



Investigating the influence of organic acid marinades, storage temperature and time on the survival/inactivation interface of *Salmonella* on chicken breast filets

Anastasia E. Lytou^a, Konstantinos Tzortzinis^a, Panagiotis N. Skandamis^b,
George-John E. Nychas^{a,*}, Efstathios Z. Panagou^a

^aLaboratory of Microbiology and Biotechnology of Foods, Department of Food Science and Human Nutrition, Faculty of Foods, Biotechnology and Development, Agricultural University of Athens (AUA), Iera Odos 75, Athens 11855, Greece

^bLaboratory of Food Quality Control & Hygiene, Department of Food Science and Human Nutrition, Faculty of Foods, Biotechnology and Development, Agricultural University of Athens (AUA), Iera Odos 75, Athens 11855, Greece

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ABSTRACT

The aim of this work was to study the influence of lemon and vinegar marinades on *Salmonella* inoculated on chicken filets and stored under different storage temperatures for nine days in the presence of indigenous microbiota. In addition to this, model development for the determination of the inactivation boundaries and the prediction of pathogens response was attempted. The different acid concentrations in the marinades, the type of acid, the storage temperature as well as the duration of storage impacted the levels of pathogens and background flora. The higher tested concentrations (2% and 4% v/v for acetic and citric acid) were more effective against *Salmonella* and spoilage microorganisms than the lower ones (0.5 and 1% v/v for acetic and citric acid), while the intermediate concentrations (1, 1.5 and 2, 3% v/v for acetic and citric acid, respectively) presented differentiations of particular interest to the microbial responses to acidic stress. The aforementioned parameters also differentiated *Salmonella* serovars persistence and spoilage microorganisms dominance. Regarding model development, the probability of inactivation of *Salmonella* was satisfactorily predicted particularly in the case of acetic acid marination while in model validation, the majority of the vinegar marinated samples were correctly classified, whereas, in case of lemon marination, a higher number of misclassifications was observed, indicating a partial weakness of the model to predict the pathogens response at interface concentrations.

1. Introduction

Salmonella is recognized as one of the most prevalent foodborne pathogens linked to several outbreaks. In 2016, a number of 94,530 confirmed salmonellosis cases were reported by the European Union. Although infections caused by *Salmonella* have been declining since 2008, in recent years (2012–2016) this decreasing trend has been stabilized. Specifically in 2016, the number of reported cases has been slightly increased according to the European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA) (EFSA, 2017). The most widespread serovar of the pathogen is *Salmonella enterica* subsp. *enterica* ser. Enteritidis or *Salmonella* Enteritidis in short (Brenner et al., 2000) followed by *Salmonella* Typhimurium, *Salmonella* Infantis and *Salmonella* Derby (EFSA, 2017). Among the products with the highest level of non-compliance

with *Salmonella* food safety criteria are ready-to-cook poultry meat preparations, including marinated chicken products (EFSA, 2017).

Marination is a food preparation process that could contribute to the mitigation of this issue by using marinades containing ingredients with antimicrobial activity. Previous studies have reported the effectiveness of several marinades that contained wine, soy sauce, essential oils or spices mixtures on decreasing either pathogen population such as *Salmonella* (Rhoades et al., 2013; Pathania et al., 2010; Nisiotou et al., 2013) and *Campylobacter* (Isohanni et al., 2010; Zakariénė et al., 2015) or extending the shelf life (Kargiotou et al., 2011; Karam et al., 2019). Marinade formulations containing organic acids could also have a significant impact on growth, inhibition or even inactivation of spoilage and pathogenic microorganisms of meat, while at the same time balancing the odor and flavor due to the presence of a variety of other ingredients, resulting in desirable final products (Birk et al., 2010; Yang

* Corresponding author.

E-mail address: gjn@aua.gr (G.-J.E. Nychas).

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et al., 2013; Yusop et al., 2010).

The growth, survival or inactivation of *Salmonella* in chicken marinated with acidic marinades could be affected by several factors such as the type of the acid, concentration of the acid in undissociated form, temperature, marinating and storage time, initial pathogen population, evolution of indigenous microbiota, etc. Inactivation boundary models could be employed for the description of these effects providing useful information on the behavior of *Salmonella* in an acidic environment as well as for the prediction of the pathogen's survival, growth or inactivation in marinated chicken.

The aim of this study was to evaluate the influence of temperature, undissociated acid (acetic and citric) concentration and storage time on the survival, growth or inactivation of a cocktail of five *Salmonella* strains (three different serotypes). For this reason, two polynomial logistic regression models were developed (one for citric and one for acetic acid marinades) to study the interactions of these factors on the probability of inactivation of *Salmonella*, also depicted as interface between survival and inactivation. Subsequently, an extended external validation of these probabilistic models was performed using various lemon and vinegar based marinades that were differentiated in acid concentration and storage temperature. In parallel, the evolution of background microbiota was monitored in order to investigate not only its influence on *Salmonella* response, but also to obtain an overall view about the microbiological status of chicken breast fillets during storage. Finally, individual *Salmonella* strain survival was monitored by applying PFGE analysis of the populations recovered on plates at selected storage temperatures, acid concentrations and storage time.

2. Materials and methods

2.1. Preparation of the inoculum

Five different *Salmonella* strains maintained in the culture collection of the Laboratory of Microbiology and Biotechnology of Foods (LMBF) were used namely: (i) *Salmonella* Typhimurium CDC 6516–60 -ATCC 14028, (ii) *Salmonella* Typhimurium DT 193 (human isolate, epidemic, multi-drug resistant), (iii) *Salmonella* Enteritidis P167807 phage type 4 (isolated from beef), (iv) *Salmonella* Enteritidis P469815 phage type 7 (isolated from beef), and (v) *Salmonella* Infantis FS117 (chicken meat isolate kindly provided by the Food Quality and Hygiene Laboratory of the Agricultural University of Athens). The strains were revived from a stock culture stored at -80°C , subcultured into 10 mL of Tryptone Soy Broth (TSB; LAB M₁) and incubated overnight at 37°C . A 10 μL aliquot of the activated culture was transferred to 10 mL of fresh TSB medium and incubated at 37°C for 18 h (initial pH 7.2, pH after growth 5.7). Bacterial cells were then harvested by centrifugation ($5000 \times g$, 10 min, 4°C), washed twice with 10 mL sterile $\frac{1}{4}$ Ringer solution (Ringer's Solution Tablets, LabM) and finally resuspended in the same Ringer solution. The strains were mixed in equal volumes to provide the final inoculum of *Salmonella* used in the experiments. Before mixing the cultures, the population of each strain was counted to ensure that the composite inoculum consisted of approximately the same cell number per strain that ranged between 8.3 and 8.5 log CFU/mL. The five strain-cocktail was further diluted to reach an inoculum level of ca. 10^6 CFU/mL.

2.2. Sample preparation, marination and storage conditions

Chicken breast fillets were purchased from a local meat market and transferred under refrigeration to the laboratory within an hour. They were aseptically cut into 10 g units and placed in stainless metal trays. Chicken samples were then inoculated with 100 μL aliquots of the 5-strain composite inoculum by dispersing it with a pipette over the chicken surface to obtain a final population of ca. 4 log CFU/g of *Salmonella* as defined by plate counting. The inoculated chicken samples were incubated at 4°C for 1 h to allow the attachment of the

inoculum on the samples surface. After inoculation, the samples were divided into three batches with duplicate samples *per* batch. The samples of the first batch were immersed into apple cider vinegar of 0.5, 1.0, 1.5, and 2.0% (v/v) concentration in acetic acid; the samples of second batch were immersed into lemon juice of 1.0, 2.0, 3.0, and 4.0% (v/v) in citric acid; the samples of third batch were not marinated and used as control. Apple cider vinegar and lemon juice used in the experiment were obtained from a local market and did not contain any other constituents that could potentially present antimicrobial activity apart from acetic and citric acid. The concentrations of these products in acetic and citric acid were determined by HPLC (High Performance Liquid Chromatography) analysis according to a slight modification of the method described by Lytou et al. (2017). These initial solutions were appropriately diluted to obtain the final concentrations. Marination was undertaken in metal trays and the volume of the solution was sufficient to ensure complete sample immersion (1300 mL of lemon juice or apple cider vinegar for 50 samples). The duration of the contact with the marinade was 1 h at 4°C . Then, the samples were removed from the trays, drained for 30 s, placed in Petri dishes, covered with air permeable polyethylene film and stored at 4, 8, 12, and 16°C for 9 days. The two samples per batch, representing the within batch variability, were repeated to have between batch variability and overall an $n = 4$.

2.3. Microbiological analysis

Microbiological analysis was undertaken before marination, after marination (0 day) and at 1, 3, 5, 7, and 9 days of storage. Ten (10) grams of chicken samples were weighed aseptically in a sterile stomacher bag and homogenized in 10 volumes of sterile $\frac{1}{4}$ strength Ringer's solution (LAB 100Z, LABM) in a stomacher (Lab Blender 400, Seward Medical, London, UK) for 60 s at room temperature. The use of Ringer's solution could not affect the pH considering its formulation [NaCl (0.225%), KCl (0.0105%), CaCl_2 (0.012%), and NaHCO_3 (0.005%)] as well as the buffering capacity of chicken meat. Decimal dilutions were prepared in the same Ringer's solution and duplicate 1 or 0.1 mL aliquots of appropriate dilutions were poured or spread on the following media: (i) Tryptone Soy Agar (LAB011, LABM) for total viable counts, incubated at 30°C for 48 h; (ii) de Man-Rogosa-Sharpe agar (401,728, Biolife) (pH adjusted to 5.7) for lactic acid bacteria (LAB), overlaid with the same medium and incubated at 30°C for 72 h; (iii) Pseudomonas agar base (LAB 108, supplemented with selective supplement X108, LAB M) for the enumeration of *Pseudomonas* spp., incubated at 25°C for 48 h; (iv) Rose Bengal Chloramphenicol Agar Base (LAB036, supplemented with X009 chloramphenicol, LABM) for the enumeration of yeasts and molds, incubated at 25°C for 72 h; (v) XLD Agar (LAB032, LAB M) for the enumeration of *Salmonella* spp., incubated at 37°C for 24 h. In order to lower the detection limit of *Salmonella* spp. with the spread-plating method, a total of 1 mL of the samples from the first dilution was spread equally on 3 XLD agar plates. For the detection of *Salmonella* spp. before inoculation as well as throughout the duration of the experiments, the process of selective enrichment was applied by suspending 25 g of chicken samples in 225 mL of buffered peptone water and incubated at 37°C for 24 h (primary enrichment). Further on, a portion of 0.1 mL of the samples was transferred to 10 mL of Rappaport-Vassiliadis selective enrichment broth and incubated at 43°C for 48 h (secondary enrichment). After each enrichment step the culture was streaked onto XLD agar plates and incubated at 37°C for 24 h. The determination of the shelf life duration was based on the time that spoilage microbiota reached the level of 7.0 log CFU/g (Ercolini et al., 2006; Höll et al., 2016).

In parallel with microbiological analyses, the pH of the samples was recorded by means of a digital pH meter (Metrohm 691 pH meter, Ion Analysis, Switzerland) after the end of microbiological analysis with direct immersion of the glass electrode in the sample homogenate.

2.4. Logistic regression model development

Logistic regression describes the relationship between the probability of an event and a set of independent (exploratory) variables. In this work, determination of “inactivation” or “growth/survival” was based on *Salmonella* spp. counts. Thus, a reduction in pathogen population of > 0.5 log CFU/g was denoted as “inactivation” and assigned the value of 1, otherwise it was denoted as “growth” or “survival” and assigned the value of 0 (Gunvig et al., 2013; Valero et al., 2009). A second order ordinary logistic regression model was fitted to the data to determine the inactivation boundaries of *Salmonella* under different storage temperatures, undissociated acid concentrations and storage time.

$$\text{logit}(P) = \ln\left(\frac{P}{1-P}\right) = a_0 + a_1 \cdot t + a_2 \cdot T + a_3 \cdot UAC + a_4 \cdot t \cdot UAC$$

+ $a_5 \cdot T \cdot UAC + a_6 \cdot t \cdot T + a_7 \cdot t^2 + a_8 \cdot T^2 + a_9 \cdot UAC^2$
) where p is the probability of growth (taking values between 0 and 1), a_i with $i = 0, \dots, 9$ are the parameters to be estimated, T is storage temperature, t is storage time (days) and UAC is the undissociated acid concentration (mmol/L). The undissociated acid concentration was related to the total acid percentage based on the Henderson-Hasselbach equation:

$$UAc = \frac{Ac}{1 + 10^{pH-pKa}} \quad (2)$$

where Ac is the total acid concentration used in the experiments (% v/v) and pKa are the dissociation constants of the acetic and citric acids, respectively, used in marination treatment.

The equation was fitted using the logistic regression procedure of Minitab version 14.1 (Minitab Inc., State College, PA). Stepwise analysis was undertaken to select the significant ($P < 0.05$) parameters. The predicted inactivation/survival or growth interfaces for $P = 0.5, 0.1,$ and 0.9 were calculated with Microsoft Excel 2010 Solver (Microsoft Corp., Redmond, WA). The goodness-of-fit of the developed models was evaluated by the concordance rate (%), correct predictions (%), fail safe and fail dangerous rates (%) (Vermeulen et al., 2007).

2.5. Model validation

The performance of the developed models was compared with data obtained from independent experiments. For this reason, different marinades based on citric or acetic acids were prepared using different types of lemon juices and vinegars (of known concentration in citric and acetic acid) adding other ingredients, such as olive oil [15% (v/v) of

total marinades volume], spices and herbs [0.2% (w/v) of each one]. The types of marinades used for model validation are detailed in Table 1. The samples were stored at the same temperatures as for model development with an additional temperature at 10 °C. The concentrations of citric or acetic acids in marinades used in validation were close to the inactivation/survival interface that was determined in model development (i.e., 2–3% v/v citric acid for lemon marinades and 1–2% v/v acetic acid for vinegar marinades). All samples were stored at one or two different temperatures as presented in Table 1 for a period of 9 days and *Salmonella* spp. levels were determined at 1st, 3rd, 5th, 7th and 9th day of storage. In total, 80 different cases (160 samples) were examined for lemon marinades and 85 (170 samples) for vinegar ones. The different cases mentioned above refer to treatments with different marinades in several storage conditions (temperature and time).

2.6. Strain differentiation of *Salmonella* spp.

Pulsed Field Gel Electrophoresis (PFGE) analysis was conducted to monitor the effect of different marinades on the differentiation of *Salmonella* strains. For this purpose, 15–20 colonies were randomly picked from XLD agar plates (one replication per batch) and stored at –80 °C in Tryptic Soy Broth (TSB) containing 30% glycerol. Samples stored at 4 and 12 °C, subjected to marination with acetic acid (0.5 and 1.0%) and citric acid (1.0 and 3.0%) were selected for PFGE analysis. As far as storage time is concerned, samples from the first day of storage (fresh samples) together with samples in which TVC was higher than 7.0 log CFU/g (i.e., near the end of shelf life) were selected for analysis. PFGE was performed as detailed elsewhere (Nisiotou et al., 2013).

3. Results and discussion

3.1. Determination of marination treatments on microbial dynamics

Organic acid concentrations with their respective, undissociated amounts and initial pH values of the different marination treatments are presented in Table 2. The pH was obviously a parameter of critical importance in this study. Even from the first experimental steps, during the process of pathogen revival and growth in TSB medium, the decrease in medium pH to 5.7, as a result of fermentative metabolism may trigger acid adaptation phenomena (Álvarez-Ordóñez et al., 2012; Wilde et al., 2000). Initial pH values, immediately after marination, varied from 4.0 to 4.9 and from 4.5 to 5.1 for citric and acetic acid treatments, respectively, while no differences could be observed among

Table 1
Types of marinades used in model validation as derived by individual ingredients.

Code	Ingredients	Organic acid concentration (%)**	Storage temperature (°C)
L1	Lemon juice 1 (40%)*	2.0	10
L2	Lemon juice 1 (60%)	3.0	10
L3	Lemon juice 1 (50%)	2.5	4, 12
L4	Lemon juice 2 (40%), olive oil, pepper, garlic powder	2.0	8, 10
L5	Lemon juice 2 (60%), olive oil, pepper, garlic powder	3.0	4, 12
L6	Lemon juice 2 (40%), olive oil, dried basil, dried thyme	2.0	4, 16
L7	Lemon juice 3 (60%), olive oil, pepper, garlic powder	3.0	8, 12
L8	Lemon juice 3 (40%), olive oil, dried basil, dried thyme	2.0	4, 8
L9	Lemon juice 3 (60%), olive oil, dried basil, dried thyme	3.0	8, 16
V1	Apple cider vinegar (17%), olive oil, dried basil, dried thyme	1.0	4, 10
V2	Apple cider vinegar (25%), olive oil, dried basil, dried thyme	1.5	8
V3	Apple cider vinegar (34%), olive oil, garlic powder, onion powder	2.0	4
B1	Balsamic vinegar (17%), olive oil, dried basil, dried thyme	1.0	4, 8
B2	Balsamic vinegar (25%), olive oil, dried basil, dried thyme	1.5	8, 10
B3	Balsamic vinegar (34%), olive oil, garlic powder, onion powder	2.0	4
Va	Apple cider vinegar (25%)	1.5	10
Vb	Apple cider vinegar (34%)	2.0	10

*Percentage (%) of the ingredient in the total composition of the marinade (derived from the dilution of the commercial product with water) to obtain a specific organic acid concentration.

**Citric acid for lemon marinades and acetic acid for vinegar marinades.

Table 2

Citric (lemon juice) and acetic (apple cider vinegar) acid concentrations (% v/v), pH values and undissociated acid concentration (UAC, mmol/L) of solutions used in chicken breast fillets marination.

	Acid concentration (% v/v)	pH	TAC (mmol/L)	UAC (mmol/L)	% UAC
CITRIC ACID (lemon juice)	0	5.90			
	1	4.93	52	21	40
	2	4.45	104	70	68
	3	4.29	156	114	73
	4	4.05	208	170	82
ACETIC ACID (apple cider vinegar)	0	5.80			
	0.5	5.13	83	24	29
	1	4.96	167	64	38
	1.5	4.70	250	132	52
	2	4.55	333	204	61

UAC: undissociated acid concentration.

TAC: total acid concentration.

pH: value after marination.

treatments with solutions of an equal concentration in citric or acetic acid. The pH values remained constant throughout storage for most treatments with the exception of certain samples immersed in marinades of lower concentrations in organic acids (0.5, 1.0 and 1.0, 2.0% v/v for acetic and citric acid, respectively) and stored at 12 and 16 °C where a noticeable increase in pH was observed (Fig. 1).

Regarding acid concentration (Table 2), total acid concentration (TAC) of acetic acid was higher compared to citric acid (e.g., 52 and 167 mmol/L for 1% citric and 1% v/v acetic acid, respectively) due to the difference in molecular weight between the two acids.

Consequently, the amount of the undissociated acid at a given concentration was higher for acetic acid. The undissociated acid concentration is of great importance for the antimicrobial effect and its contribution to the antimicrobial activity is well documented. It is widely accepted that the undissociated part of weak organic acids penetrates bacterial cell membranes and their intracellular dissociation reduces cytoplasmatic pH, affecting the metabolic activity of the cell (Davidson, 2001; Greer and Dilts, 1992).

All tested acid concentrations were effective against the indigenous microbiota and resulted in noticeable delay in microbial growth (Fig. 2). As expected, a scaling in growth inhibition was observed from the lower to the higher acid concentrations and from the higher to lower temperatures. In both citric and acetic acid marinades, a significant extension in shelf life could be observed compared to non-marinated samples even for the treatments of low concentrations in organic acid (i.e., 0.5 and 1.0% v/v for acetic and citric acid, respectively). Moreover, there was a distinct difference in the microorganisms that induced spoilage from the lower to higher acid concentrations and temperatures (Tables 3 and 4). The dominance of *Pseudomonas* spp. was pronounced in lower acid concentrations compared to LAB and yeasts that predominated at higher acid concentrations. In the samples with high *Pseudomonas* spp. levels, the pH was considerably increased -as mentioned above- probably due to the proteolytic activity of these microorganisms (Nicodème et al., 2005; Ercolini et al., 2009). The growth of LAB was enhanced in acetic acid marination compared to citric acid, probably due to their better adaptation potential to acetic acid environment, since acetic acid is one of the main metabolic products of LAB. The diversity in the dominant microbiota could affect the survival, inactivation or growth of *Salmonella*, since it is considered a

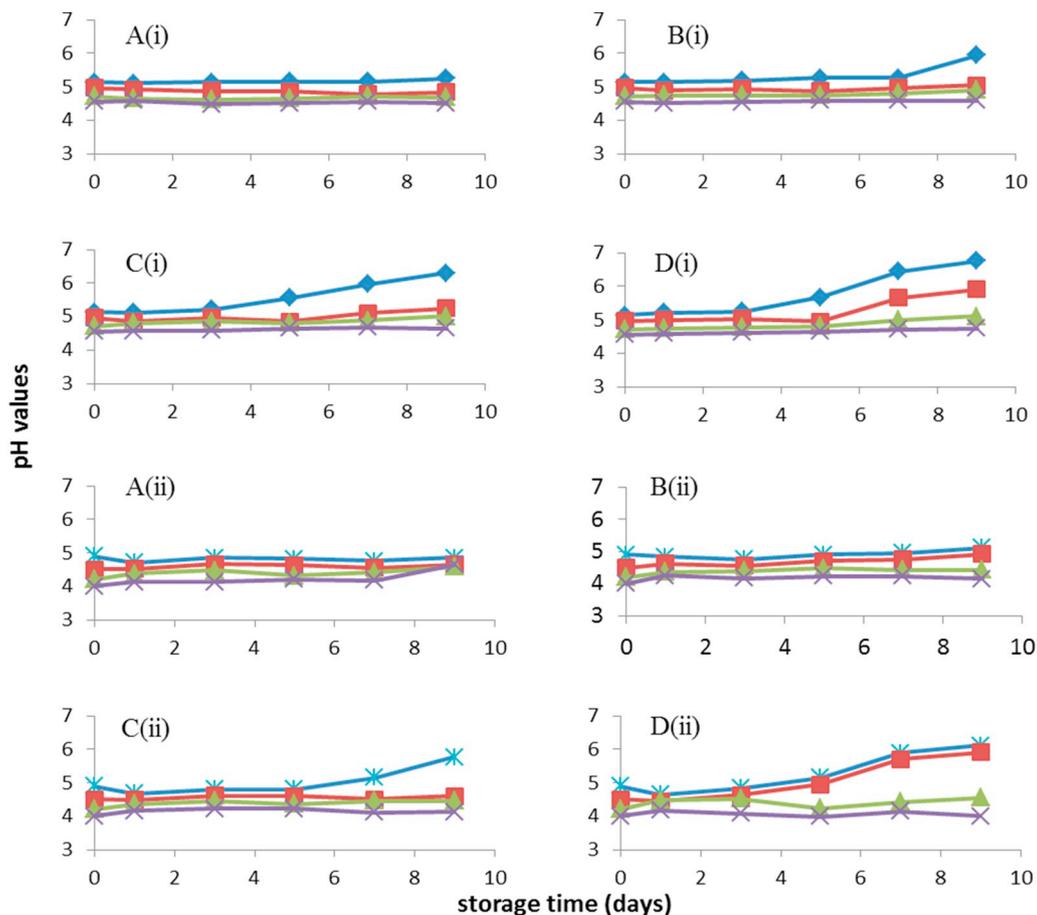


Fig. 1. Evolution of pH during storage of chicken samples immersed in apple cider vinegar (i) and lemon juice (ii) of different concentrations in acetic or citric acid, stored at (A) 4 °C, (B) 8 °C, (C) 12 °C, and (D) 16 °C for 9 days. (Standard deviation was < 0.2).

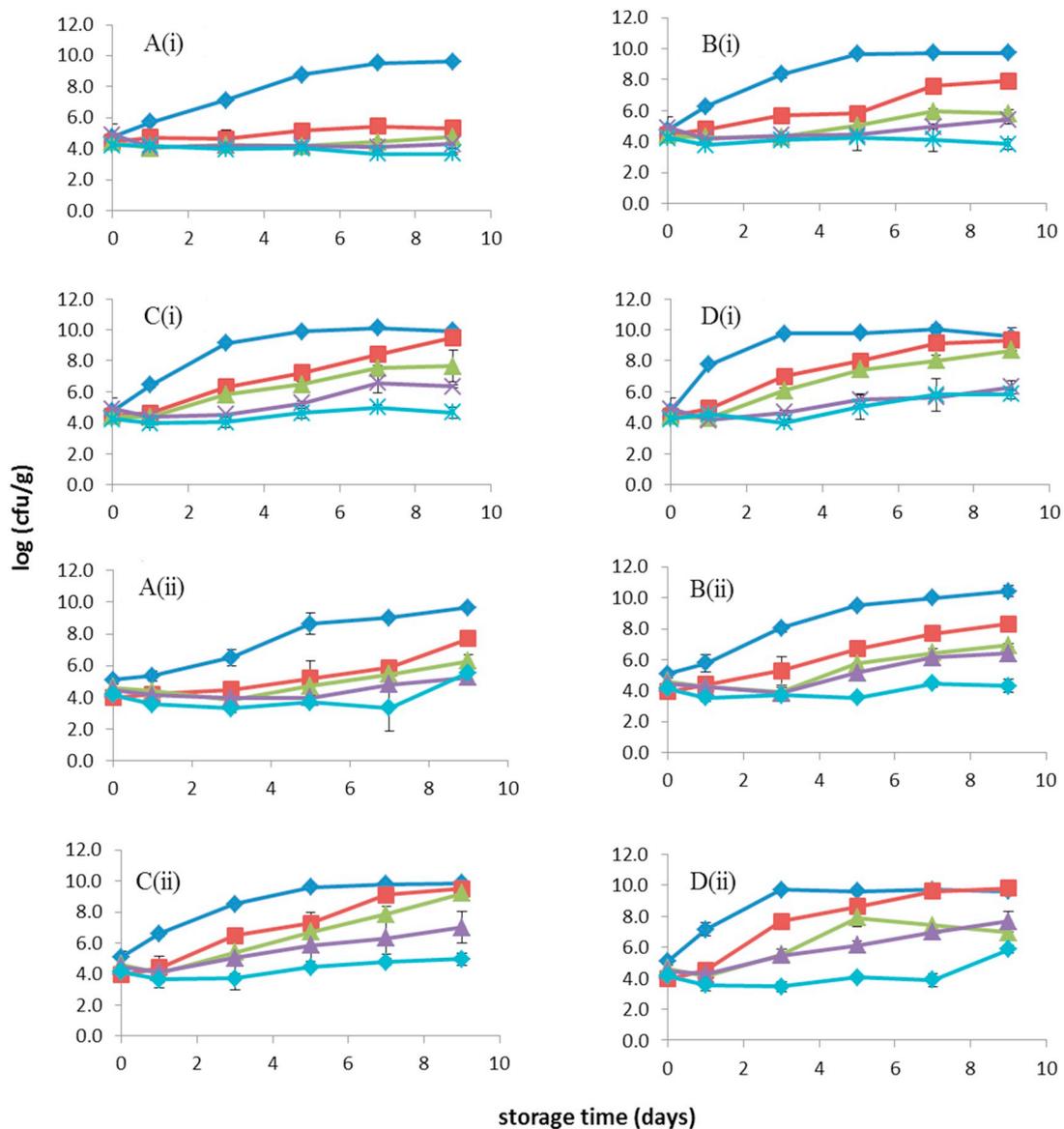


Fig. 2. Total aerobes population (TVC) of chicken samples immersed into apple cider vinegar (i) and lemon juice (ii) of different concentrations in acetic and citric acid respectively, stored at (A) 4 °C, (B) 8 °C, (C) 12 °C, and (D) 16 °C for 9 days. (Error bars < 0.2 are not shown).

Table 3

Microbiological shelf life (ca. 7.0 log CFU/g) of chicken marinated in apple cider vinegar and dominant microbial group at the end of shelf life (SL).

Acid (%)	4 °C		8 °C		12 °C		16 °C	
	SL (days)	dominant m/o**	SL (days)	dominant m/o	SL (days)	dominant m/o	SL (days)	dominant m/o
0	3	<i>Pseudomonas</i> spp.	1–3	<i>Pseudomonas</i> spp.	1–3	<i>Pseudomonas</i> spp.	1	<i>Pseudomonas</i> spp.
0.5	9+	Yeasts	7	<i>Pseudomonas</i> spp.	5	<i>Pseudomonas</i> spp.	3	<i>Pseudomonas</i> spp.
1	9+	Yeasts	9+	Yeasts-LAB	7	Yeasts-LAB	5	Yeasts-LAB
1.5	9+	Yeasts	9+	Yeasts	9	LAB	9	LAB
2	9+	Yeasts	9+	Yeasts	9	Yeasts	9	Yeasts

*(+): > 9 days.

**m/o: microorganism.

weak competitor of the spoilage microbiota (Oscar, 2007) and at the same time it could be also influenced by pH changes caused by different spoilage microorganisms. The evolution of *Salmonella* counts of chicken samples immersed in lemon or vinegar solutions is presented in Fig. 3. A wide range of responses of the pathogen (growth/survival/inactivation) to acidic stress could be observed, depending on the type of the

acidulant, temperature, acid concentration, and storage time. Specifically, in lemon marinades, the concentration of 2.0% v/v citric acid is of particular interest since the behavior of *Salmonella* presented fluctuations not only among different storage temperatures and storage durations but also among replicates. At 8 and 12 °C, *Salmonella* increased within the first 5 days of storage followed by a decrease at the

Table 4
Microbiological shelf life (ca. 7.0 log CFU/g) of chicken marinated in lemon juice and dominant microbial group at the end of shelf life (SL).

Acid (%)	4 °C		8 °C		12 °C		16 °C	
	SL (days)	Dominant m/o*	SL (days)	Dominant m/o	SL (days)	Dominant m/o	SL (days)	Dominant m/o
0	3–5	<i>Pseudomonas</i> spp.	1–3	<i>Pseudomonas</i> spp.	1–3	<i>Pseudomonas</i> spp.	1	<i>Pseudomonas</i> spp.
1	8–9	<i>Pseudomonas</i> spp.	5–7	<i>Pseudomonas</i> spp.	5	<i>Pseudomonas</i> spp.	3	<i>Pseudomonas</i> spp.
2	9+	Yeasts	7	Yeasts	5–7	Yeasts - LAB	5	<i>Pseudomonas</i> spp.
3	9+	Yeasts	9+	Yeasts	9	Yeasts	7	Yeasts
4	9+	Yeasts	9+	Yeasts	9+	Yeasts	9+	Yeasts

*(+): > 9 days.

**m/o: microorganism.

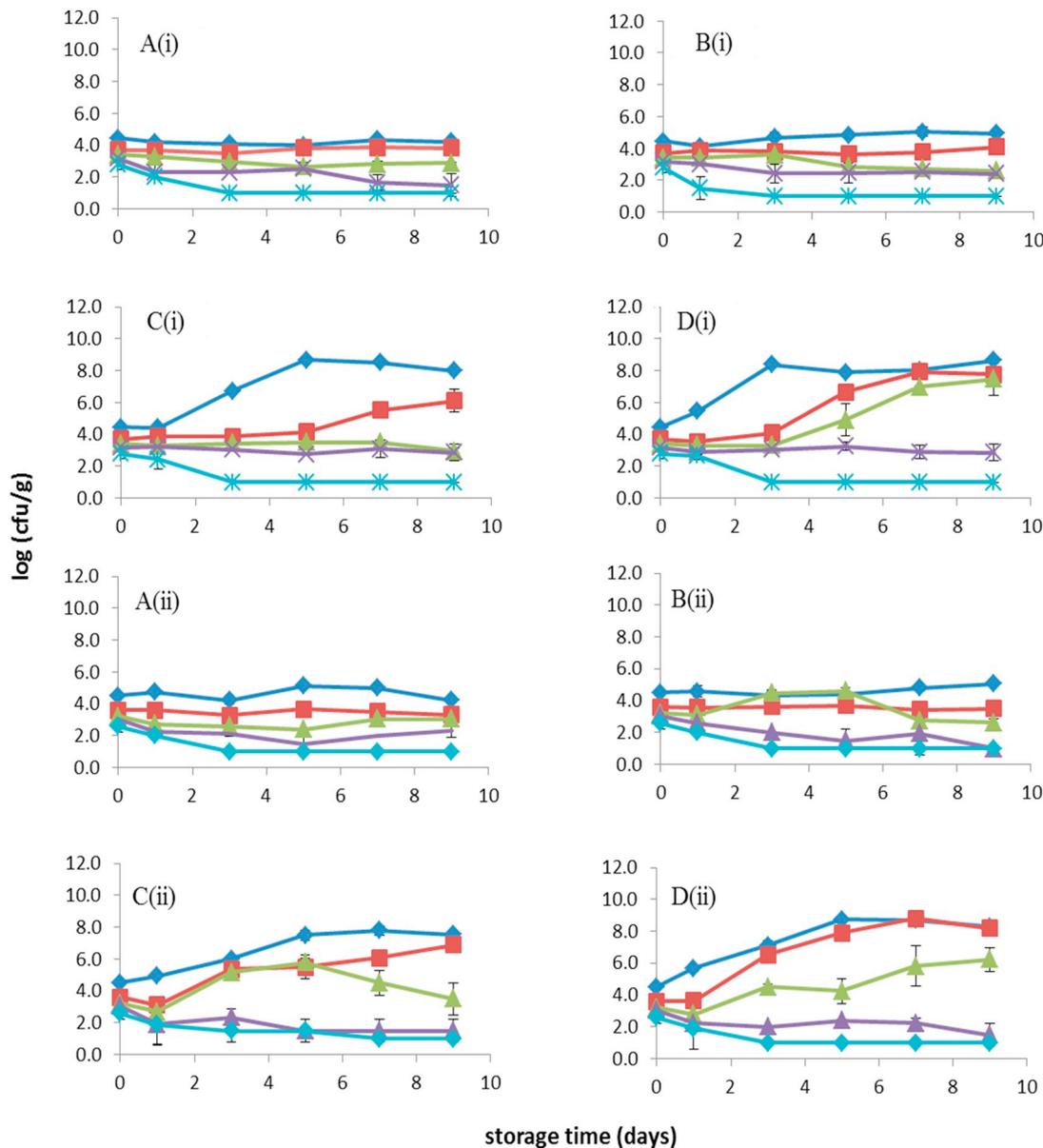


Fig. 3. *Salmonella* counts of chicken samples immersed into apple cider vinegar (i) and lemon juice (ii) of different concentrations in acetic and citric acid, stored at (A) 4 °C, (B) 8 °C, (C) 12 °C, and (D) 16 °C for 9 days. (Error bars < 0.2 are not shown).

7th and 9th days for this specific condition. In this case the microbiological profile of samples was differentiated by storage temperature. Yeasts were dominant at 4 and 8 °C, yeasts and LAB at 12 °C and *Pseudomonas* spp. at 16 °C. It is noteworthy that *Salmonella* counts in

2.0% v/v citric acid at 8 and 12 °C of the 3rd and the 5th day of storage were equal or higher compared to the population enumerated in 1% v/v citric acid. The subsequent decrease of the pathogen could be associated with the growth of LAB after the 7th day that were finally co-dominated

with yeasts (Table 4). The acidic metabolites produced by LAB could probably contribute to the inactivation of *Salmonella*. Marination in vinegar, also affected *Salmonella* counts resulting in increases or decreases depending on the intensity of acid stress, mainly dependent on the concentration of the organic acids, and storage conditions (Fig. 3). It is noteworthy that in treatments with 2.0% v/v acetic acid, absence of *Salmonella* was observed in certain temperatures and storage days (mainly at late storage and at 8, 12, and 16 °C). Finally, by comparing the effect of citric and acetic acids on *Salmonella* survival and growth it is clear that acetic acid was more effective compared to citric acid at a given concentration and chicken meat pH, since for acetic acid a higher amount was in the undissociated form. In the case where the amount of undissociated acid was equal (e.g., lemon juice 2% v/v in citric acid and apple cider vinegar 1% v/v in acetic acid that contained both 65–70 mmol UAC/L), the effect of acetic acid on the pathogen was more pronounced since apart from the total acid concentration and concentration of undissociated acid, the type of acid contributed in the antimicrobial effect as well (Booth and Stratford, 2000). It must be underlined that in this work standard methods for *Salmonella* determination were employed and it must be taken into account that with other recovery methods higher determined levels of the pathogen could be obtained.

3.2. Survival/inactivation interface of *Salmonella*

Parameter estimates and performance statistics of the developed logistic regression models for the inactivation of *Salmonella* for acetic and citric acid treatments are presented in Table 5. Backward stepwise regression eliminated the insignificant ($P > 0.05$) terms from the logistic models, leaving a simplified model consisting of a limited number of terms (linear, quadratic or interaction). The predictive power statistics (concordance, discordance) indicated a good model fit, since concordance rate was 97.6 and 98.1% for acetic and citric acid model, respectively. However, misclassification was higher in the citric acid model since 11.6% of the observed growth/inactivation (GS/INACT) cases were on the wrong side of the boundary (either fail safe or fail dangerous), whereas the respective percentage for acetic acid model was 5.2%. In the case of citric acid, 8.6% of false predictions were “fail safe” (i.e., inactivation was observed but growth or survival was predicted), while in the acetic acid model, the “fail safe” and “fail dangerous” (i.e., growth or survival was observed but inactivation was

predicted) predictions were equal (2.6%). The erroneous predictions were observed in the concentrations of 70 and 114 mmol/L UAC, which are both critical for the interface between survival/growth and inactivation. The majority of “fail dangerous” predictions (83%) were observed at the 1st day of storage whereas the majority of “fail safe” predictions (71%) were observed at the 7th and 9th days of storage. Regarding storage temperature, 65% of false predictions were obtained at 12 and 16 °C. For acetic acid, 75% of the wrong predictions were observed at the 7th and 9th days of storage. The observed GS/INACT responses against undissociated acetic or citric acid concentration and storage temperature at different days are represented in Figs. 4 and 5, respectively, along with the different probability boundaries. In citric acid, the concentrations of 70 and 114 mmol/L UAC determined the inactivation boundaries, whereas the lack of data between these two concentrations led to this abrupt interface. *Salmonella* in samples treated with citric acid containing 21 mmol/L undissociated acid presented growth at all temperatures and storage days, whereas the concentration of 170 mmol/L resulted in inactivation in all tested cases. In acetic acid treatments, the interface was configured by the contribution of the 3 (64, 132 and 204 mmol/L) undissociated acid concentrations and 4 storage temperatures. Only the concentration of 24 mmol/L undissociated acetic acid presented growth regardless of storage temperature and time. Taking into consideration the aforementioned observations, a grouping of false predictions is evident at specific storage days, temperatures or acid concentrations. This is probably due to biological phenomena associated with adaptation mechanisms or interactions between microorganisms that were present on the food matrix which is characterized by high complexity (Pinon et al., 2004). It is obvious that statistical analysis and model development is not possible to take into account the various parameters affecting microorganisms, the so-called completeness error (McMeekin and Ross, 2002). Moreover, the observed missclassifications at 12 and 16 °C could be attributed to the fact that the susceptibility of *Salmonella* spp. to acid stress depends on temperature. It has been reported (Álvarez-Ordóñez et al., 2010) that temperatures as low as 10 °C, remarkably decreased acid resistance and increased the pH boundary for growth of *S. Typhimurium* while the most acid resistant cells were those grown at temperatures closer to the optimum (37 °C).

One representative example is the concentration of 70 mmol/L undissociated citric acid (2% total citric acid), where a shift in the microbial association of spoilage microbiota was recorded by increasing

Table 5

Estimated parameters and performance statistics for the logistic regression models fitted to the growth/survival or inactivation data of *Salmonella* for (A) acetic acid and (B) citric acid model.

A. Acetic acid	Estimate	S.E.	P value
α_0	-11.63	3.33	0.000
α_1	3.730	0.945	0.000
α_2	-0.512	0.169	0.01
α_3	0.103	0.027	0.000
α_4	-0.0034	0.0012	0.048
α_7	-0.300	0.081	0.000
Concordance (%)	97.6		
Correct predictions (%)	94.8		
Fail safe (%)	2.6		
Fail dangerous (%)	2.6		
B. Citric acid	Estimate	S.E.	P value
α_0	-1.676	0.801	0.036
α_1	-0.342	0.103	0.001
α_2	-0.195	0.064	0.002
α_3	0.056	0.008	0.000
Concordance (%)	98.1		
Correct predictions (%)	88.4		
Fail safe (%)	8.57		
Fail dangerous (%)	3.03		

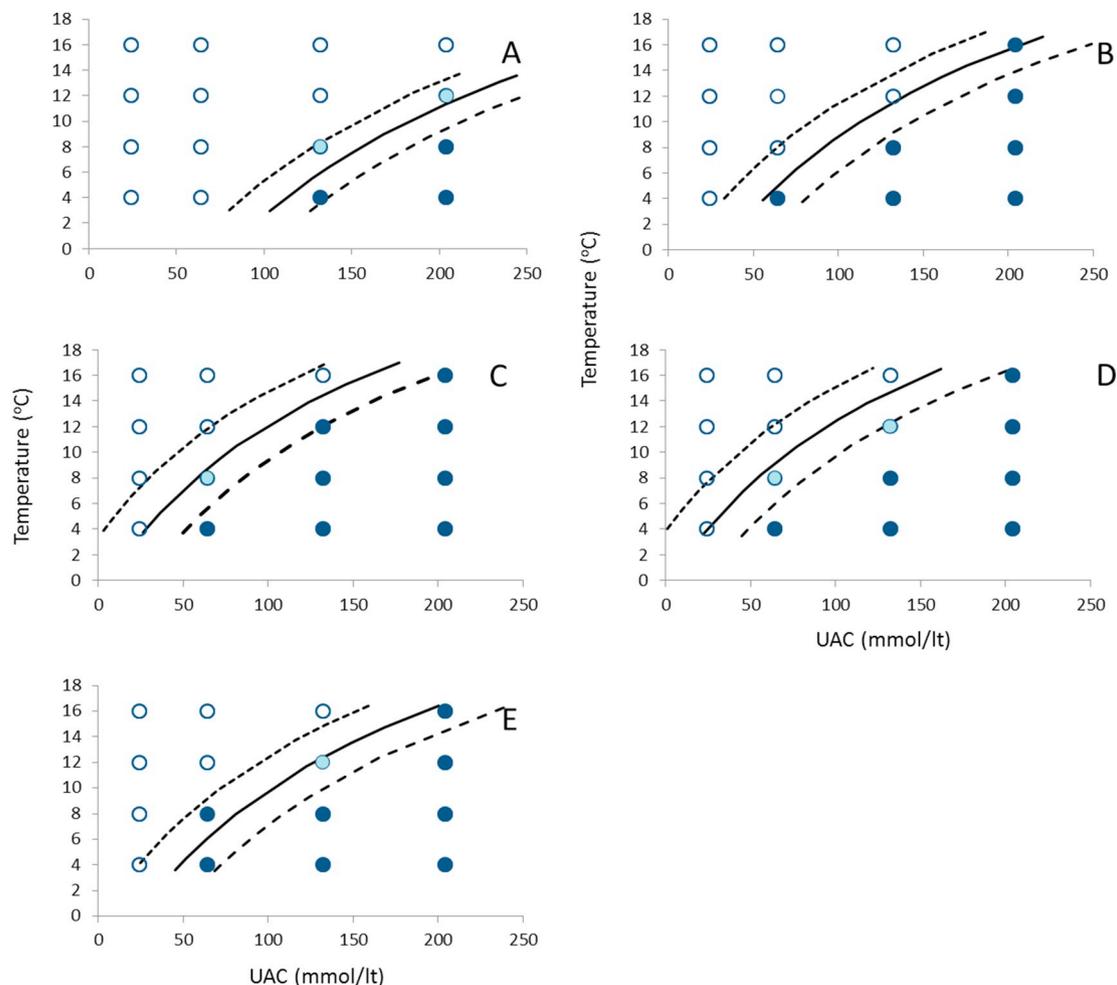


Fig. 4. Inactivation boundaries of *Salmonella* on chicken fillets marinated in different undissociated acetic acid concentrations and stored at different temperatures at the (A) 1st, (B) 3rd, (C) 5th, (D) 7th, and (E) 9th days of storage. Open symbols: growth/survival (GS); dark blue solid symbols: inactivation (INACT); light blue solid symbols: growth/survival (GS) in at least one of the replicates; dashed line: $P = 0.9$; solid line: $P = 0.5$; dotted line: $P = 0.1$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

storage temperature (Table 4). Consequently, the response of *Salmonella* was difficult to predict since different microbial groups (pseudomonads, yeasts and/or LAB) interacted in a different way with the pathogen. It needs to be noted that most misclassified predictions were observed at the first or last (9th) days of storage. This could be attributed to the diverse responses of microorganisms in the adverse, acidic environment after their first, short-term contact (1 day) with the organic acids of the marinades, while for day 9 fluctuations in *Salmonella* could be attributed to several reasons such as adaptation to acid phenomena, microbial weakening and homeostasis disturbance due to the long-time exposure to acidic conditions (Greenacre et al., 2003) or competition with the background microbiota which was in higher populations at that day compared to *Salmonella* (Figs. 2 and 3).

3.3. Validation of the developed model

An external validation using a more complex dataset was undertaken to evaluate the performance of the developed models. This is a process of critical importance for assessing the applicability of models to real situations and identify the potential areas or needs for fine tuning (Pinon et al., 2004). For the validation of the model developed for lemon marinades, 160 samples (80 different cases with 2 replicates each) of various lemon marinades were examined (Table 2). Results showed that 110 out of 160 samples (69%) were correctly predicted, whereas 50 samples were erroneously estimated (31%), with 36 (72%)

and 14 (28%) samples being “fail safe” and “fail dangerous”, respectively. With regard to storage time, 48% of the erroneous predictions were observed in days 7 and 9 while, 26% of them were observed in the 1st day. The majority (11/14 cases) of “fail dangerous” predictions were observed in day 1, while most “fail safe” predictions (23/36) were observed in day 9. The lowest number of wrong predictions was recorded in 114 mmol/L UAC being the highest concentration used in validation. The type of lemon juice or other marinade ingredients did not affect model validation. The response of *Salmonella* in marinades with high concentration in citric acid (114 mmol/L UAC) was successfully predicted under all tested conditions. Concerning temperature, no particular association could be established with the erroneous predictions. In the case of vinegar marinades, 170 samples (85 different cases with two replicates each) were used in validation. In this case, 140 out of 170 samples (82.4%) were correctly predicted, whereas only 30 samples (17.6%) were falsely predicted, with “fail safe” and “fail dangerous” cases being 27 and 3, respectively. Regarding storage time, 21/30 of wrong predictions were observed in the 1st and 9th day of storage. The misclassification trend of predictions was similar to model development and could be attributed to factors such as the complexity of the substrate (chicken meat), the diversity in the background microbiota or the development of mechanisms resulting in variable microbial responses to stresses. The selection of acid concentrations that were similar to the concentrations that were used when fitting the logistic regression model was also a reason for the higher percentage of

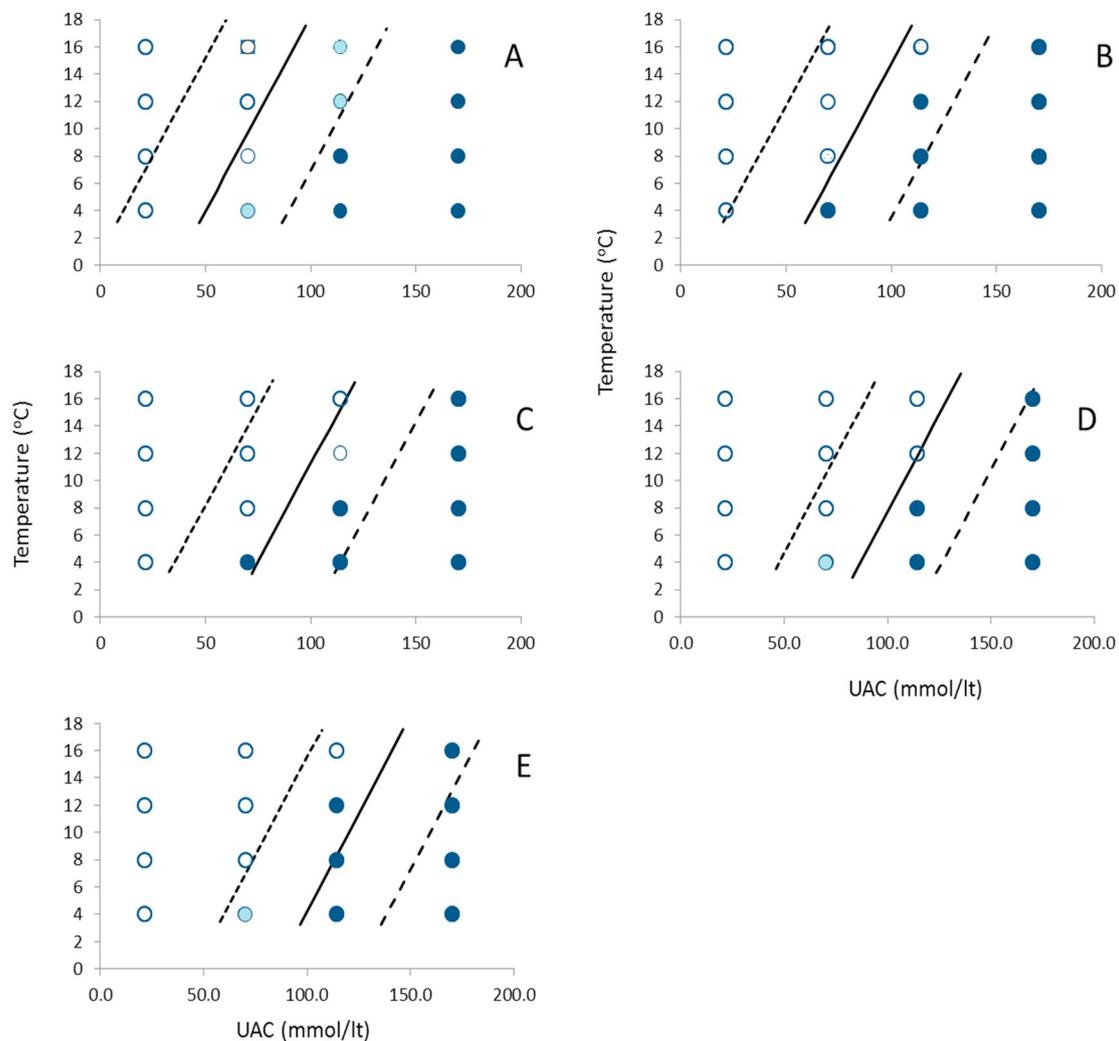


Fig. 5. Inactivation boundaries of *Salmonella* on chicken fillets marinated in different undissociated citric acid concentrations and stored at different temperatures at the (A) 1st, (B) 3rd, (C) 5th, (D) 7th, and (E) 9th days of storage. Open symbols: growth/survival (GS); dark blue solid symbols: inactivation (INACT); light blue solid symbols: growth/survival (GS) in at least one of the replicates; dashed line: $P = 0.9$; solid line: $P = 0.5$; dotted line: $P = 0.1$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

erroneous predictions in validation compared to those of model fitting.

3.4. Survival of *Salmonella* strains

The PFGE analysis in control (non-marinated) samples showed an almost equal distribution ($\pm 10\%$ deviation) of the strains on the first day of experiments. This slight deviation was observed probably due to the random selection of the microbial colonies picked for identification. At the end of shelf life of control samples and especially at 12°C , *S. Infantis* clearly dominated (80%) (Fig. 6). *S. Infantis* was the only serovar isolated from chicken meat and probably it was better adapted to the chicken meat. The complex matrix of a chicken fillet sample and the diversity of the indigenous microbiota are two factors that could differentiate strain inactivation, survival or growth (Singer et al., 2009). Regarding acetic acid treatments, the strain that managed to survive in most cases was *S. Enteritidis* P167807 phage type 4. In citric acid treatments, *S. Typhimurium* CDC 6516-60-ATCC 14028 was the most persistent microorganism even from the 1st day of storage and its dominance was gradually increased from lower to higher acid concentrations and from the early storage to the end of shelf life. This strain differentiation between acetic and citric acid treatments confirmed the fact that strains may respond differently to environmental stresses.

Previous studies have shown that the type of organic acid influences the induction of resistance mechanisms, while different strains present differences in tolerance to specific acids (Greenacre et al., 2003). Álvarez-Ordóñez et al. (2009) reported that *S. Typhimurium* was more resistant to citric compared to lactic and acetic acids after acidification at different pH values and in several growth and challenge media, while Malheiros et al. (2009) showed that *S. Enteritidis* presented increased resistance to acetic acid treatments compared to *S. Typhimurium*. However, generalization of these observations is high-risk since different strains (even from the same serovar) probably present a differentiated response.

4. Conclusion

In this study, the behavior of *Salmonella* in acidic marinades was described taking into consideration the acid concentration (expressed as both total acid and undissociated acid concentration), temperature, storage duration and relative growth of background microbiota. The individual effect of these factors as well as their combination affected the response of the pathogen to acidic stress. The probability of inactivation of the pathogen could be satisfactorily predicted during model development given the complexity of the dataset and the

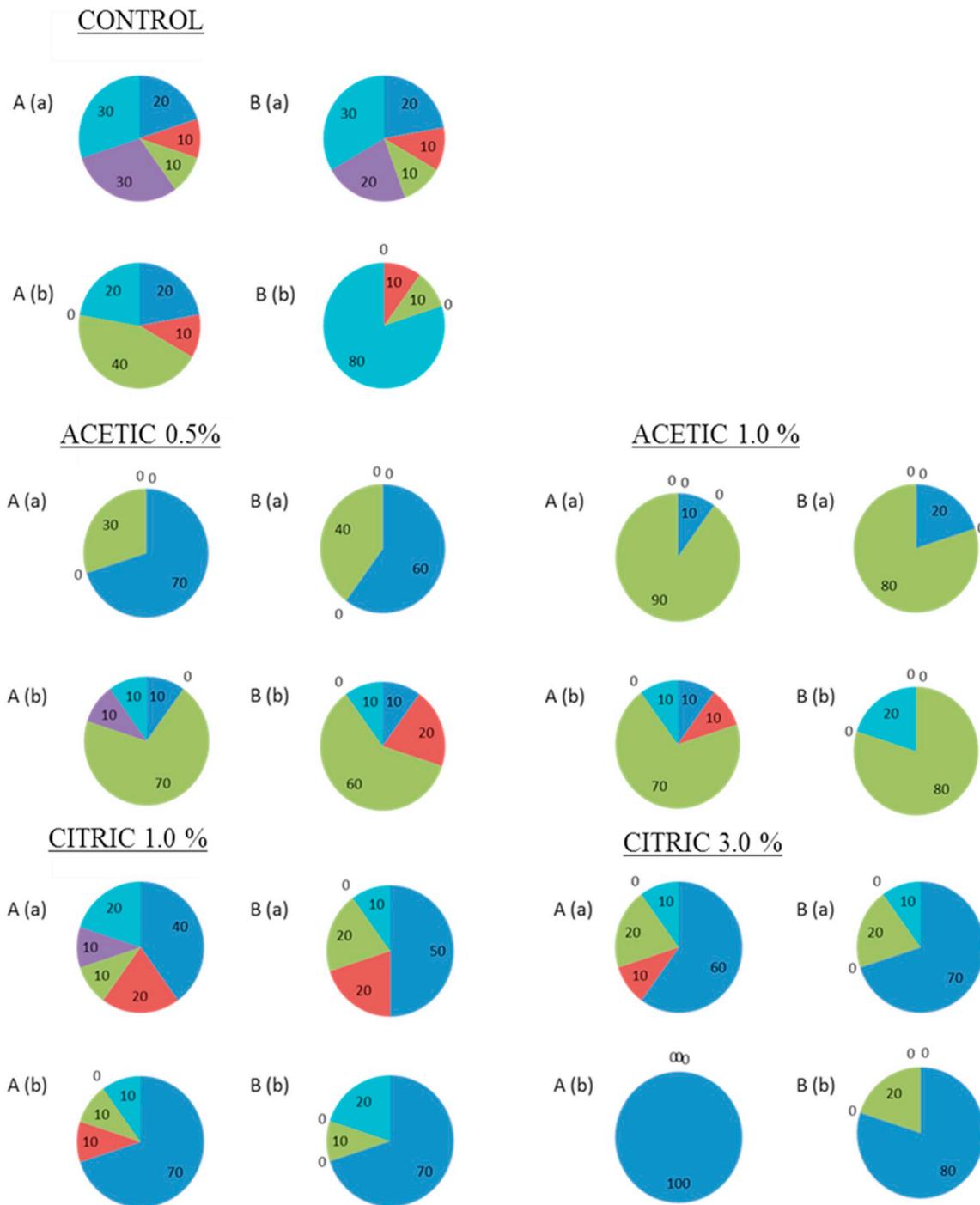


Fig. 6. Percentages (%) of *Salmonella* strain survival in untreated samples (control) and after treatment with acetic and citric acids stored at 4 °C (A) and 12 °C (B) at the early storage (a) and the end of shelf life (b) as defined by TVC counts exceeding 7.0 log CFU/g.

presence of several biological phenomena such as protective mechanisms against acid stress, microbial antagonism and impact of food matrix on bacterial survival and growth. In model validation, the *Salmonella* response in the majority of the cases with vinegar marination was correctly predicted while the misclassification rate was higher in lemon marination. In both vinegar and lemon marination, the trend of the erroneous predictions was similar to that observed in models development. The PFGE results revealed the differentiated response of *Salmonella* to acidic stress since the acid concentration and the type of acid were strain-dependent, while based on these results, it is evident that the developed models described the behavior of the dominant strains.

References

Álvarez-Ordóñez, A., Fernandez, A., Bernardo, A., Lopez, M., 2009. Comparison of acids on the induction of an acid tolerance response in *Salmonella typhimurium*, consequences for food safety. *Meat Sci.* 81 (1), 65–70.

Álvarez-Ordóñez, A., Fernández, A., Bernardo, A., López, M., 2010. Acid tolerance in *Salmonella typhimurium* induced by culturing in the presence of organic acids at different growth temperatures. *Food Microbiol.* 27, 44–49.

Álvarez-Ordóñez, A., Prieto, M., Bernardo, A., Hill, C., López, M., 2012. The acid tolerance response of *Salmonella* spp.: an adaptive strategy to survive in stressful environments prevailing in foods and the host. *Food Res. Int.* 45 (2), 482–492.

Birk, T., Gronlund, A.C., Christensen, B.B., Knochel, S., Lohse, K., Rosenquist, H., 2010. Effect of organic acids and marination ingredients on the survival of *Campylobacter jejuni* on meat. *J. Food Prot.* 73 (2), 258–265.

Booth, I.R., Stratford, M., 2000. Acidulants and low pH. In: Gould, G.W., Russell, N.J.

- (Eds.), *The Molecular Basis of Food Preservative Action*. Blackie.
- Brenner, F.W., Villar, R.G., Angulo, F.J., Tauxe, R., Swaminathan, B., 2000. *Salmonella* nomenclature. *J. Clin. Microbiol.* 38 (7), 2465–2467.
- Davidson, P.M., 2001. Chemical preservatives and natural antimicrobial compounds. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), *Food Microbiology – Fundamentals and Frontiers*, second ed. American Society for Microbiology, Washington DC, pp. 593–627.
- EFSA European Food Safety Authority, 2017. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA J.* 15 (12), 5077. <https://doi.org/10.2903/j.efsa.2017.5077>. (228 pp).
- Ercolini, D., Russo, F., Torrieri, E., Masi, P., Villani, F., 2006. Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. *Appl. Environ. Microbiol.* 72, 4663–4671.
- Ercolini, D., Russo, F., Nasi, A., Ferranti, P., Villani, F., 2009. Mesophilic and psychrotrophic bacteria from meat and their spoilage potential in vitro and in beef. *Appl. Environ. Microbiol.* 75, 1990–2001.
- Greenacre, E.J., Brocklehurst, T.F., Waspe, C.R., Wilson, D.R., Wilson, P.D.G., 2003. *Salmonella enterica* serovar typhimurium and *Listeria monocytogenes* acid tolerance response induced by organic acids at 20 degrees C: optimization and modeling. *Appl. Environ. Microbiol.* 69 (3945–3951), 0099–2240.
- Greer, G.G., Dilts, B.D., 1992. Factors affecting the susceptibility of meatborne pathogens and spoilage bacteria to organic acids. *Food Res. Int.* 25, 355–364.
- Gunvig, A., Hansen, F., Borggaard, C., 2013. A mathematical model for predicting growth/no-growth of psychrotrophic *C. botulinum* in meat products with five variables. *Food Control* 29, 309–317.
- Höll, L., Behr, J., Vogel, R.F., 2016. Identification and growth dynamics of meat spoilage microorganisms in modified atmosphere packaged poultry meat by MALDI-TOF MS. *Food Microbiol.* 60, 84–91.
- Isohanni, P., Alter, T., Saris, P., Lyhs, U., 2010. Wines as possible meat marinade ingredients possess antimicrobial potential against *Campylobacter*. *Poult. Sci.* 89, 2704–2710.
- Karam, L., Roustom, R., Abiad, M.G., El-Obeid, T., Savvaidis, I., 2019. Combined effects of thymol, carvacrol and packaging on the shelf-life of marinated chicken. *Int. J. Food Microbiol.* 291, 42–47.
- Kargiotou, C., Katsanidis, E., Rhoades, J., Kontominas, M., Koutsoumanis, K., 2011. Efficacies of soy sauce and wine base marinades for controlling spoilage of raw beef. *Food Microbiol.* 28 (1), 158–163.
- Lytou, A., Panagou, S., Nychas, G.J., 2017. Effect of different marinating conditions on the evolution of spoilage microbiota and metabolomic profile of chicken breast fillets. *Food Microbiol.* 66, 141–149.
- Malheiros, P.S., Brandelli, A., Noreña, C.P.Z., Tondo, E.C., 2009. Acid and thermal resistance of a *Salmonella* Enteritidis strain involved in several foodborne outbreaks. *J. Food Saf.* 29, 302–317.
- McMeekin, T.A., Ross, T., 2002. Predictive microbiology: providing a knowledge based framework for change management. *Int. J. Food Microbiol.* 52, 1–27.
- Nicodème, M., Grill, J.P., Humbert, G., Gaillard, G.L., 2005. Extracellular protease activity of different *Pseudomonas* strains: dependence of proteolytic activity on culture conditions. *J. Appl. Microbiol.* 99, 641–648.
- Nisiotou, A., Chorianopoulos, N.G., Gounadaki, A., Panagou, E.Z., Nychas, G.J.E., 2013. Effect of wine-based marinades on the behavior of *Salmonella* typhimurium and background flora in beef fillets. *Int. J. Food Microbiol.* 164, 119–127.
- Oscar, T.P., 2007. Predictive model for growth of *Salmonella* typhimurium DT104 from low and high initial density on ground chicken with a natural microflora. *Food Microbiol.* 24, 640–651.
- Pathania, A., McKee, S.R., Bilgili, S.F., Singh, M., 2010. Antimicrobial activity of commercial marinades against multiple strains of *Salmonella* spp. *Int. J. Food Microbiol.* 139 (3), 214–217.
- Pinon, A., Zwietering, M., Perrier, L., Membré, J.M., Leporq, B., Mettler, E., Thuault, D., Coroller, L., Stahl, V., Vialette, M., 2004. Development and validation of experimental protocols for use of cardinal models for prediction of microorganism growth in food products. *Appl. Environ. Microbiol.* 70, 1081–1087.
- Rhoades, J., Kargiotou, C., Katsanidis, E., Koutsoumanis, K.P., 2013. Use of marination for controlling *Salmonella enterica* and *Listeria monocytogenes* in raw beef. *Food Microbiol.* 36 (2), 248–253.
- Singer, R.S., Mayer, A.E., Hanson, T.E., Issacson, R.E., 2009. Do microbial interactions and cultivation media decrease the accuracy of *Salmonella* surveillance systems and outbreak investigations? *J. Food Prot.* 72 (4), 707–713.
- Valero, A., Pérez-Rodríguez, F., Carrasco, E., Fuentes-Alventosa, J.M., García-Gimeno, R.M., Zurera, G., 2009. Modelling the growth boundaries of *Staphylococcus aureus*: effect of temperature, pH and water activity. *Int. J. Food Microbiol.* 133, 186–194.
- Vermeulen, A., Smigic, N., Rajkovic, A., Gysemans, K., Bernaerts, K., Geeraerd, A., Van Impe, J., Debevere, J., Devlieghere, F., 2007. Performance of a growth-no growth model for *Listeria monocytogenes* developed for mayonnaise-based salads: influence of strain variability, food matrix, inoculation level, and presence of sorbic and benzoic acid. *J. Food Prot.* 70, 2118–2126.
- Wilde, S., Jorgensen, F., Campbell, A., Rowbury, R., Humphrey, T., 2000. Growth of *Salmonella enterica* serovar Enteritidis PT4 in media containing glucose results in enhanced *RpoS*-independent heat and acid tolerance but does not affect the ability to survive air-drying on surfaces. *Food Microbiol.* 17, 679–686.
- Yang, J., Lee, D., Afaisen, S., Gadi, R., 2013. Inactivation by lemon juice of *Escherichia coli* O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* in beef marinating for the ethnic food kelaguen. *Int. J. Food Microbiol.* 160, 353–359.
- Yusop, S.M., O'Sullivan, M.G., Kerry, J.F., Kerry, J.P., 2010. Effect of marinating time and low pH on marinade performance and sensory acceptability of poultry meat. *Meat Sci.* 85 (4), 657–663.
- Zakariénė, G., Rokaitytė, A., Ramonaitė, S., Novoslovskij, A., Mulkytė, K., Zaborskienė, G., Malakauskas, M., 2015. The antimicrobial effect of spice-based marinades against *Campylobacter jejuni* on contaminated fresh broiler wings. *J. Food Sci.* 80 (3), M627–M634.