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Evaluation of the spoilage potential of bacteria isolated from chilled chicken *in vitro* and *in situ*

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ABSTRACT

Microorganisms play an important role in the spoilage of chilled chicken. In this study, a total of 53 isolates, belonging to 7 species of 3 genera, were isolated using a selective medium based on the capacity to spoil chicken juice. Four isolates, namely *Aeromonas salmonicida* 35, *Pseudomonas fluorescens* H5, *Pseudomonas fragi* H8 and *Serratia liquefaciens* 17, were further characterized to assess their proteolytic activities *in vitro* using meat protein extracts and to evaluate their spoilage potential *in situ*. The *in vitro* studies showed that *A. salmonicida* 35 displayed the strongest proteolytic activity against both sarcoplasmic and myofibrillar proteins. However, the major spoilage isolate *in situ* was *P. fragi* H8, which exhibited a fast growth rate, slime formation and increased pH and total volatile basic nitrogen (TVBN) on chicken breast fillets. The relative amounts of volatile organic compounds (VOCs) originating from the microorganisms, including alcohols, aldehydes, ketones and several sulfur compounds, increased during storage. In sum, this study demonstrated the characteristics of 4 potential spoilage bacteria on chilled yellow-feather chicken and provides a simple and convenient method to assess spoilage bacteria during quality management.

1. Introduction

Meat is the most valuable livestock product. From a nutritional point of view, the importance of meat is derived from its high-quality protein, which contains all essential amino acids, and its highly bioavailable minerals and vitamins. In particular, chicken is one of the most traded and consumed meats worldwide. The yellow-feather chicken, as a special species in Asia, has a more distinctive flavor than many other commercial broilers (Zhang et al., 2015). However, to reduce the ongoing outbreaks of animal influenza, particularly the H7N9 strain, live poultry markets have currently been restricted in the majority of cities in China. Consumers now buy fresh chilled chicken, which originates from slaughter plants, through shops and supermarkets. Hence, the demand for these products has increased markedly, and safety problems have become a public health concern.

The safety issues associated with chilled chicken have been based mostly on the presence of toxicant and pathogenic bacteria in food, which may influence public health. It is noteworthy that according to Regulation 178/2002 of the European Parliament and Commission, a foodstuff is regarded as unsafe not only if it is harmful to consumer health but also if it is not fit for human consumption (Nowak et al., 2012). In this sense, spoiled food, which means food with an appearance, taste or flavor leading to its rejection, is also considered unsafe.

Chicken meat is prone to deterioration in a short time, even under chilled conditions (Patsias et al., 2008). Microbiological contamination is one of the most important factors contributing to quality loss, resulting in slime, colony formation, compromised food texture, off-flavors and off-odors (Grama et al., 2002; Hyldgaard et al., 2015). Many studies have reported that the specific spoilage organisms (SSOs) in refrigerated poultry are *Pseudomonas* spp., *Enterobacteriaceae*, lactic acid bacteria, and *Brochotrix thermosphacta* (Chaillou et al., 2015; Grama et al., 2002; Meredith et al., 2014). However, few studies have examined microbial organisms with strong catabolic capacities on chicken meat at refrigeration temperatures. These bacteria may destroy the cell

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structure, promoting the outflow of nutrients that can be utilized by other spoilage bacteria. Therefore, it is necessary to characterize these organisms to better understand chilled yellow-feather chicken spoilage.

The aims of the present work were to identify potential spoilage bacteria from chilled yellow-feather chicken using selective medium based on the capacity to spoil chicken juice and to assess the spoilage potential of these isolates both *in vitro* and *in situ*. Such information may eventually be used to better inform processing and preservation strategies to enhance the quality and shelf-life of chilled chicken.

2. Materials and methods

2.1. Preparation of the selective medium and samples

The selective medium was prepared by mixing raw chicken juice (the fresh chicken breasts were homogenized with deionized water and then filtered through two layers of gauze) and 1% agar. Both ingredients were maintained at 45 °C before mixing. This raw-chicken juice agar (RJA) was subsequently sterilized by irradiation at a dose of 6 KGy (Kazanas, 1968) via the ^{60}Co source at Hangyu (Hangyu Irradiation Technology Co., LTD, Nanjing, China).

Yellow-feather chicken meat was collected from slaughterhouses and supermarkets. Immediately after collection, the samples were aseptically transferred to the laboratory in an ice box within 3 h. The samples were then stored at 8 °C until spoilage before isolation was performed.

2.2. Isolation and identification of spoilage bacteria

Fifty-four surface samples (25 g) from each muscle were aseptically weighed and homogenized in 225 mL sterilized 0.85% NaCl solution. Decimal dilutions were prepared in the same solution, and 0.1 mL of each of the appropriate dilutions was plated on RJA. The plates were then incubated aerobically at 25 °C for 48 h. Colonies with a large decomposition zone were selected from each selective medium and subsequently streaked on the same medium twice to obtain a single colony. The isolates were identified based on 16S rRNA gene sequences, which were amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR products were purified and sequenced by Invitrogen (Invitrogen Biotechnology Co., Ltd, Shanghai, China). The sequences were compared with those in GenBank using the BLAST function, and the closest matches to each clone were determined based on specific probable identities and then confirmed using a VITEK2 automated system (BioMerieux, France).

2.3. Selection of spoilage bacteria via the RJA assay

A total of 53 isolates that belonged to different genera or species were identified: *Aeromonas* spp. (*Aeromonas salmonicida*, *Aeromonas hydrophila* and *Aeromonas media*), *Chryseobacterium shigense* and *Pseudomonas* spp. (*Pseudomonas fluorescens, Pseudomonas fragi* and *Pseudomonas putida*). All strains were cultured in tryptone soy broth (TSB) for 24 h at 25 °C. The cell density was adjusted to an optical density of 600 nm (OD600) of 0.4. Next, four replicate 2-μL aliquots of the isolates were spotted onto RJA, and decomposition was measured after 3 days of incubation at 25 °C. The strains *Pseudomonas fragi* H8, *Aeromonas salmonicida* 35 and *Pseudomonas fluorescens* H5 were selected because they provided the three largest decomposition zone diameters (DZDs) among the species described above. The spoilage potentials of these strains were compared with that of a well-known spoilage bacterium, *Serratia liquefaciens* 17, which was isolated previously (data not shown).

2.4. In vitro proteolytic and lipolytic assays

The proteolytic activities of the chicken sarcoplasmic and myofibrillar proteins were estimated based on SDS-PAGE analysis. In brief, each isolate was incubated in TSB. At the end of the exponential phase of growth, the cultures (1 mL) were centrifuged at 10,000 g for 5 min. The supernatant was used as the microbial extract for further assays. Sarcoplasmic and myofibrillar proteins were extracted as described by Mauriello et al. (2002) and then adjusted to a concentration of 1 mg/mL. The microbial extracts (200 μL) were incubated with sarcoplasmic and myofibrillar proteins (1 mL) for 20 h at 25 °C. Next, SDS-PAGE was performed based on the method of Paramithiotis et al. (2000). Control samples containing sarcoplasmic or myofibrillar proteins were incubated for 20 h at 25 °C as described above. The assessment of proteolytic activity was performed by comparing the protein profiles of control samples with those of the samples that had been incubated with the proteolytic strains.

An overnight culture of each strain was inoculated onto nutrient agar plates supplemented with 10 g/L triolein (Drosinos et al., 2007), and the plates were incubated at 25 °C for 10 days. The appearance of a clear zone surrounding the colonies indicated the occurrence of lipolytic activity.

2.5. In situ spoilage potential evaluation

2.5.1. Meat contamination

A total of 620 samples sliced from chilled yellow-feather chicken breasts were prepared in two batches. The first batch, containing both raw and cooked chicken, was used for microbiological analysis. The second batch, containing only raw chicken, was used for biochemical analysis. All samples were sterilized via irradiation as described above. Each inoculation mixture of the four isolates was spread onto the surface of the chicken breast pieces at a concentration of 3 log CFU/g. Uninoculated breasts were included as a control. All samples were stored at 8 °C for 7 days. The microbiological and biochemical analyses were performed after 2, 3, 4, 5, 6 and 7 days.

2.5.2. Microbiological analysis

At each sampling date, samples were transferred aseptically to a stomacher bag and diluted 10 times in 0.85% NaCl solution. The mixture was homogenized using a stomacher and 1 mL of the homogenized solution was transferred from the stomacher bag for additional serial-dilution steps. Aliquots of the appropriate dilutions (0.1 mL each) were spread on TSA in duplicate, and the agar plates were incubated aerobically at 25 °C for 24 h before counting.

2.5.3. Total volatile basic nitrogen (TVBN) and pH analysis

TVBN was determined according to China National Food Safety Standard methods - Method for analysis of hygienic standard of meat and meat products (GB/T 5009.44–2003). TVBN contents were expressed as mg of TVBN per 100 g of chicken. The pH was determined according to the method described by Kang et al. (2014). Briefly, 10 g of chicken breast sample was homogenized (Ultra Turrax T25, IKA, Germany) with 40 mL of pre-cooled distilled water at 15,000 rpm for 10 s. The pH was then determined using a digital pH meter (Hanna, Italy).

2.5.4. Sensory analysis

The samples were evaluated by five experienced panelists who participated in sensory tests from the National Center of Meat