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Effects of nisin, EDTA and salts of organic acids on *Listeria monocytogenes*, *Salmonella* and native microflora on fresh vacuum packaged shrimps stored at 4 °C

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ABSTRACT

Nisin (500 IU ml^{-1}), EDTA (0.02 M), potassium sorbate (PS) (3%, w/v), sodium benzoate (SB) (3%, w/v) or sodium diacetate (SD) (3%, w/v); alone or in combination were used to dip uninoculated shrimps and shrimps inoculated with *Listeria monocytogenes* or *Salmonella* (~4.0–5.0 log CFU g^{-1}). Shrimps were then drip-dried, vacuum packaged and stored at 4 °C for 7 days. Untreated shrimps were used as a control. Numbers of *L. monocytogenes*, *Salmonella* and native background microflora were determined on uninoculated and inoculated shrimps on days 0, 3 and 7. Nisin–EDTA–PS and nisin–EDTA–SD significantly reduced ($p < 0.05$) *L. monocytogenes* numbers by 1.07–1.27 and 1.32–1.36 log CFU g^{-1}, respectively, on day 0 and 3. However, all treatments failed to significantly reduce ($p > 0.05$) *Salmonella* counts on shrimps throughout storage. On day 7, numbers of aerobic bacteria, psychrotrophic bacteria and *Pseudomonas* on combined nisin–EDTA–salt of organic acids treated shrimps were significantly lower ($p < 0.05$) by 4.40–4.60, 3.50–4.01, and 3.84–3.99 log CFU g^{-1} respectively, as compared to the control. Dipping in organic acids solutions followed by vacuum packaging and chilled storage can help reduce *L. monocytogenes* and native microflora, but not *Salmonella*, on fresh shrimps.

1. Introduction

Shrimps represent a favourable environment for microbial growth and are therefore highly susceptible to spoilage and harbouring high levels of pathogenic bacteria. Spoilage and the presence of pathogens, such as *Listeria monocytogenes* and *Salmonella*, are among the most common reasons for shrimp product detentions and recalls (Ababouch et al., 2005). Furthermore, cases of both listeriosis and salmonellosis have been associated with the consumption of shrimps (NACMCF, 2008). For these reasons there is an interest in reducing surface microbial contamination of shrimps with a particular focus on reducing levels of pathogens.

Nisin and salts of organic acids used in dip treatments may potentially inhibit pathogens and extend the shelf life of shrimps (Al-Dagal and Bazaraa, 1999; Luo and Pan, 2004). Nisin is a bacteriocin produced by *Lactococcus lactis* and has been used as a food preservative since the 1940s (Mattick and Hirsch, 1947). It has been well studied and is effective not only against a wide range of Gram-positive bacteria, including strains of *L. monocytogenes* and spore-formers (Delves-Broughton et al., 1996), but also towards Gram-negative bacteria in combination with food grade chelators such as ethylenediamine tetraacetic acid (EDTA) (Delves-Broughton, 1993). Nisin is currently approved as a food preservative in over 50 countries (Surekha and Reddy, 2000). The salts of organic acids such as potassium sorbate (PS), sodium benzoate (SB) and sodium diacetate (SD) also act as antimicrobials in food (Thomas, 2000; Food and Drug Administration (FDA), 2009). They are commercially available, inexpensive and also widely approved as food additives (Surekha and Reddy, 2000).

The application of nisin and/or salts of organic acids to increase shelf life and reduce pathogens in dairy products, processed and ready-to-eat food has been extensively studied for decades (Delves-Broughton et al., 1996). Studies on fresh food have increased recently due to consumer demands for fresh and lightly preserved food. For example, research has demonstrated the ability of nisin and/or salts of organic acids to extend shelf life of and/or inhibit *L. monocytogenes* or *Salmonella* on fresh chicken (González-Fanzos and Dominguez, 2007; Economou et al., 2009), beef (Avery and

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Buncic, 1997; Zhang and Mustapha, 1999; Ariyapitipun et al., 2000; Lim and Mustapha, 2004), buffalo meat (Pawar et al., 2000), cabbage, broccoli, bean sprout (Bari et al., 2005) and cantaloupe (Ukuku and Fett, 2004). The application of nisin and/or salts of organic acids to control bacteria, and in particular pathogens, on fresh seafood is relatively limited. A combination of nisin and PS has been shown to maintain the quality of minced shrimps (Luo and Pan, 2004), while PS alone has been shown to prolong the shelf life of fresh gutted pearl-spot (*Etroplus suratensis*, Bloch) (Manju et al., 2008), *Lethrinus lutjan* fillets (Shalini et al., 2001) and whole peeled shrimps (Al-Dagal and Bazaraa, 1999).

This study was undertaken to investigate the effectiveness of using nisin, EDTA and salts of organic acids alone or in combination as an aqueous dipping solution in reducing the surface numbers of *L. monocytogenes*, *Salmonella* and native microflora on vacuum-packed raw (headless and peeled) shrimps stored at 4 °C.

2. Materials and methods

2.1. Bacterial strains

L. monocytogenes Scott A (4b) and *L. monocytogenes* V7 (1/2a) were obtained from the Department of Health and Human Services, Food and Drug Administration, Cincinnati, Ohio, USA. *Salmonella* Typhimurium ATCC 14028 was obtained from the American Type Culture Collection (Manassas, USA) and *S.* Senftenberg 1734b was supplied by Dr. Narelle Fegan, CSIRO Food and Nutritional Sciences (Brisbane, Australia). For long term maintenance the cultures were stored in 20% glycerol at −80 °C. All bacterial growth media were supplied by Oxoid (Basingstoke, UK) and prepared according to the manufacturer's instructions. Working cultures were kept on Trypticase Soy Agar (TSA) slants at 4 °C and subcultured every 2 weeks on the same agar.

2.2. Preparations of antimicrobial solutions

A commercial preparation of nisin (2.5% nisin in denatured milk solids and salt, Sigma Aldrich St. Louis, Mo.) was used in the present work. A nisin stock was prepared by dissolving 0.5 g of nisin in 10 ml of 0.02 N HCl, centrifuging at 5000×*g* for 15 min to separate the insoluble whey proteins and sterilizing by filtration through 0.22 μm filters (Millipore, Bedford, MA) before storing at 4 °C. For experiments, the stock solution was diluted with sterilized 0.02 N HCl to obtain a nisin working solution of 500 IU/ml (pH 1.98). A stock solution of 2 M EDTA (Amresco, Ohio, US) solution was prepared in double distilled water (ddH_2O) and the final pH was adjusted to 7.0 before autoclaving for 15 min at 121 °C. Three concentrations of PS (Sigma Aldrich, Steinheim, Germany), SB (Sigma Aldrich) and SD (Sigma Aldrich) solutions were prepared in ddH_20 and autoclaved for 15 min at 121 °C. All antimicrobial solutions were freshly prepared before use. Final concentrations of the antimicrobials used alone or in combinations were 0.02 N HCl, 500 IU ml^{-1} nisin, 0.02 M EDTA, 3.0% PS, 3.0% SB and 3.0% SD.

2.3. Preparation and inoculation of shrimps

Fresh shrimps (*Penaeus monodon*) were obtained from a retail fish market in Brisbane one day before treatment. They were transported on ice to the laboratory and processed immediately. At the laboratory the shrimps were washed with sterile distilled water (dH_2O), beheaded, peeled, washed with tap water, rinsed with sterile dH_20 and weighed. The mean weight of the shrimps was 14.3 g. Large (>16.0 g) and small (<11.0 g) shrimps were not used in the study in order to minimize the effects of size on the results. Shrimps were divided into three groups; two groups were inoculated with *Listeria* and *Salmonella* and the third was uninoculated and acted as a control. Shrimps were then stored at −4 °C until use the next day.

The bacterial strains *L. monocytogenes* Scott A, *L. monocytogenes* V7, *S.* Typhimurium ATCC 14028 and *S.* Senftenberg 1734b were grown separately in 10 ml Trypticase Soy Broth (TSB) at 37 °C for 20 h to give ~9.0 log CFU ml^{-1}. Cells were harvested by centrifugation at 9000×*g* for 10 min at 4 °C. The supernatant was decanted and the pellet resuspended in sterile 0.85% saline by vortexing and the cells were washed twice in the same solution. A 500 μl volume of each *L. monocytogenes* or *Salmonella* culture was added to 500 ml of 0.85% saline solution to provide a 2-strain mixture (~6.0 log CFU ml^{-1}) resulting in a concentration of ~4.0–5.0 log CFU g^{-1} of shrimp immediately after inoculation.

For the two groups of shrimps that will be inoculated with *Listeria* and *Salmonella*, each side of the shrimps were treated with UV light in a biosafety cabinet for 15 min to reduce competition with native microflora. To be able to determine the native microflora, another group was not treated with UV. The first two groups then was inoculated with a 2-strain mixture of *L. monocytogenes*, the second with a 2-strain mixture of *Salmonella*. The third group remained uninoculated in order to determine the growth of natural microflora during storage. Inoculation of shrimps was achieved by dipping them for 5 min at 25 °C in the bacterial suspension with agitation by a glass rod to ensure an even distribution of the organisms. After inoculation the shrimps were removed and air dried for 15 min in a biosafety cabinet to allow bacterial attachment.

2.4. Treatment of shrimps

Each set of inoculated and uninoculated shrimps was further separated into 10 groups. One group was left untreated (control) while each of the other 9 groups were dipped in 150 ml of one of the nine antimicrobial solutions for 10 min at 25 °C and drip-dried for 15 min. Shrimps were placed in vacuum bags (Micris Pty. Ltd, Queensland, Australia) that had an oxygen transmission rate of 99.6 ml m^{-2} 24 h^{-1} 1^{-1} atm at 23 °C, a water vapour transmission rate of 12.6 g m^{-2} 24 h^{-1} at 38 °C and 90% relative humidity. The bags were vacuum sealed (Multivac, Wolfertschwenden, Germany) at 80 mm Hg, and stored at 4 °C for up to 7 days. Shrimp samples were analysed microbiologically and for pH immediately after inoculation (day 0) and on days 3 and 7 of storage.

2.5. Microbial enumeration

Inoculated and treated shrimps samples were transferred into individual sterile stomacher bag and 0.85% saline was added to give a 1:10 (w/v) dilution before pummelling for 60 s in a stomacher (Seward 400, United Kingdom). Serial dilutions of the resultant bacterial suspensions were prepared with 0.85% saline and 0.1 ml of dilutions was inoculated in duplicate onto appropriate media using the spread plate technique. Specifically, dilutions from the *L. monocytogenes* inoculated samples were plated on PALCAM agar and incubated at 37 °C for 48 h. Dilutions from the *Salmonella* inoculated samples were plated on Xylose Lysine Deoxycholate (XLD) agar and incubated at 37 °C for 24 h. Selected colonies from countable PALCAM and XLD plates were streaked onto TSA for purity and confirmation tests appropriate to the species were performed including: Gram stain, oxidase test, umbrella motility in SIM medium, carbohydrate fermentation pattern and hydrogen sulphide production in TSI (Triple Sugar Iron Agar). For the uninoculated shrimps, the following groups of native microflora were enumerated: (i) Aerobic bacteria on Plate Count Agar (PCA) after incubation at 30 °C for 2 days; (ii) Enterobacteriaceae on Violet Red