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Evaluation of bioactivity of pomegranate fruit extract against *Alicyclobacillus acidoterrestris* DSM 3922 vegetative cells and spores in apple juice

Celenk Molva, Ayse Handan Baysal*

Department of Food Engineering, Izmir Institute of Technology, Urla, 35437, Izmir, Turkey

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ABSTRACT

This research evaluated the antimicrobial activity of commercial pomegranate extract (POMELLA®, PE) against *Alicyclobacillus acidoterrestris* vegetative cells and spores (approximately 10^5 log CFU/mL) in apple juice (pH 3.82, °Brix 11.3) during storage at 37 °C. After 240 h, the cell counts were reduced from the initial log count (CFU/mL) by 2.84, 3.26, 3.32, 3.46 and 3.56 in the apple juice with PE at the concentrations of 2.5, 5, 10, 20 and 40 μg/mL, respectively. On the other hand, counts of the control reached 7.36 log CFU/mL after 24 h. The Weibull model satisfactorily described the survival curves of cell inactivation kinetics ($R^2 > 0.983$). While the growth of all spores obtained from different sporulation media (potato dextrose agar, malt extract agar, *Bacillus acidoterrestris* agar, and *Bacillus acidocaldarius* agar) was inhibited in the apple juice with PE (2.5–40 μg/mL), the control spores increased by 1.9–2.2 log CFU/mL after 336 h. Based on the scanning electron microscopy (SEM) imaging, vegetative cells indicated substantial damage and spore germination was inhibited in the apple juice with PE. The results showed that PE can have possible uses as a natural antimicrobial to control the growth of *A. acidoterrestris* vegetative cells and spore germination in the apple juice.

1. Introduction

Pomegranate (*Punica granatum* L.) is widely consumed as fresh fruit and juice (Patel, Dadhaniya, Hingorani, & Soni, 2008). Interest in pomegranate juice and its products has increased in recent years because of their beneficial health effects attributed to the antioxidant potential of its constituents, mainly hydrolyzable ellagitannins, anthocyanins and other polyphenols (Patel et al., 2008; Vegara, Martí, Mena, Saura, & Valero, 2013). Pomegranate juice production yield is 40% of the whole fruit. Remaining of the waste products can be used to produce pomegranate peel extracts (Hayrapetyan, Hazeleger, & Beumer, 2012). Nowadays, pomegranate fruit extracts are consumed as dietary supplements (Patel et al., 2008). Pomegranate extracts possess health benefits such as anti–inflammatory, cardioprotective, free radical scavenging, hepatoprotective, tyrosinase inhibition and anti–diabetic (Mphahlele, Fawole, Stander, & Opara, 2014). Polyphenolic compounds in these extracts are classified as anthocyanins (derived from delphinidin, cyanidin, and pelargonidin) that give red color and hydrolyzable tannins (punicalagin, pedunculagin, punicalin, gallic and ellagic acids). Tannins are responsible for 92% of the antioxidant potential of the whole fruit (Haidari, Ali, Ward Casscells, & Madjid, 2009). In addition to antioxidant properties, gallic acid, ellagic acid and punicalagin possess antimicrobial activities (Ismail, Sestili, & Akhtar, 2012).

* Corresponding author. Tel.: +90 232 7506187; fax: +90 232 7506196.
E-mail address: handanbaysal@iyte.edu.tr (A.H. Baysal).

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Alicyclobacillus acidoterrestris is a Gram–positive, thermoacidophilic, endospore–forming and non–pathogenic spoilage microorganism. It has ability to grow at 26–60 °C (optimum 42–53 °C) and from pH 2.0 to 6.0 (optimum 3.5–5.0) (Smit, Cameron, Venter, & Witthuhn, 2011). Commercial fruit juices are normally pasteurized at temperatures between 80 °C and 100 °C (Huertas, Esteban, Antolinos, & Palop, 2014). On the other hand, *A. acidoterrestris* spores can survive thermal pasteurization. After germination process, heat resistant spores can grow and produce taint compounds leading to spoilage of fruit juices and acidic food products (Bevilacqua, Sinigaglia, & Corbo, 2009; Chang & Kang, 2004). The chemical taint compounds have been identified as 2–methoxyphenol (guaiacol), 2,6–dibromophenol, and 2,6–dichlorophenol. Among these metabolites, guaiacol is the predominant compound related to spoilage. In addition, the vegetative cell concentration for guaiacol production to spoil fruit juices has been reported to be between 10^5 and 10^6 CFU/mL (Gocmen, Elston, Williams, Parish, & Rouseff, 2005; Pettipher, Osmundson, & Murphy, 1997).

Since synthetic antimicrobials and food additives can cause negative effects, the use of natural antimicrobial compounds is an increasing trend among consumers (Aleksic & Knezevic, 2014). Nowadays, considerable effort has been performed to discover natural antimicrobials for the inhibition of bacterial and fungal growth in foods in order to improve their quality and shelf–life (Gyawali & Ibrahim, 2014). In the literature, the investigation of the antimicrobial activity of plant extracts against *A. acidoterrestris* is very limited. To the best of our knowledge, the extracts of *Piperaceae* (Ruiz et al., 2013), *Eucalyptus* leaf (Takahashi, Kokubo, & Sakaino, 2004), and grape seed (Molva & Baysal, 2015) have been tested to inhibit the growth of *A. acidoterrestris*. It is of interest to discover natural plant extracts that have the potential to be used against *A. acidoterrestris*. Therefore, the objectives of this study were to evaluate the antimicrobial activities of commercial pomegranate fruit extract (POMELLA®) on *A. acidoterrestris* cells and spores in apple juice; to mathematically model the cell inactivation behavior in the apple juice with extract, to determine the effect of sporulation media on the antimicrobial resistance of the spores, and finally to examine the ultrastructural changes of the cells and spores by scanning electron microscopy.

2. Material and methods

2.1. Microorganism and spore production

A. acidoterrestris DSM 3922 was kindly provided by Karl Poralla (Deutsche Sammlung von Mikroorganismen und Zellkulturen's collection, Braunschweig, Germany). For spore preparation, aliquots of 100 μL 4 h–grown culture in *Bacillus acidoterrestris* broth (BATB, Döhler, Germany) were spread plated onto *Bacillus acidoterrestris* agar (BATA, Merck), *Bacillus acidocaldarius* agar (BAA) (Darland & Brock, 1971), potato dextrose agar (PDA, BD Difco) and malt extract agar (MEA, Oxoid) (Molva & Baysal, 2015). The plates were incubated at 43 °C until 85–90% of the sporulation was achieved. The sporulation was monitored by phase–contrast microscopy (Olympus CX31, Japan). Spore suspensions were prepared based on the protocol developed by other researchers (Murray, Gurtler, Ryu, Harrison, & Beuchat, 2007) and stored at −20 °C for further use.

2.2. Apple juice

Concentrated apple juice (70.3 °Brix) was provided by ASYA Fruit Juice and Food Ind. Inc. (Isparta, Turkey) and reconstituted to 11.30 ± 0.1 °Brix by a refractometer (Mettler Toledo, USA). Measurements were performed at 20 °C. The pH of the reconstituted juice was measured as 3.82 ± 0.01 (Hanna instruments, Hungary).

2.3. Pomegranate extract

The PE contained >30% punicalagins, <5% ellagic acid, and trace levels of gallic acid as determined by HPLC. According to the data sheet provided by the manufacturer, the polyphenol content was measured as >50% spectrophotometrically. The appropriate concentrations of PE were prepared aseptically by dissolving in sterile deionized water and used immediately.

Total phenol content (TPC) of the PE was determined using Folin–Ciocalteu method (Al-Zoreky, 2009) and the results were expressed in terms of gallic acid equivalents (GAE) in mg/g extract using gallic acid standard curve. The antioxidant activity of the PE was determined in terms of radical scavenging activity using the stable free radical DPPH (1,1–diphenyl–2–picryhydrazyl) and ferric reducing antioxidant power (FRAP) based on the protocol described previously (Bi et al., 2013). DPPH reacts with the compounds, which are able to donate a hydrogen atom (Fernandes, Dias, Carvalho, Souza, & Oliveira, 2014). The results of the hydrogen donating ability were expressed as IC_{50} concentration (the extract concentration to obtain 50% inhibition of DPPH radical). In FRAP assay, the reduction of Fe^{3+}–2,4,6–tri [2–pyridyl–s–triazine] (TPTZ) to blue–colored Fe^{2+}–TPTZ was determined (Nono et al., 2014). The results of FRAP assay were expressed as μmol Trolox equivalents (TE)/g PE. All measurements were performed in triplicate.

2.4. Antimicrobial activity

The antimicrobial activity of PE on both *A. acidoterrestris* cells and spores in apple juice was determined according to Molva and Baysal (2015). Briefly, overnight cultures of *A. acidoterrestris* cells were standardized to a bacterial density of McFarland 2.0 (10^7 CFU/mL) in 10 mL Maximum Recovery Diluent (MRD, Oxoid) using a Densitometer (Den–1, HVD Life Sciences, Austria). Also, the stock suspensions of spores (10^7 CFU/mL) were thawed, heat–activated at 80 °C for 10 min and then cooled on ice. Inocula were prepared from cells or spores after centrifugation (14,000 rpm/3 min) and suspension in 10 mL sterile apple juice. Each of the tubes containing 9 mL apple juice with PE (0–40 μg/mL) was inoculated with 1 mL inoculum (cells or spores) to a final concentration of 10^5 CFU/mL. After inoculation, the initial population was determined for each concentration immediately. Next, the inoculated samples were incubated at 37 °C with shaking at 120 rpm. At 24 h time intervals, the viable counts were determined by spread plating onto the surface of PDA (pH 3.5). The plates were incubated at 43 °C for 48 h.

2.5. Modeling of inactivation data

Survival curves were obtained by plotting the logarithm of survivors vs. treatment time (h). Inactivation data were fitted by the GlnaFiT tool (Geeraerd, Valdramidis, & Van Impe, 2005) using log–linear tail (Geeraerd, Herremans, & Van Impe, 2000) and the Weibull models (Mafart, Couvert, Gaillard, & Leguerinel, 2002).

The Weibull model was used with the following equation (Mafart et al., 2002);

$$\log N_t = \log N_0 (t/\delta)^{\beta} \quad (1)$$

where N_t represents the number of surviving cells at any given time (t, h); N_0 is the initial level of surviving cells at the beginning of the treatment (t = 0); β describes the shape of the survival curve; if

$\beta < 1$, the curve is concave (tailing), if $\beta > 1$, the curve is convex (shoulder) (Sampedro, Rodrigo, & Martínez, 2011). Upward concave curves are associated with the adaptation of the remaining cells to the applied stress. Downward concave indicates the increased damage of the remaining cells in applied stress (van Boekel, 2002). Also, δ (h) is the time for the first decimal reduction (De Oliveira, Soares, & Piccoli, 2013). The time to obtain 4–log reduction (t_{4D}) was calculated from the model parameters (δ and β) by employing Eq. (2) (Levy, Aubert, Lacour, & Carlin, 2012).

$$t_{4D} = \delta \bullet (4)^{1/\beta} \quad (2)$$

The log–linear tail model (Geeraerd et al., 2000) is as follows;

$$logN_t = log\left(\left(10^{logN_o} - 10^{logN_{res}}\right)\right) \bullet e^{(-k_{max}t)} + 10^{logN_{res}} \quad (3)$$

where N_{res} is the residual population density (log CFU/mL) that characterizes tailing of inactivation kinetics (Hereu, Dalgaard, Garriga, Aymerich, & Bover-Cid, 2012) and k_{max} is the inactivation rate of the log–linear part of the curve (h^{-1}) (Izquier & Gómez-López, 2011).

The inactivation models were evaluated by root mean square error (RMSE), and coefficient of determination (R^2) values. Using the RMSE value, statistical deviations in the model are predicted with smaller RMSE values indicating better fit to the experimental data. On the other hand, higher values show large deviations in predicted value (Scanlon et al., 2015). Therefore, the best fit of the data is obtained when RMSE is the smallest and R^2 value is close to 1 (Berney, Weilenmann, Simonetti, & Egli, 2006).

2.6. Scanning electron microscopy

The controls and treated samples were prepared according to a previously published method (Molva & Baysal, 2015). The samples were gold–coated at a vacuum of 0.09 mbar, for 90 s, at 15 kV, in argon gas at a power of 15 mA, followed by analysis using a scanning electron microscope (Phillips XL–30S FEG).

2.7. Statistical analysis

Data were measured in triplicates (n = 3) and expressed as means ± standard deviations (Microsoft Excel 2003, Microsoft corp., USA). The Tukey–Kramer test was used to compare the means of treated groups ($p < 0.05$) (Minitab 16, Minitab Inc., UK).

3. Results and discussion

3.1. Total phenolics in pomegranate extract and antioxidant activity

In the current study, the TPC of PE was found as 573 ± 34.5 mg GAE/g extract by Folin–Ciocalteu method. The TPC in the pomegranate pulp and peel extracts varied from 11.62 ± 0.63 to 21.03 ± 1.51 and 98.28 ± 4.81 to 226.56 ± 18.98 mg GAE/g extract, respectively among different pomegranate cultivars (Shams Ardekani et al., 2011). The antioxidant capacity of plant extracts is directly related to phenolic content. The phenolic phytochemical constituents are responsible for this activity that is not a property of a single phenolic component (Aleksic & Knezevic, 2014).

According to the results of the studies on antioxidant potential of fruit and vegetable extracts using different methods (FRAP, DPPH, TRPA, ORAC and LDL oxidation etc.), different trends between these assays have been observed. Therefore, several methods should be evaluated and compared instead of a single assay to determine the antioxidant capacity of these extracts (Suárez-Jacobo et al., 2011). In the present study, the antioxidant activities of PE were tested through free radical scavenging activity by DPPH and FRAP methods. Based on the FRAP, the extract at the concentration of 10 μg/mL had high power reduction of 6570 ± 198.9 μmol TE/g extract. The reducing power in PE was reported to be 4.7–fold higher than seed extract and 10.5–fold higher than juice extract (Teixeira da Silva et al., 2013). The IC_{50} value of the extract was 0.21 ± 0.00 mg/mL. In the related literature, the DPPH scavenging activity values in PE were 23.4–fold higher than the juice extracts, and the seed extracts had 2.3–fold higher than juice extract (Teixeira da Silva et al., 2013).

3.2. Antimicrobial activity on vegetative cells and modeling inactivation data

Table 1 presents the counts of *A. acidoterrestris* vegetative cells inoculated into the apple juice with different concentrations of PE during storage at 37 °C. Significant differences in cell counts were observed at each PE treatment compared to the control ($p < 0.05$). At initial time (t = 0), the vegetative cells were inoculated into the apple juice without PE at the concentration of approximately 5.6 log CFU/mL. On the other hand, the control reached approximately 7.36 log CFU/mL at the 24th h due to sporulation of vegetative cells in the apple juice.

The number of PE treated cells decreased significantly ($p < 0.05$) increasing the treatment time and extract concentration. Differences were found between the treated samples and control after 24 h of treatment ($p < 0.05$). There were no significant differences ($p > 0.05$) between the treatments using 2.5 and 5 μg/mL PE after 48 h. The counts in the apple juice with all tested concentrations of PE were statistically different after 96 h treatment ($p < 0.05$). Similar to the results after 48 h, the cell counts in the presence of 20 and 40 μg/mL PE were statistically similar after 168 h ($p > 0.05$). At the end of 240 h, reductions of 3.46 and 3.56 log CFU/mL were obtained in the apple juice with PE at the concentrations of 20 and 40 μg/mL, respectively. In the apple juice supplemented with 2.5, 5 and 10 μg PE/mL, the cell

Table 1
Counts (log CFU/mL) of *A. acidoterrestris* vegetative cells in the apple juice with PE.

Treatment time (h)	PE concentration in the apple juice (μg/mL)					
	2.5	5	10	20	40	Control
0	$5.56(0.07)^{Abc}$	$5.65(0.01)^{Aab}$	$5.64(0.04)^{Aab}$	$5.73(0.04)^{Aa}$	$5.75(0.01)^{Aa}$	$5.57(0.04)^{Ac}$
24	$4.41(0.09)^{Bb}$	$4.34(0.19)^{Bb}$	$4.34(0.04)^{Ab}$	$4.19(0.07)^{Bb}$	$4.10(0.25)^{Bb}$	$7.36(0.09)^{ABa}$
48	$4.17(0.11)^{Cb}$	$4.05(0.05)^{BCb}$	$3.94(0.13)^{Bbc}$	$3.66(0.11)^{Cc}$	$3.57(0.09)^{Cc}$	$7.21(0.22)^{ABa}$
96	$3.87(0.07)^{Dbc}$	$3.67(0.18)^{Cbcd}$	$3.56(0.04)^{Ccde}$	$3.47(0.01)^{Cde}$	$3.28(0.21)^{Ce}$	$7.59(0.12)^{ABa}$
144	$3.44(0.01)^{Ebc}$	$3.19(0.04)^{Dcd}$	$3.13(0.20)^{Dcde}$	$2.99(0.06)^{Dde}$	$2.83(0.12)^{De}$	$7.54(0.11)^{ABa}$
168	$3.28(0.04)^{Ebc}$	$3.04(0.03)^{Dcd}$	$2.89(0.03)^{Ede}$	$2.72(0.06)^{Ee}$	$2.66(0.15)^{De}$	$7.50(0.19)^{Ba}$
192	$3.26(0.07)^{Eb}$	$3.12(0.22)^{Dbc}$	$2.85(0.11)^{Ecd}$	$2.71(0.11)^{Ede}$	$2.48(0.09)^{DEe}$	$7.64(0.07)^{Ba}$
240	$2.72(0.08)^{Fb}$	$2.39(0.06)^{Ebc}$	$2.32(0.07)^{Ecd}$	$2.27(0.09)^{Fde}$	$2.19(0.02)^{Ee}$	$7.21(0.17)^{Ca}$

Results are expressed as mean ± standard deviation of three replicates (n = 3). Values with different capital letters in the same column are significantly different ($p < 0.05$). Values with different lower case in the same row are significantly different ($p < 0.05$).

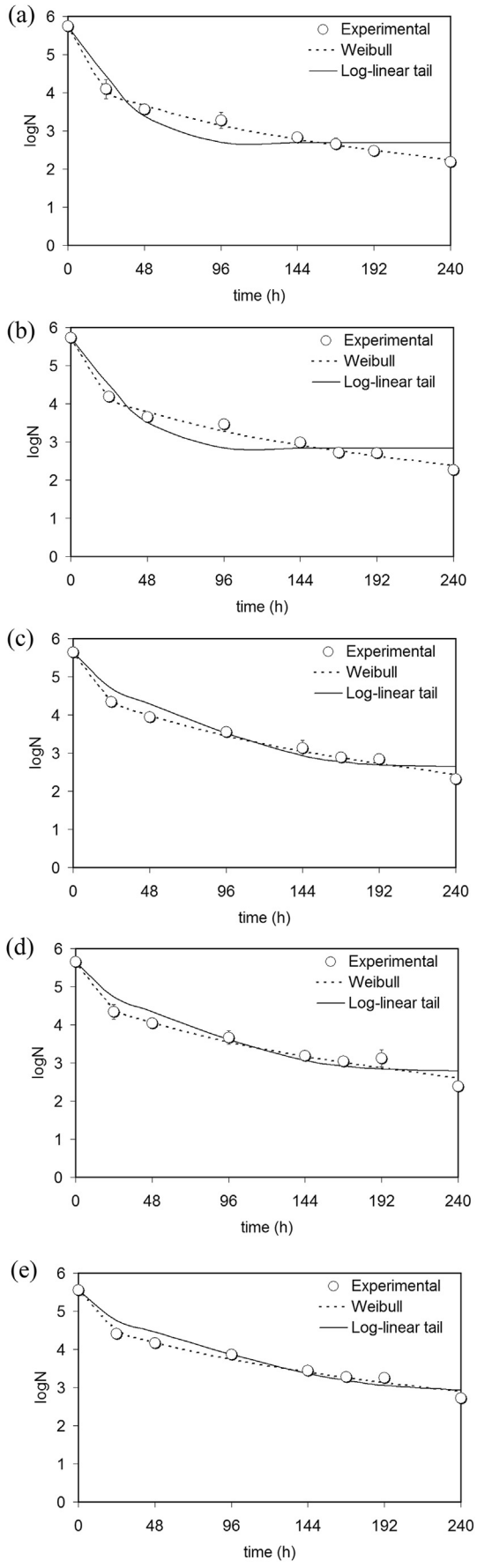

Table 2
Statistical parameters of the Weibull and the log–linear tail models.

μg PE/mL	Weibull				Log–linear tail		
	δ (h)	β	[a]t_{4D} (h)	Statistical indices	k_{max} (h^{-1})	log N_{res} (CFU/mL)	Statistical indices
2.5	23.70	0.42	643.0	[b]RMSE = 0.128 [c]R^2 = 0.985	0.03	2.89	RMSE = 0.330 R^2 = 0.900
5	16.74	0.41	492.3	RMSE = 0.165 R^2 = 0.981	0.04	2.78	RMSE = 0.397 R^2 = 0.889
10	15.88	0.42	430.9	RMSE = 0.106 R^2 = 0.993	0.04	2.63	RMSE = 0.379 R^2 = 0.908
20	7.16	0.34	422.4	RMSE = 0.127 R^2 = 0.991 R^2–adj = 0.987	0.10	2.83	RMSE = 0.425 R^2 = 0.893 R^2–adj = 0.851
40	5.28	0.33	352.4	RMSE = 0.083 R^2 = 0.996	0.11	2.69	RMSE = 0.408 R^2 = 0.910

[a] Predicted time for 4–log reduction calculated by the Weibull model.
[b] Root mean square error.
[c] Coefficient of determination.

counts were reduced from the initial count by 2.84, 3.26 and 3.32 log CFU/mL after 240 h.

There are several studies showing that PE or its pure components have the antibacterial activity of against both Gram–positive and Gram–negative bacteria (Ahmad & Aqil, 2007; Burapadaja & Bunchoo, 1995; De, De, & Banerjee, 1999; Hayrapetyan et al., 2012; Machado et al., 2002; Navarro, Villarreal, Rojas, & Lozoya, 1996; Negi, Jayaprakasha, & Jena, 2003; Prashanth, Asha, & Amit, 2001; Rani & Khullar, 2004). The antimicrobial activity of plant antimicrobials depends on the type of the target organism, extraction procedure, culture medium, size of the microbial inoculum and the method used for the determination of antimicrobial activity (Tajkarimi, Ibrahim, & Cliver, 2010). According to Hayrapetyan et al. (2012), there was also a correlation between the total phenol content and bacterial growth inhibitory effect of PE. These researchers also suggested that the extraction procedures, isolation source, solubility of the extract and incubation temperature could affect the antimicrobial activity. In another study, the antibacterial activity of phenolics was shown to be dependent on the pH (Friedman, Henika, & Mandrell, 2003).

Fig. 1 shows the inactivation kinetics of *A. acidoterrestris* cells in the apple juice with PE (2.5–40 μg/mL) during storage at 37 °C. The survival curves were fitted by the Weibull and the log–linear tail models as a function of time (h). Table 2 presents the statistical parameters for the fitted models to the inactivation data of *A. acidoterrestris* cells. The goodness of fit of these models was determined by comparing R^2 and RMSE values (Table 2). The values of R^2 for the Weibull model (0.981–0.996) were higher than that of the log–linear tail model (0.889–0.910). Also, the RMSE values of the Weibull model ranged from 0.083 to 0.165. The RMSE values of the log–linear tail model were between 0.330 and 0.425. Therefore, the Weibull model was found to describe satisfactorily the survival curves of *A. acidoterrestris* cells with higher R^2 and lower RMSE values compared to the log–linear tail model. Shape parameters (β) of the Weibull model indicate that the treatments were upward concave ($\beta < 1$) which can also be seen from Fig. 1. As the antimicrobial concentration increased, time–to–4 log reductions (t_{4D}) predicted by the Weibull model also decreased; t_{4D} values were calculated as 643, 492, 430, 422, and 352 h for 2.5, 5, 10, 20, 40 μg PE/mL juice, respectively.

Fig. 1. Survival curves of *Alicyclobacillus acidoterrestris* DSM 3922 vegetative cells in the reconstituted apple juice with PE (a) 40 μg/mL, (b) 20 μg/mL, (c) 10 μg/mL, (d) 5 μg/mL and (e) 2.5 μg/mL. Curves are fitted using the log–linear tail and the Weibull models.

3.3. Antimicrobial activity on spores

The inhibitory activity of PE against spores was evaluated in relation to sporulation media. PDA, BATA, BAA and MEA were used for sporulation. Fig. 2 represents the log numbers of *A. acidoterrestris* spores in the apple juice with different concentrations of PE (2.5–40 μg/mL). Spore suspensions from different sporulation media exhibited lower sensitivity to PE than vegetative cells. At all tested concentrations, the extract was found to inhibit the germination of spores from all sporulation media in the apple juice when compared to the control spores. At the beginning of the incubation (t = 0), the spore counts from each sporulation media was approximately 10^5 log CFU/mL. At the end of the incubation period, the total counts in the controls increased (approximately 10^8 CFU/mL after 48 h or 96 h of incubation) due to spore germination. After inoculation of spore suspensions into the apple juice with PE, a reduction of spores was observed due to the reduction in spore germination activity. Approximately, 1.9, 2.1, 2.2, and 2.1 log CFU/mL increases in spore counts were obtained among untreated controls from PDA, BATA, BAA and MEA, respectively after 336 h. At the highest tested concentration (40 μg/mL), inactivations of 1.6, 1.65, 1.4, and 2.6 log were observed among spores from PDA, BATA, BAA and MEA, respectively. When PE was applied at the concentration of 20 μg/mL, 1.7, 1.4, 1.4 and 2.3 log reductions were obtained from spores produced on PDA, BATA, BAA and MEA, respectively. At the lowest concentration tested (2.5 μg/mL), the spore counts from PDA, BATA, BAA and MEA ranged from 0.6 to 1.3 log reductions (Fig. 2).

3.4. Scanning electron microscopy

The SEM imaging of control cells revealed intact cell walls and cell membranes with smooth surfaces. In the control samples, the production of forespore due to cell division is clearly observed after 240 h (Fig. 3f). In response to nutrient depletion, vegetative cells produce the mother cell and forespore by asymmetric cell division. The forespore differentiates into the endospores while the mother cell lyses to release mature spore (Meisner, Wang, Serrano, Henriques, & Moran, 2008). In contrast, damages to the cell wall and membrane were seen among the PE treated cells (Fig. 3a). Also, PE treated cells indicate cell elongation to their multiple cell size most probably due to the effect of the PE on the cell division (Fig. 3b–d). Moreover, during growth in the apple juice with 2.5 μg PE/mL, shrinkage and leakage of the cellular materials were observed (Fig. 3e). Therefore, it can be concluded that PE might act on the cell membrane, interrupt the cell functions most probably the cell division and thereby prevent the forespore formation. The mechanism of antimicrobial activity of pomegranate peel phenolics involves precipitation of membrane proteins resulting in microbial lysis. Phenolics react with microbial cell membrane proteins and/or protein sulfhydryl groups resulting in bacterial cell death due to the membrane precipitation and inhibition of enzymes such as glycosyltransferases (Ismail et al., 2012).

Based on the representative SEM images, the treated spores produced on PDA and BATA did not display visible damage to the surface compared to the untreated spores after 336 h. The spore controls in apple juice without PE contain both vegetative cells and spores (Fig. 4c and f). On the other hand, the PE treated samples indicate only the presence of spores (Fig. 4). In the related literature, the addition of grape seed extract into the apple juice was found to inhibit the endospore germination of *A. acidoterrestris* (Molva & Baysal, 2015). In the previous study, the antimicrobial activity of grape extract was found sporulation media dependent and the spores produced on mineral containing media such as BATA and BAA were more sensitive to treatments. But, the sporulation media composition was found to have no effect on the antimicrobial activity of PE against *A. acidoterrestris* spores.

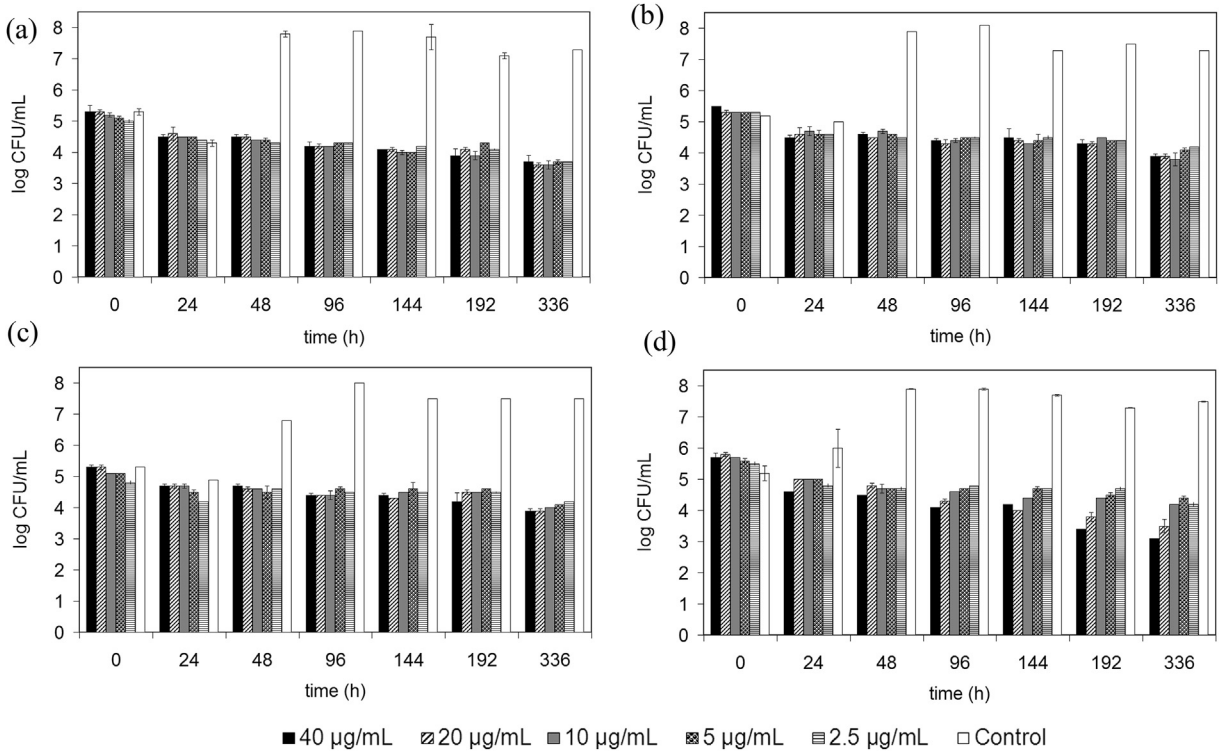

Fig. 2. Counts of *A. acidoterrestris* spores (log CFU/mL) produced on (a) PDA, (b) BATA, (c) BAA, and (d) MEA after treatment with PE (2.5–40 μg/mL) during 336 h. Each spore suspension from different media was inoculated separately into the apple juice and was used as control.

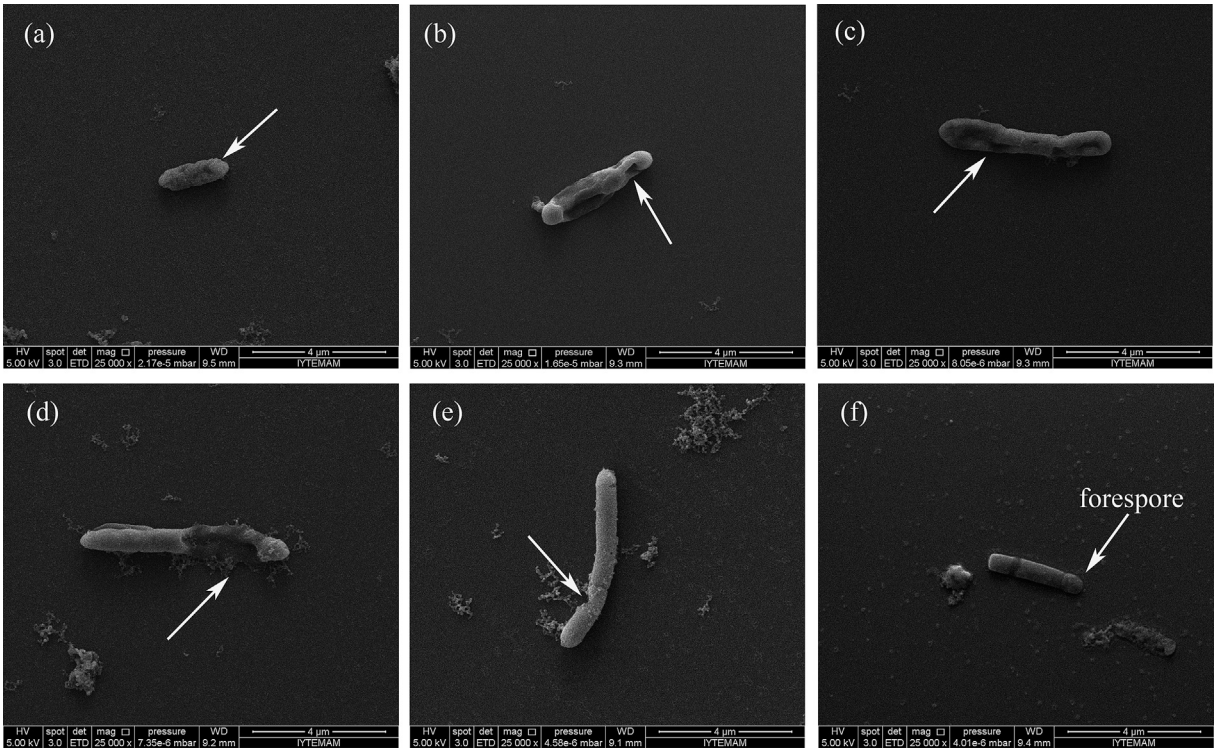

Fig. 3. Scanning electron microscopy images of treated *A. acidoterrestris* cells in the apple juice after 240 h (a) 40 μg/mL, (b) 20 μg/mL, (c) 10 μg/mL, (d) 5 μg/mL, (e) 2.5 μg/mL, (f) untreated control. Samples inoculated in the apple juice without PE were used as controls. White arrows indicate the damage on the cell structure after PE treatments. Bars = 4 μm (magnification ×25,000).

4. Conclusions

There is limited published data on the antimicrobial activity of natural plant extracts against *A. acidoterrestris* in fruit juices and the present study is the first report on the antimicrobial activity of PE against *A. acidoterrestris* in the apple juice. The use of natural plant extracts in the fruit juice industry is an increasing trend. Therefore, the results from this study will be useful to reveal the potential of

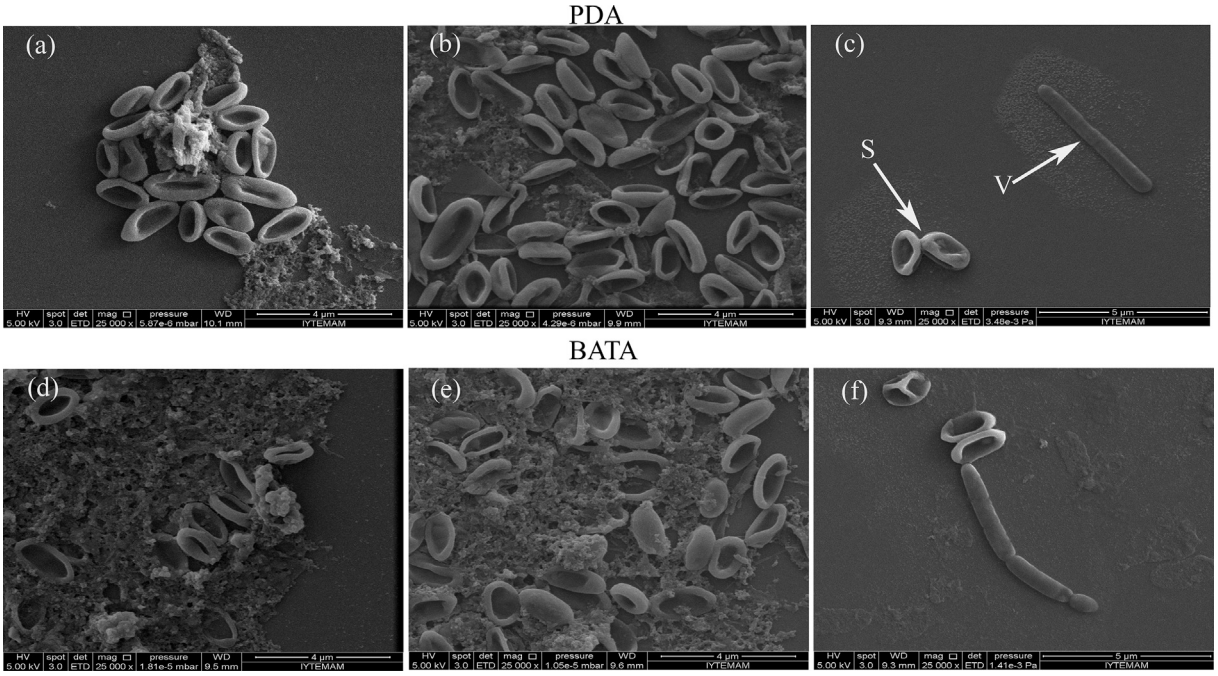

Fig. 4. Scanning electron microscopy images of PE–treated *A. acidoterrestris* spores in the apple juice after 336 h. PDA (a) 20 μg/mL (b) 10 μg/mL (c) untreated control; BATA (d) 20 μg/mL (e) 10 μg/mL (f) untreated control inoculated in the apple juice. V and S represent vegetative cell and spore, respectively. Bars = 4 μm for treated samples and 5 μm for untreated controls (magnification ×25,000).

this extract as a natural antimicrobial to inhibit the growth of vegetative cells of *A. acidoterrestris* and spore germination/outgrowth in the apple juice and also its products even if it is added at lower concentrations.

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[1] *The selected key references explain the importance of the spoilage caused by *A. acidoterrestris* for the fruit juice industry and the use of pomegranate extract as a natural preservative to control the microbial growth in foods.