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Short communication

Effects of pomegranate and pomegranate–apple blend juices on the growth characteristics of *Alicyclobacillus acidoterrestris* DSM 3922 type strain vegetative cells and spores

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ABSTRACT

The present study examined the growth characteristics of *Alicyclobacillus acidoterrestris* DSM 3922 vegetative cells and spores after inoculation into apple, pomegranate and pomegranate–apple blend juices (10, 20, 40 and 80%, v/v). Also, the effect of sporulation medium was tested using mineral [*Bacillus acidoterrestris* agar (BATA) and *Bacillus acidocaldarius* agar (BAA)] and non-mineral containing media [potato dextrose agar (PDA) and malt extract agar (MEA)]. The juice samples were inoculated separately with approximately 10^5 CFU/mL cells or spores from different sporulation media and then incubated at 37 °C for 336 h. The number of cells decreased significantly with increasing pomegranate juice concentration in the blend juices and storage time ($p < 0.001$). Based on the results, 3.17, 3.53, and 3.72 log cell reductions were observed in 40%, 80% blend and pomegranate juices, respectively while the cell counts attained approximately 7.17 log CFU/mL in apple juice after 336 h. On the other hand, the cell growth was inhibited for a certain time, and then the numbers started to increase after 72 and 144 h in 10% and 20% blend juices, respectively. After 336 h, total population among spores produced on PDA, BATA, BAA and MEA indicated 1.49, 1.65, 1.67, and 1.28 log reductions in pomegranate juice; and 1.51, 1.38, 1.40 and 1.16 log reductions in 80% blend juice, respectively. The inhibitory effects of 10%, 20% and 40% blend juices varied depending on the sporulation media used. The results obtained in this study suggested that pomegranate and pomegranate–apple blend juices could inhibit the growth of *A. acidoterrestris* DSM 3922 vegetative cells and spores.

1. Introduction

Fruit juices are traditionally pasteurized for the inactivation of spoilage and pathogen organisms. After pasteurization, products are stored at refrigerated or ambient temperatures (Smit et al., 2011). However, thermal resistance studies indicated the ability of *Alicyclobacillus acidoterrestris* to survive pasteurization applied to fruit juices and acidic products. Due to acidophilic nature, their endospores can germinate and increase in products to cell counts high enough to produce taint compounds leading to spoilage (Gocmen et al., 2005; Orr et al., 2000; Pettipher et al., 1997; Vieira et al., 2002; Wisotzkey et al., 1992).

Taint compounds responsible for off-flavor are guaiacol, 2,6-dibromophenol, and 2-methoxyphenol. Among these compounds, guaiacol is the major compound related to taint production (Witthuhn et al., 2012). It is formed from ferulic acid via vanillin and can be detected by smell in fruit juices at 2 ppb (Bevilacqua et al., 2008; Chang and Kang, 2004). Detectable taint production in fruit juices is generally reported when the levels of *A. acidoterrestris* CFU/mL reach about 10^4-10^5 CFU/mL (Komitopoulou et al., 1999).

The growth characteristics of *A. acidoterrestris* strains depend on the juice type and the isolation source (Goto, 2007). Single strength juice is an ideal environment for spore germination and growth leading to spoilage under certain conditions (Walls and Chuyate, 2000). The growth has been detected in various spoiled commercial pasteurized fruit juices such as apple, orange, tomato, white grape, pear, and grapefruit but not observed in red grape juice, pineapple juice, Amazonian fruit Cupuaçu, salsa, and apple–cranberry blend juices (Silva and Gibbs, 2001; Splittstoesser et al., 1994; Tokuda, 2007; Wisse and Parish, 1998). The reason for growth and spoilage in specific juices is unclear, but these juices might contain different concentrations of guaiacol precursors or growth inhibitors (Walls and Chuyate, 2000). These inhibitory compounds might inhibit either spore germination or vegetative cell growth (Tokuda, 2007).

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Pomegranate (*Punica granatum* L.) is one of the important fruits grown in Turkey, Iran, USA, Middle East, Mediterranean and Arabic countries (Maskan, 2006). Pomegranate juice contains water, organic acids, sugars, pectin, tannins (punicalagin, ellagic acids and gallic acid), flavonoids (catechol, catechins, epigallocatechins) and anthocyanins (3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin) (Boggia et al., 2013; López-Rubira et al., 2005; Tanveer et al., 2014). Pomegranate juice has much more antioxidant activity than other fruit juices and beverages (Gil et al., 2000). Nowadays, interest in pomegranate juice and its products has increased significantly for health benefits and placed them in the first line of functional juice market (Vegara et al., 2013). Due to high acidity, astringency, bitterness and other factors found in some fruits and vegetables having high nutritional values, the production of processed products from these plants is limited. Therefore, blending of two or more fruit or vegetable juices for the production of ready-to-drink beverages may be used as alternative. Blending of fruit juices provides improved flavor, taste, and nutritive value, decreases the production cost and leads to new product development (Bhardwaj and Pandey, 2011).

In our previous work (Molva and Baysal, 2014), we found that the wet-heat resistance of *A. acidoterrestris* DSM 3922 spores varied depending on the sporulation medium used. Such variations need to be considered when evaluating the survival and inactivation of *Alicyclobacillus* spp. in fruit juices.

Therefore, the objective of this study was to determine the inhibitory effects of pomegranate juice and pomegranate–apple blend juices (10, 20, 40 and 80%, v/v) on the vegetative cells of *A. acidoterrestris* DSM 3922 and spores prepared from different sporulation media.

2. Material and methods

2.1. Bacterial strain and preparation of spore suspensions

A. acidoterrestris DSM 3922 type strain was kindly provided by Karl Poralla (Deutsche Sammlung von Mikroorganismen und Zellkulturen's collection, Braunschweig, Germany) and used as a test organism in this study. For sporulation, 100 μL of 4 h-grown culture at 43 °C in *Bacillus acidoterrestris* broth (BATB, Döhler, Germany) was spread onto *B. acidoterrestris* agar (BATA, Merck), *Bacillus acidocaldarius* agar (BAA) (Darland and Brock, 1971), potato dextrose agar (PDA, BD Difco) and malt extract agar (MEA, Oxoid). All inoculated plates were incubated at 43 °C for 10 days until 85–90% of cells sporulated as determined under phase-contrast microscope (Olympus CX31, Japan). Spore suspensions were prepared based on the protocol developed by other researchers (Murray et al., 2007) and stored at −20 °C for further use.

2.2. Juice samples

Concentrated apple juice (70.3 °Brix) and pomegranate juice (65 °Brix) were kindly provided by ASYA Fruit Juice and Food Ind. Inc. (Isparta, Turkey) and reconstituted to 11.30 and 14.26 °Brix as determined by a refractometer (Mettler Toledo, USA). For the preparation of blend juices, pomegranate juice was mixed with apple juice aseptically with the ratio of 80%, 40%, 20% and 10% (v/v). Diluted juice samples were tested for the presence of *Alicyclobacillus* spp. using membrane filtration method. Briefly, the membranes (0.45 μm pore size, Sartorius) that had been used to filter the reconstituted juice samples (50 mL) were transferred aseptically onto BATA. Then, the membranes on the plates were incubated at 43 °C for up to seven days (Molva and Baysal, 2014).

2.3. Total phenol content

The total phenol content (TPC) of the juice samples was determined according to Folin–Ciocalteu method as described previously (Al-Zoreky, 2009). A calibration curve was prepared using standard solutions of gallic acid (0–0.09 mg/mL) and the results were expressed in gallic acid equivalents (GAE) in mg/100 mL juice.

2.4. Antioxidant activity

Ferric reducing antioxidant power (FRAP) and free radical scavenging activity of the juice samples were determined according to the method described previously (Bi et al., 2013). The results of FRAP assay were expressed as Trolox equivalents (TE) using a calibration curve in the range of 0–0.1 mM. Free radical scavenging activity was determined using a stable 2,2-diphenyl-2-picrylhydrazyl radical (DPPH) and calculated as % inhibition of DPPH using the equation below (Katalinić et al., 2010).

$$(\%\ \text{Inhibition of DPPH}) = \left[\left(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}\right)/\text{OD}_{\text{control}}\right] \cdot 100.$$

2.5. Inhibitory effect on A. acidoterrestris vegetative cells

A. acidoterrestris cells were grown overnight on PDA at 43 °C. The colonies were suspended in 10 mL Maximum Recovery Diluent (MRD, Oxoid) to obtain a bacterial density of McFarland 1.0 (10^6 CFU/mL) by using a densitometer (Den-1, HVD Life Sciences, Austria). After centrifugation at 16,000 ×*g* for 5 min, the pellet was suspended in 10 mL apple; pomegranate and pomegranate–apple blend juices [80%, 40%, 20%, 10% (v/v)]. The initial cell population was determined for each juice sample. Next, the inoculated samples were incubated at 37 °C by shaking (Lab-Line MaxQ 4450 incubated shaker) at 120 rev/min. At regular time intervals, the samples were serially diluted in MRD; 10-fold dilutions were then spread onto the surface of PDA (pH 3.5). Finally, the plates were incubated at 43 °C for 48 h.

2.6. Inhibitory effect on A. acidoterrestris spores from different media

Since there is no standardized medium for the production of *A. acidoterrestris* spores (Molva and Baysal, 2014), different media such as PDA, BATA, BAA, and MEA were used for sporulation. Before inoculation into juice samples, each spore suspension (10^6 CFU/mL) was heat shocked at 80 °C/10 min and cooled on ice. After centrifugation at 16,000 ×*g* for 5 min, the pellet was suspended in 10 mL apple; pomegranate and pomegranate–apple blend juices [80%, 40%, 20%, 10% (v/v)]. Then, the initial spore population was determined. After that, the inoculated samples were incubated at 37 °C by shaking (Lab-Line MaxQ 4450 incubated shaker) at 120 rev/min. The total population (cells and spores) was evaluated at regular time intervals without heat shock treatments. The samples were serially diluted in MRD; then 10-fold dilutions were spread onto the surface of PDA (pH 3.5). Inoculated plates were incubated at 43 °C for 48 h.

2.7. Statistical analysis

Each experiment was performed in triplicate. The mean values and standard deviations were calculated by Excel (Microsoft Corp., USA). Data from three replicates were analyzed by one way analysis of variance (ANOVA) using Minitab 16.0 (Minitab Inc., UK). The Tukey–Kramer test was used to compare the means of treated groups. The confidence interval used to determine statistical significance was set at 95% ($p < 0.05$).

3. Results and discussion

3.1. Juice samples

The pH, °Brix, TPC, FRAP and DPPH-radical scavenging activities of juice samples used in this study are presented in Table 1.

Table 1
pH, °Brix, TPC, FRAP and DPPH-radical scavenging activities of juice samples.

Juice[a]	pH	°Brix	TPC mg GAE/100 mL	FRAP mmole TE/L	% inhibition of DPPH
PJ	3.36 (0.00)^{A}	14.26 (0.02)^{A}	400.48 ± 9.41^{A}	40.05 ± 1.05^{A}	80.41 ± 0.06^{B}
80% BJ	3.42 (0.00)^{B}	13.66 (0.05)^{B}	243.23 ± 3.40^{B}	35.31 ± 1.93^{B}	83.81 ± 0.94^{B}
40% BJ	3.60 (0.00)^{C}	12.55 (0.01)^{C}	157.78 ± 5.23^{C}	16.85 ± 1.15^{C}	88.79 ± 1.91^{A}
20% BJ	3.74 (0.00)^{D}	12.00 (0.03)^{D}	89.41 ± 2.54^{D}	8.21 ± 0.63^{D}	89.74 ± 1.55^{A}
10% BJ	3.82 (0.01)^{E}	11.63 (0.01)^{E}	67.21 ± 1.47^{E}	4.65 ± 0.07^{E}	83.91 ± 0.94^{B}
AJ	3.96 (0.00)^{F}	11.30 (0.10)^{F}	26.48 ± 1.13^{F}	0.93 ± 0.03^{F}	19.01 ± 1.16^{C}

All data were the means ± standard deviation from three experiments ($n = 3$). Values with different capital letters in the same column are significantly different ($p < 0.05$).
[a] PJ: pomegranate juice; BJ: blend juice; AJ: apple juice.

3.1.1. Total phenol content

Phenolic content of the compounds are important for their antimicrobial activity. Total phenol content of the apple juice was found to be 26.48 ± 1.13 mg GAE/100 mL juice (Table 1) and this finding was in good agreement with previously published data (Caminiti et al., 2012). Also, TPC of the pomegranate juice was 400.48 ± 9.41 mg GAE/100 mL juice (Table 1) which was quite high compared to the literature from the commercial juices in Turkey (Tezcan et al., 2009). Similar to apple juice, the differences between TPC are related to the fruit processing method. Especially, some extraction methods can involve rubbing the internal part of the rind, and may cause the extraction of the phenolic contents (Vázquez-Araújo et al., 2011). Also, the TPC of the blend juices ranged from 67.21 to 243.23 mg GAE/100 mL juice (Table 1). There was a strong correlation between the TPC and the concentration of pomegranate juice in the blend juice samples ($R^2 > 0.956$).

3.1.2. Antioxidant activity

Pomegranate juice displayed higher antioxidant capacity (40.05 mM TE) than apple juice (0.93 mM TE) according to FRAP method (Table 1) since commercial pomegranate juice obtained by pressing this fruit contains water soluble punicalagin which is a potent antioxidant in significant amounts (Patel et al., 2008). The FRAP values of blend juices were strongly correlated with the concentration of pomegranate juice in the apple juice ($R^2 > 0.993$) and ranged from 4.65 to 35.31 mM TE (Table 1). Based on the DPPH method, the apple and pomegranate juices were found to exhibit 19.01% and 80.41% inhibition, respectively (Table 1). And, % inhibition of pomegranate juice, 80% and 10% blend juices were not statistically different ($p > 0.05$). In addition, 20% and 40% blend juices yielded the highest inhibition with 89.74% and 88.79%, respectively.

3.2. Inhibitory effect on A. acidoterrestris vegetative cells

The average initial cell population was about 5.43, 5.49, 5.53, 5.55, 5.44 and 5.62 log CFU/mL for 10%, 20%, 40%, 80% blend, apple and pomegranate juices, respectively (Table 2). The pH (3.36–3.96) and °Brix values (11.30–14.26) of juice samples (Table 1) do not appear to be limiting on *A. acidoterrestris* DSM 3922, since its survival and growth at these values have been demonstrated (Tokuda, 2007). While *A. acidoterrestris* cells reached 7.27 log CFU/mL within 24 h incubation in the apple juice, the number of cells decreased significantly with increasing pomegranate juice concentration in the blend juices and storage time ($p < 0.001$). After 336 h, 3.17, 3.53 and 3.72 log CFU/mL reductions were observed in 40%, 80% blend juices and pomegranate juice, respectively (Table 2). On the other hand, the growth was inhibited for a certain time in 10% and 20% blend juices, and then the cell numbers started to increase after 72 and 144 h storage, respectively. The population attained approximately 7.17 log CFU/mL in apple juice at the end of the incubation while the cell numbers reached 7.12 and 7.00 log CFU/mL in 10% and 20% blend juices, respectively (Table 2).

The antimicrobial activity of pomegranate juice was reported to be due to tannins such as ellagitannins and other high molecular weight tannins (Al-Zoreky, 2009; Machado et al., 2003; Türkyılmaz et al., 2013). More specifically, tannins in pomegranate juice could exhibit antimicrobial activity in different mechanisms. First, they might form complexes with proteins found in cell walls decreasing both cell permeability and substrate transportation into the cells. Secondly, they might inhibit essential bacterial enzymes. Also, they might form stable complexes with metal ions such as Fe^{2+} and Cu^{2+}. Therefore, they decrease the availability of these ions for bacteria and affect the activity of metalloenzymes (Puupponen-Pimiä et al., 2005). In a recent study (Türkyılmaz et al., 2013), antimicrobial activity of pomegranate juice was tested against Gram-positive (*Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Lactobacillus plantarum*) and Gram-negative bacteria (*Pseudomonas* spp., *Escherichia coli*, *E. coli* O157:H7, *Salmonella* Enteritidis, *Enterobacter cloacae*, and *Citrobacter freundii*) and molds (*Aspergillus niger*, *Aspergillus* spp., *Penicillum* spp.). They found that pomegranate juice showed higher antimicrobial activity on Gram-positive than Gram-negative bacteria.

Structural deformations such as leakage of cellular constituents; perforations and blebs on the cell surface during growth in 40%, 80% pomegranate–apple blend and pomegranate juices were observed by scanning electron microscopy (SEM) at the end of the incubation period (data not shown). Since pomegranate juice has higher TPC than apple juice, the antimicrobial mechanism of the pomegranate juice on the cell structure is very likely to have been caused by the action of phenolics as observed in previously published data (Molva and Baysal, 2015).

Table 2
Counts of vegetative cells (log CFU/mL) in the juice samples during storage at 37 °C.

Storage (h)	PJ	80% BJ	40% BJ	20% BJ	10% BJ	AJ[a]
0	5.62(0.09)^{Aa}	5.55(0.07)^{Aa}	5.53(0.00)^{Aa}	5.49(0.05)^{Da}	5.43(0.18)^{Ca}	5.44(0.06)^{Ba}
24	4.42(0.19)^{Bb}	4.19(0.03)^{Bb}	4.51(0.12)^{Bb}	4.29(0.05)^{Eb}	4.53(0.09)^{Db}	7.27(0.67)^{Aa}
48	4.02(0.04)^{Cd}	3.89(0.04)^{Ce}	4.01(0.01)^{Cd}	4.15(0.03)^{Ec}	4.78(0.01)^{Db}	7.30(0.00)^{Aa}
72	3.87(0.21)^{Cb}	3.72(0.01)^{Db}	3.92(0.04)^{Cb}	4.09(0.18)^{Eb}	7.78(0.07)^{Aa}	7.59(0.12)^{Aa}
144	3.07(0.10)^{Dc}	2.99(0.08)^{Ec}	3.12(0.00)^{Dc}	6.59(0.08)^{Cb}	6.84(0.16)^{Bb}	7.17(0.08)^{Aa}
168	2.93(0.00)^{DEb}	2.91(0.00)^{Eb}	2.95(0.04)^{Eb}	7.56(0.08)^{Aa}	7.67(0.13)^{Aa}	7.54(0.13)^{Aa}
240	2.67(0.13)^{Eb}	2.71(0.02)^{Fb}	2.69(0.00)^{Fb}	7.14(0.09)^{Ba}	6.87(0.04)^{Ba}	6.95(0.24)^{Aa}
336	1.90(0.08)^{Fc}	2.02(0.09)^{Gbc}	2.36(0.01)^{Gb}	7.00(0.16)^{Ba}	7.12(0.06)^{Ba}	7.17(0.19)^{Aa}

Values with different capital letters in the same column are significantly different ($p < 0.05$). Values with different lowercase letters in the same row are significantly different ($p < 0.05$).
[a] The experiments were repeated three times, and data are expressed as mean ± standard deviation.

3.3. Inhibitory effect on A. acidoterrestris spores from different media

Spores prepared from BATA and BAA media did not germinate and were only slightly inactivated during incubation in pomegranate juice and blend juices. The spores produced on BATA were inoculated into pomegranate juice, 80%, 40%, 20% and 10% blend juices at initial levels of 5.65, 5.54, 5.43, 5.38 and 5.31 log CFU/mL and the levels were 3.99, 4.16, 4.20, 4.15, and 4.30 log CFU/mL, respectively at the end of the incubation. For spores produced on BAA, the initial levels were 5.48, 5.31, 5.42, 5.36 and 5.15 log CFU/mL in pomegranate juice, 80%, 40%, 20% and 10% blend juices and the levels were 3.81, 3.91, 4.12, 4.33 and 4.41 log CFU/mL, respectively after 336 h (Fig. 1). However, in apple juice only, both spores germinated after 24 h leading to an increase to approximately 10^7 CFU/mL by the end of incubation. On the other hand, the germination and inactivation of spores prepared on PDA and MEA differed depending on the type of juice sample analyzed. Similarly, spores prepared from MEA did not germinate and were slightly inactivated during incubation in pomegranate juice and blend juices ranging from 20% to 80%. The initial levels of spores from MEA were 5.38, 5.32, 5.40, and 5.45 log CFU/mL in pomegranate juice, 80%, 40%, and 20% blend juices and the levels were 4.10, 4.16, 4.20, and 4.78 log CFU/mL, respectively after 336 h (Fig. 1). However, in 10% blend juice and apple juice, spores from MEA media germinated after 96 h and 24 h, respectively leading to an increase to approximately 10^7 CFU/mL by the end of incubation. Spores obtained from PDA did not germinate and were inactivated during incubation in pomegranate juice and 80% blend juice. The inoculum levels were 5.59 and 5.55 log CFU/mL in pomegranate and 80% blend juices, respectively. After 336 h, the levels were 4.10 and 4.04 log CFU/mL, respectively. However, the germination of spores was observed in 40%, 20%, and 10% blend juices and apple juice after 240 h, 96 h, 48 h and 24 h, respectively attaining to approximately 10^7 CFU/mL after 336 h.

The presence of certain transition metals in the sporulation media is important for spore properties such as spore formation, heat resistance and dormancy (Kihm et al., 1988). It has been well documented that stabilization of Ca–DPA and the ability to strongly bind divalent cations contribute to the heat resistance (Yamazaki et al., 1997). In another study, heat resistances of *A. acidoterrestris* DSM 3922 spores obtained from mineral containing media (BATA and BAA) were found to be higher than those of spores from non-mineral containing media (PDA and MEA) (Molva and Baysal, 2014). However, very limited data is available on the sporulation medium dependency of antimicrobial activity. It was only reported that the antimicrobial activity of grape seed extract in the apple juice against spores was variable depending on the concentration tested and type of sporulation medium used (Molva and Baysal, 2015). In accordance with the results of this study, the spores produced on mineral containing media (BATA and BAA) were found to be sensitive to all tested concentrations of grape seed extract. Finally, we suggest that the presence or absence of certain metal ions in the sporulation medium might be important for the antimicrobial activity of phenolic compounds against spores due to the interactions between tannins in pomegranate juice and metal ions (Fe^{2+} and Cu^{2+}) by forming stable complexes and thereby decreasing the availability of these ions (Puupponen-Pimiä et al., 2005).

The spores from PDA and BATA were also examined by SEM to visualize the spore morphology that occurred during growth in apple juice and pomegranate juice after 336 h (data not presented). Similar to our previous findings (Molva and Baysal, 2015), both vegetative cells and spores were present during growth in apple juice but no vegetative cells were formed during growth in pomegranate juice possibly due to the action of certain compounds inhibiting spore germination/outgrowth. In the related literature, the growth inhibition of *A. acidoterrestris* spores in red grape juice has also been reported (Splittstoesser et al., 1994). This inhibitory effect was found to be not only due to the synergistic effects of polyphenols. Also, phenolic compounds in red grape juice showed stronger antimicrobial activity at lower pH values, lower activity in the presence of fibers and no activity at the higher pH values (Tokuda, 2007).

4. Conclusions

Under the light of the results of this study, it may be concluded that the blending of pomegranate and apple juices can inhibit the growth of *A. acidoterrestris* cells and spore germination/outgrowth. On the other hand, further research should be evaluated to fully understand the antimicrobial mode of activity of pomegranate juice or its components on *A. acidoterrestris* spores.

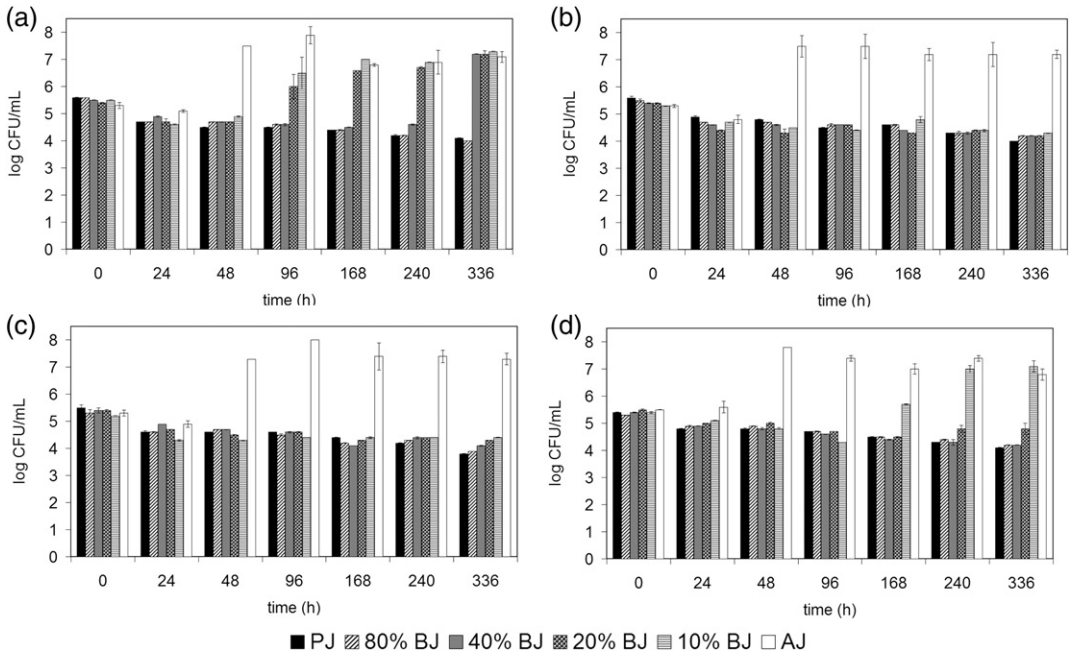

Fig. 1. Survival and germination of *A. acidoterrestris* spores in pomegranate, apple and pomegranate–apple blend juices during 336 h of storage as affected by sporulation medium used to prepare the spores. PDA (a), BATA (b), BAA (c), and MEA (d).

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