typhimurium DMST 0562, *Listeria monocytogenes* DMST 11256, *Pseudomonas fluorescens* DMST 20076, and *Escherichia coli* DMST 4212 (obtained from the culture collection of the Department of Medical Sciences, Ministry of Public Health, Thailand). The isolates inhibiting at least one indicator bacteria (observed by a clear zone) were selected for further screening based on suitable criteria to be used as meat starter cultures.

Nitrate reductase activity: The nitrate reductase activity of the selected LAB isolates was tested by a colorimetric method, as described by Miralles *et al.* (12). Briefly, the cell suspension (30 µL, 10⁷ CFU/mL) of the selected LAB isolates was inoculated into a 6-mm agar well of MRS agar supplemented with 1 g/L potassium nitrate.

After incubation at 37°C for 24 h, the surface of this agar plate was flooded with a mixture of two solutions (NIT1 and NIT2 solutions in the ratio of 1:1 v/v). The NIT1 solution was prepared by dissolving 0.8 g sulphanilic acid in 100 mL of 5 N acetic acid solution, whereas the NIT2 solution was prepared by dissolving 0.6 g N,N-dimethyl-1-naphthylamine in 100 mL of 5 N acetic acid solution. The red color surrounding the agar wells indicates the presence of nitrite or positive reaction of nitrate reductase.

Catalase test: Each LAB culture from the MRS slant was tested by emulsifying the colony in 1 drop of 3.0% hydrogen peroxide on a glass slide. Immediate gas bubbling indicated a positive reaction for the catalase test (13).

Amino acid decarboxylase activity: The selected LAB isolates were tested for amino acid decarboxylase activity as described by Joosten and Northolt (14) and Bover-Cid and Holzapfel (15) with a slight modification. In order to promote the enzyme induction before the actual screening test, the LAB isolates were cultivated and subcultured for 5 times in MRS broth containing 0.1% of each precursor (L-tyrosine, L-histidine monohydrochloride, ornithine monohydrochloride, and L-lysine monohydrochloride) supplemented with 0.005% of pyridoxal-5-phosphate. These LAB cultures were then streaked onto the surface of decarboxylation medium plates (15) with and without amino acids (as control) and incubated at 37°C for 4 days under anaerobic conditions. The purple halos around the colonies indicate the growth of decarboxylating strains, whereas the yellow halos indicate the growth of non-decarboxylating strains. The isolates with no amino acid decarboxylase activity were selected for further characterization.

Analysis of some probiotic properties

Acid, sodium chloride, and bile salt tolerance: Each selected LAB isolate was cultivated in MRS broth at 37°C for 24 h. Cells were harvested by centrifugation (3,000×g at 4°C for 20 min), washed twice and resuspended in 0.1% peptone water. The turbidity of the cell suspension was adjusted to reach the turbidity corresponding to a McFarland standard value of 2. The final cell concentration was 10⁵ CFU/mL.

To study lactic acid or hydrochloric acid tolerance, MRS broth (190 µL) acidified with 85% (w/v) lactic acid or 5 M hydrochloric acid was adjusted to six different pH values (pH 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0) and added into each well of a microtiter plate. To study bile salt tolerance, MRS broth supplemented with porcine bile salts (0.3, 0.5, 1.0, and 1.5 % w/v) adjusted to pH 8.0 was used, whereas MRS broth with sodium chloride (1.0–11.0% w/v) was used to study salt tolerance. The inoculum suspension (10 µL) of each LAB strain was then added into each well, and subsequently incubated at 37°C for 24 h. Turbidity was measured at 620 nm using a microplate reader (N12648 Thermo Labsystem iEMS Reader MF Type 1401, Finland). The lowest pH value of lactic acid and hydrochloric acid or the highest concentration of porcine bile salts and sodium chloride at which the isolates were able to grow were recorded (16).

Identification of the selected lactic acid bacteria

Morphological and biochemical identification: The selected LAB isolates were initially tested for the characteristics of Gram staining, cell and colony morphology, motility, carbon dioxide production from glucose, growth at 10 and 45°C, growth in the presence of 6.5% and 18% NaCl, and growth at pH 4.4 and 9.6 (17). Then, the isolates were characterized by their carbohydrate fermentation patterns using the API-50 CH system (bioMerieux, France). The reference strain *P. pentosaceus* TISTR 414 was used to compare in this study.

Molecular identification: The genomic DNA of the selected LAB isolate was extracted using the MasterPure™ Gram Positive DNA Purification kit (Epicentre, Madison, WI, USA). The 16S rRNA gene was amplified by a polymerase chain reaction using primers F16SrDNA-27bac (5'-AGAGTTTGATCCTGGCTCAG-3') and R16SrDNA-1492bac (5'-GGTTACCTTGTT ACGACTT-3'). Polymerase chain reaction (PCR) was conducted in a total volume of 50 µL containing 1X PCR buffer, 100 ng genomic DNA, 1.5 mM of MgCl₂, 0.2 mM dNTPs, 0.25 mM of each primer, and 2.5 U of *Taq* DNA polymerase (Promega, Fitchburg, WI, USA). The PCR conditions comprised an initial denaturing step of 94°C for 10 min, followed by 35 cycles of denaturing for 30 s at 94°C, annealing for 30 s at 55°C, and 90 s of primer extension at 72°C. A final extension at 72°C for 10 min was then performed. The 1,500 bp PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and ligated to TA-cloning vector (Qiagen PCR Cloning kit, Qiagen). The recombinant plasmids were transformed into competent cells of *E. coli* DH5α and the transformants were selected with a blue-white screening on LB agar containing 50 µg/mL of kanamycin, 50 µM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and 80 µg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Plasmid DNA of the transformants was isolated using QIAprep® Spin Miniprep kit (Qiagen). It was then sequenced by a Big dye terminator reaction method using an ABI PRISM® 3700 DNA analyzer (First Base Laboratories Sdn Bhd, Seri Kembangan, Malaysia). Nucleotide sequences were analyzed using the BLAST program (18).

Characterization of the selected lactic acid bacteria

Antibiotic resistance: To determine the antibiotic susceptibility of the selected LAB, the minimum inhibitory concentrations (MICs) of seven antibiotics, including penicillin, tetracycline, rifampicin, erythromycin, gentamicin, cefuroxime, and chloramphenicol, against the selected LAB were determined by an agar dilution method (19). MRS agar slants with each of the antibiotics at the final concentrations of 0.064–1,000 µg/mL were prepared. After surface drying, a loopful of the selected LAB was streaked onto the surface of each MRS agar slant. Then, all tubes were incubated at 37°C for 48 h. The lowest concentration of the antibiotic wherein the visible growth of the selected LAB was completely inhibited was recorded as the MIC.

Inhibitory activity of the antimicrobial compounds: MRS broth (10 mL) was inoculated with the selected LAB culture and incubated at 37°C for 24 h. The cell-free supernatant was obtained by

centrifugation (3,000×g) at 4°C for 20 min, and then the pH was adjusted to 6.5 using 1 M NaOH, followed by filter sterilization through a 0.2-µm cellulose acetate filter (Whatman, Maidstone, UK). Furthermore, the inhibitory activity of the antimicrobial compounds in this culture supernatant was examined by an agar disc diffusion assay against some pathogenic bacteria, including *Listeria monocytogenes* DMST 11256, *Salmonella* Typhimurium DMST 0562, and *Staphylococcus aureus* TISTR 118, using the modified method of Schillinger and Lücke (20). Briefly, the bacterial cell suspension (100 µL) was swabbed onto the surface of Brain Heart Infusion agar (Difco Laboratories). Sterile 6-mm filter paper discs (Whatman) were aseptically placed on the surfaces of each agar medium. Then, the sterile cell-free supernatant (15 µL) was immediately added to the paper disc. The plates were incubated at 37°C for 24 h. The antimicrobial activity was evaluated by measuring the inhibition zone diameters. The experiments were conducted in triplicate.

Inhibitory activity of the partially purified antimicrobial compounds:

Briefly, the selected LAB strain was cultured in MRS broth at 37°C for 24 h. Then, the overnight culture was centrifuged (8,000×g) at 4°C for 30 min. The cell-free supernatant was adjusted to pH 6.5 and then filtered through a 0.2 µm cellulose acetate membrane filter (Whatman). Two methods of partial purification of antimicrobial compounds were compared: i) ammonium sulfate precipitation method and ii) ammonium sulfate precipitation plus a dialysis method.

a) Ammonium sulfate precipitation: The cell-free culture supernatant was precipitated with ammonium sulfate to 60% saturation and stirred at 4°C. Then, the mixture was centrifuged (8,000×g) for 30 min at 4°C. The precipitate was resuspended in 50 mL of 0.05 M potassium phosphate buffer (pH 7.0) and divided into three portions: i) 10 mL for antimicrobial compound titer analysis (21), ii) 2 mL for protein content analysis (22) and iii) 38 mL for further purification by dialysis.

The partially purified antimicrobial compound titer analysis of the precipitate was determined by a critical dilution method (21). First, the inoculum suspension (0.1 mL, 10⁸ CFU/mL) of the pathogenic bacterium inhibited by the antimicrobial compounds in the culture supernatant was swabbed on the entire surface of TSA (Difco Laboratories). Sterile 6-mm filter paper discs (Whatman) were aseptically placed on this TSA surface. Then, an aliquot of the partially purified antimicrobial compounds (10 µL) at each dilution (1:2¹ to 1:2¹⁸) was placed into the paper disc. All the plates were incubated at 37°C for 24 h and the inhibition zone was examined. The antagonistic activity was expressed as arbitrary units (AU/mL). It is proportional to the reciprocal of the highest dilution factor producing zone of inhibition (DFi). The inhibitory activity of the antimicrobial compounds was calculated as follows:

$$\text{Activity of the antimicrobial compounds (AU/mL)} \\ = 1/\text{DFi} \times 1000/\text{volume spotted in } \mu\text{L}$$

b) Ammonium sulfate precipitation plus dialysis: The antimicrobial compound containing an aliquot of the obtained after ammonium sulfate precipitation was dialyzed in a tubular cellulose membrane (Spectrapor, 1,000 dalton MWCO; Thermo Fisher Scientific, Waltham, MA, USA) against 5 L of 0.05 M potassium phosphate buffer (pH 7.0) for 24 h. After dialysis, the dialysate was evenly divided into two portions for antimicrobial compound titer analysis (21) and protein content determination (22).

Effect of hematin on the growth and catalase activity of the selected lactic acid bacterium during fermentation In this study, the effect of hematin added in SMB on the growth and catalase activity of the selected LAB strain during fermentation at 37°C for 48 h was studied. First, SMB (containing 55 g MRS broth powder (Difco Laboratories), 20 g glucose, 2 g sodium ascorbate, 15 g sodium chloride, 2% garlic extract (prepared according to the method of Swetwathana *et al.* (23)) and 1,000 mL distilled water) with or without addition of 65 µmol/L hematin (Hematin porcine, Sigma-Aldrich, St. Louis, MO, USA) was prepared. Then, the cell suspension (10^7 CFU/mL) of the selected LAB was inoculated into each SMB flask (120 mL). All the flasks were incubated at 37°C for 48 h. The optical density at 600 nm and pH value were measured at 0, 24, and 48 h of incubation. Whole cell catalase activity (catalase activity in a cell-free culture supernatant and cell pellets) was analyzed at the end of incubation.

Catalase activity assay: After 48-h incubation, the culture broth was centrifuged, and the supernatant was analyzed for catalase activity by a colorimetric assay method (24) using the catalase assay kit (CAT 100; Sigma-Aldrich). This assay is based on the catalytic activity of catalase in the decomposition, as described in the technical bulletin of this assay kit. Activity of catalase is expressed as µmoles (H_2O_2)/min/mL of the sample. One unit of catalase will decompose 1.0 µmole of hydrogen peroxide to oxygen and water (per minute at pH 7.0 at 25°C) at a substrate concentration of 50 mM hydrogen peroxide.

To prepare the sample for catalase activity analysis in the cell pellets, the method described by Pine *et al.* (25) was used. Briefly, the cell pellets were washed twice. Then, the cells were mixed with buffer in the ratio of 1:8 (v/v) and disintegrated using a sonifier operating at 100 W for 2 min. Exposure to sonic energy was at a 30-s interval in an ice bath. Whole cells and cell debris were separated by centrifugation at $8,000\times g$ /min for 30 min. The supernatant was centrifuged at $8,000\times g$ /min for 30 min. The clear supernatant was collected to analyze the catalase activity using CAT 100 (Sigma-Aldrich). The results were expressed as µmoles (H_2O_2)/min/mL of the sample. All data were analyzed by ANOVA and Duncan's multiple range test at 95% confidence level using the IBM SPSS 22.0 version statistical package (IBM, North Castle, NY, USA).

Results and Discussion

Screening of lactic acid bacteria from raw and fermented pork

The pH and a_w values of the raw pork samples were 5.54–6.21 and 0.97–0.99, respectively. *Nham* and *saigongyisan* samples had a more acidic pH (4.37–5.89) with a_w in the range of 0.93–0.97. Total LAB counts in raw pork samples ranged from 1.7×10^8 to 2.0×10^{10} CFU/g. In *nham*, the total LAB counts ranged from 1.2×10^7 to 1.8×10^{10} CFU/g, whereas those in *saigongyisan* ranged from 3.3×10^9 to 2.5×10^{10} CFU/g. Seventy-three isolates (41.9%) of 174 LAB isolates inhibited at least one of the seven tested organisms (Table 1).

Characterization of the selected lactic acid bacteria Only three LAB isolates (P0201, P0804, and P0805) from raw pork could produce nitrate reductase. Two isolates (P0804 and P0805) out of which were catalase positive, whereas only one (the isolate P0805) was amino acid decarboxylase negative. In terms of the ability to tolerate acid, bile salts, and sodium chloride, the isolate P0805 could tolerate the low pH of hydrochloric acid (pH 2.0) and lactic acid (pH 2.5) compared to the other two isolates (Table 2). Thus, the isolate P0805

Table 1. Screening of inhibitory substance producing lactic acid bacteria isolated from raw pork and fermented pork products

Samples (number of samples)	pH	A_w (30°C)	Total LAB counts (CFU/g)	Selected LAB (isolates)	LAB with antimicrobial activity ¹⁾ (isolates)
Raw pork (10)	5.54–6.21	0.97–0.99	1.7×10^8 – 2.0×10^{10}	53	23
<i>Nham</i> (16)	4.37–5.89	0.93–0.96	1.2×10^7 – 1.8×10^{10}	84	34
<i>Saigongyisan</i> (6)	4.66–4.79	0.95–0.97	3.3×10^9 – 2.5×10^{10}	37	16
Total (32)	-	-	-	174	73

¹⁾The LAB isolates inhibited the indicator bacteria of at least one species by an agar spot test.

Table 2. Acid, sodium chloride, and bile salt tolerance of lactic acid bacteria isolated from raw pork

Source of isolates	LAB isolates	pH ¹⁾ of HCl	pH ¹⁾ of Lactic acid	NaCl (% w/v) ²⁾	Bile salts (% w/v) ²⁾
Raw Pork	P0201	3.0	4.0	11.0	1.5
	P0804	3.0	4.0	11.0	1.5
	P0805	2.0	2.5	11.0	1.5

¹⁾The lowest pH of acid at which the isolates were able to grow.

²⁾The highest concentration of sodium chloride and bile salts at which the isolates were able to grow.

was the only isolate that showed the highest tolerance to acids, bile salts, and sodium chloride. Moreover, the antimicrobial compounds produced by this LAB isolate could inhibit the growth of *S. Typhimurium* with an inhibition zone diameter of 12 mm. Therefore, the isolate P0805 was the most suitable LAB isolate to be used as starter cultures for fermented meat products. This isolate P0805 was further identified and characterized.

Identification of the isolate P0805 The isolate P0805 was Gram-positive cocci. This isolate could grow at 6.5% NaCl, at 10 and 45°C, and at pH 4.4 and 9.6, but could not grow in the presence of 18% NaCl. In addition, this isolate did not produce carbon dioxide from glucose. Therefore, this LAB isolate was a homofermentative lactic acid bacterium. Based on the key characteristics and test results, the isolate P0805 could be presumptively identified as genus *Pediococcus* or *Enterococcus* (17).

The API 50 CH biochemical profiles of the isolate P0805 showed 99.6% similarity with those of *P. pentosaceus*. The carbohydrate fermentation tests showed that the isolate P0805 could ferment L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-trehalose, and D-tagatose, but not glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, D-lactose (bovine origin), D-melibiose, D-saccharose, inulin, D-melzitose, D-raffinose, amido (starch), glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, and potassium 5-ketogluconate. The molecular identification by 16S rRNA sequence analysis confirmed that the isolate P0805 was *P. pentosaceus* (99% similar to *P. pentosaceus* ATCC 25745 (CP000422.1), *P. pentosaceus* LM2632 (AY675245.1), *P. pentosaceus* SL4 (AY675243.1), and *P. pentosaceus* NGRI 0305 (AB362987.1).

Antibiotic resistance of *P. pentosaceus* P0805 *P. pentosaceus* P0805 was most sensitive to erythromycin (MIC: 4 µg/mL), followed by ampicillin and chloramphenicol (MIC: 16 µg/mL), but quite resistant to rifampicin and gentamicin (MIC: 128 µg/mL) and cefuroxime (MIC: 256 µg/mL) (Table 3). These results are in agreement with those reported by Vidhyasagar and Jeevaratnam

Table 3. Antibiotic resistance of *Pediococcus pentosaceus* P0805

Antibiotics	Minimum inhibitory concentration (µg/mL)
Ampicillin	16
Tetracycline	64
Cefuroxime	256
Rifampicin	128
Erythromycin	4
Gentamicin	128
Chloramphenicol	16

(26). LAB used as starter cultures could possibly contain an antibiotic-resistant gene, which might be transferred to the pathogenic bacteria (27).

Inhibitory activity of antimicrobial compounds produced by *P. pentosaceus* P0805

P. pentosaceus P0805 could produce antimicrobial compounds that could inhibit the growth of *S. Typhimurium*. The antimicrobial compounds of this bacterium were partially purified and tested for their activity. The antimicrobial compounds purified by ammonium sulfate precipitation plus dialysis had a higher inhibitory activity against the growth of *S. Typhimurium* (409,600 AU/mL) compared to the other fraction (Table 4). These results corresponded to the specific activity of the antimicrobial compounds, which increased from 8.89 AU/µg in ammonium sulfate precipitation fraction to 801.83 AU/µg in ammonium sulfate precipitation plus dialysis fraction. The increase of total activity after ammonium sulfate precipitation plus dialysis may be due to the increasing concentration of the antimicrobial compounds during these purification steps. The increasing concentration of ammonium sulfate to a sufficiently high concentration leads to aggregation and ultimately, to precipitation of the protein from the solution (28). On the other hand, protein purification dialysis is aimed to separate the compounds with lower molecular weight out of those with higher molecular weight, which are stored in a cellulose membrane bag. Thus, protein molecules with high molecular weight may have been trapped in a cellulose bag. The other compounds with low molecular weight as well as residual ammonium sulfate may diffuse through the cellulose bag, thereby increasing the purity of the antimicrobial compounds. These antimicrobial compounds could be bacteriocins. Some LAB produce bacteriocins, the antimicrobial peptides inhibiting

Table 4. Characteristic of the partially purified antimicrobial compounds from *Pediococcus pentosaceus* P0805

Purification stage	Volume (mL)	Activity (AU/mL)	Total activity (AU) ¹⁾	Protein content ²⁾ (µg/mL)	Specific activity ³⁾ (AU/µg)
Ammonium sulfate Precipitation	50	6,400	320,000	720	8.89
Ammonium sulfate Precipitation+Dialysis	38	409,600	15,564,800	510.83	801.83

¹⁾Total activity was determined by multiplication of volume by activity.

²⁾Protein content was determined by the Bradford method (µg/mL).

³⁾Specific activity (AU/µg) is the activity unit divided by the protein concentration (µg/mL).

Table 5. Effect of hematin on the growth and fermentation of *Pediococcus pentosaceus* P0805 during fermentation in the sausage model broth

Fermentation time (h)	Hematin concentration ($\mu\text{mol/L}$) ¹⁾			
	0		65	
	OD ₆₀₀	pH	OD ₆₀₀	pH
0	0.02±0.02 ^A	6.34±0.08 ^A	0.03±0.02 ^A	6.28±0.01 ^A
24	1.68±0.22 ^A	3.92±0.04 ^A	1.79±0.17 ^A	3.89±0.02 ^A
48	2.11±0.04 ^A	3.80±0.08 ^A	2.17±0.04 ^A	3.75±0.04 ^A

¹⁾Data are mean of three replications.**Table 6.** Effect of hematin on the catalase production of *Pediococcus pentosaceus* P0805 at 48-h fermentation in the sausage model broth

Hematin concentration ($\mu\text{mol/L}$)	Catalase activity ($\mu\text{mol/min/mL}$) ¹⁾	
	Cell-free supernatant	Cell pellet
0	32.67±6.4 ^A	8.12±5.67 ^A
65	44.44±5.7 ^B	19.0±15.63 ^B

¹⁾Data are mean of three replications.

the growth of other bacteria. *P. pentosaceus* has been reported to produce bacteriocins (29). *P. pentosaceus* strain K34 isolated from alheia, a traditional Portugal meat product, was found to produce bacteriocins bacPPK 34 with the activity of 12,800 AU/mL (29). Maragkoudakis *et al.* (30) reported that pediocin AcH-PA1 gene of *P. pentosaceus* PCD 237 isolated from sausages was found.

Catalase activity of *P. pentosaceus* P0805 The cell turbidity of *P. pentosaceus* P0805 in both SMB with and without hematin increased, whereas their pH values decreased as fermentation time increased. Both the cell turbidity and pH value of these culture broths rapidly changed during the first 24 h of fermentation. However, addition of hematin in SMB did not significantly affect the growth and fermentation of this bacterium ($p>0.05$) (Table 5). Differently, supplementation of 65 $\mu\text{mol/L}$ hematin in SMB significantly enhanced the catalase activity of this bacterium ($p<0.05$) (Table 6). Mares *et al.* (7) reported that the addition of more than 60 μmol hematin per liter of growth medium resulted in a high catalase activity of *L. plantarum*.

In the current study, *P. pentosaceus* P0805 grown in both SMB with 65 $\mu\text{mol/L}$ hematin and without hematin exhibited catalase activity in both the cell-free supernatant and cell pellets (Table 6). These results confirmed that the addition of hematin in the culture broth was not necessary for catalase production by *P. pentosaceus* P0805. Hematin is molecule of iron-containing porphyrin linked with hydroxide. It is a part of some redox enzymes such as catalase and peroxidase (8). In general, most LAB are catalase negative due to their inability to synthesize heme compounds. However, certain strains of LAB, such as *Pediococcus*, *Enterococcus*, and *Leuconostoc*, possess a heme-dependent catalase. They have the potential to establish aerobic respiration when exogenous sources of heme are available. On the other hand, few LAB such as some strains of *Pediococcus*, *Leuconostoc*, *Enterococcus*, and *L. plantarum* are able

to produce a heme-independent catalase (non-heme catalase or pseudocatalase) (7). Therefore, catalase produced by *P. pentosaceus* P0805 may be pseudocatalase. The activity of this non-heme catalase does not need to be induced by addition of hematin. Engesser and Hammes (31) reported similar results. They investigated non-heme and heme catalase activity of 71 LAB species and found that non-heme catalase activity was restricted to *L. plantarum*, *L. mali*, and *P. pentosaceus* strains.

In conclusion, *P. pentosaceus* P0805 had some desirable properties such as production of inhibitory substances against *S. Typhimurium*; production of catalase that can remove hydrogen peroxide accumulated in fermented meat products; no production of amino acid decarboxylase, which may not cause accumulation of biogenic amines; and production of nitrate reductase, which may contribute to the formation of pink color in the fermented meat products. Moreover, this bacterium was found to be homofermentative. Lactic acid is the main product of fermentation. These factors would make this bacterium an eligible candidate for good starter cultures in meat fermentation. In fact, *P. pentosaceus* has been used as a commercial meat starter culture (32). Thus, *P. pentosaceus* P0805 can be used as a new specific strain for a meat starter culture. For further studies, the technological properties of this bacterium as a starter culture are needed to be evaluated during real meat fermentation.

Disclosure The authors declare no conflict of interest.

References

- Caplice E, Fitzgerald GF. Food fermentations: Role of microorganisms in food production and preservation. *Int. J. Food Microbiol.* 50: 131–149 (1999)
- Spano G, Russo P, Lonvaud-Funel A, Lucas P, Alexandre H, Grandvalet C, Coton E, Coton M, Barnavon L, Bach B, Rattray F, Bunte A, Magni C, Ladero V, Alvarez M, Fernández M, Lopez P, de Palencia PF, Corbi A, Trip H, Lolkema JS. Biogenic amines in fermented foods. *Eur. J. Clin. Nutr.* 64: S95–S100 (2010)
- Smith LJ, Palumbo AS. Use of starter cultures in meats. *J. Food Protect.* 46: 997–1006 (1983)
- Ammor MS, Mayo B. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. *Meat Sci.* 76: 138–146 (2007)
- Arena MP, Russo P, Capozzi V, López P, Fiocco D, Spano G. Probiotic abilities of riboflavin-overproducing *Lactobacillus* strains: A novel promising application of probiotics. *Appl. Microbiol. Biot.* 98: 7569–7581 (2014)
- Erkkilä S, Petäjä E. Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. *Meat Sci.* 55: 297–300 (2000)
- Mares A, Neyts K, Debevere J. Influence of pH, salt and nitrite on the heme-dependent catalase activity of lactic acid bacteria. *Int. J. Food Microbiol.* 24: 191–198 (1994)

8. Pshezhetskii VS, Jaroslavov AA. Activation of hematin catalase function by ethylenediamine. *FEBS Lett.* 49: 29–32 (1974)
9. Hammes WP, Bantleon A, Min S. Lactic acid bacteria in meat fermentation. *FEMS Microbiol. Lett.* 87: 165–173 (1990)
10. Fleming HP, Etchells JL, Costilow RN. Microbial inhibition by an isolate of *Pediococcus* from cucumber brines. *Appl. Microbiol.* 30: 1040–1042 (1975)
11. Tichaczek PS, Nissen-Meyer J, Nes IF, Vogel RF, Hammes WP. Characterization of the bacteriocins curvacin A from *Lactobacillus curvatus* LTH1174 and sakacin P from *L. sake* LTH673. *Sys. Appl. Microbiol.* 15: 460–468 (1992)
12. Miralles MC, Flores J, Perez-Martinez G. Biochemical tests for the selection of *Staphylococcus* strains as potential meat starter cultures. *Food Microbiol.* 13: 227–236 (1996)
13. Bennett RW, Lancette GA. Bacteriological Analytical Manual Chapter 12 *Staphylococcus aureus*. U.S. Food and Drug Administration, Silver Spring, USA (2001). Available from: <http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071429.htm> Accessed Dec. 12, 2013.
14. Joosten HMLJ, Northolt MD. Detection, growth, and amine-producing capacity of lactobacilli in cheese. *Appl. Environ. Microb.* 55: 2356–2359 (1989)
15. Bover-Cid S, Holzapfel WH. Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int. J. Food Microbiol.* 53: 33–41 (1999)
16. Paludan-Müller C, Madsen M, Sophanodora P, Gram L, Møller PL. Fermentation and microflora of plaasom, a Thai fermented fish product prepared with different salt concentrations. *Int. J. Food Microbiol.* 73: 61–70 (2002)
17. Axelsson L. Lactic acid bacteria: Classification and physiology. pp.1–66. In: *Lactic Acid Bacteria: Microbiological and Functional Aspects*. Salminen S, von Wright A, Ouwehand A (eds). Marcel Dekker Inc., New York, NY, USA (2004)
18. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J. Mol. Biol.* 215: 403–410 (1990)
19. Jorgensen JH, Turnidge JD, Washington JA. Antibacterial susceptibility test: Dilution and disk diffusion methods. pp. 1526–1562. In: *Manual of Clinical Microbiology*. Murray PR, Barron ER, Praller MA, Tenover FC, Tenover RH (eds). ASM Press, Washington DC, USA (1999)
20. Schillinger U, Lücke FK. Antibacterial activity of *Lactobacillus sake* isolated from meat. *Appl. Environ. Microb.* 55: 1901–1906 (1989)
21. Yousef AE, Carlstrom C. *Food Microbiology: A Laboratory Manual*. John Wiley & Sons Inc, Hoboken, NY, USA. pp. 231–248 (2003)
22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254 (1976)
23. Swetiwathana A, Leutz U, Lotong N, Fischer A. Controlling the growth of *Salmonella anatum* in Nham - Effect of meat starter cultures, nitrate, nitrite and garlic. *Fleischwirtschaft* 9: 124–128 (1999)
24. Fossati P, Prencipe L, Berti G. Use of 3,5-dichloro-2-hydroxybenzenesulfonic acid/ 4-aminophenazone chromogenic system in direct enzymic assay of uric acid in serum and urine. *Clin. Chem.* 26: 227–231 (1980)
25. Pine L, Hoffman PS, Malcolm GB, Benson RF, Keen MG. Determination of catalase, peroxidase, and superoxide dismutase within the genus *Legionella*. *J. Clin. Microbiol.* 20: 421–429 (1984)
26. Vidhyasagar V, Jeevaratnam K. Evaluation of *Pediococcus pentosaceus* strains isolated from *Idly* batter for probiotic properties *in vitro*. *J. Funct. Foods* 5: 235–243 (2013)
27. Hummel AS, Hertel C, Holzapfel WH, Franz CMAP. Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. *Appl. Environ. Microb.* 73: 730–739 (2007)
28. Doonan S. Concentration of extracts. pp. 85–90. In: *Protein Purification Protocols*. Cutler P (ed). Humana Press Inc., Totowa, NJ, USA (2004)
29. Cleveland J, Montville TJ, Nes IF, Chikindas ML. Bacteriocins: Safe, natural antimicrobials for food preservation. *Int. J. Food Microbiol.* 71: 1–20 (2001)
30. Maragkoudakis PA, Mountzouris KC, Psyras D, Cremonese S, Fischer J, Cantor MD, Tsakalidou E. Functional properties of novel protective lactic acid bacteria and application in raw chicken meat against *Listeria monocytogenes* and *Salmonella enteritidis*. *Int. J. Food Microbiol.* 130: 219–226 (2009)
31. Engesser DM, Hammes WP. Non-heme catalase activity of lactic acid bacteria. *Syst. Appl. Microbiol.* 17: 11–19 (1994)
32. Domínguez R, Munekata PE, Agregán R, Lorenzo JM. Effect of commercial starter cultures on free amino acid, biogenic amine and free fatty acid contents in dry-cured foal sausage. *LWT-Food Sci. Technol.* 71: 47–53 (2016)