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Effectiveness of a pomegranate peel extract (PGE) in reducing *Listeria monocytogenes in vitro* and on fresh-cut pear, apple and melon.

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Abstract

Pomegranate peel extract (PGE) is a new promising natural alternative control substance with large spectrum of activity against wide range of pathogenic microorganisms. In the present study, PGE was firstly investigated as natural antimicrobial against *Listeria monocytogenes* both *in vitro* and on fresh-cut fruits. The *in vitro* results showed quick and strong bactericidal and bacteriostatic activity against 5 different strains which were almost completely inhibited by the extract. Furthermore, it significantly decreased growth rate and maximum growth of all tested strains. *In vivo* trials, confirmed a strong antibacterial activity of the extract that significantly reduced the bacterial load on fresh-cut apple, melon and pear and maintained the population at low levels throughout the storage period (7 days). PGE at 12 g/l reduced *L. monocytogenes* by 1.24, 1.89, and 0.91 log units soon after treatment and by 3.81, 1.53, and 2.99 log units, after 7 days of storage on apple, pear and melon, respectively. This high antibacterial activity could be mainly related to the high content of polyphenols (ellagitannins) in the extract. Overall, results of this study suggest a potential industrial application of PGE to reduce the growth of the pathogenic microorganisms in fresh-cut fruit and ensure a microbial safety in case of contamination.

Keywords: PGE, *Listeria monocytogenes*, antimicrobial activity, fresh-cut fruits.

Introduction

In recent years, the demand for healthy and ready-to-eat fresh-cut products has highly increased and, therefore, the industry is in continuous search for new and improved methods to maintain the quality and extend the shelf-life of products. Fresh-cut fruits and vegetables are minimally processed products (trimmed, peeled and/or cut) that offer to consumers high nutritional value, freshness, convenience and flavour similar to the original raw intact product [1,2]. However, these products deteriorate faster than the unprocessed raw material, mainly due to the damages caused by peeling operation as well as the other minimally processing operations [3,4]. This alters the processed product and makes it more vulnerable to microbial contamination and colonization with the consequent reduction of quality and shelf life [3].

Microbial contamination may represent a direct critical risk for human health because of the proliferation of important pathogens such as *Listeria monocytogenes* [5]. This bacteria is an important human pathogen that can contaminate fresh-cut produces in any step of the processing chain [6]. Therefore, several methods and strategies have been developed and

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used by the fresh-cut industries in attempt to reduce the occurrence and the risk associated to foodborne diseases. Sanitizers, including chlorine [7], organic acids [8], heat treatments [9], ultraviolet (UV) light [10], and ozone [11] have been widely applied to disinfect and reduce the initial bacterial load on fruits and vegetables. However, these methods have shown several drawbacks such as the formation of potential carcinogenic by-products from using chlorine, low efficiency in reducing the bacterial population, chemical residues, destruction of nutrients and the alteration of sensory characteristics [6,12]. This, together with the increase of the consumer awareness in food safety and healthy living, has increased the interest to safe and environmentally friendly alternative control means and mainly plant substances including essential oils and plant extracts.

Recently, a pomegranate peel extract (PGE) proved to be very effective in controlling fungal postharvest rots on different fruit species [13]. Experiments demonstrated a complex mechanism of action which include the induction of resistance in treated host tissues and a strong antimicrobial activity against both fungi and bacteria [14-16]. The high antimicrobial activity was associated to the high content of phenolic compounds in PGE [17]. Although PGE has never been tested against potential human bacterial pathogens, other extracts from pomegranate by-products were able to reduce the germination and growth of several pathogenic bacteria including *Listeria monocytogenes, L. innocua, Staphylococcus aureus, Escherichia coli, Yersinia enterocolitica, Pseudomonas aeruginosa*, and *Salmonella* spp. [18,19,14]. Furthermore, edible coatings formulated with a pomegranate peel extract and other anti-browning agents were used to extend the shelf life of fresh-cut persimmon fruits [20].

The aim of the present study was to evaluate the potential use of PGE as natural antimicrobial to reduce the growth of foodborne pathogens using *L. monocytogenes* as a model pathogen *in vitro* and on fresh-cut melons, apples and pears.

Material and Methods

Pomegranate peel extract (PGE) and Bacterial strains

All experiments were conducted using a stock solution of an aqueous pomegranate peel extract (PGE) prepared according to Romeo et al. (2015). The solution was stored, before use, at 5±1 °C and diluted to have 3 concentrations of PGE containing 12 (PGE-12), 2.4 (PGE-2.4), and 1.2 (PGE-1.2) g/l of dry matter. Since the pH of these solutions was very low (2.7, 2.8, 3.1, respectively), PGE-12 was adjusted with phosphate buffer to increase the pH to 4.4 (aPGE-12) and included in experiments with fresh-cut fruit plugs in order to evaluate the potential impact of solution acidity on the antimicrobial activity.

Four strains of *L. monocytogenes* belonging to the Spanish Type Culture Collection (CECT 4031, serovar 1/2, CECT 933, serovar 3a, CECT 940, serovar 4d, CECT 4032, serovar 4b) and a strain that was previously isolated from fresh-cut lettuce (Lm 230, serovar 1/2 a,[21]) were used in the present study. Strains were grown individually in tryptone soy broth (TSB, Biokar Diagnostics, Beauvois, France) supplemented with 6 g/l of yeast extract (TSYEB). After 24 h of incubation at 37±1 ºC, bacterial cells were harvested by centrifugation (9800 × *g* for 10 min at 10 ºC) and resuspended in a saline solution (8.5 g/l NaCl) to obtain single-strain stock suspensions. The concentration of each strain suspension was determined by plating duplicate 10-fold serial dilutions on TSA media (TSA, Biokar Diagnostics, Beauvois, France) enriched with 6 g/l of yeast extract, 2.5 g/l glucose and 2.5 g/l K_2HPO_4, TSAYE) and incubated at 37 ºC for 24 h.

In vitro assays

To evaluate the bactericidal activity of PGE, 50 µl of *L. monocytogenes* suspensions (10^9 UFC/ml) were added to 5 ml of PGE at three different concentrations (12, 2.4 and 1.2 g/l). Sterile water was used as control. For each strain and concentration three replicates were used. After 2, 5, 10 and 30 min of contact time at 20°C, bacterial suspensions of *L. monocytogenes* were 10-fold serially diluted in saline peptone (8.5 g L^{-1} NaCl and 1 g L^{-1} peptone) and plated on TSA

(TSA, Biokar Diagnostics, Beauvois, France) enriched with 6 g/l of yeast extract, 2.5 g/l glucose and 2.5 g/l K_2HPO_4, TSAYE). After 24 h of incubation at 37 ºC, the number of colony forming units was recorded and converted to CFU/ml. To evaluate the impact of PGE on the growth parameters of *L. monocytogenes*, 20 µl of bacterial suspensions containing approximately 10^5 CFU/ml were added to 180 µl of TSBYE to obtain final PGE concentrations of 2.4 or 1.2 g/l in a round-bottomed 96-well microplate (Greiner, Frickenhausen, Germany). TSBYE without PGE served as a control and each treatment was replicated four times. The microplate was incubated for 36 h at 37±1ºC and the absorbance of suspensions was recorded every 30 min using a spectrophotometer (Epoch Microplate Spectrophotometer, Biotek-Instruments, Winooski, USA) set at λ = 700 nm. Plates were automatically agitated before measurements.

In vivo assays

Experiments were performed on apples (cv. *Golden Delicious*) and pears (cv. *Conference*) obtained from local packinghouses in Lleida (Catalonia, Spain) and on melons (cv. *Cantaloupe*), purchased from a local supermarket. Fruits were preliminary washed with tap water, surface disinfected with 70% ethanol and dried at room temperature. Fruits were peeled and cut with a sterilized cork-borer to have cylindrical plugs of 1.2 cm diameter × 1.0 cm long (weighting approximately 1 g).

Fruit plugs were inoculated with *L. monocytogenes* by dipping in a bacterial suspension (10^6 CFU/mL) containing the five strains of the pathogen, for 2 min. The bacterial suspension was obtained by mixing equal volumes of the single-strain stock solutions. Inoculated fruit plugs were air dried at room temperature for 30 min and incubated overnight at 5ºC. Plugs of each fruit were then divided into 6 uniform groups and subjected to different treatments including PGE-12, PGE-2.4, PGE-1.2, aPGE-12, and distilled water. Other plugs did not receive any treatment. Treatments were performed by dipping the inoculated plugs for 10 min at 150 rpm. After drying for 30 min at room temperature, plugs from each treatment were further divided into two sub-groups, each consisting of 6 replicates. Sub-groups were used to determine the concentration of bacterial cells soon after the treatment or after 7 days of storage at 10±1ºC. To determine the bacterial population, plugs were put in a sterile bag containing 9 ml of buffered peptone water (BPW, Oxoid, LTD, Basingstoke, Hampshire, England) and blended in a homogenizer (Minimix® 100, Interscience, France) for 120 s at 12 strokes/s. The homogenized mixtures were then serially diluted in saline peptone, plated on duplicate plates of selective Palcam agar (Biokar Diagnostics, Beauvois, France) and incubated at 37±1ºC for 48 h. The bacterial concentration was expressed as log CFU/g. The reductions in bacteria were calculated by subtracting the initial mean bacteria population of the untreated samples from the bacteria population after each treatment.

Statistical analysis

Prior analyses, all CFU g^{-1} data were transformed to $\log_{10}$ CFU g^{-1}. For the bacterial growth experiment, primary models were fitted using the DMFit 3.5 Excel add-in provided by ComBase predictive modelling tool (https://www.combase.cc) and growth parameters (lag time, growth rate, and maximum population density) were determined using the re-parameterized Gompertz model described by Zwietering *et al*. (1990) based on the equation $y = A\exp\{-\exp\left(\frac{\mu_{max}\, e}{A}(\lambda - t) + 1\right)\}$ where y, μ_m, t, λ, and A represent the absorbance (OD) at time t, maximum growth rate (h^{-1}), incubation time (h), lag time (h), and asymptotic value, respectively.

Data were analysed using general linear model analysis with JMP®8, 2004 software (JMP®8, SAS Institute, Cary, NC, USA). After analysis of variance (ANOVA), significant differences between treatments were determined according to Tukey's test at a significance level of $P < 0.05$.

Results

In vitro assays

In vitro experiment showed a strong bactericidal activity of PGE. The number of viable cells (log CFU/mL) of *L. monocytogenes* was always significantly reduced by the extract (Table 1). No significant differences were observed among the 3 tested concentrations of PGE. In addition, the incubation time did not have a relevant influence on the bactericidal activity as similar results were achieved after 2, 5, 10 and 30 min of contact. On the contrary, important differences were observed among *L. monocytogenes* strains. Strains CECT 4031 and CECT 933 were the most sensitive since their population was always below the detection limit for almost all tested concentrations and incubation times (Table 1). A slightly higher tolerance was revealed for the strain CECT 4032. Strains CECT 940 and Lm230 showed the highest rates of survival, but still their population was reduced at least by 3.3 log units after 2 minutes of incubation with all the PGE doses.

Table 1 Concentration of *L. monocytogenes* cells (log_{10} CFU/ml) after 2, 5, 10 or 30 min of incubation in PGE solutions at three different concentrations (1.2, 2.4 or 12.0 g/l) or in water (control). Separate statistical analyses were conducted for each strain, contact period, and incubation time. Different letters indicate significantly different values according to Tukey's test ($P < 0.05$).

Strain	**Treatment**	**Incubation period (min)**							
		2		**5**		**10**		**30**	
CECT 933	Water	7.11	a	7.04	a	7.08	a	7.11	a
	PGE 1.2 g/l	<dl	b	<dl	b	<dl	b	<dl	b
	PGE 2.4 g/l	<dl	b	<dl	b	<dl	b	<dl	b
	PGE 12 g/l	<dl	b	<dl	b	<dl	b	<dl	b
CECT 940	Water	7.26	a	7.08	a	6.97	a	6.63	a
	PGE 1.2 g/l	3.59	b	3.49	b	3.32	b	3.23	b
	PGE 2.4 g/l	3.67	b	3.48	b	3.11	b	3.00	b
	PGE 12 g/l	3.61	b	3.54	b	3.43	b	3.30	b
Lm230	Water	6.93	a	6.90	a	6.93	a	6.90	a
	PGE 1.2 g/l	3.52	b	3.49	b	3.38	b	3.34	b
	PGE 2.4 g/l	3.48	b	3.41	b	3.30	b	3.28	b
	PGE 12 g/l	3.61	b	3.45	b	3.40	b	3.23	b
CECT 4032	Water	7.28	a	7.18	a	7.08	a	7.28	a
	PGE 1.2 g/l	2.28	b	1.89	b	1.37	b	1.30	b
	PGE 2.4 g/l	2.56	b	0.92	b	1.74	b	0.23	b
	PGE 12 g/l	1.56	b	<dl	b	<dl	b	<dl	b
CECT 4031	Water	6.70	a	6.85	a	6.74	a	6.65	a
	PGE 1.2 g/l	0.20	b	0.52	b	<dl	b	<dl	b
	PGE 2.4 g/l	<dl	b	<dl	b	<dl	b	<dl	b
	PGE 12 g/l	<dl	b	<dl	b	<dl	b	<dl	b

< dl: below detection level

The analysis of the growth parameters of *L. monocytogenes* in TSBYE showed a significant impact of PGE on the maximum cell growth of all investigated strains (Table 2). Interestingly, the effect of PGE was directly correlated to its concentration since significant differences were always revealed between the two tested concentrations. In particular, PGE-2.4 reduced the maximum cell growth between 46.9% (CECT 9333) and 62.9% (CECT 4031) as compared to the control (TSBYE without PGE). While, PGE-1.2, reductions ranged between 18.4% (strain CECT 4032) and 35.3% (CECT 933).

PGE-2.4 significantly decreased also the growth rate of all strains with reductions ranging between 41.6% (CECT 4032) and 63.9% (CECT 933) as compared to the control. Lower, but still significant reductions were also achieved with PGE at 1.2 g/l for 4 out of 5 strains. Similarly, the duration of the lag phase was increased by PGE.

Table 2 Growth kinetic parameters (lag time, growth rate, and max absorbance) of the five tested strains of *L. monocytogenes* cultured in standard TSBYE (control) or in TSBYE amended with PGE at 1.2 and 2.4 g/l. For each parameter and strain, different letters indicate statistically different values according to Tukey's test ($P < 0.05$).

Strains	Medium	Lag time, λ (h)		Growth rate, μ (Absorbance at λ = 700 nm)		Max absorbance	
Lm230	TSBYE	7.5	c	0.002229	a	0.564225	a
	TSBYE+PGE 1.2 g/l	7.9	b	0.001435	b	0.370295	b
	TSBYE+PGE 2.4 g/l	8.4	a	0.001005	c	0.242917	c
CECT 933	TSBYE	9.3	c	0.001122	a	0.491362	a
	TSBYE+PGE 1.2 g/l	11.3	b	0.000610	b	0.317862	b
	TSBYE+PGE 2.4 g/l	13.0	a	0.000405	c	0.261084	c
CECT 940	TSBYE	7.9	c	0.002180	a	0.492187	a
	TSBYE+PGE 1.2 g/l	8.3	b	0.001366	b	0.359077	b
	TSBYE+PGE 2.4 g/l	8.9	a	0.000972	c	0.234525	c
CECT 4031	TSBYE	9.0	b	0.001738	a	0.402777	a
	TSBYE+PGE 1.2 g/l	9.3	ba	0.001000	b	0.313139	b
	TSBYE+PGE 2.4 g/l	9.9	a	0.000685	c	0.149649	c
CECT 4032	TSBYE	6.7	c	0.002335	a	0.495242	a
	TSBYE+PGE 1.2 g/l	7.4	b	0.002658	a	0.403998	b
	TSBYE+PGE 2.4 g/l	8.2	a	0.001364	b	0.216037	c

In vivo trials

Effect of PGE on *L. monocytogenes* population on fresh-cut apple

Initial population of *L. monocytogenes* soon after the inoculation was 5.65 log CFU/g (Fig. 1). Without PGE treatment, the population of *L. monocytogenes* greatly increased during the cold storage and reached 7.18 and 7.43 Log CFU/g after 7 days of cold storage on untreated and water treated fresh-cut apple samples, respectively.

PGE proved to be very effective in reducing the population of *L. monocytogenes*. Soon after treatments, significant reductions of 1.57, 1.24, 1.21, and 0.85 log units were recorded, comparing to the untreated samples, with aPGE-12, PGE-12, PGE-2.4, and PGE-1-2, respectively. These reductions greatly increased after 7 days of cold storage to reach 3.81, 3.31, and 2.19 log units in apple plugs treated with PGE-12, PGE-2.4, and aPGE-12, respectively. However, PGE with the lowest concentration (PGE-1.2) did not show any significant effect in reducing the bacterium population comparing to the controls.

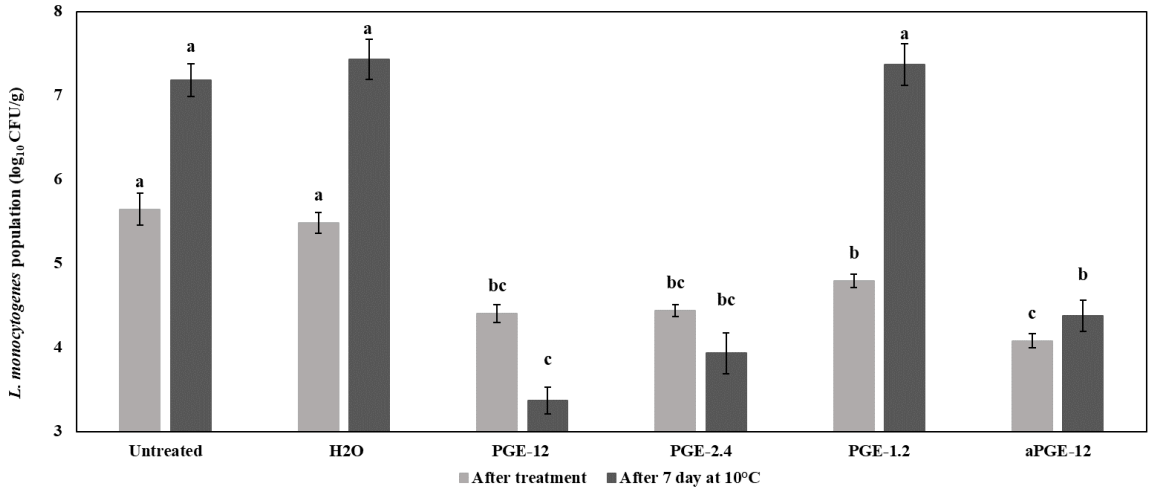

Fig. 1 Population of *L. monocytogenes* (log CFU/g) determined on fresh-cut apple plugs soon after the treatment (blue column) and after 7 days of storage at 10°C (orange column). Inoculated plugs were dipped in different PGE solutions or in distilled water (control). Furthermore, untreated plugs were also used as control. Bars indicate standard errors of the means. For each assessment time, different columns with different letters indicate significant differences between treatments according to Tukey's test ($P < 0.05$).

Effect PGE treatments on *L. monocytogenes* population on fresh-cut pear

Soon after the inoculation, the population of *L. monocytogenes* on untreated pear plugs and on plugs dipped in water was estimated at approximately 6.40 log CFU/g (Fig. 2). On these samples, the bacterium greatly proliferated reaching approximately 8.50 log CFU/g, after seven days of cold storage.

Except of PGE-1.2, all other PGE treatments significantly reduced the populations of *L. monocytogenes*, soon after the treatment (Fig. 2). In particular, PGE-12, PGE-2.4, and a-PGE12 reduced the bacterium by 1.89, 1.08, and 1.22 log units, respectively. However, after 7 days of cold storage, only PGE-12 significantly reduced the growth of the bacterium with a reduction of 1.53 log units compared to untreated fresh-cut pear.

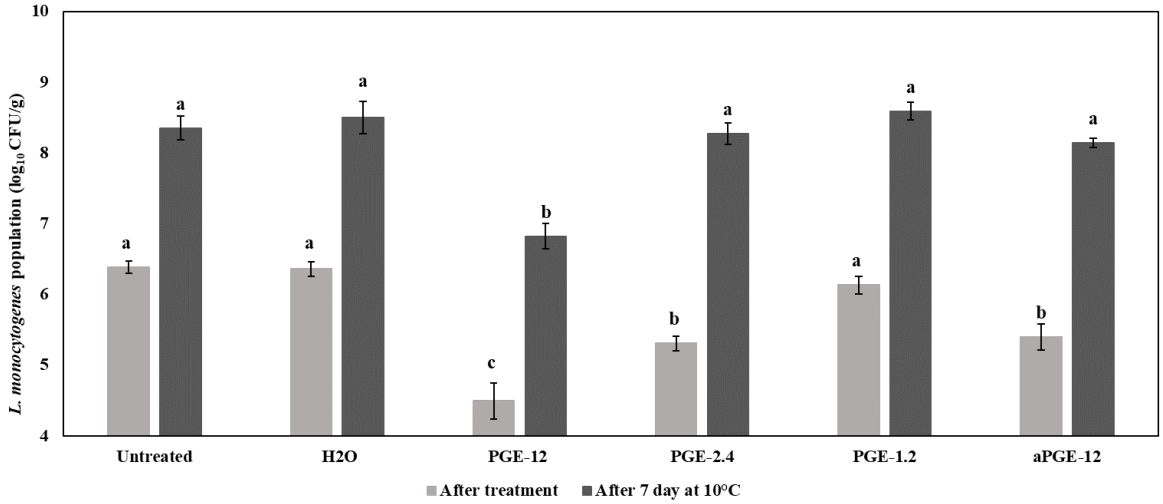

Fig. 2 Population of *L. monocytogenes* (log CFU/g) on fresh-cut pear plugs soon after treatments (blue column) and after 7 days of storage at 10°C (orange column). Inoculated plugs were dipped in different PGE solutions or in distilled water (control). Furthermore, untreated plugs were also used as control. Bars indicate standard errors of the means. For each

assessment time, different columns with different letters indicate significant differences between treatments according to Tukey's test ($P< 0.05$).

Effect of PGE on *L. monocytogenes* population on fresh-cut melon

Soon after the inoculation, the population of *L. monocytogenes* on untreated and water treated melon plugs was 6.9 and 6.74 log CFU/ml, respectively. On both samples, the bacterium population increased during the storage at 10 ºC, reaching approximately 9.0 log CFU/g (Fig. 3).

Soon after treatments, a significant reduction of *Listeria* population was achieved with all PGE treatments except of PGE-1.2. In particular, compared to untreated fresh-cut melons, PGE-12, PGE-2.4 and aPGE-12, reduced the bacterial population by 0.91, 0.58, and 0.85 log units, respectively.

After 7 days of storage, the bacterium population on melon plugs was reduced by 2.99, 1.93, and 1.88 log units with PGE-12, PGE-2.4 and aPGE-12, respectively. However, PGE at the lowest concentration (1.2 g/l) did not show any significant effect.

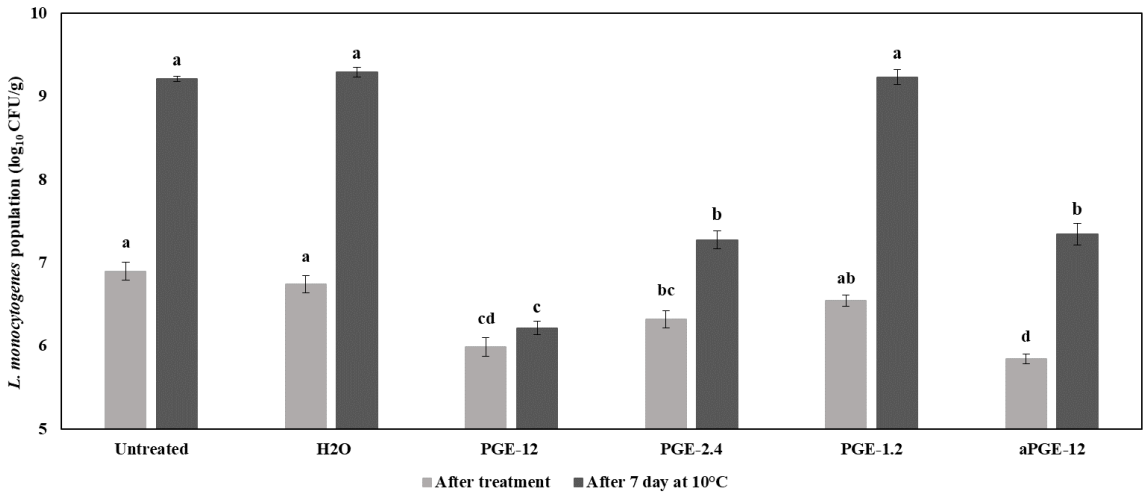

Fig. 3 Population of *L. monocytogenes* (log CFU/g) on fresh-cut melon plugs soon after treatments (blue column) and after 7 days of storage at 10°C (orange column). Inoculated plugs were dipped in different PGE solutions or in distilled water (control). Furthermore, untreated plugs were also used as control. Bars indicate standard errors of the means. For each assessment time, different columns with different letters indicate significant differences between treatments according to Tukey's test ($P< 0.05$).

Discussion

The present study represents the first investigation of PGE as natural antimicrobial to reduce and control the growth of foodborne pathogens on ready-to-eat fresh-cut fruits. Experiments were conducted using *L. monocytogenes* as a model species in light of its primarily importance as food contaminant, and future investigations will be needed to evaluate the efficacy of PGE against other foodborne microorganisms. Overall, *in vitro* and *in vivo* results showed high bactericidal and bacteriostatic effects of PGE against *L. monocytogenes*. In particular, the *in vitro* results (Table 1) revealed that regardless of the tested concentration, PGE exerted a quick and high significant inhibitory activity against all the *L. monocytogenes* tested strains by reducing their population by at least 3.3 log units after a short contact time (2 minutes). This high antibacterial activity could be explained by the composition of the extract. In fact, PGE is rich in polyphenols (ellagitannins) mainly represented by punicalagins, ellagic acid and its derivatives that have been reported to exert a

strong antimicrobial activity against Gram-negative and Gram-positive bacteria [22]. In any case, the absence of the outer membrane in *L. monocytogenes*, as a Gram-positive bacterium, makes it easier for the extract to alter and, therefore, causing a loss of the bacterium cellular components [23].

The antimicrobial activity of other extracts from pomegranate by-products against a variety of food-borne pathogens including *L. monocytogenes*, *E. coli*, and *S. aureus* has been already reported [22,19,23]. However, PGE, seems to be more effective due to its higher content in polyphenols and, being obtained with food grade solvent, it can be considered a safe and eco-friendly antimicrobial preparation [18,17]. Furthermore, PGE bactericidal activity seems to be stronger comparing to other plant extracts such as cherry pomace and plum extracts [24,25].

PGE also revealed strong bacteriostatic effect and its activity was significantly influenced by the concentration of the extract (Table 2). In particular, the log phases of the tested bacteria strains grown in broth media containing PGE were significantly longer. This delayed response of the growth indicates that PGE can negatively modify the growth environment making it longer for the bacteria to adjust [26]. More importantly, PGE showed high efficiency in reducing the growth rate as well as the maximum growth of *L. monocytogenes*. This effect may be attributed to the richness of the extract in tannins that may combine with proteins and cause their precipitation [22,27]. Likely tannins of the extract may combine with proteins of the bacterial membrane as well as with protein of the culture media forming complexes that lead to the lysis and death of the bacteria. Moreover, the high concentration of polyphenols of PGE causes the decrease of pH gradient around the cell membrane and the increase of its permeability, leading to cell death (Singh et al., 2018).

In vivo results confirmed a strong antibacterial activity of PGE that significantly reduced the bacterial load on fresh-cut apples, pears and melons and was able to maintain the population at low levels throughout the storage period (7 days). However, the reduction of the bacterial population in the *in vivo* experiments was overall lower as compared to *in vitro* conditions. This could be mainly explained by the presence of organic matter as well as to the presence of a solid matrix that increase the bacterial survival and decrease the contact between the treatment and the bacteria [28,29]. For the same reason, a higher concentration of PGE seems to be needed to control the bacterium in practical *in vivo* conditions as confirmed by the low efficacy of the lowest tested concentration of PGE (1.2 g/L). Interestingly, aPGE-12 (pH4.4) showed a similar efficacy as compared to normal PGE-12 (pH 2.7), both soon after treatments and after 7 days of storage at 10 ºC. This result confirms that the composition of PGE, rather than its low pH, was the main determinant factor for its activity. In this context, the higher concentration of polyphenols compared to other plant extracts, make pomegranate peel extracts particularly promising for future applications especially that it already proved major beneficial effects on human health [30,31]. However, the slight lower efficacy of aPGE-12 may suggest a secondary role of pH in modulating the level of efficacy, due to the influence of pH on chemical structure and functions of polyphenols. This aspect needs to be taken into account in future applications and/or in the development of commercial formulations. On a practical point of view, PGE seem to provide higher levels of reductions of *L. monocytogenes* populations compared to other widely used alternative compounds. For instance, on fresh cut apples, PGE proved higher efficacy comparing to vanillin, citrox, hydrogen peroxide and peroxyacetic acid that are generally recognized by the scientific community as effective sanitizers of fresh-cut fruits [32]. In particular, after storage, the microbial reductions observed with PGE treated apple plugs were almost double the reduction obtained after treatment with hydrogen peroxide [32].

Although the impact of PGE on the sensory quality of the fruits still needs to be evaluated, available data suggest a high potential of the extract as a natural antimicrobial against foodborne pathogens. Furthermore, the antimicrobial activity of PGE and/or its spectrum of activity could be further enhanced by combining it with other alternative control means.

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Compliance with ethical standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Compliance with ethics requirements: This article does not contain any studies with human or animal subjects.

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