Combined effect of anaerobiosis, low pH and cold temperatures on the growth capacities of psychrotrophic Bacillus cereus

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Combined effect of anaerobiosis, low pH and cold temperatures on the growth capacities of psychrotrophic *Bacillus cereus*

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A B S T R A C T

Psychrotrophic strains of the foodborne pathogen *Bacillus cereus* can multiply during the refrigerated storage of food products. The aim of this study was to determine the impact of anaerobiosis on the growth of two psychrotrophic *B. cereus* strains exposed to acidic pH at a cold temperature in a laboratory medium. At 10 °C, growth occurred at pH values equal to or higher than 5.7 during anaerobiosis, whereas aerobic growth was observed from pH 5.4. Growth rates during aerobiosis were similar at pH 5.4 and pH 7. No growth was observed for the two tested strains at 8 °C without oxygen regardless of the pH; however, both strains grew at this temperature from pH 5.4 in the presence of oxygen. These pH growth limits in aerobiosis are consistent with those reported for different strains and different foods or media, but no other studies have described anaerobic growth at acidic pH values. The maximal *B. cereus* concentration was approximately 6.0 $\log_{10}$ CFU/ml for cultures in the absence of oxygen and approximately 8.0 $\log_{10}$ CFU/ml for cultures in the presence of oxygen. In conclusion, we found that the combination of anaerobiosis, pH < 5.7 at 10 °C, or anaerobiosis and temperatures ≤8 °C prevent psychrotrophic *B. cereus* growth.

Keywords:
Acid
Low temperature
Oxygen
Growth limits

1. Introduction

Bacillus cereus was reported as the second leading cause of foodborne illnesses between 2006 and 2010 in France, and it is an increasing cause of foodborne outbreaks in the EU (EFSA, 2012, 2013, 2014, 2015). *B. cereus* produces heat-resistant spores (Luu-Thi et al., 2014), and some strains are able to grow at refrigeration temperatures; thus, these bacteria can contaminate different types of food, such as Refrigerated Processed Foods of Extended Durability (REPFEDs), particularly those containing vegetables (Choma et al., 2000; Daelman et al., 2013; Del Torre et al., 2001; Soares et al., 2012).

B. cereus psychrotrophic strains are able to survive to pasteurization treatments (Luu-Thi et al., 2014). The risk depends on their ability to grow at cold temperatures during product storage and transport (Carlin et al., 2013; Guinebretière et al., 2008). However, food products containing fruits and vegetables have different levels of acidity. The capacity of several *B. cereus* strains to grow at cold temperatures was studied, at various pH values, in laboratory medium or in food products such as natural and acidified carrot substrates or zucchini broth at pH 6.5 (Valero et al., 2000, 2003). These studies were done in the presence of air, but food products can also be packaged in the absence of oxygen to prevent oxidative deterioration. Some studies previously showed that oxygen limitation can reduce the growth of *B. cereus* (de Sarrau et al., 2012; Samapundo et al., 2011), but these studies only evaluated growth at neutral pH values. The following combinations of pH, temperature and anaerobiosis have been examined: i) low temperatures and anaerobiosis at neutral pH (de Sarrau et al., 2012) (Samapundo et al., 2011) and ii) pH and temperatures in aerobic conditions (Fernández et al., 2002; Valero et al., 2000, 2003). Moreover, models of *B. cereus* growth as a function of pH, temperature, a_w were developed in the presence of oxygen (Olmez and Aran, 2005; Sutherland et al., 1996).

The goal of the present study was to determine the impact of anaerobic conditions (e.g., by vacuum packaging) on the potential development of *B. cereus* in foods varying in pH and stored at low temperatures. The impact of acidification on *B. cereus* growth at cold temperature and anaerobic conditions was also assessed.

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2. Materials and methods

2.1. Strains and media

Two *B. cereus* strains able to grow at cold temperatures, as described by (Carlin et al., 2013), were used. Strain INRA KBAB4 was isolated from forest soil (Sorokin et al., 2006; Vilas-Boas et al., 2002), and strain ADRIA I21 was isolated from food by ADRIA Normandie (Villier Bocage, France).

Growth experiments were performed in Brain Heart Infusion (BHI; Biokar) broth acidified with HCl 1 N or 6 N after autoclaving to achieve final pH values of 5.0, 5.3, 5.4, 5.5, 5.6, 5.7 and 7.0.

2.2. Growth conditions

The stock of cultures for the whole study consisted in suspensions of exponential phase cells (OD_{600} of 0.5) in 30% final concentration of glycerol, stored at −80 °C.

10 ml of BHI were inoculated in KIMAX tubes with 100 µl of the frozen cultures and incubated at 20 °C under shaking at 200 rpm. When this culture reached an OD_{600} of 0.5, 100 µl were used to start a new culture of 10 ml BHI that was incubated at 10 °C with shaking at 200 rpm to reach an OD_{600} of 0.5. Then, 100 µl of this culture was diluted and used to inoculate tubes containing 10 ml of BHI medium at various pH values at a final concentration of 10^2 CFU/ml. These tubes were then incubated at 8 or 10 °C, with or without oxygen, and shaken at 200 rpm. The CFU were enumerated once or twice a day by sampling 100 µl of each of the different cultures and plating serial dilutions on Luria Bertani (LB; Biokar) agar plates incubated at 30 °C. The drop in pH medium at the end of aerobic growth was of 1 unit for an initial pH of 7.0 and decreased with the decrease of the initial pH value.

Anaerobic cultures were performed in 20 ml Hungate tubes (Dutscher) with butyl septa. Oxygen was eliminated from BHI medium by boiling under a flow of nitrogen passed through a Hungate column to remove any trace of oxygen. Hungate tubes were filled under the flow of oxygen-free nitrogen and autoclaved. Cultures were inserted through the septum with 1 ml sterile syringes (BD Plastipak). For each replicate culture, at each sampling time, one Hungate tube was opened to enumerate bacterial CFU and then discarded. Aerobic cultures were done in KIMAX tubes. For each condition and replicate, the same tube was used for all sampling times. The drop in pH medium at the end of anaerobic growth was of 0.3 unit for an initial pH of 7.0 and decreased with the decrease of the initial pH value.

All growth curves were performed in triplicate with three independent inocula.

2.3. Estimation of lag times, growth rate, and maximal population

Growth curves were established by plotting the $\log_{10}$ CFU/ml as a function of time. Lag times, maximal specific growth rates (μ_{max}), and maximal populations (N_{max}) were determined with the models of Rosso et al. (Rosso et al., 1995), Baranyi and Roberts (Baranyi et al., 1993) and Gompertz (Zwietering et al., 1990).

2.4. Criteria to define growth versus no growth

Growth was defined by an increase in CFU of more than 1 $\log_{10}$ (Pujol et al., 2012), considering that it should be twice the commonly accepted microbiological experimental error of 0.5 $\log_{10}$ CFU. In one of the conditions tested in our study, successive slight increases in counts, approx. 1.5 $\log_{10}$, followed by decreases, were observed and considered as "erratic growth".

2.5. Statistical analysis

The results are expressed as the means of three independent biological replicates. A Student's T-test was used to compare mean values. The null hypothesis was rejected for $P < 0.05$.

3. Results

We studied the combined effect of pH and absence of oxygen on the growth capacity of two strains of *B. cereus* at cold temperatures. We first investigated the effect of the inoculum on growth at low temperature in BHI medium. Inoculum prepared through one subculture at 10 °C always led to lag times of unpredictable durations (up to several days) of the subsequent culture at 10 °C. One subculture at 20 °C reduced the lag time of the subsequent culture at 10 °C. Combining two subcultures, one at 20 °C followed by one at 10 °C, led to growth with the shortest lag time for the subsequent culture at 10 °C (Fig. 1). These two successive subculture steps allowed the best cold adaptation of the two *B. cereus* strains and were applied to all growth kinetic studies.

Table 1 shows growth parameters obtained with the model of Rosso. Growth parameters obtained with the two other models were similar and are not presented. Without oxygen at 10 °C, growth of strain *B. cereus* KBAB4 was observed between pH 5.7 and 7.0 (Fig. 2A). For both pH values, maximal specific growth rates and lag phases were similar ($p > 0.05$) (Table 1). The maximal populations were 5.4 $\log_{10}$ CFU/ml and 6.2 $\log_{10}$ CFU/ml at pH 5.7 and pH 7.0, respectively (Table 1). At pH 5.6, cells survived without significant decline or growth over the duration of the experiment (Fig. 2A). At 8 °C and in absence of oxygen, KBAB4 cells survived at pH 7.0 but not at the other tested pH values (Fig. 2B). In contrast, in the presence of oxygen, KBAB4 cells grew similarly at pH 5.4 and 7.0 at 10 °C and 8 °C (Fig. 2C and D), with maximal populations of approximately 8 $\log_{10}$ CFU/ml (Table 1). At 10 °C and pH values of between 5.4 and 7.0, the maximal specific growth rates were the same, approximately 0.13 h^{-1} ($p > 0.05$) (Table 1) and lag phases were comprised between 5 h and 10 h. In summary, growth of KBAB4 with and without oxygen shows that maximal population achieved without oxygen were markedly lower, but that the maximal specific growth rates, at permissive pH values (≥5.7), during the initial phase of growth, were similar in both conditions ($p > 0.05$) (Table 1). In contrast, lag phases of anaerobic and aerobic cultures were different, with a 30 h-delay before growth in absence of oxygen at 10 °C, compared to growth in presence of oxygen

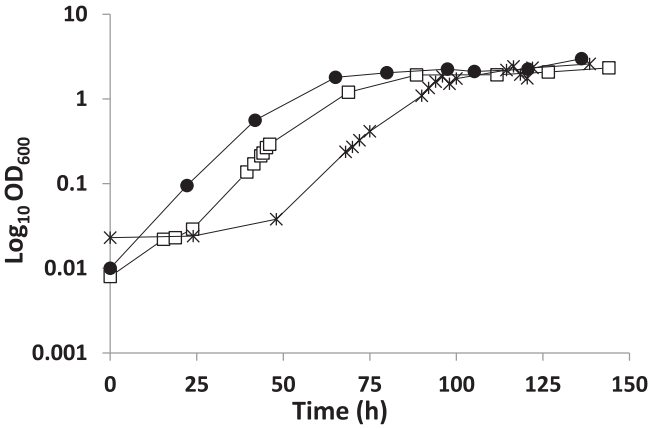

Fig. 1. *B. cereus* KBAB4 growth in BHI at pH 7.0 at 10 °C with oxygen after (●) a subculture at 20 °C followed by subculture at 10 °C, (□) a subculture at 20 °C, and (×) a subculture at 10 °C.

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Table 1
Calculated parameters of growth curves obtained with *B. cereus* KBAB4 and *B. cereus* ADRIA I21 in brain heart infusion (BHI) broth at different pH values and at 10 °C or 8 °C, using the model of Rosso (Rosso et al., 1995). Data shown are the means (in bold) of three replicate growth curves ± range. NT: Not tested; S: Survival; D: Death; EG: Erratic Growth.

pH	Anaerobic						Aerobic					
	KBAB4			ADRIA I21			KBAB4			ADRIA I21		
	Lag time (h)	Maximal specific growth rate (h^{-1})	Nmax (log_{10} UFC/ml)	Lag time (h)	Maximal specific growth rate (h^{-1})	Nmax (log_{10} UFC/ml)	Lag time (h)	Maximal specific growth rate (h^{-1})	Nmax (log_{10} UFC/ml)	Lag time (h)	Maximal specific growth rate (h^{-1})	Nmax (log_{10} UFC/ml)
10 °C												
7.0	**37.81** ± 9.39	**0.13** ± 0.02	**6.22** ± 0.04	**25.95** ± 6.75	**0.08** ± 0.02	**6.03** ± 0.18	**6.80** ± 3.38	**0.13** ± 0.03	**8.52** ± 0.10	**2.03** ± 2.92	**0.13** ± 0.01	**8.31** ± 0.16
5.7	**38.16** ± 10.44	**0.11** ± 0.04	**5.45** ± 0.02	S	S	S	**10.03** ± 4.76	**0.12** ± 0.00	**8.15** ± 0.10	**8.21** ± 3.41	**0.11** ± 0.02	**7.87** ± 0.09
5.6	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT
5.5	S	S	S	S	S	S	**5.90** ± 0.53	**0.13** ± 0.00	**8.07** ± 0.05	**6.11** ± 15.12	**0.08** ± 0.01	**7.71** ± 0.14
5.4	S	S	S	S	S	S	**5.15** ± 0.61	**0.13** ± 0.01	**8.03** ± 0.05	**24.46** ± 1.72	**0.07** ± 0.01	**7.65** ± 0.09
5.3	NT	NT	NT	NT	NT	NT	S	S	S	S	S	S
5.0	D	D	D	NT	NT	NT	D	D	D	NT	NT	NT
8 °C												
7.0	EG	EG	EG	**0.00** ± 0.00	**0.04** ± 0.01	**4.79** ± 0.40	**0.00** ± 0.00	**0.08** ± 0.00	**8.39** ± 0.13	**0.00** ± 0.00	**0.06** ± 0.01	**7.54** ± 0.39
5.7	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
5.6	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
5.5	S	S	S	NT	NT	NT	**4.43** ± 13.17	**0.06** ± 0.01	**7.79** ± 0.10	S	S	S
5.4	NT	NT	NT	NT	NT	NT	**13.40** ± 1.80	**0.07** ± 0.01	**7.77** ± 0.05	S	S	S
5.3	S	S	S	NT	NT	NT	S	S	S	D	D	D
5.0	NT	NT	NT	NT	NT	NT	NT	NT	NT	ND	ND	NT

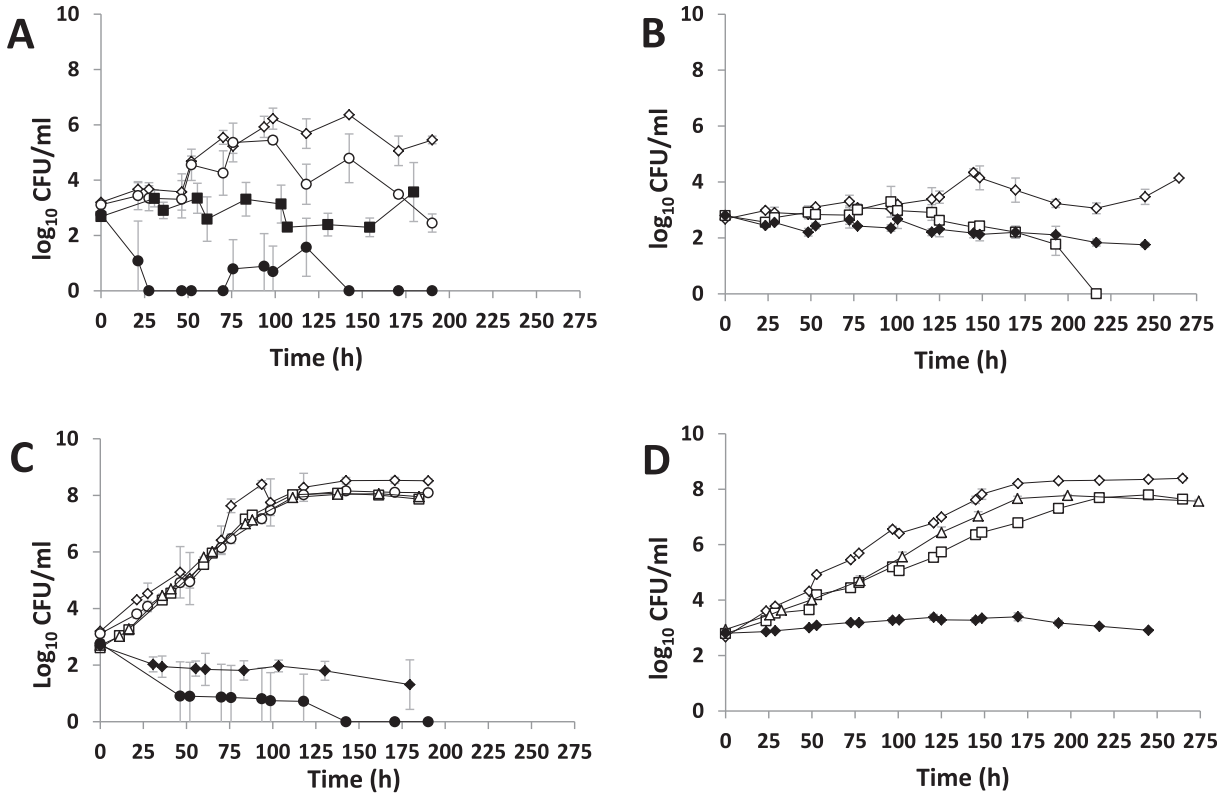

Fig. 2. Growth kinetics of *B. cereus* KBAB4 in BHI at 10 °C (A and C) and 8 °C (B and D), without oxygen (A and B) or with oxygen (C and D) and at pH (◇) 7.0, (○) 5.7, (■) 5.6, (□) 5.5, (Δ) 5.4, (◆), 5.3 and (●) 5.0. Error bars represent standard deviations and can be smaller than symbols. They are comprised between 0.005 and 1.4 log_{10} CFU/ml.

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(Table 1). At 8 °C and in aerobiosis, the maximal specific growth rates at pH 5.4 and 5.5 were slightly lower than at pH 7.0 (0.06 h^{-1} compared to 0.08 h^{-1}) ($p < 0.05$). Lag phases varied between 0 h at pH 7.0 and 13 h at pH 5.4. At both 10 °C and 8 °C, cell survival was observed at pH 5.3 throughout the experiment (Fig. 2C and D).

Without oxygen, the *B. cereus* ADRIA I21 strain grew only at pH 7.0 (Fig. 3A and B). At 10 °C, the maximal population was approximately 6 log_{10} CFU/ml with a maximal specific growth rate of 0.08 h^{-1} and a lag phase of 26 h (Table 1). At 8 °C, cells grew very slightly during the first 100 h before declining. In contrast, with oxygen, growth of strain ADRIA I21 was observed between pH 5.4 and 7.0 at 10 °C (Fig. 3C) with maximal specific growth rates increasing from 0.07 h^{-1} at pH 5.4 to 0.13 h^{-1} at pH 7.0, without any lag phases (Table 1). The strain survived at pH 5.3 (Fig. 3C). Maximal populations of ADRIA I21 at 10 °C were approximately 8 log_{10} CFU/ml at pH 7.0 but tended to be lower for other growth permissive conditions. At 8 °C, growth was observed only at pH 7.0 with a maximal specific growth rate of 0.06 h^{-1}, a maximal population of 7.5 log_{10} CFU/ml, and survival was observed at pH 5.5 (Fig. 3D). As for KBAB4, absence of oxygen markedly reduced maximal populations achieved by ADRIA I21.

4. Discussion

The absence of oxygen increased the lower pH limit for growth at cold temperature. At 10 °C, both KBAB4 and ADRIA I21 were able to grow at pH 5.4 in the presence of oxygen, whereas they did not grow below 5.7 without oxygen. In addition, ADRIA I21 did not grow at pH 5.7 and was thus less able to adapt to a low pH than KBAB4 in absence of oxygen. Absence of oxygen also increased the lower temperature limit for growth. At pH 7.0 and 8 °C, both KBAB4 and ADRIA I21 strains grew in aerobic conditions to final populations close to 8 log CFU/ml, which is consistent with predictive T_{min} values at 3.9 °C and 3.3 °C, respectively (Carlin et al., 2013), whereas KBAB4 did not grow and ADRIA I21 grew slightly before rapidly declining without oxygen. In conditions permitting growth, the absence of oxygen reduced the maximal population (Nmax) compared to aerobic cultures, as observed with a mesophilic strain of *B. cereus* grown at higher temperatures in laboratory media (de Sarrau et al., 2012) and with a psychrotrophic strain of *B. cereus* grown in vegetable purées Samapundo et al. (2011). Absence of oxygen reduced the maximal specific growth rate for ADRIA I21 but not for KBAB4. Absence of oxygen increased lag phases of both KBAB4 and ADRIA I21. Probably, the lack of oxygen leads the cells to a significantly longer adaptation phase before exponential growth. In presence of oxygen, KBAB4 was less affected by pH reduction at cold temperatures than ADRIA I21, as it grew at similar rates and N_{max} values at all pH values permitting growth, in contrast to ADRIA I21 in which the growth rate decreased with acidification.

No previous studies have described *B. cereus* growth in anaerobic conditions at cold temperatures and acidic pH, restricting the comparison of our results with those of the literature to neutral pH. Our results in BHI are consistent with those obtained with a psychrotrophic *B. cereus* strain in vegetable purées ranging in pH from 6.15 to 6.3 in which the authors observed growth rates of approximately 0.03 h^{-1}, similar to that of ADRIA I21 (0.04 h^{-1}), at 8 °C, pH 7.0, and N_{max} values between 4.49 log_{10} CFU/g and 5.35 log_{10} CFU/g depending on the purée, which is similar to the N_{max} of 4.79 log_{10} CFU/ml for ADRIA I21 (Samapundo et al., 2011).

In the presence of oxygen, the pH limit of 5.4 we found in BHI at 10 °C and 8 °C for KBAB4 was the same as for a mixture of two psychrotrophic *B. cereus* strains in carrot purée (Valero et al., 2003). A strain of *B. cereus* was previously reported to grow at pH 5.53 in BHI (Jaquette and Beuchat, 1998). However, the specific growth rates reported previously were lower than those found in our study. For instance, at 8 °C the growth rates of the *B. cereus* strains in the

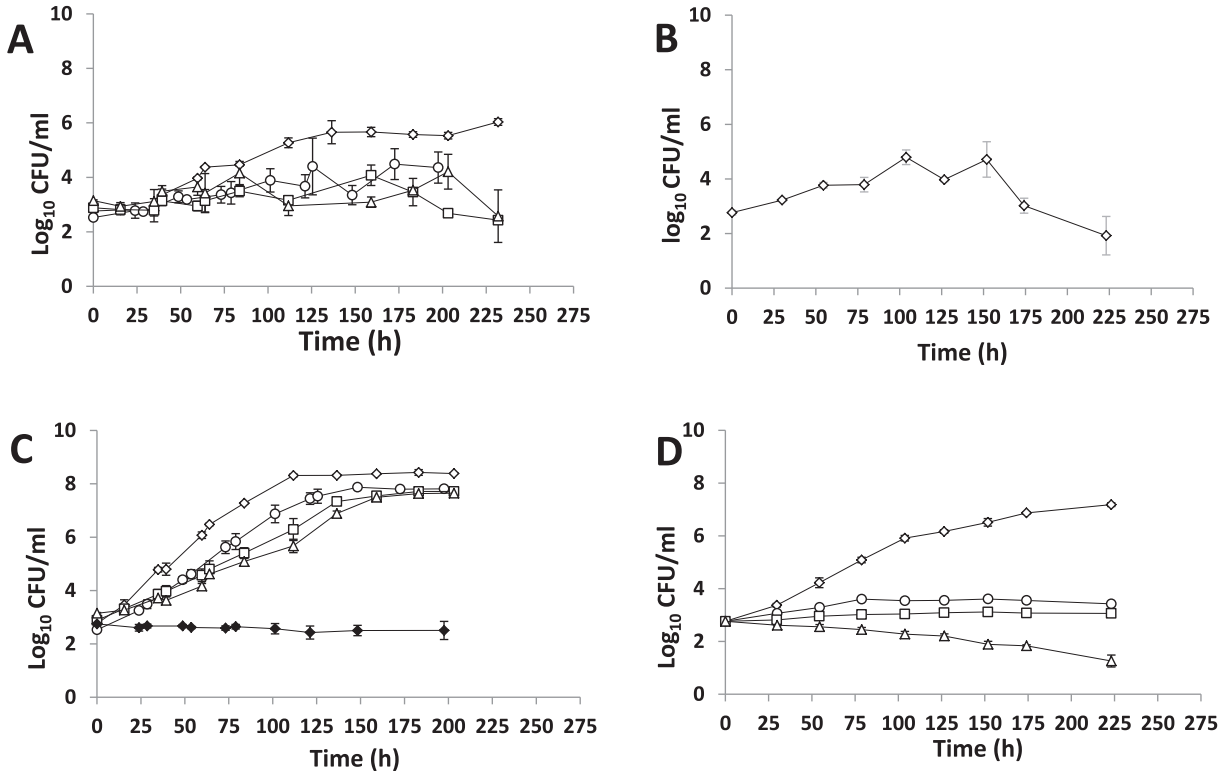

Fig. 3. Growth kinetics of *B. cereus* ADRIA I21 in BHI medium at 10 °C (A and C) and 8 °C (B and D), without oxygen (A and B) or with oxygen (C and D), and at different pH (◇) 7.0, (○) 5.7, (□)5.5, (Δ) 5.4, and (◆) 5.3. Error bars represent standard deviations and can be smaller than symbols. They are comprised between 0.01 and 0.96 log_{10} CFU/ml.

Food Microbiology (2016), Vol. 59, p. 119-123, DOI: 10.1016/j.fm.2016.05.015
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studies of Valero et al. (2000 and 2003) were of 0.047 h^{-1} at pH 6.2 and 0.01 h^{-1} at pH 5.5, compared to 0.07 h^{-1} at pH 5.4 for KBAB4 in the present study (Table 1). No growth was observed by Benedict et al., 1993 at pH below 6.5 and 8 °C. Growth rates presumably differ because of differences in growth media and strains in these different studies. For studies that used BHI (Benedict et al., 1993) the higher growth rate of KBAB4 may reflect a particularly good adaptation of this strain to the combined low pH and cold temperature.

The results from this study and from the previous studies cited above show that at 10 °C and during anaerobiosis, the pH limit for growth of psychrotrophic *B. cereus* is between 5.7 and 5.6, whereas it is between 5.4 and 5.3 during aerobiosis. At pH values between 6.15 and 7.0 and in anaerobiosis, the temperature growth limit is between 8 °C and 7 °C with no or limited growth, depending on strains and growth media, whereas it is below 5 °C with oxygen.

It can therefore be concluded that the combination of cold temperature, low pH and the absence of oxygen could help to control the growth of psychrotrophic *B. cereus* strains in food products.

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