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Analysis of the variability in microbial inactivation by acid treatments

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ABSTRACT

The variability in microbial inactivation through acid treatments was evaluated in the present study. *Enterococcus faecalis*, *Listeria innocua*, *Salmonella enterica*, and *Pseudomonas fluorescens* were inoculated in buffered peptone water, chicken soup, and citrate solution, and were subjected to acid treatments of various intensities to reach a microbicidal effect of 0, 2, 3, and 4 logarithmic cycles. The variability in the number of survivors was greater in treated than in untreated samples. Furthermore, the effect of acid treatment on survival variability depended on the intensity of the acidification. More specifically, as the intensity of the applied acid treatment increased, the number of viable microorganisms, although smaller, was more variable. The results of this study indicate that the inactivation behaviour of microbial cells within a population is subject to variation; such variability must be quantified and taken into account in predictive food microbiology, and it can be valuable for risk assessment purposes when acidification of food is involved.

1. Introduction

Given the increasing consumer demand for ready-to-eat (RTE) or minimally-processed food, the food industry has applied and continues to develop strategies aimed at providing a variety of high-quality, safe, and nutritionally balanced food products. With particular reference to the microbiological quality and safety of foods, a method that has been used extensively for food preservation is acidification through either fermentation or addition of weak acids. These organic acids (e.g., acetic, ascorbic, citric, lactic and malic), in addition to their contribution to the development of desirable organoleptic/technological traits, have been commonly used in the food industry due to their microbicidal activity (Hartwing & McDaniel, 1995).

Despite the fact of the effectiveness of the acidification, biological systems are extremely complex, particularly with regard to the application of preservation treatments where many variables are involved. For instance, a non-homogeneous inoculum size in a product batch before its treatment will lead to a distributions of number of survivors, although many zeros will be found, units with "1, 2, 3" etc., survivors can be detected, even if it is assumed a similar initial microbial load, the survivors after a treatment is inherent not the same per each unit. This variability response depends on the intensity of the treatment (Aguirre, Pin, Rodriguez, & García Fernando, 2009). More specifically, the greater the intensity of the applied treatment, the smaller the number of viable cells, with the latter number being highly variable (Aguirre et al., 2009, Aguirre, Rodriguez, & García Fernando, 2011).

Biological variability may be associated with strain variability (Lianou, Stopforth, Yoon, Wiedmann, & Sofos, 2006) and/or intrinsic cell-to-cell variability (i.e. the variable manner in which individual cells respond to environmental conditions) (Nauta, 2000). This intrinsic variability may reflect the differential physiological states of single cells, which are affected by the environmental conditions (including potential stress conditions) that they were previously exposed to (Augustin, Carlier, & Rozier, 1998; Robinson et al., 2001; Whitell, 1942), and, such variability is expected to have a considerable impact on the kinetic behaviour of

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microorganisms, its modelling, and on the processing design to control them (Aguirre et al., 2011). The variation may be even more relevant, especially when a "minimal" treatment is applied, expecting to inactivate all the cells; for instance, in foods in which the presence of a pathogen in a determined weight (usually 25 g) is unacceptable. Knowledge of only the mean population decline is unlikely to be a sufficient basis for processing design. On the other hand, the use of the 'worst-case' scenario approach in food processing leads to unrealistic estimations with negative impacts on food quality (Aspridou & Koutsoumanis, 2015), although it may be desirable from a safety point of view.

Ultimately, the number of survivors and its variability will affect and determine the shelf-life of foods during storage. Given that both the variability and uncertainty of each parameter involved in overall risk estimation should be taken into account (Delignette-Muller & Rosso, 2000), characterizing the variability in microbial inactivation is expected to be useful in quantitative microbial risk assessment (QMRA) approaches. Nevertheless, although the biological variability of the number of viable bacteria has been studied with regard to inactivation caused by heat (Aguirre et al., 2009; Mackey, Kelly, Colvin, Robbins, & Fryer, 2006; Metris, George, Mackey, & Baranyi, 2008), irradiation (Aguirre et al., 2011), and high pressure (Cuevas-Muñoz et al., 2013), as well as with respect to individual cell lag phases and growth rate after acidification (Lianou & Koutsoumanis, 2011, 2013; Metris, George, & Baranyi, 2006; Rasch, Metris, Baranyi, & Bjorn Budde, 2007), heat (Metris et al., 2008), osmotic stress (Niven, Morton, Fuks, & Mackey, 2008), and irradiation (Aguirre et al., 2011), few data are available on the effect of acidification on inactivation variability.

The aim of this work was to quantify and analyse the variability in the number of survivors during inactivation through acidification of Gram-positive bacteria *Enterococcus faecalis* and *Listeria innocua*, as well as to the Gram-negative bacteria *Salmonella enterica* serovar Enteritidis, and *Pseudomonas fluorescens*. To do this, two substrates were used: buffered peptone water as a model system, and chicken soup as food system. The latter was chosen because the poultry industry has been involved in several recalls and outbreaks. Both systems were acidified using acetic acid. Citrate solution was used as a control.

2. Materials and methods

2.1. Bacterial strains and preparation of inocula

E. faecalis (LM24), *L. innocua* (ATCC33090), *S. enterica* serovar Enteritidis (ATCC 13076), and *P. fluorescens* (CECT 378) were kept frozen (−20 °C) in tryptic soy broth (TSB, Pronadisa, Madrid, Spain) supplemented with 10% glycerol (Panreac Chemistry S.A., Barcelona, Spain). The bacterial strains were activated and subcultured in sterilised TSB and incubated at 37 °C for 24 h, with the exception of *Pseudomonas fluorescens* which was incubated at 25 °C for 36 h. The strains' cultures were centrifuged (8000 × *g* for 9 min at 5 ± 1 °C) in a Sorvall RC5B refrigerated centrifuge. Pellets were washed with 10 ml of sterile saline solution (0.80% NaCl [Panreac Chemistry S.A.]), centrifuged again at the same conditions, and the pellets were resuspended in sterile saline solution (diluted in 40 ml of saline solution), resulting in a final inoculum concentration of approximately 10^8 cfu/ml.

2.2. Acid challenge trials

The acid challenge trials were undertaken in duplicate test tubes containing three different substrates: (i) sterile citrate solution (citric acid) 0.1 N ($C_6H_8O_7$ [Panreac Chemistry S.A.] and $C_6H_5O_7Na_3 \cdot 2H_2O$ [Panreac Chemistry, S.A.]), (ii) chicken soup (Don Simon™, Almeria, Spain), and (iii) sterile buffered peptone water (Oxoid LTD., Hampshire, United Kingdom). The ingredients of the chicken soup are water, chicken, carrots, onions, parsley, olive oil, salt, corn starch, and aromas, with a composition of 0.4% fat, 0.7% carbohydrates, and 0.6% protein, as stated by the manufacturer. The pH of Chicken soup and buffered peptone water were adjusted with glacial acetic acid (Panreac Chemistry S.A.). The pH values used for acidification treatments, shown in Table 1, were determined in aliquots after sterilization. Each tube containing 9 ml of substrate was inoculated with 1-ml of the bacterial suspensions, and at regular time intervals (1–5 min) 100 μl-aliquots were removed and immediately mixed in tubes with 900 μl of sterile phosphate buffer solution 0.2 N (pH 7.0 Na_2HPO_4 [Scharlau, Barcelona, Spain] and $NaH_2PO_4 * H_2O$ [Merck, Darmstadt, Germany]) in order to stop the inactivation treatment. Bacterial suspensions were surface-plated on tryptic soy agar (TSA, Pronadisa, Madrid, Spain) using a spiral platter system (model Eddy Jet, IUL Instruments, Barcelona, Spain), and surviving bacteria were enumerated, using an image analyser (model Countermath Flash, IUL Instruments, Barcelona, Spain), after incubation of the plates at 37 °C for 48 h (*L. innocua*, *E. faecalis*, *S.* Enteritidis) or at 25 °C for 72 h (*P. fluorescens*).

2.3. Inactivation kinetic parameters

The characterization of the inactivation kinetic behaviour of the tested organisms was based on the estimation of (i) the time required for an 1−log population reduction (D_{pH} value) and (ii) the difference in pH needed to increase or decrease the D_{pH} value by 10 times (z_{pH} value). Both of the above inactivation kinetic parameters were estimated from the inactivation curves of the bacterial strains corresponding to the different treatments applied. More specifically, the D_{pH} values were calculated as the inverse of the absolute value of the slope of the graphic representing the log of the number of survivors as a function of time. Accordingly, the z_{pH} values were estimated as the inverse of the slope of the straight line describing log D_{pH} as a function of pH.

2.4. Variability in inactivation

In order to quantify the variability of the number of survivors to acidification treatments, 4.5-ml portions of each one of the three substrates, contained in test tubes, were adjusted to different pH values (see Table 2), and then inoculated with 0.5-ml aliquots of the bacterial suspensions, as described above. In total, 75 tubes were used for each treatment, while 30 tubes, containing the same substrates with pH adjusted to 7.0, were used as controls. By using the D_{pH} values (Table 1), the times required to obtain from 0 to 4 logarithmic reductions in the populations of each bacterium in the above substrates were applied. When the acid treatment was completed, an aliquot of 100 μl from each tube was removed and mixed with 900 μl of sterile phosphate buffer solution 0.2 N (pH 7.0) in Eppendorf tubes in order to stop the microbicidal effects. To minimize the error due to processing a high number of samples, the order of the tubes was the same for inoculation and for sampling, and the time spent on inoculation was kept as close as possible to the time spent for sampling.

Forty microlitres of each sample (from each Eppendorf tube) was further diluted, if needed, and plated onto TSA by using a spiral platter system. The plates were incubated at 37 °C for 48 h (*L. innocua*, *E. faecalis*, *S.* Enteritidis) or at 25 °C for 72 h (*P. fluorescens*), and colonies were counted with a digital colony counter.

All experiments described in Sections 2.3–2.5 were carried out in triplicated.

Table 1
Estimated D_{pH} and z values of selected bacterial species in different substrates.

Bacterium	Acid challenge medium	pH	D value[a] (min)	SE[b]	R^2	z values	SE[b]	R^2
E. faecalis	Peptone water/acetic acid	3.03	1.16	0.0337	0.98	0.27		
	Peptone water/acetic acid	3.49	47.39	0.0018	0.67			
	Chicken soup/acetic acid	2.59	1.55	0.0459	0.90	0.38	0.32	0.99
	Chicken soup/acetic acid	3.07	51.02	0.0009	0.86			
	Chicken soup/acetic acid	3.52	454.55	0.0009	0.10			
	Citrate solution	2.39	1.86	0.0096	1.00	0.78		
	Citrate solution	3.51	6.12	0.0072	0.97			
L. innocua	Peptone water/acetic acid	3.51	20.12	0.0049	0.83	0.55		
	Peptone water/acetic acid	4.00	158.73	0.0012	0.54			
	Chicken soup/acetic acid	3.09	5.69	0.0195	0.93	0.55	0.28	0.98
	Chicken soup/acetic acid	3.50	52.91	0.0005	0.99			
	Chicken soup/acetic acid	4.01	270.27	0.0006	0.68			
	Citrate solution	2.63	2.78	0.0198	0.97	0.65	0.17	0.98
	Citrate solution	3.09	7.15	0.0152	0.92			
	Citrate solution	3.55	73.53	0.0008	0.94			
	Citrate solution	4.04	322.58	0.0006	0.57			
P. fluorescens	Peptone water/acetic acid	3.84	4.36	0.0098	0.96	0.28		
	Peptone water/acetic acid	4.08	32.57	0.0025	0.76			
	Chicken soup/acetic acid	3.33	1.06	0.0828	0.91	0.32	0.21	1.00
	Chicken soup/acetic acid	3.51	3.22	0.0217	0.91			
	Chicken soup/acetic acid	3.82	37.04	0.0009	0.93			
	Citrate solution	3.01	3.39	0.1142	0.53	0.45	0.24	0.98
	Citrate solution	3.57	34.36	0.0007	0.97			
	Citrate solution	4.08	769.23	0.0004	0.15			
S. Enteritidis	Peptone water/acetic acid	3.52	6.67	0.0107	0.96	0.45		
	Peptone water/acetic acid	4.07	114.94	0.0014	0.67			
	Chicken soup/acetic acid	3.03	2.97	0.0094	1.00	0.44	0.83	0.88
	Chicken soup/acetic acid	3.57	285.71	0.0005	0.72			
	Chicken soup/acetic acid	4.06	666.67	0.0005	0.37			
	Citrate solution	2.56	5.62	0.0122	0.92	0.45	0.23	0.98
	Citrate solution	3.06	109.89	0.0007	0.91			
	Citrate solution	3.52	769.23	0.0001	0.90			

R^2 Coefficient of the determination.
[a] D_{pH} value.
[b] Standard error of the parameter.

2.5. Statistical tests

Normal distribution fitting of the numbers of survivors was carried out using the Microsoft Excel Add-in Varifit (BACANOVA, 2005), kindly donated by the programmers from the Computational Microbiology Group of the Institute of Food Research (Norwich, United Kingdom). To analyse the variability, the variances in the distributions of the concentrations of survivors were compared using the Bartlett test (Zar, 1999).

3. Results

3.1. Inactivation kinetic parameters

The estimated D_{pH} and z_{pH} values, obtained for each bacterium from the acid inactivation curves in the different substrates, are shown in Table 1. The experimental data confirmed that a decrease in the applied treatment's pH resulted in a corresponding decrease in the decimal reduction times.

Both tested Gram-positive bacteria (i.e. *L. innocua* and *E. faecalis*), as well as *S.* Enteritidis (Gram-negative) are more acid-tolerant than *P. fluorescens*, which was more susceptible to acidic pH values (Table 1). Also, the estimated z_{pH} values indicate that acetic acid holds a stronger inactivation potential than citric acid (Table 1). On the other hand, the substrate significantly affected the resistance of the microorganisms to the applied acidification treatments (Table 1). Complex substrates, such as the chicken soup, protected the organisms against the acidification.

3.2. Variability in inactivation

Times required to obtain various degrees of inactivation are summarized in Table 2. These times were calculated based on the D_{pH} and z_{pH} values presented in Table 1. Despite the fact that the D_{pH} values in Tables 1 and 2 should theoretically coincide, they showed, in fact, a certain degree of variability, possibly due to the difficulty of adjusting the pH to an exact value and the effect of the autoclaving on the pH.

With regard to the acid tolerance exhibited by the tested bacteria, there were remarkable differences among the substrates used. In general, for the same acidity and similar treatment times, the degree of inactivation was lower in food than that observed in peptone water and citrate solution (Table 2). Indeed, the treatment times required to attain the same degree of inactivation followed the order of magnitude chicken soup/acetic acid > peptone water/acetic acid > citrate solution (Table 2), demonstrating the well-recognized microbicidal activity of acetic acid.

The values of the standard deviation of the number of survivors in the untreated samples were always smaller than those observed in the acidified samples. In addition, as the intensity of the acid treatments increased, the number of surviving bacteria decreased and the standard deviation of the obtained distributions increased proportionally (Table 2). Table 3 shows the regression equations and the corresponding coefficients of determination (R^2), describing the effect of the degree of inactivation on the variability in the number of survivors in terms of standard deviation, for each microorganism and substrate. Although in some cases (*E. faecalis* and *L. innocua* in chicken soup/acetic acid and *E. faecalis* and *P. fluorescens* in citrate buffer), the R^2 are far from the unit, the rest

Table 2
Bacterial survival after the application of various acid treatments in different substrates.

Bacterium	medium/acidulant	pH	Expected inactivation	Length of treatment (min)	Nº of samples	Mean of survivors (log cfu/ml)	SD	Mean inactivation achieved (log cfu/ml)	Min[a]	Max[b]	Coefficient of variation (%) (100 × SD/mean)
E. faecalis	Peptone/acetic acid	3.08	0	0.0	30	7.4	0.08	0.0	7.3	7.6	1.1
			2	5.3	61	5.2	0.32	2.3	4.6	5.8	6.2
			3	7.9	63	4.6	0.37	2.9	3.8	5.3	8.0
			4	10.6	67	3.9	0.48	3.5	3.0	4.8	12.3
	Chicken soup/ acetic acid	2.80	0	0.0	24	7.6	0.07	0.0	7.4	7.7	0.9
			2	13.9	70	5.4	0.08	2.2	5.3	5.6	1.4
			3	20.9	75	4.9	0.14	2.6	4.7	5.2	2.8
			4	27.8	70	4.0	0.29	3.6	3.4	4.6	7.4
	Citrate solution	3.59	0	0.0	30	7.7	0.08	0.0	7.6	7.9	1.0
			2	13.3	68	5.6	0.44	2.1	4.7	6.5	7.9
			3	20.0	60	5.0	0.50	2.7	4.0	6.0	9.9
			4	26.7	66	4.0	0.60	3.8	2.8	5.1	15.2
L. innocua	Peptone/acetic acid	3.48	0	0.0	24	7.7	0.15	0.0	7.4	8.0	1.9
			2	36.6	76	5.1	0.39	2.6	4.3	5.9	7.7
			3	54.8	76	4.4	0.44	3.4	3.5	5.2	10.2
			4	73.1	76	3.7	0.60	4.0	2.5	4.9	16.2
	Chicken soup/ acetic acid	3.13	0	0.0	15	8.5	0.04	0.0	8.4	8.6	0.5
			2	16.1	75	6.5	0.10	1.9	6.3	6.7	1.6
			3	24.2	76	5.7	0.26	2.8	5.2	6.2	4.6
			4	32.3	76	4.7	0.29	3.8	4.1	5.3	6.2
	Citrate solution	2.97	0	0.0	58	7.7	0.14	0.0	7.4	7.9	1.8
			2	14.8	76	5.9	0.63	1.8	4.7	7.1	10.8
			3	22.3	76	4.9	0.69	2.8	3.5	6.2	14.2
			4	29.7	76	4.1	0.57	3.5	3.0	5.3	13.7
P. fluorescens	Peptone/acetic acid	4.03	0	0.0	30	6.7	0.12	0.0	6.5	7.0	1.8
			2	32.8	73	4.6	0.35	2.1	3.9	5.3	7.6
			3	49.2	75	3.3	0.37	3.4	2.6	4.0	11.3
			4	65.6	76	2.7	0.42	4.0	1.9	3.5	15.4
	Chicken soup/ acetic acid	3.80	0	0.0	11	6.7	0.07	0.0	6.5	6.8	1.0
			2	60.0	75	4.8	0.32	1.8	4.2	5.5	6.6
			3	90.1	75	3.6	0.38	3.2	2.9	4.4	10.5
			4	120.1	75	2.5	0.43	4.2	1.6	3.3	17.4
	Citrate solution	3.77	0	0.0	28	7.3	0.12	0.0	7.1	7.6	1.6
			2	189.3	69	5.1	0.55	2.3	4.0	6.1	10.9
			3	284.0	54	3.3	0.64	4.0	2.1	4.6	19.2
			4	378.7	61	2.8	0.57	5.0	1.3	3.5	20.1
S. Enteritidis	Peptone/acetic acid	3.80	0	0.0	11	7.2	0.13	0.0	7.0	7.5	1.8
			2	57.4	76	5.9	0.23	1.3	5.4	6.3	3.9
			3	243.5	76	3.9	0.48	4.1	2.9	4.8	12.4
			4	324.7	76	3.6	0.47	4.3	2.7	4.6	12.9

SD: Standard deviation (log cfu/ml).
[a] Minimum concentration in a sample (log cfu/ml).
[b] Maximum concentration in a sample (log cfu/ml).

of the R^2 are higher than 0.91.

The increasing variability was proportional to the lethality of the acidification, as can be seen in Fig. 1, which shows the effect of the microbial inactivation on the *CVs*, a dimensionless parameter that considers both the mean and the standard deviation, and is independent of the unit in which the measurement has been taken. The values of the *CVs* of the number of bacterial survivors after the most intense treatments were between 5 and 17 times higher than those of untreated samples, particularly in the case of *P. fluorescens* in chicken soup (Table 2). Independently of the *CV* values, we consistently observed a positive linear relationship between *CV* (variability) and the intensity of the acidification treatment.

As illustrated in Fig. 2, in which the distributions of the survivors of all microorganisms in each of the tested substrates are presented, the data dispersion becomes greater as the treatment is intensified, increasing the variability of the number of surviving bacteria. Although the achieved inactivation was relatively close to that expected, this was not always the case. For example, the reduction observed in practice for *E. faecalis* in chicken soup acidified with acetic acid was lower than the theoretically expected reduction of 4 logarithmic cycles (Fig. 2B); however, in other cases, the actual reduction was greater than the expected, for instance, for *P. fluorescens* in the three substrates (Fig. 2G–I).

A Bartlett test showed that the variability in the number of viable cells was significantly smaller for the control than for any of the acid-treated organisms (Fig. 2). In most of the cases, the variances in the final bacterial concentrations were significantly different ($p < 0.05$) for the different acid treatments (Fig. 2). In general, the distributions of the logarithms of the final bacterial concentrations did not show tails and the shapes were nearly symmetrical (Fig. 2). In contrast, the distributions of *E. faecalis*, *L. innocua* and *P. fluorescens* concentrations were bimodal for the most severe acid treatments in both chicken soup and peptone, which may be explained by the different acid-resistance patterns within the cell populations.

A multi-test-comparison, using the Bartlett test, showed the significant differences within and in-between intensities, substrates for the same microorganism (Table 4). In general, there were significant differences ($p < 0.05$) between substrates and intensities of the treatments.

4. Discussion

In this study, the effect of pH on the inactivation variability of four microorganisms relevant to food safety was investigated through the application of various acidification (pH) intensities

Table 3
Equations of regression lines relating the standard deviation of the number of viable bacteria (y) to the inactivation achieved (x).

Organisms	Substrate	Equation	SE[a]	R^{2b}
Enterococcus faecalis	Peptone/acetic acid	$y = 0.108x + 0.0789$	0.023	0.99
	Chicken soup/acetic acid	$y = 0.054x + 0.0309$	0.079	0.62
	Citrate solution	$y = 0.142x + 0.0992$	0.042	0.98
Listeria innocua	Peptone/acetic acid	$y = 0.104x + 0.1333$	0.045	0.96
	Chicken soup/acetic acid	$y = 0.057x + 0.1273$	0.152	0.37
	Citrate solution	$y = 0.136x + 0.2345$	0.176	0.68
Pseudomonas fluorescens	Peptone/acetic acid	$y = 0.072x + 0.1436$	0.046	0.92
	Chicken soup/acetic acid	$y = 0.085x + 0.1045$	0.053	0.93
	Citrate solution	$y = 0.096x + 0.2010$	0.137	0.78
Salmonella Enteritidis	Peptone/acetic acid	$y = 0.724x + 0.1328$	0.010	0.99
	Chicken soup/acetic acid	$y = 0.070x + 0.1615$	0.019	0.99
	Citrate solution	$y = 0.912x + 0.1042$	0.033	0.98

[a] Standard error of the regression.
[b] Values of coefficient of determination.

using two acidulants (acetic acid and citrate solution) on two substrates (soup chicken and buffered peptone water). The results showed that the higher the intensity of the applied acid treatments

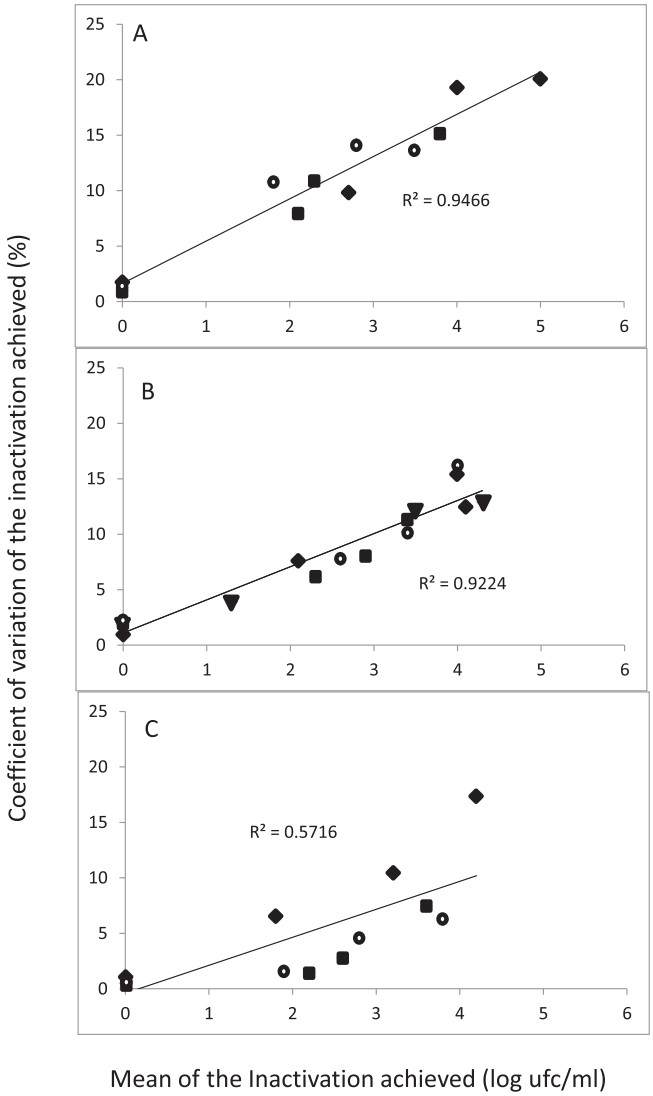

Fig. 1. Effect of the inactivation degree achieved through acidification on the variability (coefficient of variation) of the inactivation of *E. faecalis* (squares), *L. innocua* (circles), *Pseudomonas fluorescens* (diamonds) and *Salmonella* Enteritidis (triangles) in citrate buffer (A), peptone/acetid acid (B) and chicken soup/acetic acid (C). R^2 of the regression are shown.

(2, 3, and 4 log reductions), the lower the number of survivors, although number of 4 log reductions is more variable than the others. Several researchers have attempted to characterize the acid stress responses of pathogenic microorganisms, and variable results have been reported (Adams & Nicolaides, 1997; Buchanan & Edelson, 1999; Koutsoumanis & Sofos, 2004; Lianou & Koutsoumanis, 2013; Nightingale, Thippareddi, Phebus, Marsden, & Nutsch, 2006). Despite the variability and sometimes the discrepancies between the published research findings, such data are of great value from a food quality/safety perspective. Janssen et al. (2007) observed that the inactivation of *L. innocua* with lactic acid was greater, the number of survivors was more variable, and that it may even cause (or not) inactivation of the entire population. These observations coincide with the results obtained in this study (Table 2). This fact has been found in other food conservation operations such as heat treatment (Aguirre et al., 2009) and electron beam irradiation (Aguirre et al., 2011).

Bacterial cells that survive acid treatments may adapt relatively fast to new conditions and have a high chance of recovery and multiplication under favourable conditions (Koutsoumanis & Sofos, 2004), potentially reaching high concentrations during storage of food products, thus compromising their quality (i.e. shelf life) and safety. Acid resistance data are, therefore, expected to be useful in the development of new or the adjustment of already-existing microbial control interventions for application in the food industry.

When microbial inactivation is the result of the application of organic acid treatments, then the estimation of the inactivation parameters (D and z values) is relatively complex (Valero, Carrasco, Pérez-Rodríguez, García-Gimeno, & Zurera, 2006) because classic deterministic inactivation models do not take into account heterogeneity in the resistance of individual cells to a lethal stress (Casolari, 1988) which, as in the case of growth, can be a significant source of variability in microbial inactivation during food processing (Aspridou & Koutsoumanis, 2015). In addition, there are certain methodological difficulties in acid inactivation studies that need to be taken into account, most of them stemming from the fact that even minor differences in pH may result in considerable differences in the estimated D values, as also demonstrated by the results of the present study (Table 1). Such an excessive effect can be attributed to the fact that minimal variations in extracellular pH may produce a corresponding change (increase or decrease) in cytoplasmic pH. Indeed, the cytoplasmic membrane, by comprising a barrier between the external environment and the cellular cytosol, regulates the substances entering and leaving the cell, permitting homoeostasis of the cytoplasmic environment (Moat & Foster, 1995). As such, cytoplasmic membranes are both specific and selective structural entities, capable of being actively modified in response to changes in the external medium (Brown, Ross, McMeekin, &

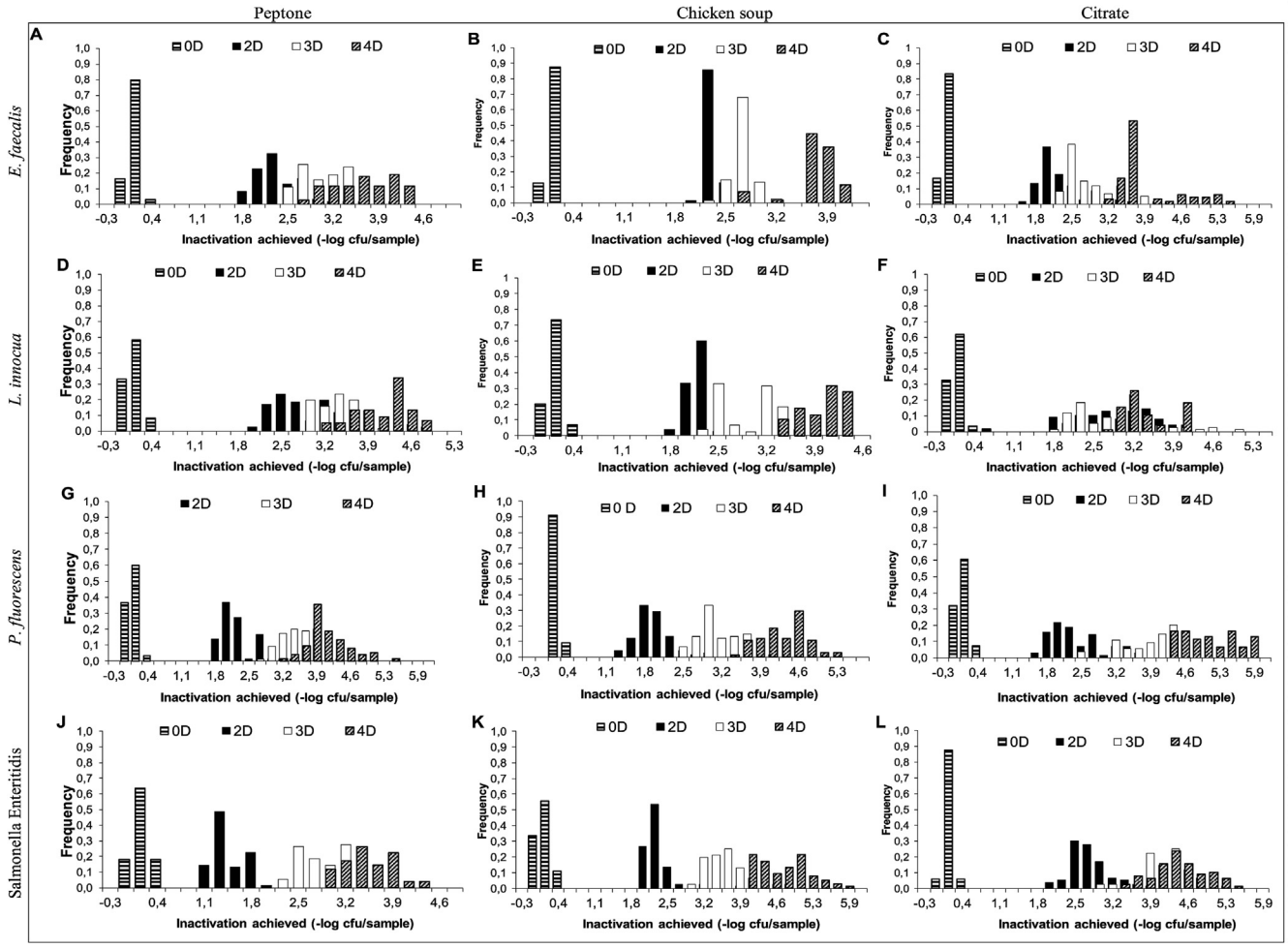

Fig. 2. Distributions of bacterial inactivation in peptone water, chicken soup and citrate solution after the application of different acid treatments. 0D–4D indicate the expected number of decimal reduction.

Nichols, 1997). Hence, increased extracellular concentrations of organic acids can result in the ample movement of the undissociated acid through the cell membrane, causing great mechanical stress to the cell wall and leading to serious cellular damage affecting bacterial survival or even to cell death (Valero et al., 2006).

The results obtained in this study indicate that the resistance intervals to pH of 4.03-2.80 follows a pattern: *E. faecalis* > *L. innocua* > *S.* Enteritidis > *P. fluorescens*, as shown in Table 2, which may be considered similar to that reported by Koutsoumanis and Sofos (2004), who observed that the bacteria with greater resistance to pH ranges of 4.0–5.5 follow this order: *Listeria monocytogenes* > *Escherichia coli* O157:H7 > *Salmonella Typhimurium*.

D_{pH} values estimated in this study are not necessarily comparable to those obtained by other investigators under similar conditions, due to potential differences in the experimental protocols and procedures. According to Férnandez, Álvarez-Ordóñez, López, and Bernardo (2009), a treatment time of 3.69 min was required in order to achieve a decimal reduction of 2.5 $\log_{10}$ of *Enterococcus faecium* in brain heart infusion (BHI) acidified to pH 5.0 with acetic acid (*D* value around 1.5 min). Álvarez-Ordóñez, Fernández, Bernardo, and López (2009) reported that *S.* Typhimurium inoculated in meat extract acidified to pH 4.5 with acetic acid, was reduced by 4 logarithmic cycles in approximately 9 min (*D* value around 2.25 min). These findings are quite different from ours, where the D_{pH} value for S. Enteritidis at pH 3.5 was estimated at 7 min, a difference that may be ascribed to strain variability. It is well known that the inherent differences among identically treated strains of the same microbial species may constitute an important source of variability in microbiological studies (Whiting & Golden, 2002).

Observations made by other researchers showed that the rate of inactivation is dependent not only on the environmental pH, but also on the type and the concentration of the acidulants used (Buchanan & Edelson, 1999; Buchanan, Golden, & Whiting, 1993). For instance, Buchanan et al. (1993) reported that both the lag (shoulder) period and the inactivation rate of *L. monocytogenes* in BHI acidified with lactic or acetic acid depended on the pH, as well as the identity and the concentration of the acidulant. More specifically, the estimated *D* values were inversely related to the levels of undissociated lactic and acetic acids (Buchanan et al., 1993). However in the present study it was not possible to use both acidulants to modify pH of the substrates, so the citrate solution was used only as blank.

In addition, the type of applied acidulant may also determine the extent of variability in the number of surviving bacteria that was seen in our study was also by observed Conner, Scott, and Bernard (1990).

In addition to the pH value and the applied acidulant, another parameter that may have an important effect on the observed acid

Table 4
Comparison of the inactivation distributions achieved by acidification treatments using the Bartlett test. The * in a square means that the distributions obtained with the two inactivation treatments were not significantly different ($p > 0.05$).

E. faecalis		Peptone				Chicken soup				Citrate			
Substrate	Inactivation	0	2	3	4	0	2	3	4	0	2	3	4
Peptone	0	■	2	3	4	0	2	3	4	0	2	3	4
	2		■	3	4								
	3		*	■	4								
	4				■	0							
Chicken soup	0	*				■	2	3	4				
	2	*				*	■	3	4				
	3							■	4				
	4		*	*					■	0	2	3	4
Citrate	0	*				*	*			■	2	3	4
	2										■	3	4
	3				*						*	■	4
	4				*							*	■

L. innocua		Peptone				Chicken soup				Citrate			
Substrate	Inactivation	0	2	3	4	0	2	3	4	0	2	3	4
Peptone	0	■	2	3	4	0	2	3	4	0	2	3	4
	2		■	3	4								
	3		*	■	4								
	4		*	*	■	0							
Chicken soup	0	*				■	2	3	4				
	2	*				*	■	3	4				
	3			*	*			■	4				
	4								■	0	2	3	4
Citrate	0	*				*	*			■	2	3	4
	2										■	3	4
	3											■	4
	4							*					■

Ps. fluorescens		Peptone				Chicken soup				Citrate			
Substrate	Inactivation	0	2	3	4	0	2	3	4	0	2	3	4
Peptone	0	■	2	3	4	0	2	3	4	0	2	3	4
	2		■	3	4								
	3		*	■	4								
	4		*	*	■	0							
Chicken soup	0	*				■	2	3	4				
	2		*	*			■	3	4				
	3			*				■	4				
	4		*	*	*				■	0	2	3	4
Citrate	0	*				*				■	2	3	4
	2										■	3	4
	3										*	■	4
	4										*	*	■

S. Enteritidis		Peptone				Chicken soup				Citrate			
Substrate	Inactivation	0	2	3	4	0	2	3	4	0	2	3	4
Peptone	0	■	2	3	4	0	2	3	4	0	2	3	4
	2		■	3	4								
	3			■	4								
	4			*	■	0							
Chicken soup	0	*				■	2	3	4				
	2						■	3	4				
	3							■	4				
	4								■	0	2	3	4
Citrate	0	*				*				■	2	3	4
	2			*	*			*			■	3	4
	3							*	*			■	4
	4							*			*	*	■

resistance profiles of microorganisms is the composition of the substrate/food matrix under study. Waterman and Small (1998) showed that bacterial resistance to extremely acidic conditions was enhanced in food matrices with a high fat or protein content. In concordance with these findings, the D_{pH} values estimated in our study were higher in chicken soup, although its content in fat is not high, but is higher than in the other two substrates (Table 2). Such differences may be attributed to the fact that fat content of foods favour an increase in the concentration of acids in the lipid phase of the bacterial cytoplasmic membrane, limiting the availability and, thus, the antimicrobial activity of acids (Sofos & Busta, 1993). On the other hand, it has been shown that the presence of certain amino acids (e.g., arginine, lysine, and glutamic acid) in the treatment medium can also have an impact on bacterial acid resistance, enabling the activation and manifestation of acid tolerance response (ATR) systems (Castaine-Cornet & Foster, 2001; Castaine-Cornet, Penfound, Smith, Elliott, & Foster, 1999; Foster, 2004; Iyer, Williams, & Miller, 2003). Nevertheless, the peptone contains free amino acids while it is doubtful that chicken soup does.

The linear regressions of Fig. 1 show the variability of the number of survivors after different acidifying treatments. It is evident that the greater the intensity of acidification, the higher is the variability. Some researchers have explained the heterogeneity of microorganisms as a response to the acidification conditions (Brown et al., 1997; Dufourc, Smith, & Jarrell, 1984; Dunkley, Guffanti, Clejan, & Krulwich, 1991).

Variability is characterized by equations (Table 3), where *y* is the standard deviation and *x* is the degree of inactivation achieved (-log cfu/sample). These regressions, in general, are characterized by acceptable coefficients of determination ($R^2 > 0.91$), showing some similarity to other variability data; f. For instance, the inactivation of *E. faecium* through heating at 70 °C in BHI ($R^2 = 0.86$) (Férnandez et al., 2009) and the irradiation of ham with electron beams irradiation of ham and TSA ($R^2 = 0.84$) experimentally contaminated with *E. faecalis*, *L. innocua*, *P. fluorescens*, and *S.* Enteritidis (Aguirre et al., 2011). The increasing variation in the response of bacteria to less favourable conditions (Fig. 1) has been widely reported in the literature (Aguirre et al., 2009; Álvarez-Ordóñez et al., 2009; Buchanan et al., 1993; Koutsoumanis & Sofos, 2004). This non-homogeneous response of the microbial population to stress conditions depends on many factors, like, the environmental pH of the identity and concentration of acidulant used to modify the pH of the substrate (Buchanan et al., 1993). This heterogeneity can also be a phenotypic response to the modification of fatty acid compositions in the cell membrane, associated with acidity (Brown et al., 1997).

The microbial response in different substrates (Table 1) makes it difficult to compare with other studies; in fact, the components of the substrate, type of acid, inoculum preparation, conditions of pH, time of habituation, and other environmental conditions, make it almost impossible to compare the behaviour of organisms with other studies (Bearson, Wilson, & Foster, 1998; Foster & Hall, 1990). However, there are studies in thermal (Aguirre et al., 2009) and irradiation treatments (Aguirre et al., 2011) that showed that the variability of the number of survivors increased as the intensity of the treatments increased, which was the same tendency observed in the present study. Aguirre et al. (2011) observed R^2 (>0.90) values in irradiated samples of ham similar to those reported here (Table 3). Our study, suggests that linear equations can be used to predict the variability in the number of survivors after the application of a specific treatment and to calculate the expected frequency in bacterial load in a batch, same finding was described by Aguirre et al. (2009; 2011).

Our results indicate that the distribution of the number of

survivors depends on the severity of the treatment (Fig. 2). This leads to the idea that the inactivation times for the cells within a population are not identically distributed and that applying acid treatments of different severities allows for the survival of different subpopulations, which can explain the bimodal distributions observed at the higher acid treatment (Fig. 2). Regarding inactivation parameters, it has been reported that the resistance of the surviving bacteria and spores increases during the inactivation treatment (Augustin et al., 1998; Aspridou & Koutsoumanis, 2015; Smelt, Bos, Kort, & Brul, 2008), which explains further the heterogeneity of the cells within a population as the result of dynamic survival strategies.

The extensive quantity of data obtained in this study allows us to conclude that microbial inactivation is not constant and that more intense condition of inactivation increase the variability of the number of surviving bacteria. This enormous versatility makes a more solid knowledge of the responses of survivors microorganisms necessary, through predictive models that evaluate and subsequently adjust the shelf-life of foods and minimize microbial hazards, to ensure food safety. Furthermore, the quantification of variability in microbial inactivation is expected to improve the accuracy of risk assessment models.

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