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Inactivation of Escherichia Coli by Ozone Treatment of Apple Juice at Different pH Levels

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Title: "**Inactivation of *Escherichia coli* in orange juice using ozone**"

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Abstract

This research investigated the efficacy of gaseous ozone for the inactivation of *Escherichia coli* ATCC 25922 and NCTC 12900 strains in orange juice. Orange juice inoculated with *E. coli* (10^6 CFU mL^{-1}) as a challenge microorganism was treated with ozone at 75-78μg mL^{-1} for different time periods (0-18 min). The efficacy of ozone for inactivation of both strains of *E. coli* was evaluated as a function of different juice types: model orange juice, fresh unfiltered juice, juice without pulp, and juice filtered through 500μm or 1mm sieves. Fast inactivation rates for total reduction of *E. coli* were achieved in model orange juice (60 seconds) and in juice with low pulp content (6 min). However, in unfiltered juice inactivation was achieved after 15-18 min. This indicated that juice organic matter interferes with antibacterial activity of gaseous ozone. The effect of prior acid (pH 5.0) exposure of *E. coli* strains on the inactivation efficacy of ozone treatment was also investigated. There was a strain effect observed, where prior acid exposure resulted in higher inactivation times in some cases by comparison with the control cells. However, the overarching influence on inactivation efficacy of ozone was related to the pulp content. Generally, the applied gaseous ozone treatment of orange juice resulted in a population reduction of 5 log cycles.

Key words**: *Escherichia coli*, ozone, non-thermal inactivation, acid exposure, orange juice, microbial kinetics**

Industrial relevance: To facilitate the preservation of unstable nutrients many juice processors have investigated alternatives to thermal pasteurisation, including un-pasteurised short shelf life juices with high retail value. This trend has continued within the European Union. However within the US recent regulations by the FDA have

required processors to achieve a 5-log reduction in the numbers of the most resistant pathogens in their finished products. Pathogenic *E. coli* may survive in acid environments such as fruit juices for long periods. This study demonstrates that the use of ozone as a non-thermal technology is effective for inactivation of *E. coli* and acid exposed *E. coli* in orange juice. Information on the design of the ozone treatment for inactivation of *E. coli* which results into safe juice products is also among the main outputs of this work. Ozone auto-decomposition makes this technology safe for fruit juice processing.

1. Introduction

Fruit juices are an important source of bioactive compounds such as phenolics (e.g flavanone glycosides, hydroxycinnamic acids), vitamin C and carotenoids (Abeysinghe, Li, Sun, Zhang, Zhou & Chen, 2007), but technologies used for their processing and subsequent storage may cause alterations in their contents so they may not provide the benefits expected by the consumer. Fruit juice producers have traditionally relied on the acidity of their products to assure microbiological safety. Nevertheless, several incidents of food borne disease have been associated with juices. In 1991, an outbreak of *Escherichia coli* O157:H7 infections and hemolytic uremic syndrome was linked to traditionally pressed apple cider. In United States 21 juice-associated outbreaks reported to the CDC (Centers for Disease Control and Prevention) between 1995 and 2005 (Vojdani, Beuchat & Tauxe, 2008). Recent outbreaks have shown that fruit juices can be vehicles for food borne pathogens (CDC, 1996, 1999). *E. coli* O157:H7 is an enteric pathogen with a low infectious dose, which usually causes hemorrhagic colitis, but has also the potential to cause hemolytic uremic syndrome in young children and the immunocompromised (Boyce, Swerdlow & Griffin, 1995).

These outbreaks led the United States Food and Drug administration (FDA) to issue hazard analysis and critical control points (HACCP) regulations for safe and sanitary processing of juice (USFDA, 2001). A primary performance standard is a minimum 5-log reduction of the pathogens of concern in the juice being processed (USFDA, 2001). A common method for preservation and processing of fruit juices is pasteurisation. Thermal pasteurisation of orange juice can cause degradation of the product's quality (non-enzymatic browning and off-flavours production), while the fresh juice flavour

(Basak & Ramaswamy, 1996) may be impaired and its vitamin content decreased. In recent years consumers have increasingly sought ready-to-use 'fresh-like' products, which are usually refrigerated. This has led the food industry to develop alternative processing technologies in order to produce foods with a minimum of nutritional, physicochemical, or organoleptic changes (Esteve & Frigola, 2007). Consumers tend to prefer recently extracted fresh juices with fresh taste and minimal flavour or vitamin losses (Bignon, 1997). The FDA's approval of ozone as a direct additive to food in 2001 triggered interest in ozone applications. A number of commercial fruit juice processors in the US and Europe began employing ozone for pasteurisation resulting in the issue of industry guidelines. These guidelines (FDA, 2004) highlight gaps in the literature with respect to the critical control parameters of ozone during microbial inactivation in liquid systems.

Ozone is a triatomic allotrope of oxygen and is characterized by a high oxidation potential that conveys bactericidal and viricidal properties (Burleson, Murray & Pollard, 1975; Kim, Yousef & Dave, 1999). Ozone inactivates microorganisms through oxidisation and residual ozone decomposes to nontoxic products (i.e., oxygen) making it an environmentally friendly antimicrobial agent for use in the food industry (Kim et al., 1999). Restaino et al. (1995) determined that ozone effectively killed Gram-positive bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis,* and Gram-negative bacteria including *Pseudomonas aeruginosa*, and *Yersinia enterocolitica* in deionized water in the absence or presence of organic material such as soluble starch (SS) and bovine serum albumin (BSA). Ozone has been shown to reduce populations of *E. coli* O157:H7 in phosphate buffer (Byun, Kwon, Yook

& Kim, 1998) while its preservation efficacy has been also evaluated in a variety of food products, including milk, gelatin, albumin, casein, and meat products (Kim et al., 1999). The antibacterial activity of ozone has been attributed to its diffusion capability (Hunt & Marinas, 1997). It reacts up to 3000 times faster than chlorine with organic material, and it readily diffuses through biological cell membranes.

Microorganisms can induce adaptation responses to environmental stresses by expressing specific sets of genes on exposure to acid, salt, heat, cold, reactive oxygen species, starvation etc. Therefore it is of great importance to evaluate the efficiency of food preservation treatments using resistant strains while developing process criteria (Johnson, 2003). The objectives of this study were (i) to determine the efficacy of continuous gaseous ozone treatment for reduction of two different strains of *E. coli* at ambient temperature (12-15 ºC) in orange juice, (ii) to evaluate how inactivation was affected by the orange juice pulp content and (iii) to investigate if prior acid exposure of the challenge microorganism significantly impacted on treatment efficacy.

2. Materials and Methods

2.1 Bacterial strains and cultural conditions

Two strains of *E. coli* were used in this study: *E. coli* ATCC 25922 (generic strain), obtained from microbiology stock culture of the School of Food Science and Environmental Health of the Dublin Institute of Technology, and *E. coli* NCTC 12900 (non-toxigenic strain of *E. coli* O157:H7), obtained from National Collection of Type Cultures of the Health Protection Agency (London, UK). Both strains were used for inactivation studies to ensure potential useful effects against this key pathogen of concern

to fruit juice processors were measured. The bacteria were maintained as frozen stocks at -70ºC in the form of protective beads, which were plated onto tryptic soy agar (TSA, Scharlau Chemie) and incubated overnight at 37 ºC to obtain single colonies before storage at 4 ºC. Working cultures were prepared by inoculating a single colony into tryptic soya broth (TSB, Scharlau Chemie) and incubating overnight at 37ºC (Cheng, Yu & Chou, 2003; Caggia, Ombretta Scifò, Restuccia & Randazzo, 2009) .

2.2 Preparation of model orange juice (MOJ)

The MOJ medium of Shinoda, Murata, Homma, and Komura (2004) without modifications was used in the experiments. The composition of MOJ per 100mL was as follows: sucrose: 5.0g; glucose: 2.5g; fructose: 2.5g; citric acid: 1.0g; ascorbic acid: 30 mg; L-serine: 7.0 mmol; L-asparagine: 5.4 mmol, L-alanine: 1.9 mmol; L-arginine: 0.75 mmol; L-glutamic acid: 0.54 mmol; L-proline: 0.42 mmol. The pH of MOJ was adjusted to pH 3.0 using 1N NaOH. MOJ was then sterilized at 121 °C for 15 min.

2.3 Preparation of orange juice

2.3.1 Fresh orange juice unfiltered

Oranges (variety: Balady, Egypt) were purchased from a local market, washed with tap water and cut into two pieces. The fresh oranges were squeezed with fruit juicer (Rowenta NEO type 8332). All juice preparations were stored at 4 ºC. The pH was measured using a pH meter with a glass electrode (Orion Model, England) and was in the range of 3.5-4.0.

2.3.2 Fresh orange juice filtered (without pulp)

Juice without pulp was prepared as above with centrifugation (SIGMA 2K15, Bench Top Refrigerated Ultracentrifuge, AGB scientific LTD) at 13000 rpm for 10 min followed by filtering the juice through Whatman No.1 filter paper, giving a 75% yield in terms of filtrate.

2.3.3 Fresh orange juice with reduced pulp content

Juice with reduced pulp was prepared as above and submitted to a finishing process by passing through sieves (Laboratory test sieve, Retsch, Germany) to reduce the pulp content. Two different sieve sizes were employed to obtain juice with different pulp levels; sieve size of 500µm {mesh no.35} and sieve size of 1mm {mesh no.18}.

2.4 Preparation of cell suspensions

Cells grown in TSB were harvested by centrifugation at 10,000 rpm for 10min at 4 ºC. The cell pellet was washed twice with sterile phosphate buffered saline (PBS, Oxoid LTD, UK). The pellet was re-suspended in PBS and the bacterial density was determined by measuring absorbance at 550 nm using McFarland standard (BioMérieux, Marcy - l'Etoile, France) to allow a working inoculum corresponding to 1.0×10^8 CFU mL^{-1} to be prepared. This was then serially diluted in maximum recovery diluent (MRD, Scharlau Chemie) to obtain approximately 10^7 CFU mL^{-1}. Adding 10 mL of cell concentration (10^7 CFU mL^{-1}) to 90 mL of orange juice yielded a final concentration of 10^6 CFU mL^{-1}. For model orange juice samples, the pellet was re-suspended in PBS and diluted into MOJ to yield the same final concentration.

2.5 Acid exposure of bacterial cultures

186 Cells were exposed to hydrochloric acid (HCl) as described by Cheng, Yu and Chou
187 (2003). Acid stress conditions were imposed for two time periods; 1 hour and 18 hours.
188 Working cultures were grown overnight in TSB at 37 ºC. Cells were then harvested by
189 centrifugation at 10,000 rpm for 10min at 4ºC. The cell pellet was washed twice
190 with sterile PBS, re-suspended in 10 mL TSB (pH5.0, adjusted with 6N HCl, at ambient
191 temperature of 12-15 ºC) and incubated at 37 ºC for 1h. For a 18-h acid exposure,
192 bacterial strains were grown directly in TSB (pH 5.0) at 37ºC. After incubation, cultures
193 were diluted in MRD (pH 5.0) to yield approximately 10^7cells mL^{-1}, with further dilution
194 in orange juice to a final concentration of 10^6 CFU mL^{-1}.
195
196 *2.6 Ozone treatment*
197 Ozone gas was generated using an ozone generator (Model OL80, Ozone services,
198 Canada, Figure 1) in a 100 mL glass bubble column. Ozone was produced by a corona
199 discharge generator. Pure oxygen was supplied via an oxygen cylinder (Air Products
200 Ltd., Dublin, Ireland) and the flow rate was controlled using an oxygen flow regulator. A
201 previously determined optimum flow rate of 0.12L min^{-1} with an ozone concentration of
202 75-78µg mL^{-1} was applied for each treatment (Patil, Cullen, Kelly, Frias & Bourke,
203 2009). Ozone concentration was recorded using an ozone analyzer (built in ozone module
204 OL80A/DLS, Ozone services, Burton, Canada). Excess ozone was destroyed by an ozone
205 destroyer unit. To prevent excess foaming, 20 µl sterile anti-foaming agent (Antifoam B
206 emulsion, Sigma Aldrich, Ireland Ltd.) was added before each ozone treatment. Two
207 bacterial strains (*E. coli* ATCC 25922, *E. coli* NCTC 12900) were investigated for their
208 response to ozone treatment. Experiments were performed with non-acid exposed control

cultures as well as a range of acid exposed cultures; namely 1 h, and 18 h acid exposed cultures. Unfiltered juice was treated for 30 minutes with sampling at 3 min intervals. All other juices were treated for 6-7 minutes with sampling at 1 min intervals. All experiments were carried out in duplicate and replicated at least twice.

2.7 Microbiological analysis

The efficacy of treatments was determined in terms of reduction in viable counts over time. Populations of challenge organism were determined by plating onto both TSA and selective media, Sorbitol MacConkey agar (SMAC, Scharlau Chemie) respectively. Samples (1mL aliquots) were withdrawn from treated juice at specific time intervals, serially diluted in MRD and 0.1mL aliquots of appropriate dilutions were surface plated on TSA and SMAC to compare recovery of *E. coli* strains. Plates were incubated at 37 ºC for 24h and then counted. Results were reported as $Log_{10}CFU\ mL^{-1}$. Data were pooled and average values and standard deviations determined. Means were compared using ANOVA followed by LSD testing at $p < 0.05$ level (SPSS, version 15.0).

2.8 Inactivation kinetics

The GInaFiT tool was employed to perform the regression analysis of the microbial inactivation data (Geeraerd, Valdramidis & Van Impe, 2005). The Weibull model was used to analyze the data:

$$\log_{10}(N) = \log_{10}(N_0) - \left(\frac{t}{\delta}\right)^p \quad (1)$$

where N is the number of microorganisms, N_0 (CFU mL^{-1}) is the initial number of microorganisms, δ [min] (time for the first decimal reduction) and p [-] are parameters related to the scale and shape of the inactivation curve, respectively. The Weibull

distribution corresponds to a concave upward survival curve if p<1 and concave downward if p >1 (Van Boekel, 2002).

The numerical values of δ and p were used to calculate a desired log reduction. The time required to obtain an x log reduction (t_{xd}) was calculated using equation 2. For this case study x was equal to 5, following the regulation of USFDA for a minimum 5-log reduction in the juice being processed (USFDA 2001).

$$t_{xd} = \delta \times (x)^{\frac{1}{p}} \qquad (2)$$

3. Results

3.1 Effect of ozone inactivation of E. coli in model orange juice

Ozone inactivation of both *E. coli* strains in model orange juice was rapid in this low pH medium. Ozone treatment at the optimum flow rate of 0.12L min^{-1} with an ozone concentration of 75-78µg mL^{-1} resulted in a 6.0 log cycle reduction within 60 seconds.

3.2 Effect of ozone on inactivation of E. coli in orange juice

The Weibull parameters δ and p are shown in Table 1. In the present study, the shape parameter p showed downward concavity for both *E. coli* strains (Fig. 2 and 3). The inactivation of *E. coli* in orange juice was fitted using the Weibull model, which provided estimations of microbial inactivation in terms of processing time required. The R^2 values of 0.93 and above (Table 1) show that the Weibull model was a good fit for the experimental data analysed. p values >1 indicate the susceptibility of the remaining cells to the treatment (van Boekel, 2002).

254 The efficacy of ozone was found to depend both on the juice type and the bacterial strain
255 (statistical indices of $p < 0.05$). Both strains of *E. coli* studied (*E. coli* ATCC 25922, *E.*
256 *coli* NCTC 12900) were sensitive to ozone ($p<0.05$). In unfiltered juice, ATCC 25922
257 and NCTC 12900 were completely inactivated after 18 and 15 min respectively (Fig. 2
258 and 3) as determined on TSA and SMAC. However, ozone treatment of ATCC 25922 in
259 orange juice without pulp and juice passed through the 500µm sieve, resulted in complete
260 inactivation within 5 min (Fig.2). The population of *E. coli* 25922 in juice passed through
261 sieve of 1mm diameter decreased by 6.0 log cycles in 6 min treatment time (Fig.2).
262 Similarly, ozone treatment of NCTC 12900 in orange juice without pulp and juice passed
263 through the 500µm sieve resulted in complete inactivation in 5 and 6 min, respectively
264 (Fig.3). NCTC 12900 decreased by 4.6 and 6.0 log cycles after 6 min treatment time in
265 juice passed through the 1mm sieve as determined on TSA and SMAC, respectively.
266 The t_{5d} (t_{5d} - the time required for a 5 log reduction) for both *E. coli* strains in the different
267 juice types are shown in Table 1. The t_{5d} values were lower as the amount of pulp present
268 in the orange juice decreased ($p<0.05$). The inactivation of *E. coli* strains in unfiltered
269 juice showed higher t_{5d} values compared to the other juice types. *3.3 Effect of acid*
270 *exposure on treatment efficacy*
271 The effect of acid exposure on ozone treatment efficacy was evaluated in orange juice
272 passed through a 1mm sieve. Ozone inactivation curves for acid-exposed *E. coli* cells at
273 the different acid exposure conditions are shown in Figure 4. For acid exposed *E. coli*
274 strains the shape parameter *p* showed downward concavity. The *p* values for 1h acid
275 exposed cells were lower by comparison with both the 18h acid exposed and control

276 populations (Table 2), indicating a lower susceptibility to the treatment with a short
277 period of acid adaptation.
278 Ozone treatment of 1h acid exposed *E. coli* ATCC 25922 resulted in a reduction of 4.8
279 and 5.5 log cycles after 7 min treatment time on TSA and SMAC, respectively. However,
280 ozone treatment of 1h acid exposed *E. coli* NCTC 12900 reduced an initial count of log
281 6.28 CFU mL^{-1} to below detectable levels after 7 min treatment time on TSA and SMAC,
282 respectively. However, with the 18h acid exposed cells, populations of *E. coli* ATCC
283 25922 and *E. coli* NCTC 12900 were decreased by 6.0 and 5.3 log cycles respectively
284 within 7 min as determined by using TSA. Similar trends were observed using SMAC
285 where 18 h acid exposed *E. coli* ATCC 25922 and *E. coli* NCTC 12900 were decreased
286 by 5.8 and 5.1 log cycles, respectively. The t_{5d} values of the acid exposed *E. coli* strains
287 are shown in Table 2. There was a strain difference observed between acid exposed and
288 control populations. The estimated time for a 5 log reduction of control (non-acid
289 exposed) *E. coli* NCTC 12900, was 6.14 min, while the estimate for the generic strain *E.*
290 *coli* ATCC 25922 was 5.62 min. When the strains were subjected to a 1h acid exposure,
291 the estimated time required for a 5 log cycle reduction in *E. coli* ATCC 25922 increased
292 to 6.46 min, while there was no similar increase for *E. coli* NCTC 12900. Conversely,
293 following 18h acid exposure, the estimated time required for a 5 log cycle reduction in *E.*
294 *coli* NCTC 12900 increased to 6.84 min, while the estimated time for *E. coli* ATCC
295 25922 was similar to that recorded for the control cells. However, there was a significant
296 difference observed for *E. coli* ATCC 25922 between 1-h acid exposed population
297 compared to the control and 18-h acid exposed population ($p>0.05$); whereas there was

298 no significant difference observed between control population of *E. coli* NCTC 12900
299 and those exposed to acid conditions for 1h or 18 h.
300
301 **4. Discussion**
302 The direct application of ozone was found to be effective for the inactivation or reduction
303 of *E. coli* in orange juice (Figures 2, 3 and 4), but the rate was dependant on the juice
304 type used. In the present study inactivation in unfiltered juice was achieved after 15-18
305 min treatment time by comparison with significantly shorter inactivation times within
306 model orange juice or juice with low pulp content. This could be ascribed to the organic
307 compounds such as sugars, fibres, ascorbic acid, present in orange juice which could
308 affect the dissolution rate of ozone in the system, thereby reducing the ozone level
309 available for inactivation of *E. coli* cells. The organic load present within the medium is
310 known to decrease the effectiveness of ozone for the inactivation of microorganisms.
311 Williams, Sumner and Golden (2005), observed a reduced efficacy of ozonation for
312 inactivation of *E. coli* in orange juice in the presence of ascorbic acid and organic matter
313 and Mielcke and Ried (2004), also reported that a high and persistent level of organic
314 substances will have a negative impact on the ozone disinfection rate. The effectiveness
315 of ozone against microorganisms depends not only on the amount applied, but also on the
316 residual ozone in the medium, various environmental factors such as medium pH,
317 temperature, humidity, additives (surfactants, sugars, etc.), and the amount of organic
318 matter surrounding the cells (Pascual, Liorca & Canut, 2007). The focus of this study was
319 to evaluate the impact of organic matter during ozone processing. However, the effect of
320 residual ozone for the specific flow rate and ozone concentration levels employed was

321 evaluated in apple juice, where non-significant microbial reduction was observed (Data
322 not shown).
323 The type of organic material may impact ozone efficacy more than the amount of organic
324 material present (Restaino, Frampton, Hemphill & Palnikar, 1995). This is in agreement
325 with Guzel-seydim, Bever and Greene (2004), who observed that the presence of food
326 components such as caseinate in whipping cream provided a high level of protection to
327 the bacterial populations against ozone treatment, whereas locust bean gum resulted in an
328 intermediate level of protection. In the present study, fast inactivation rates were achieved
329 in the model orange juice and the filtered juices which may be attributed to the absence of
330 high ozone demanding substances. Komanapalli and Lau (1998) found that the cidal
331 activity of ozone was greatly affected by the dose applied, the presence of ozone-
332 quenching proteins, and the type of challenge microorganisms. Williams et al., (2004)
333 reported *E. coli* O157:H7 was inactivated in orange juice after a 75 min ozone treatment
334 applied at ambient temperature, while in the present study faster inactivation rates within
335 a period of 6 to 18 min were achieved. The possible reason for this could be the different
336 ozone system as well as the different control parameters (i.e., flow rate of of 2.4 L min^{-1}
337 and ozone concentration of 0.9g h^{-1}) that were used for the Williams et al., (2004)
338 inactivation studies. In the present study, a previously optimized ozone flow rate was
339 used which was lower than that employed by Williams et al., (2004). Flow rate was
340 previously determined to be a critical factor, at high flow rates a small number of large
341 bubbles are produced, which rise to the liquid surface quickly, thereby escaping the
342 medium quickly. The resulting poor gas dissolution reduces the contact time, leading to a
343 lower inactivation rate (Patil et al., 2009). The antibacterial efficacy of ozone was greater

344 when target microorganisms were suspended in pure water or simple buffers than in
345 complex systems (Khadre, Yousef & Kim, 2001). The mechanism for inactivation of
346 microorganisms by ozone is due to its high oxidation-reduction potential. Ozone is
347 capable of oxidizing the constituent elements of microbial cell walls before penetrating
348 inside the organism and oxidizing certain essential components such as unsaturated
349 lipids, proteins, enzymes and nucleic acids. When a large part of the membrane barrier is
350 destroyed, it causes lysis and leakage of bacterial cells and results in their immediate
351 destruction (Muthukumarappan, O'Donnell & Cullen, 2008). Decreasing pH and
352 temperature are associated with increasing stability of ozone molecules (Kim et al.,
353 1999). Tiwari, O'Donnell, Muthukumarappan & Cullen (2009) recently studied the
354 effects of ozone on quality and nutritional parameters for a range of fruit juices,
355 highlighting significant losses in nutritional quality which were dependent on ozone
356 control parameters of ozone concentration and gas flow rate. However, achieving rapid
357 microbial inactivation using optimised control parameters may mitigate losses in
358 nutritional quality.

359 When microorganisms are stressed, an adaptive response may follow which can
360 increase the organisms' tolerance to the same or to a different type of stress (Yousef &
361 Courtney, 2003). Many bacteria react to stress by inducing the synthesis of various
362 proteins (Herendeen, Vanbogelen & Neidhardt, 1979; Jones & Inouye, 1994). Buchanan
363 and Edelson (1999), reported a cross protective effect of acid shocking and acid
364 adaptation of enterohaemorrhagic *E. coli* (EHEC) against heat or other stresses but also
365 observed that the determination of survival of EHEC in acidic foods should consider the
366 strain and its ability to induce stress responses. The resistance or adaptation of

367 microorganisms to acid conditions can have implications for food safety. Additionally,
368 Johnson (2003) observed that challenge studies in food systems are required to
369 adequately assess growth or survival of pathogens. The acid adaptation responses of food
370 borne pathogens were previously examined at different pH conditions and pH 5.0-5.5
371 lead to the highest level of acid resistance for *E. coli* O157:H7 (Koutsoumanis & Sofos,
372 2004). In this study both *E. coli* strains were subjected to acid exposure at pH 5.0 to
373 examine the effect of prior acid exposure on the efficacy of ozone treatment in orange
374 juice. Increased inactivation time of acid exposed *E. coli* cells of both strains to ozone
375 treatment over the control cells was observed in the present study. The t_{5d} values of acid
376 exposed *E. coli* cells were higher than the t_{5d} values of control cells in some cases. Acid
377 exposure of *E. coli* ATCC 25922 for 1h and longer acid exposure (18h) for NCTC 12900
378 resulted in increased acid resistance, potentially giving a cross - protective effect against
379 ozone treatment. Treatment of *E. coli* O157:H7 with acid has been reported to increase
380 acid resistance after exposure to moderate acid environments (Kroll & Patchett, 1992;
381 Leyer, Wang & Johnson, 1995) and was also shown to confer cross resistance to salt and
382 heat (Rowe & Kirk, 1999). In beef processing, prior acid adaptation negatively
383 influenced the efficacy of a 2% acetic acid decontamination treatment for reduction of *E.*
384 *coli* O157:H7 on carcasses (Berry & Cutter, 2000) and acid adaptation prolonged the
385 survival of *E. coli* O157:H7 in various food systems, including apple cider, sausages
386 (Leyer et al, 1995) and acid fruit juice (Hsin-Yi & Chou, 2001).

387 Acid habituation of pathogens may enhance survival in acidic food (e.g. fruit
388 juice) or in the stomach and subsequently cause infection after ingestion (Goodson &
389 Rowbury, 1989). In an environment with changing pH, acid sensitive *E. coli* O157

390 cultures can become acid-resistant within 17 min (de Jonge, Takumi, Ritmeester & van
391 Leusden, 2003). Acid resistance and survival of pathogens have significant implications
392 for food safety and the virulence of pathogenic microorganisms and the ability of non-
393 acid adapted *E. coli* O157 to adapt within a very short period under extreme conditions
394 further contribute to their virulence (Beales, 2004). Our results also showed that the
395 extent of increased acid resistance varied with the strain and acid exposure conditions.
396 When *E. coli* ATCC 25922 was acid exposed for 1 h, an increased resistance to ozone
397 treatment was observed. In the case of *E. coli* NCTC 12900 only the longer acid exposure
398 time (18h) showed an increased t_{5d} value compared to the control cells. However, while
399 increased resistance of acid stressed *E. coli* cells to ozone treatment was observed, 5 log
400 cycle reductions in populations were still achieved in less than 7 min. Buchanan, Edelson
401 and Boyd (1999) also reported that while pH during exposure had little effect on survival
402 of *E. coli* O157:H7, acid-resistance consistently enhanced radiation resistance.
403 Therefore, acid resistance should be considered when determining t_{5d} values in foods.
404 Additional studies could be conducted in order to further elucidate the role of strains and
405 stress exposure time on the inactivation efficacy of direct ozone treatments. Such studies
406 could include comparison of the behaviour of acid-stressed *E. coli* strains with that of
407 unadapted control cells in orange juice, through measurement of the in vivo expression of
408 stress-related genes.

409 **5. Conclusions**

410 This work has shown that direct ozone treatment can be used to inactivate *E. coli*
411 in orange juice. The efficacy of ozone treatment was found to be a function of juice type,
412 strain of *E. coli* and duration of acid exposure conditions. Inactivation times for a 5 log

cycle reduction ranged between 60 sec and 18 min. Therefore ozone treatment could be used as a potential alternative to traditional thermal pasteurization for control of *E. coli* populations as a safety issue in fresh orange juice.

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Figure Captions

Figure 1: Schematics of Ozone generator

Figure 2: Microbial survival curve of *Escherichia coli* ATCC 25922 for the different orange juice types. Curves are fitted using the Weibull model.

Figure 3: Microbial survival curve of *Escherichia coli* NCTC 12900 for the different orange juice types. Curves are fitted using the Weibull model.

Figure 4: Microbial survival curve of acid exposed *Escherichia coli* strains of the reduced pulp orange juice (1mm sieve size).

. Curves are fitted using the Weibull model.

a) 1h acid exposed *Escherichia coli* ATCC 25922
b) 18h acid exposed *Escherichia coli* ATCC 25922
c) 1h acid exposed *Escherichia coli* NCTC 12900
d) 18h acid exposed *Escherichia coli* NCTC 12900

567 Table 1: Parameters of the Weibull model and the time required to reach a 5 log reduction for *Escherichia coli* strains in orange juice

568 after treatment with ozone (Different letters indicate a significant difference at the 0.05 level between each juice type for each strain

569 and between both strains for each juice type.)

Microorganism	Juice type	Condition	δ (min) ± SE	p ± SE	R^2	t_{5d} (min)
E.coli ATCC 25922	Unfiltered	control	1.58^{a}±0.84	0.80^{f}±0.16	0.96	11.86^{k}
	1mm sieve	control	3.28^{b}±0.33	2.98^{g}±0.49	0.98	5.62^{l}
	500 µm sieve	control	2.77^{c}±0.39	3.14^{g}±0.73	0.97	4.63^{m}
	Without pulp	control	2.91^{d}±0.35	3.26^{g}±0.69	0.98	4.76^{m}
E.coli NCTC 12900	Unfiltered	control	2.55^{ea}±0.91	1.08^{hf}±0.21	0.98	11.30^{nk}
	1mm sieve	control	3.24^{eb}±0.43	2.52^{ig}±0.52	0.97	6.14^{ol}
	500 µm sieve	control	3.12^{e}±0.26	2.81^{j}±0.35	0.98	5.53^{p}
	Without pulp	control	3.41^{e}±0.55	4.44^{j}±1.81	0.93	4.90^{pm}

570 * SE- standard error

571 Table 2: Parameters of the Weibull model and the time required to reach a 5 log reduction for acid exposed *Escherichia coli* strains in

572 orange juice after treatment with ozone (* Different letters indicate a significant difference at the 0.05 level between each juice type

573 for each strain and between both strains for each juice type.)

Microorganism	Juice type	Condition	δ (min) ± SE	p ± SE	R^2	t_{5d} (min)
E.coli ATCC 25922						
	1mm sieve	Control	3.28^{a}±0.33	2.98^{d}±0.49	0.98	5.62^{h}
	1mm sieve	1h acid adapatation	2.41^{b}±0.52	1.63^{e}±0.31	0.97	6.46^{i}
	1mm sieve	18 h acid adaptation	3.08^{a}±0.20	2.63^{f}±0.25	0.99	5.67^{h}
E.coli NCTC 12900						
	1mm sieve	Control	3.24^{ca}±0.43	2.52^{gd}±0.52	0.97	6.14^{jh}
	1mm sieve	1h acid adapatation	2.49^{cb}±0.36	1.81^{ge}±0.25	0.98	6.06^{ji}
	1mm sieve	18 h acid adaptation	3.47^{ca}±0.37	2.37^{gf}±0.35	0.98	6.84^{j}

574 * SE- standard error

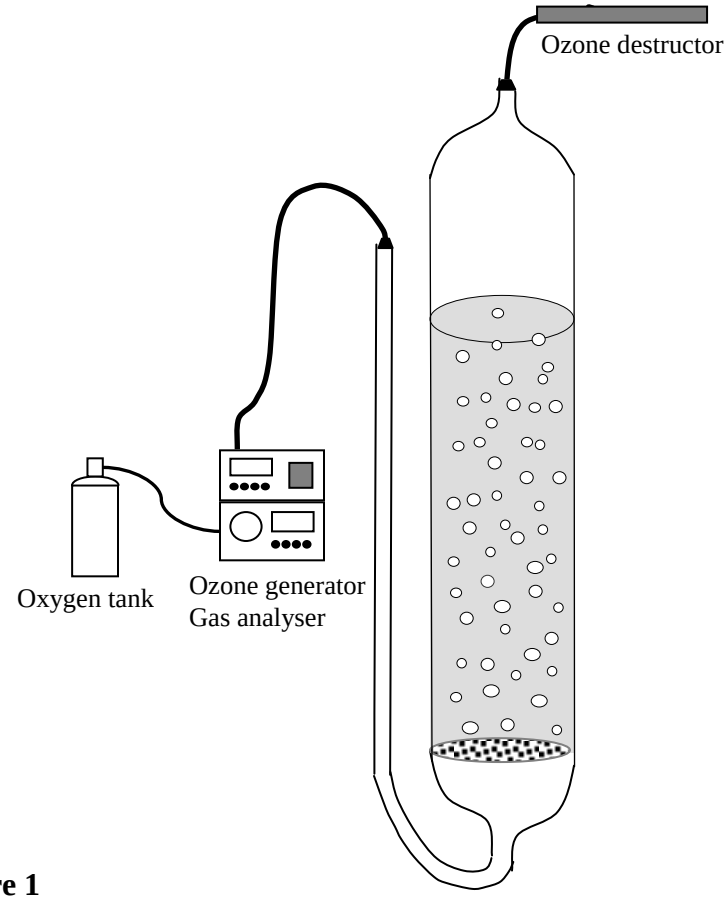
Ozone destructor
Oxygen tank
Ozone generator
Gas analyser

Figure 1

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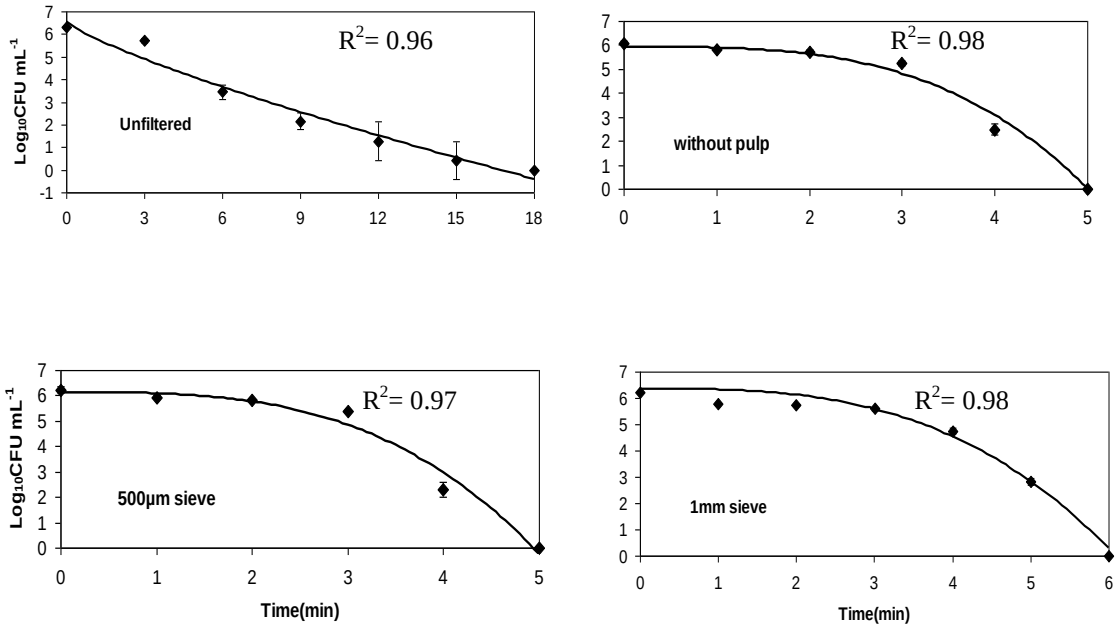

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600 **Figure 2**

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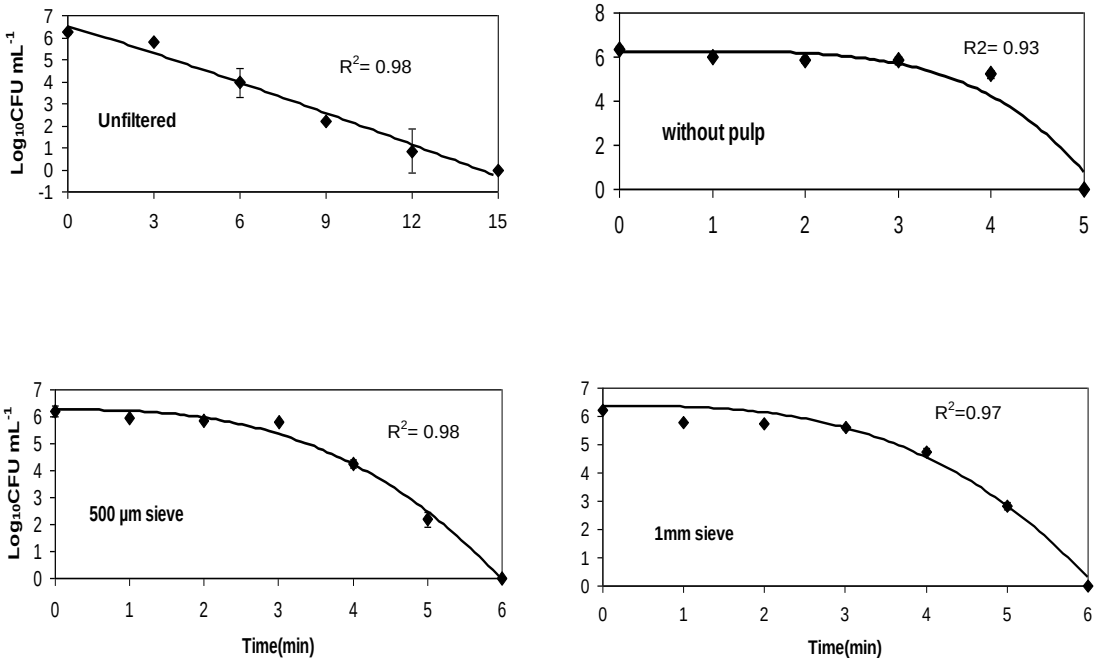

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613 **Figure 3**

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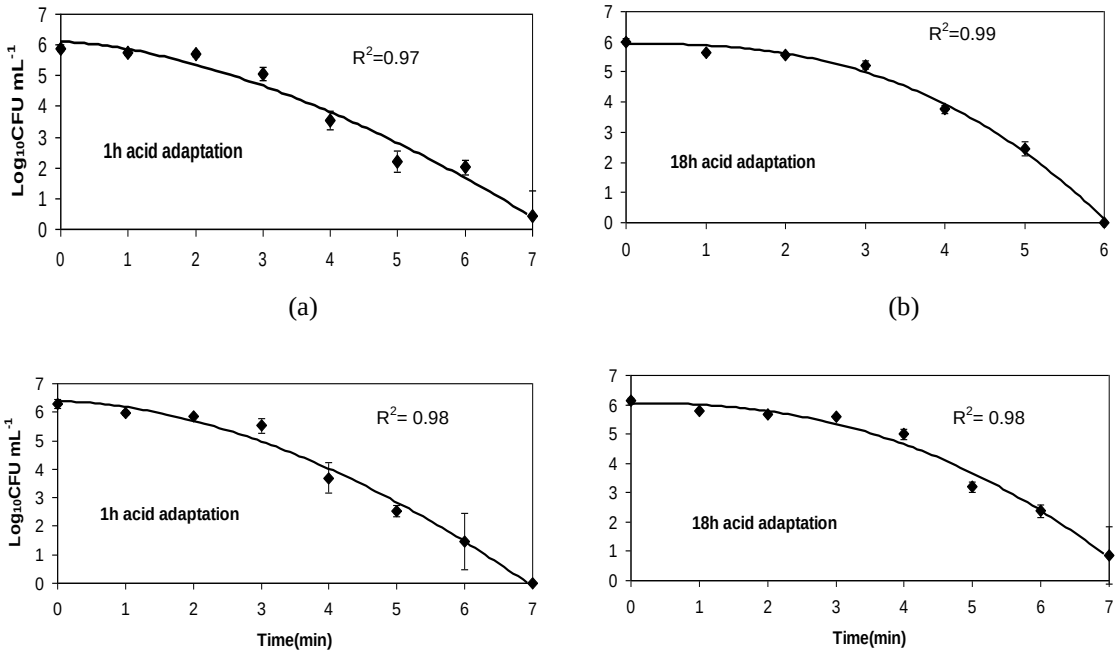
Log10CFU mL-1
R2=0.97
1h acid adaptation
(a)
R2=0.99
18h acid adaptation
(b)
Log10CFU mL-1
R2= 0.98
1h acid adaptation
Time(min)
(c)
R2= 0.98
18h acid adaptation
Time(min)
(d)

Figure 4

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