LWT - Food Science and Technology 66 (2016) 369–377

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Analysis of the variability in microbial inactivation by acid treatments

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ARTICLE INFO

Article history:
Received 15 July 2015
Received in revised form
23 October 2015
Accepted 23 October 2015
Available online 28 October 2015

Keywords:
Variability
Inactivation
Acid treatment
Acetic acid
Chicken soup

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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http://dx.doi.org/10.1016/j.lwt.2015.10.056
0023-6438/

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.