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Amir H.P. Anvarian, Madeleine P. Smith, Tim W. Overton

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

Amir HP Anvarian[1†], Madeleine P Smith[1], Tim W Overton[1,2]*

[1]Bioengineering, School of Chemical Engineering, and [2]Institute of Microbiology & Infection, The University of Birmingham, Birmingham B15 2TT

*Corresponding author: t.w.overton@bham.ac.uk

Telephone: +44 121 4145306

† Current address: Teagasc, Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

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 hours 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 hours 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 hours, decreases in TVC were observed between 0-48 hours 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.

Keywords: Orange Juice; Filtration; Flow Cytometry; *E. coli*; Viable but non-culturable

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, 2005).

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 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 hypothesized 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 minutes 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 analyzer (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 deionized H_2O was stirred at 2,500 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 µL 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} ≈ 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 3,256 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 seconds. 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 hours 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 2,500 events/seconds. Since *E. coli* and OJ cloud particles have similar scatter properties, the concentration of cloud particles in filtered OJ was determined (see Supplementary information for full details of methodology). Fluorescence was detected using 533/30 BP and 670 LP filters corresponding to GFP/BOX and PI fluorescence respectively. Data was analysed using CFlow (BD).

Statistical analysis was done using the Student's t-test for independent samples as described in figure legends.

3. Results & Discussion

3.1. Characterization of OJ Cloud Particles

In order to clarify OJ using filtration, the pulp was first removed by centrifugation. Pulp-free OJ was then filtered using filters with different pore sizes, and the particle size distribution of the resultant filtered OJ samples analysed by Mastersizer (Supplementary Fig. 1a). Following filtration, the filter papers were also visually examined (Supplementary Fig 1b). Depending on the filtration regime, three cloud populations of descending size could be observed. First, large particles of greater than 3 µm diameter most likely lightly-coloured large rag fragments, visible on 8 µm and 11 µm filters and giving rise to little colour change (Mizrahi and Berk, 1970). Second, small to medium size particles of between 0.7 µm and 3 µm, most likely the carotenoid-containing chromoplastids and small rag fragments; these bright orange particles were visible on the 1.6 µm and 1.2 µm filters (Supplementary Fig. 1b). Third, small particles (< 0.7 µm) which were assumed to be mainly needle-like crystals of hesperidin. Filtration of the 1.2 µm filtered OJ with a 0.7 µm filter paper resulted in the retention of fewer chromoplastids as well as a white layer on the surface of the filter, most likely crystals of hesperidin. The volume median diameter (D_{50}) of particles in filtered OJ decreased with decreasing pore size down to 1.2 µm (Supplementary Fig. 1a); however, D_{50} increased for 0.7 µm-filtered samples, probably due to the tendency of the laser diffraction technique for overestimating the size of non-spherical needle-like hesperidin particles.

It was hypothesized that autoclaving filtered OJ samples containing particles of larger than 0.7 µm (proteinous rag fragments, fat globules and chromoplastids) would result in the degradation of these

components, hence generating dark browning products; this was indeed the case (Supplementary Fig. 1c). After autoclaving, OJ filtered with a 0.7 µm filter was bright orange. There was also a large amount of white precipitate at the bottom of the test tube typical of hesperidin crystals (Ben-Shalom and Pinto, 1999). There was a smaller quantity of precipitate in autoclaved 0.22 µm-filtered OJ.

3.2 The Effects of OJ Cloud on TVC of E. coli at 4 °C and 37 °C

The role of different size cloud components on the TVC of *E. coli* MG1655 was determined. 3×10^9 *E. coli* MG1655 cells were added to 15 mL of OJ that had been either centrifugally clarified or centrifugally clarified then filtered through 0.7 µm, 1.6 µm or 11 µm filters and incubated at 4 °C or 37 °C. At 4 °C the presence of OJ cloud particles greater than 0.7 µm in size had an adverse effect on the TVC of *E. coli* MG1655 (Fig. 1a). TVC were similar in centrifugally-clarified, 11 µm- and 1.6 µm-filtered OJ. These data suggest that cloud particles between 1.6 µm and 0.7 µm in size have the greatest negative effect on *E. coli* TVC at 4 °C; these are thought to include hesperidin crystals. At 37 °C (Fig. 2b), removal of the cloud particles did not affect the TVC of *E. coli*, most likely because at higher temperatures, *E. coli* is far more sensitive to the acidic conditions in OJ. It should be noted that the exponential growth phase of the *E. coli K-12* used in these experiments have heightened acid sensitivity; stationary-phase *E. coli* tend to be more acid-resistant (Chung *et al*., 2006).

As clarified OJ was amenable to analysis using FCM due to the removal of large particulates, this technique was used to interrogate bacterial viability in a non-growth based manner and physiology in more detail.

3.3. Use of FCM to determine effects of OJ Cloud on physiology of E. coli K-12 MG1655 at 4 °C

The effect of cloud particles of different sizes on the physiology of *E. coli* K-12 MG1655 was investigated using FCM with the green dye Bis-oxonol (BOX), which only enters cells with a collapsed membrane potential, signifying injury, and Propidium Iodide (PI), a red DNA dye that can only enter cells with compromised membranes, thus signifying dead cells. Due to rapid death observed at 37 °C, this experiment was performed at 4 °C to mimic refrigerated storage of OJ (Fig. 2).

Numbers of healthy bacteria (stained with neither PI nor BOX) as detected using FCM decreased up to 4.5 hours but then increased in most samples. Large numbers (~ 10^8 bacteria/mL) of injured bacteria (stained with BOX, signifying collapse of membrane potential, but not stained with PI) were present between 1.5 and 24 hours incubation in OJ; a relatively small dead population (~ 10^7 bacteria/mL; stained with PI) increased to around 10^8 bacteria/mL at 48 hours. These dynamics reflect the initial response of *E. coli* to the harsh conditions in OJ; initial injury and loss of membrane potential, followed by recovery of a subpopulation of bacteria after 24 hours and death of a second subpopulation after 48 h. These rapid changes during early stages of incubation in OJ make FCM an ideal technique to monitor physiology. The total number of bacteria in each OJ sample did not vary by more than 1% during incubation.

Comparing the numbers of viable bacteria determined using FCM (defined as those not stained with PI; Fig. 2d) and TVC data (Fig. 1a) reveals that although cell viability as measured by FCM did not substantially decrease until 48 hours, decreases in TVC were observed between 0-48 hours incubation. This is caused by a subset of injured (PI^- BOX^+) bacteria being unable to grow on agar plates, the VBNC (Viable But Non-Culturable) phenotype often encountered in bacteria exposed to stress (Müller and Nebe-von-Caron, 2010).

Differences between bacteria incubated in OJ filtered with different filter papers were less marked than when analysed using TVC. Bacteria incubated in 0.22 µm-filtered OJ showed a significantly

lower number of healthy cells (PI^- BOX^-) and a significantly higher number of injured cells (PI^- BOX^+) after 24 hours than other OJ samples; likewise, the number of dead bacteria (PI^+) after 24 hours was significantly higher in 8 µm- and 11 µm-filtered OJ than in other samples. However, these differences were not reflected in samples taken after 48 hours incubation and so probably represent transient changes in physiology during incubation.

3.4. Use of FCM to determine effects of OJ Cloud on physiology of E. coli K-12 SCC1 at 22.5 °C

The effect of cloud particles on the physiology of *E. coli* in OJ samples incubated at 22.5 °C was investigated to model improper storage of OJ. For this experiment, the GFP-generating *E. coli* strain K-12 SCC1 was used as it enables investigation of the bacterial pH_i; decreases in pH_i have been shown to deactivate GFP (Kneen et al., 1998). SCC1 therefore displays four sub-populations when exposed to OJ (Supplemental Figure 2): A healthy population (retaining GFP fluorescence and not staining with PI); a dead population (having lost GFP fluorescence and staining with PI); and intermediate between these two populations, two stressed populations that were viable (defined here as not staining with PI) but had intermediate or low green fluorescence caused by partial or complete inactivation of GFP due to decreases in pH_i (Supplemental Fig. 2c). Some SCC1 cells entered the stressed and dead populations immediately on addition to OJ (Supplemental figure 2b).

Counts of healthy GFP^+ cells decreased over time (Fig. 3a). The greatest mean rate of decrease in the number of healthy cells was observed during the first one and a half hours for all samples, suggesting that the initial shock of entering OJ caused the largest effect on pH_i and physiology; this decrease is mirrored by increases in numbers of injured and dead (PI^+) bacteria (Fig. 3b&c). There were significant differences between the number of healthy bacteria in 0.7 µm-filtered OJ and to other samples incubated for 3, 4.5 and 6 hours, and between the 0.7 µm-filtered OJ and 0.22 µm- and 1.2 µm-filtered OJ at 24 hours (Fig. 3a). This points to a role for components in the OJ cloud

between 0.7 µm and 0.22 µm in size being damaging to bacteria; this is likely to comprise hesperidin crystals. Presence of cloud particles larger than 0.7 µm appears to protect bacteria against these harmful effects.

Numbers of injured (GFP^- PI^-) cells increased rapidly between 0 and 1.5 hours incubation, then decreased up until 6 h incubation (Fig. 3b). The number of dead (PI^+) cells also increased rapidly in the first 1.5 hours, then steadily increased with incubation time (Fig. 3c). Numbers of injured (therefore not dead) bacteria increased after 24 hours incubation in samples of OJ containing particles larger than 0.7 µm. Again, this points to a protective effect of cloud components; in addition, it suggests that after 24 hours, a subpopulation of the bacteria have adapted to conditions within the OJ.

4. Conclusion

FCM has been demonstrated to be a technique amenable to investigation of the physiology of *E. coli* in OJ. Although not the most common infectious agent present in OJ, *E. coli* has previously been reported as causing outbreaks of OJ-related foodborne disease (Singh *et al.,* 1995) and the interaction of *E. coli* and orange fruit has also been investigated (Eblen *et al*., 2004).

Clarification of OJ by centrifugation and filtration was shown to have significant effects on the viability and physiology of *E. coli* K-12 as measured using TVC and FCM. Compared to 11 µm- and 1.6 µm-filtered OJ, the presence of OJ cloud particles greater than 0.7 µm in size led to a significant reduction in TVC at 4 °C (Fig. 1). Moreover, FCM revealed a significantly lower number of healthy cells and a significantly higher number of injured cells in 0.22 µm-filtered OJ at 24 hours post-inoculation at 4 °C compared to other filtered OJ samples (Fig. 2). Differences in viable count as determined using FCM and TVC indicated the induction of VBNC state in cells exposed to OJ. Similarly, at 22.5 °C, there was a significant difference between the number of healthy cells in the 0.7

µm-filtered OJ and the 0.22 µm-filtered and 1.2 µm-filtered OJ samples at 24 hours. This indicated that OJ cloud between 0.7 µm and 0.22 µm in size might be being damaging to bacteria. The results suggest that different components of the OJ cloud have different modes of bacterial killing, and that interactions between cloud and serum components affect bacterial viability. A major potential implication of this study for OJ manufacturers is that clarification can potentially increase the viability of microorganisms in OJ. Taking into consideration the requirements by the FDA regulations to achieve 5-log reduction of the pertinent pathogens in fruit juices, the results of this study could have a potential impact on the type of control measures chosen by the manufacturers for the clarification step of their HACCP plan. Future work could utilise FCM to investigate the physiology of pathogenic *E. coli* strains such as O157:H7 in fruit juices, and *E. coli* in stationary phase, which would be expected to be more acid-resistant.

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Figure captions

Figure 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 hours. 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)

Figure 2. The physiology of *E. coli* K-12 MG1655 in filtered OJ incubated at 4 °C

3×10^9 *E. coli* MG1655 were incubated in 15 mL of filtered OJ at 4 °C for 48 hours and analysed by flow cytometry at regular intervals. Numbers of (a) healthy cells (BOX^- PI^-), (b) injured cells (BOX^+ PI^-), (c) dead cells (BOX^+ PI^+) and d) viable cells (defined here as being PI^-) are plotted; error bars are the ± standard deviation of the mean value obtained at each time point. The experiment was repeated twice in duplicate; data from a representative experiment are shown (n=2). *: $p < 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; all compared to 0.22 µm filtered OJ at the marked timepoint and calculated using 2-tailed assuming equal variance t-test. CFU/mL values were normalized by calculating their log values before performing the statistical analysis.

Figure 3. The physiology of *E. coli* K-12 SCC1 in filtered OJ incubated at 22.5 °C.

3×10^9 *E. coli* SCC1 were incubated in 15 mL of filtered OJ at 22.5 °C for 24 hours and analysed by flow cytometry at regular intervals. Numbers of (a) healthy GFP^+ cells, (b) injured cells (intermediate and low green fluorescence, PI^-), (c) dead cells (PI^+) and d) viable cells (defined here as being PI^-) are

plotted; error bars are the ± standard deviation of the mean value obtained at each time point. The experiment was repeated three times (n=5). *: $p < 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; all compared to 0.7 µm filtered OJ at the marked timepoint and calculated using 2-tailed assuming equal variance t-test. CFU/mL values were normalized by calculating their log values before performing the statistical analysis.

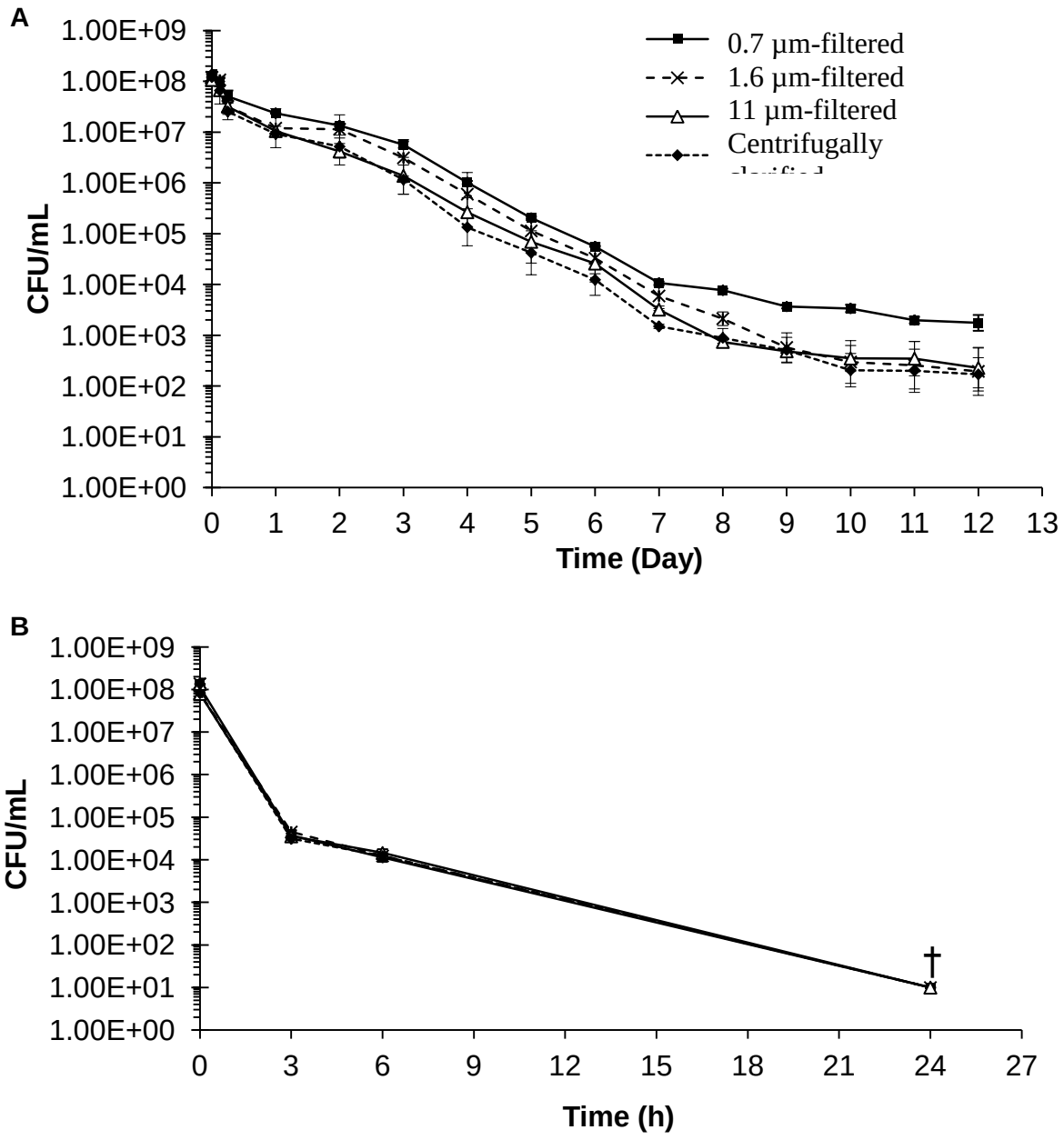
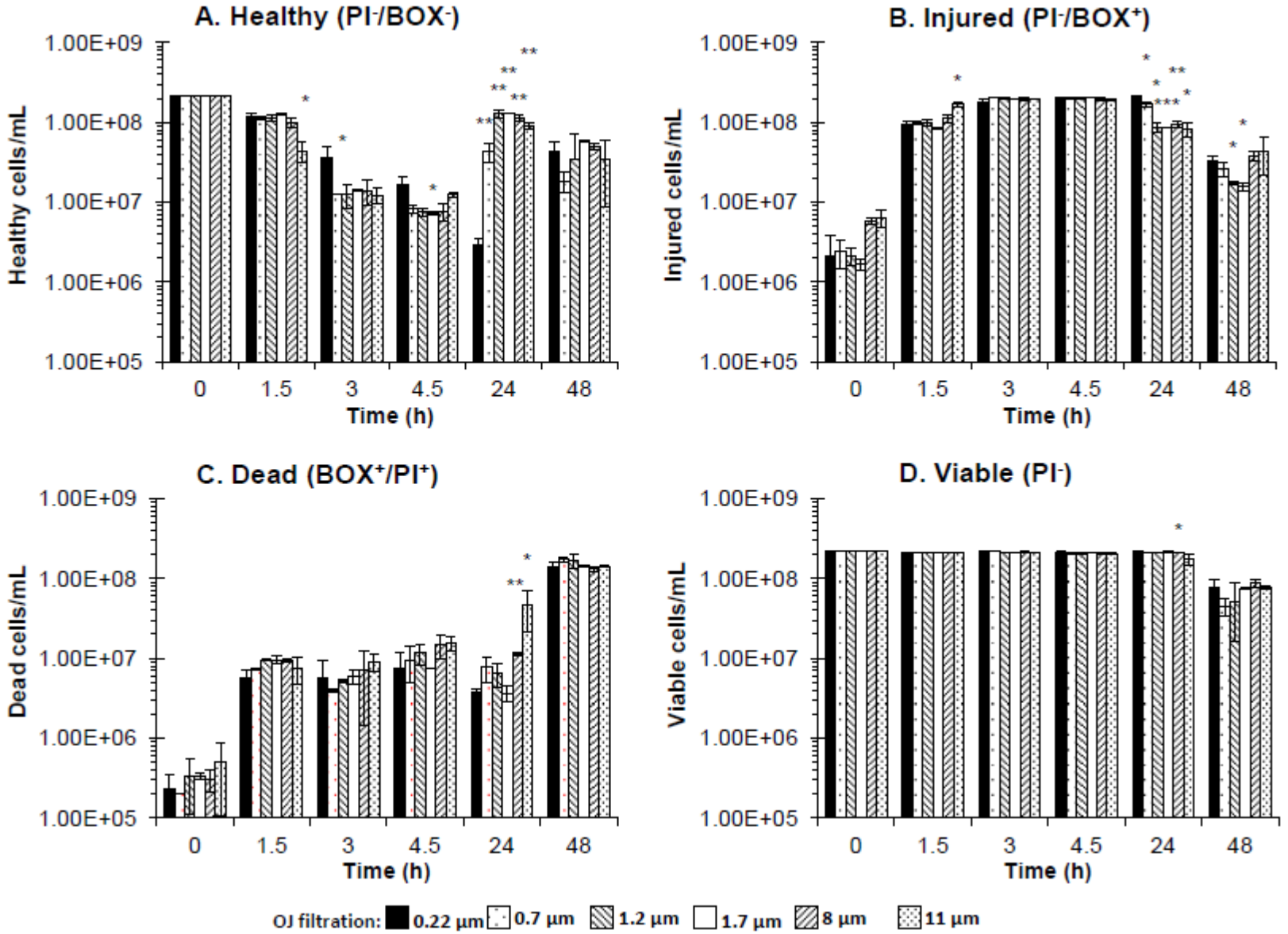


Figure 1.

Figure 2



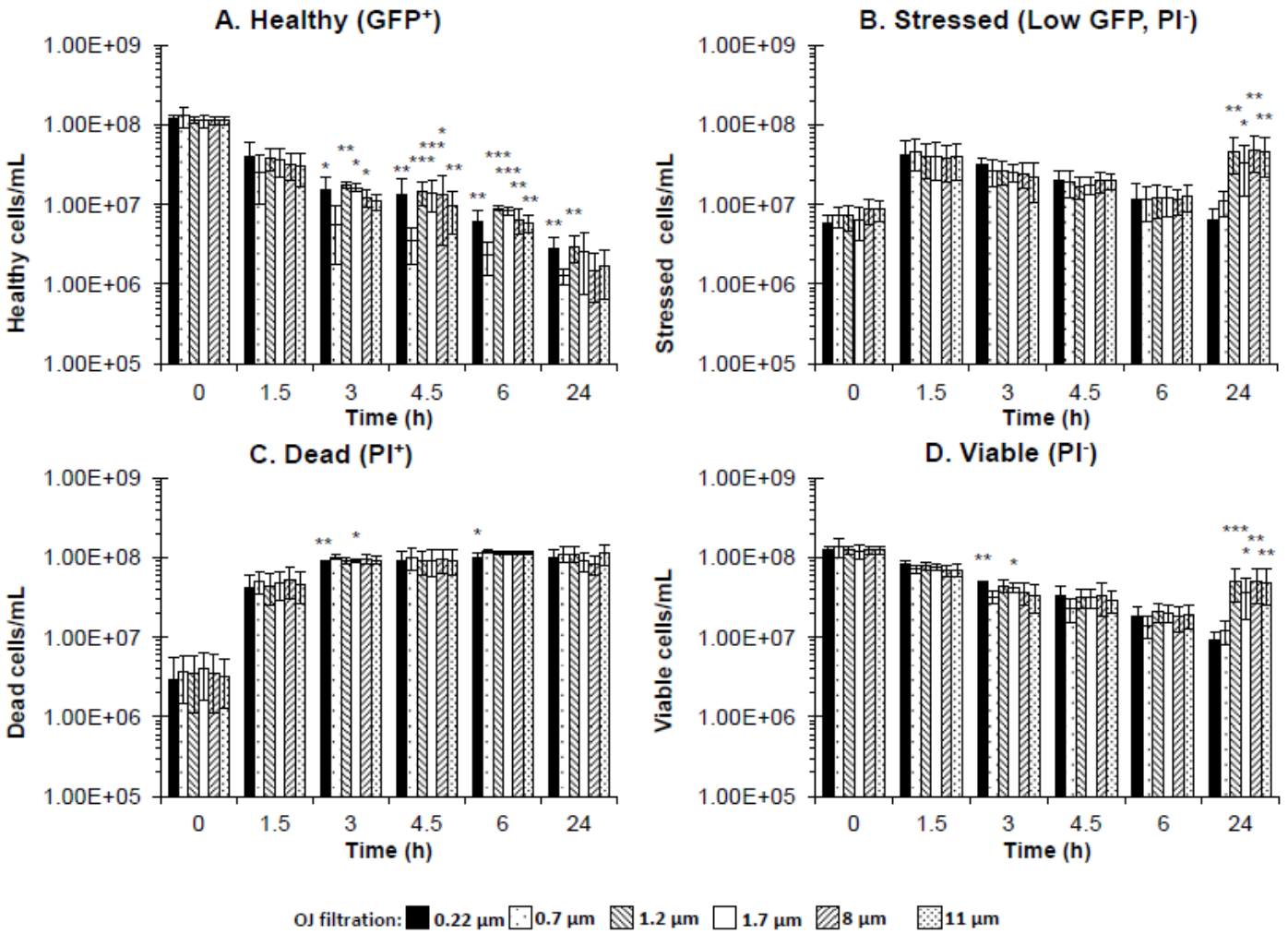


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Fig. 3

Highlights

- The effect of clarification of orange juice on the culturability and physiology of *Escherichia coli* K-12 was tested
- Flow cytometry was used to rapidly detect changes in physiology and intracellular pH
- Clarification of orange juice alters both culturability and viability of *E. coli*