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Dipicolinic Acid Release and the Germination of *Alicyclobacillus acidoterrestris* Spores under Nutrient Germinants

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Abstract

The presence of *Alicyclobacillus*, a thermoacidophilic and spore-forming bacterium, in acidic fruit juices poses a serious problem for the processing industry. A typical sign of spoilage in contaminated juices is a characteristic phenolic off-flavour associated with the production of guaiacol. Spores are formed in response to starvation and in a natural environment re-access the nutrients, *e.g.*: L-alanine and AGFK – a mixture of asparagine, glucose, fructose and potassium, triggers germination. The aim of this study was to estimate the impact of L-alanine and AGFK on the germination of the spores of two *Alicyclobacillus acidoterrestris* strains and to evaluate the relationship of the germination rate with dipicolinic acid (DPA) release. The spores were suspended in apple juice or in buffers at pH 4 and pH 7, followed by the addition of L-alanine and AGFK. Suspensions were or were not subjected, to a temperature of 80°C/10 min and incubated for various periods of time at 45°C. Optical density (OD_{660}) was used to estimate the number of germinated spores. The amount of DPA released was determined using HPLC. The results indicate that the degree of germination of *A. acidoterrestris* spores depended on the strain and time of incubation and the nutritious compounds used. The data obtained show that the amount of DPA released correlated to the number of *A. acidoterrestris* spores germinated.

Key words: *Alicyclobacillus acidoterrestris*, AGFK mixture, dipicolinic acid, L-alanine, spore germination

Introduction

Alicyclobacillus acidoterrestris, a gram-positive, thermoacidophilic, spore forming bacterium, is a frequent contaminant of juices and is a common spoilage microorganism in the processing industry. Due to its ability to undergo sporulation in an acidic environment, and to spoil juices by producing undesirable off-flavours (Sokołowska, 2014; Tianli *et al.*, 2014), comprehensive knowledge about the germination of *A. acidoterrestris* spores is of general interest. The germination of *A. acidoterrestris* spores, and their subsequent outgrowth or inactivation, can be induced by external factors such as high hydrostatic pressure (Wuytack *et al.*, 2000; Vercammen *et al.*, 2012; Sokołowska *et al.*, 2013; 2015; Porębska *et al.*, 2015a) or supercritical carbon dioxide (Bae *et al.*, 2009; Porębska *et al.*, 2016). The data indicate that within *Bacillus* species, the germination of spores can also be induced by various nutrients: amino acids, purine nucleosides, sugars (Lovdal *et al.*, 2012), L-alanine (Parades-Sabja *et al.*, 2011; Kuwana and Takamatsu, 2013; Cruz-Mora *et al.*, 2015), ions and combinations of these, and a mixture of asparagine, glucose, fructose and potassium ions (AGFK) (Gosh *et al.*, 2012; Stewart *et al.*, 2012).

The mechanism of spore germination is very complex and has been the subject of many studies (Setlow *et al.*, 2008; Parades-Sabja *et al.*, 2011; Luu and Setlow, 2014; Bevilacqua *et al.*, 2015; Luu *et al.*, 20 15; Troiano *et al.*, 2015). Nutrients initiate spore germination by binding to the germination receptors (GRs), located in the spore's inner membrane which rapidly degrades the cortex peptidoglycan. Water is taken up, calcium dipicolinate (Ca-DPA) is released and a variety of spore constituents are degraded by hydrolytic enzymes. Although proteins which are likely candidates for DPA channels in the spore membranes have been identified, as yet there is no understanding of how the *Ger* receptors interacti on triggers the earliest events in spore germination, including the release of DPA and cations (Setlow *et al.*, 2006).

Each *Ger* receptor can detect a specific germinant, including amino acids, nucleosides, sugars and cations.

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Thus, expressing multiple *Ger* receptor operons allows spores to respond to structurally diverse compounds. Different *Ger* receptors can cooperate to recognize a single germinant or multiple germinants (Moir *et al*., 2002; Moir, 2006; Ross and Abel-Santos, 2010; Mongkolthanaruk *et al*., 2013).

The mechanism whereby individual GRs can cooperate to recognize a single germinant or multiple germinants to allow or accelerate germination is not known. However, it has been suggested that either various individual GRs form complexes in the spore's inner membrane or there is a mechanism which integrates signals from various individual GRs or GR complexes and that this integration determines the ultimate rate of germination (Yi *et al*., 2011). For example L-alanine and AGFK mixture as the nutrient germinants in the spore germination process may be involved in integrating signals from different GRs (Parades-Sabja *et al*., 2011; Wang *et al*., 2015). During spore germination, Ca-DPA release is preceded by the loss of resistance to heat, the release of Na^+, K^+, H^+, and Zn^{2+}, and a significant change in the elastic light-scattering intensity from individual spores (Luu and Setlow, 2014).

In the case of *Bacillus subtilis*, the GRs are encoded by homologous tricistronic *GerA*, *GerB* and *GerK* operons. Each of these GRs contains A, B and C sub-units, all of which are required for the function of the individual GR. The *GerA* receptor responds to either L-alanine or valine, while the *GerB* and *GerK* receptors together are essential for germination in a AGFK mixture (Yi *et al*., 2011; Stewart *et al*., 2012; Chen *et al*., 2014).

In general, the model for bacterial spore germination for *Bacillus* and *Clostridium* is known, and is associated with the expression of *Ger* proteins. The *Ger* family germination protein can also be found in *Alicyclobacillus* [http://www.ncbi.nlm.nih.gov/protein/YP_003184683.1?report=genpept].

L-alanine is a common germinant for both *Bacillus* and *Clostridia* species. L-alanine-mediated germination has been characterized mostly in *B. subtilis* spores (McCann *et al*., 1996; Ramirez-Peralta *et al*., 2012). However, L-alanine can also induce the germination of *B. cereus* (Barlass *et al*., 2002), *B. anthracis* (Fisher and Hanna, 2005), *B. megaterium* (Christie and Lowel, 2007), *B. lichenoformis* (Lovdal *et al*., 2012), *C. botulinum* (Broussolle *et al*., 2002), *C. sporogenes* (Broussolle *et al*., 2002), *C. perfringes* (Parades-Sabja *et al*., 2008) and *C. sordellii* (Ramirez and Abel-Santos, 2010). In fact L-alanine seems to be the most ubiquitous germinant for bacterial spores. There are data indicating that high hydrostatic pressures of 200 MPa – 400 MPa also trigger germination through the *GerA*, *GerB* and *GerK* receptors (Parades-Sabja *et al*., 2011).

To understand the molecular determinants of *Ger* receptor interactions and their effect on germinant recognition, the kinetic method can be used to study bacterial spore germination (Abel-Santos and Dodatko, 2007; Akoachere *et al*., 2007; Ramirez and Abel-Santos, 2010; Zhang J. *et al*., 2011; Zhang P. *et al*., 2010; 2014; Brunt *et al*., 2014). Spore germination can be analysed by optical density decreases (Terano *et al*., 2005; Akoachere *et al*., 2007; Brunt *et al*., 2014; Nagler *et al*., 2015; Porębska *et al*., 2015a).

The aim of this study was to characterize the process of spore germination in two *A. acidoterrestris* strains, initiated by L-alanine and AGFK, and to evaluate the relationship between DPA release and the germination of *A. acidoterrestris* spores induced by these biochemical substances. This study is an attempt to expand the current state of knowledge concerning the mechanism of the *A. acidoterrestris* spore germination process, the variations in spore population and the factors stimulating this process.

Experimental

Materials and Methods

Tested organisms. The *A. acidoterrestris* strains TO-169/06 and TO-117/02 used in this study were isolated from Polish concentrated apple juice, using the International Federation of Fruit Juice Producers' method (2004/2007). These strains were selected from among eight wild strains tested previously (Skąpska *et al*., 2012; Porębska *et al*., 2015a; 2015b; 2016). TO-117/02 was the strain highly resistant to temperature and HHP and TO-169/06 was the sensitive one.

Spore production. Spores were produced based on a method described by Sokołowska *et al.* (2012). Just before the experiments, the spores (> 95% phase bright – ungerminated) were suspended in apple juice (11.2 Bx, pH 3.4) or in a McIlvain buffer solution of pH 4.0 and pH 7.0. L-alanine (50 mM) or AGFK (50 mM) were then added to the samples, which were afterwards subjected, or not subjected, to a temperature of 80°C/10 min and incubated at 45°C (Bevilacqua *et al*., 2014). The number of spores in the suspensions was approximately 6 log cfu/ml for determining spore germination using the drop in optical density, and approximately 9 log cfu/ml for determining the release of dipicolinic acid.

Optical density measurement. The optical density (OD_{660}) of the spore suspensions was measured at a wavelength of 660 nm in a UV/Visible spectrophotometer Ultrospec 2000 (Pharmacia Biotech Ltd., England). To estimate the effect of the process parameters on the dynamics of spore germination, the drop in optical density was expressed as the ratio (OD_{660} during germination/OD_{660} before germination) × 100% (Terano *et al*., 2005; Kato *et al*., 2009; Pandey *et al*., 2013;

Bevilacqua *et al*., 2014; Porębska *et al*., 2015a). The optical density was measured 10 min after the addition of L-alanine or AGFK, after incubation for 30 min, for 4 h, and after overnight incubation of the same samples at 45°C (optimum growth temperature).

Determining the release of dipicolinic acid. Quantification of the DPA concentration in the samples 10 min after the addition of nutrient germinants was performed using the HPLC method (Warth, 1979). A Waters 2695 Separations Module with Waters 2996 Photodiode Array Detector system and SunFire C8 Column, (5 µm, 4.6 mm × 250 mm) with SunFire C8 Guard Pre-column, (5 µm, 4.6 mm × 20 mm) were used.

Samples containing 50 to 1000 µM DPA (0.1 to 2 mg of spores per ml) in 0.2 M potassium phosphate, pH 1.75, were prepared and centrifuged at 17000 × *g* for 10 min at 4°C and filtered through a 0.2-µm membrane filter. All the samples were stored at −70°C prior to the HPLC analysis. Ten µl samples were injected onto the column at approximately 12-min intervals. Elution was carried out with 1.5% tertamylalcohol in 0.2 M potassium phosphate, pH 1.75, at a flow rate of 1.0 ml/min at 25°C. The eluent was filtered and degassed. The stock solution of pH 1.75 buffer contained 3 M H_3PO_4 and 1 M KH_2PO_4. The peak heights at 271 nm were measured at 0.02 or 0.20 absorbance units, full scale.

To determine the total amount of DPA in the spore suspensions, 3 ml of each individual batch was sterilized at 121°C for 20 min and then analysed (Reineke *et al*., 2013a).

Data analysis. Analysis of variance and Duncan's multiple-range test, using StatSoft® Statistica 7.1, was used to test the significance of the differences ($p < 0.05$) between the drop in optical density. The assays were performed using two independent samples. Microsoft Office Excel 2010 was used for linear regression and to calculate the coefficient of determination (R^2) and coefficient of correlation (r).

Results and Discussion

According to the literature, optical density measurement allows a rapid assessment of spore germination (Terano *el al*., 2005; Porębska *et al*., 2015a). When spores begin to germinate, optical density decreases, and begins to rise again when the outgrowth stage begins. Previously, Porębska *et al.* (2015a) showed that 1 log cfu/ml germination of *A. acidoterrestris* spores under high hydrostatic pressure resulted in a 4.5% decrease in optical density ($R^2 = 0.84$). The subsequent rapid release of Ca-DPA under external factors is accompanied by the activation of the spores CLEs which degrade the cortex and cause a loss in the optical density of the spore (Reineke *et al*., 2013b). The germination of *A. acidoterrestris* spores induced by nutrient germinants was assessed by measuring the optical density and monitoring DPA release. Porębska *et al.* (2015b) showed that 2 log cfu/ml germination of *A. acidoterrestris* spores under high hydrostatic pressure resulted in 8.7 µM DPA being released from the spore suspension ($R^2 = 0.89$). Fig. 1 shows the dynamics of the germination of *A. acidoterrestris* 169/06 spores in apple juice with nutrient germinants – L-alanine and AGFK.

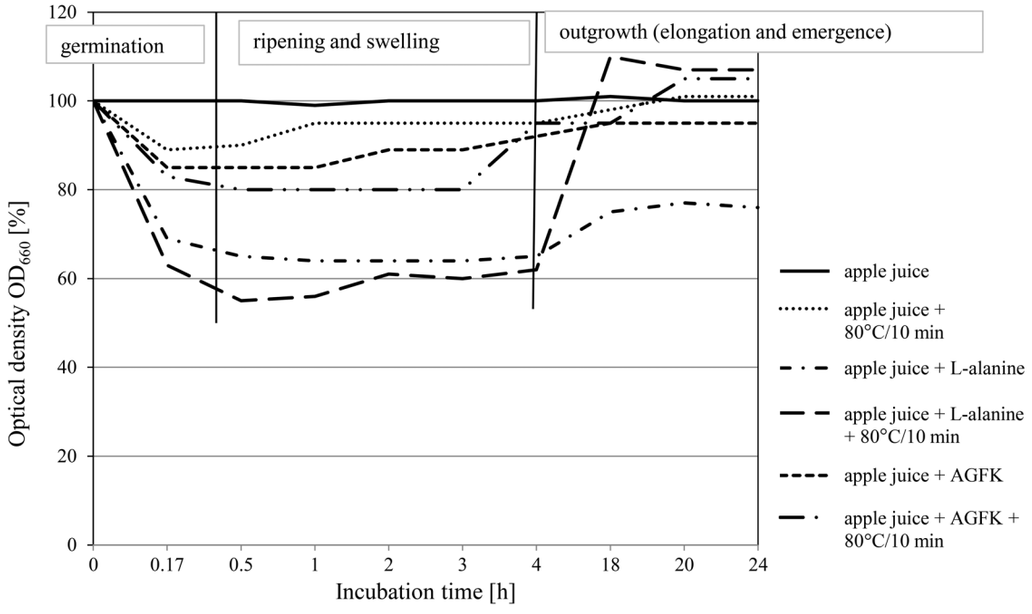

Fig. 1. Dynamics of the germination of *A. acidoterrestris* 169/06 spores in apple juice with nutrient germinants.

Germination occurred and there was also a significant drop (of 15%) in optical density after 10 min incubation at 45°C in the samples treated with AGFK. A higher (17%) drop in optical density was observed when the samples were additionally subjected to a temperature of 80°C/10 min and incubated at 45°C. The samples with L-alanine indicated a 31% drop in optical density. The best results (a 37% decrease in optical density) were observed when the samples of apple juice with L-alanine were additionally subjected to a temperature of 80°C/10 min and incubated at 45°C. It was expected that thermal treatment would determine a reduction in absorbance, as it is known that slight heat shocks can additionally activate spores (Byun *et al*., 2011). Similar results were obtained by Bevilacqua *et al.* (2014), who reported promoting effect of L-alanine on spore germination. During incubation at 45°C for a further 4 hours, no change in optical density was observed. After further incubation overnight, in favourable conditions, a significant increase in the optical density of apple juice with germinants and heat treatment was achieved. The highest increase was noticed in apple juice with L-alanine. This indicates that L-alanine and heat treatment promoted spore germination. The germinated spores transformed into vegetative cells and a subsequent growth of *A. acidoterrestris* in apple juice was observed (Fig. 1).

The specificity of the nutrient germinants is strain and species specific and probably reflects the adaptation of spore formers to their specific environmental niches. Some pathogenic spore formers require specific nutrient germinants. In models for bacterial spore germination the release of DPA through a DPA channel, presumably composed at least partly of SpoVA proteins, which leads to the activation of CwIJ, whereas changes in the cortex strain might activate SleB. These two redundant CLEs (cortex lytic enzymes) degrade the PG cortex, allowing the completion of germination and initiation of spore outgrowth (Parades-Sabja *et al*., 2011). In our study, to complete the germination of the spores and return them to a vegetative form, incubation with nutrient germinants and treatment with heat shock was necessary. Similar results were observed by Terano *et al.* (2005) and Brunt *et al.* (2014). They found that germination was initiated following the recognition of small molecules (germinants) by GRs located in the spore's inner membrane, and that the addition of L-alanine initiated spore germination but had no effect on the rate or overall germination process in the case of *Clostridium* sp.

Germination as a decrease in the optical density of a suspension of *A. acidoterrestris* 169/06 spores in apple juice and buffers with different pH after 10 min incubation at 45°C is presented in Figure 2. The highest germination and highest DPA release was observed in apple juice and buffer pH 4 with L-alanine and after heat treatment. In buffer pH 7, germination was suppressed. This could be associated with the acidophilic nature of these bacteria. The results indicate that the nutrients present in apple juice can promote the germination of *A. acidoterrestris* spores in the presence of L-alanine. These results confirmed that L-alanine induces spore germination (Barlass *et al*., 2002).

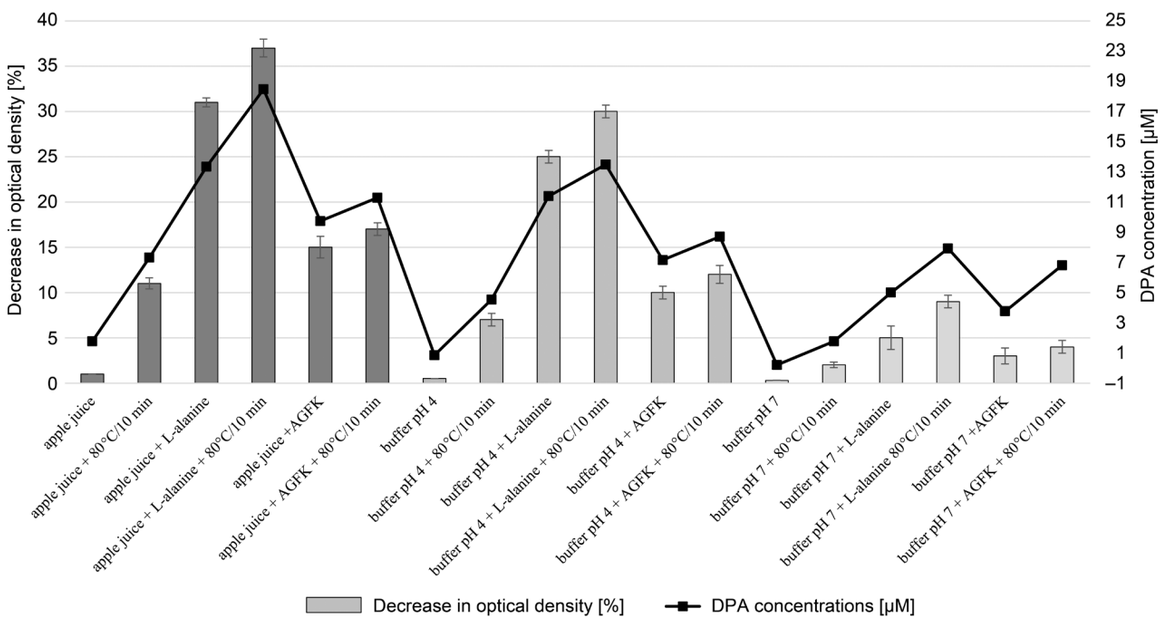

Fig. 2. Germination as a decrease in optical density and DPA released from *A. acidoterrestris* 169/06 spores in buffers at low and neutral pH and in commercial apple juice.

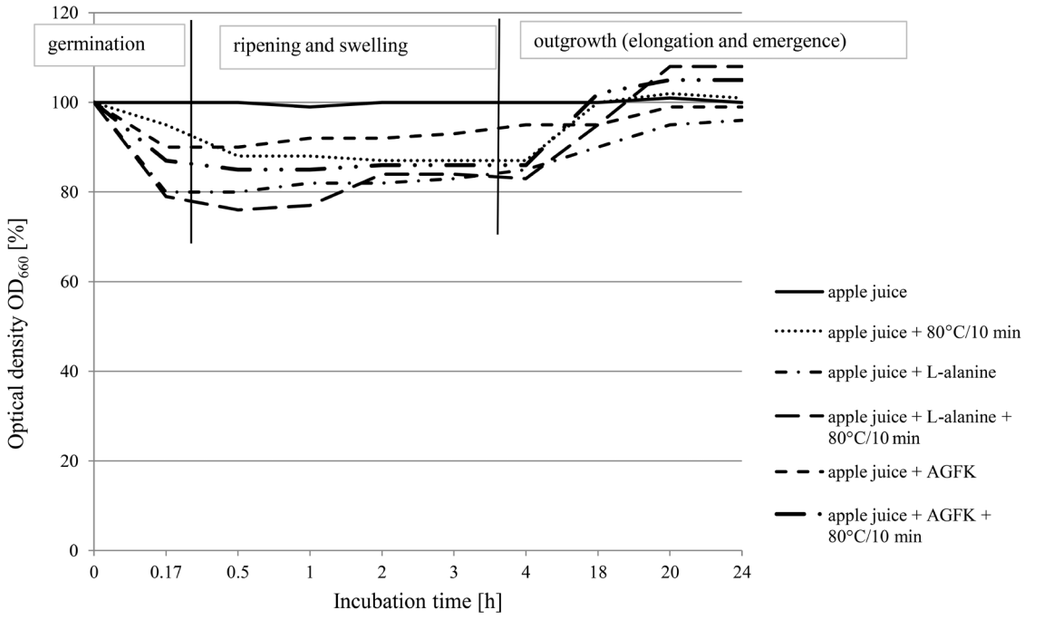

Fig. 3. Dynamics of the germination of *A. acidoterrestris* 117/02 spores in apple juice with nutrient germinants.

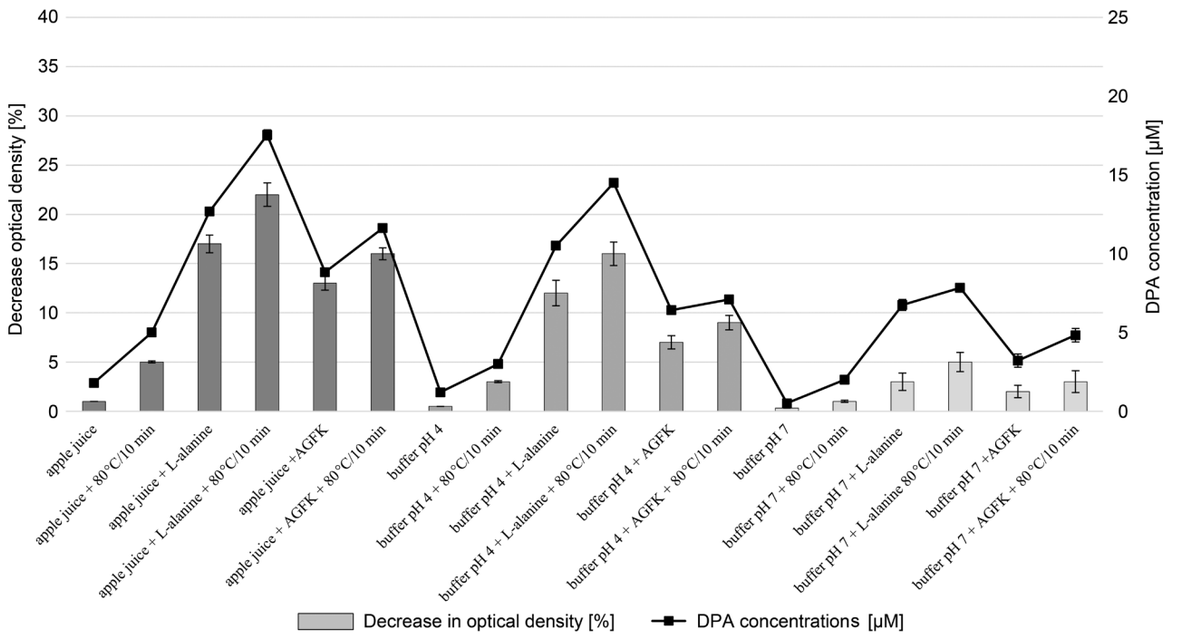

Fig. 4. Germination as a decrease in optical density and DPA released from *A. acidoterrestris* 117/02 spores in buffers at low and neutral pH and in commercial apple juice.

Some aspects of *A. acidoterrestris* spore germination triggered by nutrients and heat treatment were investigated by Terano *et al.* (2005). *A. acidoterrestris* spores showed efficient germination after heat activation in potato dextrose medium (pH 4) and commercial fruit juices. The same treatment was lethal for germinated spores. Germination was weaker in buffer pH 4, but increased to levels comparable to growth in apple juice in the presence of L-alanine. In phosphate buffer pH 7, germination was suppressed.

The results obtained under the same conditions for spores of the second *A. acidoterrestris* TO-117/02 strain, showed the same trend, but at lower values, indicating a weaker germination process (Fig. 3–4).

The germination of *A. acidoterrestris* 117/02 spores in apple juice and buffers of different pH is presented in

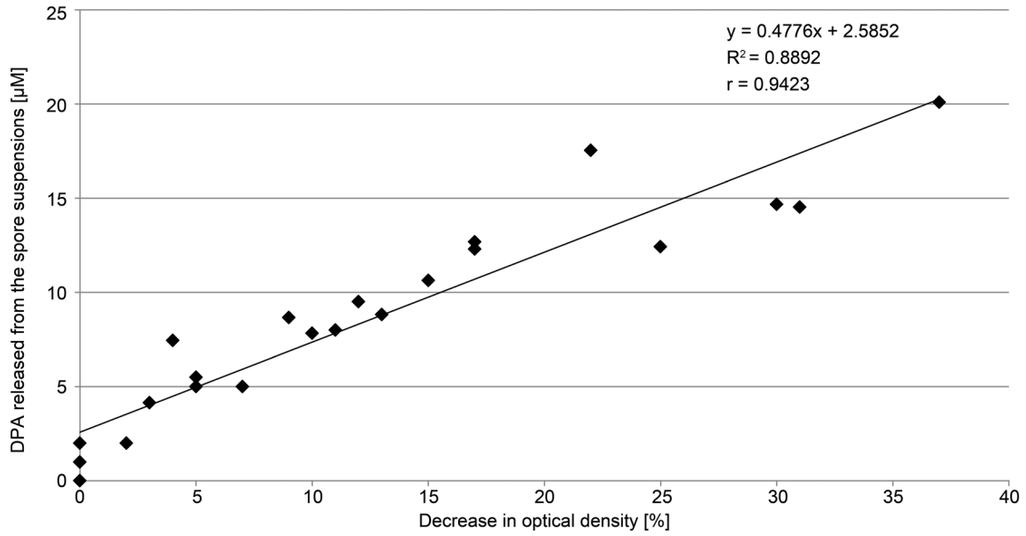

Fig. 5. DPA released from the spore suspensions *vs* decrease in optical density as a measure of the number of germinated *A. acidoterrestris* spores.

Figure 4. The results indicate that the nutrients present in apple juice can promote the germination of *A. acidoterrestris* spores with the addition L-alanine. In many cases, nutrients such as L-alanine are those found in environments favoured by growing bacteria derived from spores. Nutrients trigger spore germination *via* their interaction with germinant receptors, proteins that recognize and respond to specific nutrients such as sugar, amino-acids and cations. Spores of the bacteria germinate better with co-germinants such as, in the case of our study, glucose from apple juice with L-alanine and AGFK or with glycine (Parades-Sabja *et al*., 2011) and lysozyme (Bevilacqua *et al*., 2014).

In the second part of our study, we focused on examining the process of DPA release. An early event in spore germination is DPA release, a process that likely requires proteins in the inner spore membrane and prior to DPA release, there is also a release of monovalent ions (Cabrera-Martinez *et al*., 2003).

The data presented in Fig. 2 and 4, showing the dynamics of DPA release, derived from processes conducted under the same conditions as the experiments in which the germination phenomenon was investigated. DPA concentrations were measured after 10 min incubation with a nutrient germinant.

The total amount of DPA present in *A. acidoterrestris* TO-169/06 spores (released during sterilization) was 50.3 µM, and 42.7 µM for the TO-117/02 strain, respectively (data not showed). The highest amount of released DPA was achieved during the incubation of spores in apple juice with L-alanine and after additional heat treatment and was 20.1 µM for TO-169/06 spores (40% of the total DPA) (Fig. 2) and 17.5 µM for TO-117/02 spores (41% of the total DPA) (Fig. 4).

The relationship between DPA release after incubation with nutrient-induced germination of *A. acidoterrestris* spores is presented in Figure 5. A good correlation ($R^2 = 0.8892$, $r = 0.9423$) between these variables was observed. A similar phenomenon was observed by Porębska *et al*. (2015b), who claimed that the amount of DPA released correlated to the amount of germinated *A. acidoterrestris* spores treated with high hydrostatic pressure. Setlow *et al*. (2003) claimed that dodecylamine may be correlated with DPA concentrations and trigger spore germination by directly or indirectly activating the release of DPA from the spore core, through the opening of channels for DPA in the spore's inner membrane.

Strains of bacteria possess an efficient germination machinery for L-alanine and AGFK germination. In our study we observed that AGFK had a less impact on germination of *A. acidoterrestris* spores than L-alanine. AGFK is often less efficient, because the GerB genes are more diverged, and the two germinant receptor operons of unknown function could have been lost from the genome in these strains (Cabera-Martinez *et al*., 2003).

The *A. acidoterrestris* strains have conserved the *GerA* receptor function, confirming its importance, at least in the natural environments of these strains. It also seems likely that *Ger* proteins have a direct effect on the release of DPA that takes places early in germination and during germination induced by nutrient germinants. This step of germination is crucial with regard to loss of resistance and it is therefore of great interest for a variety of food preservation techniques and further research.

Until now, no studies have been reported on the DPA release of *A. acidoterrestris* spores in L-alanine and AGFK.

Conclusions

The presence of L-alanine and AGFK in the environment stimulated spore germination. The results indicate that the degree of germination of *A. acidoterrestris* spores depended on the strain and medium. Optical density is a fast and effective method for estimating the dynamics of spore germination. The results showed a greater decrease in optical density after incubation with L-alanine than with AGFK, and a subsequent increase of this parameter after overnight incubation at 45°C. It is worth noting that heat treatment additionally stimulated the increase in the rate of germination of spores. This indicates the start of the next phase, outgrowth. These results may show that the strains of *A. acidoterrestris* tested are dominated by the presence of *GerA* receptors. A slight decrease in the germination of spores was observed with an increase in the pH, while a lower pH was conducive to germination. The nutrients in apple juice also can stimulate *A. acidoterrestris* spores to germinate. The process of DPA release from the spores depended on the strain and biochemical substances. The amount of DPA released correlated to the amount of *A. acidoterrestris* spores germinated.

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