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Growth kinetics of heated *Bacillus cereus* spores in extracts acidified with different acidulants

Wachstumskinetik erhitzter, mit verschiedenen Säuerungsmitteln angesäuerter, Bacillus cereus Sporen

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Summary

The influence of acidification on the growth of *Bacillus cereus* ATCC 7004 spores after heat treatment was studied. *B. cereus* spores were submitted to heat treatment (90 °C, 10 min) and later they were incubated in nutrient broth, chicken and rice extracts acidified to various pH levels with different acids. The organisms that survived heating were able to recover, germinate, and grow at low pH values. The effect of the pH of the recovery medium on the growth of heated *B. cereus* was influenced significantly by its composition and the acidifying agents. Lactic acid resulted in higher minimum pHs allowing growth of spores of *B. cereus.* However, citric acid was the most effective acidulant, inhibiting the spores grown in rice and chicken extracts. Rice extract was more stressful to the spores than chicken extract and nutrient broth without acidifying.

Keywords: *Bacillus cereus,* acidity, pH, recovery, growth parameters

Zusammenfassung

In der vorliegenden Arbeit wurde der Einfluss von Säuerung auf das Wachstum von *Bacillus cereus* ATCC 7004 Sporen nach einer Hitzebehandlung untersucht.

Zunächst wurden die *B. cereus* Sporen einer Hitzebehandlung (10 min. bei 90 °C) unterzogen. Im Anschluss wurden die Sporen in einer Nährbouillon inkubiert; Huhn und Reisextrakt wurden zu unterschiedlichen pH-Werten mit verschiedenen Säuren angesäuert.

Die Organismen, die bei der Hitzebehandlung nicht abgetötet wurden, konnten sich erholen, auskeimen und bei niedrigen pH-Werten wachsen. Die Auswirkungen des pH-Wertes des Wiederherstellungsmediums auf das Wachstum der erhitzten *B. cereus* Sporen wurde durch seine Zusammensetzung und Säuerungsmittel stark beeinflusst.

Milchsäure führte zu höheren pH-Minimalwerten, was das Wachstum von *B. cereus* Sporen ermöglichte. Dennoch stellte sich Zitronensäure als effektivstes Säuerungsmittel heraus, da sie die Sporen auf Reis- und Hühnerfleischextrakt am Wachstum hinderte. Reisextrakt stellte sich als strapazierender für die Sporen heraus als das Hühnerfleischextrakt und die nicht gesäuerte Nährbouillon.

Schlüsselwörter: *Bacillus cereus,* Säuregehalt, pH-Wert, Wiederherstellung, Wachstumsparameter

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Introduction

Bacillus cereus is a spore-forming bacterium that has been recognized as a causative agent of spoilage of foods and foodborne pathogen (Stenfors Arnesen et al., 2008). *B. cereus* is found in wide variety of environments and different substrates including meats, milk, vegetables, and fish (Mosupye and Von Holg, 2000; Nyati, 2000; Rukure and Bester, 2001). *B. cereus* spores are highly resistant to heat, and they are capable of germination and growth at refrigerated temperatures. Minimally processed refrigerated foods are a particular interest with respect to *B. cereus* food poisoning because these mild processing conditions inactivate vegetative cells, but do not inactivate spores. On the other hand, the number of vegetative bacterial formed during the cooling phase of these products could increase, because the heat treatment can produce activation of endospores rather than inactivation (Collado et al., 2003).

Survival of spores after heat treatment is greatly influenced by the characteristics of recovery medium, which determine the capacity of bacteria to repair heat damage and, in this regard, pH medium is a very important factor. The spores which survived the heat treatment usually do not germinate under acidic conditions (Blocher and Busta, 1983; Leguerinel et al., 2005). Therefore, low pH is recognised as an important factor in reduction and inactivation of bacteria. Some acids are often added to foods to prevent or delay the growth of bacteria (Dziezak, 1986; Podolak et al., 1996). However, it is necessary to bear in mind that different microorganisms are showing different rankings for the inhibitory effects of acids (Matsuda et al., 1994).

There are several workers who have shown the effects of composition and pH of the recovery medium against *B. cereus* (Fernández et al., 1999; Hsiao and Siebert, 1999; Gaillard et al., 2005). However, many studies have been carried out on laboratory medium, and insufficient information is available about different foods. Furthermore, pH of medium is often considered constant, but it may vary extensively throughout food processing.

Because of the widespread distribution of *B. cereus* in food products, it is important to understand the capacity of these microorganisms to survive and multiply under conditions associated with food processing, storage, and distribution, and to be able to predict the ability of the *B. cereus* spores to survive heat treatment, and the influence of environmental conditions on their growth in foods.

On the other hand, the incidence of *B. cereus* is higher in some foods such as meat or vegetables containing foods after cooking. The rice and chicken meat products are frequently used as ingredients in processed foods and they can be the source of *B. cereus*.

The aim of this investigation was to study the ability of *B. cereus* ATCC 7004 to grow in different media (nutrient broth and rice and chicken extracts) acidified with different acidulants after mild heat treatment. Assessment of the growth kinetic after heat treatment in different acidified foods may provide useful data for designing commercial processes that ensure microbiological safety.

Materials and Methods

Microorganism and preparation of spore suspensions

The strain 7004 of *Bacillus cereus* from the American Type Culture Collection (ATCC) (Rockville, MD, USA) was used. The spores were obtained on nutrient agar (NA) (Oxoid Ltd., Basingstoke, UK), collected and washed according to the procedures described by Mazas et al. (1995). Spores were resuspended in 180 mM McIlvaine buffer (pH 7.0) (anhydrous dibasic sodium phosphate and monohydrate citric acid, Panreac Quimica SA, Barcelona, Spain) prepared as described previously McKenzie and Dawson (1969), to achieve a stock spores suspensions containing 10^7–10^8 colony forming units (cfu/ml). Cultures were maintained at 2 ± 1 ºC until used. The viability of the spores was very stable.

Growth media

Nutrient broth (NB) (Oxoid) was prepared according to the manufacturer's instructions.

In order to prepare the chicken extracts, meat without fat (350 g), purchased at a local market, was cut into 1–2 cm cubes and minced with 750 ml of distilled water into flasks which were sealed, and heated at 121 ºC for 15 minutes.

For the preparation of the rice extracts, 100 g of rice were blended with 900 ml of distilled water into flasks, and heated at 100 ºC for 5 minutes.

The extracts were filtered through several layers of sterile gauze.

The pH values of nutrient broth were adjusted to 7.0; 6.5; 6.0; 5.5; 5.0; 5.0; 4.5 and 4.2 using 1 N hydrochloric, ascorbic, citric, and lactic acids. The rice and chicken extract were used at natural pH (6.6 and 6.3, respectively) and acidified to pH 6.0, 5.0 and 4.2 with 1 N citric, ascorbic and lactic acids.

The flasks were capped with foam plugs and sterilized by autoclaving for 15 min at 121ºC. After preparation, the extracts were stored frozen prior to use.

Heat injury

0.1 ml of the stock spore suspension was transferred to 5 ml test tube containing 1.0 ml of sterile rice and chicken extracts or 1.0 ml sterile nutrient broth. The heat treatment was performed in a stirred water bath WB22 (Memmert GmB-H Co., Schwabach, Germany). The temperature chosen for this study was 90 ºC. After 10 min heat treatment, tubes were removed and rapidly cooled in ice water.

Growth curves

Growth curves were obtained in nutrient broth with different pH values and in natural and acidified chicken and rice extracts.

0.3 ml of the heat-treated spore mixture were transferred to 150 ml of the final medium (rice or chicken extracts or nutrient broth tempered overnight at the appropriate incubation temperature), with the different initial pH values tested. Triplicate flasks were used for each cultural condition. Flasks were incubated aerobically at 30 ºC. The temperature was recorded and was kept within ± 1 ºC the desired temperature.

At appropriate intervals (0, 1, 2, 4, 6, 8, 12, 16, 24, 48, 72, 96, 120, 144, 168, 216 h), samples of the cultures (1.0 ml) were taken from each flask representing a particular condition and decimally diluted in sterile 0.1 % (w/v) peptone solutions (Oxoid). Appropriate dilutions were plated in duplicate on nutrient agar (Oxoid). Plates were incubated at 30 ºC during 24 hours, and the numbers of colonies (cfu/ml) were enumerated.

At the same time, 5.0 ml aliquots were taken for pH testing.

Growth curves were constructed and the flasks were kept incubating until the beginning of the death phase or 50 days if growth was not detected.

Modelling of growth curves

For each combination of cultural conditions the bacterial numbers obtained were plotted against time to produce growth curves.

Growth curves were generated from each variable condition by fitting the Gompertz equation as previously described (Gibson et al., 1987; Buchanan et al., 1997), in conjunction with the Program GraphPad.Prism (GraphPad Software Inc., San Diego, California, USA), a non-linear curve-fitting regression program. Gompertz parameters were used to calculate exponential growth rate (EGR, log (cfu/ml)/h), generation time (GT, h), lag-phase duration (LPD, h) and maximum population density (MPD, log cfu/ml). The program also calculates the goodness of fit (R^2) for each growth curve performed.

Results and discussion

A mild heat treatment of the foods kills any vegetative *B. cereus* cells but not the spores. These spores may activate, geminate and grow if the foods or the recovery mediums are stored at temperature abuse, and the conditions are favourable.

The heat resistance of *B. cereus* spores is very variable. It has been described D-values (time of decimal reduction) ranging from 5 to 108 min at 85 °C and from 0.3 to 27 min at 100 °C (Bergère and Cerf, 1992; Picoche et al., 1993). In a previous study on the heat resistance of *B. cereus* ATCC 7004, it has showed for this microorganism D-values (min) ranging from 1.2 (in carrot extract at pH 4.0) to 19.5 (in phosphate buffer at pH 5.2) (Moussa-Boudjemaa et al., 2006).

ATCC 7004 strain was able to survive processing at 90 °C for 10 min and germinate and grow after heating when recovery conditions were favourable.

The surviving populations were determined as described in Materials and methods. Growth parameters calculated for heated *Bacillus cereus* ATCC 7004 incubated in nutrient broth with initial pH 7.0, 6.5, 6.0, 5.5, 5.0, 4.5 or 4.2 are shown in Table 1. The pH was regulated at desired value by addition of 1 N hydrochloric, ascorbic, citric and lactic acids.

Growth curves obtained fitted well to the Gompertz function, with high R^2 values.

Growth of *B. cereus* surviving heat treatment was dependent not only on pH but also on the type of acidulant and medium. Our results show that for the same pH values, lactic acid was more effective than ascorbic, citric and hydrochloric acids at lower pH values. *B. cereus* cells were not able to grow in nutrient broth acidified with lactic acid to pH 4.2, 4.5 and 5.0 beyond 50 days at 30 °C.

However, increasing the pH resulted in a reduction in the effectiveness of lactic acid. Our result showed that at pH values ≥ 5.5, lactic acid causes higher lag phases than citric and ascorbic acids however it reduces the growth rate.

Citric acid prevents growth of survivors at pH 4.2 and 4.5, but its inhibitory effect disappears at higher pH (pH 5.0). During growth in nutrient broth acidified with citric acid at pH values between 7.0 and 5.0, no significant differences in growth parameters were observed.

The lowest pH at which our strain was able to grow in nutrient broth acidified with ascorbic acid, was 4.5. In these conditions, the lag phase and generation time were 19.5 and 3.5 h, respectively. The minimum lag times were observed in nutrient broth acidified with ascorbic acid at pH 7.0 and 6.5. Hernández-Herrero et al. (2008) observed that the lowest pH for growth of *B. cereus* in nutrient broth acidified with citric acid at 16 °C was 5.0. Biesta- Peters et al. (2010) observed that the lower pH values for growth of *B. cereus* in BHI broth acidified with sulphuric acid was 4.8.

The type of acidulant determines the capacity to grow at low pH values of *B. cereus*. Mols and Abee (2011) showed that the growth rate and lag phase of *B. cereus* are highly dependent on acidulant used. Usually, organic acids inhibit microorganism growth more than inorganic acids because of their lipophilic nature (Corlett and Brown, 1980). In our case, acidification at pH 4.2 was unable to prevent growth of heated *B. cereus* in nutrient broth acidified with hydrochloric acid. Heated *B. cereus* exhibited lengthy lag phase compared to other conditions (178.5 h), and the maximum population density detected was lower (5.28 log cfu/ml).

TABLE 1: *Growth parameters for heated* B. cereus *spores ATCC 7004 in nutrient broth acidified with different acidulants, at 30 °C.*

Type of acid	pH	MPD	EGR	GT	LPD	R^2
Without acid	7.4*	7.91	0.79	0.38	5.47	0.97
Hydrochloric acid	4.2	5.28	0.09	3.65	178.50	0.99
	4.5	7.91	0.14	2.08	21.83	0.99
	5.0	7.95	0.69	0.44	5.16	0.97
	5.5	8.26	0.61	0.50	5.44	0.98
	6.0	8.11	0.75	0.41	4.91	0.99
	6.5	7.79	0.82	0.35	5.23	0.98
	7.0	7.82	0.79	0.38	5.57	0.99
Ascorbic acid	4.2	NG	NG	NG	NG	NG
	4.5	8.57	0.09	3.52	19.55	0.98
	5.0	8.56	0.43	0.71	5.09	0.99
	5.5	8.41	0.44	0.69	3.21	0.98
	6.0	8.59	0.47	0.63	3.69	0.99
	6.5	8.86	0.46	0.66	0.70	0.99
	7.0	8.79	0.67	0.45	0.68	0.99
Citric acid	4.2	NG	NG	NG	NG	NG
	4.5	NG	NG	NG	NG	NG
	5.0	8.24	0.52	0.54	3.26	0.97
	5.5	8.31	0.52	0.57	2.83	0.97
	6.0	8.23	0.54	0.56	2.65	0.98
	6.5	8.71	0.56	0.54	2.59	0.97
	7.0	8.43	0.63	0.48	2.48	0.99
Lactic acid	4.2	NG	NG	NG	NG	NG
	4.5	NG	NG	NG	NG	NG
	5.0	NG	NG	NG	NG	NG
	5.5	8.15	0.80	0.37	4.88	0.99
	6.0	8.35	0.79	0.38	3.96	0.99
	6.5	8.24	0.67	0.45	3.88	0.98
	7.0	8.24	0.71	0.43	3.73	0.98

*: pH nutrient broth without acid; MPD: Maximum population density (log CFU/ml); EGR: Exponential growth rate (log (CFU/ml)/h); GT: Generation time (h); LPD: Lag phase duration (h); NG: No growth within 50 days.

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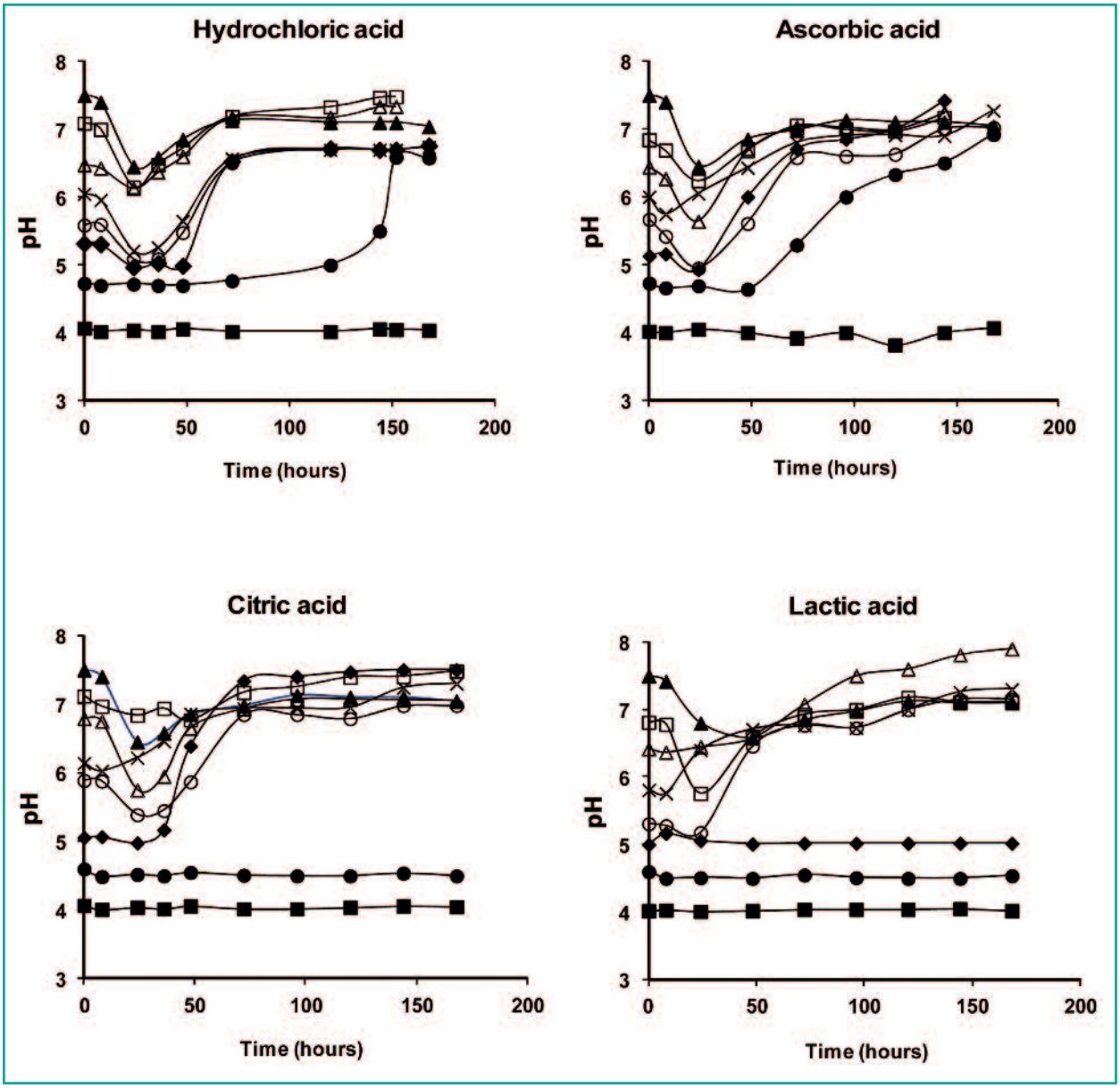

FIGURE 1: *pH evolution during the growth of* B. cereus *ATCC 7004 in nutrient broth acidified with different acids at initial pH 7.4 (▲), 7.0 (□), 6.5 (△), 6.0 (x), 5.5 (○), 5.0 (◆), 4.5 (●) and 4.2 (■).*

It has been suggested that heat treatment decreases the capacity of spores to recover and grow at low pH values and that growth does not occur at pH 4.8 in media acidified with HCl (Anonymous, 1996). However, *B. cereus*, ATCC 7004 strain was able to grow after a heat treatment of 10 minutes at 90 ºC in nutrient broth acidified at pH 4.2 with hydrochloric acid.

The pH of the different medium was followed in time to compare the different recovery conditions and determine the pH change. A relationship was observed between final pH medium and the capacity of recovery and growth of *B. cereus* ATCC 7004. As seen in Figure 1, heated *B. cereus* spores were able to change the pH of the medium during growth. The changes were more pronounced with higher initial pH values, and they were dependant on the type of acidulant.

The pH of the nutrient broth remained constant whenever the initial pH was 4.2 coinciding with the absence of growth in most of cases. At initial pH ≥ 5.0, the pH decreases quickly in the first hours during growth, except when nutrient broth was acidified with lactic acid at pH 5.0. After, the pH of the medium gradually increased over the end of the incubation period.

The lower recovery rate caused by low pH differed with the medium. Table 2 shows growth parameters for heated *B. cereus* spores in natural rice and chicken extract (pH 6.6 and 6.3, respectively), and in both extracts acidified at different pH values with ascorbic, citric and lactic acids.

Growth parameters of heated *B. cereus* did not only depend on pH but also on the type of acidulant and medium.

In comparison with growth in nutrient broth, growth of heated *B. cereus* in food extracts was more affected by the acidification.

The chicken extract appeared to have a stimulating effect, not being detected the lag phase (< 30 minutes), when heated *B. cereus* was incubated in this extract without acid.

Acidification was able to prevent growth of heated spores of *B. cereus* ATCC 7004 in food extracts, regardless

of the acidulant used, when the initial pH was 4.2. In general, citric acid was the most effective acid in both extracts.

Ascorbic acid was more effective in rice extract than chicken extract. An increase in the LPD and a descent in MPD were observed in rice extract at pH 5.0. These suggest that ascorbic acid is more effective than lactic acid in rice extract. However, ascorbic acid at pH 5.0 was less inhibitory than lactic acid in chicken extract.

At 24 hours, the pH of rice extract in which growth was observed had decreased, and then the pH increased approximately until initial values (Fig. 2). No pH changes were observed in extracts in which the population did not increase. The decrease in pH for growth in chicken extract was not as great as for growth in rice extract during the first few hours.

The specific effect of lactic and citric acid on *B. cereus* has been made available previously. Oscroft et al. (1990) indicated that citric acid was less effective than lactic acid in inhibiting *B. cereus* growth. By contrast, Hsiao and Siebert (1999) did not find any differences in the growth of *B. cereus* in medium acidified with citric or lactic acid, and they indicated that a similar concentration of citric and lactic acids was able to prevent growth of *B. cereus*. Del Torre et al. (2001) reported that lactic acid was more effective in inhibiting *B. cereus* growth than citric acid.

TABLE 2: *Growth parameters for heated* B. cereus *spores ATCC 7004 in rice and chicken extracts acidified with different acidulants, at 30 ºC.*

Medium / Type of acid	pH	MPD	EGR	GT	LPD	R^2
Rice extract						
Ascorbic acid	4.2	NG	NG	NG	NG	NG
	5.0	5.44	0.46	0.66	8.09	0.96
	6.0	5.97	0.80	0.38	6.71	0.98
	6.6[1]	7.90	0.54	0.55	4.30	0.99
Citric acid	4.2	NG	NG	NG	NG	NG
	5.0	NG	NG	NG	NG	NG
	6.0	7.57	0.63	0.48	4.92	0.98
	6.6[1]	7.90	0.54c	0.55	4.30	0.99
Lactic acid	4.2	NG	NG	NG	NG	NG
	5.0	7.36	0.67	0.45	5.22	0.96
	6.0	7.82	0.61	0.49	4.87	0.98
	6.6[1]	7.90	0.54	0.55	4.30	0.99
Chicken extract						
Ascorbic acid	4.2	NG	NG	NG	NG	NG
	5.0	6.94	0.32	0.95	5.92	0.97
	6.0	8.78	0.33	0.90	2.37	0.99
	6.3[1]	8.92	0.39	0.77	ND	0.98
Citric acid	4.2	NG	NG	NG	NG	NG
	5.0	7.29	0.08	3.69	46.91	0.99
	6.0	8.52	0.20	0.42	4.96	0.99
	6.3[1]	8.92	0.39	0.77	ND	0.98
Lactic acid	4.2	NG	NG	NG	NG	NG
	5.0	8.15	0.07	4.58	16.23	0.99
	6.0	8.68	0.60	0.50	3.33	0.99
	6.3[1]	8.92	0.39	0.77	ND	0.98

[1]: Natural pH rice and chicken extracts; MPD: Maximum population density (log CFU/ml); EGR: Exponential growth rate (log (CFU/ml)/h); GT: Generation time (h); LPD: Lag phase duration (h); NG: No growth within 50 days; ND: Not detected.

Young and Foegeding (1993) showed that for given pH values between 4.7 and 6.0, the order of effectiveness of organic acids inhibiting the growth of *Listeria monocytogenes* in BHI (brain heart infusion broth) on an equimolar

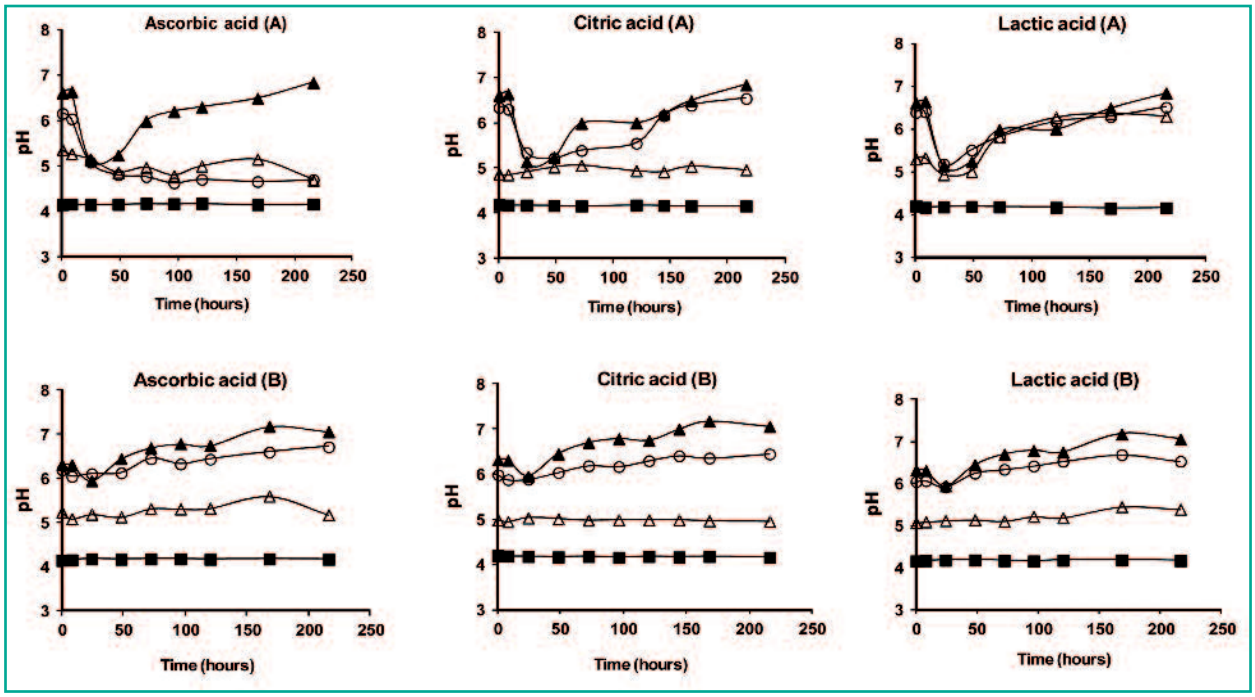

FIGURE 2: *pH evolution during the growth of* B. cereus *ATCC 7004 in rice extract (A) and chicken extract (B) without acidifying (▲) and acidified with different acids at initial pH 6.0 (○), 5.0 (△) and 4.2 (■).*

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basic was acetic > lactic > citric acid. However, with the same data, if the effectiveness was based on initial concentration of undissociated acid, the order was reversed.

For *B. coagulans,* it has been shown that differences between citric and lactic acids could be due to the bigger cation exchange capability of citric acid (Palop et al., 1997).

It is important to point out that the differences among the inactivating effect of lactic and citric acids disappeared with increasing pH.

B. cereus ATCC 7004 was able to change pH medium during its growth. This observation is in agreement with that published previously by García Arribas and Kramer (1990) and Del Torre et al. (2001). These authors showed that *B. cereus* is able to increase pH of an acid environment to values which were optimum to grow and to produce toxins. Cronin and Wilkinson (2009) also observed that the pH values decreased during the growth of *B. cereus* in rice stored at 18 ºC. Chung et al. (1976) related these changes with the glucose metabolism of *B. cereus*. Mols et al. (2010) observed that the pH of the cultures of unstressed *B. cereus*, HCl-shocked *B. cereus* and lactic acid-shocked *B. cereus* increased. They showed that this fact could be due to amino acid catabolism resulting in the production of ammonio. This fact might explicate that no significant changes in *B. cereus* growth were observed above a certain pH (≥ 5.5), regardless of the acidulant used and initial pH values. The changes in pH medium might affect the growth of *B. cereus* spores, and therefore this possibility would have to be considered.

It has also been reported that the inhibitory effect of an extreme pH is different in laboratory medium than in foods. Acidification was able to prevent growth of heated spores of *B. cereus* ATCC 7004 in rice and chicken extracts, but only when the pH was 4.2, regardless of the acidulant used. Both food extracts were more stressful than nutrient broth.

Acidification of rice extract to pH 5.0 with citric acid was sufficient to inhibit heated *B. cereus* growth. In a previous study, it was showed that the starch in the product could stimulate the growth and toxin production by *B. cereus* (García-Arribas and Kramer, 1990). Our result, disagree with this thesis, at least for growth of heated spores of ATCC 7004 strain. Rice extract was more stressful than chicken extract or nutrient broth.

Valero et al. (2000, 2003) reported that citric acid or lemon citric were effectives in inhibiting *B. cereus* growth in carrot and zucchini broth acidified to pH 5.0. However, Del Torre et al. (2001) observed that citric acid was not effective in a REPFED of Italian origin acidified to pH 5.0.

The contradictory results could be due to the physical and chemical characteristics of media. González et al. (1996) showed that the effect caused by low pH on the recovery of *B. cereus* spores, differed with the medium. Other authors have also observed that there is an interaction between the composition and the pH of the recovery medium (Moussa-Boudjemaa et al., 2006).

On the other hand, organic acid (type and concentration) and pH can both independently affect the growth and death of bacterial cells. Palop et al. (1997) suggested that differences in the amount of acidulant used to adjust the pH to required values, could determine the different effect observed. In this case, it is possible that the magnitude of the effect is small, if small amounts of acids are required or the food has low buffer capacity.

The effect of organic acids on the growth of bacteria in acidified foods is also conditioned by hydrogen ion concentration in media. Undissociated acids can diffuse across bacterial cell membranes and they can cause a more important decrease in intracellular pH. This fact can inhibit cellular metabolic enzymes, transport proteins or membrane function (Breidt et al., 2004), and impede the growth, the recovery and toxin production.

Mols et al. (2010) showed that *B. cereus* when it was exposed to pH 5.5 had different physiological and genetic responses to acid shocks depending on the acidulant used.

The effect of pH on growth kinetics is a complex phenomenon. The data presented in this paper indicate that the effect of the acidification is directly related with type of acid, and the medium composition and the conditions should be studied in each case.

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