JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2014. Vol. **8**(Spl. Edn. 1), p. 525-531

Identification of Spoilage Bacteria Isolated from Cooked Ham and its Related Food Preservatives Evaluation

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(Received: 08 January 2014; accepted: 24 March 2014)

From spoiled cooked ham, six kinds of spoilage bacteria were isolated and three isolates were identified as *Bacillus* through matrix-assisted laser desorption/ionization time-off light mass spectrometry (MALDI-TOF MS). The results were further confirmed by 16S rRNA gene sequencing method at the species level. All these strains were found to be highly resistant to potassium sorbate and sodium nitrite, but greatly sensitive to both ε-poly-L-lysine and nisin. Biopreservatives, especially, ε-poly-L-lysine exhibited excellent inhibition on food spoilage microbial growth, significantly superior to a single chemical preservative, presenting a potential of improving food safety.

Key words: ε-poly-L-lysine; Nisin; Potassium sorbate; Sodium nitrite; 16S rRNA; Biotyper.

Nowadays, huge amount quantities of excessive foods are lost due to microbial spoilage even with after modern food processing and with preservation techniques. Since food spoilage is a complex process, the thermal treatment is not always sufficient to inactivate all bacteria in food materials. During slicing and packaging operations, ready to eat (RTE) meat products, like cooked ham, could be contaminated by various bacteria due to exposure to the ambient[1]. Although good handling during post-processing, careful control of the temperature and modified atmosphere packaging had been reported to increase the safety and extend the shelf life of processed cooked products, some pathogens could still grow on RTE products during the days of storage[2]. In an effort to find proper protecting solutions, it is necessary and important for the food industry to develop a rapid and low-cost method for spoilage microorganisms identifications[3], since the classical methods through molecular biology tools, such as 16S rRNA gene sequencing and real-time PCR, showed remarkably high complexities and cost for daily production. Lately, a new fast and inexpensive technique, MALDI-TOF MS for microbial identification, was developed and widely used to identify clinical pathogens, for instance, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Achromobacter xylosoxidans* etc.[4-7]. The premise of bacterial identification by the MALDI-TOF MS approach is the spectral profile establishment of abundant bacterial proteins, the majority of which are likely ribosomal[8]. The spectral profile is referenced to a compiled database, allowing for comparison and differentiation of bacterial isolates

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via their protein profiles. However, there were few reports about MALDI-TOF MS applied on food-microorganism classifications[9]. Thus, MALDI-TOF MS microbial identification technology needed to be introduced and further verified in the food area.

Generally, different food preservation techniques have been used to improve the microbial safety and extend the shelf life of food, including freezing, heat, high pressure, and chemical preservation[2, 10-12]. However, previous researches demonstrated that these traditional preservation methods lead to decreasing nutritional value of the products or enhancing the possible risk of poisoning and carcinogenesis[13-15]. Therefore, there is a clear and urgent necessity for a development of new effective and safe preservatives which permit shelf life extension of ready to eat (RTE) products, specifically herein, cooked ham.

In this study, six bacterial isolates from cooked ham were identified using MALDI-TOF MS technology. Subsequently, the results were further verified by 16S rDNA sequencing technology. In the meantime, four food preservatives (two bio-oriented, nisin and ε-poly-L-lysine; two chemically oriented, potassium sorbate and sodium nitrite) were employed to evaluate their inhibitions on the growth of spoilage microorganisms.

MATERIALS AND METHODS

Isolation of strains

The spoiled cooked ham samples (25 g) were homogenized with 225 mL of sterile water, submitted to serial ten-fold dilutions in sterile water, and spread on the surface of Luria–Bertani (LB) agar (proteose peptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, and agar 15 g/L, pH 7.5). After aerobic incubation at 37 °C for 24 - 48 h, the colonies were randomly selected and streaked on LB medium to obtain pure cultures. Then, the cultures were stored at -80 °C in the presence of 20 % w/v glycerol as a protector reagent for future experimental uses.

Sample preparation and MALDI-TOF MS identification

Bacterial proteins were extracted using an ethanol/formic acid method. Briefly, a colony (5-10 mg) was suspended in 300 µL of molecular-grade water and vortexed, and then 900 µL of anhydrous ethanol (Merck, Germany) was added. The samples were mixed and centrifuged (13,000 × g) for 2 min. The supernatant was discarded and 50 µL of 70 % formic acid (Merck, Germany) was added and mixed. Finally, 50 µL of acetonitrile (Merck, Germany) was added, and the mixture was carefully mixed. After centrifuging (13,000 × g) for 2 min, 1 µL of the supernatant protein sample was overlaid with 1 µL of a chemical matrix (saturated solution of a-cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 2.5 % trifluoroacetic acid). MALDI-TOF MS measurements were made using a Microflex III instrument (Bruker Daltonik, Bremen, Germany) for the automatic acquisition of mass spectra in the linear positive mode within a range of 2-20 kDa, according to the manufacturer's instructions.

16S rRNA gene sequencing and sequence analysis

Nucleic acids of the 6 samples for PCR were extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) following manufacturer specifications. For the amplification of the 16S rRNA gene fragment, the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used. PCR amplification was performed on MyCycler equipment (Bio-Rad, USA). The sequence data for the 16S rRNA genes were compared with those of the GenBank database using the BLAST software (http://blast.ncbi.nlm. nih.gov/Blast.cgi). A homology level of >99 % was considered as a threshold value for species identification.

Antimicrobial activity

Nisin and ε-poly-L-lysine (ε-PL) were provided by Zhejiang Silver Elephant Bioengineering Co., Ltd (China). Spoilage microorganism was incubated at 37 °C for 8-10 h to obtain approximately 10^6-10^7 CFU/mL in LB liquid medium previously. A culture (20 µl) was added to 20 mL LB medium containing ε-poly-L-lysine, nisin, potassium sorbate, and sodium nitrite, respectively, and incubated for 24 h at 37 °C with 180r/min. Concentrations of each antimicrobial used were as follows (µg/mL): ε-poly-L-lysine, 0, 31.25, 62.5, 125, 250, 500, and1000; Nisin, 0, 15.625, 31.25, 62.5, 125, 250, and 500; potassium sorbate, 0, 31.25, 62.5, 125, 250, 500, and 1000; Sodium nitrite,0, 4.6875, 9.375, 18.75, 37.5, 75, and 150. After incubation, the antimicrobial activities against bacteria were determined by measuring the optical density (OD) at a wavelength of 600 nm using a

UV–vis spectrophotometer (UVmini - 1240, Shimadzu, Japan). LB medium with added inhibitors, incubated under the same conditions, was used as a blank.

RESULTS AND DISCUSSIONS

Strains isolation

Six strains were isolated from spoiled cooked ham and classified by Gram staining method. Fig. 1 showed that all isolated strains were Gram-positive rods. These spoilage bacteria could survive after treated with heat and preservatives, might be due to forming endospores which can survive under extreme conditions and make a serious threat to food safety.

MALDI-TOF MS identification

MALDI-TOF MS identifications of the 6 strains was performed on Biotyper 3.0 Software. According to the demonstrations on score values in Table 2, the results are listed in Table 1. Specifically, one isolate was confirmed as *Bacillus licheniformis* due to score values >2.0; two scored from 1.700 to 1.999 were identified at genus level; the remaining three could not be recognized, since scored from 0 to 1.699. MALDI-TOF MS was partly successful in the identification of isolates from spoiled cooked hams. As previously described[16], the results strongly indicate that MALDI-TOF MS combined with other approaches might be a useful and powerful tool for viable bacterial detection in the meat industrials.

16S rRNA gene sequencing identification

All the isolates were identified at the species level by the 16S rRNA gene sequencing technique which is considered as the "gold standard" identification method. Three isolates were identified as *Bacillus licheniformis*, shown in Table 3, which well matches the results from MALDI-TOF MS identification. The other three

Table 1. MALDI-TOF identification of six isolates

Strain	Organism (best match)	Score Value
A	no reliable identification	1.586 (-)
B	no reliable identification	1.367 (-)
C	*Bacillus licheniformis* 992000432 LBK	1.848 (+)
D	no reliable identification	1.529(-)
E	*Bacillus licheniformis* DSM 13T DSM	2.167(++)
F	*Bacillus licheniformis* DSM 13T DSM	1.883(+)

Table 2. Meaning of Score Values

Range	Description	Symbols
2.300 ... 3.000	highly probable species identification	(+++)
2.000 ... 2.299	secure genus identification, probable species identification	(++)
1.700 ... 1.999	probable genus identification	(+)
0.000 ... 1.699	no reliable identification	(-)

Table 3. Comparison of the results obtained by use of MALDI-TOF MS-based methods and the 16S rRNA method

Strain	MALDI -TOF MS method	16S rRNA method
A	no reliable identification	*Bacillus subtilis*
B	no reliable identification	*Bacillus subtilis*
C	*Bacillus*	*Bacillus licheniformis*
D	no reliable identification	*Bacillus subtilis*
E	*Bacillus licheniformis*	*Bacillus licheniformis*
F	*Bacillus*	*Bacillus licheniformis*

strains which could not be classified in MALDI-TOF MS were identified as *Bacillus subtilis* by 16s rRNA gene sequence method. The failure identifications by MALDI-TOF MS might be ascribed to lack of certain strains' mass spectrometry (MS) peak profiles in the database of Biotyper 3.0. In other words, an up-to-date and comprehensive database is critical for accurately identifying isolates by MALDI-TOF MS[5]. Although the database is proprietary to the commercially available systems, it might be still updated and expanded through addition of in-house spectra from those identified strains. Therefore, the impact of our study is to improve the Biotyper 3.0 database with the spectra of all the strains identified through 16S rDNA, enhancing the reliability and recognition rate of MALDI-TOF MS method for identification of spoilage bacteria.

Antimicrobial activities of various food preservatives

Antimicrobial activities of potassium sorbate, sodium nitrite, nisin, and ε-poly-L-lysine against spoilage microorganism isolated from cooked ham were studied. *B. subtilis* A, *B.*

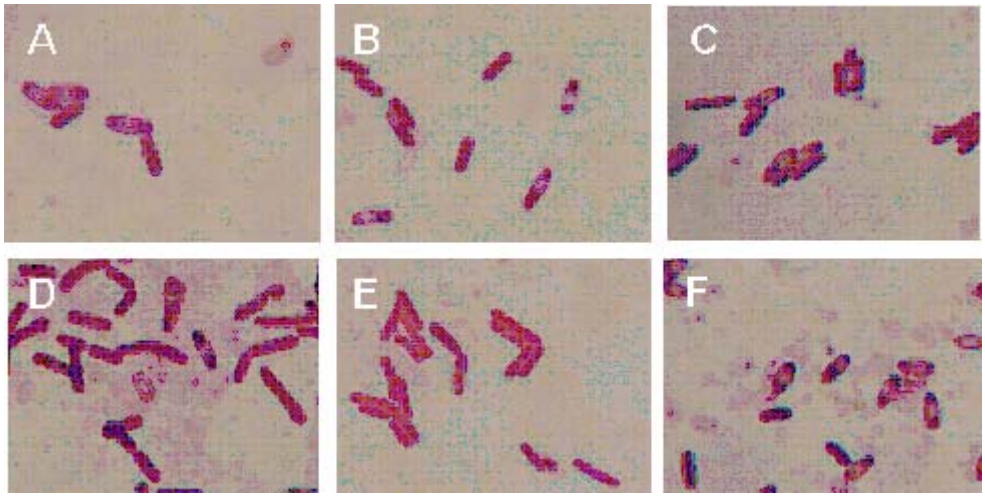

Fig. 1. Isolates from spoiled cooked ham stained by Gram staining (×1,000)

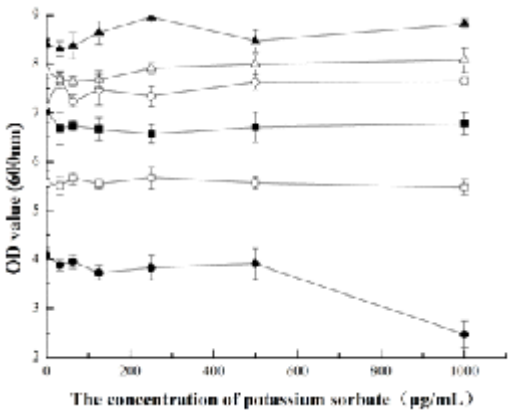

Fig. 2. Growth curves of different bacterial strains cultured respectively in medium containing different concentrations of potassium sorbate: 1000, 500, 250, 125, 62.5, 31.25, and 0 μg/mL. (■) indicates *B. subtilis* A, (●) indicates *B. subtilis* B, (▲) indicates *B. licheniformis* C, (□) indicates *B. subtilis* D, (○) indicates *B. licheniformis* E, (Δ) indicates *B. licheniformis* F.

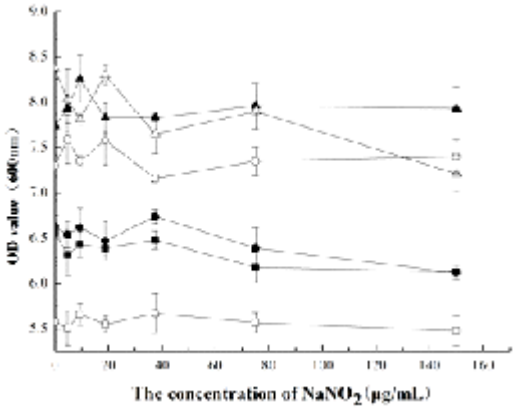

Fig. 3. Growth curves of different bacterial strains cultured respectively in mediums containing different concentrations of $NaNO_2$: 150, 75, 37.5, 18.75, 9.375, 4.6875, and 0 μg/mL. (■) indicates *B. subtilis* A, (●) indicates *B. subtilis* B, (▲) indicates *B. licheniformis* C, (□) indicates *B. subtilis* D, (○) indicates *B. licheniformis* E, (Δ) indicates *B. licheniformis* F.

licheniformis C, *B. subtilis* D, *B. licheniformis* E and *B. licheniformis* F exhibited strong resistance towards potassium sorbate (Fig. 2). Potassium sorbate could not inhibit the growth of isolated strains, when its concentrations no more than 500 µg/mL. Moreover, *B. subtilis* B was more sensitive than *B. subtilis* A and *B. subtilis* D to high concentration (1000 µg/mL) potassium sorbate. Oloyede (1994) reported that the combination of heat, potassium sorbate and NaCl at pH 5.5 to 6.5 did not cause any significant inhibition on the spores of either species[17]. This study also indicated that the inhibitory effect of potassium sorbate against *Bacillus* is very limited.

Nitrate and nitrite have been used for centuries in curing and preserving meats and fish, even in the manufacture of certain cheeses[14]. As known, nitrite sodium can efficiently inhibit the growth of food spoilage bacteria, especially, *Clostridium botulinum* which thrives under anaerobic conditions and produces a neurotoxin, one of the most lethally natural products[15].The adding amount of nitrite into foods is, however, progressively being restricted, in view of the possible risk of its toxicity and carcinogenesis[14, 15]. From Fig. 3, it is clearly presented that the antimicrobial activities against isolated *B. licheniformis* and *B. subtilis* are limited, even under the maximum permitted use level (150 µg/mL).

The antibacterial activity of nisin on spoilage microorganism is showed in Fig. 4. Strain *B. subtilis* D exhibits higher sensitivity to nisin than other strains and the MIC value is 250 µg/mL. Meanwhile, the MIC value of *B. subtilis* A, *B. subtilis* B, *B. licheniformis* C, *and B. licheniformis* F is 500 µg/mL, but at the same concentration strain *B. licheniformis* E still showed the high resistance and its growth could not be inhibited. The bactericidal effect of nisin is specifically exerted at the cytoplasmic membrane[18]. In our case, though nisin presented antibacterial activity against most of Gram positive bacteria, some nisin-resistance strains still survived and grew. Therefore, it is necessary for professionals to develop new food preservatives with broader antibacterial spectrum.

The antibacterial activity of ε-poly-L-lysine against isolated *B. licheniformis and B. subtilis strains* was studied with different concentrations. The susceptible range of ε-PL for *B. subtilis* A, *B. subtilis* B, and *Bacillus subtilis* D was between 125 and 250 µg/mL, shown in Fig. 5. Furthermore, all isolates could be completely inhibited when the concentration of ε-PL up to 500 µg/mL. Thus, it is concluded that ε-PL has an excellent antibacterial activity and is remarkably superior to other preservatives.

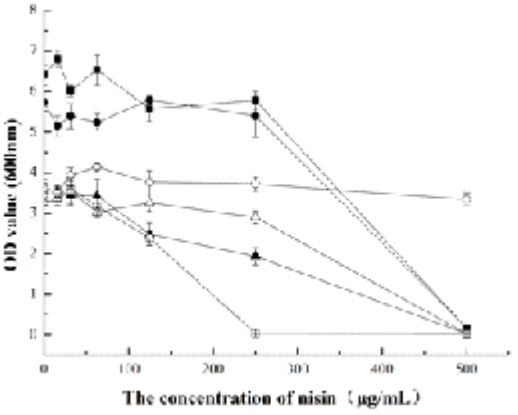

Fig. 4. Growth curves of different bacterial strains cultured respectively in medium containing different concentrations of nisin: 500, 250, 125, 62.5, 31.25, 15.625, and 0 µg/mL. (■) indicates *B. subtilis* A, (●) indicates *B. subtilis* B, (▲) indicates *B. licheniformis* C, (□) indicates *B. subtilis* D, (○) indicates *B. licheniformis* E, (Δ) indicates *B. licheniformis* F

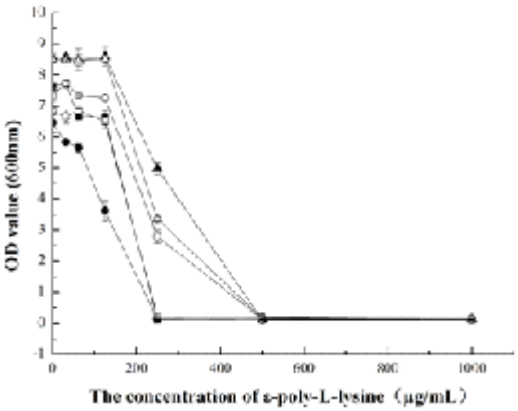

Fig. 5. Growth curves of different bacterial strains cultured respectively in medium containing different concentrations of ε-poly-Llysine: 1000, 500, 250, 125, 62.5, 31.25, and 0 µg/mL. (■) indicates *B. subtilis* A, (●) indicates *B. subtilis* B, (▲) indicates *B. licheniformis* C, (□) indicates *B. subtilis* D, (○) indicates *B. licheniformis* E, (Δ) indicates *B. licheniformis* F.

The antibacterial activity evaluations indicate that different strains or even the strains within same species show different resistances to various food preservatives. In general, the inhibition of potassium sorbate and sodium nitrite against spoilage bacteria was very limited, especially, some *Bacillus* strains that can survive and induce food spoilage, even after heat and chemical treatments. In contrary, nisin and ε-poly-L-lysine are natural antimicrobials and showed excellent antimicrobial activity. Noticeably, ε-poly-L-lysine is widely used as an antibacterial agent due to its broad antimicrobial spectrum. Ye R. et al. reported that the antibacterial mechanism of ε-PL against *E. coli* O157:H7 may be attributed to disturbance on membrane integrity, oxidative stress by reactive oxygen species (ROS), and influence on various gene expressions[19]. However, the mechanism of ε-PL against *Bacillus* at the molecular level has not been clearly elucidated.

In conclusion, six spoilage bacteria strains were isolated and identified by MALDI-TOF MS and 16S rRNA gene sequencing technique. MALDI-TOF MS is a rapid identification method and might result in the potential application of spoilage bacteria identification. In the meantime, in comparison of different food preservatives, nisin and ε-poly-L-lysine show superior inhibition effects against spoilage microorganism from cooked ham. Therefore, our results disclose that food-preservative selection play a key role on the final antibacterial performance. In this study, ε-poly-L-lysine inhibited all spoilage bacteria and showed huge potential of being a highly safe and efficient preservation for cooked ham products.

ACKNOWLEDGMENTS

This work was financially supported by the National High Technology Research and Development Program of China (2013AA102106), the Agriculture Science Technology Achievement Transformation Fund (2012GB2A100023), the National Natural Science Foundation of China (21276197), the National Key Technology R&D Program (2011BAD23B05-3), and the Natural Science Foundation of Tianjin University of Science & Technology (20120113). Excellent technical assistance was contributed by Zhao Hua-Bing and Hu xiao.

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