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Ozone Inactivation of Acid Stressed Listeria Monocytogenes and Listeria Innocua in Orange Juice Using a Bubble Column

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Title: “**Ozone inactivation of acid stressed *Listeria monocytogenes and Listeria innocua* in orange juice using a bubble column**”

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Abstract

Orange juice inoculated with *Listeria monocytogenes* strains ATCC 7644, NCTC 11994 and *Listeria innocua* NCTC 11288 (10^6 CFU/ml) as challenge microorganisms was treated with direct ozone at 0.098mg/min/ml for different time periods (0-8 min) using an ozone bubble column. Ozone treatment of mild acid stressed and mild acid stress-habituated (pH 5.5) cells of *L. monocytogenes* resulted in higher inactivation times compared to control non-acid stressed cells. Additionally acid stressed cells habituated in orange juice (ATCC 7644 & NCTC 11288), showed higher inactivation times during ozonation by comparison with the control as well as the mild-acid stressed cells. Overall the gaseous ozone treatment applied to orange juice resulted in a population reduction of 5 log cycles within a time range that varied between 5 to 9 min.

Key words**: *Listeria monocytogenes*, ozone, bubble column, non-thermal inactivation, acid stress, orange juice, microbial kinetics**

1 Introduction

Listeria monocytogenes is a Gram positive, psychrotrophic pathogen ubiquitous in the environment and has been found in fruits and vegetables. *L. monocytogenes* is capable of growing at refrigeration temperatures in high salt and acid foods. *L. innocua* is often selected for inactivation studies because it is non pathogenic but still closely related to *L. monocytogenes* (Picart, Dumay & Cheftel, 2002). No outbreaks involving *L. monocytogenes* in fruit juices have been reported; however this pathogen has been isolated from unpasteurised apple juice (pH 3.78) and apple-raspberry juice blend (pH 3.75) after 1 day storage at 5 ºC (Sado, Jinneman, Husby, Sorg & Omiecinsky, 1998). This pathogen is a vehicle of human listeriosis which survived well beyond the normal shelf life of unsterile orange juices (Ryser & Marth, 1991). Oyarzábal, Nogueira and Gombas (2003) studied the survival of *L. monocytogenes* and other foodborne pathogens in apple, orange, pineapple, and white grape juice concentrates and showed that these pathogens were recoverable from all concentrates through 12 weeks of storage at -23 °C. The low pH of fruit juices plays an important role in survival of food borne pathogens. The ability of *L. monocytogenes* to respond to low pH conditions plays an integral role in its survival and resistance to acidic foods (Cotter, Gahan & Hill, 2000), thus affecting the food processing and preservation protocols. The organism can become highly resistant to even extremely acidic conditions due to stress hardening (Lou & Yousef, 1997). Some studies have shown that Acid Tolerance Response (ATR) of *L. monocytogenes*, as a consequence of stress hardening, can result in its increased thermal tolerance in apple, orange and white grape juice (Mazzotta, 2001). Strategies to meet consumer demands for better quality food products include minimal processing, which could introduce potential for pathogen survival. Caggia, Ombretta, Restuccia and Randazzo (2009) reported that orange juice and minimally processed orange juice slices can support the growth of acid adapted *L. monocytogenes*. In food processing technologies, there is an extensive use of low pH environments (decontamination by acetic acid in beef processing, fermentation etc.) which can result in the alteration of the cellular physiology of the pathogen either by *de novo* protein synthesis or by changes in the fatty acid composition of the cell membrane (Foster 1991, Phan-Thanh, Mahouin, & Alige, 2000). This can lead to enhanced resistance to any further or subsequent acid stress which may be part of a processing treatment. This acid tolerance is also termed as acid habituation which is the increased resistance to extreme pH conditions after adaptation to sublethal acidic environments (Koutsoumanis & Sofos, 2004). *L. monocytogenes* is more resistant than many foodborne pathogens to organic acids and can be difficult to control in food processing facilities (Johnson, 2003), therefore it is necessary to evaluate responses of *Listeria* cells exposed to different acidic conditions.

The US Food and Drug Administration (US FDA) issued a final rule requiring fruit and vegetable juice producers to apply a 5-log pathogen reduction process (US FDA, 2004_a). 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, to produce foods with a minimum of nutritional, physicochemical, or organoleptic changes induced by these technologies (Esteve & Frigola, 2007), whilst maintaining safety profiles with respect to pathogens of concern. The FDA’s approval of ozone as a direct additive to food in 2001 triggered interest in ozone applications, with a number of commercial fruit juice processors in the US and

Europe employing ozone for pasteurization, resulting in industry guidelines being issued by the FDA (USFDA, 2004_b). Ozone is a triatomic allotrope of oxygen and is characterized by a high oxidation potential that conveys bactericidal and viricidal properties (Burleson, Murray & Polard, 1975; Kim, Yousef & Dave, 1999). Ozone inactivates microorganisms through oxidization 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). Ozone as an oxidant is used in natural water treatment, washing and disinfecting of fruits and vegetables, and juice processing to inactivate pathogenic and spoilage microorganisms (Muthukumarappan, Halaweish & Naidu, 2000). In a gas or aqueous phase, ozone has been used to inactivate microorganisms and decontaminate meat, poultry, eggs, fish, fruits, vegetables and dry foods (Fan, Song, McRae, Walker & Sharpe, 2007). Tiwari, Muthukumarappan, O'Donnell and Cullen (2008, 2009_a) and Tiwari, O'Donnell, Patras, Brunton and Cullen (2009_b) recently highlighted that nutritional quality depends on the ozone control parameters of concentration and gas flow rate. Achieving rapid microbial inactivation using optimized control parameters while retaining the nutritional quality is of overall importance.

The objectives of this study were to investigate (i) the efficacy of gaseous ozone treatment for reduction of *L. monocytogenes* and *L. innocua* at ambient temperature in orange juice, (ii) ozone treatment efficacy in orange juice inoculated with the acid stressed *Listeria* population, using a range of acid stress conditions, namely mild acid stressed, mild acid stress-habituated and acid stressed but habituated in orange juice.

2. Materials and Methods

2.1 Bacterial strains

Three strains of *Listeria* were used in this study. *L. monocytogenes* ATCC 7644, *L. monocytogenes* NCTC 11994, and *L. innocua* NCTC 11288 obtained from microbiology stock culture, School of Food Science and Environmental Health, Dublin Institute of Technology. Strains were maintained as frozen stocks at -70 ºC in the form of protective beads, which were plated onto tryptic soy agar (TSA, Barcelona, Scharlau Chemie) and incubated overnight at 37 ºC to obtain single colonies before storage at 4 ºC.

2.2 Preparation of orange juice

Oranges (variety: Navalate, Peru) were purchased from a local market and squeezed with a fruit juicer (Rowenta PA4002NEO). The fresh orange juice was then submitted to a finishing process by passing through a sieve (Laboratory test sieve, Retsch, Germany) of 1mm diameter (mesh no. 18) to reduce the pulp content (Patil, Bourke, Frias, Tiwari & Cullen, 2009_a). 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 found to be in the range of 3.5-3.7.

2.3 Experimental design

In order to investigate the efficacy of ozone against *L. monocytogenes* and *L. innocua* microbial populations, four different conditions were investigated;

a) To obtain a non acid stressed control *Listeria* population, cells were grown in TSB without glucose (TSB-G). TSB-G was used as the basic medium for obtaining control cells as presence of glucose in the medium results in mild acid stress of cells by reducing the pH of TSB to 4.9.

b) To obtain mild acid stressed *Listeria* population, cells were grown in TSB with glucose (TSB+G, 0.25%).

c) To obtain 1 h mild acid stress-habituated *Listeria* population, cells were grown in TSB+G, 0.25% and then habituated at pH 5.5 (adjusted using 80% lactic acid) for 1 h and to obtain 18 h mild acid stress-habituated *Listeria* population, cells were grown in TSB+G, 0.25% (pH 5.5).

d) To obtain a *Listeria* population habituated in orange juice, cells were grown in TSB+G, 1.25% leading to acid stressed cells which were then habituated in orange juice for 90 min at 37 ºC. Cells prepared under these different conditions were then treated with ozone in orange juice.

2.4 Preparation of cell suspensions and culture conditions

For the first (a) and second investigation (b), a single isolated colony of each strain was inoculated separately either in TSB-G or in TSB+G, 0.25% to produce non acid stressed cells (control sample) and mild acid stressed cells, respectively. Cultures were then incubated overnight at 37 ºC and were then harvested by centrifugation (SIGMA 2K15, Bench Top Refrigerated Ultracentrifuge, AGB scientific LTD.) 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 550nm using McFarland standard (BioMérieux, Marcy -l'Etoile, France). The inoculum was then diluted in maximum recovery diluent (MRD, Scharlau Chemie) to obtain approximately 10^7cells/ml. For each investigation, the cell concentration was further diluted in orange juice to yield a final concentration of 10^6 cells/ml and then ozone treatment was applied.

For the third investigation (c), two acid stress-habituation conditions were imposed, i.e., 1 hour and 18 hours. For the 1 hour habituation environment, working cultures were grown overnight in TSB+G, 0.25% at 37 ºC (thus creating a mild acid stress environment). Cells were then harvested by centrifugation at 10,000 rpm for 10min at 4 ºC. The cell pellet was washed twice with sterile PBS, re-suspended in 10 ml TSB adjusted to pH 5.5, and incubated at 37 ºC for 1h (Cheng, Yu & Chou, 2003; Caggia et al., 2009). To prepare 18 h habituated cells, bacterial strains were grown directly in TSB+G, 0.25% (pH 5.5) at 37 ºC. The mild acid stress-habituated cells were diluted in MRD (pH 5.5) to yield approximately 10^7cells/ml, with further dilution in orange juice (pH 3.5-3.7) to a final concentration of 10^6cells/ml and then ozone treatment was applied. For the fourth investigation (d) the working cultures were incubated overnight in TSB+G, 1.25% at 37 ºC. This was performed to produce a more acid stressed population, as described by Buchanan and Edelson (1996) with some modifications. The pH of the culture following overnight incubation was measured using a pH meter with a glass electrode and was found to be in the range of 4.4-4.6. Cultures were then centrifuged as described above and cell pellet was resuspended directly in 10ml orange juice (pH 3.5-3.7) and incubated at 37 ºC for 90 min. Cultures were further diluted in orange juice to yield an approximate final concentration of 10^6 – 10^7cells/ml and then ozone treatment was applied.

2.5 Ozone treatment

Ozone gas was generated using an ozone generator (Model OL80, Ozone services, Burton, Canada, Fig. 1). Ozone was produced by a corona discharge generator. Pure oxygen was supplied via an oxygen cylinder (Air Products Ltd., Dublin, Ireland) and the flow rate was controlled using an oxygen flow regulator. A previously determined optimum flow rate of 0.12L/min with an ozone concentration of 0.098mg/min/ml was applied for each treatment (Patil, Cullen, Kelly, Frias & Bourke, 2009_b). Excess ozone was destroyed by an ozone destroyer unit. To prevent excess foaming, 20 µl sterile anti-foaming agent (Antifoam B emulsion, Sigma Aldrich, Ireland Ltd.) was added before each ozone treatment. The treatment of all orange juice samples previously inoculated with *Listeria* strains (as described in section 2.4) was carried out for 7-8 minutes with sampling intervals of 1 min. All experiments were performed in duplicate and replicated at least twice.

2.6 Microbiological analysis

The efficacy of treatment was determined in terms of reduction in viable counts over time. Populations of challenge organism were determined by plating onto TSA and selective media (Palcam), 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 Palcam agar. Plates were incubated at 37 ºC for 48 h and then colony forming units were counted. Results were reported as Log_{10}CFU/ml. Data were pooled and average values and standard deviations were determined. Means were compared using ANOVA followed by LSD testing at $p < 0.05$ level (SPSS, version 15.0).

2.7 Microbial 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 (Mafart, Couvert, Gaillard & Leguerinel, 2002) was used to analyze the data:

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

where N (CFU/ml) is the number of microorganisms at time t, N_0 (CFU/ml) 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 estimates of δ and p were used to calculate a desired log reduction. The time required to obtain a 5 log reduction (t_{xd}) was calculated using equation 3. For this case study x was equal to 5

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

2.8 Determination of degree of injury and recovery index

The non-selective medium TSA was expected to support the growth of both uninjured and ozone injured cells whereas the selective medium, Palcam agar was expected to support growth of uninjured populations. The difference from selective to non-selective media gives an indication of cell injury during the ozone treatment. Percent injury was calculated by using equation 3 (Hansen & Knochel, 2001). It was calculated by choosing the time intervals of samples which resulted in colony formation on both the media used.

$$\%\ \text{injured cells} = \frac{\text{cfu/ml on TSA} - \text{cfu/ml on Palcam}}{\text{cfu/ml on TSA}} \times 100 \qquad (3)$$

A recovery index was defined as the t_{5d} (time required to obtain a 5 log reduction) determined from the counts on the Palcam divided by t_{5d} determined from the counts on TSA (Hansen & Knochel, 2001).

3. Results

The inactivation kinetics of *Listeria* in orange juice were fitted using the Weibull model, which provided estimations of microbial inactivation parameters in terms of the processing times required. The Weibull parameters δ and p are shown in Table 1. The shape parameter p, gave downward concavity for the kinetic curves of all the *Listeria* strains (Figs. 2, 3 and 4). p values of >1 indicates a greater susceptibility of microorganisms to the treatment (van Boekel, 2002).

3.1 Inactivation of *Listeria monocytogenes* NCTC 11994

The inactivation curves of *L. monocytogenes* NCTC 11994 are shown in Fig. 2. Ozone treatment of mild acid stressed population required a longer treatment time to achieve reduction by 5 log cycles (t_{5d}) compared to control non acid-stressed cells. For these test conditions, significant differences were observed for recovery index as well as for t_{5d} ($p<0.05$) (Table 1). Ozone treatment of 18 h acid stress-habituated population recorded the highest time required for achieving t_{5d} compared to other test conditions investigated (Table 1). Recovery index and t_{5d} values for acid stress-habituated cells showed significant difference compared to the other test conditions ($p<0.05$). In the case of acid stressed cells habituated in orange juice, t_{5d} was achieved in comparatively less time than that required for mild acid stressed and 1 h or 18 h acid stress-habituated cells (Table 1). In the case of cells habituated in orange juice, lower % injury was obtained (Table 1) and for the precise estimation of the uninjured vs. the injured population, counts on Palcam agar were recorded for up to 6 min of ozone treatment by which time the detection limit was not reached for both media used.

3.2 Inactivation of *Listeria monocytogenes* ATCC 7644

Survivor curves for *Listeria* strain ATCC 7644 following ozone treatments are presented in Fig. 3. In the case of control non acid-stressed, mild acid stressed and acid stressed cells habituated in orange juice, t_{5d} was achieved in less than 6 min of ozone treatment with no significant differences obtained with the recovery index for any of the test conditions studied (Table 1).

In the case of acid stress-habituated populations (1 h and 18 h), a significant difference was observed in t_{5d} values compared to the three other test conditions investigated ($p<0.05$). At all test conditions where acid stress was applied, ≥ 97.4% injury was observed indicating the efficacy of ozone in conjunction with applied acid stress conditions (Table 1). However, for the control non acid stressed cells, a smaller % injury was observed.

3.3 Inactivation of *Listeria innocua* NCTC 11288

Ozone inactivation curves of *L. innocua* cells for different test conditions are shown in Fig. 4. The control non acid-stressed and mild acid stressed cells were reduced by 5 log cycles in short treatment times (Table 1).

Mild acid stress-habituation of cells for the longer duration (18h) followed by ozone treatment resulted in significantly higher t_{5d} value compared to other test conditions investigated (Table 1). However, a significant difference was observed in t_{5d} values for orange juice habituated cells, compared with mild acid stressed cells and control non acid-stressed cells ($p<0.05$).

The lower % injury observed for acid stressed cells habituated in orange juice after 7 min ozone treatment underlines the importance of investigating the efficacy of ozone in real product formulations in addition to simulated stress conditions in model media.

4. Discussion

The direct application of ozone was found to be effective for the inactivation of *Listeria* in orange juice (Figs. 2, 3, and 4). However, there were some significant effects of bacterial cell pre-treatment and condition observed on inactivation efficacy. The pre-treatments and conditions employed were designed to mimic the environment that a contaminating population could be exposed to in orange juice and other food processing scenarios. Literature studies on the efficiency of ozone for inactivating *Listeria* in food products vary (Olmez & Akbas, 2009; Rodgers, Cash, Siddiq & Ryser, 2004; Vaz-Velho, Silva, Pissoa & Gibbs, 2006; Yuk, Yoo, Yoon, Moon, Marshall & Oh, 2006). Olmez & Akbas (2009), stated that the efficiency of ozone treatment can be related to the delivery method.

Applying a mild acid stress actually increased the ozone treatment time required for a 5 log reduction for both strains of *L. monocytogenes* by comparison with the control population. However, in the case of *L. innocua*, applying a mild acid stress did not significantly effect the ozone treatment time required by comparison with the control. Leistner (2000) reported that simultaneous exposure of bacteria to different stress factors requires increased energy consumption and leads bacteria to cellular death through metabolic exhaustion.

Foodborne bacteria encounter organic and inorganic acids in foods or in the gastrointestinal tract and cells of the host (Yousef & Courtney, 2003). Adaptation of *L.*

monocytogenes to sublethal stresses has been demonstrated to protect the pathogen to a variety of normally lethal conditions present in certain foods (Lou and Yousef, 1997). The resistance or adaptation of microorganisms to acid conditions can have implications for food safety. In this study, acid stress-habituated *Listeria* cells had an increased resistance to ozone treatment and also recorded the highest time for achieving 5 log (t_{5d}) reductions. Similar findings of significantly increased resistance of *L. monocytogenes* to heat were reported by Mazzotta (2001) after acid adaptation of *Listeria* in single strength apple, orange and white grape juices adjusted to pH 3.9. Caggia et al. (2009) recorded the highest acid tolerance response of *L. monocytogenes* OML 45 strain, after 3h treatment in TSB adjusted to pH 5.7, thus concluding that cells adapted to acidic environments can grow in normally lethal pH conditions.

It has been reported that the heat and acid resistance of *L. monocytogenes* are strain dependant (Skandamis, Yoon, Stopforth, Kendall & Sofos, 2008). Phan-Thanh et al. (2000) reported the lowest pH value which *L. monocytogenes* could resist was dependant on the strain and the kind of acid used. Our results also showed that the extent of increased acid resistance varied with the bacterial strain and acid stress conditions. Strain NCTC 11994 was the most resistant strain independent of the applied conditions.

In orange juice production, low acidic conditions are present before the pasteurization process and may induce an ATR that can result in increased thermal tolerance (Caggia et al., 2009). The exposure to sequential acid stressors such as a prior acid stress followed by an acid environment in the product may result in cross protection to a subsequent processing treatment as observed here. In the case of all 18 h acid stress-habituated populations, the highest t_{5d} values were estimated, however, lower recovery indices were reported, where greater recovery of cells was evident on non-selective media by comparison with selective media (Table 1). The applied acid stress did not promote recovery on selective medium (Palcam) at the same rate of the recovery on non-selective medium (TSA), however the injured sub-population may have a greater resistance to ozone. Therefore, to mimic the stresses encountered in food processing environments, conditions like acid stress-habituation and habituation in actual orange juice should be considered for determining inactivation parameters (e.g., t_{xd}, %injury, recovery index) and process design in foods.

From the present study and based on the different inactivation responses to ozone treatment it was also observed that inactivation responses of *L. innocua* NCTC 11288 were closer to those of *L. monocytogenes* ATCC 7644 than *L. monocytogenes* NCTC 11994.

5. Conclusions

This work has shown that direct ozone treatment can be used to inactivate *L. monocytogenes* and *L. innocua* in orange juice. The efficacy of ozone treatment was found to be a function of strain and duration of acid stress-habituation conditions. The data also indicate that adaptive stress responses should be taken into account for process design or method development for the inactivation of *L. monocytogenes*. Inactivation times for a 5 log cycle reduction were achieved in between 5.08 and 8.44 min. Therefore, direct ozone diffusion treatment could be used as a potential alternative to traditional thermal pasteurisation for control of *Listeria* populations in fruit juices or other liquid foods.

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Figure captions

Figure 1 Schematics of the ozone processing equipment.

Figure 2 Ozone inactivation of *Listeria monocytogenes* NCTC 11994

(a) Control non acid-stressed cells

(b) Mild acid-stressed cells

(c) 1 h acid stress-habituated cells

(d) 18 h acid stress-habituated cells

(e) Habituated cells in orange juice

Figure 3 Ozone inactivation of *Listeria monocytogenes* ATCC 7644

(a) Control non acid-stressed cells

(b) Mild acid-stressed cells

(c) 1 h acid stress-habituated cells

(d) 18 h acid stress-habituated cells

(e) Habituated cells in orange juice

Figure 4 Ozone inactivation of *Listeria innocua* NCTC 11288

(a) Control non acid-stressed cells

(b) Mild acid-stressed cells

(c) 1 h acid stress-habituated cells

(d) 18 h acid stress-habituated cells

(e) Habituated cells in orange juice

Table 1: Parameters of the Weibull model and the time required to reach a 5 log reduction for *Listeria* strains in orange juice (Different letters indicate a significant difference at the 0.05 level between each type of condition).

Microorganism	Condition	δ(min) ± STE	p± STE	R^2	t_{5d} (min)	Recovery index	% injury
L. monocytogenes NCTC 11994	Control non-acid stressed	3.48±0.64	3.17±1.04	0.93	5.78^{a}	0.99^{k}	95.9
	mild-acid stressed cells	3.07± 0.55	1.97± 0.41	0.96	6.95^{b}	0.76^{l}	99.7
	1h acid stress-habituation	4.05± 0.40	2.64± 0.38	0.98	7.45^{c}	0.79^{l}	97.8
	18 h acid stress-habituation	4.45± 0.69	2.52± 0.65	0.93	8.44^{d}	0.60^{lm}	99.9
	Habituated cells in orange juice	2.96± 0.73	1.97± 0.48	0.94	6.69^{ab}	0.89^{k}	76.6
L. monocytogenes ATCC 7644	Control non-acid stressed	2.99±0.47	2.84±0.64	0.94	5.27^{e}	0.98^{n}	91.6
	mild-acid stressed cells	3.17± 0.30	2.89± 0.42	0.98	5.53^{e}	1.00^{n}	99.8
	1h acid stress-habituation	4.12± 0.90	2.74± 0.89	0.90	7.41^{f}	0.75^{o}	99.3
	18h acid stress-habituation	4.54± 0.52	3.00± 0.60	0.95	7.77^{f}	0.80^{n}	99.2
	Habituated cells in orange juice	1.43± 0.56	1.14± 0.24	0.95	5.87^{e}	0.86^{n}	97.4
L. innocua NCTC 11288	Control non-acid stressed	2.94±0.66	2.66±0.82	0.91	5.38^{h}	0.96^{p}	74.6
	mild-acid stressed cells	3.44± 0.47	4.14± 1.45	0.94	5.08^{h}	1.0^{q}	99.8
	1h acid stress-habituation	4.17± 0.34	4.33± 0.96	0.97	6.05^{i}	0.85^{pr}	98.4
	18h acid stress-habituation	4.12± 0.42	2.62± 0.40	0.97	7.60^{j}	0.80^{r}	89.5
	Habituated cells in orange juice	1.82± 0.88	1.30± 0.40	0.91	6.26^{i}	0.83^{r}	66.7

δ – time for the first decimal reduction
STE - standard error
p - parameters related to the scale and shape of the inactivation curve
R^2 - coefficient of determination
% injury- calculated using equation 1
Recovery index- t_{5d} determined on Palcam divided by t_{5d} determined on TSA

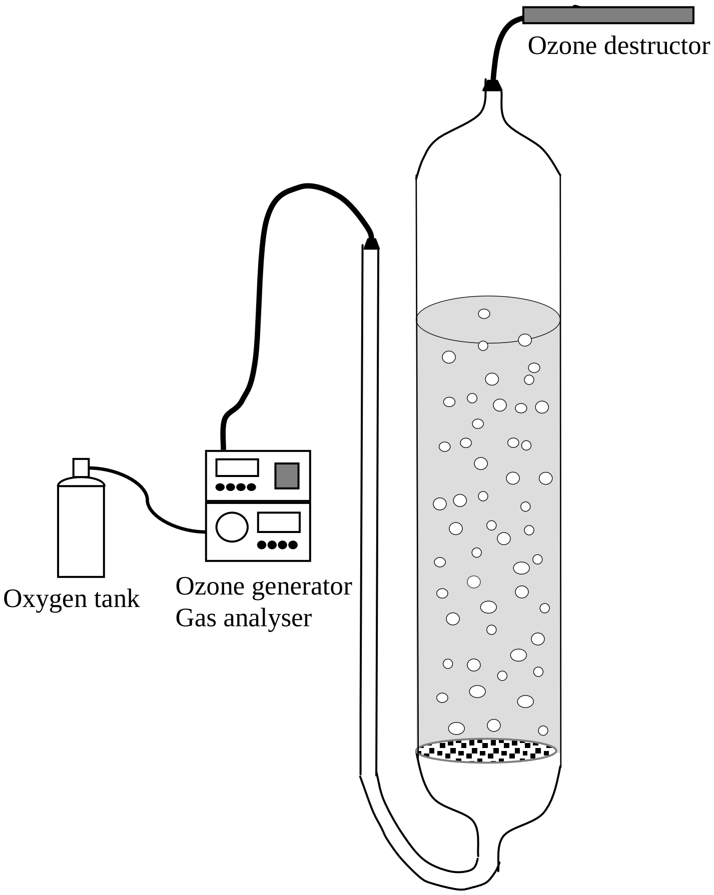
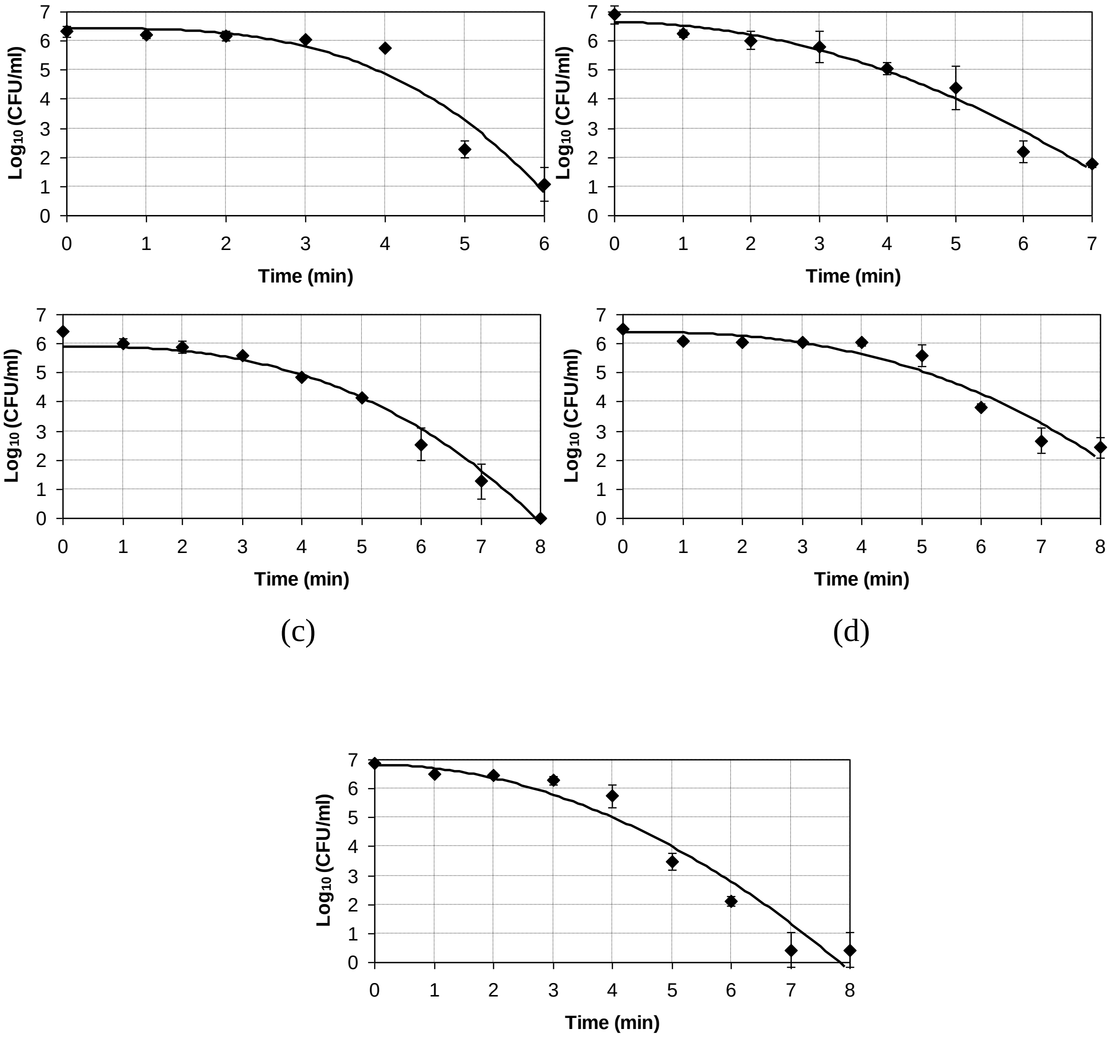

Ozone destructor
Oxygen tank
Ozone generator
Gas analyser

Fig. 1



(a) (b)

(c) (d)

(e)

Fig. 2

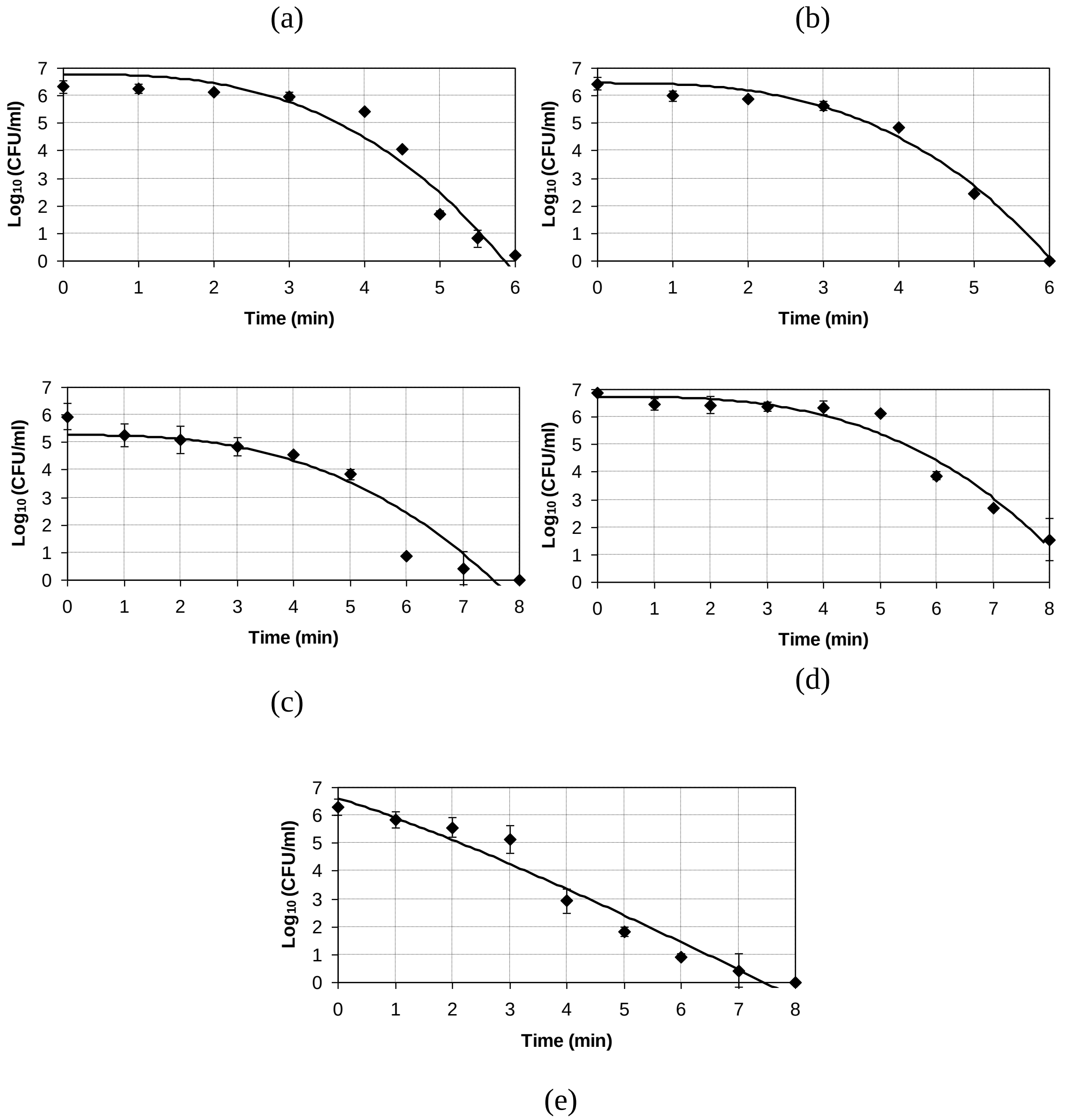
(a)
(b)
Log10(CFU/ml)
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
Time (min)
(c)
(d)
0
1
2
3
4
5
6
7
8
Time (min)
(e)

Fig. 3

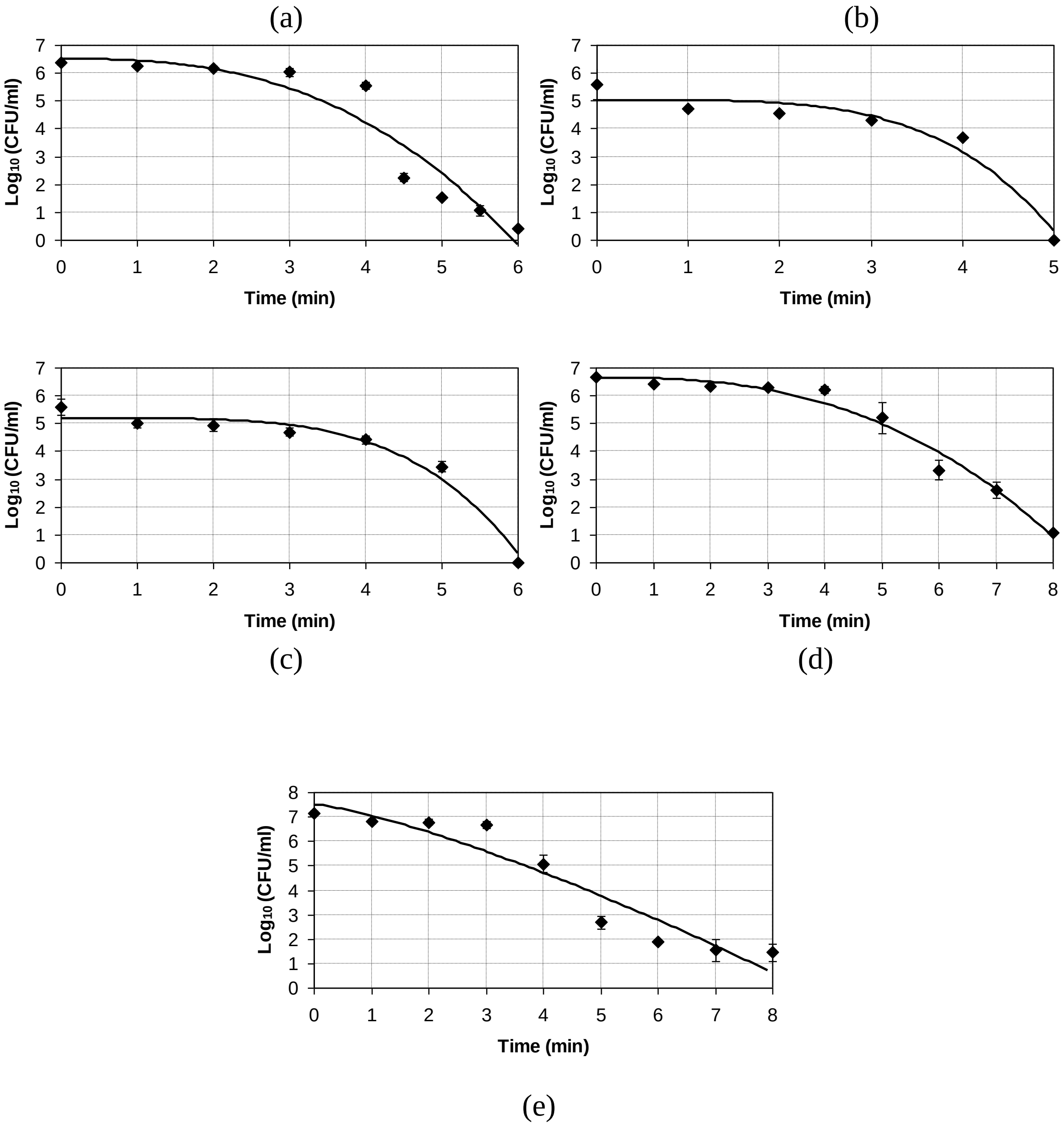


Fig. 4