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Effects of *Salmonella* bacteriophage, nisin and potassium sorbate and their combination on safety and shelf life of fresh chilled pork

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ABSTRACT

Salmonella is an important foodborne pathogen and a serious threat to human health worldwide. This study was to reduce *Salmonella* and the spoilage bacteria on fresh chilled pork using bacteriophage, nisin, and potassium sorbate (PS) along with their combinations. Microbial, chemical, and sensory qualities of the fresh chilled pork (artificially contaminated with *Salmonella* 3 log CFU/g) treated with bacteriophage (9 log PFU/g), nisin (5000 IU/g), PS (2 mg/g) and their combinations were evaluated. The result showed that all the samples treated with phage could significantly ($P < 0.05$) reduce *Salmonella* population on fresh chilled pork. The combination treatment of nisin, PS and phage (N-PS-P) could significantly lower total viable counts (TVC), TVB-N and TBARS of the chilled pork during the storage period. The TVC of sample treated by N-PS-P was reduced by 2.3 log CFU/g at 7th day. It was also found through the electronic nose detection that the N-PS-P treatment was able to significantly reduce odour and maintain good sensory of the chilled pork. Hence, the N-PS-P treatment extended the shelf life of fresh chilled pork up to 14 days. No adverse effect of the phage on the chilled pork was observed. In conclusion, this study suggests that the phage and its combination with nisin and PS have great potential to be used as a good preservative for fresh chilled pork.

1. Introduction

According to the National Bureau of Statistics of China (NBSC, 2016), the production of pork in China was 54,870,000 tonnes in 2015, and the consumption of chilled pork has been increasing rapidly in China for its better taste and nutrition (Wang et al., 2015). However, the spoilage microorganisms and foodborne pathogens contaminated on meat shorten the shelf-life of chilled pork and often cause economic loss (Malakar, 2013; Tang et al., 2013; Wang et al., 2015). The problems associated with cross-contamination and the resistance of pathogens to antibiotics during processing, storage and distribution, are currently one of the highest meat safety risks (Domenech et al., 2015; Li et al., 2016; Majowicz et al., 2010; Newell et al., 2010). *Salmonella* is one of the most common food pathogens that contaminated pork products (Rajic et al., 2007; Yang et al., 2016a). Thus, contaminated pork products become a major source of human *Salmonella* infections in many countries (EFSA, 2011; Scallan et al., 2011). The risk of *Salmonella* infection and illness increased with the elevated amount of consumed pork products (Møller et al., 2013; Teunis et al., 2010).

Constant efforts have been made to reduce *Salmonella* population on pork from farm to consumption (Albino, Rostagno, Húngaro, & MendoncA, 2014; Hooton, Atterbury, & Connerton, 2011; Smid, Heres, Havelaar, & Pielaat, 2012). Bacteriophage (phage) is bacterial virus that has great potential for use as biocontrol agent in foods. Previous studies have demonstrated that phages can be used to successfully reduce *Salmonella* spp. in foods, especially meat and poultry products (Bigwood, Hudson, Billington, Carey-Smith, & Heinemann, 2008; Guenther, Herzig, Fieseler, Klumpp, & Loessner, 2012; Hooton et al., 2011; Hungaro, Mendonça, Gouvêa, Vanetti, & Pinto, 2013). However, the narrow antimicrobial spectrum of phage limits its application for extending the shelf life of meat. One method of extending the shelf life of fresh meat is to use natural antimicrobials such as nisin on meat. Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. Lactis, is the first antimicrobial peptide with a “generally recognized as safe” status in the United States Food & Drug Administration (FDA, 2009), but it only could inhibit gram-positive bacteria and their spores. In addition, potassium sorbate (PS) is the potassium salt of sorbic acid and primarily used

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as a food preservative. The antimicrobial activity of PS is generally used to inhibit gram-negative bacteria or molds and yeasts. PS is effective for its applications on many foods, such as meats, drinks, and chees (FDA, 2009). To date, a few publications have revealed increased effectiveness of combinative natural antimicrobial against both Gram-positive and Gram-negative bacteria and/or pathogens on meat products (Branen & Davidson, 2004; Gill and Holley, 2003). The *Salmonella* lytic phage preparation (SalmoFresh™) in combination with LAE or CPC significantly reduced (0.5–1.3 log CFU/g) *Salmonella* counts on chicken breast fillets (Sukumaran, Nannapaneni, Kiess, & Sharma, 2015). The *Listeria monocytogenes* lytic phage preparation (P100) had been tested individually and in combination with LAE and other chemical antimicrobials (Soni, Desai, Oladunjoye, Skrobot, & Nannapaneni, 2012).

However, the combined application of phage, nisin and PS to reduce *Salmonella* population on fresh chilled pork has remained elusive. This study is undertaken to investigate the possible effect of using phage, nisin and PS individually or their combination to inhibit *Salmonella* and total viable counts and assess the efficacy of phage on fresh chilled pork.

2. Material and methods

2.1. Salmonella strain and isolation

Salmonella Typhimurium (CMCC 50115) was obtained from the National Center for Medical Culture Collections (Beijing, China). The strain was reactivated from a glycerol stock culture, in vials containing 30% (v/v) glycerol in Luria-Bertani (LB) borth kept at −70 °C. The isolate was inoculated into 50 mL LB and incubated for 24 h at 37 °C with constant and gentle shaking (180 r/min) to obtain ~10^{8-9} CFU/mL concentration of cells prior to experiment.

2.2. Preparation of antimicrobial solutions

The lytic *Salmonella* phage fmb-p1 was isolated from sewage and kept by our lab in 2014. The phage fmb-p1 could lyse seven serotypes of *Salmonella* such as *S.* Typhimurium, *S.* Enteritidis, *S.* Anatum, *S.* Miami, *S.* Agona, *S.* Saintpaul and *S.* Paratyphi-C. It was stable under different temperature (40–75 °C), pH (4–10) and NaCl solutions (1–11%). High titer stocks of phage were prepared by adding 100 μL of phage (~10^{8-9} PFU/mL) and 100 μL of *Salmonella* overnight culture (~10^{7-9} CFU/mL) to 100 mL of LB broth. The LB broth mixture was incubated for 12 h at 37 °C to allow amplification of the phage. The culture was centrifuged at 10,000 × *g* for 10 min and filtered through 0.22 μm filters (Agela, USA), and the filtrate was stored at 4 °C until further use. The phage concentration was determined to be 10^{11} PFU/mL by soft agar overlay technique (Soni & Nannapaneni, 2010). The phage was diluted by SM buffer (10 mM NaCl, 10 mM MgSO4, 50 mM Tris•HCl, pH 7.5) to certain concentration before use.

A stock solution of nisin (activity of 1 × 10^6 IU/g according to the manufacturer, Sigma) was prepared by dissolving 5 g product in 100 mL volumetric flask with 0.02 mol/L HCl solution and sterilized by filtration through 0.22 μm filters and stored at 4 °C before use. The lower concentrations of nisin solutions were prepared by diluting with HCl solution (0.02 mol/L) from the stock solution. The stock solution of potassium sorbate (98%, aladdin) was prepared by dissolving 10.2 g product with sterile ddH_2O in 100 mL volumetric flask and sterilized by filtration through 0.22 μm filters. The lower concentrations of PS solutions were prepared with sterile ddH_2O from the stock solution. All antimicrobial solutions were freshly prepared before use.

2.3. Preparation of fresh chilled pork

Fresh chilled pork was purchased from a local grocery store and then was aseptically cut into pieces (4 cm × 4 cm) of approximately 10 g. The meat samples were stored at 4 °C for 1 h, then inoculated with 400 μL of *S.* Typhimurium culture (to achieve the final inoculum level in meat ~ 3 log CFU/g) and kept under biosafety cabinet (room temperature) for 30 min for proper bacterial attachment. Each sample was then surface treated (treatment solutions were uniformly applied over the surface of samples using micropipette) with the assigned phage, antimicrobial, or their combinations (10 log PFU/mL phage, 5% PS, 5% nisin) as shown in Table 1. The combined antimicrobial treatments followed this turns: phage, PS, nisin. Control samples were surface treated with 400 μL of sterile distilled water. All the samples were stored at 4 °C for 21 days and taken at 0, 1, 4, 7, 14 and 21 days for microbiological, chemical and sensory evaluation.

2.4. Determination of Salmonella count, total viable counts and phage titer on fresh chilled pork

Salmonella count, Total viable counts (TVC, CFU/g) and phage concentrations (PFU/g) were determined immediately after 1, 4, 7, 14 and 21 days of incubation. The *Salmonella* count was determined as described by Sukumaran et al. (2015) with a few modifications. 10 g samples were homogenized in 90 mL of saline using a stomacher blender (Luqiao, China). To avoid phages from being plated, 10 mL homogenate was centrifuged at 10,000 × g for 5 min, supernatant was discarded and pellets were resuspended in 10 mL of sterile saline. 250 μL of the homogenate was plated on to four XLT4 plates, and incubated at 37 °C for 24 h. For quantitative determination of TVC, decimal dilutions of aliquots (100 μL) of the homogenates were directly placed on 90 mm Plate Count Agar plates, and incubated at 37 °C for 48 h. Infective phage particles recovered from the samples were enumerated as described earlier (Carlton, Noordman, Biswas, Meester, & Loessner, 2005) with some modifications. Aliquots of 100 μL of decimal dilutions from the food samples were mixed with 100 μL host cells and 5 mL LB soft agar. The suspension was poured on solid agar plates and incubated at 37 °C for 12 h until plaques could be counted. After incubation, *Salmonella* count, TVC or plaques were counted and results were reported as log CFU/g. The tests were performed in triplicate.

2.5. Determination of total volatile basic nitrogen (TVB-N)

TVB-N content in pork was measured by a stream distillation method according to Chinese standard GB/T 5009.44 (2003). Briefly, all samples for analysis were ground individually using a Panting homogeneous bag (Luqiao, China). 10 ± 0.1 g of the ground pork was treated with 100 mL distilled water for 30 min and shook the beaker every 10 min. After filtration, 5 mL of filtrate and 5 mL of 10 g/L Magnesia (MgO) were added into a Kjeldahl distillation unit (ZLQ03, East China Glass, China). Steam distillation was distilled for 5 min. The distillate was absorbed by 10 mL of 20 g/L boric acid, and then titrated with 0.01 mol/L HCl. The amount of TVB-N was calculated using the following equation. The result stated for each sample is the mean value of three measurements:

$$TVB-N(mg/100gmeat) = \frac{(V_1 - V_2) \times c \times 14}{m \times 5/100} \times 100$$

Where V_1 is the titration volume for the tested sample (mL), V_2 is the titration volume of blank (mL), and c is the actual concentration of HCl (mol/L), m is the weight of ground pork sample (g).