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The effects of orange juice clarification on the physiology of *Escherichia coli*; growth-based and flow cytometric analysis

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ABSTRACT

Orange juice (OJ) is a food product available in various forms which can be processed to a greater or lesser extent. Minimally-processed OJ has a high consumer perception but presents a potential microbiological risk due to acid-tolerant bacteria. Clarification of OJ (such as removal of cloud) is a common processing step in many OJ products. However, many of the antimicrobial components of OJ such as essential oils are present in the cloud fraction. Here, the effect of clarification by filtration on the viability and physiology of *Escherichia coli* K-12 was tested using total viable count (TVC) and flow cytometric (FCM) analysis. The latter technique was also used to monitor intracellular pH during incubation in OJ. Removal of the OJ cloud fraction was shown to have dramatic effects on bacterial viability and physiology during storage at a range of incubation temperatures. For instance, at 4 °C, a significantly lower number of healthy cells and a significantly higher number of injured cells were observed in 0.22 μm-filtered OJ at 24 h post-inoculation, compared to filtered OJ samples containing particles between 0.22 μm and 11 μm in size. Similarly, there was a significant difference between the number of healthy bacteria in the 0.7 μm-filtered OJ and both 0.22 μm-filtered and 1.2 μm-filtered OJ after 24 hour incubation at 22.5 °C. This indicated that OJ cloud between 0.7 μm and 0.22 μm in size might have an adverse effect on the viability of *E. coli* K-12. Furthermore, FCM allowed the rapid analysis of bacterial physiology without the requirement for growth on agar plates, and revealed the extent of the viable but non-culturable (VBNC) population. For example, at 4 °C, while the FCM viable count did not substantially decrease until 48 h, decreases in TVC were observed between 0 and 48 hour incubation, due to a subset of injured bacteria entering the VBNC state, hence being unable to grow on agar plates. This study highlights the application of FCM in monitoring bacterial physiology in foods, and potential effects of OJ clarification on bacterial physiology.

1. Introduction

When compared to conventionally-processed foods (such as pasteurised or UHT foods), many minimally-processed foods have more desirable organoleptic properties and greater nutritional value, and thus are of higher value for consumers (Pasha et al., 2014; Ragaert et al., 2004). However, elimination of harsh processing steps shortens shelf life by failing to eliminate spoilage microorganisms. As such, it is desirable to understand the effects of the composition of minimally processed foods on bacterial physiology, such that the food might be engineered to decrease growth of spoilage microorganisms and thus increase product shelf life.

Orange juice (OJ) is a foodstuff available in a range of forms, from highly processed to minimally-processed. One of the major production stages of OJ is clarification, intended to remove excess seeds, pieces of orange fruit and membrane as well as bitter essential oils such as limonene present in the freshly squeezed OJ (Rutledge, 1996). Depending on the OJ product, up to 12% (*w*/*v*) pulp is added back to the clarified juice before packaging (Berlinet et al., 2007). OJ can also be supplemented with homogenised pulp in order to meet the demand of the consumers for smoother OJ products (Sorenson and Bogue, 2003).

Flow cytometry (FCM) is a rapid technique that can be used to enumerate and determine optical and fluorescent properties of particles (Müller and Nebe-von-Caron, 2010). Although most commonly used for analysis of mammalian cells, it has many applications in microbiology and can be used to determine bacterial viability and physiology. An advantage of FCM over the total viable count (TVC) method is that it does not rely upon microbial growth for analysis, allowing detection of bacteria that are unable to grow on agar plates, the so-called VBNC (viable but non-culturable) phenotype (Oliver, 2005). Bacteria readily enter the VBNC state when exposed to many stresses. FCM has been used in some food processing applications (Comas-Riu and Rius, 2009), including analysis of apple juice (Yamaguchi et al., 2003), but it is often not well-suited to foods containing large numbers of particles of similar size to the bacteria under study. As such, pre-treatment is

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often required to remove particles of similar size to bacteria (such as colloidal fats and proteins in milk; Gunasekera et al., 2000) or additional techniques are required to differentiate bacteria from particles present in the food matrix, such as antibody staining (Clarke and Pinder, 1998) or the use of fluorescently-labelled bacteria. This also allows detection of lower numbers of bacteria using FCM methods.

OJ contains many compounds with antimicrobial activities such as essential oils, primarily limonene (Fernández-Vázquez et al., 2013) and flavanones, primarily hesperidin (Bisignano and Saija, 2002; Di Pasqua et al., 2007; Garg et al., 2001). It has also been shown that the majority of these compounds, especially the limonene and hesperidin, are mainly present in the pulp (defined as particulate matter >2 μm in size; Brat et al., 2003) and cloud (particulates <2 μm in size) of OJ (Ben-Shalom and Pinto, 1999; Brat et al., 2003). As a result, it was hypothesised that OJ clarification and the resultant removal of these compounds could lead to improved survival of microbes in OJ and thereby an alteration of the microbiological risk posed by OJ products of different formulations (Sampedro et al., 2011). The main aims of the current study were to test this hypothesis, and to investigate the utility of FCM as a technique of monitoring physiology of bacteria in orange juice. The effects of OJ filtration on the physiology of *E. coli* K-12 MG1655 (an enteric marker strain; Valdramidis et al., 2007) were determined using TVC, indicating the viability of bacteria as determined by their ability to grow on agar plates, and FCM using the dye propidium iodide (PI) which stains dead *E. coli* (Shi et al., 2007) indicating viability in a non-growth dependent manner. Although having different acid tolerance characteristics to pathogenic *E. coli* strains, *E. coli* K-12 is very well-characterised in terms of stress responses and physiology. FCM was also used to determine membrane potential and intracellular pH (pH_i) of bacteria in OJ. As far as the authors are aware, this is the first study of the effects of OJ clarification on bacterial viability or physiology.

2. Materials and methods

2.1. OJ clarification and filtration

Freshly-squeezed OJ was obtained from a local retailer and centrifuged at 17,696 *g* for 40 min to remove pulp. The supernatant (pulp-free OJ) was then filtered through sterile filter papers with pore sizes of 11 μm, 8 μm, 1.6 μm or 1.2 μm. The 1.2 μm-filtered OJ was also then filtered through sterile 0.7 μm filter paper or 0.22 μm syringe filters.

2.2. Particle size distribution

The size distribution of cloud particles in OJ was measured by laser diffraction using a Malvern Mastersizer 2000 equipped with a Malvern Hydro 2000SM particle size analyser (Malvern, UK). The refractive indices of cloud particles and dispersed phase and the absorption index of cloud particles were set at 1.73, 1.33 and 0.1 respectively as described by Corredig et al. (2001). A mixture of 10 mL of filtered OJ and 100 mL of deionised H_2O was stirred at 2500 rpm as it was passed through the optical cell. In total ten measurements were recorded per sample for particles of between 0.02 μm and 2 mm.

2.3. Bacterial strains and methods

Two *E. coli* K-12 strains were used: MG1655 (F^- λ^- *ilvG*$^-$ *rfb-50 rph-1*); and SCC1 (MG1655 $P_{A1/04/03}$-*gfp*mut3*) which constitutively expresses green fluorescent protein (GFP; Miao et al., 2009). A single colony was picked from an agar plate and used to inoculate 20 mL of 2 × LB (20 g/L tryptone, 10 g/L yeast extract (both Difco) and 10 g/L NaCl (Sigma)), which was grown overnight in a 250 mL Erlenmeyer flask at 37 °C with shaking at 150 rpm. Fifty microliters of the overnight culture was added to 50 mL of fresh 2 × LB medium in a 500 mL Erlenmeyer flask and allowed to grow in the same conditions until it reached mid-exponential phase ($OD_{650} \approx 0.5$), whereupon 3×10^9 *E. coli* cells (estimated by measuring the OD_{650} of the culture, where an OD_{650} of 1 is equivalent to a concentration of 10^9 bacteria/mL) were transferred to 50 mL plastic centrifuge tubes and centrifuged at 3256 *g* for 10 min in a Jouan C4.22 centrifuge (Jouan, Saint-Mazaire, France). The pellet was dispersed in 50 μL of Dulbecco's Phosphate-Buffered Saline (PBS; pH 7.3; Oxoid) by vortexing and the cell suspension added to 15 mL of the OJ sample in a 25 mL universal bottle to achieve a final concentration of 2×10^8 cells/mL. Cells were dispersed in the OJ by vortexing the bottle for 12 s. OJ samples were stored at 4 °C, 22.5 °C or 37 °C without agitation and samples were taken at regular intervals. TVC was determined by serial dilution in maximum recovery diluent (MRD; Sigma), plating onto nutrient agar (Oxoid) plates and incubation at 37 °C for 48 h before enumeration.

2.4. Flow cytometry analysis

Bacteria were analysed using a BD Accuri C6 flow cytometer (BD, Oxford, UK). Samples were stained with PI (Sigma) and Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol ($DiBAC_4(3)$) or BOX; Life Technologies) to determine viability. A 200 μg/mL stock solution of PI was made up in distilled water and added to samples at a final concentration of 4 μg/mL. A 10 mg/mL stock solution of BOX was made up in Dimethyl Sulphoxide (DMSO) and added to samples at a final concentration of 4 μg/mL. EDTA was also added at a final concentration of 400 μM in order to facilitate staining with BOX. Samples were excited using a 488 nm solid state laser. Particulate noise was eliminated using a Forward scatter height (FSC-H) threshold. 20,000 data points were collected at a maximum rate of 2500 events/s. Since *E. coli* and OJ cloud particles have similar scatter properties, the concentration of

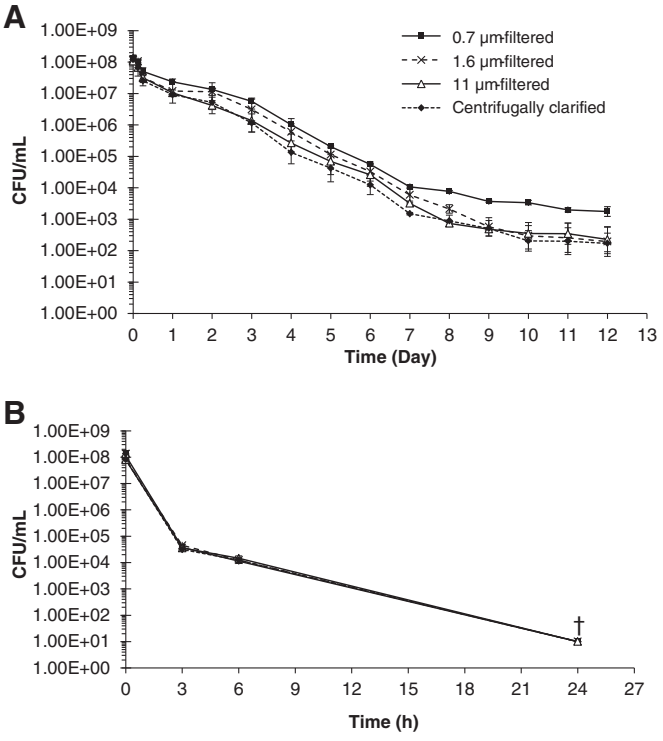

Fig. 1. Total viable counts of *E. coli* K-12 MG1655 incubated in filtered OJ with different cloud contents at (A) 4 °C and (b) 37 °C. 3×10^9 cells of mid-logarithmic phase *E. coli* were added to 15 mL of clarified or filtered OJ. Samples were stored at (A) 4 °C for 12 days or (B) 37 °C for 24 h. The number of culturable cells was determined by serial dilution in maximum recovery diluent (MRD) and plating on nutrient agar plates. Error bars are the standard deviation of the mean value obtained at each time point. The experiment was repeated at least twice in duplicate; data from a representative experiment are shown (n = 2). †At the marked data point, in case of all samples, no colony grew on nutrient agar plates when 100 μL of neat samples were plated (<10 CFU/mL).