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

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1 Title: “**Inactivation of *Escherichia coli* in orange juice using**
2 **ozone**”

3

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24

25 **Abstract**

26 This research investigated the efficacy of gaseous ozone for the inactivation of
27 *Escherichia coli* ATCC 25922 and NCTC 12900 strains in orange juice. Orange juice
28 inoculated with *E. coli* (10^6 CFU mL⁻¹) as a challenge microorganism was treated with
29 ozone at 75-78µg mL⁻¹ for different time periods (0-18 min). The efficacy of ozone for
30 inactivation of both strains of *E. coli* was evaluated as a function of different juice types:
31 model orange juice, fresh unfiltered juice, juice without pulp, and juice filtered through
32 500µm or 1mm sieves. Fast inactivation rates for total reduction of *E. coli* were achieved
33 in model orange juice (60 seconds) and in juice with low pulp content (6 min). However,
34 in unfiltered juice inactivation was achieved after 15-18 min. This indicated that juice
35 organic matter interferes with antibacterial activity of gaseous ozone. The effect of prior
36 acid (pH 5.0) exposure of *E. coli* strains on the inactivation efficacy of ozone treatment
37 was also investigated. There was a strain effect observed, where prior acid exposure
38 resulted in higher inactivation times in some cases by comparison with the control cells.
39 However, the overarching influence on inactivation efficacy of ozone was related to the
40 pulp content. Generally, the applied gaseous ozone treatment of orange juice resulted in a
41 population reduction of 5 log cycles.

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

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

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

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71 **1. Introduction**

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

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

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

106 Ozone is a triatomic allotrope of oxygen and is characterized by a high oxidation
107 potential that conveys bactericidal and viricidal properties (Burlison, Murray & Pollard,
108 1975; Kim, Yousef & Dave, 1999). Ozone inactivates microorganisms through
109 oxidisation and residual ozone decomposes to nontoxic products (i.e., oxygen) making it
110 an environmentally friendly antimicrobial agent for use in the food industry (Kim et al.,
111 1999). Restaino et al. (1995) determined that ozone effectively killed Gram-positive
112 bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*,
113 *Enterococcus faecalis*, and Gram-negative bacteria including *Pseudomonas aeruginosa*,
114 and *Yersinia enterocolitica* in deionized water in the absence or presence of organic
115 material such as soluble starch (SS) and bovine serum albumin (BSA). Ozone has been
116 shown to reduce populations of *E. coli* O157:H7 in phosphate buffer (Byun, Kwon, Yook

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

122 Microorganisms can induce adaptation responses to environmental stresses by expressing
123 specific sets of genes on exposure to acid, salt, heat, cold, reactive oxygen species,
124 starvation etc. Therefore it is of great importance to evaluate the efficiency of food
125 preservation treatments using resistant strains while developing process criteria (Johnson,
126 2003). The objectives of this study were (i) to determine the efficacy of continuous
127 gaseous ozone treatment for reduction of two different strains of *E. coli* at ambient
128 temperature (12-15 °C) in orange juice, (ii) to evaluate how inactivation was affected by
129 the orange juice pulp content and (iii) to investigate if prior acid exposure of the
130 challenge microorganism significantly impacted on treatment efficacy.

131

132 **2. Materials and Methods**

133 *2.1 Bacterial strains and cultural conditions*

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

140 to fruit juice processors were measured. The bacteria were maintained as frozen stocks at
141 -70°C in the form of protective beads, which were plated onto tryptic soy agar (TSA,
142 Scharlau Chemie) and incubated overnight at 37 °C to obtain single colonies before
143 storage at 4 °C. Working cultures were prepared by inoculating a single colony into
144 tryptic soya broth (TSB, Scharlau Chemie) and incubating overnight at 37°C (Cheng, Yu
145 & Chou, 2003; Caggia, Ombretta Scifò, Restuccia & Randazzo, 2009) .

146

147 *2.2 Preparation of model orange juice (MOJ)*

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

154

155 *2.3 Preparation of orange juice*

156 *2.3.1 Fresh orange juice unfiltered*

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

162 *2.3.2 Fresh orange juice filtered (without pulp)*

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

167 *2.3.3 Fresh orange juice with reduced pulp content*

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

172

173 *2.4 Preparation of cell suspensions*

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

184

185 *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 °C. Cells were then harvested by
189 centrifugation at 10,000 rpm for 10min at 4°C. 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 °C) and incubated at 37 °C for 1h. For a 18-h acid exposure,
192 bacterial strains were grown directly in TSB (pH 5.0) at 37°C. After incubation, cultures
193 were diluted in MRD (pH 5.0) to yield approximately 10^7 cells 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 $75\text{-}78\mu\text{g mL}^{-1}$ 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

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

213

214 *2.7 Microbiological analysis*

215 The efficacy of treatments was determined in terms of reduction in viable counts over
216 time. Populations of challenge organism were determined by plating onto both TSA and
217 selective media, Sorbitol MacConkey agar (SMAC, Scharlau Chemie) respectively.
218 Samples (1mL aliquots) were withdrawn from treated juice at specific time intervals,
219 serially diluted in MRD and 0.1mL aliquots of appropriate dilutions were surface plated
220 on TSA and SMAC to compare recovery of *E. coli* strains. Plates were incubated at 37 °C
221 for 24h and then counted. Results were reported as Log₁₀CFU mL⁻¹. Data were pooled
222 and average values and standard deviations determined. Means were compared using
223 ANOVA followed by LSD testing at p < 0.05 level (SPSS, version 15.0).

224 *2.8 Inactivation kinetics*

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

$$228 \quad \log_{10}(N) = \log_{10}(N_0) - \left(\frac{t}{\delta}\right)^p \quad (1)$$

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

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

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

238

$$239 \quad t_{xd} = \delta \times (x)^{\frac{1}{p}} \quad (2)$$

240 **3. Results**

241 *3.1 Effect of ozone inactivation of E. coli in model orange juice*

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

245

246 *3.2 Effect of ozone on inactivation of E. coli in orange juice*

247 The Weibull parameters δ and p are shown in Table 1. In the present study, the shape
248 parameter p showed downward concavity for both *E. coli* strains (Fig. 2 and 3). The
249 inactivation of *E. coli* in orange juice was fitted using the Weibull model, which provided
250 estimations of microbial inactivation in terms of processing time required. The R^2 values
251 of 0.93 and above (Table 1) show that the Weibull model was a good fit for the
252 experimental data analysed. p values > 1 indicate the susceptibility of the remaining cells
253 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 500 μ m 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 500 μ m 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 residual
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 2.4 L min^{-1}
337 and ozone concentration of 0.9 g 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

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

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548 **Figure Captions**

549 Figure 1: Schematics of Ozone generator

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

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

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

556 . Curves are fitted using the Weibull model.

557 a) 1h acid exposed *Escherichia coli* ATCC 25922

558 b) 18h acid exposed *Escherichia coli* ATCC 25922

559 c) 1h acid exposed *Escherichia coli* NCTC 12900

560 d) 18h acid exposed *Escherichia coli* NCTC 12900

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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) \pm SE	$p \pm$ SE	R^2	t_{5d} (min)
<i>E.coli</i> ATCC 25922	Unfiltered	control	1.58 ^a \pm 0.84	0.80 ^f \pm 0.16	0.96	11.86 ^k
	1mm sieve	control	3.28 ^b \pm 0.33	2.98 ^g \pm 0.49	0.98	5.62 ^l
	500 μ m sieve	control	2.77 ^c \pm 0.39	3.14 ^g \pm 0.73	0.97	4.63 ^m
	Without pulp	control	2.91 ^d \pm 0.35	3.26 ^g \pm 0.69	0.98	4.76 ^m
<i>E.coli</i> NCTC 12900	Unfiltered	control	2.55 ^{ea} \pm 0.91	1.08 ^{hf} \pm 0.21	0.98	11.30 ^{nk}
	1mm sieve	control	3.24 ^{eb} \pm 0.43	2.52 ^{ig} \pm 0.52	0.97	6.14 ^{ol}
	500 μ m sieve	control	3.12 ^e \pm 0.26	2.81 ^j \pm 0.35	0.98	5.53 ^p
	Without pulp	control	3.41 ^e \pm 0.55	4.44 ^j \pm 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) \pm SE	$p \pm$ SE	R^2	t_{5d} (min)
<i>E.coli</i> ATCC 25922						
	1mm sieve	Control	3.28 ^a \pm 0.33	2.98 ^d \pm 0.49	0.98	5.62 ^h
	1mm sieve	1h acid adaptation	2.41 ^b \pm 0.52	1.63 ^e \pm 0.31	0.97	6.46 ⁱ
	1mm sieve	18 h acid adaptation	3.08 ^a \pm 0.20	2.63 ^f \pm 0.25	0.99	5.67 ^h
<i>E.coli</i> NCTC 12900						
	1mm sieve	Control	3.24 ^{ca} \pm 0.43	2.52 ^{gd} \pm 0.52	0.97	6.14 ^{jh}
	1mm sieve	1h acid adaptation	2.49 ^{cb} \pm 0.36	1.81 ^{ge} \pm 0.25	0.98	6.06 ^{ji}
	1mm sieve	18 h acid adaptation	3.47 ^{ca} \pm 0.37	2.37 ^{gf} \pm 0.35	0.98	6.84 ^j

574 * SE- standard error

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588 **Figure 1**

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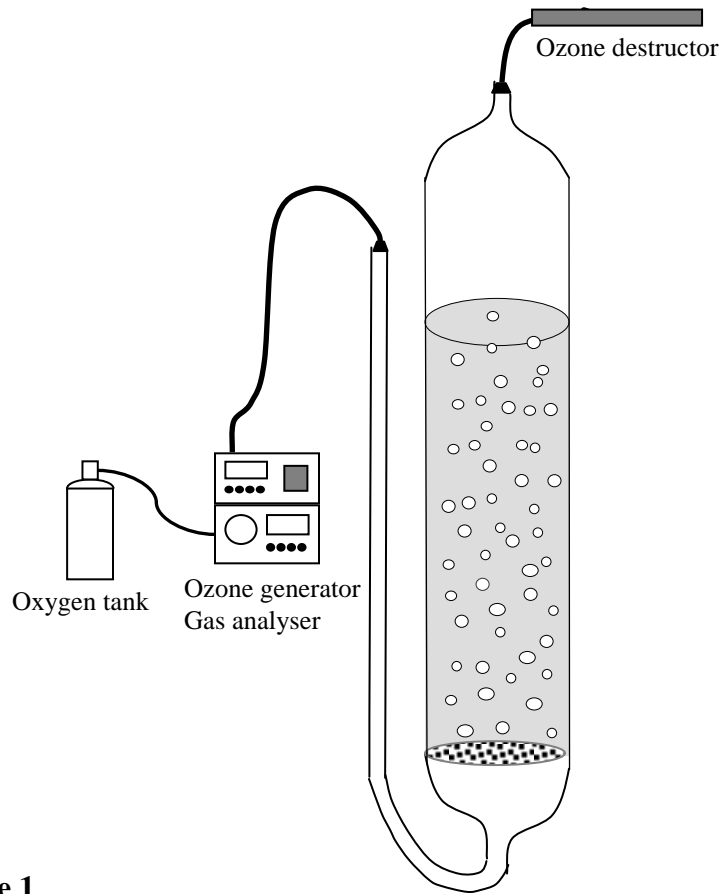
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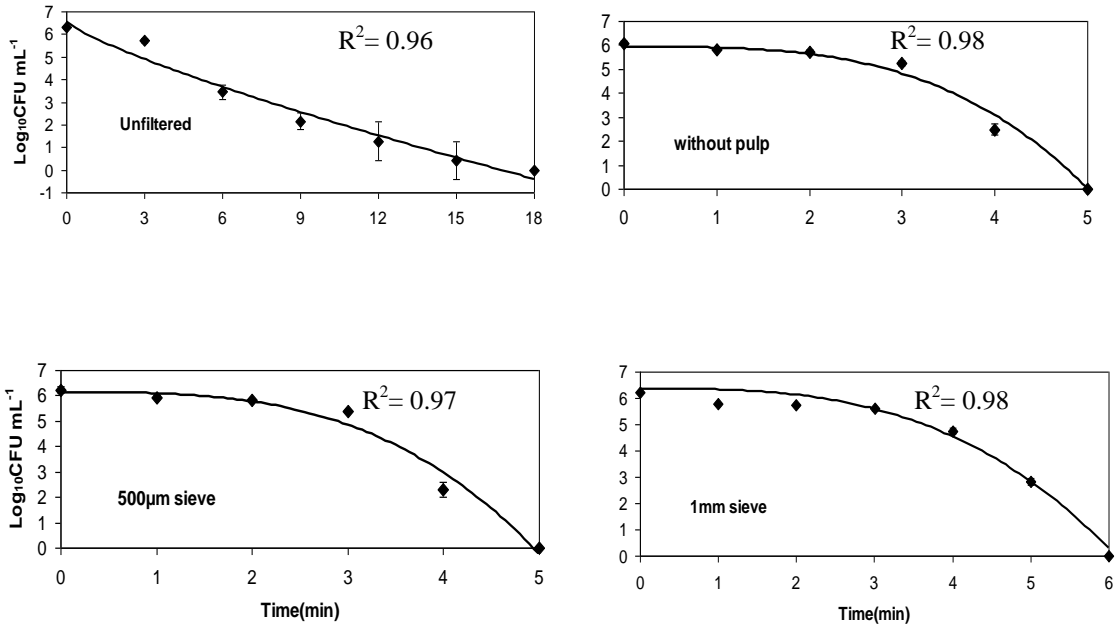
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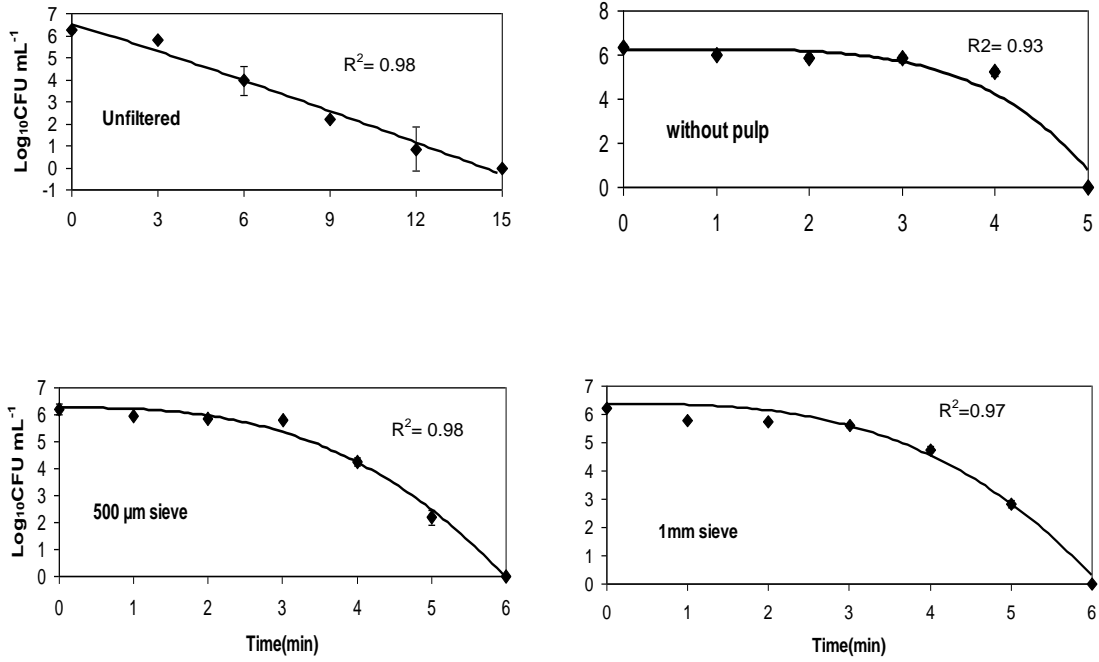
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600 **Figure 2**

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613 **Figure 3**

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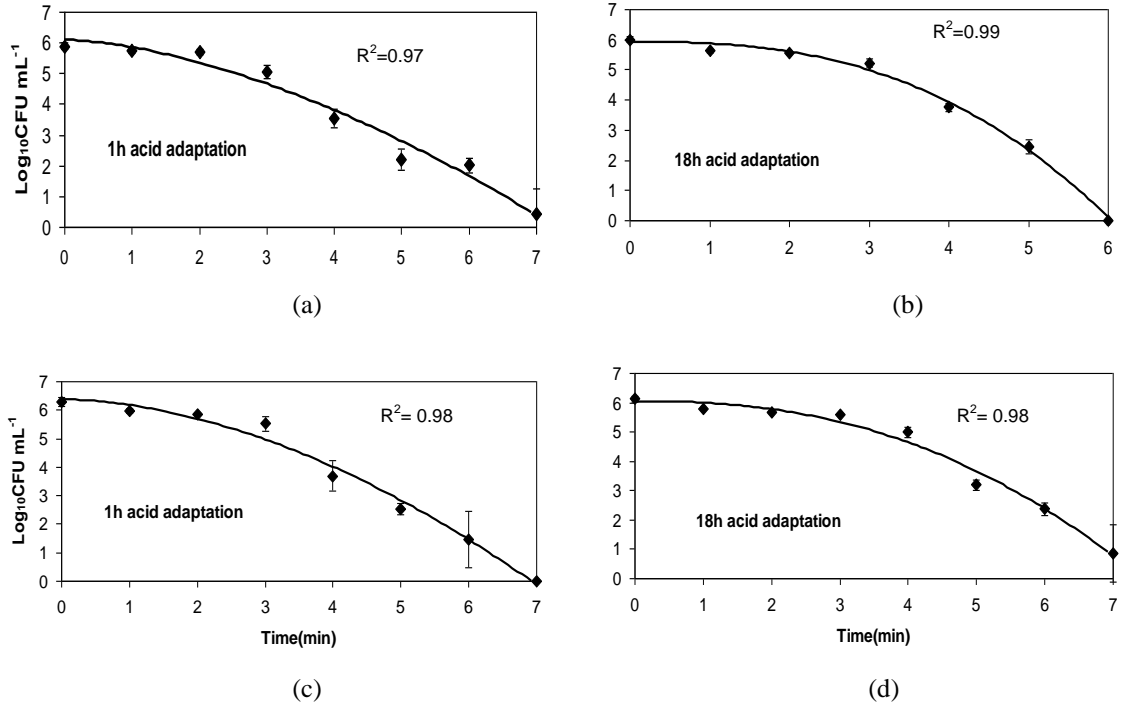
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624 **Figure 4**

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