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PEF based hurdle strategy to control *Pichia fermentans, Listeria innocua* and *Escherichia coli* k12 in orange juice

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ABSTRACT

The combination of pulsed electric fields (PEF) and bacteriocins in a hurdle approach has been reported to enhance microbial inactivation. This study investigates the preservation of orange juice using PEF in combination with nisin (2.5 ppm), natamycin (10 ppm), benzoic acid (BA; 100 ppm), or lactic acid, (LA; 500 ppm). *Pichia fermentans,* a spoilage yeast frequently isolated from orange juice, *Escherichia coli* k12 or *Listeria innocua* were inoculated into sterile orange juice (OJ) with, and without, added preservatives. The antimicrobial activity over time was evaluated relative to an untreated control. The effect of PEF treatment (40 kV/cm, 100 μs; max temperature 56 °C) was assessed on its own, and in combination with each antimicrobial.
The acidic environment of OJ inactivated *E. coli* k12 (1.5 log reduction) and *L. innocua* (0.7 log reduction) slightly but had no effect on *P. fermentans*. PEF caused a significant decrease ($P<0.05$) in the viability of *P. fermentans, L. innocua* and *E. coli* k12 achieving reductions of 4.8, 3.7 and 6.3 log respectively. Nisin combined with PEF inactivated *L. innocua* and *E. coli* k12 in a synergistic manner resulting in a total reduction to 5.6 and 7.9 log respectively. A similar synergy was shown between LA and PEF in the inactivation of *L. innocua* and *P. fermentans* (6.1 and 7.8 log reduction), but not *E. coli* k12. The BA–PEF combination caused an additive inactivation of *P. fermentans*, whereas the natamycin–PEF combination against *P. fermentans* was not significantly different to the effect caused by PEF alone. This study shows that combining PEF with the chosen preservatives, at levels lower than those in current use, can provide greater than 5 log reductions of *E. coli* k12, *L. innocua* and *P. fermentans* in OJ. These PEF–bio–preservative combination hurdles could provide the beverage industry with effective non-thermal alternatives to prevent microbial spoilage, and improve the safety of fruit juice.

1. Introduction

Fruit juice, has been linked with a number of incidences of food borne disease between 1995 and 2005 (Vojdani et al., 2008). While enterotoxigenic *Escherichia coli* O157:H7 has been most often linked to unpasteurized apple cider, orange juice has been specifically implicated as a transmission vehicle in many reported *Salmonella* and Hepatitis A virus outbreaks over several decades (Parish, 1997; Cook et al., 1998; Mead et al., 1999). The initial microbial levels in the fresh juice can vary from 1.3 to 5.3 log cfu/ml (Fellers, 1988; Pao et al., 1998; Parish, 1997), the typical microbiota comprising acidolactic bacteria, molds, and yeasts. Species from the genus *Rhodotorula*, *Pichia*, *Hanseniaspora*, and *Metschnikowia* are commonly found in citrus juices (Arias et al., 2002). In the USA, the current requirement of the Food and Drug Administration (FDA) is a 5-log reduction of "pertinent" pathogen numbers in processed juice (USFDA, 2002).

Thermal processing is widely used to inactivate microorganisms, as well as enzymes, in orange juice but, while satisfying the FDA requirements, this method can impair the sensory and nutritional qualities (Moshonas and Shaw 1989; Parish, 1997). Modern consumers demand orange juice that is safe and has a good shelf life, but is also fresh in terms of its sensory characteristics. In a bid to satisfy these demands, manufacturers have shown increased interest in novel non-thermal technologies such as irradiation, pulsed electric fields (PEF) and ultra high pressure treatments, often in conjunction with other hurdles, to improve the shelf-life nutritional value and flavour (Manas and Pagan, 2005; Patil et al., 2009).

PEF is a non-thermal preservation method which applies high voltage electric field (20–50 kV/cm) in short electric pulses (1–10 μs), resulting in microbial inactivation and enzyme activity reduction in the product (Zhang et al., 1995). PEF has the advantage of retaining fresh quality attributes of food (Charles-Rodriguez et al., 2007; Hodgins et al., 2002; Min et al., 2007) and, in comparison with unprocessed juices, has been reported to extend the microbiological shelf-life of fruit juices such as orange (Min et al., 2006), tomato (Aguilo-Aguayo et al., 2008), apple, and cranberry (Raso et al., 1998). The antimicrobial effectiveness of PEF against *Salmonella* spp (Liang et al., 2002a,b), *E. coli* (Evrendilek et al., 1999) and *Listeria monocytogenes* (Mosqueda-Melgar et al., 2007) populations in some fruit juices has been reported. Inactivation by PEF is dependent on multiple factors relating to the process conditions,

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medium, and microbial species (Aronsson et al., 2001; Wouters and Smelt, 1997), which may limit the application of PEF as a sole preservation method. Because PEF inhibits microorganisms by causing temporary or permanent loss of the integrity of the microbial cell membrane, the potential exists to enhance the effects of some antimicrobial compounds due to their increased uptake by the cell (Tsong, 1991).

The hurdle approach, as described by Leistner (1978), is used to produce minimally processed food by applying several sub-lethal treatments to achieve microbial stability, rather than focusing solely on one lethal preservation method. The microbial stability is achieved by combining these hurdles to increase destruction of the microbial cytoplasmic membrane as well as preventing cell repair of survivors from treatments (e.g. PEF), such as sub-lethally injured cells or bacterial endospores (Galvez et al., 2007; Leistner, 2000). Previous studies report that combining PEF with natural antimicrobials such as bacteriocins, antifungal peptides, essential oils, spices and organic acids can enhance its killing effect on microorganisms in fruit juices (Liang et al., 2002b; Mosqueda-Melgar et al., 2008; Nguyen and Mittal, 2007).

The objective of this study was to evaluate the effectiveness of the PEF treatment on spoilage (*Pichia fermentans)* and indicator (*E. coli* k12 and *Listeria innocua)* microorganisms. The inactivation of *P. fermentans* by PEF has not been previously reported. Further investigations to develop a hurdle approach to control these microorganisms in orange juice using PEF in conjunction with the bio-preservatives nisin, natamycin, benzoic acid and lactic acid used at levels consistent with the maintenance of high sensory quality were also carried out.

2. Material and methods

2.1. Microbial culture preparation

Pure cultures of *E. coli* k12 HB101 and *L. innocua* IMD 11288, were routinely grown in Tryptone Soya Broth, (TSB: CM129; Oxoid Ltd. Basingstoke, Hampshire, U.K.) at 37 °C for 24 h. *P. fermentans* CBS was propagated in Universal Medium for Yeasts (YM; code No. 189; DSMZ, GmbH, Braunschweig, Germany) at 37 °C with continuous shaking at 130 rpm for 72 h. Short-term cultures were maintained on agar slants of Tryptone Soya Agar (TSA:CM0131; Oxoid)/YM agar (YM broth + 15% Technical Agar, LP0013) and stored at 4 °C. Long-term microbial stock cultures were prepared in 80% glycerol and stored at −20 °C. Prior to experiments the final microbial load in the growth media was in the range of 10^9–10^{11} cfu/ml and 10^7–10^9 cfu/ml for the bacteria and the yeast respectively.

2.2. PEF processing

The experimental system used in this study, consisting of a peristaltic pump (SR25 S300, Esska GmbH, Hamburg, Germany), a PEF treatment unit and a cooling coil, were as described by Walkling-Ribeiro et al. (2008). The processing conditions used were an electric field strength of 40 kV/cm, and a treatment time of 100 μs (flow rate, 16 ml/min). Maximum outlet temperature reached was 56 °C. Before and after each treatment, the chamber was cleaned with 5% solution of sodium hydroxide, 1% sterilising solution of sodium hypochlorite, potable rinse water and de-ionised sterile water.

2.3. Orange juice preparation

A pulp-free model orange juice (OJ) was prepared by centrifuging 800 ml of commercially available reconstituted OJ at 9000 rpm for 10 min at 4 °C. The supernatant was autoclaved (110 °C for 10 min) and cooled to 4 °C prior to inoculation and subsequent processing. The pH of the OJ was determined using a pH meter (Model SP10P Symphony VWR International, Bristol CT, USA) with a glass combination electrode (VWR International).

2.4. Preparation of antimicrobials

The antimicrobials used for the various microorganisms are summarised in Table 1. Stock solutions of lactic acid (LA), benzoic acid (BA) and nisin were made up in OJ. Stock solutions of natamycin were prepared in distilled water to maintain full activity (Delves-Broughton, 2007). The organic acids were filter-sterilized using Ministart Single Filter 17597 (Sartorius AG 37070, Goettingen Germany), while both nisin and natamycin were sterilized by autoclaving at 110 °C for 10 min to maintain the maximum antimicrobial activity (Delves-Broughton, 2007).

2.5. Determination of antimicrobial activity in OJ

For the determination of antimicrobial activity, fresh microbial cultures, OJ, 1/4 strength Ringers solution (BR00529; Oxoid) and TSA/YM agar were prepared and sterilized. The overnight cultures were harvested by centrifuging at 9000 rpm for 10 min, washing with sterile Ringers solution and re-centrifuging. The cells were re-suspended in 1 ml of sterile OJ which was used to inoculate 30 ml of OJ. A sample was taken to confirm the initial microbial load and the appropriate bio-preservative was aseptically added. Survival curves were constructed by taking samples after 0, 1, 3, 4, 5, 7, 9 and 24 h of incubation at 30 °C and at 4 °C. Serial decimal dilutions were prepared in 1/4 strength Ringers solution and the appropriate dilutions were spread plated and incubated at the appropriate optimum temperature for each microorganism. The effect of the preservatives on the population level was calculated as the increase or decrease in microbial population and expressed as log cfu/ml. Each assay was performed in duplicate.

2.6. The effectiveness of the PEF treatment and the combination treatments

A fresh culture of 1% inoculum was prepared as previously described and an aliquot of this was used to inoculate 1 l of the same medium and the organisms were grown at 30 or 37 °C, with or without aeration, for the appropriate time (Table 1). A pellet was prepared and re-suspended in OJ as previously described. The microbial suspension was added to 800 ml of OJ at 4 °C to give a final concentration of approx 10^8–10^{11} cfu/ml. An appropriate volume of the sterilized bio-preservative was aseptically added to the OJ, approximately 10 min prior to the PEF

Table 1
Summary of the antimicrobial treatments and growth conditions used for each test Microorganism.

Microorganism	Growth requirements	Antimicrobial	Level tested
E. coil K12 Ashtown Food Research Centre, (AFRC), Teagasc	Growth with aeration at 37 °C in, Tryptone Soya Broth (TSB: CM; 030; Oxoid) for 24 h	Nisin (Nisaplin), Danisco A/S DK-7200 Crindsted, Denmark	2.5 ppm
		Lactic acid (27714), Riedel-de haën, Sigma- Aldrich	500 ppm
L. innocua AFRC	Growth with-out aeration at 30 °C in TSB for 24 h	Nisin	2.5 ppm
P. fermentans DSMZ	Growth with aeration at 30 °C in universal medium for yeast (YM: 189, DSMZ) for 72 h	Lactic acid	500 ppm
		Benzoic acid (18101), Riedel-de haën, Sigma- Aldrich	100 ppm
		Nantamycin (Natamax), 104417, Danisco	10 ppm
		Lactic acid	500 ppm