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Formation of red myoglobin derivatives and inhibition of spoilage bacteria in raw meat batters by lactic acid bacteria and *Staphylococcus xylosus*

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ABSTRACT

The conversion of metmyoglobin ($MbFe^{III}$) to red myoglobin derivatives by bacteria and their effect on the inhibition of spoilage organisms in raw meat batters as protective cultures were investigated. Culture-dependent and culture-independent (PCR-denaturing gradient gel electrophoresis) methods were employed to reveal the microbial populations. The results from visible absorption and electron spin resonance spectroscopy analysis showed that *Staphylococcus xylosus* and *Lactobacillus fermentum* could convert $MbFe^{III}$ into nitrosylmyoglobin ($MbFe^{II}NO$), while oxymyoglobin ($MbFe^{II}O_2$) was formed by *Lactobacillus curvatus* and *Lactobacillus plantarum* in model systems. And the red colour was also observed in the meat batters inoculated with *L. fermentum* and *S. xylosus*. *Staphylococcus* spp., *Carnobacterium* sp., *Lactobacillus* sp., *Brochothrix thermosphacta* and *Enterococcus faecalis* were prevalent in control and nitrite-cured meat batters in tray packaging during refrigeration, whereas lactic acid bacteria and *Staphylococcus* were observed to be the predominant microbial flora in the samples inoculated with *Lactobacillus sakei* and *S. xylosus*, respectively. Most of the spoilage bacteria were inhibited by the two bacterial species. This study provides a potential method for nitrite substitution by improving meat colour and inhibiting spoilage bacteria in meat curing.

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1. Introduction

Nitrite is a key ingredient in meat curing. It can react with myoglobin (Mb) to form nitrosylmyoglobin ($MbFe^{II}NO$) which is responsible for the characteristic pink colour of cured meat, the inhibition of unwanted bacteria, the retardation of lipid oxidation and the development of desired meat flavours (Sebranek & Bacus, 2007). Unfortunately, nitrite addition can also result in the formation of carcinogenic, teratogenic and mutagenic N-nitrosamines due to its reaction with secondary or tertiary amines in meat proteins, unless antioxidant such as ascorbate is used to restrain the reaction (Li, Shao, Zhu, Zhou, & Xu, 2013). Since there is an increasing consumer's demand for natural products with less chemical additives (Messina, Bono, Renda, La Barbera, & Santulli, 2015), many attempts have been made to replace nitrite in the meat industry (Armenteros, Aristoy, & Toldrá, 2012; Djeri & Williams, 2014). However, only few substitutes have been used widely in meat products until now (Feng, Shao, & Chen, 2012).

Recently, microbial conversion of metmyoglobin ($MbFe^{III}$) to red myoglobin derivatives was observed and has the potential to be used for improving the red colour of meat. Many bacteria, such as *Lactobacillus fermentum* and *Staphylococcus xylosus*, were proved to have the ability to convert metmyoglobin to red myoglobin derivatives (Morita, Sakata, & Nagata, 1998; Møller, Jensen, Skibsted, & Knöchel, 2003). Our previous study also revealed that *L. fermentum* could cause the formation of $MbFe^{II}NO$, and therefore the cured meat colour in Harbin red sausage without nitrite addition (Zhang, Kong, & Xiong, 2007). These findings reveal that microbes can play a “reddening” role in meat products with reduced nitrite.

Many types of bacteria can inhibit pathogens and/or spoilage microorganisms in food matrices and are referred to “protective cultures”. “Cultures” are the live microorganisms that, once added

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to food products, can inhibit pathogens and/or prolong the shelf-life of food products (Ghanbari, Jami, Domig, & Kneifel, 2013). Because meat and meat products are excellent substrates for microorganisms, it is important to explore the potential of protective cultures in meat products to inhibit the growth of spoilage and pathogenic bacteria. Indeed, some lactic acid bacteria (LAB) species have been added to meat and successfully inhibit pathogens and/or spoilage microorganisms (Gaggìa, Mattarelli, & Biavati, 2010). However, to our knowledge, there is no report on the dual functions of the microbes, conversion of $MbFe^{III}$ and inhibition of unwanted bacteria, which are two main roles played by nitrite during meat curing. It has been assumed that if bacteria species could be found to perform both functions, or if species that perform a single function individually were mixed together as a mixed starter culture, the implications would be interesting and beneficial for meat manufacturing.

The objective of this study was to assess the ability of LAB and *S. xylosus* to convert $MbFe^{III}$ in a model system and to inhibit spoilage bacteria in raw meat batters without nitrite addition by culture-dependent and culture-independent methods.

2. Materials and methods

2.1. Bacteria species and culture media

Lactobacillus curvatus, *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *S. xylosus* were isolated from Harbin dry sausage and stored at the Northeast Agricultural University. *Lactobacillus sakei* and *L. fermentum* were obtained from the China General Microbiological Culture Collection Center (Beijing, China). De Mann-Rogosa-Sharp (MRS) broth was used to cultivate the six species. The glucose content was adjusted to 2.0 g/L in model systems to prevent Mb from denaturing and precipitating (Arihara et al., 1993). MRS broth without $MnSO_4$ was prepared when subjected to electron spin resonance (ESR) spectroscopy. All reagents were purchased from Hopebio Co., Ltd (Qingdao, China).

2.2. $MbFe^{III}$ conversion in model system

A 20-mg/mL $MbFe^{III}$ solution was prepared as described by Arihara et al. (1993). The solution was sterilised by filtration (0.22-μm pore size; Pall, USA) and then added to the MRS broth to yield a final $MbFe^{III}$ concentration of approximately 2.0 mg/mL to formulate a model system. Bacteria cultures (40 μL) in the exponential growth phase were inoculated into 2 mL of MRS broth containing $MbFe^{III}$. After 18 h of anaerobic cultivation at 37 °C, the media culture was centrifuged to remove the bacterial cells and then the colour, visible absorption and ESR spectroscopy of the supernatant were determined.

2.3. Visible absorption spectroscopy

Visible absorption spectra were measured according to the method of Møller et al. (2003) with modified absorption scans obtained at wavelengths ranging from 450 to 700 nm in 1-nm intervals using a UV–Vis spectrophotometer (UV-6000PC, Shanghai Metash Instruments Co., Ltd., China).

2.4. Electron spin resonance (ESR) spectroscopy

Each MRS broth (300 μL) with bacterial cells removed was submitted directly to ESR spectroscopy using the method described by Gøtterup et al. (2007).

2.5. Preparation of raw meat batters

Pigs were slaughtered at the slaughtering plant of the Heilongjiang Beidahuang Meat Processing Co., in compliance with ethical guidelines for animal care published by the Ministry of Science and Technology of the People's Republic of China in 1988. Raw meat obtained within 12 h of slaughter was trimmed of fat and connective tissue and then passed through a meat chopper in a cold room (4 °C). Eight groups of different meat batters were prepared, including the control (C), the nitrite treatment (N) and six treatments each inoculated with one of the bacteria species (Table 1). According to our preliminary experiments, LAB species and *S. xylosus* were inoculated at levels of 10^7 and 10^6 CFU/g meat, respectively. Glucose was added to provide a carbon source to samples. Colour formation assay was done as described by Li, Kong, Chen, Zheng, & Liu (2013). The meat samples for microbiological analysis and DNA extraction were placed in polypropylene trays, wrapped with an oxygen-permeable polyvinyl chloride film (Weiguang Plastic Co., Ltd., Jiangsu, China) and stored at 4 °C for 12 d.

2.6. Colour measurement

Colour difference was determined using a ZE-6000 colourimeter (Nippon Denshoku, Kogyo Co., Tokyo, Japan), using D65 as the standard illuminant. It was calibrated using a white standard plate ($L^* = 95.26$, $a^* = -0.89$, $b^* = 1.18$). For the samples in the broth model systems, after the bacteria cells were removed from the culture media by centrifugation at 10,000 × *g* for 5 min at 4 °C, the colour of the supernatant was measured in the transmittance mode using a quartz cuvette with a 2-mm path length. For meat batters, the colour difference was analysed using the reflectance mode with a 3-cm port sample-plate.

2.7. Microbiological analysis

Microbiological analysis of meat batters was conducted at day 0, 3, 6, 9 and 12. Total aerobic count, LAB count and *Staphylococcus* count were determined. Each sample (25 g) was homogenised in 225 mL of sterile peptone saline and shaken at 200 rpm for 10 min with a stomacher. Further decimal dilutions were made, and the following analyses were carried out: total aerobic bacterial counts on Plate Count Agar (PCA, Hopebio Co., Ltd) incubated at 37 °C for 48 h; LAB on MRS agar incubated anaerobically at 30 °C for 48 h; *Staphylococcus* spp. on Mannitol Salt Agar (MSA, Hopebio Co., Ltd) incubated at 30 °C for 48 h. Colonies developing on plates were counted.

2.8. DNA extraction

Total bacterial pellets were obtained directly from eight meat batter samples at day 12 as described by Han et al. (2011). DNA was extracted using the TIANamp Bacteria DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China). The DNA was then eluted with Tris-EDTA (TE) buffer and stored at −20 °C for subsequent polymerase chain reaction (PCR).

2.9. PCR

Primers U968f-GC (CGCCCGGGGCGCGCCCCGGGCGGGGCGG GGGCACGGGGGGAACGCGAAGAACCTTAC) containing a GC clamp and L1401r (GCGTGTGTACAAGACCC) were used to amplify the V6–V8 regions of the bacterial 16S rRNA gene (Lu, Ji, Wang, Li, Li, & Xu, 2015). The PCR reaction system (50 μL) included 0.5 μL of Taq polymerase (1.25 U), 0.5 μL of primers U968f-GC and L1401r (10 μM), 2.5 μL of DNA extraction solution (approximately 1 μg),

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