Universidade de Aveiro Departamento de Biologia
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Microbial warfare: the role of valinomycin and cereulide

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Prof. Drª. Ângela Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Cereulida, valinomicina, ionóforos de potássio, actividade antimicrobiana, potencial de membrana

resumo

As estirpes eméticas de *Bacillus cereus* produzem uma toxina altamente resistente, denominada cereulida. Esta toxina é o agente responsável por uma intoxicação alimentar caracterizada por emese. A cereulida é um dodecadepsipéptido cíclico com uma estrutura semelhante à da valinomicina, um composto antimicrobiano produzido por *Streptomyces* spp. Ambos os compostos são ionóforos de potássio, facilitando o movimento the K^+ através das membranas celulares com concomitante dissipação do potencial de membrana. Devido às suas propriedades ionofóricas, a valinomicina e a cereulida são ambas tóxicas para as mitocôndrias. As similaridades com a valinomicina sugerem que a cereulida possa ser um composto antimicrobiano. A actividade antimicrobiana da cereulida e da valinomicina foi testada avaliando o seu efeito no crescimento das bactérias seleccionadas sob diferentes condições de crescimento. A valinomicina e a cereulida exibiram actividade antimicrobiana contra as bactérias Gram positvo testadas. Por outro lado, as bactérias Gram negativo revelaram-se insensíveis a estes ionóforos de potássio sob as condições testadas. À excepção de *Enterococcus faecalis*, o crescimento bacteriano foi fortemente inibido pela valinomicina e pela cereulida a pH 8.5. Enquanto que a pH 6.5 estes ionóforos foram ineficazes. O oxigénio revelou-se um importante factor para a eficiência dos ionóforos. As células aeróbicas foram mais sensíveis à acção da valinomicina e da cereulida do que as células anaeróbicas. As estirpes eméticas revelaram uma menor suscepibilidade aos ionóforos testados. Curiosamente, a estirpe não emética *Bacillus cereus* ATCC 14579 mostrou, ao contrário das outras estirpes não eméticas, alguma resistência à valinomicina.

keywords Cereulide, valinomycin, potassium ionophores, antimicrobial activity, membrane potential

abstract Emetic *Bacillus cereus* produce an highly resistant toxin, named cereulide. This toxin is the causative agent of a food-borne disease characterized by emesis. Cereulide is a cyclic dodecadepsipeptide with a structure similar to the one of valinomycin, an antimicrobial compound produced by *Streptomyces* spp. Both of these compounds are potassium ionophores, facilitating the movement of K^+ across cell membranes with concomitant dissipation of the membrane potential. Due to their ionophoric properties, valinomycin and cereulide are both toxic to mitochondria. The similarities with valinomycin suggest that cereulide may be an antimicrobial compound. The antimicrobial activity of cereulide and valinomycin was evaluated in relation to growth of selected bacteria under different growth conditions. Valinomycin and cereulide were found to exhibit antimicrobial activity effective against the Gram positive bacteria tested. Gram negative were insensitive to these potassium ionophores in the conditions used. With the exception of *Enterococcus faecalis*, bacterial growth was strongly inhibited by valinomycin and cereulide at pH 8.5. While at pH 6.5 these potassium ionophores were inefective**.** Oxygen was found to be an important effector of ionophore efficiency. Aerobic cells were more sensitive to valinomycin and cereulide than anaerobic cells. The emetic *Bacillus* strains were found to be less susceptible to the tested ionophores. Interestingly, the non-emetic *Bacillus cereus* ATCC 14579 showed, unlike the other tested non-emetic strains, some resistance to valinomycin.

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1. INTRODUCTION

1.1. Microbial warfare

The availability of nutrients and space is limited, which leads to competition between microorganisms. To gain the competition, microorganisms developed several strategies. One of those strategies is the synthesis of antibiotics, which are compounds that can inhibit the growth or even kill other microorganisms. Antibiotics can be specific for some microorganisms or have a broad-spectrum, according to their mode of action (Riley and Wertz, 2002). These compounds are usually synthesized during post-logarithmic growth after exhaustion of nutrients and space for bacterial multiplication (Martin and Demain, 1980).

1.2. Cell metabolism and energetics

Movement of anions, nutrients and metabolites across cytoplasmic membranes is mediated by a proton motive force, originated from the electrochemical gradient of protons (Mitchell, 1966; Gale, 1971; Harold, 1972). According to the chemiosmotic theory of Mitchell (Mitchell, 1966), the proton motive force is composed of a transmembrane pH gradient (alkaline inside) and of a transmembrane electrical potential (negative inside). On respiring and fermentative bacteria the proton motive force is established by different mechanisms, schematized in Figure 1. Bacterial respiration conducts through the extrusion of protons, which creates an imbalance and charge difference between the intra and extracellular milieu - proton motive force (Kashket, 1981). Due to the proton-motive force, protons return to the cytoplasm by passing through an ATPase in the membrane, with concomitant generation of ATP. In fermentative bacteria, ATP is produced by substrate-level phosphorylation (Nakano and Zuber, 1998). Thus, the proton motive force is generated by the extrusion of protons through ATPase, during the hydrolysis of ATP (Harold, 1972; Kashket, 1981).

There are many factors that can interfere with the pH gradient and with the membrane potential across membranes. The metabolic processes associated with growth and metabolism are the main cause of perturbation of the pH gradient across the membrane (Booth, 1985). While the membrane potential is, essentially, disturbed by compounds such as antibiotics that disturb the balance of ions in the cell. A decrease or even the collapse of

the proton motive force can be fatal for cells. Therefore, bacteria usually can compensate the dissipation of one of the components of the proton motive force with the increase of the other component (Abee *et al*., 1988).

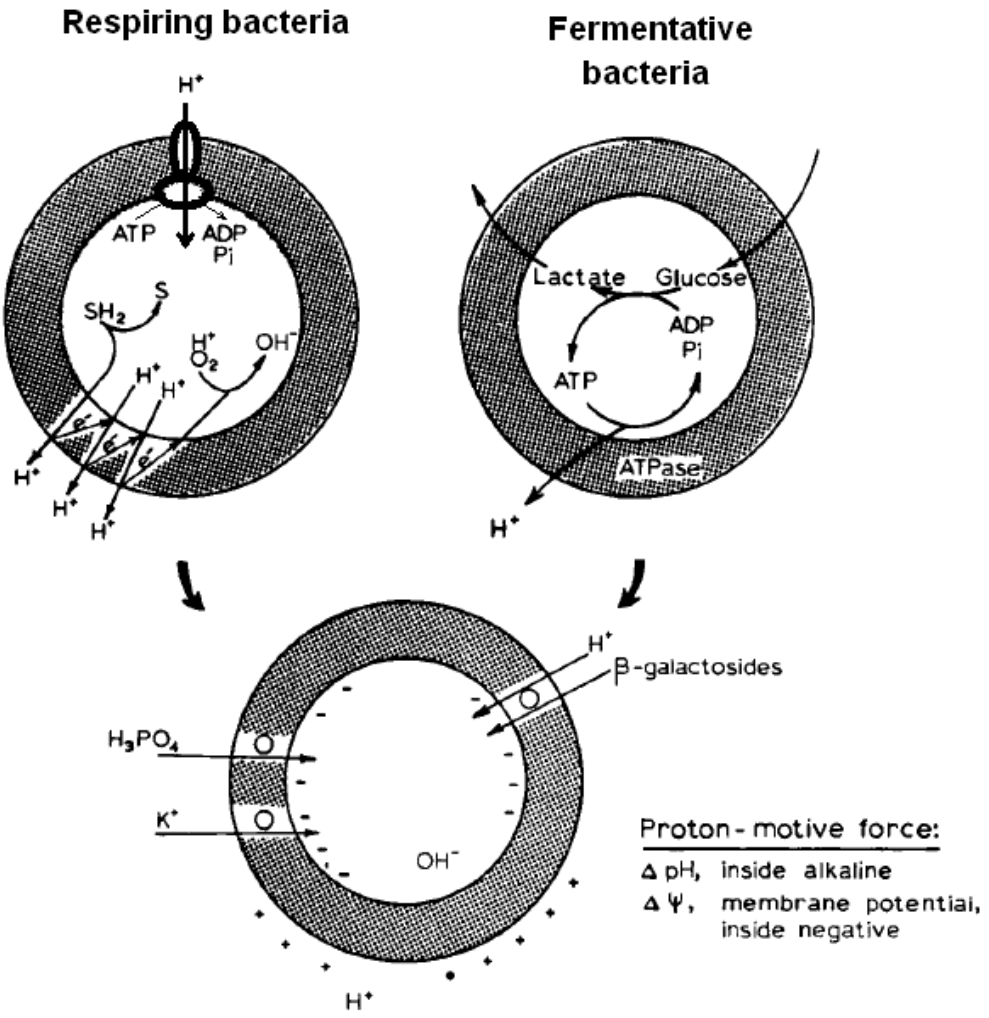

Figure 1. Schematic representation of cellular mechanisms involved in the generation of a proton motive force in respiring and fermentative bacteria. The driving force is generated by extrusion of protons by the respiratory chain (respiring bacteria) or by the ATPase (fermentative bacteria). *Adapted from Harold, 1974.*

1.3. Valinomycin

Valinomycin is a well known antibiotic produced by species of the genus *Streptomyces* (Mikkola *et al*., 1999, Magarvey *et al*., 2006), which is a cyclic dodecadepsipeptide containing the three-repeat sequence D-hydroxyisovaleric acid-D-valine-L-lactic acid-L-valine (Pressman, 1965; Magarvey *et al*., 2006), of molecular

weight 1111.3218 (http://www.ncbi.nlm.nih.gov/). This peptide forms a central hydrophilic cavity in which a monovalent cation, generally a potassium ion, can be accommodated (Daniele and Holian, 1976). Coating this polar cage, valinomycin has hydrophobic side chains of valine and hydroxyisovaleric acid, (Figure 2) which allow its diffusion through the hydrophobic interior of cell membranes (Lehninger *et al*., 1993). Valinomycin is classified as a potassium ionophore (Lehninger *et al*., 1993) which means that when the K^+-valinomycin complex is formed, as the charge of the potassium ion is shielded (Morrison *et al*., 2005), the K^+ is carried by valinomycin across the hydrophobic interior of cell membranes down its electrochemical gradient (Lehninger *et al*., 1993; Morrison *et al*., 2005). The transport of K^+ by valinomycin across cell membranes dissipates the transmembrane electrical potential (Muchl and Peschek, 1984) and consequently, the proton motive force.

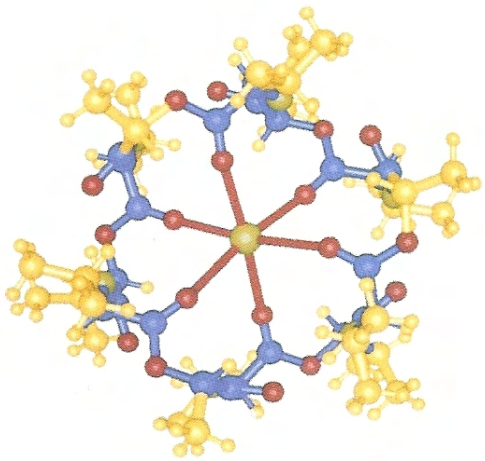

Figure 2. Valinomycin structure. The central hydrophilic cavity is formed by the oxygen atoms (red) that bind K^+ (atom in the center). Coating this cavity, there are the hydrophobic side chains (yellow). *Lehninger et al., 1993.*

1.4. *Bacillus cereus* and cereulide

Bacillus cereus is a Gram positive, facultative anaerobic, spore-forming, motile and an ubiquitously present rod (Kotiranka *et al*., 2000), which belongs to the *B. cereus* group, also known as *B. cereus sensu lato*. This group comprises the species *B. cereus*, *B. weihenstephanensis*, *B. mycoides, B. pseudomycoides, B. anthracis* and *B. thuringiensis* (Kotiranka *et al*., 2000, Ehling-Schulz *et al.,* 2004, Arnesen *et al*., 2008). *Bacillus cereus* is found in bulk soil, in the rhizosphere (Vilain *et al*., 2000), in the air (Arnesen *et al*.,

2008), in the gut of invertebrates (Jensen *et al*., 2003) and is commonly present in food (Granum *et al*., 1997, Ehling-Schulz *et al.,* 2004).

There are two distinct food-borne disease types associated with *B. cereus* named diarrheal and emetic syndromes. The diarrheal syndrome is caused by enterotoxins that are produced during the growth of vegetative cells in the small intestine, after the ingestion of contaminated food (Adams and Moss, 2008). The emetic syndrome is an intoxication caused by cereulide, a toxin produced in food by emetic *B. cereus* (Granum *et al*., 1997, Arnesen *et al*., 2008) and by the psychrotolerant *B. weihenstephanensis* (Thorsen *et al*., 2006). The two types of food-borne illness are connected to different types of food. While meat products, soups, vegetables, puddings and sauces are often implicated in the diarrheal syndrome (Adams and Moss, 2008), the emetic syndrome is commonly associated with starchy products such as rice, pasta (Agata *et al*., 2002; Adams and Moss, 2008), noodles, pastry (Ehling-Schulz *et al*., 2004) and potatoes (Agata *et al*., 2002; Altayar and Sutherland, 2006) in spite of the inability of emetic *B. cereus* to hydrolyze starch (Ehling-Schulz *et al*., 2004).

The emetic toxin is produced by emetic strains in the beginning of their stationary phase (Finlay *et al*., 2000; Thorsen *et al*., 2009), at temperatures ranging from 12 to 37°C (Finlay *et al*., 2000), with the exception of the psychrotrophic *B. weihenstephanensis* which is able to produce cereulide at 8°C (Thorsen *et al*., 2006). Cereulide is a very resistant toxin, maintaining its stability at temperatures up to 121°C, extreme pH and being also resistant to proteolysis (Granum *et al*., 1997; Kotiranta *et al.*, 2000; Arnesen *et al*., 2008). Therefore, cereulide can be a serious problem concerning food safety.

Cereulide, represented on Figure 3, has been shown to cause emesis in primates (Agata *et al*., 1995) and mice (Turnbull *et al*., 1979), swelling of mitochondria in HEP-2 cells (Agata *et al*., 1994), in boar spermatozoa (Andersson *et al*., 1998) and in natural killer cells of the immune system (Paananen *et al*., 2002). The toxic effect of cereulide was also described for hepatocytes after two cases of lethal food poisoning by this toxin (Mahler *et al*., 1997; Dierick *et al*., 2005)

The function of cereulide in *B. cereus* growth and ecology is unknown. It is known that cereulide causes emesis (Arnesen *et al*., 2008) but it has no apparent benefit for emetic *B. cereus*. Synthesis of secondary metabolites have a cost for cells (Kell *et* al., 1995), thus it must be profitable for their producers. The toxic effect of cereulide in mammalian cells is

most likely just a collateral effect and not its main function.

The synthesis of cereulide occurs during the same growth phase as sporulation (Finlay *et al*., 2000; Häggblom *et al*., 2002), the absence of both events under anaerobiosis (Jääskeläinen *et al*., 2004), the association between the main regulator for sporulation with cereulide synthesis (Lücking *et al*., 2009) and the association of enterotoxin production with sporulation in other microorganisms (Duncan *et* al., 1972) seems to indicate a correlation between synthesis of cereulide and sporulation. However there is no evidence of this association. There is no relation between the amount of spores and the amount of cereulide produced (Finlay *et al*., 2000; Häggblom *et al*., 2002). The emetic strains are able to sporulate even without the production of cereulide (Finlay *et al*., 2000). Furthermore, the sporulation sigma factor H (σ^H) which plays an important role in post-exponential-phase gene expression, showed no relation with cereulide expression (Lücking *et al*., 2009).

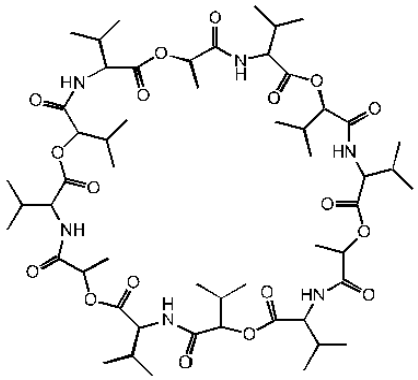

Figure 3. Schematic representation of cereulide.

Several factors indicate the possibility of cereulide being an antimicrobial compound. Valinomycin and cereulide have similar structures (Agata *et al*., 1994), differing only in two of the four residues that form the three-repeat sequence. Both compounds are potassium ionophores (Mikkola *et al*., 1999) carrying potassium ions across the membrane down an electrochemical gradient. Moreover, these potassium ionophores both inhibit mitochondrial activity by dissipation of the membrane potential (Mikkola *et al*., 1999). Most likely, they also have the same function playing a role in microbial warfare for nutrients. As observed with the majority of the antibiotics, synthesis of cereulide starts during the stationary phase (Finlay *et al*., 2000; Thorsen *et al*., 2009). Its production is

enhanced at 20°C (Finlay *et al*., 2000) and is oxygen dependent (Jääskeläinen *et al.*, 2004) suggesting that it is not an human pathogenic agent. Cereulide may be targeted against bacteria, but due to biochemical similarities between bacterial cells and mitochondria, these are also affected by this ionophore.

1.5. Objectives

The toxic character, the high stability and the optimal synthesis conditions of cereulide are known, but the main question, "why is cereulide produced?", still does not have an answer. The present study tried to give an answer to that question.

As mentioned above, there are several factors pointing to the fact that cereulide may be an antimicrobial compound. Therefore, the goal of this study was to test if cereulide could be or not an antimicrobial compound.

Since cereulide is similar to valinomycin, which is already an well studied compound, both compounds were studied in parallel in order to better evaluate the antimicrobial activity of cereulide.

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

The antimicrobial activity of valinomycin and cereulide was tested on selected Gram positive and Gram negative bacteria which are shown in Table 1.

Stock cultures grown in Brain Heart Infusion broth (BHI, Becton Dickinson, France) were stored at -80°C in ~26 % v/v of glycerol.

Table 1. Bacterial strains and their characteristics.

Microorganisms	Gram	Oxygen Requirement	Observations
Escherichia coli ECO 016	-	Facultative anaerobic	
Salmonella thyphimurium II 505	-	Facultative anaerobic	
Enterococcus faecalis ATCC 29212	+	Microaerophilic	without functional electron transport chain
Staphylococcus aureus ATCC 25923	+	Facultative anaerobic	
Listeria innocua	+	Facultative anaerobic	
Listeria monocytogenes	+	Facultative anaerobic	
Bacillus subtilis 168	+	Facultative anaerobic	
Bacillus cereus ATCC 10987	+	Facultative anaerobic	non-emetic strain (cereulide non-producer)
Bacillus cereus ATCC 14579	+	Facultative anaerobic	non-emetic strain (cereulide non-producer)
Bacillus cereus F4810/72	+	Facultative anaerobic	emetic strain (cereulide producer)
Bacillus cereus A529	+	Facultative anaerobic	emetic strain (cereulide producer)

Stock cultures were streaked on BHI agar plates and incubated overnight at 30°C. A single colony was used to inoculate 6 or 8 ml of BHI broth in tubes, which were grown at 30°C at 200 rpm (Innova 4335; New Brunswick Scientific, The Netherlands) for 17 to 27h.

The cultures of *B. cereus* ATCC 14579, *B. cereus* 10987 and *B. cereus* F4810/72 used for the survival and recovery assay were prepared by inoculating a single colony in 20

ml of BHI broth in a 100 ml Erlenmeyer flask, followed by 14 to 17 hours of growth in a shaking water bath (Julabo SW23) at 30°C and 200 rpm.

2.2. Preparation of valinomycin and cereulide solutions

Stock solutions of valinomycin were prepared using valinomycin ready made solution in DMSO (Sigma-Aldrich, 1 mg/ml) and valinomycin powder (Sigma-Aldrich) according to the description in Table 2.

Synthetic cereulide, stored in methanol at -20°C, after evaporation of methanol by air drying, was used to prepare four different stock solutions. Cereulide was dissolved in DMSO and dilutions were made with H_2O as described in Table 3.

All stock solutions were stored at 4°C.

Table 2. Preparation of stock solutions of valinomycin and their concentrations.

Final concentration		**Stock solution**		
Valinomycin (µM)	**DMSO (%)**	**Concentration of valinomycin (µM)**	**Concentration of DMSO (%)**	**Preparation**
0.09	1	1.80	20	ss (89.98 µM) + 20 % DMSO (1:9)
0.90	1	18.00	20	ss (179.97 µM) + 20 % DMSO (1:9)
4.50	1	89.98	20	ss (179.97 µM) + 20 % DMSO (1:1)
9.00	1	179.97	20	rms + H_2O (1:4)
22.50	5	449.92	100	rms + 100 % DMSO (1:1)
44.99	5	899.83	100	rms
n.d.	n.d.	9.00	1	rms + H_2O (1:99)
n.d.	n.d.	179.97	4	vp + 4 % DMSO
n.d.	n.d.	899.83	4	vp + 4 % DMSO

n.d.: not determined

ss: stock solution

rms: ready made solution of valinomycin in DMSO

vp: valinomycin powder

Table 3. Preparation of stock solutions of cereulide and their concentrations.

Final concentration		Stock solution		
Cereulide (µM)	**DMSO (%)**	**Concentration of cereulide (µM)**	**Concentration of DMSO (%)**	**Preparation**
8.39	1	167.84	20	cereulide dissolved in 100 % DMSO + H_2O (1:4)
8.39	1	839.18	100	cereulide dissolved in 100 % DMSO
9.00	1	179.97	20	cereulide dissolved in 100 % DMSO + H_2O (1:4)
n.d.	n.d.	167.84	4	cereulide dissolved in 100 % DMSO + H_2O (1:24)

n.d.: not determined

2.3. Microtiter plate assay

The antimicrobial effect of valinomycin and cereulide on the growth of selected bacteria was assessed by the generation of spectrophotometric growth curves in 96-well microtiter plates (greiner bio-one, Germany). The effect of the pH and the availability of O_2 on these ionophores effectiveness were evaluated, as well as the concentration of ionophore used.

To have the same initial optical density (OD) of bacterial cells in all performed tests, cells of overnight cultures were resuspended in phosphate buffer (PPi, 0.01 M, pH 7.0) on the right proportion to obtain an OD_{600nm} of 2. After inoculation, this OD resulted in an OD_{600nm} of 0.1.

The microtiter plates were prepared as described below and incubated at 30°C in a plate reader (SpectraMax plus^{384}) for 24 h. The OD600nm of each well was automatically recorded for every 5 minutes with shaking for 30 seconds before the first read and between each read. The changes in OD over time were used to generate growth curves.

2.3.1. Effect of pH and O_2 on valinomycin effectiveness

The effect of pH on valinomycin effectiveness as an antibiotic was evaluated by measuring bacterial growth at a range of pH values, 5.5, 6.5, 7.5, 8.5 and 9.5. The 96-well microtiter plates were prepared by dispensing 180 µl of BHI broth, with the intended pH, into a well to which an inoculum of 10 µl of bacterial cells resuspended in PPi (OD_{600nm}=2) was added. Finally, 10 µl of valinomycin stock solution (179.97 µM in 20 % DMSO) was

added to each well, resulting in the final concentrations of 9 µM of valinomycin with 1 % v/v DMSO. As a blank control, DMSO solution (20 %) without valinomycin was used, obtaining a final concentration of 1 % v/v DMSO. Each condition was tested in triplicate.

The effect of O_2 was evaluated by growing bacterial cells under aerobic and anaerobic conditions. Anaerobiosis was created by covering the 96 well plates with optical adhesive film (MicroAmp).

2.3.2. Dose effect of valinomycin

Effect of 0.09, 0.90, 4.50, 9.00, 22.50 and 44.99 µM of valinomycin on the growth of *B. cereus* strains was evaluated at pH values of 5.5, 7.5, 8.5 and 9.5.

The 96-well microtiter plates were prepared by dispensing 180 µl of BHI broth, with the intended pH, into a well to which an inoculum of 10 µl of bacterial cells resuspended in PPi (OD_{600nm}=2) was added. Finally, 10 µl of valinomycin stock solution (1.80, 18.00, 89.98 and 179.97 µM in 20 % DMSO and 449.92 and 899.83 µM in 100 % DMSO) was added to each well resulting in the final concentrations of 0.09, 0.90, 4.50 and 9.00 of valinomycin with 1 % v/v DMSO and 22.50 and 44.99 µM of valinomycin with 5 % v/v DMSO.

2.3.3. Effect of cereulide on bacterial growth

The effect of pH on cereulide effectiveness as an antibiotic was evaluated by measuring growth of *B. subtilis*, *L. monocytogenes*, *B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *B. cereus* F4810/72 and *L. innocua* at a range of pH values, 5.5, 6.5, 7.5, 8.5 and 9.5. The procedure was followed as above (), but, instead of valinomycin, cereulide stock solution was used (179.97 µM in 20 % DMSO).

2.3.4. Effect of H_2O on cereulide effectiveness

In order to evaluate the effect of H_2O on the activity of cereulide, a stock solution of cereulide in 20 % DMSO (167.84 µM) and another in 100 % DMSO (839.84µM) were prepared. Both solutions were used to assess the effect of cereulide on the growth of *B. cereus* F4810/72, *B. cereus* 10987 and *B. subtilis,* at pH 8.5.

A 96-well microtiter plate was prepared by dispensing 180 µl of BHI broth (pH 8.5) into a well to which an inoculum of 10 µl of bacterial cells resuspended in PPi (OD_{600nm}=2) was added. Then, wells were filled with 10 µl of cereulide stock solution in 20 % DMSO (167.84 µM) or with 8 µl of H_2O and 2 µl of cereulide stock solution in 100% DMSO

(839.84μM), obtaining, in both cases, a final concentration of 8.39 μM of cereulide with 1 % v/v DMSO in each well. This was performed with fresh and 24 hours old solutions of cereulide. As a blank control, DMSO solution (20 %) without cereulide was used, obtaining a final concentration of 1 % v/v DMSO. Each condition was tested in triplicate.

2.3.5. Data processing and statistical analysis

The OD_{600nm} measurements from the plate reader run were imported into Microsoft Office Excel 2003 and corrected by subtracting the background OD_{600nm} of the growth media from the data of each well. The average growth curve was calculated and then plotted.

For each growth curve generated, the specific growth rate (μ) and the duration of the lag phase (lag) were fitted and determined using the Zwietering growth model (Zwietering *et al*. , 1990), referred as Equation 1. In this equation the A represents the difference between the maximum OD and the initial OD ($OD_{t=0}$), whereas t stands for the time. Logarithmically transformed growth curves were fitted according to this model using the solver from Microsoft Office Excel 2003. The average μ and lag for each condition were then calculated, as well as their standard error.

$$\log OD = \log OD_{t=0} + A * \exp\left(-\exp\left(\left(\frac{\mu * \exp(1))}{A}\right) * (lag - t) + 1\right)\right) \qquad \text{Equation 1}$$

Growth rates (μ) in presence of valinomycin and cereulide were determined as percentage of the control growth rate values (Equation 2), the acquired value was termed "relative growth rate (RGR)". It was also used to calculate the difference between the length of the lag phase of the growth control and the growth with ionophores (Equation 3).

$$RGR = \frac{\mu_{test}}{\mu_{control}} \times 100 \qquad \text{Equation 2}$$

$$\Delta\, lag = lag_{test} - lag_{control} \qquad \text{Equation 3}$$

Statistical significance of differences between values of the growth parameters obtained from test and control conditions were determined using a two-tailed t-test and were considered significant when $p<0.05$. The selection between a paired or unpaired t-test was based on the F-test, considering $p<0.05$.

2.4. Paper disk assay

The evaluation of the susceptibility of *B. subtilis, L. innocua*, *L. monocytogenes, E. faecalis*, *S. aureus*, *S. thyphimurium* and *E. coli* to valinomycin and cereulide (supernatant of *B. cereus* A529 culture) was performed with a filter paper disk method.

The cereulide extract was obtained by spinning 1 ml of *B. cereus* A529 overnight culture at 13000 rpm for 1 minute. Then the supernatant was filtered with a 0.2 μm cellulose acetate filter (Whatman, Germany).

Each bacterial strain was tested by pour-plating 1 ml of overnight culture in BHI agar (1.5 % w/v agar). After solidification, a paper disk of 6 mm (Becton Dickinson, France) was placed on the surface of the plate. The disks were saturated with 5 μl aliquots of valinomycin stock solution (9.00 μM in 1 % DMSO) or with filtered supernatant of *B. cereus* A529 culture. The effect was quantified after 20 hours of incubation at 30°C by measuring the diameter of the inhibition zone.

2.5. Drop dilution assay

2.5.1. Effect of valinomycin and cereulide

A 1000-fold dilution of overnight cultures of *B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *B. cereus* A529, *B. cereus* F4810/72, *B. subtilis*, *L. innocua*, *L. monocytogenes* and *E. coli,* was prepared using phosphate buffered saline (PBS, pH 7.37).

The sensitivity of the microorganisms to valinomycin and cereulide was tested on BHI agar with pH 7.5, 8.5 and 9.5. Briefly, 1 ml of overnight culture was pour-plated in 14 ml of BHI agar (1.5% w/v agar). After solidification, 5 μl of valinomycin (179.97 and 899.83 μM in 4 % DMSO) or cereulide (167.84 μM in 4 % DMSO) stock solutions were spotted on the agar surface, in duplicate. As control, 5 μl of 4 % DMSO were spotted on the agar surface, also in duplicate. Petri dishes were prepared in duplicate and incubated overnight at 30°C. The antimicrobial activity was evaluated by measuring the diameter of the inhibition zone.

2.5.2. Effect of *B. cereus* A529

The sensitivity of test strains to cereulide, likely to be produced by the emetic *B. cereus* A529 strain, was tested by growing those strains in the presence of *B. cereus* A529, using two different methods.

Sensitivity of *B. cereus* ATCC 10987, *B. cereus* F4810/72, *L. monocytogenes*, *E. faecalis* and *S. aureus* to cereulide was evaluated by growing these strains, at different pH values (7.5, 8.5 and 9.5), over colonies of *B. cereus* A529. No growth or reduced growth of test strains over the colonies of *B. cereus* A529 might indicate growth inhibition by cereulide.

A 5 µl aliquot of *B. cereus* A529 culture was spotted, in quadruplicate, on the surface of 7.5 ml of BHI agar (1.5% w/v agar), in a Petri dish. After overnight incubation at 30°C, 4 ml of melted BHI soft agar (0.5% w/v agar) inoculated with 1ml of overnight culture of test strains were overlaid on top of the BHI agar with the grown colonies. The BHI soft agar used had different pH values (7.5, 8.5 and 9.5). The plates were incubated again at 30°C for ~16h.

Growth of *B. cereus* A529 occurred on top of cultures of *B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *B. cereus* F4810/72, *B. subtilis*, *L. innocua*, *L. monocytogenes* and *E. coli*. Strain sensitivity was evaluated at different pH values (7.5, 8.5 and 9.5).

Overnight cultures of the test strains were diluted 1000-fold in PBS. Afterwards, 1 ml of each dilution was pour plated in 14 ml of BHI agar (1.5% w/v agar) (pH 7.5, 8.5 and 9.5). After solidification, 5 µl of *B. cereus* A529 overnight culture were spotted on the agar surface, in duplicate. Petri dishes were prepared in duplicate and incubated overnight at 30°C. Absence of growth or reduced growth of test bacteria around *B. cereus* A529 colonies might be an indicator of antimicrobial activity of cereulide.

2.6. Survival and recovery assay

The effect of potassium ionophores on survival of bacteria (bacterial-cidal or bacterial-static) was evaluated by determining CFU counts of bacterial cultures with simultaneous OD_{600nm} measurement. A decrease in the number of CFU may indicate a bacterial-cidal effect, while a bacterial-static effect may result in a constant number of CFU. A negative effect on cell survival followed by normal viable counts show a cell recovery.

2.6.1. Effect of valinomycin on bacterial cell survival

B. cereus ATCC 10987, *B. cereus* ATCC 14579 and *B. cereus* F4810/72 were

exposed to valinomycin (9 μM) with 1 % v/v DMSO, at pH 8.5 and 9.5. A 2.5 ml aliquot of valinomycin stock solution (179.97 μM in 20 % DMSO) was pipetted into 250 ml Erlenmeyer flasks containing 45 ml of BHI broth (pH 8.5 or 9.5) previously inoculated with 2.5 ml of bacterial suspension in PPi (OD_{600nm}= 2). The prepared culture had an OD_{600nm} of 0.1. As a control, 20 % DMSO without test compound was added, resulting in a final concentration of DMSO of 1 % v/v.

Each flask was prepared in duplicate and was incubated at 30°C in a shaking water bath at 200 rpm.

At several time points, between 30 min and 24 hours of incubation, 2 ml samples were taken aseptically from each culture and 100-, 10000- and 1000000-fold dilutions were prepared in PBS. In each sampling time point, the OD_{600nm} of each culture was measured.

2.6.2. Effect of cereulide on bacterial cell survival

B. cereus ATCC 10987 and *B. cereus* F4810/72 were exposed to cereulide (8.39 μM) with 1 % v/v DMSO, at pH 8.5. A 0.1 ml aliquot of cereulide stock solution (839.18 μM in 100 % DMSO) was dispensed into 50 ml Erlenmeyer flasks containing 0.4 ml of sterile H_2O and 9 ml of BHI (pH 8.5) previously inoculated with 0.5 ml of bacterial suspension in PPi (OD_{600nm}= 2). The prepared culture had an OD_{600nm} of 0.1. As a control, cereulide and H_2O were replaced by 0.5 ml of 20 % DMSO, resulting in the final concentration of 1 % v/v.

Each flask was prepared in duplicate and was incubated at 30°C in a shaking water bath at 200 rpm.

At several time points, between 30 min and 24 hours of incubation, 1ml samples were taken aseptically from each flask and 10-, 100-, 1000-, 10000-, 100000- and 1000000-fold dilutions were prepared in PBS. In each sampling time point, the OD_{600nm} of each culture was measured.

2.6.3. Plating and calculations

The appropriate dilutions were plated, in duplicate, on BHI agar plates by using a spiral plater (Eddy Jet; IUL Instruments) set in E-mode, plating 50 μl aliquots. After overnight incubation at 30°C, colonies were enumerated and the number of CFU ml^{-1} and respective standard error were determined.

The antimicrobial effect of valinomycin and cereulide on the growth of selected bacteria was assessed by the generation of spectrophotometric growth curves in 96-well microtiter plates (greiner bio-one, Germany).

3. RESULTS

3.1. Antimicrobial activity of valinomycin

Growth of test bacteria exposed to valinomycin in BHI broth was monitored by measurement of their OD_{600nm}, generating growth curves (see Appendix 1 and Appendix 2). These growth curves showed that all Gram positive strains tested, *E. faecalis, S. aureus, L. innocua, L. monocytogenes*, *B. subtilis, B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *B. cereus* F4810/72 and *B. cereus A*529 suffered growth inhibition by 9.00 μM of valinomycin. On the other hand, Gram negative bacteria tested (*E. coli* and *S. thyphimurium*) were resistant to valinomycin.

3.1.1. Effect of pH on valinomycin effectiveness

Growth of bacteria in a range of pH values (5.5 to 9.5) showed that there is a relation between the pH of growth media and the intensity of the growth inhibition caused by valinomycin. Excluding *E. faecalis*, growth inhibition of test bacteria by valinomycin increased with the increase of the pH. At pH 5.5 and 6.5, this ionophore was less effective in growth inhibition than at alkaline pH. At pH 9.5, excluding *E. faecalis*, none of the Gram positive strains tested was able to grow in presence of valinomycin, aerobically, for at least 24h.

Although *B. cereus* strains ATCC 14579 and ATCC 10987 are both non-emetic strains, they exhibited different responses to valinomycin. While *B. cereus* ATCC 10987 showed high sensitivity to valinomycin, *B. cereus* ATCC 14579 expressed much lower sensitivity to this compound. Figure 4 illustrates the relative growth rate (RGR) and the increase on length of the lag phase caused by valinomycin (Δlag) of *B. cereus* ATCC 10987 (A), *cereus* ATCC 14579 (B) and Figure 5 illustrates the RGR and the Δlag of *B. cereus* F4810/72. The relative growth rate values are proportionally inverse to the inhibition values, a relative growth rate of 100 % indicates no inhibition and vice versa.

The response of *B. cereus* ATCC 14579 to valinomycin was found to be comparable with the response of the emetic strains. The emetic strains (*B. cereus* F4810/72 and A529) and the non-emetic *B. cereus* ATCC 14579 strain were the ones that showed less sensitivity to valinomycin. Their growth inhibition was similar at pH 5.5, 6.5 and 7.5, with all exhibiting a decrease of around 20 % in their growth rate. At pH 7.5, beside the decrease of

the growth rate, valinomycin also caused a small increase of the lag phase (≤ 3 hours). At pH 8.5, these strains were the only ones that did not experience a decrease of growth rate by the action of valinomycin. Although not negatively affected in growth rate, at this pH, strains suffered a lag phase of 9 to 13 hours (Figure 4B, Figure 5). Interestingly, *B. cereus* ATCC 14579 (Figure 4B) and *B. cereus* A529, after experiencing the lag phase, underwent a significant increase in their growth rate after exposure to valinomycin. See Appendix 3 for more details.

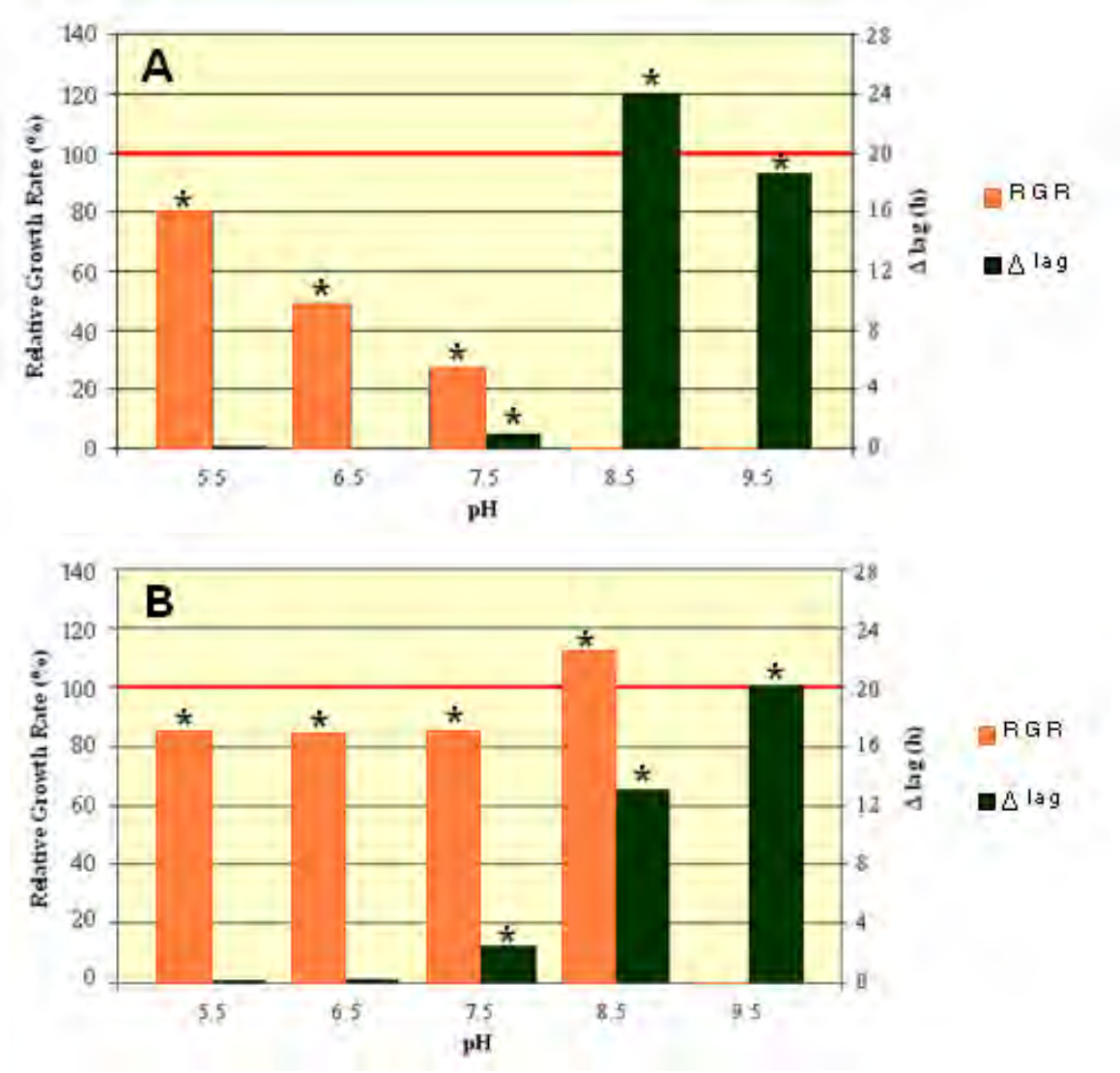

Figure 4. Relative growth rate (RGR) and difference between the length of the lag phase of the growth control and the growth with valinomycin (Δlag) of *B. cereus* ATCC 10987 **(A)**, and *B. cereus* ATCC 14579 **(B)** exposed to 9 µM of valinomycin and 1 % DMSO in BHI broth at different pHs, at 30°C, with aeration. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), $p<0.05$.

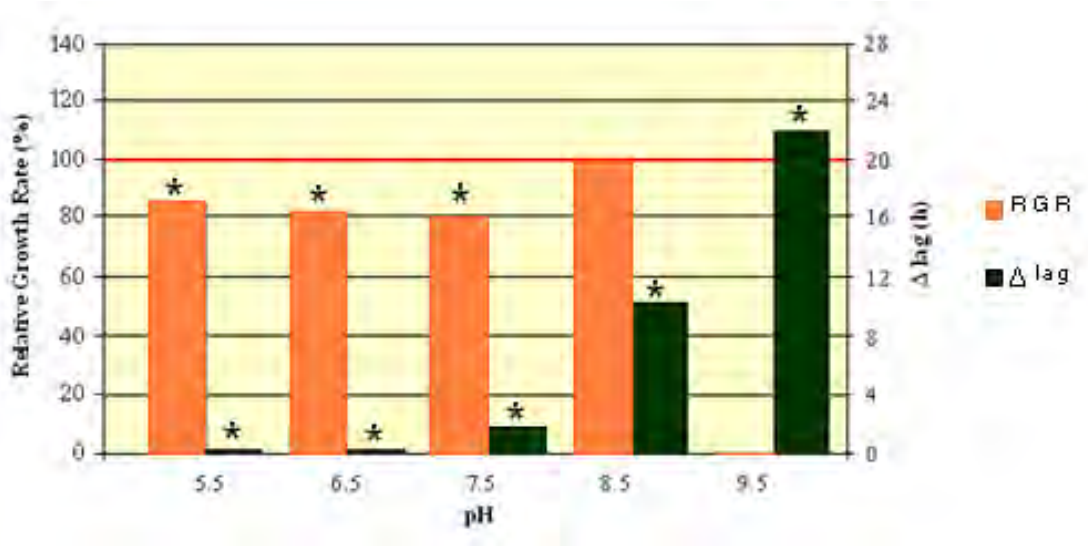

Figure 5. Relative growth rate (RGR) and difference between the length of the lag phase of the growth control and the growth with valinomycin (Δlag) of *B. cereus* F4810/72 exposed to 9 µM of valinomycin and 1 % DMSO in BHI broth at different pHs, at 30°C, with aeration. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), $p<0.05$.

The reaction of the non-emetic *B. cereus* ATCC 10987 to valinomycin was similar to that of *B. subtilis*, *L. monocytogenes, L. innocua* and *S. aureus* which all exhibited high sensitivity to valinomycin. At pH 6.5, exposure to valinomycin resulted in a 50 % decrease in growth rate of *B. cereus* ATCC 10987 (Figure 4A) and an inhibition of approximately 30 % for the other sensitive strains. At pH 7.5, excluding *B. subtilis* which was more affected on its lag time, the sensitive strains suffered an inhibition of 40 to 70% on their growth rate after exposure to 9.00 µM of valinomycin. See Appendix 3 for more details.

E. faecalis showed a response to valinomycin completely different from the other Gram positive bacteria. It was at acidic pH that valinomycin caused the highest growth inhibition (9.00 µM valinomycin, pH 5.5) suffering a decrease of more than 80% on its growth rate. At alkaline pH, its growth rate suffered an inhibition of around 30% (Figure 6). Exposure of *E. faecalis* to valinomycin resulted also in a significant decrease of the maximum OD600 reached, indicating a lower cell density. Its lag phase was not affected by valinomycin. See Appendix 1 for more details.

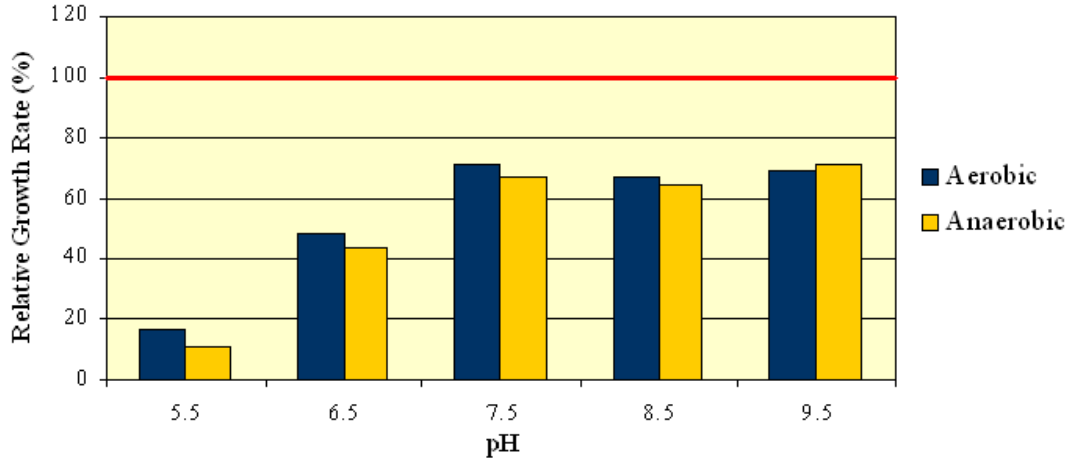

Figure 6. Relative growth rate (RGR) of *E. faecalis* after exposure to 9 µM of valinomycin in BHI agar at different pHs, at 30°C, under aerobic and anaerobic conditions. Red line (RGR = 100 %) indicates no inhibition. Growth rates from growth control and growth test were statistical different ($p<0.05$).

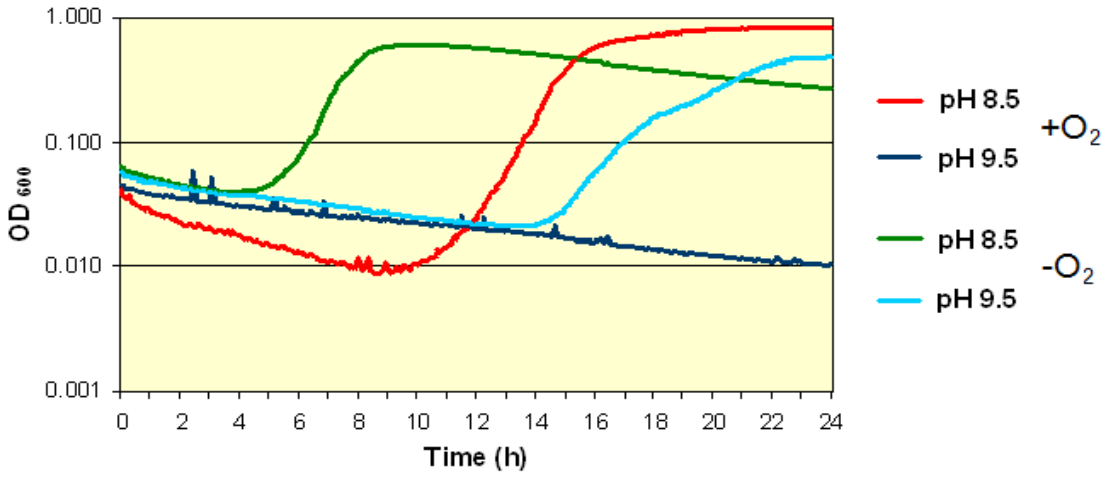

Figure 7. Anaerobic growth of B. cereus F4810/72 in BHI broth, at pH 8.5 and 9.5, with and without 9 µM of valinomycin and with 1% DMSO.

3.1.2. Effect of O_2 on valinomycin effectiveness

The antimicrobial activity of valinomycin was evaluated under aerobic and anaerobic conditions. Under both conditions, addition of 9.00 µM of valinomycin exhibited growth effects against Gram positive strains tested. However, excluding *E. faecalis*, the level of inhibition by valinomycin under anaerobic conditions was found to be lower than under

aerobic conditions. These results are illustrated on Figure 7 that shows the difference between growth curves of oxygenated and non-oxygenated *B. cereus* F4810/72 cells. Control growth was similar under both conditions. See Appendix 1, Appendix 2 and Appendix 3 for more details.

In opposition to the other test strains, *E. faecalis* suffered more growth inhibition under anaerobic conditions that under aerobiosis (Figure 6).

3.1.3. Dose effect of valinomycin

Growth of *B. cereus* strains was evaluated in the presence of a range of valinomycin concentrations. The results exhibited by the sensitive *B. cereus* ATCC 10987, in the form of growth comparatively to the control, are shown in Figure 8. Results from the other strains can be seen in Appendix 4. Aerobic growth of *B. cereus* strains was not affected by concentrations of valinomycin lower than 0.90 μM. However, when submitted to the presence of 4.5 μM of valinomycin or higher concentrations, these strains suffered some growth inhibition. The highest concentrations of valinomycin tested (22.50 and 45.00 μM) were responsible for the highest antimicrobial activity against *B. cereus* strains, preventing outgrowth, at pH 9.5, for at least 24 hours. Nevertheless, these two solutions of valinomycin contained 5 % of DMSO which caused, by itself, some growth inhibition.

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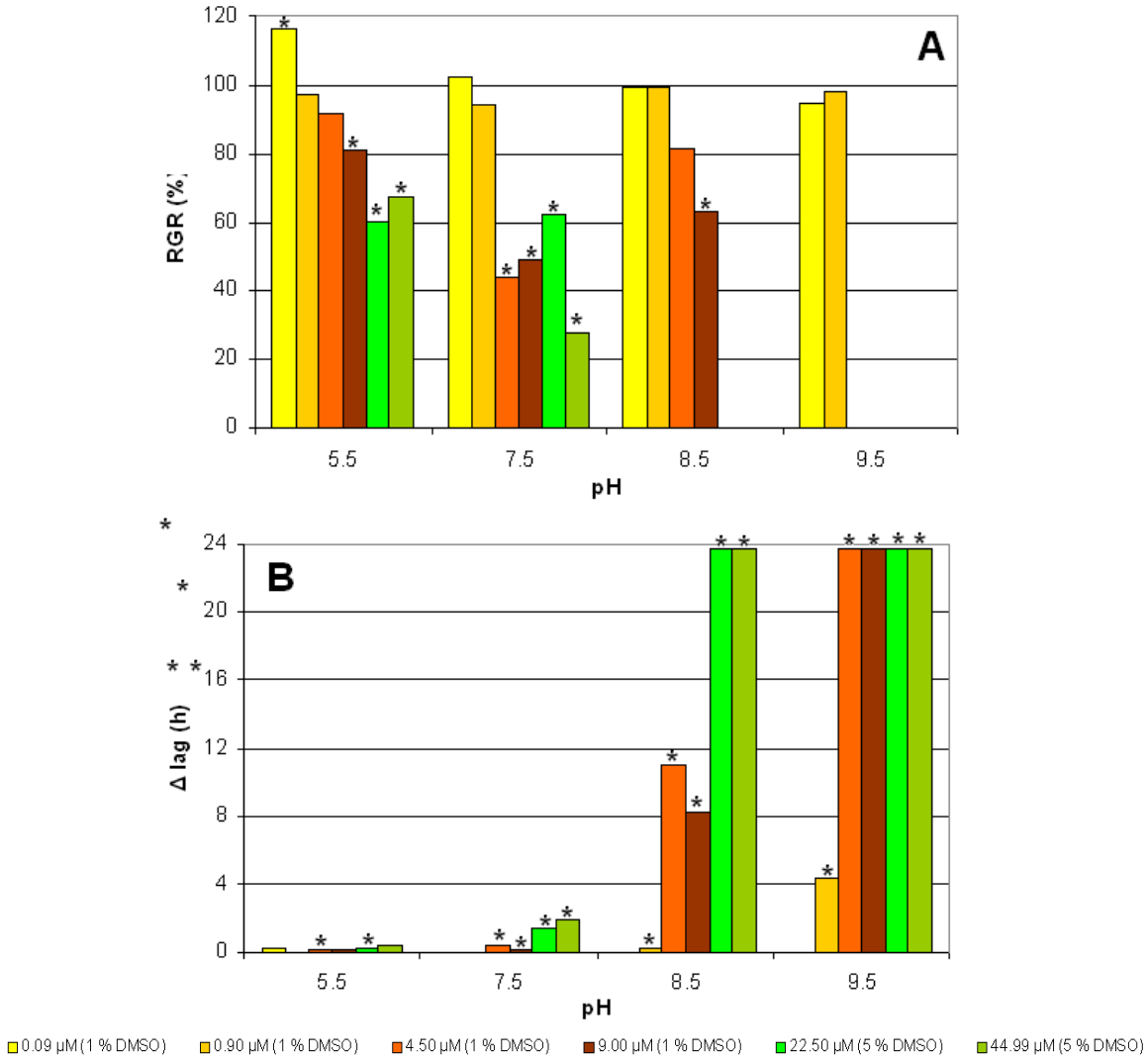

Figure 8. Relative growth rate – RGR **(A)** and difference between the length of the lag phase of the growth control and the growth with valinomycin – Δlag **(B)** of *B. cereus* ATCC 10987 exposed to a range of valinomycin concentrations (0.09 to 44.99 µM) and 1 or 5 % of DMSO, in BHI broth at different pHs, at 30°C, with aeration. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), $p<0.05$.

3.1.5. Paper disk and drop dilution assays

The susceptibility of bacteria to valinomycin was also evaluated in solid media, by two different approaches. A drop of valinomycin solution was placed on paper disks (9 µM of valinomycin with 1% DMSO) or directly on the bacterial culture (179. 97 and 899.83 µM of valinomycin with 4 % DMSO).

Using the method of paper disk diffusion, inhibition of tested bacteria (*B. subtilis, L. innocua, L. monocytogenes, E. faecalis, S. aureus, S. thyphimurium* and *E. coli*) was not

observed.

The sensitivity of *B. cereus* F4810/72, *B. cereus* A529, *B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *B. subtilis, L. monocytogenes* and *L. innocua* to valinomycin was observed after this compound (179.97 and 899.83 µM) was spotted on the surface of the bacterial cultures. The diameter of inhibition zones is shown in Table 4. In general, the largest inhibition of test strains was caused by the higher concentration of valinomycin used (899.83 µM) and at pH 8.5.

In *B. cereus* strains, valinomycin was able to prevent the development of colonies at the surface of medium, but inside the medium, in the less oxygenated layer, development of colonies was observed. Emetic strains showed to be less susceptible to valinomycin, being resistant to 179.97 µM of valinomycin at pH 7.5. On the other hand, at pH 8.5, the non-emetic *B. cereus* ATCC 10987 strain was quite sensitive to valinomycin. On Figure 9, inhibition caused by valinomycin in *B. cereus* F4810/72 (A) and in *B. cereus* ATCC 10987 (B) can be compared. See Appendix 5 for more figures.

B. subtilis showed higher sensitivity to valinomycin, exhibiting no growth at pH 7.5 (Figure 10A) and 8.5 (Figure 10B) in the presence of valinomycin. At pH 8.5, this strain exhibited the largest inhibition zone observed (Figure 10).

At pH 9.5, only *Listeria* strains and *B. cereus* F4810/72 showed uniform growth in the plate. All other strains exhibited insufficient growth at this pH making it impossible to determine the growth effects of valinomycin at this pH.

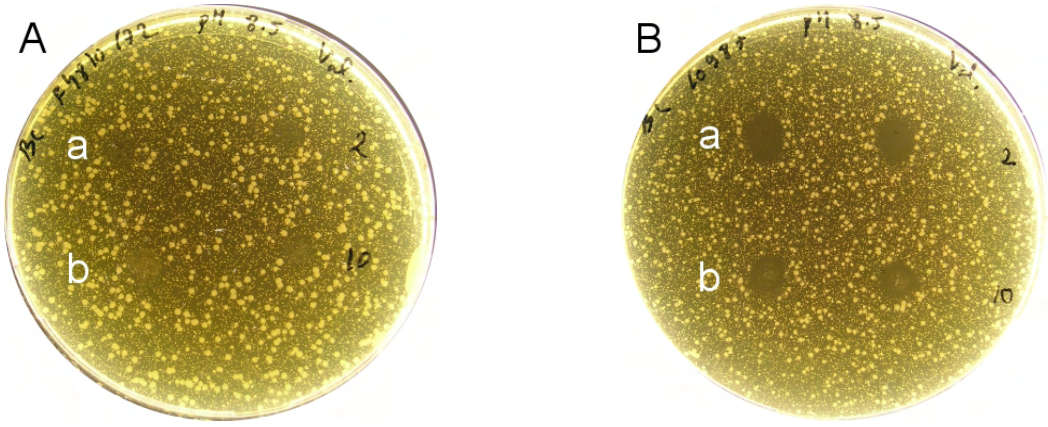

Figure 9. Inhibition zones caused by 179.97 µM (a) and 899.83 µM (b) of valinomycin in *B. cereus* F4810/72 ATCC **(A)** and *B. cereus* ATCC 10987 **(B)** cultures in BHI agar, at pH 8.5, after 14 h of growth at 30°C.

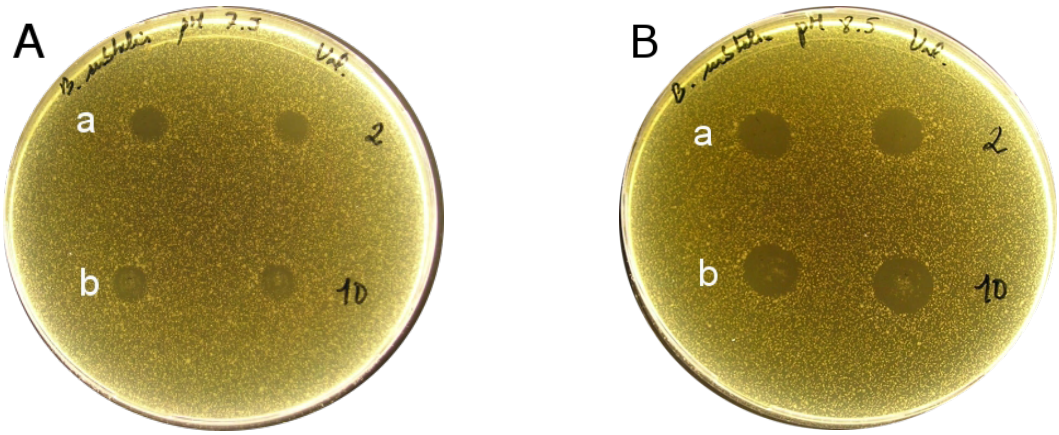

Figure 10. Inhibition zones caused by 179.97 µM (a) and 899.83 µM (b) of valinomycin in *B. subtilis* cultures in BHI agar, at pH 7.5 **(A)** and at pH 8.5 **(B)**, after 14 h of growth at 30°C.

Table 4. Inhibition zone (mm±SE) resultant from exposure of test bacteria to valinomycin in BHI agar, after 14h of incubation at 30ºC.

	Valinomycin					
	179.97µM			**899.83µM**		
Microorganism	**pH 7.5**	**pH 8.5**	**pH 9.5**	**pH 7.5**	**pH 8.5**	**pH 9.5**
***B. cereus* 10987**	5.4±0.6 [1]	8.4±0.0	n.d.	7.5±0.3 [1]	7.8±0.0	n.d.
***B. cereus* 14579**	4.0±0.0 [2]	6.5±1.3[1]	n.d.	4.7±0.0 [2]	6.5±0.0[1]	n.d.
B. cereus F4810/72	0	6.9±0.0[1]	11.2±0.4	6.2±0.0 [1]	6.9±0.0[1]	10.7±0.0
***B. cereus* A529**	0	7.2±0.3[1]	n.d.	0	8.1±0.0[1]	0
B. subtilis	7.7±0.3	10.5±0.0	n.d.	7.3±0.0	11.9±0.5	0
L. monocytogenes	7.3±0.0 [2]	7.6±0.3 [1]	1.00±0.0	7.3±0.0[2]	9.4±0.3	10.3±0.3
L. innocua	9.0±0.6 [1]	8.8±0.0	1.20±0.0	9.6±0.0	10.8±0.7	12.0±0.2
E. coli	0	0	0	0	0	0

n.d. (not determined): insufficient growth of test bacteria

[1]: not complete inhibition, some colonies present

[2]: slight inhibition, many colonies present

3.2. Strain sensitivity to *B. cereus* A529

The susceptibility of *B. cereus* ATCC 10987, *B. cereus* F4810/72, *E. faecalis, L. monocytogenes,* and *S. aureus* to cereulide, produced by growing cultures of *B. cereus* A529, was evaluated by growing these strains in semi-solid BHI top-agar (pH 7.5, 8.5 and 9.5), which was applied over the pre-grown (27 hours) colonies of *B. cereus* A529 . All of the strains tested showed some sensitive to *B. cereus* A529, but it was not significant.

In another method, the growth of test bacteria and of emetic strain started at same time. Colonies of the emetic *B. cereus* A529 strain were spotted and grown on the BHI agar surface (pH 7.5, 8.5 or 9.5) previously inoculated with approximately 10^5 CFU/ml of *B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *B. cereus* F4810/72, *B. subtilis*, *L. innocua*, *L. monocytogenes* or *E. coli*. The results showed that the emetic *B. cereus* F4810/72 strain was unable to grow around the colonies of *B. cereus* A529 (Figure 11) and *L. monocytogenes* (Figure 12) and *L. innocua* (Figure 13) exhibited a reduced density of cells around that colonies. The inhibition caused by the presence of *B. cereus* A529 was dependent of the media pH, which increased at higher pH. The other strains tested were not affected by the presence of the colonies of *B. cereus* A529.

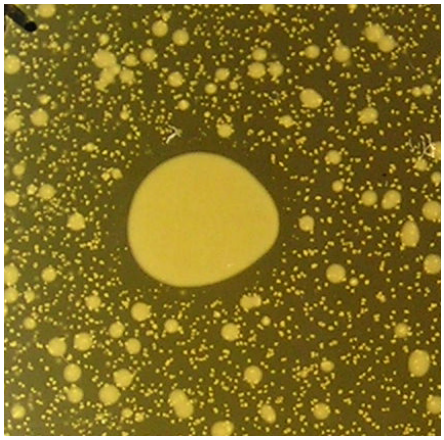

Figure 11. Inhibition of *B. cereus* F4810/72 growth by *B. cereus* A529 (center), on BHI agar, at pH 7.5.

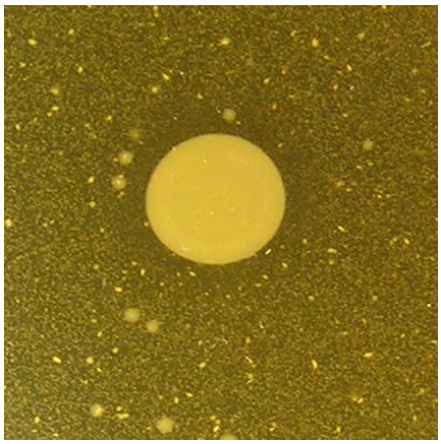

Figure 12. Inhibition of *L. monocytogenes* growth by *B. cereus* A529 (center), on BHI agar, at pH 8.5.

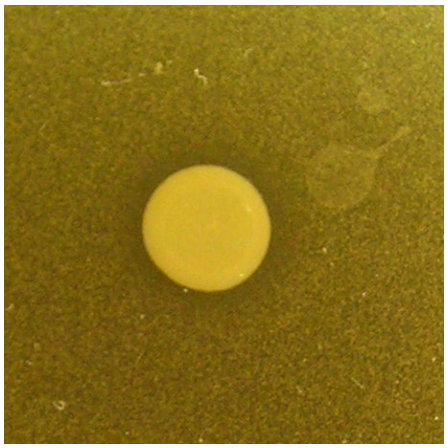

Figure 13. Inhibition of *Listeria innocua* growth by *Bacillus cereus* A529 (center), on BHI agar, at pH 8.5.

3.3. Antimicrobial activity of cereulide

3.3.1. Effect of supernatant of *B. cereus* A529

The susceptibility of *B. subtilis, L. innocua*, *L. monocytogenes, E. faecalis*, *S. aureus*, *S. thyphimurium* and *E. coli* to cereulide was initially evaluated by dropping filter sterilized supernatant of *B. cereus* A529 on paper disks, previously placed on bacterial cultures on BHI agar. After a period of 20 hours at 30°C, no inhibition of these strains by the supernatant was observed.

3.3.2. Effect of cereulide

The variation of OD_{600nm} in aerobic bacterial growth in BHI broth, with addition of

cereulide (179.97 µM in 20 % DMSO) from a fresh stock solution, showed that *B. cereus* ATCC 10987, *B. subtilis* and *L. monocytogenes* experienced growth inhibition by 9 µM of this ionophore. On the other hand, *B. cereus* F4810/72, *B. cereus* A529, *B. cereus* ATCC 14579 and *L. innocua,* which were submitted to cereulide from a solution stored for 24-48 hours, did not suffer any growth inhibition.

3.3.3. Effect of H_2O on cereulide effectiveness

Growth of *B. cereus* F4810/72, *B. cereus* 10987 and *B. subtilis* occurred simultaneously in presence of cereulide from two different stock solutions (167.84 µM in 20 % DMSO and 167.84 µM in 100 % DMSO). Growth was recorded with fresh and 24 hours old solutions of cereulide.

The results obtained revealed that the 24 hours old solution of cereulide in 20 % DMSO had no effect on the growth of *B. cereus* 10987 and *B. subtilis*, in opposition to the solution of cereulide in 100% DMSO. Both fresh solutions of cereulide caused growth inhibition of *B. cereus* 10987 and *B. subtilis*, but the solution of cereulide in 20 % DMSO was less effective. Figure 14 and Figure 15 represent the growth curves of *B. cereus* ATCC 10987 and *B. subtilis,* respectively, resultant from their growth with cereulide dissolved in 20 % DMSO and dissolved in 100 % DMSO.

In opposition to *B. cereus* 10987 and *B.* subtilis, *B. cereus* F4810/72 was resistant to 8.39 µM of cereulide, at pH 8.5 (Figure 16).

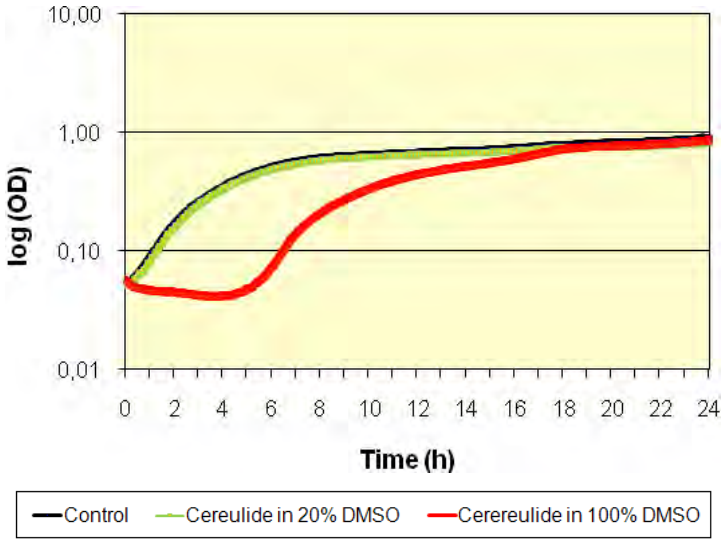

Figure 14. Aerobic growth of *B. cereus* ATCC 10987 in BHI broth, at pH 8.5, with addition of cereulide in 20 and 100% DMSO from a 24 hours old solution (final concentration 1% DMSO with 8.39 µM of

cereulide).

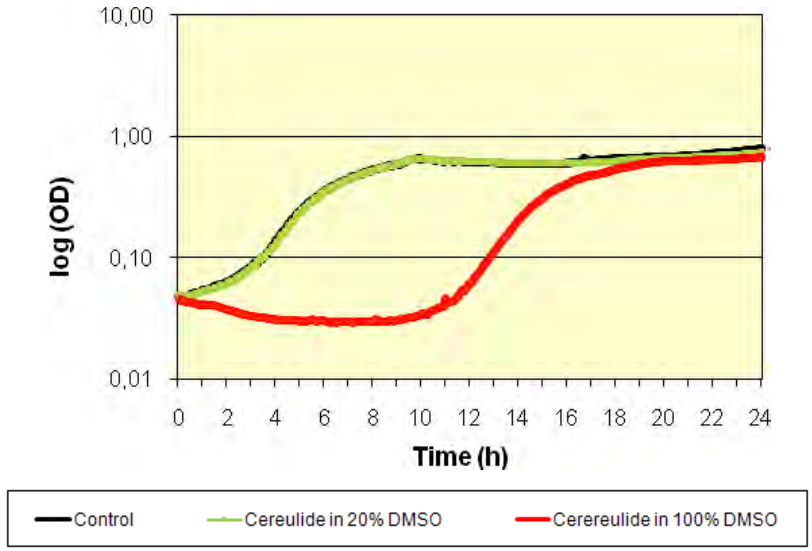

Figure 15. Aerobic growth of *B. subtilis* in BHI broth, at pH 8.5, with addition of cereulide in 20 and 100% DMSO from a 24 hours old solution (final concentration 1% DMSO with 8.39 µM of cereulide).

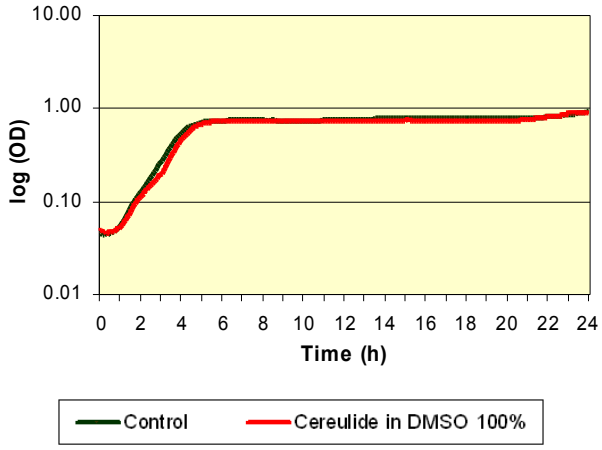

Figure 16. Aerobic growth of *B. cereus* F4810/72 in BHI broth, at pH 8.5, with and without 8.39 µM of cereulide and 1% DMSO.

3.4. Drop dilution assay

Cereulide (167.84 µM in 4 % DMSO), spotted on BHI agar inoculated with test bacteria, caused some inhibition on the growth of the Gram positive non-emetic strains. Diameter of inhibition zones is shown in Table 5. However, this ionophore was only able

to cause a decrease in cell density without complete inhibition of growth. Inhibition of *B. cereus* ATCC 10987 (Figure 17A), *B. cereus* ATCC 14579 (Figure 17B) and *B. subtilis* (Figure 18) was observed at pH 8. Both *Listeria* strains were inhibited at pH 7.5 (Figure 19A), 8.5 (Figure 19B) and 9.5. At pH 8.5, all the inhibited strains exhibited similar inhibition zones, without significant differences between the results of the non-emetic strains, *B. cereus* ATCC 10987 and ATCC 14579.

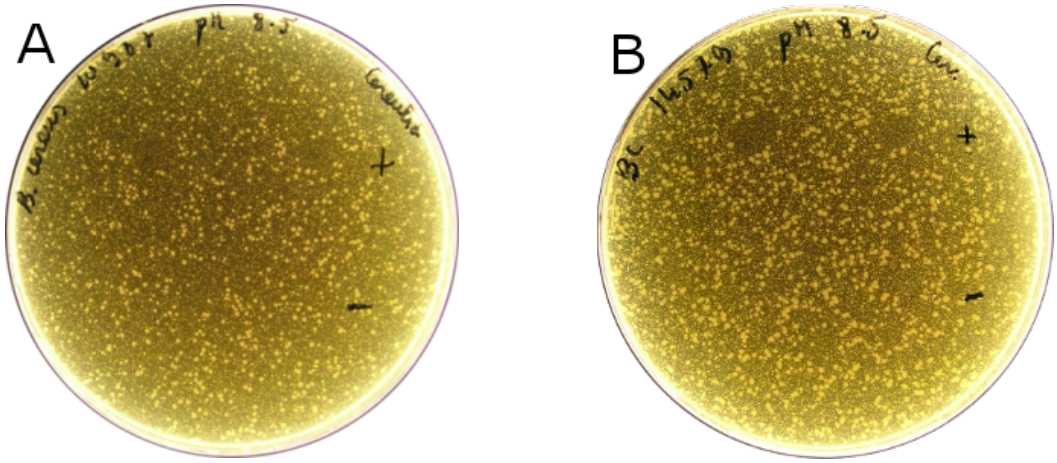

Figure 17. Inhibition zones caused by cereulide (167.84 µM) in cultures of *B. cereus* ATCC 10987 **(A)** and *B. cereus* ATCC 14579 **(B)** in BHI agar, at pH 8.5, after 14 h of growth at 30°C.

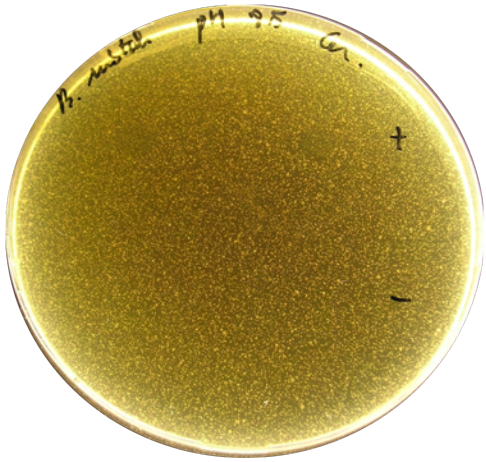

Figure 18. Inhibition zones caused by cereulide (167.84 µM) in *B. subtilis* culture in BHI agar, at pH 8.5,

after 14 h of growth at 30°C.

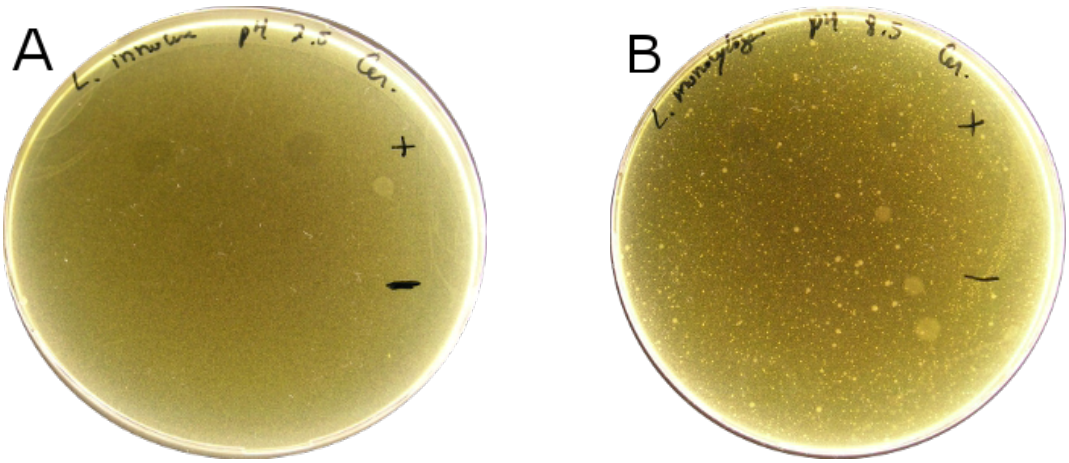

Figure 19. Inhibition zones caused by cereulide (167.84 µM) in cultures of *L. innocua* at pH 7.5 **(A)** and *L. monocytogenes* at pH 8.5 **(B)**, both in BHI agar, after 14 h of growth at 30°C.

Table 5. Inhibition zone (mm±SE) resultant from exposure of test bacteria to cereulide in BHI agar, after 14h of incubation at 30ºC.

	Cereulide (167.84µM)		
Microorganism	**pH 7.5**	**pH 8.5**	**pH 9.5**
***B. cereus* 10987**	0	6.6±0.3 [2]	n.d.
***B. cereus* 14579**	0	6.3±0.0 [2]	n.d.
B. cereus F4810/72	0	0	0
***B. cereus* A529**	0	0	n.d.
B. subtilis	0	6.5±0.0 [2]	n.d.
L. monocytogenes	5.1±0.0 [2]	6.5±0.3 [2]	5.3± 0.3[1]
L. innocua	6.5± 0.0[2]	6.8±1.4 [1]	6.6±0.3 [1]
E. coli	0	0	0

n.d. (not determined): insufficient growth of test bacteria

[1]: not complete inhibition, some colonies present
[2]: slight inhibition, many colonies present

3.5. Effect of potassium ionophores on bacterial survival

In growth curves that exhibited long lag phases, a drop in absorbance was generally

observed (See Appendix 1 and Appendix 2). To verify if that drop in absorbance was a consequence of cell death, the number of CFU of cultures of *B. cereus* ATCC 10987, *B. cereus* ATCC 14579 and *B. cereus* F4810/72 was determined.

3.5.1. Effect of valinomycin

Growth of *B. cereus* ATCC 10987, *B. cereus* ATCC 14579 and *B. cereus* F4810/72 occurred in BHI, at pH 8.5 and 9.5, in presence of 9 µM of valinomycin in 1 % DMSO.

At pH 8.5, valinomycin caused a decrease of the number of CFU of all strains tested. The same was observed at pH 9.5, but, at this pH, CFU counts of control growth also suffered a decrease.

The non-emetic *B. cereus* ATCC 10987 was the most susceptible strain to valinomycin. At pH 8.5, in 2 hours of exposure to valinomycin, it suffered more than 3.5 log reduction of the initial number of CFU/ml. Recovery of CFU occurred more than 8 hours after the beginning of exposure to the ionophore (Figure 20). On the other hand, the emetic *B. cereus* F4810/72 undered a low percentage of mortality induced by valinomycin, suffering less than 1 log reduction of the initial number of CFU/ml (Figure 21). In 6 hours of exposure to valinomycin, non-emetic *B. cereus* ATCC 14579 experienced a 2.4 log reduction of the initial number of CFU/ml. Recovery of CFU occurred immediately after that period. Decrease of the number of CFU/ml of cultures of *B. cereus* F4810/72 and *B. cereus* A529 with valinomycin was found only at the moment that CFU numbers of the control growth started increasing.

All strains were able to recovery from reduction of the number of CFU caused by valinomycin, at pH 8.5. After a 24 hours period of incubation, there were no significant differences between the number of CFU/ml of cultures with and without valinomycin.

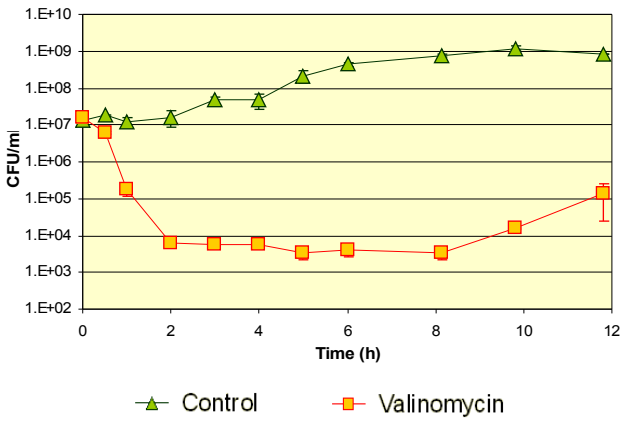

Figure 20. CFU counts of *B. cereus* ATCC 10987 cultures exposed to 9 µM of valinomycin in BHI broth, at pH 8.5.

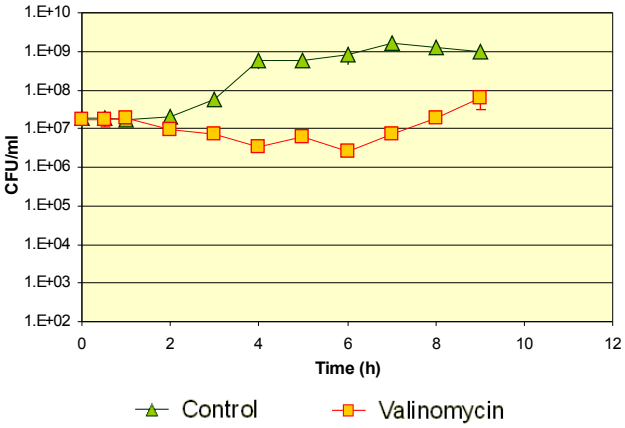

Figure 21. CFU counts of *B. cereus* F4810/72 cultures exposed to 9 µM of valinomycin in BHI broth, at pH 8.5.

3.5.2. Effect of cereulide

The effect of cereulide (8.39 µM in 1% DMSO) on survival of *B. cereus* ATCC 10987 and *B. cereus* F4810/72 growing in BHI broth, at pH 8.5, was also evaluated by determining the number of CFU. The viable counts of *B. cereus* ATCC 10987 showed that cereulide caused at least 2.5 log reduction of the initial number of CFU/ml, but recovery of CFU occurred after less than 6 hours of exposure to cereulide (Figure 22). After a 24 hours period of incubation, cultures with and without cereulide had identical numbers of CFU/ml. Contrarily to the non-emetic strain, number of CFU/ml of cultures of *B. cereus* F4810/72 was not significantly affected by cereulide, being similar with the control ones (Figure 23).

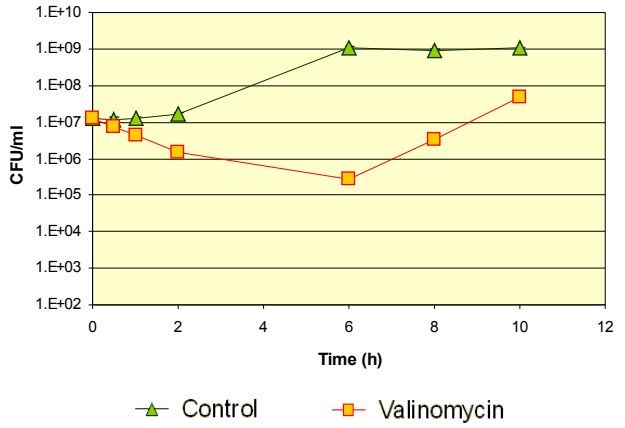

Figure 22. Viable counts of *B. cereus* ATCC 10987 cultures exposed to 8.39 µM of cereulide in BHI broth, at pH 8.5.

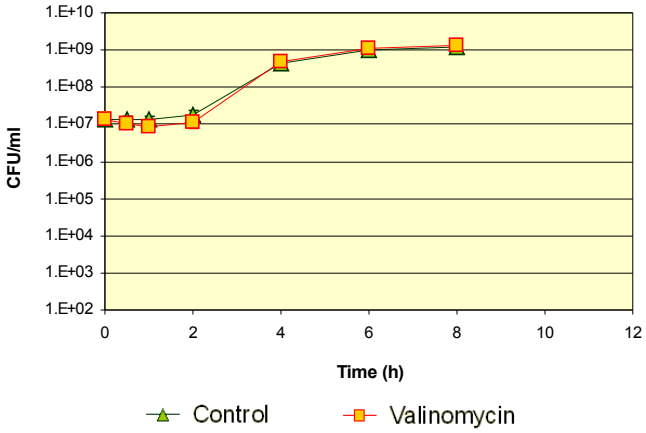

Figure 23. Viable counts of *B. cereus* F4810/72 cultures exposed to 8.39 µM of cereulide in BHI broth, at pH 8.5.

4. DISCUSSION

4.1. Antimicrobial activity of valinomycin

Valinomycin is a potassium ionophore with known antimicrobial activity against Gram positive bacteria, as confirmed in this study. The inhibition of *E. faecalis* by this ionophore was already reported (Harold and Baarda, 1967; Seschachalem *et al.*, 1973; Ryabova *et al*., 1975) as well as the inhibition of *B. subtilis* (Seschachalem *et al.*, 1973 and Ryabova *et al*., 1975). *Bacillus subtilis* also experienced reversal loss of its motility induced by this ionophore (Shioi *et al*., 1978). *Staphylococcus aureus,* which, in this study, was sensitive to 9 μM of valinomycin (pH 7.5), has shown, in previous studies, different susceptibility to valinomycin. According to Seschachalem *et al.* (1973), *S. aureus* growing in trypticase soy broth media is not sensitive to at least 90 μM of valinomycin. On the other hand, Ryabova and colleagues (1975) observed the inhibition of *S. aureus* at pH 7.2 exposed to 0.2 (200 mM of K^+) and 10 μM (5 mM of K^+) of ethanolic valinomycin in a broth medium.

The resistance of Gram negative bacteria to valinomycin has been observed in other studies (Seschachalem *et al.*, 1973, Ryabova *et al*., 1975). This resistance is probably due to the presence of an outer membrane that may prevent the entry of ionophores in the cell. This hypothesis is supported by the study of Pavlasova and Harold (1969), in which *E. coli* became sensitive to valinomycin after treatment with Tris-EDTA, which increases the permeability of the outer membranes.

Bactericidal activity of valinomycin, observed in this study, is a novelty. Until now, bactericidal effects of valinomycin were not known. However, only a partial cell death was observed, since all strains, after a period of cell death, were able to reach identical cell numbers to control growth. Although bactericidal activity of valinomycin had not been tested at a pH lower than 8.5 it is unlikely that, in relation to the results obtained, this ionophore causes cell death. From the tested bacteria, *B. subtilis* is the only one that may suffer cell death by valinomycin at lower pH predicted from the characteristics of its growth curve, at pH 7.5. Shioi and colleagues (1978) did not find a significant loss of viability of *B. subtilis* cells submitted to the presence of valinomycin (9 μM), but the analysis was done only during the first 10 minutes of exposure to this ionophore. In this study, bactericidal effects of valinomycin were observed only after 30 minutes of exposure

to valinomycin.

The apparent relation between cell death of *B. cereus* ATCC 14579 and F4810/72 strains and cell growth suggests that the survival of these strains is not affected when they are in a stage of low energy consumption. However, as soon as cell division starts, cells start dying. Cell division involves synthesis of all the components of a cell (e.g. cell membrane and DNA), which requires a high consumption of ATP and nutrients (Goehring and Beckwith, 2005). Valinomycin, due to its ionophoric properties, dissipates the membrane potential which, at pH 8.5, will result in the decrease or dissipation of the proton motive force. Most likely, these cells can maintain a minimal proton motive force required for cell survival. However, this driving force should be not enough to allow the synthesis of ATP and uptake of nutrients required for a stage of high energy consumption, like cell division is. Therefore, with the beginning of division, cells could experience exhaustion of ATP and nutrients which were required for their survival, and will dye.

In opposition to the drop dilution assay, evaluation of antimicrobial activity of valinomycin by performing the disk assay did not generate good results. Two factors can be associated with these bad results. The diffusion of the concentration of valinomycin (9 μM) used in agar media could have result in a harmless concentration for cells. Moreover, the use of paper disks allows the observation of a possible inhibition only around the disk.

4.2. Antimicrobial activity of cereulide

Cereulide is known for its ability to inhibit mitochondrial activity (Mikkola *et al*., 1999) and even for induction of apoptosis in cell lines (Morrison *et al*., 2005) but there are no references to cereulide as an antimicrobial compound, although this had already been a subject of study (Altayar and Sutherland, 2006).

The results obtained show that cereulide, like valinomycin, exhibits antimicrobial activity, which was expressed against *B. cereus* ATCC 10987, *B. subtilis* and *L. monocytogenes,* at pH higher than 7.5. Cereulide was even found to cause cell death, at least of *B. cereus* ATCC 10987 cells growing in BHI broth at pH 8.5. Tests with *B. cereus* F4810/72 suggest that cereulide does not enhance the growth of its producer. Although *B. cereus* A529, *B. cereus* ATCC 14579 and *L. innocua* had shown resistance to cereulide, those results are not trustable, since cereulide used on those tests was dissolved and stored in H_2O. As it was later on confirmed, the antimicrobial activity of cereulide is lost when

this hydrophobic compound is dissolved in H_2O. The hydrophobic character of cereulide, when coming in contact to water, most likely result in aggregation of the compound or starts sticking to tube walls, consequentially losing its activity before adding it to a cell culture.

Although cereulide had shown antimicrobial activity, the pH at which it was observed is an uncommon pH for bacterial growth. Substrate colonized by *B. cereus* such as food, has a pH below 7.5 (Adams and Moss, 2008). The soil may be the substrate where a more alkaline pH can be found, since its normal pH values are in a range of 3 to 8 (Breemen and Buurman, 2002). However, it was observed that the non emetic *B. cereus* ATCC 14579 strain has the ability to increase the extracellular milieu to a value of approximately 8 (Nakata and Halvorson, 1960; de Vries *et al*., 2004). The increase of the media pH starts during the early stationary phase (de Vries *et al*., 2004), which is the moment at which production of cereulide takes place. In this study, *B. cereus* ATCC 14579 revealed a response to valinomycin similar to the response of emetic strains. Thereby, emetic strains may also cause an increase of extracellular pH, creating the perfect conditions for cereulide to inhibit the growth of competitors. Moreover, it was reported that cereulide production occurs in BHI at pH 6.0-6.8 but, when compared with its production at pH 7.4, the synthesis is slower (Rajkovic *et al*., 2006). This may support the hypothesis of cereulide being an antimicrobial compound. If cereulide inhibits bacterial growth only at alkaline pH, its production must be enhanced at this pH. The delay in cereulide production at acidic pH can also be related with a longer time needed to increase the pH to an optimal pH for cereulide activity.

4.3. Antimicrobial activity of cereulide likely produced by *B. cereus* A529

The lack of susceptibility of *B. subtilis, L. innocua*, *L. monocytogenes, E. faecalis* and *S. aureus* to the filtered supernatant of *B. cereus* A529 culture might be due to the absence of cereulide in the supernatant. It was reported that, after centrifugation of an emetic strain culture, the majority of cereulide produced remains in the pellet, whereas the filter-sterilized supernatant contains no cereulide (Rajkovic *et al*., 2006).

The effect of cereulide likely produced by the emetic *B. cereus* A529 strain on cell growth of other bacterial strains was evaluated by growing cultures of *B. cereus* A529 together with cultures of *B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *B. cereus*

F4810/72, *B. subtilis*, *L. innocua*, *L. monocytogenes, E. faecalis* or *S. aureus*. The results obtained do not elucidate if *in vivo* cereulide acts or not as an antibiotic. Inhibition of *L. monocytogenes* and of *L. innocua* was observed. However, *B. cereus* F4810/72 strain, which showed resistance to cereulide, was also inhibited by *B. cereus* A529. Therefore, it is not clear if the inhibition observed was caused or not by cereulide.

4.4. Effect of pH on growth inhibition by valinomycin and cereulide

In this study, growth inhibition by valinomycin and cereulide was greatly enhanced by raising the alkalinity of growth media. In opposition to acidic medium, at neutral and alkaline medium the pH gradient across the cytoplasmic membrane of bacterial cells disappears. Hence, at these conditions the proton motive force depends mainly on the membrane potential (Weerkamp *et* al., 1977). Potassium ionophores stimulate the K^+ movements across cytoplasmic membrane, down a concentration gradient (Lehninger *et al*., 1993; Morrison *et al*., 2005). Then, treatment of bacterial cells with valinomycin and cereulide causes dissipation of membrane potential, but without affecting the pH gradient (Weerkamp *et* al., 1977; Morrison *et al*., 2005). Therefore, in presence of potassium ionophores, the proton motive force is abolished at neutral and alkaline pH values of growth media, but not at acidic pH. Since the proton motive force is the driving force for proton movements which in turn drives nutrient uptake and ATP synthesis (respiring bacteria) (Harold, 1977), its dissipation will disturb these two essential processes for cell growth and survival and, consequently, bacterial growth is inhibited at neutral end alkaline pH.

4.5. Effect of O_2 on growth inhibition by valinomycin

Although valinomycin had caused growth inhibition of bacteria growing aerobically and anaerobically, with the exception of *E. faecalis,* this potassium ionophore was more effective against bacteria growing under aerobic conditions. The availability of O_2 (Kashket, 1981; Tran and Unden, 1998) as well as the presence or absence of a functional electron transport chain (ETC) determine how ATP is synthesized, if by aerobic respiration ($+O_2$, +ETC), by anaerobic respiration ($-O_2$, +ETC) or by fermentation ($-O_2$, -ETC). With the exception of *E. faecalis*, which lacks a functional electron transport chain (Harold and

Baarda, 1967; Harold, 1972), all the tested strains perform aerobic respiration under aerobic conditions. Under anaerobiosis, all of these strains are able to ferment. Since respiring and fermentative bacteria establish a proton motive force by different mechanisms (Kashket, 1981), this driving force acts differently in both types of cells. Respiring bacteria require a proton motive force to synthesize ATP and to uptake nutrients, while for fermentative bacteria this driving force is directly required only for nutrient uptake. Therefore, the abolishment of the proton motive force by valinomycin causes higher inhibition in respiring bacteria than in fermentative cells.

4.6. Effect of valinomycin on *E. faecalis* growth

In agreement with previous studies (Gale, 1971; Ryabova *et al*., 1975), *E. faecalis* exhibited a response to valinomycin opposite to the one exhibited by the other bacterial strains. This strain suffered stronger inhibition at acidic pH, while the inhibition of the other strains was enhanced by raising the alkalinity of growth media. As already mentioned, this microorganism has the peculiarity of lacking a respiratory chain. Thereby, the proton motive force is generated by the extrusion of protons through the ATPase whether in anaerobiosis or aerobiosis (Harold and Baarda, 1967; Harold, 1972). Ryabova and colleagues (1975) demonstrated that valinomycin induces a decrease of the internal concentration of potassium in *E. faecalis* cells, in contrast to respiring cells. Due to the positive charge of K^+ and to the membrane potential (inside negative), an influx of K^+ induced by valinomycin would be expected, not an efflux. However, fermentative bacteria have a lower proton motive force when compared with respiring bacteria (Kashket, 1981; Booth, 1985; Tran and Unden, 1998). These facts suggest that the proton motive force, generated during fermentation, may be insufficient to cause the influx of K^+ against its concentration gradient. Therefore, the complex K^+-valinomycin will flow according to the gradient concentration of potassium. The efflux of K^+ will cause the hyperpolarization of its membrane with consequent induction of the influx of protons. The influx of protons is facilitated at acidic media pH, but is reduced during alkalinization of the external milieu (Harold and Baarda, 1967; Ryabova *et al*., 1975). Then, the higher inhibition of *E. faecalis* at acidic pH must be due to the higher influx of protons at this pH resulting in inhibition of the glycolysis by lowering internal pH (Harold and Baarda, 1967).

4.7. Effect of valinomycin on *B. cereus* growth

In this study, emetic strains showed less sensitivity to valinomycin than the non-emetic strains tested (excluding *B. cereus* ATCC 14579). As expected, cereulide producer strains were resistant to this ionophore, in opposition to non-emetic strains studied. These results suggest that emetic strains must possess some mechanism to enable self-protection against the inhibitory effect of cereulide. This mechanism (most likely a multidrug resistance/efflux pump) could be specific to cereulide but, probably because of the structural similarities, works also against valinomycin, although less efficiently. Lactic acid bacteria, which produce bacteriocins, have ABC-transport proteins that give them self-protection against the bacteriocin by transporting its molecules back to the extracellular medium, when they enter the cell (Kraaij *et al*., 1999; Chen and Hoover, 2003). Sequence analysis of the cereulide synthetase (*ces*) gene cluster, which is responsible for the biosynthesis of cereulide, shows that 2 of the open reading frames encoded in this gene cluster, *cesC* and *cesD*, seem to encode an ABC transporter (Ehling-Schulz *et al*., 2006). This suggests that emetic strains might have a similar mechanism of self-protection like the one of lactic acid bacteria, which may act alone or in combination with other mechanisms.

The non-emetic *B. cereus* ATCC 14579 exhibited less sensitivity to valinomycin than the other non-emetic strains, being its response to valinomycin more comparable with the one of the cereulide producer strains. But the most interesting results were obtained from the comparison of the inhibition of *B. cereus* ATCC 14579 by valinomycin with the inhibition of *B. cereus* ATCC 10987. Despite their similarities, *B. cereus* ATCC 10987 exhibited much higher sensitivity to valinomycin than *B. cereus* ATCC 14579. *B. cereus* ATCC 14579, contrary to ATCC 10987 strain, can metabolize a large number of carbohydrates (Mols *et al*, 2007) which could supply a growth advantage for this strain in starchy foods. As cereulide has been found essentially in starchy foods (Ehling-Schulz *et al*., 2004; Adams and Moss, 2008), it is possible that emetic strains colonize mainly this type of food. Thereby, the competition between *B. cereus* ATCC 14579 and emetic strains for the same habitat and nutrients could have resulted, by a process of natural selection, in a non-emetic strain able to minimize the inhibitory effect of ionophores on its growth. Bacteria have developed multidrug resistance pumps which provide them protection against toxins and antibiotics (Stavri *et al*., 2007). *B. cereus* ATCC 14579, in opposition to *B. cereus* ATCC 10987**,** could have a multidrug transporter with some affinity to

valinomycin and cereulide. Since the genomes of *B. cereus* ATCC 10987 (Rasko *et al*., 2004) and *B. cereus* ATCC 14579 (Ivanova *et al*., 2003) are completely sequenced, a microarray platform comparison could be done to try to find out which mechanism(s) of *B. cereus* ATCC 14579 confers some tolerance to potassium ionophores and that seems to be absent in *B. cereus* ATCC 10987.

5. METHODOLOGICAL RECOMMENDATIONS

Cereulide is a very hydrophobic compound, with very low solubility in H_2O. The dilution in H_2O caused loss antimicrobial activity. Therefore dilution in H_2O must be avoided. Dissolution of cereulide in 100% DMSO showed to be a better approach.

Although valinomycin is more easily dissolved in H_2O, it is also an hydrophobic compound which is more stable if dissolved in 100% DMSO. In 20% DMSO, the stability may decrease and it must be used within a short period of time.

The drop dilution assay must be preferred to the disk assay, since the use of paper disks only allows the observation of a possible inhibition around the disk, while the direct spotting of the antibiotic solution on the bacterial cultures allows a better observation of that effect.

6. CONCLUSION AND FUTURE RESEARCH

The present study shows that, comparatively to valinomycin, cereulide has antimicrobial activity against Gram positive bacteria. However, it is not clear if that is or not the main function of cereulide.

Potassium ionophores studied were found to be more effective against bacterial growth in presence of O_2 and at alkaline pH. Since pH is an important factor for antimicrobial effect of cereulide, the effect of extracellular pH on cereulide production could be analyzed in order to verify if there is a relation between the production of cereulide and its activity.

The loss of the membrane potential is assumed as the cause of bacterial growth inhibition by cereulide, but it was not proved yet. For this reason, the impact of cereulide on the membrane potential should be determined using fluorescent probes.

Emetic strains exhibited low sensitive to valinomycin and resistance to cereulide. Curiously, the non-emetic *B. cereus* ATCC 10987 and *B. cereus* ATCC 14579 strains exhibited different sensitivity to valinomycin. To try to find out which system in *B. cereus*

ATCC 14579 confers some tolerance to valinomycin and that seems to be absent in *B. cereus* ATCC 10987 a microarrray platform comparison should be performed. Unfortunately, the results obtained do not allow the analysis of the effect of cereulide on the growth of *B. cereus* ATCC 14579. Thereby, the effect of cereulide on the growth of this strain should be evaluated.

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Appendix

Appendix 1. Growth curves (OD600nm) of bacterial growth with and without valinomycin under aerobic conditions

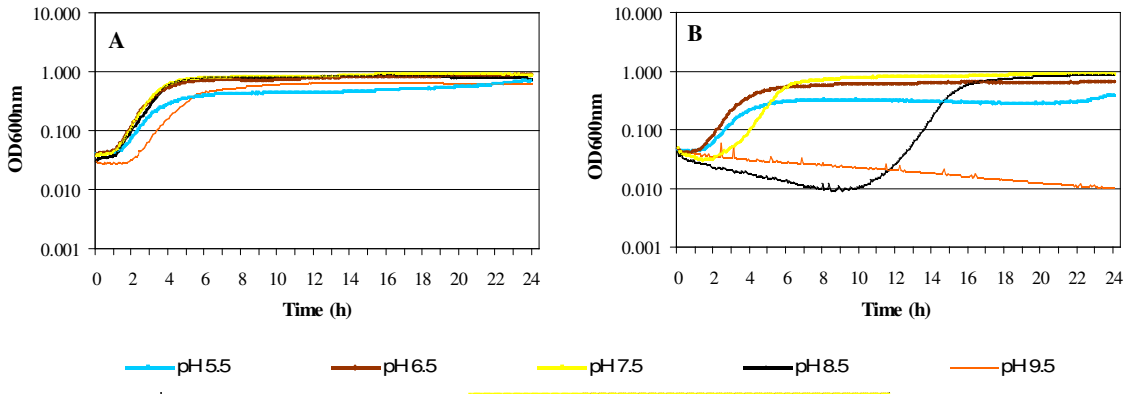

Figure A-1. Aerobic growth of *B. cereus* F4810/72 in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B).**

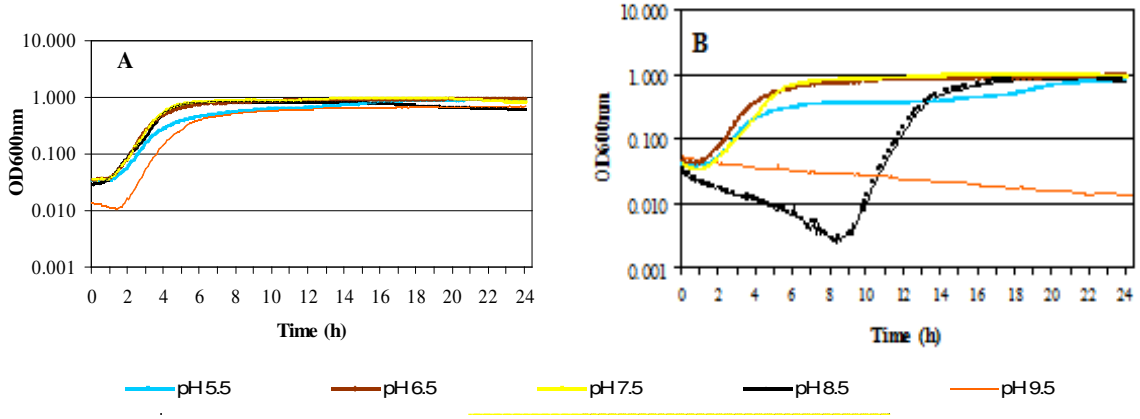

Figure A-2. Aerobic growth of *B. cereus* A529 in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

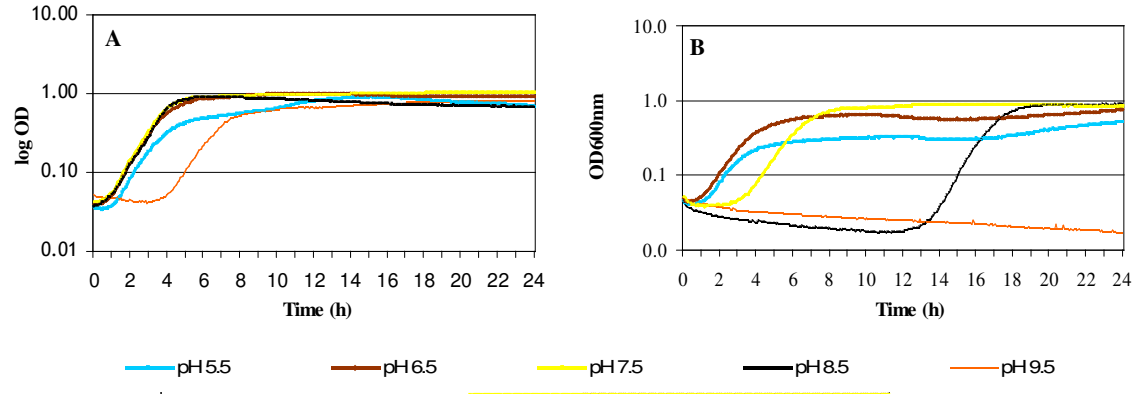

Figure A-3. Aerobic growth of *B. cereus* ATCC 14579 in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

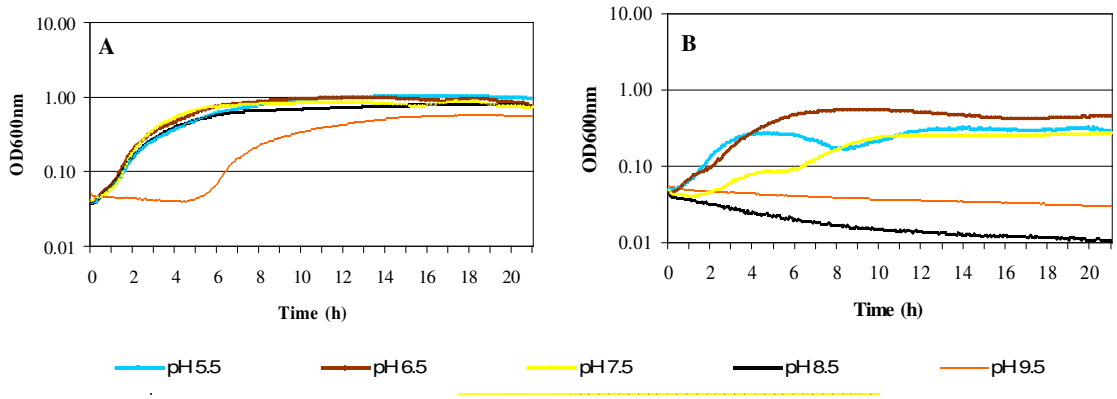

Figure A-4. Aerobic growth of *B. cereus* ATCC 10987 in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

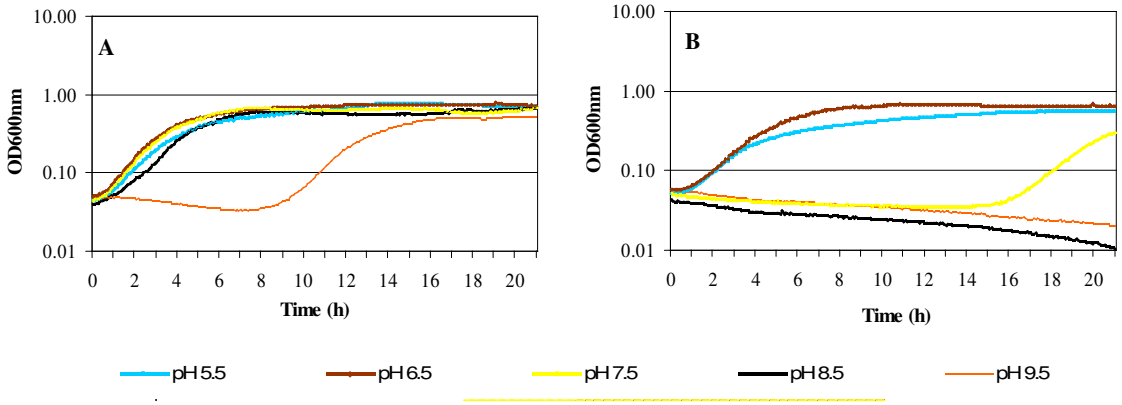

Figure A-5. Aerobic growth of *B. subtilis* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

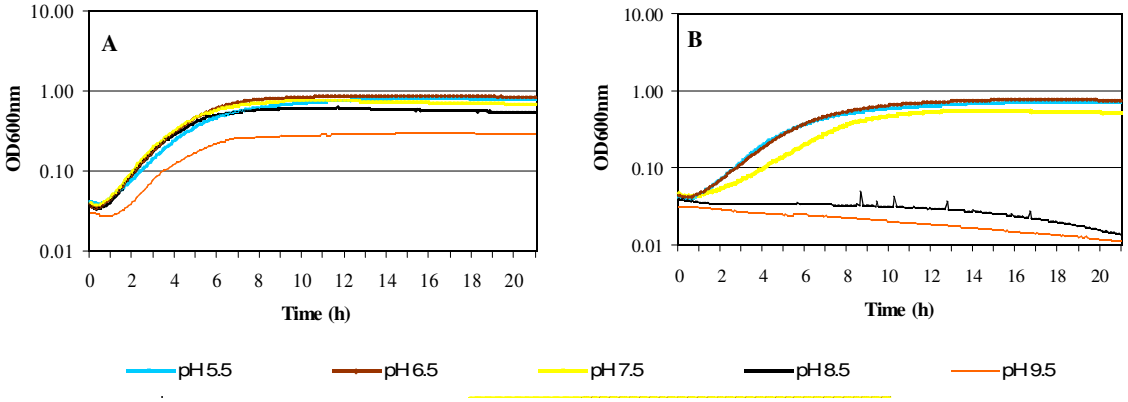

Figure A-6. Aerobic growth of *L. monocytogenes* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

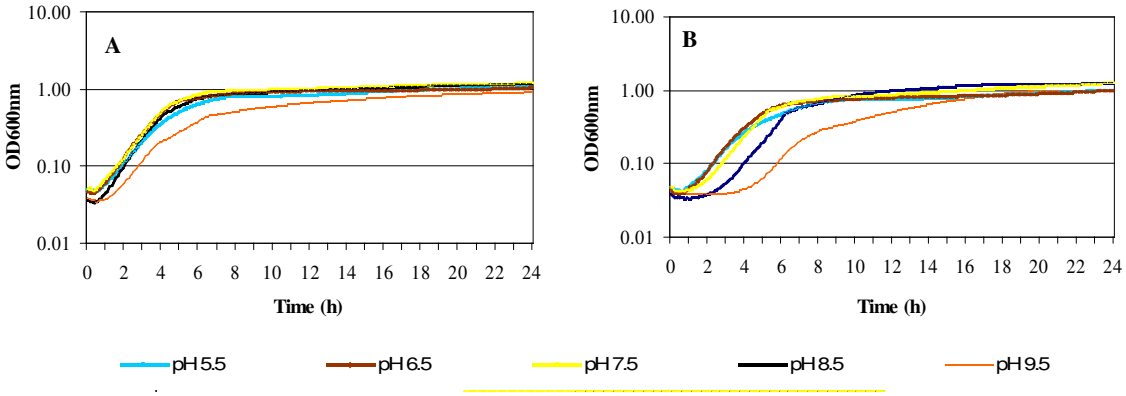

Figure A-7. Aerobic growth of *L. innocua* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

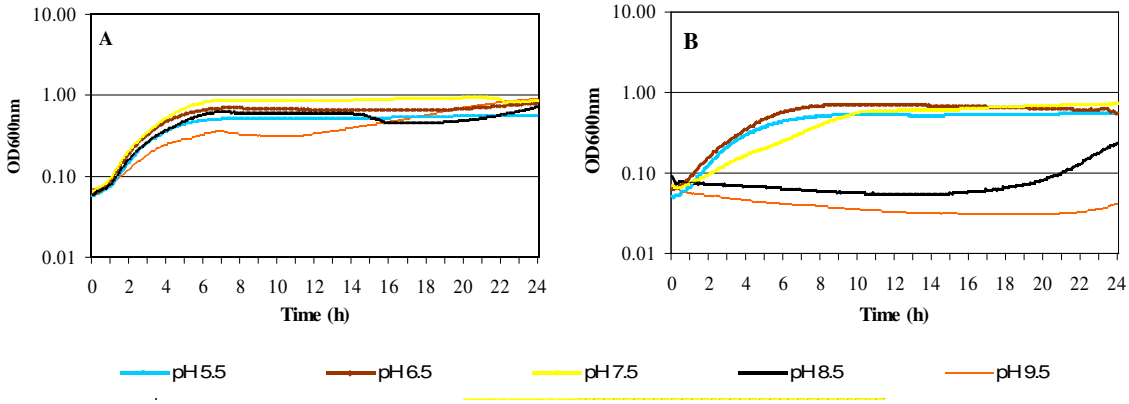

Figure A-8. Aerobic growth of *S. aureus* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

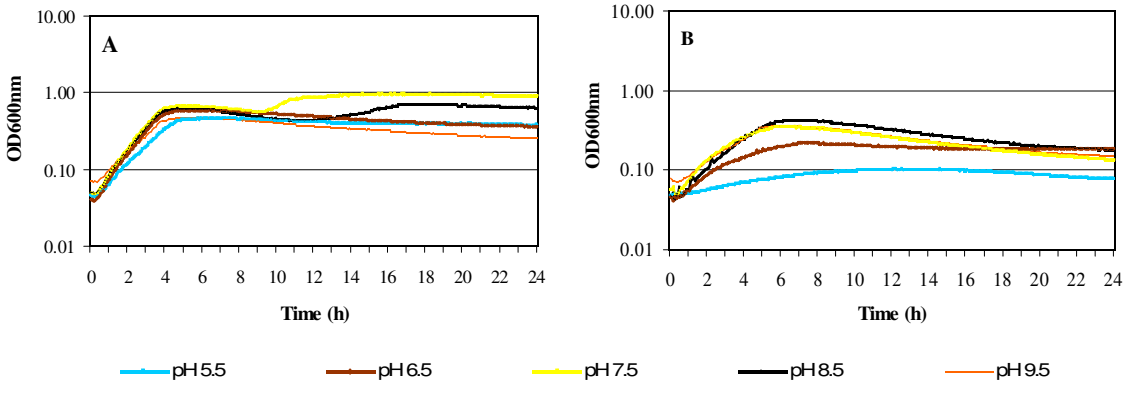

Figure A-9. Aerobic growth of *E. faecalis* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

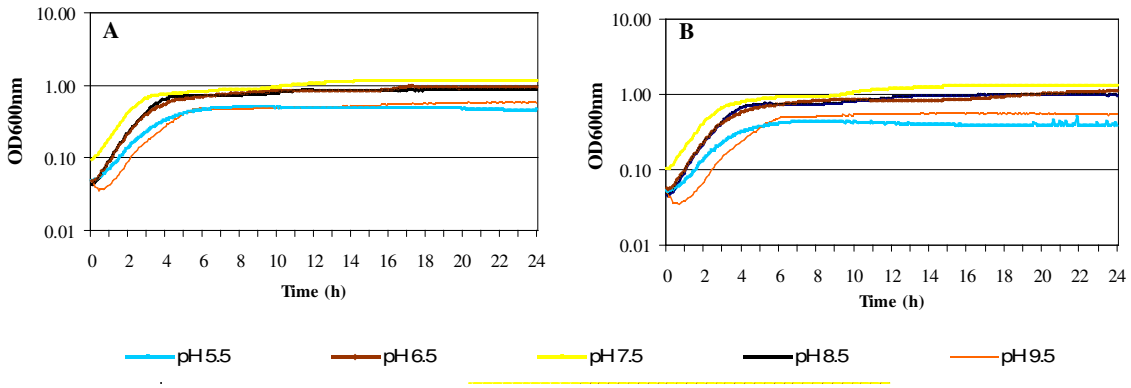

Figure A-10. Aerobic growth of *E. coli* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

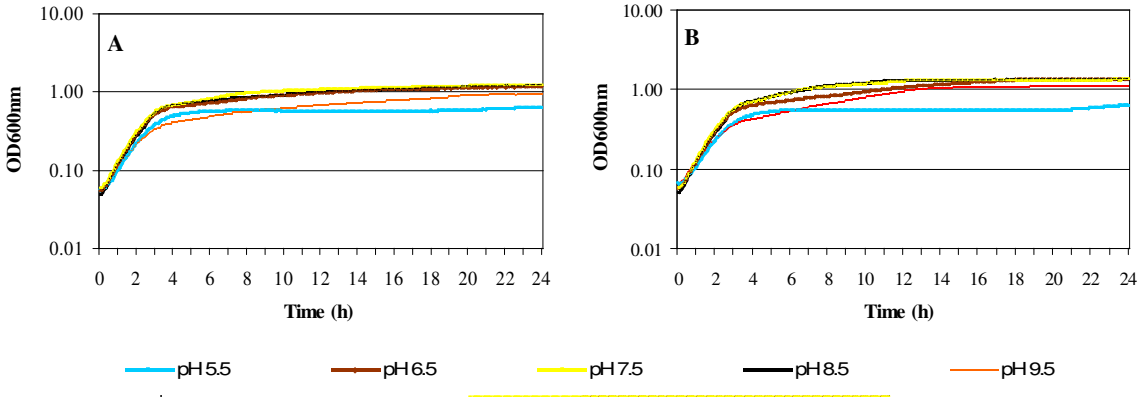

Figure A-11. Aerobic growth of *S. thyphimurium* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

Appendix 2. Growth curves (OD600nm) of bacterial growth with and without valinomycin under anaerobic conditions

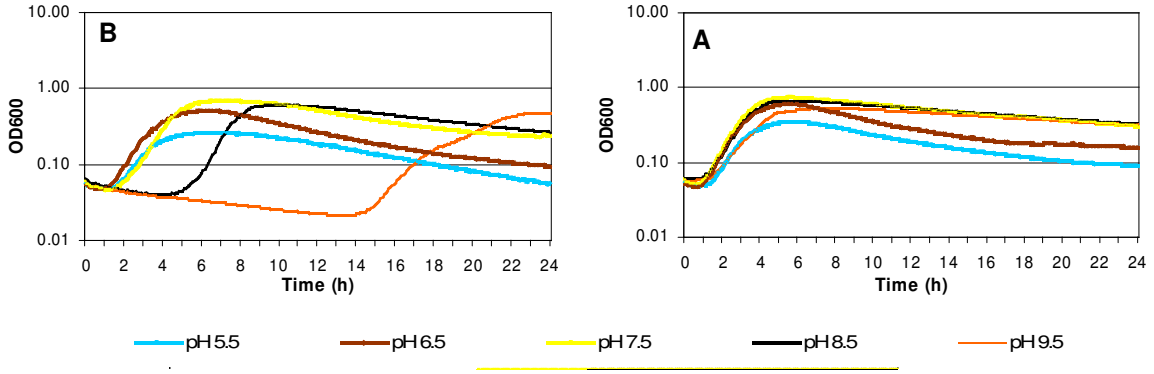

Figure A-12. Anaerobic growth of *B. cereus* F4810/72 in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

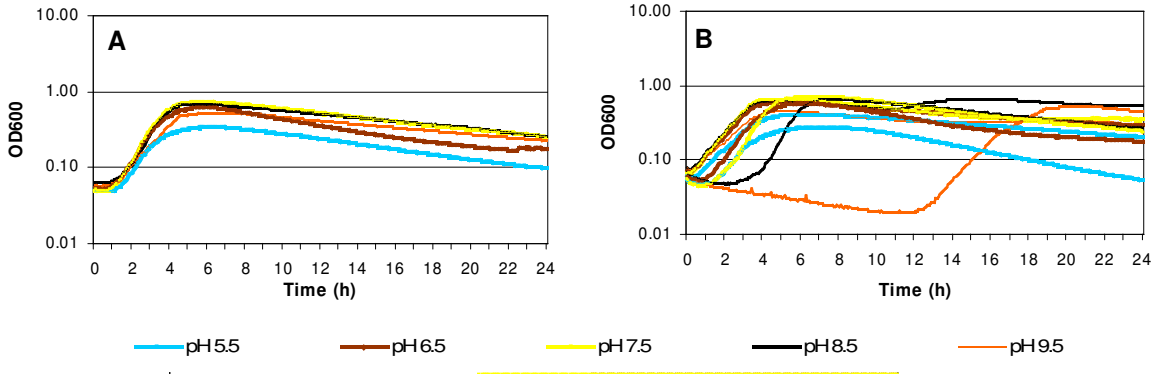

Figure A-13. Anaerobic growth of *B. cereus* A529 in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

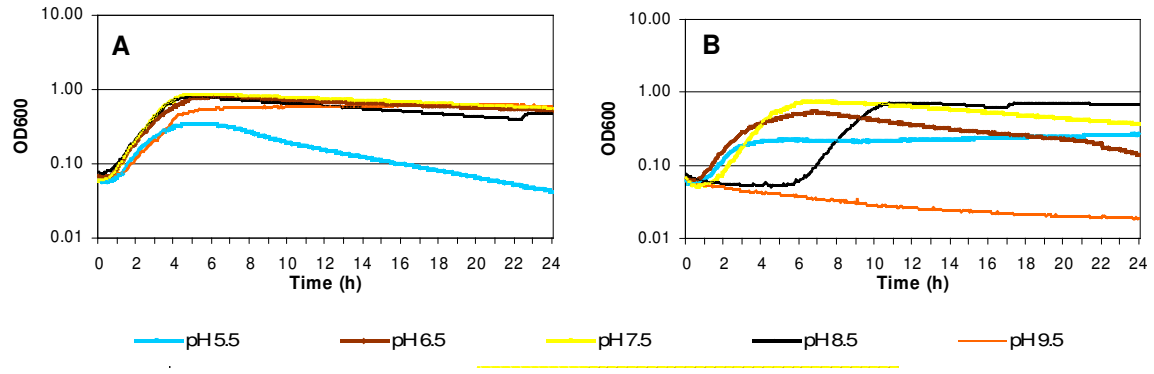

Figure A-14. Anaerobic growth of *B. cereus* ATCC 14579 in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

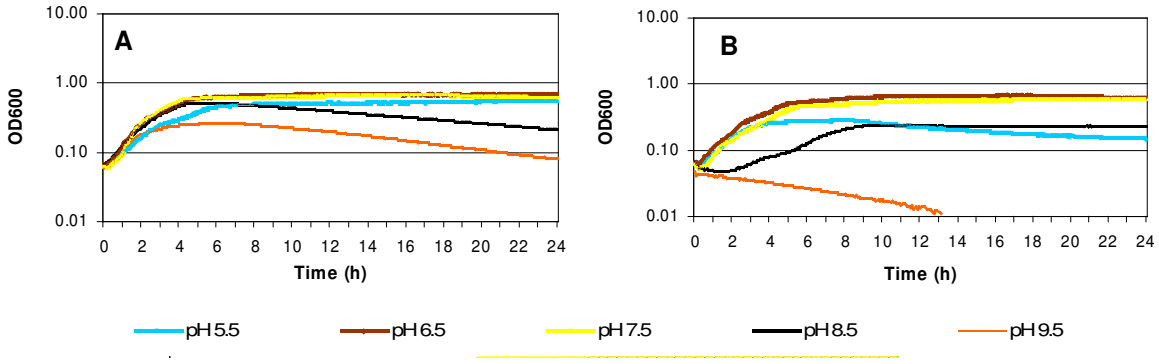

Figure A-15. Anaerobic growth of *B. cereus* ATCC 10987 in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

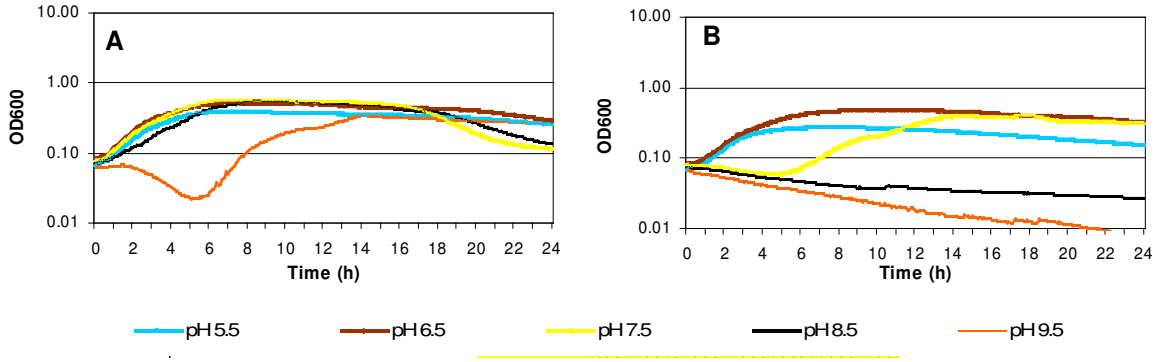

Figure A-16. Anaerobic growth of *B. subtilis* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

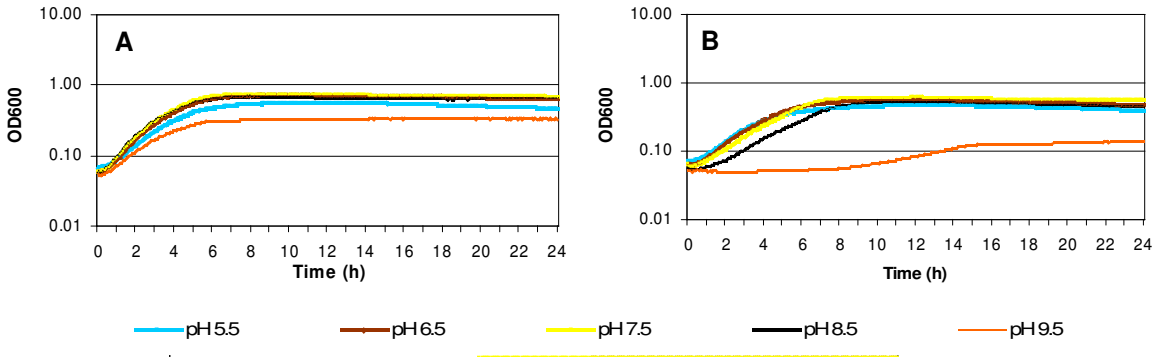

Figure A-17. Anaerobic growth of *L. monocytogenes* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

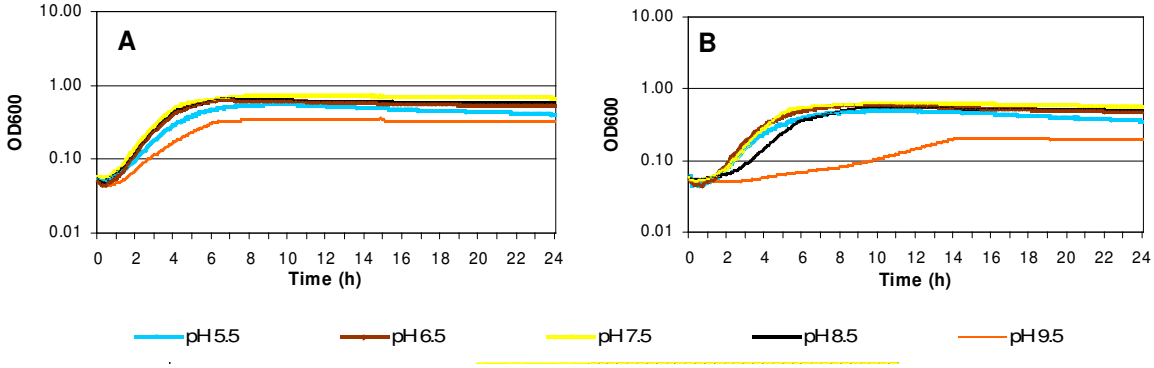

Figure A-18. Anaerobic growth of *L. innocua* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMS18 **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

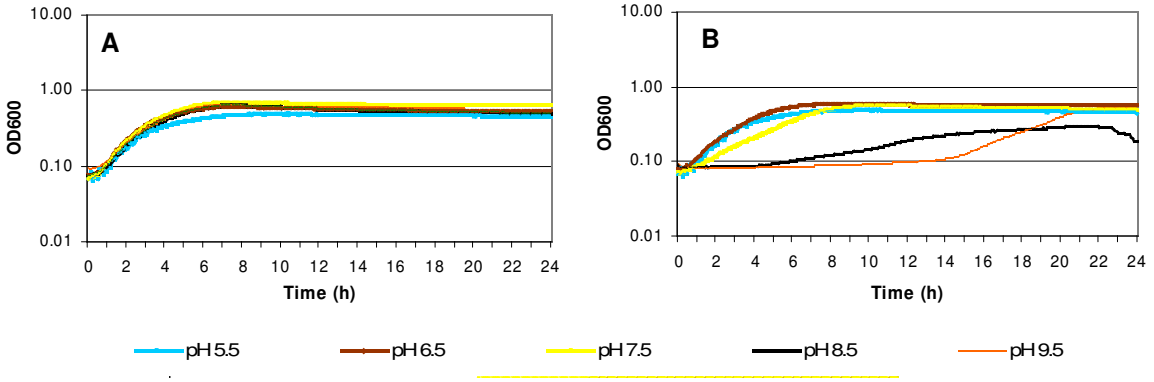

Figure A-19. Anaerobic growth of *S. aureus* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

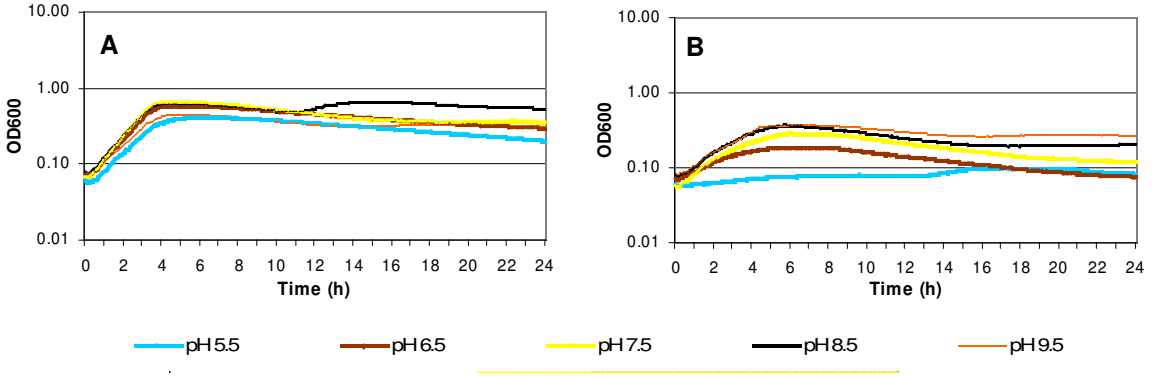

Figure A-20. Anaerobic growth of *E. faecalis* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

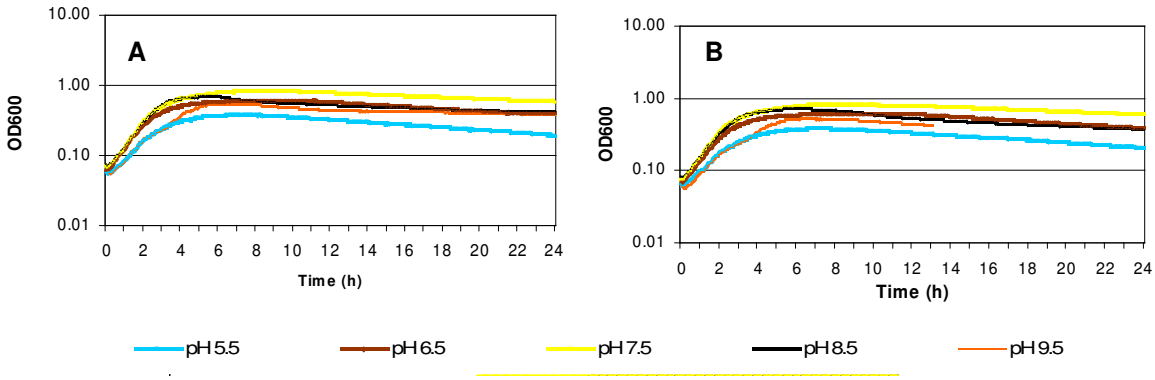

Figure A-21. Anaerobic growth of *E. coli* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

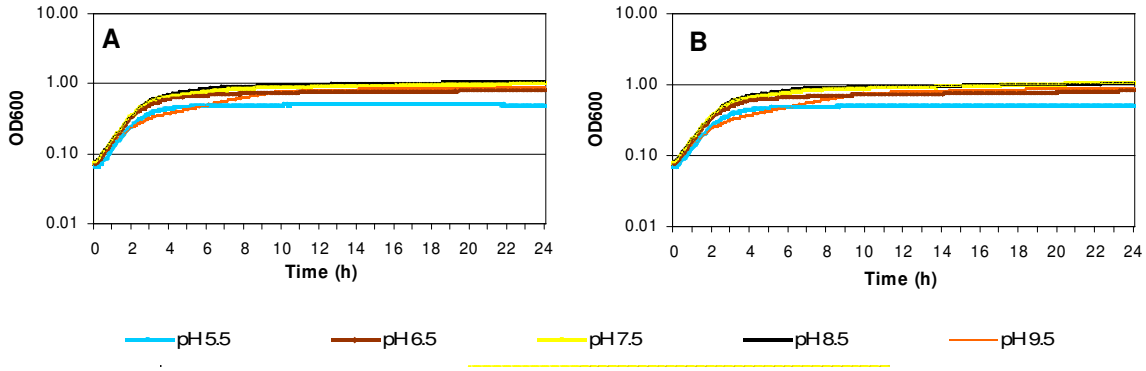

Figure A-22. Anaerobic growth of *S. thyphimurium* in BHI broth, at a range of pH values (5.5 to 9.5) in presence of 1% DMSO **(A)** and in presence of valinomycin (9 µM) with 1% DMSO **(B)**.

Appendix 3. Comparison of inhibition induced by valinomycin in aerobic and anaerobic conditions

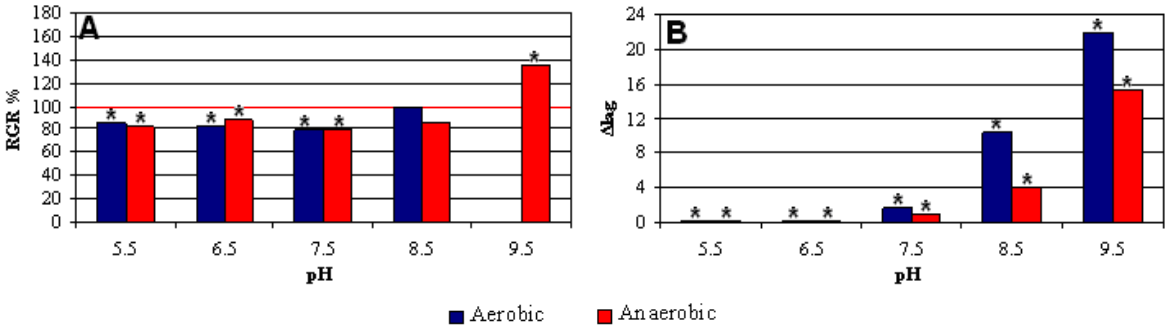

Figure A-23. Relative growth rate – RGR **(A)** and difference between the length of the lag phase of the growth control and the growth with valinomycin – Δlag **(B)** of *B. cereus* F4810/72 exposed to 9 µM of valinomycin and 1 % DMSO in BHI broth at different pHs, at 30°C, under aerobic and anaerobic conditions. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), $p<0.05$.

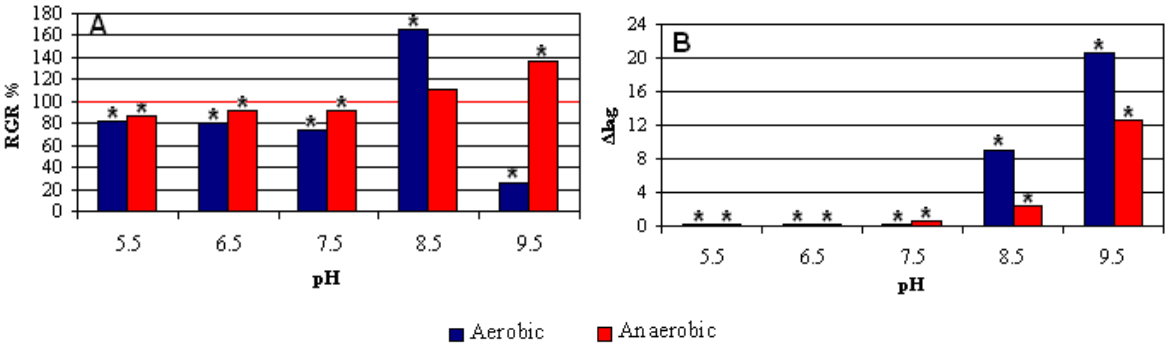

Figure A-24. Relative growth rate – RGR **(A)** and difference between the length of the lag phase of the growth control and the growth with valinomycin – Δlag **(B)** of *B. cereus* A529 exposed to 9 µM of valinomycin and 1 % DMSO in BHI broth at different pHs, at 30°C, under aerobic and anaerobic conditions. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), $p<0.05$.

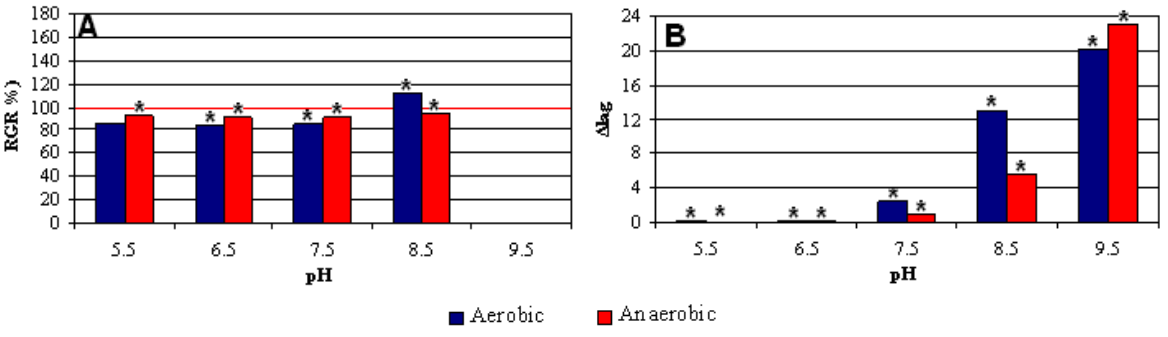

Figure A-25. Relative growth rate – RGR **(A)** and difference between the length of the lag phase of the growth control and the growth with valinomycin – Δlag **(B)** of *B. cereus* ATCC 14579 exposed to 9 µM of valinomycin and 1 % DMSO in BHI broth at different pHs, at 30°C, under aerobic and anaerobic conditions. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), $p<0.05$.

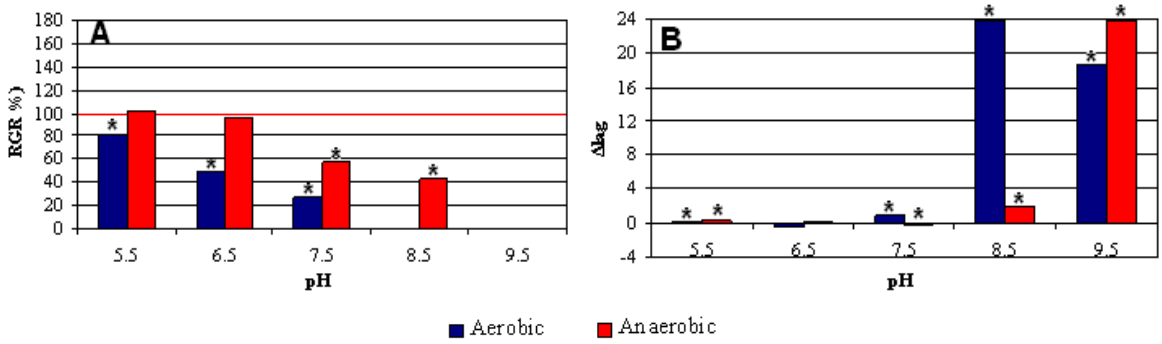

Figure A-26. Relative growth rate – RGR **(A)** and difference between the length of the lag phase of the growth control and the growth with valinomycin – Δlag **(B)** of *B. cereus* ATCC 10987 exposed to 9 µM of valinomycin and 1 % DMSO in BHI broth at different pHs, at 30°C, under aerobic and anaerobic conditions. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), $p<0.05$.

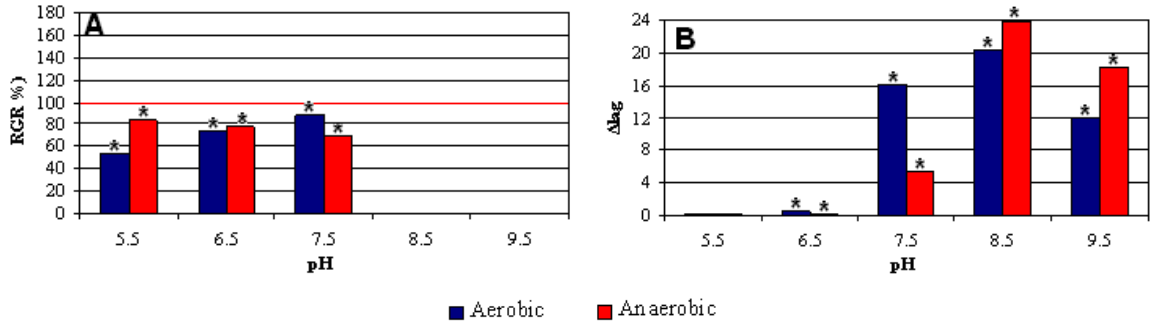

Figure A-27. Relative growth rate – RGR **(A)** and difference between the length of the lag phase of the growth control and the growth with valinomycin – Δlag **(B)** of *B. subtilis* exposed to 9 µM of valinomycin and 1 % DMSO in BHI broth at different pHs, at 30°C, under aerobic and anaerobic conditions. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), $p<0.05$.

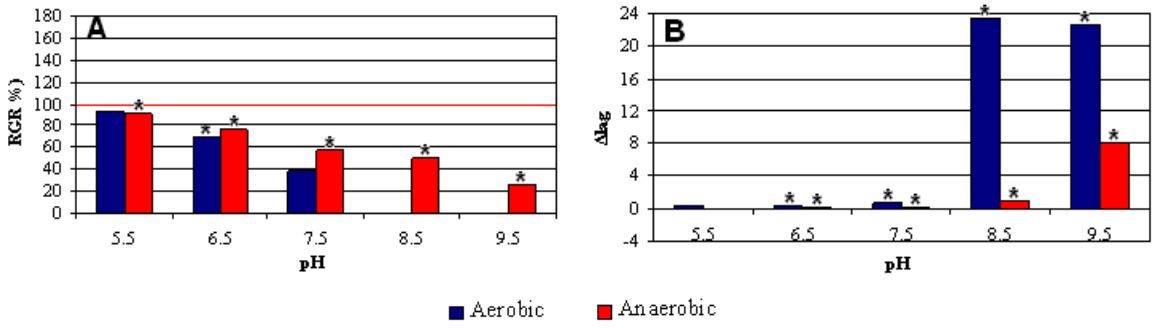

Figure A-28. Relative growth rate – RGR **(A)** and difference between the length of the lag phase of the growth control and the growth with valinomycin – Δlag **(B)** of *L. monocytogenes* exposed to 9 µM of valinomycin and 1 % DMSO in BHI broth at different pHs, at 30°C, under aerobic and anaerobic conditions. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), $p<0.05$.

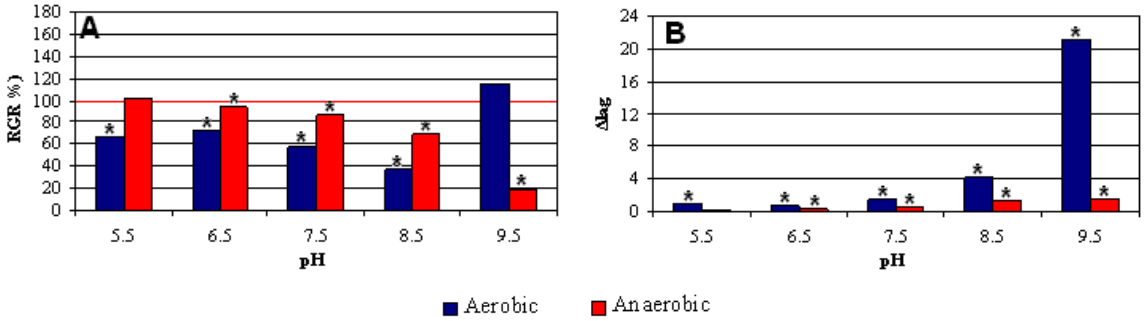

Figure A-29. Relative growth rate – RGR **(A)** and difference between the length of the lag phase of the growth control and the growth with valinomycin – Δlag **(B)** of *L. innocua* exposed to 9 µM of valinomycin and 1 % DMSO in BHI broth at different pHs, at 30°C, under aerobic and anaerobic conditions. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), $p<0.05$.

Appendix 4. Growth inhibition caused by a range of concentrations of valinomycin

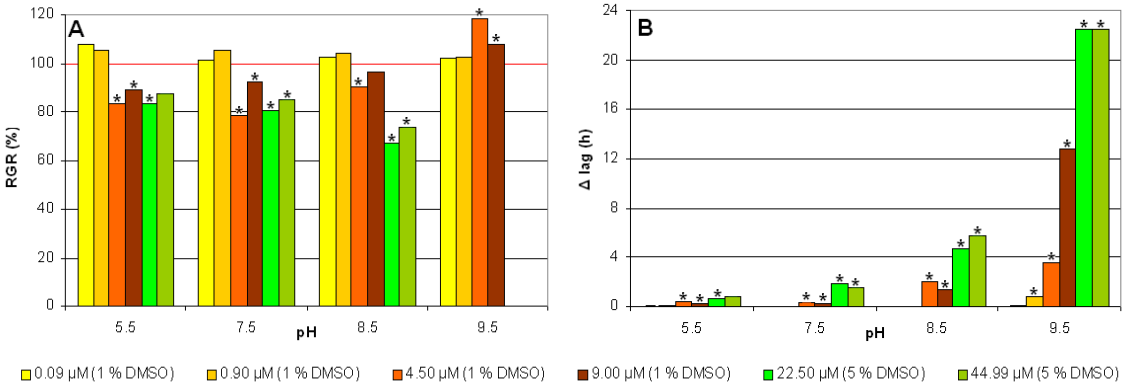

Figure A-30. Relative growth rate – RGR **(A)** and difference between the length of the lag phase of the growth control and the growth with valinomycin – Δlag **(B)** of *B. cereus* F4810/72 exposed to a range of valinomycin concentrations (0.09 to 44.99 µM) and 1 or 5 % of DMSO, in BHI broth at different pHs, at 30°C, with aeration. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), p<0.05.

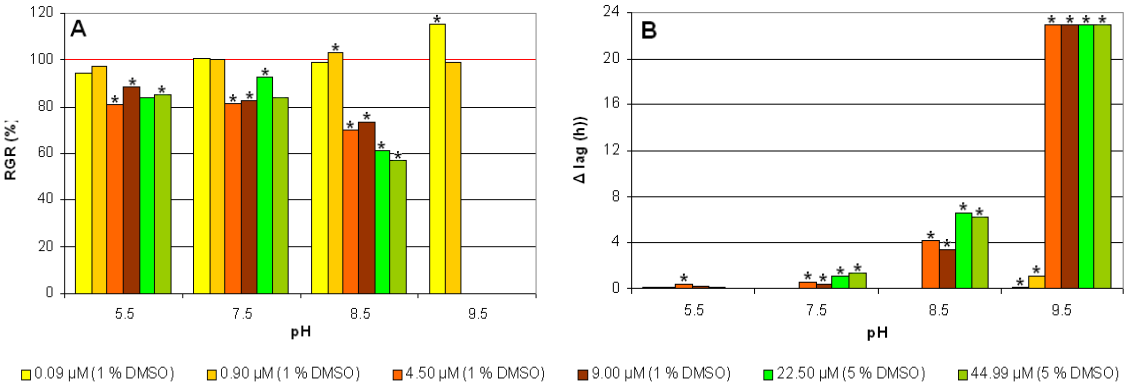

Figure A-31. Relative growth rate – RGR **(A)** and difference between the length of the lag phase of the growth control and the growth with valinomycin – Δlag **(B)** of *B. cereus* A529 exposed to a range of valinomycin concentrations (0.09 to 44.99 µM) and 1 or 5 % of DMSO, in BHI broth at different pHs, at 30°C, with aeration. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), p<0.05.

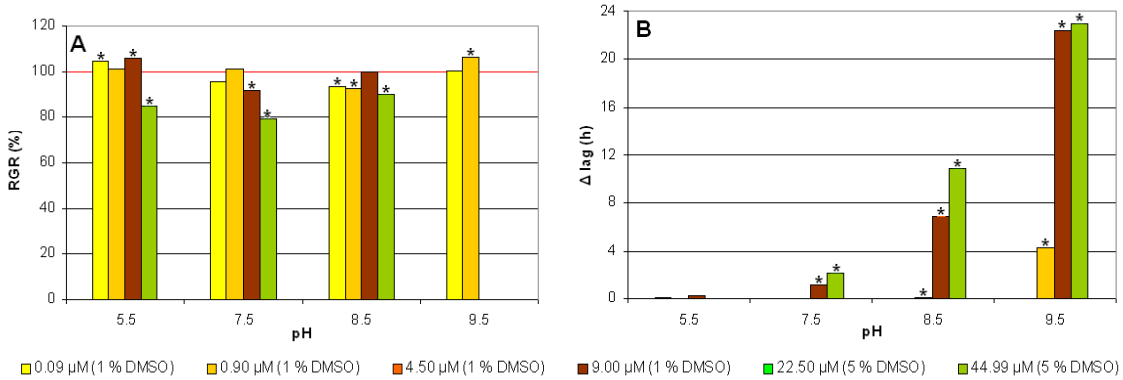

Figure A-32. Relative growth rate – RGR **(A)** and difference between the length of the lag phase of the growth control and the growth with valinomycin – Δlag **(B)** of *B. cereus* ATCC 14579 exposed to a range of valinomycin concentrations (0.09 to 44.99 µM) and 1 or 5 % of DMSO, in BHI broth at different pHs, at 30°C, with aeration. Red line (RGR = 100 %) indicates no inhibition. Statistical significant difference between growth control and growth test (*), $p<0.05$.

Appendix 5. Results of the survival & recovery assay

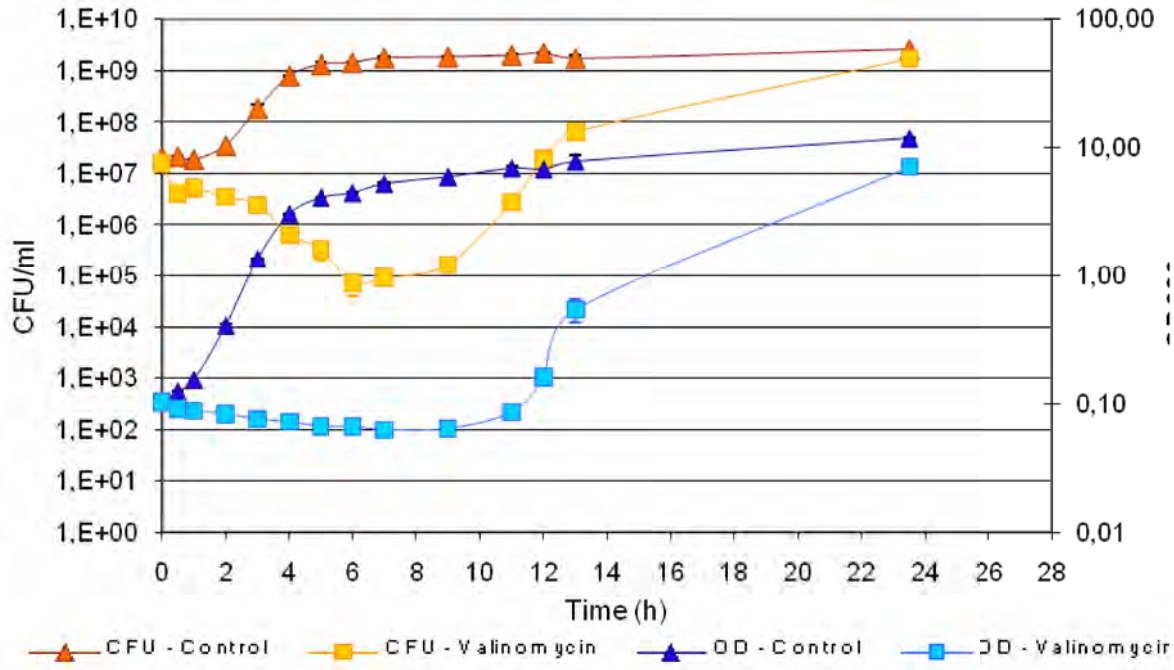

Figure A-33. Viable counts of *B. cereus* ATCC 14579 cultures exposed to valinomycin (9 µM) with 1% DMSO or 1% DMSO in BHI (pH 8.5) and respective OD600nm.

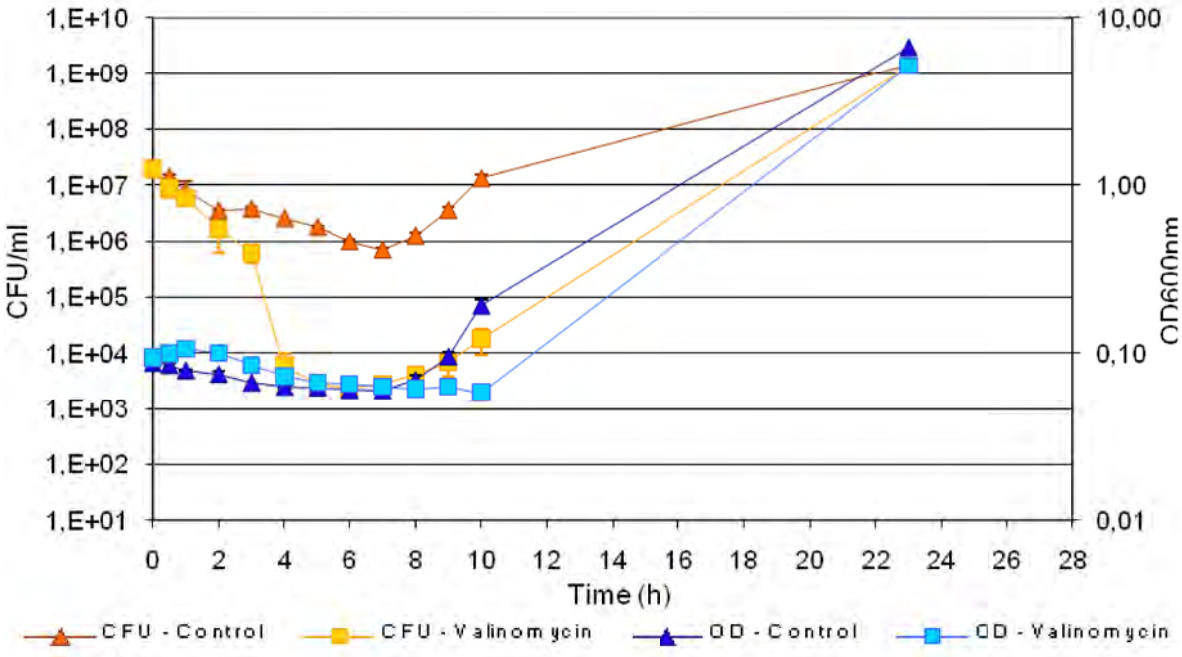

Figure A-34. Viable counts of *B. cereus* F4810/72 cultures exposed to valinomycin (9 µM) with 1% DMSO or 1% DMSO in BHI (pH 9.5) and respective OD600nm.

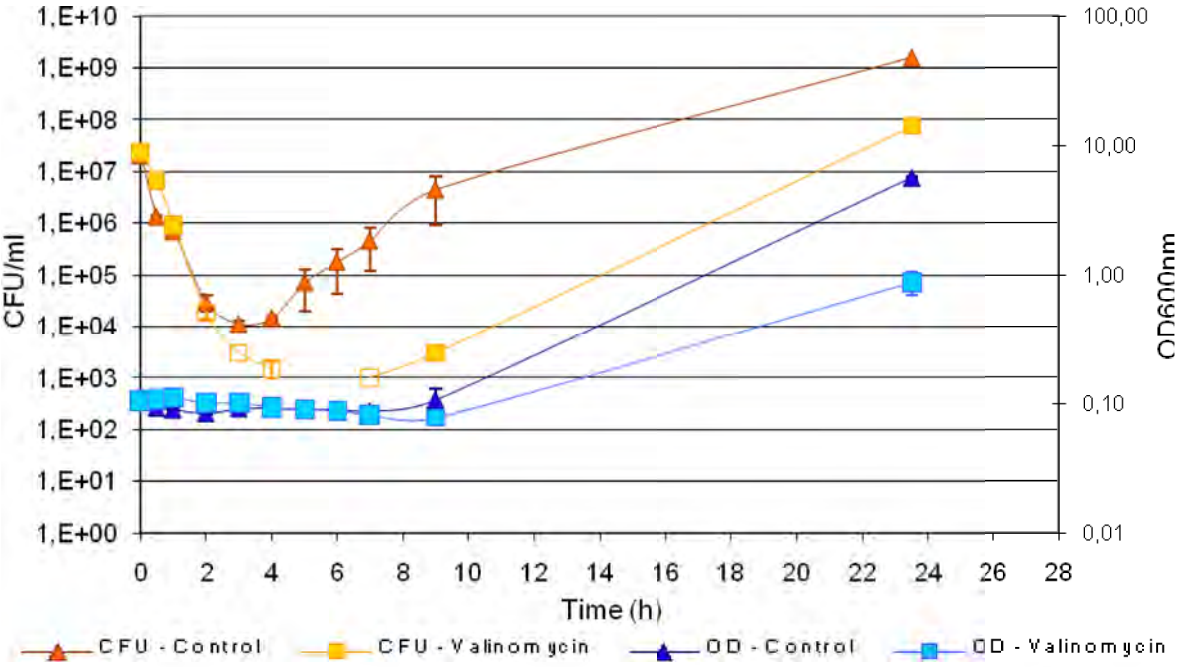

Figure A-35. Viable counts of *B. cereus* ATCC 14579 cultures exposed to valinomycin (9 µM) with 1% DMSO or 1% DMSO in BHI (pH 9.5) and respective OD600nm.

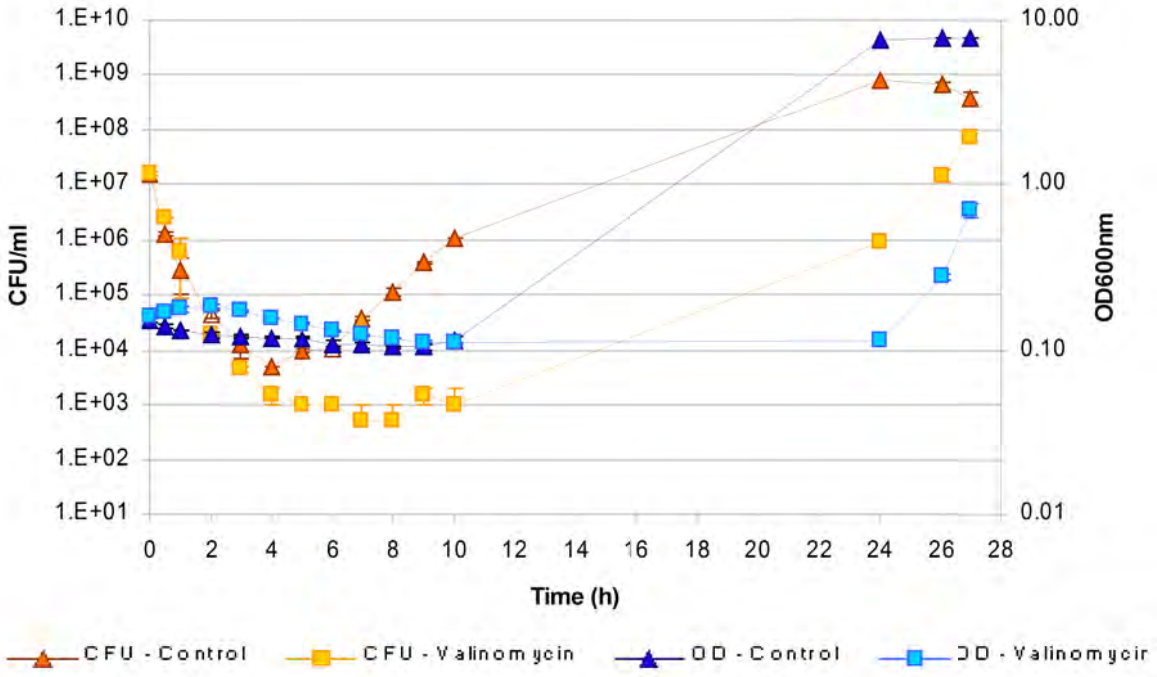

Figure A-36. Viable counts of B. cereus ATCC 10987 cultures exposed to valinomycin (9 µM) with 1% DMSO or 1% DMSO in BHI (pH 9.5) and respective OD600nm.

Appendix 6. Results of the drop dilution assay (valinomycin)

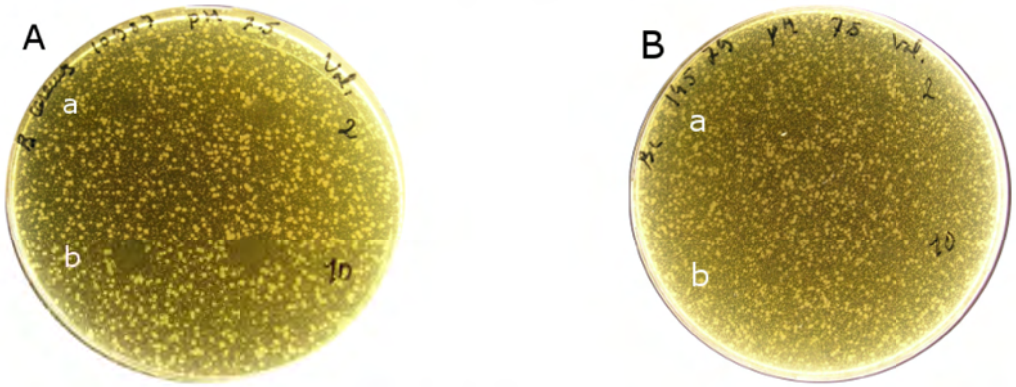

Figure A-37. Inhibition zones caused by 179.97 µM **(a)** and 899.83 µM **(b)** of valinomycin in cultures of *B. cereus* ATCC 10987 **(A)** and *B. cereus* ATCC 14579 **(B)** in BHI agar (pH 7.5), after 14 h of growth at 30°C.

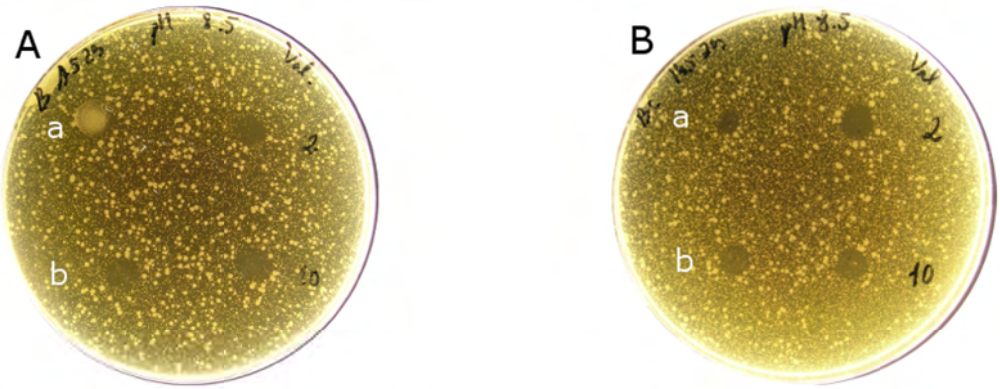

Figure A-38. Inhibition zones caused by 179.97 µM (a) and 899.83 µM (b) of valinomycin in cultures of B. cereus A529 **(A)** and B. cereus ATCC 14579 **(B)** in BHI agar (pH 8.5), after 14 h of growth at 30°C.

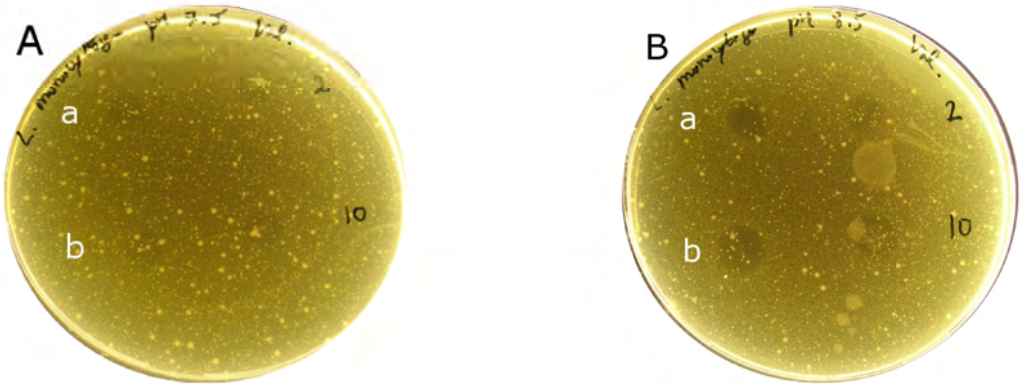

Figure A-39. Inhibition zones caused by 179.97 µM (a) and 899.83 µM (b) of valinomycin in cultures of L. monocytogenes in BHI agar at pH 7.5 **(A)** and pH 8.5 **(B)**, after 14 h of growth at 30°C.

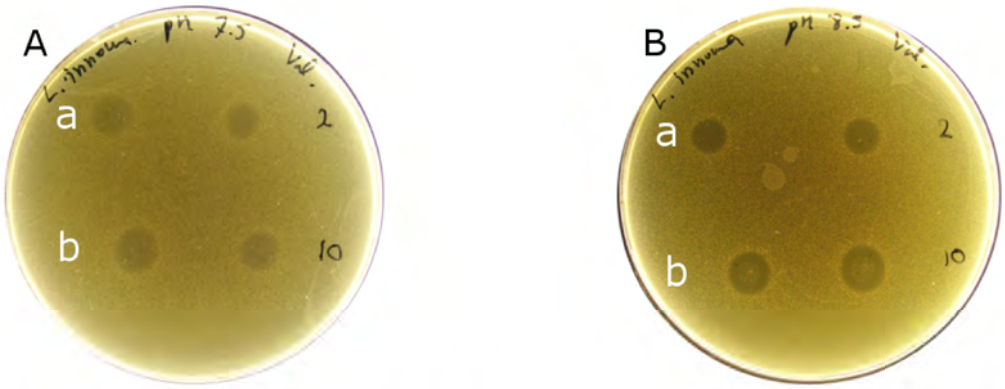

Figure A-40. Inhibition zones caused by 179.97 µM **(a)** and 899.83 µM **(b)** of valinomycin in cultures of *L. innocua* in BHI agar at pH 7.5 **(A)** and pH 8.5 **(B)**, after 14 h of growth at 30°C.

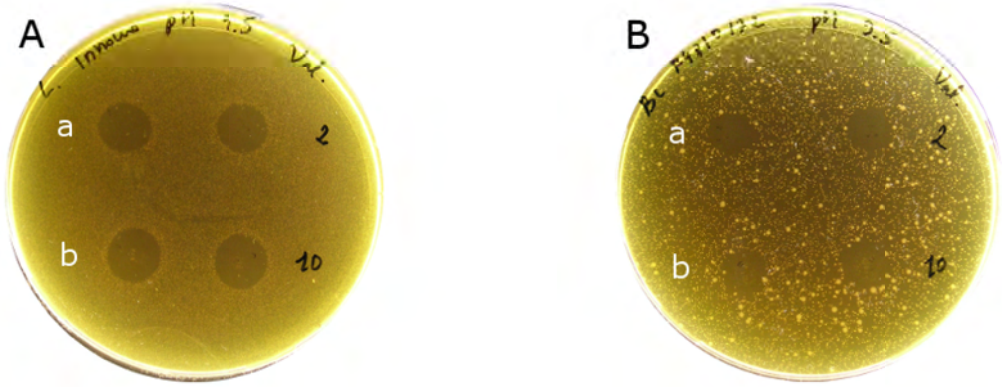

Figure A-41. Inhibition zones caused by 179.97 µM **(a)** and 899.83 µM **(b)** of valinomycin in cultures of *L. innocua* **(A)** and *B. cereus* F4810/72 **(B)** in BHI agar pH 9.5 after 14 h of growth at 30°C.