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Identification of Two Selected Lactic Acid Bacteria Strains Isolated From Dry-Cured Fish and Their Behaviors in Fermented Fish Sausage

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Abstract: The aim of this study was to identify the two selected LAB strains (ZY-40 and GY-23) isolated from dry-cured fish, and to evaluate their effects on the qualities of fermented grass carp sausage. Based on the 16s rRNA gene sequences, strains ZY-40 and GY-23 were identified as L. plantarum and P. pentosaceus, respectively. During the 48 h fermentation of grass carp sausages, L. plantarum ZY-40 and P. pentosaceus GY-23 significantly inhibited the growth of undesired bacteria, rapidly decreased the pH to the range of 4.6-4.7 with a progressive increase in titratable acid and markedly reduced the formation of total volatile base nitrogen (TVB-N). The lipid oxidation of grass carp sausage was prevented by L. plantarum ZY-40, whereas enhanced by P. pentosaceus GY-23. In addition, the accumulations of biogenic amines in grass carp sausages were significantly reduced by the two LAB strains, particularly L. plantarum ZY-40. These results suggested that L. plantarum ZY-40 might be used as a potential starter for fish fermentation.

Keywords: Lactic acid bacteria; Identification; Grass carp sausage; Quality.

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Introduction

Fermented fish products are common in South-East Asia. The composition and quality of these products vary considerably since they are mostly manufactured on a small scale. And the traditional process depends on a spontaneous fermentation initiated by natural fortuitous microorganisms, mainly lactic acid bacteria (LAB). LAB have been found to be the predominant microorganisms in many fermented fish products (Paludan-Mu¨ller et al., 2002; Lee et al., 2005; Kuda et al., 2009). They can ferment the available carbohydrates to produce organic acids (mainly lactic acid) and thereby cause a rapid decrease in pH, which is considered the main preservation factor in fermented products (Saithong et al., 2010). *Lactobacillus*, *Leuconostoc* and *Pediococcus* species have been identified as the main LAB in some fermented fish products (Paludan-Mu¨ller et al., 2002; Olympia et al., 1992; Thapa et al., 2004).

In recent years, the impact of LAB starter culture on fermented fish products' properties has received considerable attention. These researches showed that controlled LAB fermentation could be a successful process for improving the quality of fermented fish product. According to Yin et al. (2001, 2002), using LAB starters to ferment mackerel mince could suppress the growth of undesired bacteria and inhibit the formation of volatile basic nitrogen. In plaa-som inoculated with *Lactobacillus plantarum* IFRPDP15 or *Lactobacillus reuter*i IFRPD P17, LAB dominated the whole fermentation process and accelerated the acidification of the raw material (Paludan-Mu¨ller et al., 2002). The best organoleptic and chemical results were obtained from inoculated sardine fillets using *Lactobacillus delbrueckii subsp. delbrueckii,* supplemented with salt and sugar (Ndaw et al., 2008). Moreover, the rapid pH decrease caused by amine-negative LAB strains would reduce biogenic amine accumulation in fermented food, resulting from the growth inhibition of amine-positive bacteria (Hu et al., 2007; Liu et al., 2010; Petäjä et al., 2000).

The development of LAB starter cultures is one of the prerequisites for the production of fermented fish foods. However, only a few studies were related to the LAB strains isolated from traditional fish products. In our preliminary study, two LAB strains (ZY-40 and GY-23) had been isolated from dry-cured fish and selected as possible starter cultures for fish fermentation. The aim of this study was to identify the two LAB strains and evaluate their effects on the qualities of fermented grass carp sausage. Quality parameters of fermented grass carp sausage, such as the growth of main bacteria, production of organic acid, formation of total volatile basic nitrogen, hydrolysis of muscle protein and oxidation of lipid, were determined. In addition, the accumulation of biogenic amine in fermented sausage was also detected.

Materials and Methods

Strain

Dry-cured fish (10 g) were aseptically homogenized in 90 mL of sterile saline solution (0.90% NaCl). The decimal dilutions were poured into de Man Rogasa Sharpe (MRS) agar, and then inoculating at 37°C for 48 h. Bacterial colonies on the plates were individually picked and streaked on MRS agar. The procedure was repeated in order to purify the isolates. Each isolate was first tested for catalase activity and gram staining. Only those isolates which were catalase-negative and gram-positive were screened according to the selection criteria described by Ammor and Mayor (2006). Two LAB strains (ZY-40 and GY-23) were selected and maintained on MRS agars at 4°C.

Identification by 16S RNA gene sequencing

Single pure bacterial colony was used as template in a PCR reaction that contained 5 μL of 10 × PCR buffer, 3 μL of 2.5 mM dNTP, 5U Taq polymerase, 1.5 μL of 50 μM each primer (27F and 1541R) and 38 μL of distilled water. PCR amplification was carried out using the following program: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45s, annealing at 50°C for 45s and extension at 72°C for 1 min; ending with a final extension at 72°C for 10 min. The amplified product was confirmed by 1.0% agarose gel electrophoresis and sequenced at Invitrogen Corporation (Shanghai, China). The 16S rRNA gene sequence obtained was compared against those deposited in GenBank using BLAST Search.

Culture preparation

Each strain was cultured twice in MRS broth at 30°C for 24 h. The cells were harvested by centrifugation, washed twice with saline solution (0.90% NaCl) and resuspended in the same solution. The cell suspension was then used as starter culture for fermented fish sausage production.

Fermentation of grass carp sausage

Grass carp mince was mixed with 2% NaCl, 0.5% glucose and 0.5% sucrose. Then the mixture was inoculated with LAB strains at a final level of about 5×10^7 CFU/g fish mince. The mixture containing no LAB strain was used as the control. After mixing thoroughly for 2 min, the resulting mince was stuffed into collage casings and incubated at 30°C and 90% RH (relative humidity) for 48 h. Samples were taken every 12 h for analysis.

Microbial analysis

Each sausage sample (10 g) was aseptically homogenized in 90 mL of sterile saline solution. The homogenate was diluted and spread on different agar plates for microbial analysis. Total aerobic bacteria, LAB, *Micrococaceae* and *Pseudomonas* were enumerated on Plate Count Agar (PCA), de Man Rogosa Sharpe agar (MRS), Mannitol Salt Phenol-Red Agar (MSA) and *Psendomonas aeromonas* selective Agar (GSP agar) at 30°C for 48 h, respectively. *Enterobacteriaceae* was determined on Violet Red Bile Dextrose Agar (VRBD) at 30°C for 24 h.

pH and titratable acid

Each of ten-gram sausage was homogenized in 100 ml of distilled water, and the pH was measured with a digital pH meter (Mettler Toledo, Schwerzenbach, Switzerland). Using the same homogenate, titratable acid was detected by titration according to the Beddows method (Beddows et al., 1979). Dilution samples (10

mL) were mixed with 50 mL of distilled water and titrated to pH 7.0 with 0.05 M NaOH. Titratable acid was expressed as percent lactic acid.

Total volatile base nitrogen (TVB-N)

TVB-N was measured by the method of steam distillation (Yao et al., 2011). Briefly, the water extraction of sausage sample was steam-distilled after the addition of magnesium oxide. Distillate was collected for 5 min into 10 mL of 2% boric acid with Tashero indicator and titrated with 0.01 M HCl. The TVB-N content was calculated and expressed in mg /100 g sample.

Tthiobarbituric acid reactive substances (TBARS)

TBARS was determined as described by Vyncke W (1970) with slight modification. Sample (5 g) was homogenized in 50 mL of 15% trichloroacetic acid. After filtration, 5 mL of the filtrate and 5 mL of 0.6% TBA solution were mixed and heated at 90°C for 30 min, followed by cooling with running water. The absorbance was read at 532 nm. The TBARS value was expressed as mg MDA/ kg sample.

Biogenic amines

Biogenic amines were detected by HPLC according to Tosukhowon, et al. (2011). Biogenic amines were extracted with 0.4 M perchloric acid and then derivatised with dansyl chloride (Mah et al., 2002). The derivatives were separated on Hypersil ODS C_{18}, 5 μm, 4.6 × 200 mm column (Thermo, Bellefonte, PA, USA). The column temperature was set at 35°C. The mobile phase was composed of 0.01 M ammonium acetate as solvent A and 0.01 M ammonium acetate in 90% acetonitrile solution as solvent B. The flow rate was 1 ml/min. The gradient program started at 45% A and 55% B and then solvent B was raised to 95% within 25 min and hold for 10 min, after that the gradient was switched to 45% A and 55% B within 8 min and held for 2 min before starting the next run. Biogenic amines were detected at wavelength 254 nm.

Statistical analysis

The data were analyzed statistically by one-way ANOVA using SPSS 17.0 and means were compared by Ducan's multiple range test.

Results and Discussion

Technological properties of strains ZY-40 and GY-23

A total of 93 LAB strains isolated from dry-cured fish products were characterized in respect to their technological properties, e.g. growth at different temperatures, tolerance to salt concentrations, acidification ability, gas from glucose, production of H_2S, hydrolysis of amino acid and inhibitory activity against pathogens (Table 1). Among them, strains ZY-40 and GY-23 grew well in MRS broth at different temperature and different salt concentration (3% and 6%), and reduced the pH to 3.67 and 3.73 in 24 h at 30°C. Ammor and Mayor (2007) reported that growth rates at different temperature and salt concentration have been the limiting factors affecting the viability of the starter culture over the fermentation process. Gas from glucose, production of H_2S, NH_3 from arginine and amino acid-decarboxylating activity were not detected in the two strains. The formation of CO_2, H_2S, NH_3 and biogenic amines can make adverse effects to the texture, flavor and safety of fermented food. In addition, one of the most important characteristics of functional starter cultures is inhibiting the growth of pathogenic microorganisms (Ammor & Mayor, 2007). The present study showed that strains ZY-40 and GY-23 displayed widest inhibitory zones against *E. coli* and *S. aureus* on agar.

Molecular identification of strains ZY-40 and GY-23

The 16s rRNA gene sequences obtained from strains ZY-40 and GY-23 were compared to those of reference strains held in GenBank, and the phylogenetic analysis was conducted with the sequences of representative strains from *Lactobacillus* species using ClustalX 1.8 and Mega 4.1 programs (Figure 1). The phylogenetic tree showed a clear division between the two species. The sequence of strain ZY-40 showed a homology of 99.0% to the type strains of *L. plantarum NRIC 1832, L. plantarum NRIC 1838 and L. plantarum LBA16SRRN1*. The sequence of strain GY-23 showed a homology of 95.0% to the type strains of *Pediococcus pentosaceus* KT3CE27 and *Pediococcus pentosaceus* RTa11. Strains ZY-40 and GY-23 were characterized as *L. plantarum* and *P. pentosaceus*, respectively.

Effects of the selected LAB strains on the qualities of fermented fish sausage

Microbial counts

The counts of total aerobic bacteria, LAB, *Micrococci*, *Pseudomonas* and *Enterobacteria* were monitored during the fermentation of grass carp sausage, as shown in Table 2. Inoculated sausages had initial LAB counts of 7.24-7.37 log CFU/g, compared to the level of 5.57 log CFU/g in the control. LAB counts in inoculated sausages increased rapidly to 8.91-9.18 log CFU/g by 12 h and thereafter maintaining the levels during the later stage. In contrast, LAB count in the control slowly increased to 6.78 log CFU/g and then decreased. The counts of total aerobic bacteria showed the same growth pattern as those of LAB during the fermentation. These data indicated that LAB dominated the fermentation process of inoculated sausage, but not in the control.

Micrococcaceae counts in inoculated sausages gradually increased from 4.60-4.75 log CFU/g to 6.41-6.57 CFU/g at the end of fermentation. Significant differences in *Micrococcaceae* counts between the control and inoculated sausages were observed in the later fermentation stages. These data indicated that *L. plantarum* ZY-40 and *P. pentosaceus* GY-23 had certain inhibitions against the growth of *Micrococcaceae*. Several authors have also documented that some LAB cultures brought about the reduction of *Micrococacceae* counts during the sausages' fermentation (Antara et al., 2004; Baka et al., 2011).

Inoculation with *L. plantarum* ZY-40 and *P. pentosaceus* GY-23 resulted in significant growth inhibition of *Pseudomonas* and *Enterobacteriaceae* in grass carp sausage. After 48 h fermentation, *Pseudomonas* counts in inoculated sausages increased from initially 1.68-1.84 log CFU/g to 4.15-4.51 log CFU/g; whereas it reached 7.29 log CFU/g in the control. And *Enterobacteriaceae* counts in inoculated sausages were obviously reduced by above 2 log cycle, compared to the control. The changes were similar to those reported in fermented mackerel mince (Yin & Jiang, 2001; Yin et al., 2002). These phenomena might be mainly due to the rapid decrease of pH and action of bacteriocins produced by LAB. The domination of LAB and the inhibition of undesired bacteria are necessary for a successful production of fermented sausage.

pH and titratable acid

The evolutions of pH value and titrable acid content in grass carp sausage are given in Figure. 2. As the fermentation processed, inoculated sausages exhibited rapid decreases in pH, which continuously decreased to 4.6-4.7; whereas the pH of the control decreased slightly and then increased gradually to 7.12. The rapid pH decrease in fermented product indicated good acidification of raw material through LAB fermentation.

The production of titratable acid coincided with the pH changes during the fermentation. An immediate and rapid production of titratable acid at the beginning of fermentation was found in the sausage inoculated with *P. pentosaceus* GY-23. It seemed that *P. pentosaceus* GY-23 have better acidification capacity than *L. plantarum* ZY-40. When the fermentation was accomplished, titratable acid contents were 0.72% and 0.68% for *L. plantarum* ZY-40 and *P. pentosaceus* GY-23 samples, respectively. These results were lower than those in sliver carp sausage (Hu et al., 2008). The main cause might be the dissimilar amount of carbohydrates in fermented system. These changes of pH value and titratable acid content during the fermentation might be due to production of organic acids and formation of alkaline nitrogenous substances (Xu et al., 2008; Tungkawachara et al., 2003).

TVB-N and TBARS

Total volatile basic nitrogen (TVB-N) is the most useful index for the microbial spoilage in various species of fish during their processing and storage (Dalgaard, 2000). As shown in Figure.3, the TVB-N content in the control increased sharply from 9.55 mg/100g to 23.41 mg/100g after 24 h fermentation and further increased to 65.30 mg/100g after 48 h fermentation, while those in inoculated sausage increased slowly to 12-17 mg/100 g. The results indicated that *L. plantarum* ZY40 and *P. pentosaceus* GY-23 could significantly inhibit the TVB-N formation during the sausage's fermentation. This change trend could also be supported by the changes of pH and undesired microorganisms (Figure.2 and Table 2). These results were in agreement with those reported by other authers (Yin et al., 2002; Glatman et al., 2000).

Lipid oxidation in grass carp sausage was monitored by the formation of TBARS (Figure. 3). TBARS values in all samples ranged from 0.25 to 0.51 mg MDA/kg, much below the allowable limit (5 mg MDA/kg) in fresh fish and fish products (Gökalp, 1986). The two selected LAB strains performed opposite effects on the lipid oxidation in grass carp sausage. Inoculation with *P. pentosaceus* GY-23 resulted in the enhancement of TBARS value, while *L. plantarum* ZY-40 might prevent the lipid oxidation. The similar results were also found in Greek fermented sausage inoculated with selected autochthonous starter cultures (Baka et al., 2011). The difference between LAB strains may be explained by their antioxidant properties and hydrogen peroxide production (Lin & Yen, 1999). In general, LAB are able to produce hydrogen peroxide which may cause the increase of oxidation of lipid (Riebroya et al., 2008). However, some LAB strains such as *L. sakei*, *L. plantarum*, *L. pentosus* and *P. acidilactici*, can deal with free radicals and hydrogen peroxide by heme-dependent catalase, superoxide dismutase, glutathione or a high internal Mn^{2+} concentration (Ammor and Mayo, 2007; Stecchini et al., 2001).

Biogenic amines

Biogenic amines are mainly produced by microbial decarboxylation of amino acids in food. The accumulation of biogenic amine has been related to *Bacillus*, *Escherichia*, *Pseudomonas* and some LAB strains (Halasz et al., 1994). Thus, various authors have proposed the absence of biogenic amine accumulation as a selection criterion for new strains used as starter cultures (Ammor and Mayo, 2007; Buckenhüskes, 1994). In the selective test, *L. plantarum* ZY-40 and *P. pentosaceus* GY-23 exhibited no decarboxylation of histidine, lysine and arginine (Table 1).

Biogenic amines contents in grass carp sausages were detected by RP-HPLC method, as presented in Table 3. The high amounts of histamine, cadaverine, putrescine and tyramine were found in the control. Histamine was the main amine formed; its amount was about 1000 mg/kg. Nout (1994) pointed out that histamine amount should be below 100 mg/kg in sausages processed according to "Good Manufacturing Practice". Compared to the control, the accumulations of the four biogenic amines in inoculated sausages were drastically inhibited by the two LAB strains, especially *L. plantarum* ZY-40. The amounts of histamine, cadaverine, putrescine and tyramine in sausage with *L. plantarum* ZY-40 were 5.28, 13.27, 1.78 and 20.01 mg/kg, respectively. The application of amine-negative LAB starter have been suggested to inhibit amine accumulation in fermented producuts (Hu et al., 2007; Kalač et al., 2000). And significant difference was also observed between the sausages with *L. plantarum* ZY-40 and *P. pentosaceus* GY-23.

Conclusion

LAB are widely used as starter cultures for improving the safety and stability of fermented food. In our study, two LAB strains (ZY-40 and GY-23) selected from dry-cured fish were identified as *L. plantarum* and *P. pentosaceus* by 16s rRNA gene sequences. The two LAB strains changed the microbial and chemical characters of grass carp sausages. In inoculated sausages, LAB dominated the fermentation process and rapidly decreased the pH values through the production of organic acids; the growth of undesired microorganisms such as *Pseudomonas* and *Enterobacteriaceae*, the formation of TVB-N and the accumulation of biogenic amines were significantly inhibited. However, *L. plantarum*

ZY-40 exhibited an inhibitory effect on lipid oxidation, while *P. pentosaceus* GY-23 tended to enhance the TBARS value. Thus, *L. plantarum* ZY-40 is considered as the suitable LAB strain for fermented fish products.

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