THE EFFECT OF HIGH PRESSURE PROCESSING ON INACTIVATION OF LISTERIA MONOCYTOGENES IN POMEGRANATE ARILS

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ABSTRACT

The pomegranate fruit and its seeds have become increasingly popular due to their high nutritional and antioxidant properties. There is an increase in consumer preference for minimally or non-thermally processed food that is nutritious and safe, with an extended shelf life and fresh-tasting sensory properties. High Pressure Processing (HPP) is a method of non-thermal processing and this study evaluates its effectiveness at 40,0000PSI (~276MPa), 60,000PSI (~414MPa) and 85,000PSI (~586MPa) for 1 and 3 minutes, with and without pomegranate juice, on inactivation of *Listeria monocytogenes* in pomegranate seeds (arils). Physicochemical characteristics of the arils like color, texture, total soluble solids (Brix), water activity (A_w) and pH were also evaluated.

The highest log reduction of *Listeria monocytogenes* after high pressure processing of pomegranate arils was 7 log and pressure treatments were enough to keep *Listeria monocytogenes* below detection limits after cold storage for 45 days. With the exception of 60K1NJ, HPP had similar treatment effects at 60K and 85K with higher log reduction compared to 40K. Effect of treatment at 60K1NJ was reduced because holding time was shorter and there was no pomegranate juice added. There was increased log reduction at 40K3J but little effect at 40K1J, 40K1NJ and 40K3NJ. This suggests that HPP inactivated *Listeria monocytogenes* at 85K but was not as effective at 40K ($p<10^{-7}$). The addition of pomegranate juice and longer holding time (3min) during pressure treatment influenced log reduction and this explains the higher log reduction at 40K3J even at a lower pressure. Treatment at 40K3J had similar effect as treatment at 60K and 85K.

Inactivation of background microflora increased as pressure increased and at the end of the 45-day storage period, there were no detectable counts. At treatment of 85K background microflora were inactivated in aril samples immediately after treatment but the effect was not as

effective at lower pressure of 40K. For example log reduction of background microflora in treated samples at 85K1J immediately after treatment was 6.7 log compared to 0.09 log at 40K1J ($p<2x10^{-7}$). Highest log reduction of 7.12 log was observed at 85K3NJ and addition of pomegranate juice and longer holding time (3min) influenced inactivation rates.

Yeasts and molds were very sensitive to pressure treatment and were inactivated at 40K. The lowest pressure, shorter holding time without pomegranate juice was 40K1NJ, however, there were no detectable yeast and mold colonies in aril samples immediately after this treatment. Samples exposed to other treatment conditions did not have any detectable colonies either.

pH, Brix , water activity were not affected by HPP. After treatment at 85K with pomegranate juice, the color of pomegranate arils became brighter and more appealing. However, there were changes in texture and the treated samples were softer after cold storage for 14 days.

Keywords: High Pressure Processing, *Listeria monocytogenes*, pomegranate arils, shelf life, physicochemical parameters

BIOGRAPHICAL SKETCH

Afua Owusu acquired her Bachelor's degree in Biochemistry at Kwame Nkrumah University of Science and Technology in Kumasi, Ghana in 1999. She worked as a food regulatory officer at Food and Drugs Authority, a government regulatory agency that regulates food, drugs, cosmetics, household chemicals and medical devices. She worked with the Food Enforcement Department as the head of food manufacturing facilities inspections unit. In 2019, she acquired her Master of Professional Studies (MPS) in Food Science with a specialization in food safety at Cornell University.

Dedicated to my children: Derrick, Karen and Kendra

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LIST OF SYMBOLS

40K -40,000PSI

60K -60,000PSI

80K -80,000PSI

w/J or J –arils samples with pomegranate juice

w/o J or NJ –aril samples without pomegranate

INTRODUCTION

Pomegranate Arils

Pomegranate (*Punica granatum*) is a shrub that produces red, round fruits. The skin of the fruit is tough and leathery, making it inedible. The soft inner part of the pomegranate fruit called arils, are edible and represents 50–70 % of the total weight of the fruit (Safa and Khazaei 2003). Arils are composed of 78% juice and 22% seed (Kurkarni & Aradhya, 2005). The chemical components of arils include 10 % sugar (mainly fructose and glucose), 1.5 % organic acids (principally ascorbic acid, citric, and malic acid) and bioactive compounds such as anthocyanins and other phenolic compounds (Safa and Khazaei 2003). [Pomegranate juice is produced by extracting the juice from the arils.] Pomegranate juice contains a considerable amount of total soluble solids, sugars, anthocyanin, polyphenols, ascorbic acid and proteins; it is also a rich source of antioxidants (Gil, Tomas-Barberan, Hess-Pierce, Holcroft, & Kader, 2000). Earlier studies conducted (Hertog and others, 1992; Lansky and others, 1998) have shown that this rich source of anthocyanin gives pomegranate chemo preventive properties like antimutagenicity, antihypertensive, antioxidative potential and reduction of liver injury. There is a lot of interest in pomegranate because it has great benefit in the human diet as it contains several groups of substances that are useful in disease risk reduction. However, consumption of pomegranate is limited by the difficulty in the extraction of arils from the fruit and the tendency of the fruit to be physically damaged. Pomegranate is very sensitive to sunburn, cracking, cuts, or bruises in the husk, as well as to chilling injury (Artes et al. 2000). These external defects render fruits with excellent internal quality unsuitable for fresh marketing and the fruits end up being diverted for industrial use or animal feed. The processing of the externally damaged pomegranates could be an excellent way to obtain a commercial profit from discarded pomegranate fruits (Lopez et al. 2005).

However, maintaining the nutritional and microbial quality of pomegranate arils is a major challenge as minimally processed arils have a short shelf life and easily deteriorate in texture, color, and overall quality(Caleb &Opara. 2012).

Minimally processed fruit can be stored for up to 14 days at 7°C without compromising quality (Kader, 2006). Lopez-Rubira,Conesa, Allende, and Artes (2005) were able to achieve a longer shelf life of pomegranate arils through the use of controlled atmosphere storage (CA). Shelf life extension of pomegranate arils has been limited to a few postharvest technologies, for example, Modified Atmosphere Packaging or MAP (Gil, Artes, & Tomas-Barberan, 1996; Sepulveda et al., 2000), CA (Holcroft, Gil, & Kader, 1998), the use of antioxidants (Gil et al, 1996; Sepulveda et al., 2000), honey coating (Ergun and Ergun, 2009), waxing (Waskar et al, 1999), film wrapping (Nanda et al, 2001; D'aqino et al, 2010), thermal treatments (Artes et al, 1998,2000; Mirdehghan et al, 2007).

Production of safe, ready-to-eat pomegranate arils with desirable sensory properties would increase the demand for pomegranate by consumers (Ghasemnezhad, Zareh, Rassa, & Sajedi, 2013). Food scientists and the food industry are therefore searching for novel methods, which can destroy undesirable microorganisms but have less adverse effects on product quality (Varela-Santos, Ochoa-Martinez,Tabilo-Munizaga, & Reyes,2012).

High Pressure Processing (HPP)

High-pressure processing (HPP), also known as high hydrostatic pressure (HHP), is a relatively new, nonthermal food processing method that subjects foods (liquid or solid) to pressures between 50 and 1000 MPa. (Considine,Kelly,Fitzgerald, Hill,& Sleator, 2008). HPP treatment has enabled the consumer to access foods with distinct advantages over thermally processed foods, such as minimally processed, fresh-tasting, high-quality convenient products

with an extended shelf life. (Considine et al, 2008). During HPP, food is exposed to pressures between 100 to 600 MPa, normally using water as a pressure-transmitting medium. The pressures applied to foods being processed is transmitted isostatically and uniformly. Unlike in thermal processing, food is treated evenly in HPP regardless of the shape or size of the food (Smelt, 1998, Considine et al, 2008) (Considine et al, 2008). Pressurization of liquid or solid foods at room temperature is usually accompanied by a moderate temperature increase (*c*. 5–15°C), termed adiabatic heating, depending on the food composition (Balasubramanian & Balasubramaniam, 2003). Foods cool down to their original temperature on decompression provided no heat is lost or gained during the pressure hold time (Hogan, Kelly & Sun,2005). Previous studies demonstrated the effectiveness of HPP in maintaining the safety and quality of foods. Houska et al.(2006) have reported that high pressure pasteurization process (500 MPa for 10 min) is capable of inactivating more than 5 log orders of the viable microorganisms (coliform bacteria, yeast, molds and salmonella) present originally in raw broccoli juice, and during the 30 days of storage at chilled room temperature conditions (temperature up to 5°C), there were no detectable colonies. At room temperature, HPP in the range of 300-500 MPa reduces the enzymatic activity and has minimal effects on taste and color molecules(Laboissière et al., 2007). HHP in combination with packaging of good barrier properties can prevent browning in minimally processed products during storage in the sealed pack (Perera, Gamage, Wakeling, Gamlath, & Versteeg, 2009). HPP, as a clean label technology, has found a growing acceptance in the food industry for producing high-quality foods. (Sonaliben, Parekh, Aparnathi, & Sreeja, 2017).

Listeria monocytogenes

Listeria genus includes Gram-positive, short rods with 0.4–0.5 μm in diameter and 1.2 μm in length, usually appearing as single cells or in short chains. It typically stains as gram-positive,

yet cells can lose their ability to retain stain with time. Belonging to *Listeriaceae* family and Bacillales order, Listeria are closely related to *Bacillus* and *Staphylococcus*. (Ludwig, Schleifer, & Whitman, 2009; Sallen, Rajoharison, Desvarenne, Quinn, & Mabilat, 1996)

Given their ubiquity, these bacteria have been isolated from a range of sources that include soil, vegetation, sewage, poultry, dairy products, seafood, plants, water, meat, feces, and decaying vegetables, which are their natural habitats as saprophytes. (Ludwig et al, 2009; Weis & Seliger,1975). *Listeria* can also be found in facilities and equipment involved in food processing and storage, due to the ability to form biofilms on surfaces. (Valderrama & Cutter 2013). Of the 10 species that are known for this genus, *L. monocytogenes* responsible for the majority of listeriosis cases in humans (Lakićević, Katić, Lepšanović, Janković, Rašeta, 2014). The severity of listeriosis can range from mild gastroenteritis to severe disease conditions (septicemia, encephalitis, meningitis, abortions and stillbirths) and can result in high fatality in immune-compromised populations (Zhu, Gooneratne, & Hussain,2017). The Center for Disease Control (CDC) estimated that about 1600 people become seriously ill with listeriosis each year in the United States alone, of which about 260 die (CDC,2019). In a 2014 multistate outbreak in the US, thirty-five people including 11 pregnant women were diagnosed with listeriosis after consuming caramel apples. Seven people died in this outbreak. The environmental testing at the company's apple packing facility confirmed the presence of the pathogen. *L. mono*cytogenes can survive for long periods of time in a seemingly inhospitable environment such as a food processing facility due, in part, to its ability to resist various stresses (Moorhead and Dykes, 2004; Zhang et al., 2011) and its ability to form biofilm (Latorre et al., 2010; Cruz and Fletcher, 2011). Since *L. monocytogenes* is ubiquitous in the environment, safe practices and conditions should be prevalent in food processing facilities in order to prevent infection to humans through food vehicles.

The objective of this study was to evaluate the effectiveness of HPP on the inactivation of *L. monocytogenes*, shelf life extension, and physicochemical characteristics (color, texture, total soluble solids or Brix, water activity, and pH) of the pomegranate arils. The following HPP parameters were evaluated in this study: pressure (40000, 60000, and 85000 PSI), holding time (1 and 3 minutes), and addition of pomegranate juice to the arils.

MATERIALS AND METHODS

Proof of Concept

To determine whether the arils could withstand the high pressure treatment without significant structural changes, samples of arils were subjected to treatment under the highest pressure/time combination (85,000PSI for 3 minutes). Based on the results of these preliminary experiments, the rest of the samples were exposed to the different pressure treatments.

Strain characterization and Inoculum preparation

A five-strain mixture of *Listeria monocytogenes* (FSL J1-103, FSL J1-109, FSL R9-0506, FSL R9-5411 and FSL R9-5506) was used in the study (see Table 1). The diversity of strains was to eliminate differences in pressure resistance among strains. Stock cultures of each strain were maintained in tryptic soy broth (TSB, Becton Dickinson, US) supplemented with 15% glycerol and stored at −80 °C. Starter cultures of each strain were prepared by inoculating individual culture tubes containing tryptic soy broth and incubating on a rotary shaker (200 rpm) for 24 hours at 37ºC. 5 ml aliquots of each strain (in early stationary phase) were combined to prepare the inoculum. Serial dilutions were prepared with 0.1% sterile peptone water to achieve initial target populations.

Table 1 Strains characterization of *L.monocytogenes*

Strain	Source	Serotype
FSL J1 - 103	Human	1/2b
FSL J1 - 109	Human	4b
FSL R9 - 0506	Cantaloupe	1/2a
FSL R9 - 5411	Caramel apples	1/2b
FSL R9 - 5506	Produce	4a

Aril preparation and inoculation

Pomegranate arils (*Punica granatum*), weighing about 1kg and packaged in two high density polyethylene bags were shipped under preservation of cold chain at 4ºC. They were kept under similar refrigerated conditions, inoculated and HPP-treated 48 hours after they were received. One bottle (16.0 fl oz) of POM Wonderful 100% Pomegranate juice was purchased from a local grocery store for the study. Twenty five (25) grams each of the arils were weighed out into twelve 24 oz. high density polyethylene bags and 25 ml of pomegranate juice was added to 6 of these bags. The other six did not have juice and all the bags were labeled to indicate their respective treatment parameters (Table 2)

Aril samples were inoculated with an equalized pool of the five-strain mixture of *Listeria monocytogenes*; 0.5ml of the appropriate dilution of combined 24-hour culture was homogeneously distributed over each 25gram sample of arils without juice to achieve an initial population density of ~7 log CFU/g and 1ml homogeneously distributed over aril samples (with juice). The relatively high concentration was chosen to achieve a bacterial load reduction of 5 log CFU/g (99.999% reduction). The samples were inoculated as quickly as possible to minimize errors due to change in temperature. After inoculation, all samples were mixed thoroughly in a standardized manner for about a minute to optimize the distribution of the inoculum. The bags with inoculated samples were sealed with an induction sealer. Bags exposed to similar pressures

and time points (e.g. 40,000 PSI, 1 minute with juice) were sealed together in larger bags into which 0.5% bleach solution was poured.

High Pressure Treatment

The packaged samples were placed in a multilayered steel vessel (Hiperbaric 55) with a capacity of 55 L and a diameter of 200 mm, pressurized as indicated in Table 2. The pressure-transmitting medium was water at the approximate temperature of 5°C. Pressurization was achieved in approximately 1 minute and depressurization took less than 5 seconds. Once the samples were pressurized, they were stored at 4°C until further analysis. The treated arils were stored under refrigerated temperature without agitation throughout the experiment. Refrigeration temperatures were monitored for highs and lows by sensors to ensure that treated samples within 4±1ºC.

Table 2 HPP conditions

Pressure (PSI)	Time (minute)	Juice/ No Juice	Label
40,000	1	Juice	40K1J
		No Juice	40K1NJ
	3	Juice	40K3J
		No Juice	40K3NJ
60,000	1	Juice	60K1J
		No Juice	60K1NJ
	3	Juice	60K3J

		No Juice	60K3NJ
85,000	1	Juice	85K1J
		No Juice	85K1NJ
	3	Juice	85K3J
		No Juice	85K3NJ
No HPP Treatment		Juice	Control w/ Juice
		No Juice	Control w/o Juice

Microbial Analysis

Microbiological analyses were carried out immediately after HPP ("day 0") and then on days 2, 7, 14, 21, 28, 35 and 45 for each pressure treatment. In addition to *L.monocytogenes*, total aerobic mesophilic bacteria, yeasts and molds counts were enumerated at a typical refrigeration temperature. For the microbiological analysis, 25 grams of each sample was aseptically transferred into a sterile stomacher bag, diluted with 225 mL of 0.1% sterile peptone water and homogenized in a Stomacher® 400 Circulator (Seward, England) at 200 rpm for 90 seconds. Serial dilutions were prepared from the homogenate using 0.1% peptone water. A 1- ml aliquot of each dilution was plated in duplicate using the pour-plate technique. For each treatment condition,, four dilutions were plated on each of the following growth media:

1. PALCAM with PALCAM selective supplement (Millipore, EMD Millipore Corp, 290 Concord Rd, Billerica, MA, 01821) for *L.monocytogenes* (aerobic incubation for 48 h at 37 °C)

2. Plate count (PC; Hardy Diagnostics, CA,USA) for total aerobic mesophilic bacteria (aerobic incubation for 48 h at 30 °C)
3. Potato Dextrose Agar with Tartaric Acid pH 3.5 (PDA; Hardy Diagnostics, CA, USA) for yeasts and molds (anaerobic incubation for 48 h at 37 °C). For each pressure/time combination, four dilutions were plated for all three media.

After incubation, colonies were counted using a manual colony counter (Quebec colony counter). sampling continued for 45 days. Microbial counts in plates containing between 15 and 300 colonies were recorded. The average of the duplicate plates were calculated and expressed as log CFU/g. The limit of detection was 1 log CFU/g. If no colonies were detected or the counts fell below the limit of detection, an arbitrary value of 0 log CFU/g was assigned for graphing the data.

Physico-chemical Analysis

pH, water activity (a_w), color and texture of all samples were determined immediately after treatment (Day 0) and on Day 14 after the inoculation. A_w was measured using AQUALAB water activity meter 3TE (METER Food, Munich, Germany). pH was measured using a Hanna pH meter. Texture was analyzed with a Brookfield CT3 analyzer.

Color was analyzed using a Hunter ColorFlex EZ (Hunter Associates Laboratory Inc, Virginia, USA) fitted with a 2.5-cm diameter aperture. The instrument was calibrated using the black and white tiles provided. Color was expressed in Hunter Lab units L*(lightness/darkness), a*(redness/greenness) and b*(yellowness/blueness). Samples of pomegranate arils were filled into glass dishes and placed under the aperture of the colorimeter. The measurements were taken and recorded and total color difference (ΔE) was calculated using the following equation:

$$\Delta E = \sqrt{(a^* - a_o^*)^2 + (b^* - b_o^*)^2 + (L^* - L_o^*)^2}$$

where L_o^*, a_o^*, b_o^* are the control values

Statistical Analysis

Analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test was used to compare mean log reductions across treatment groups at a significance level of 0.05. All statistical tests were run using a statistical software R version 3.5.3.

RESULTS AND DISCUSSION

Results from proof of concept experiment

In this study, the pomegranate arils exposed to the highest pressure of 85K PSI for 3 minutes remained intact on observation. A previous study found that the physical structure of most high-moisture food products remains unchanged after HPP exposure as the pressure exerted does not generate shear forces (Hogan et al., 2005). For the treated samples containing both pomegranate arils and juice, the juice had penetrated the arils through the porous membranes (Fig 1C, 1D). This change occurred because there is an increased permeability in the pressurized cells during HPP (Jun et al., 2009).

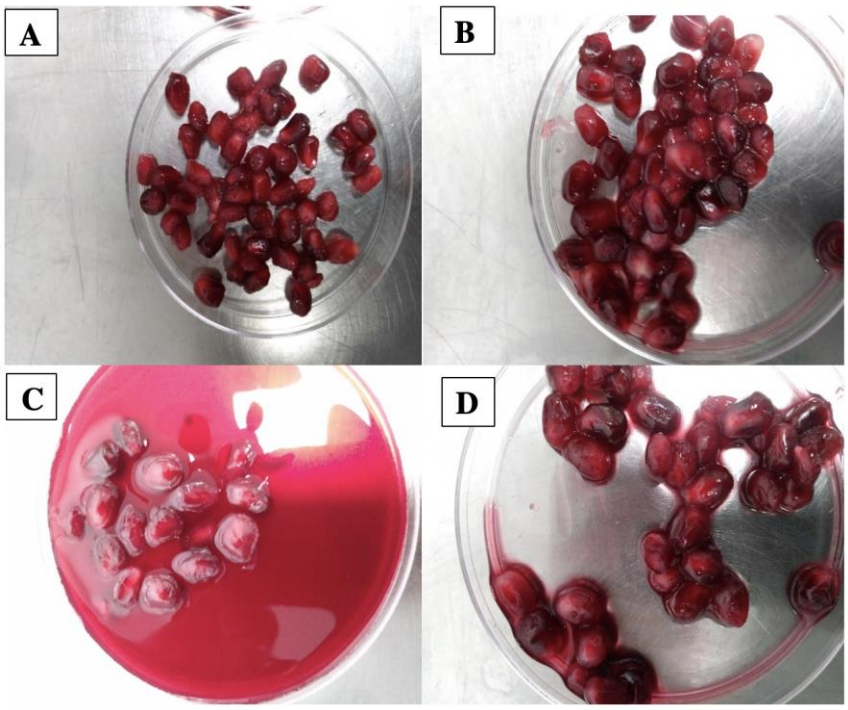


Fig 1
(A) Control w/o juice
(B) 85K3NJ
(C) Control w/ juice
(D) 85K3J

Effect of HPP treatment on microbial behavior, shelf life extension and *Listeria monocytogenes* inactivation in aril samples

The mean initial population of *L.monocytogenes* in Control w/ Juice and Control w/o Juice were 6.7 and 7.07 log CFU/g, respectively. Table 3 shows the changes in *L.monocytogenes* counts in treated samples during storage at 4 °C. *L.monocytogenes* counts immediately after treatment is shown in Fig 2A & 2B. Colonies were detected in samples treated at 40K1NJ, 40K3NJ and 60K1NJ. Samples treated at 40K1NJ was the only treatment with *L.monocytogenes* counts beyond day 0. Samples of 40K1J had 0.8 log reduction of *L.monocytogenes* immediately after treatment (Fig 2B), however, there was inactivation during storage and there were no detectable colonies after 45 days in cold storage. The highest log reduction was ~7 log at 60K3NJ and 85K3NJ and there were no detectable colonies after storage period (Fig 3 top). There were no detectable colonies of *L.monocytogenes* at 40K3J, 60K1J, 60K3J, 85K1J, 85K1NJ and 85K3J immediately

after treatment and during storage. Fig 4 shows that HPP had similar effects on inactivation of *L.monocytogenes* at 40K3J, 60K1J, 60K3J, 85K1J and 85K3J. This observation is consistent with previous studies on HPP effects on log reduction of some *Listeria spp*. Evrendilek and Balasubramaniam (2011) observed over 5-log reduction of *L.monocytogenes and L.innocua* in ayran (savory yoghurt drink) treated at ~87,000 PSI for 5 minutes at ambient temperature. A study by Chen, Neetoo, Ye and Joerger (2009) also demonstrated reductions ranging from 1.9 to 7.1 log (10) CFU/ml in tryptic soy broth with 6% yeast extract (TSBYE) of *Listeria monocytogenes* during screening for their pressure tolerance phenotype at 400 MPa for 2 min at 21 degrees C.

Background microflora, yeast and mold inactivation in aril samples after HPP treatment

Many countries demand that minimally processed or fresh cut produce not carry more than 7-log CFU/g aerobic bacteria (Ergun, M., 2010). Long term storage of pomegranate fruit is limited by spoilage that could be caused by yeasts, molds or other spoilage bacteria. Due to sub-lethal stress or injury to bacteria from exposure to pressure, non-selective microbiological medium was used to allow detection of all viable organisms during post treatment storage. Table 4 shows the changes in counts of background microflora in treated samples during storage at 4 °C. The mean initial population of background microflora in Control w/Juice and Control w/o Juice were 6.75 and 7.12 log CFU/g, respectively. Background microflora were detected immediately after treatment at 40K1NJ, 40K1J, 40K3NJ, 40K3J, 60K1NJ and 60K3NJ. HPP had similar effect on treatment at 40K1NJ, 40K3NJ and 40k1J as shown in Fig 3(bottom) and the highest log reduction was 7.12 log at 85K3NJ treatment (Fig 3A). There were no detectable background microflora at this treatment.

The main indicator of spoilage or deterioration in fruits and vegetables are yeasts and molds which are generally not associated with food borne illnesses. In the present study, yeasts and molds were under the limit of detection in every treatment on day 0 and throughout the storage period. Table 5 shows the changes in yeast and mold counts in treated samples during storage at 4 °C. This is consistent with earlier findings that yeasts and molds are relatively sensitive to HPP. Hite and others (1914) concluded from previous studies that because yeasts and other spoilage organisms were susceptible to pressure, especially in acidic foods, high pressure treatment was one of the most effective ways to extend the shelf life of fruits and fruit juices (Patterson, 2005). Landl, Abadias, Sárraga, Viñas, and Picouet (2010) demonstrated 3.2-log reduction in yeasts and molds in apple puree under HPP treatment of 400-600 MPa 15 min 20◦C. Most vegetative species are inactivated within a few minutes when pressurized at 300-400MPa at room temperature. Varela-Santos et al (2012) found in a study that during HPP of pomegranate juice, applying pressure of 350 MPa for 30s led to 1.8 log reduction and 2.1 log reduction in aerobic mesophilic bacteria and yeasts and molds respectively. These findings led to other successful experiments that achieved 4-log reductions at 400MPa for 5min.

Table 3 *Listeria monocytogenes* Count (log CFU/g) in HPP-treated Pomegranate Arils

Treatment	**Day 0**	**Day 2**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**	**Day 45**
40k1J	5.9	0	0	0	0	0	0	0
40k1NJ	6.8	6.09	4.4	4.54	2.95	2.78	2.35	0
40k3J	0	0	0	0	0	0	0	0

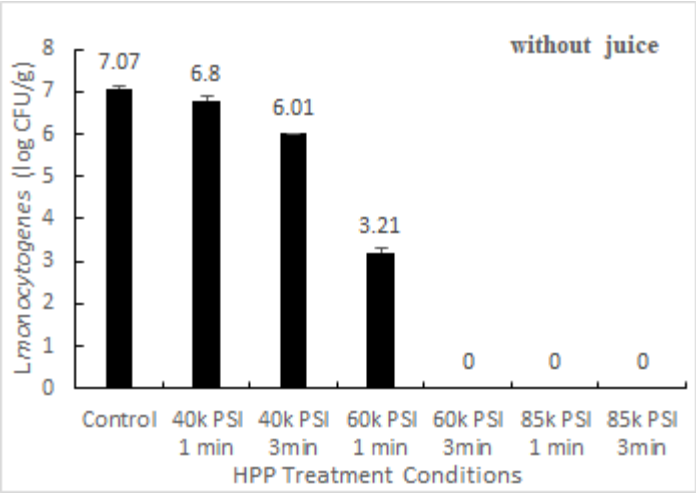
40k3NJ	6.01	0	0	0	0	0	0	0
60k1J	0	0	0	0	0	0	0	0
60k1NJ	3.21	0	0	0	0	0	0	0
60k3J	0	0	0	0	0	0	0	0
60k3NJ	0	0	0	0	0	0	0	0
85k1J	0	0	0	0	0	0	0	0
85k1NJ	0	0	0	0	0	0	0	0
85k3J	0	0	0	0	0	0	0	0
85k3NJ	0	0	0	0	0	0	0	0

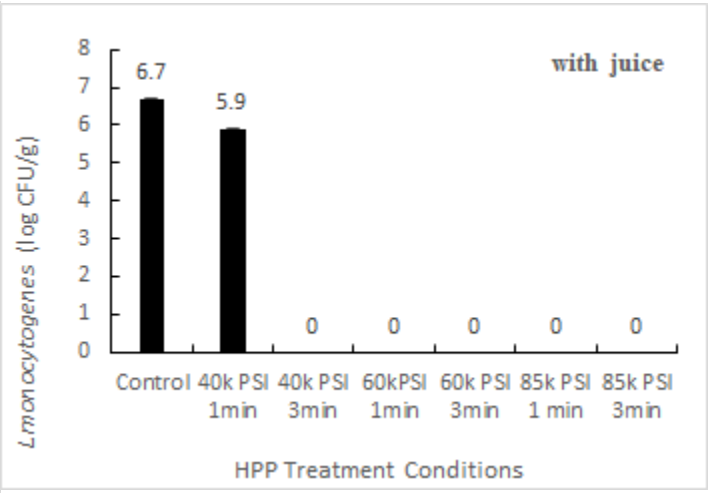
Table 4 Background Microflora Count (log CFU/g) in HPP-treated Pomegranate Arils

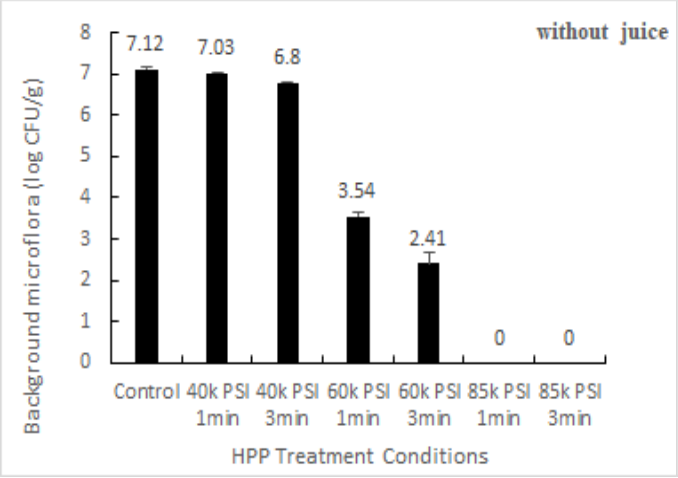
Treatment	**Day 0**	**Day 2**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**	**Day 45**
40K1J	6.66	5.76	2.04	3.73	3.4	0	2.38	2.08
40K1NJ	7.03	7.06	7.17	6.74	6.65	7.8	8.02	8.45
40K3J	4.41	3.62	2.13	0	0	0	0	0
40K3NJ	6.8	6.76	6.17	5.79	5.43	5.79	3.88	5.02
60K1J	0	2.08	0	0	0	0	0	0
60K1NJ	3.54	3.97	3.18	2.64	2.46	2.47	0	0
60K3J	2.61	0	0	0	0	0	0	0
60k3NJ	2.41	5.28	0	0	0	0	0	0
85k1J	0	2.54	0	0	0	0	0	0
85k1NJ	0	3.29	0	0	0	0	0	1.4
85k3J	0	0	0	0	0	0	0	0
85k3NJ	0	0	0	0	0	0	0	0

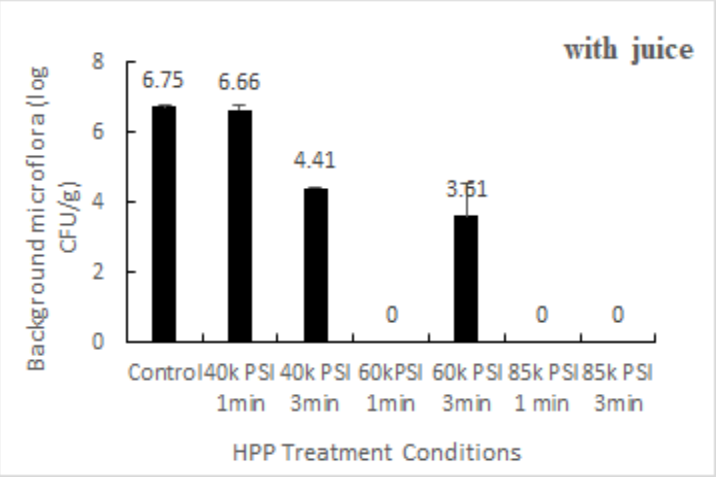
Table 5 Yeasts and Molds Count (log CFU/g) in HPP-treated Pomegranate Arils

Treatment	Day 0	Day 2	Day 7	Day 14	Day 21	Day 28	Day 35	Day 45
40k1J	0	5.34	0	0	0	2.04	2.67	0
40k1NJ	0	5.43	0	0	0	6.74	7.49	8.02
40k3J	0	2.96	0	0	0	0	0	2.23
40k3NJ	0	3.42	0	0	0	0	2.39	3.85
60k1J	0	0	0	0	0	3.02	0	0
60k1NJ	6.01	3.54	0	0	0	2.54	0	0
60k3J	0	2.85	0	0	0	0	0	0
60k3NJ	0	0	0	0	0	0	0	0
85k1J	0	0	0	0	0	0	0	0
85k1NJ	0	0	0	0	0	0	0	0
85k3J	0	2.54	0	0	0	0	0	0
85k3NJ	0	0	0	0	0	0	0	0









(Top Left)Fig 2A *L.monocytogenes* survivors in arils without juice immediately after treatment compared to untreated
(Top Right)Fig 2B*: L.monocytogenes* survivors in arils with juice immediately after treatment compared to untreated control
(Bottom Left)Fig 2C-Background microflora survivors in arils without juice immediately after treatment compared to untreated control
(Bottom Right) Fig 2D- Background microflora survivors in arils with juice immediately after treatment compared to untreated control

Holding time during HPP treatment

The holding time is one of the most important parameters of high pressure, considering that there is a direct relation between the time during which pressure is applied and the inactivation factor (Ferreira, Almeida, Delgadillo, Saraiva, & Cunha, 2016). When aril samples were treated at 40K1J, log reduction in *L.monocytogenes* was 0.8 log in day 0. However, at 40K3J, when holding time is increased to 3mins (Fig 3) there is a significantly increased log reduction of 2.37 log ($p<10^{-7}$). Also, there was a log reduction of 3.8 and 7.07 ($p<10^{-7}$) in *L.monocytogenes* in

samples treated at 60K1NJ and 60K3NJ respectively indicating that log reduction increased with holding time. A similar trend in log reduction of background microflora was observed when samples are treated at 40K1J.(Fig 3D). Immediately after treatment, log reduction was 0.09 log on day 0 in treated samples. However, at 40K3J, an increased log reduction of 2.37 log was achieved. These results demonstrated the influence of holding time during HPP on pomegranate arils and shows higher inactivation of *L.monocytogenes* in pomegranate arils can be achieved by increasing holding time when all other parameters(pressure,with/without juice) are kept constant. In a study to determine the effectiveness of HPP in activation of *L. monocytogenes* in fruit extracts, Barba, Criado, Belda-Galbis., Esteve and Rodrigo (2014) demonstrated that an increase in holding time from 5 to 15 mins resulted in enhanced inactivation of ~5 log . Similarly, an increase from 5 to 30 minutes at 350 MPa caused a greater *L. innocua* inactivation in poultry meat of ~5 log. (source?)

Addition of Pomegranate Juice to Arils

The inactivation of *L. monocytogenes* can be conditioned by the overall chemical composition of the food product and by particular growth factors such as pH or water activity (Ferrira et al, 2016). In general, microorganisms are more sensitive to pressure and the survival of pressured damaged cells is reduced in lower pH environments (Smelt, 1998). Immediately after treatment (day 0), samples treated at 60K1NJ had 3.86 log reduction in *L.monocytogenes*. However, samples treated at 60K1J had 6.7 log reduction ($p<10^{-6}$) and there were no detectable colonies after that treatment. In addition, samples treated at 40K3J had 6.2-log reduction in *L.monocytogenes* and 40K3NJ had 1.05-log reduction ($p< 10^{-7}$). The results suggested that higher inactivation of *L.monocytogenes* in pomegranate arils can be achieved by adding pomegranate juice. This observation could be explained by

1. Microbial inactivation is enhanced in fruit juices due to the low pH. Dugan and Erkman (2004) observed an improvement in the inactivation of *L. monocytogenes* in orange and peach juices compared to when the inactivation medium was brain-heart infusion broth (BHI) or milk. Higher inactivation of *L. monocytogenes* was also observed when pH was reduced from 7 to 4 and when a combination of pH and different concentrations of solutes were used (Koseki and Yamamoto, 2006)

2. In a study, Haymen et al (2008) postulated that a low water activity results in protein stabilization, which prevents protein denaturation and cell death during HPP.

Aril samples with low a_w (without juice) seem to have more baroprotective effect on microorganisms than those without juice. A low water activity protects microorganisms against the effects of pressure (Palou, Lopez-Malo, Barbosa-Canovas, Welti-Chanes, & Swanson, 1997). Oxen and Knorr (1993**)** reported that reducing a_w of the medium from 0.98–1.0 down to 0.94–0.96 resulted in a better survival of *Rhodotorula rubra* when it was subjected up 200–400 MPa for 15 mins at 25°C. Additionally, the nature of the solute is important. For example, at the same a_w, cells were more sensitive to pressure in glycerol than in monosaccharides and disaccharides. A sodium chloride solution is less protective to *L. monocytogenes* than a sucrose solution adjusted to the same a_w. (Koseki and Yamamoto, 2006).

Pressure variation, pressurization and depressurization

The required pressure treatment for microbiologically safe and stable products is dependent on the target microorganism to be inactivated. Vegetative cells of bacteria, yeasts and molds are sensitive to pressures between 200 and 700 MPa (Varela-Santos et al., 2012). Various factors influence the pressure resistance of microorganisms, including the strain of microorganism and its

physiological state, the intrinsic properties of the menstruum, and the processing temperature, time and magnitude of pressure treatment (Bull M.K et al., 2004).

When aril samples were exposed to pressure of 40,000PSI (40K1J), there was 0.8 log reduction in *L. monocytogenes* immediately after treatment. At 60K1J when arils were treated with pomegranate juice and pressure was increased to 60,000PSI, log reduction was 6.7 log ($p<10^{-7}$). Also, log reduction of *L.monocytogenes* on day 0 after treatment at 60K1NJ was 3.86 log. With increased pressure 80,000PSI and after treatment at 80K1NJ, log reduction was 7.07 log ($p<10^{-7}$). For 1-minute processing of arils without juice, increasing the pressure from 40,000 to 60,000 and 85,000 PSI improved the immediate log reduction of background microflora from 0.09 to 4.58 and 7.12, respectively. At 85,000 PSI, there were no detectable microorganisms in treated samples. Increased pressure treatments reduce survival rates of microorganisms. A study by Huang ,Lung, Yang, and Wang (2014) on the effect of variation in pressure on microorganisms during HPP indicated that increasing pressure to 300MPa or more induces bacterial cell death through denaturation of enzymes and proteins, rupturing of bacterial cell membrane and finally excretion of internal substances. Effects of pressurization and depressurization rates on inactivation of *L. monocytogenes* (Chapleau et al, 2006) and *L. innocua* (Ferreira et al, 2015; Rademacher et al., 2002) have been studied. Chapleau et al (2006) observed the most significant bacteria reduction with fast pressurization/depressurization. In contrast, Ferreira et al. (2015) stated that "the application of a slow pressurization/depressurization led to enhanced inactivation compared with medium or fastest pressurization/depressurization, as already demonstrated for B. subtilis spores. However, at 500 MPa, the concentration of viable bacteria was quickly reduced to levels below the detection limit and no When pressure values of 300 MPa were applied to a fruit extract matrix inoculated with *L. monocytogenes*, an increase in holding time from 5 to 15 minutes resulted in an

enhanced inactivation of ~5 log. differences on inactivation efficiency were detected between different holding times"

Change in *L.monocytogenes* and background microflora counts in HPP-treated Arils During Storage

L. monocytogenes counts were detected on days 0, 2, 7, 14, 21 and 28 in 40K1NJ samples (Fig 4). This treatment was the only treatment condition that had *L. monocytogenes* counts beyond day 0. However, while in storage, there was gradual log reduction until counts fell below the detection limit on day 45. After an initial reduction of 0.27 log compared to control on day 0, an additional reduction of 0.71 log and 1.69 log were observed on day 2 and day 7, respectively. At the end of the storage period, *L.monocytogenes* log reduction of 6.8 log had been achieved in pomegranate arils treated at 40K1NJ.

Background microflora were detected in 40K1NJ, 40K3NJ, 60K1NJ, 60K1NJ and 60K3NJ samples, immediately after treatment and while in storage (Fig 5). For treated arils with juice, background microflora were detected only in samples treated at 40K1J and 40K3J. Of the six (6) treatment conditions that showed background microflora counts while in storage, 40K1NJ samples had the highest microbial counts. For example, on day 0, the microbial count of 40K1NJ was **6.03 log CFU/g** in comparison to **5.8 log CFU/g, 2.54 log CFU/g** and **1.41 log CFU/g** at 40K3NJ, 60K1NJ and 60K3NJ, respectively. There was gradual inactivation of microorganisms while in storage and the highest decrease observed was 4.58-log reduction. A possible reason for this observation could be cold storage of treated samples at refrigerated temperatures that could lead to inactivation because microorganisms can only survive at certain optimum temperatures.

Also, variation in pressure sensitivity of the different microorganisms and recovery from sublethal injury after low-pressure treatment could account for this observation. Most bacteria associated

with fresh fruits and vegetables include *Salmonella* and *Shigella spp*., *E.coli* O157:H7, *Listeria monocytogenes*, *Campylobacter jejuni* and *Yersinia enterocolitica*. Alpas, Kalchayanand, Bozoglu and Ray (2000) observed that different microorganisms have different degrees of resistance to HPP and that there was vast sensitivity to HPP even among bacterial species and strains. Patterson, Quinn, Simpson, and Gilmore (1995) also confirmed from a study that sensitivity of microorganisms was mostly dependent on the medium used. They also observed that growth conditions of the culture and sublethal injury at pressures lower than that required for cell death could also influence bacterial response to HPP treatment.

Low pressure treatment could also trigger germination of bacterial spores. Wuytack, Boven and Michiels (1998) reported that germination of spores could be achieved using both low- and high-pressure treatments.

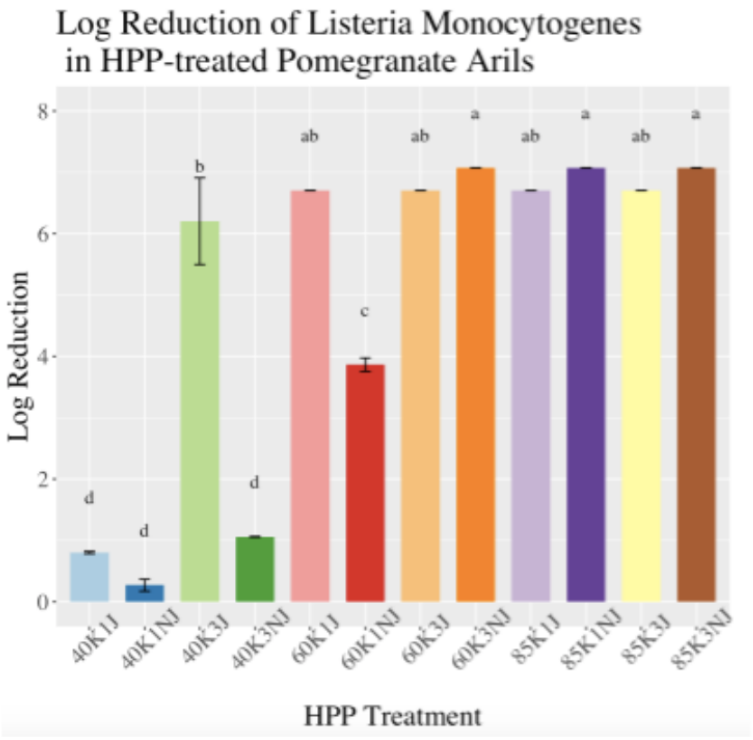


Fig 3

(Top)

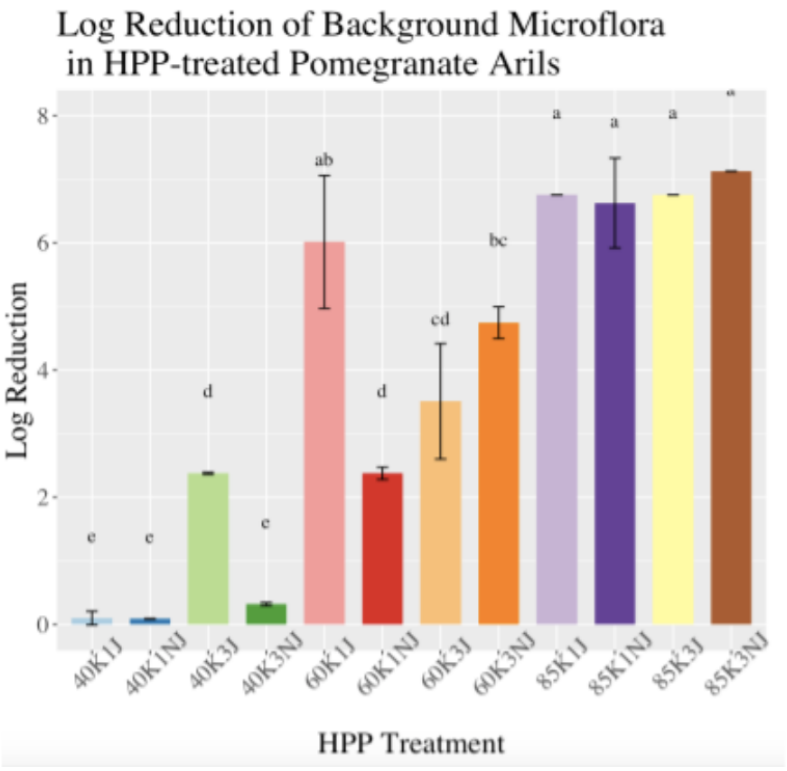
Effect of HPP

treatment on the initial

log reduction of

Listeria

monocytogenes.



(Bottom)

Effect of HPP

treatment on the initial

log reduction of the

background microflora

Temperature plays an important role in microbial inactivation after HPP treatment. Smelt (1998) postulated that at temperatures beyond the optimum for growth of microorganisms, membrane fluidity could become more susceptible to damage and hence microorganisms could be easily inactivated. Low temperature storage and good control of refrigeration temperatures resulted in further damage to cells and limited the growth of *L.monocytogenes,* background microflora and yeasts and molds**.** Han and Linton (2004) demonstrated that *L.monocytogenes* was inactivated over a 45-day period of storage at 4ºC. According to Jordan and others (2001), microorganisms lose the ability to survive at low temperatures after pressure treatment.

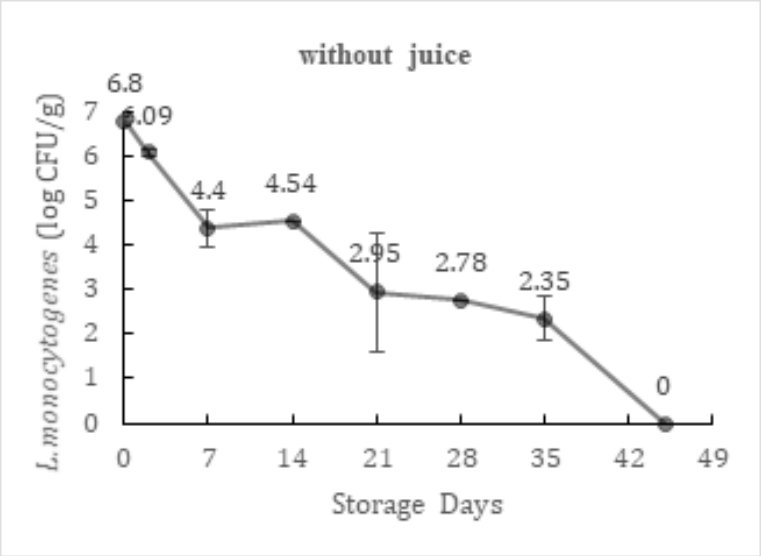


Fig 4 - *Listeria monocytogenes* counts in 40K1NJ samples during 45-day storage period

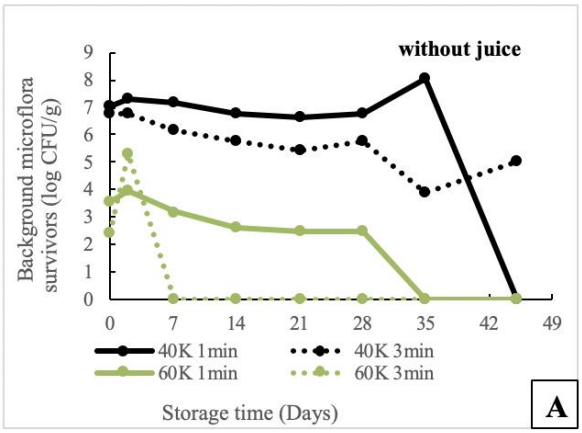


Fig 5 A- Background microflora survivor curves at 40k PS I (1min, 3mins) and 60k (1min, 3min) without juice during 45- day storage time

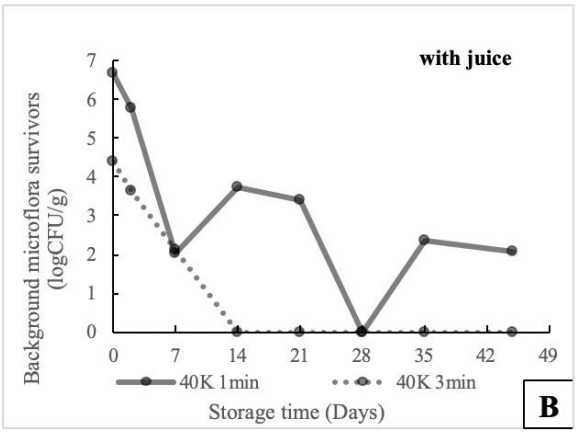


Fig 5 B- Background microflora survivor curves at 40k PSI (1min, 3mins) in arils with juice during 45-day storage time.

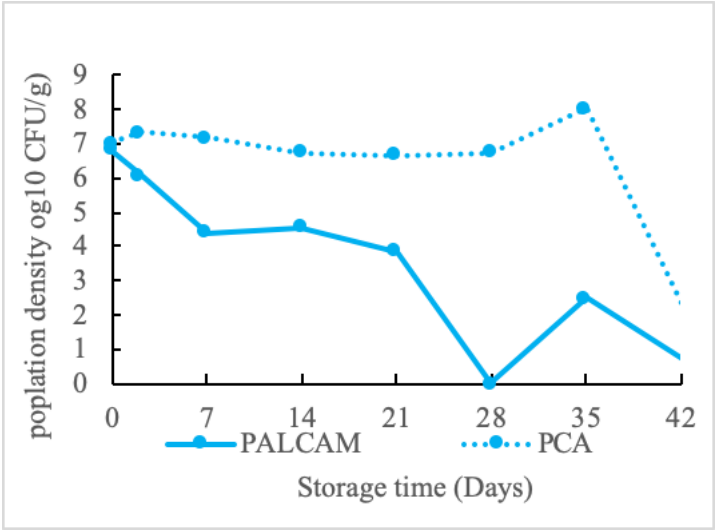


Fig 6-Comparison of log counts of *L.monocytogenes* (PALCAM)

Fig 6 shows a compares log reduction of *L.monocytogenes* (detected with PALCAM) and background microflora (detected with PCA) in pomegranate arils after treatment at 40K1J. The high level of phytochemicals in arils could lead to higher inactivation of *L.monocytogenes* than the background microflora. Previous work on plant antimicrobials by Kurosaki and Nishi (1983) supports this observation. Based on results of a study, they suggested that phytoalexins mainly target fungi and Gram-positive bacteria, but not the Gram-negative microbiota of the plants.

L.monocytogenes might have not grown on PALCAM selective medium but might have survived on PCA. Some authors suggested that, when microorganisms suffer sublethal structural damage at the level of cytoplasmic membrane, cells can no longer grow in a selective medium and will only grow in a non-selective medium. (Bull, Hayman, Stewart,. Szabo, E.A., & Knabel, 2005; Cheftel & Culioli, 1997; Han et al, 2004; Bozoglu., Alpas, & Kaletunç, 2004). Scolari et al (2015) were able to revive *L.monocytogenes* from pressurization by culturing the injured cells in a non-selective tryptic soy agar supplemented with yeast extract (TSAYE). However, in the present study, all microorganisms were inactivated at 85,000PSI (85K1NJ, 85K1J, 85K3NJ, 85K3J) and *L.monocytogenes* and background microflora that survived lower pressure treatments (40K1NJ, 40K1J, 40K3NJ, 40K3J, 60K1NJ, 60K3NJ,) were inactivated during cold storage.

Physico-Chemical Parameters

Fig 7 (A, B, C, D, E and F) shows the effect of HPP on pH, Brix, and water activity of treated pomegranate arils, on day 0 and day 14, compared with the controls during storage at 4ºC. Immediately after treatment and after 14 days in cold storage, treated and untreated samples did not show much difference in pH, Brix, and water activity. There have been conflicting reports on the effect of HPP on physico-chemical parameters of foods. Alighourchi and Barzegar (2009) reported an increase in pH from 3.21 to 3.39 in HPP-treated pomegranate. González-Molina, Moreno, and García-Viguera (2009) observed no significant changes in quality parameters in HPP-treated pomegranate. According to Varela-Santos et al. (2012), the physico-chemical properties could vary depending on cultivars, growing seasons, agricultural practices, variations in physico-chemical assays as well as the composition and structure of the food in general. Patterson postulated in 2005 that the pH of acidic conditions decreases as pressure increases. When the treatment pressure is released, reversion to the original pH value occurs. However, the effect of sudden changes in pH on microbial survival is unknown.

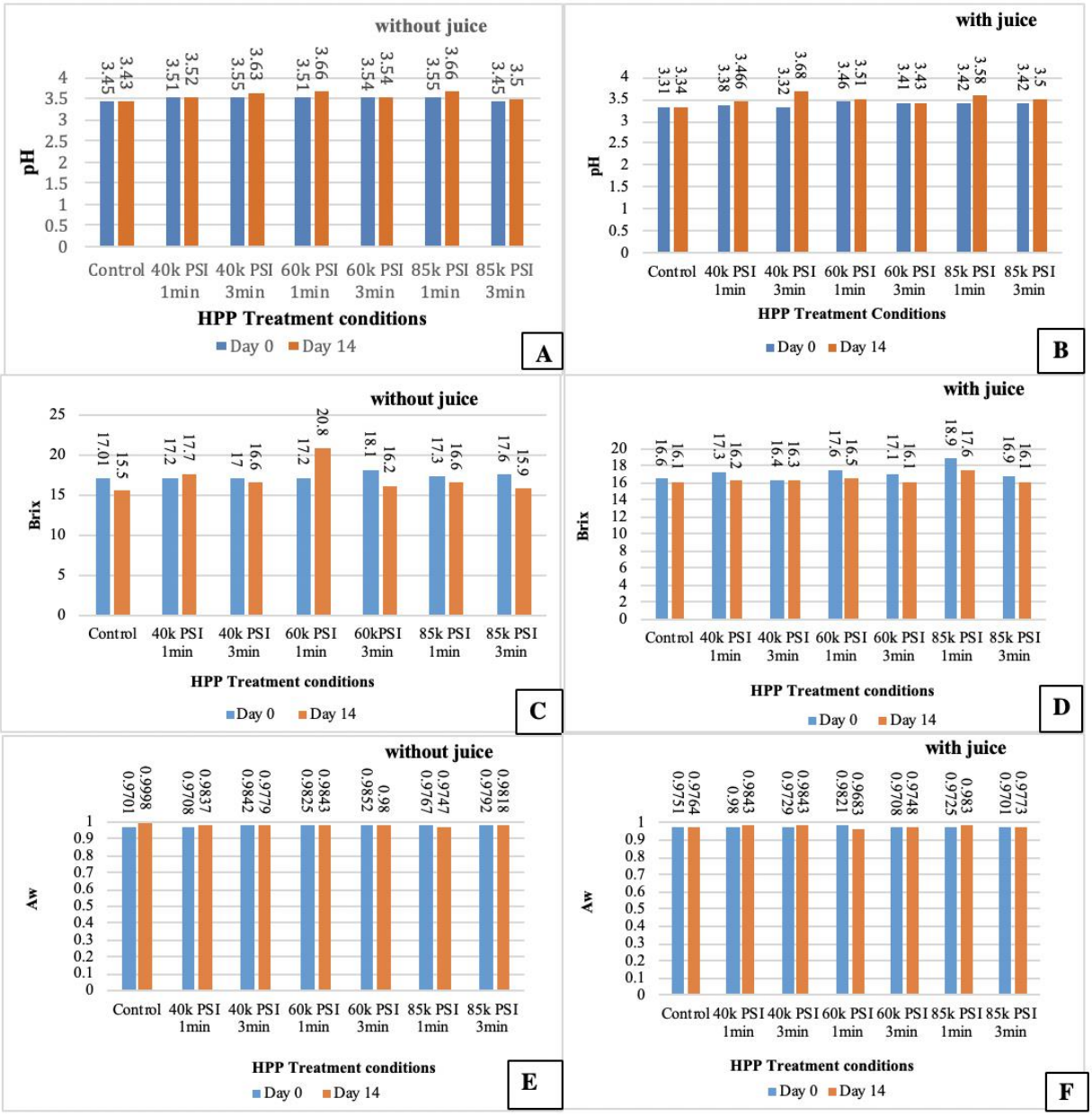


Fig 7A *pH* of treated aril samples (without juice) on day 0 and day 14
Fig 7B *pH* of treated aril samples (with juice) on day 0 and 14
Fig 7C *Brix* of treated aril samples (without juice) on day 0 and day 14
Fig 7D *Brix* of treated aril samples (with juice) on day 0 and day 14
Fig 7E *Aw* of treated aril samples (without juice) on day 0 and day 14
Fig 7F *Aw* of treated aril samples (with juice) on day 0 and day 14

Color

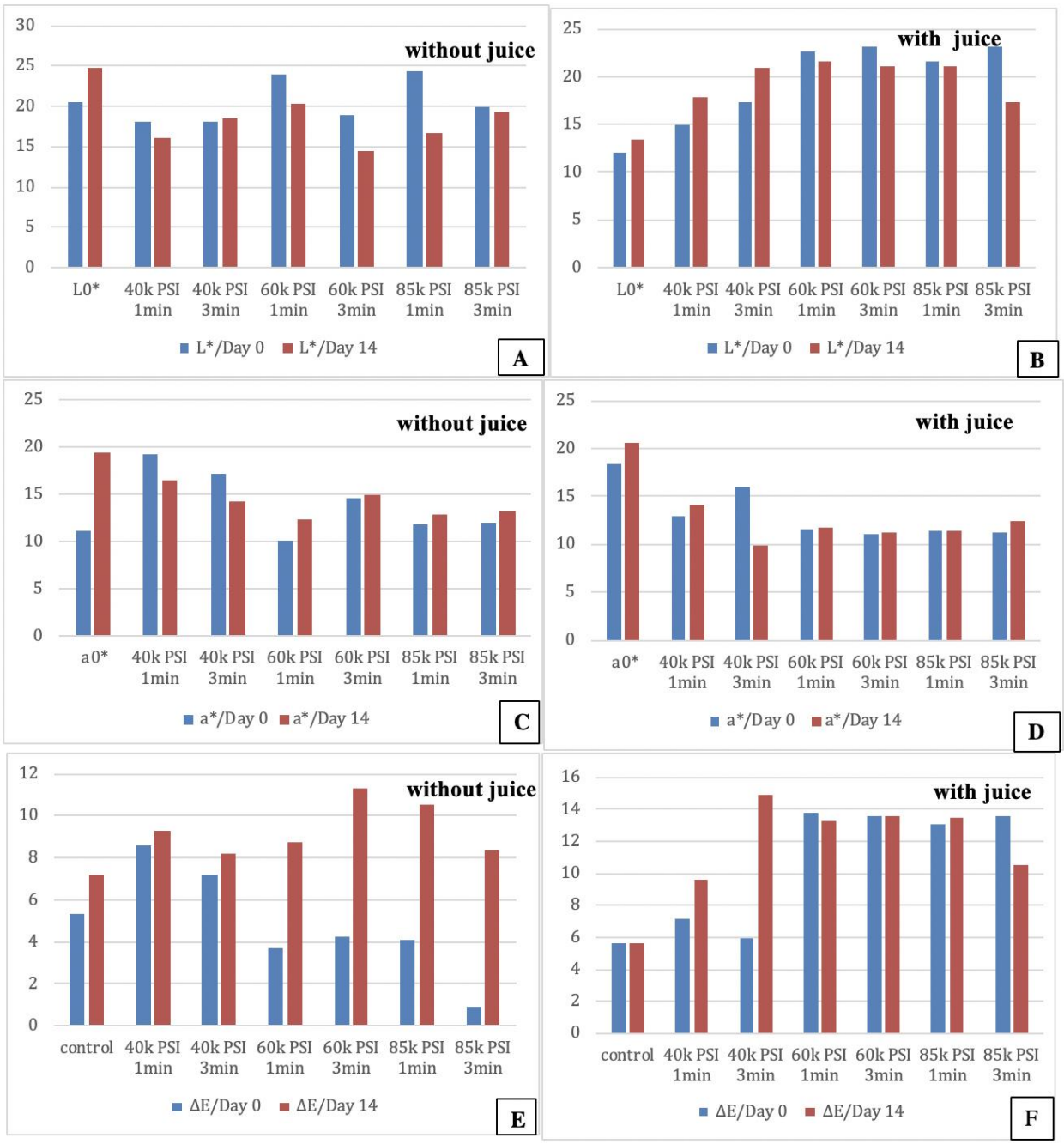


Fig 8A-Changes in brightness (L*) of treated arils (without juice) compared to control during 14 day storage
Fig 8B-Changes in brightness (L*) of treated arils (with juice) compared to control during 14 day storage
Fig 8C-Changes in redness (a*) of treated arils (without juice) compared to control during 14-day storage period
Fig 8D-Changes in redness (a*) of treated arils(with compared) to control during 14-day storage period
Fig 8E -Changes in total color difference (ΔE) of treated arils (without juice) compared to control during 14-day storage period
Fig 8F -Changes in total color difference (ΔE) of treated arils (with juice) compared to control during 14-day storage period

Color is an important quality attribute in the food and bioprocess industries, and it influences consumer's choice and preferences (Pathare et al., 2012). On day 0, untreated aril samples without juice and with juice had L* value (degree of brightness) of 20.47 and 12, respectively. The a* values (degree of redness) of untreated samples without juice was 11.17 and the value of untreated samples with juice was18.45

Pressure had an effect on the color characteristics of pomegranate arils (Fig 9). With increased pressure and pomegranate juice, samples appeared brighter (higher L* values) and less red (lower a* values) immediately after treatment. There was not much difference in b* values (degree of yellowness) as compared to the control. A decrease in lightness (L* value) was detected in samples treated without juice; however, lightness increased in samples treated with juice while in storage for 14 days. a* values also decreased as compared to control when treated at 40K1NJ,40K1J, 40K3NJ, 40K3J ,60K1NJ, 60K1J, 60K1J, 60K1NJ with pomegranate juice. Some browning reaction could have occurred from breakdown of enzymes during storage. The oxidation of phenolic substrates by polyphenol oxidase (PPO) is thought to be the major cause of the brown coloration of many fruits and vegetables during storage and processing (Vidhan, Ara,& John, 2010).

Artes, Tudela, and Villaescusa, (2000) reported that the CIE L*, a*, and b* color parameters were higher in pomegranate fruit husk than in arils and juice at harvest period. However, the authors observed no significant color difference in fruit husk and arils after 80 days of cold storage at 0°C and 5°C, respectively. A study by Ghasemnezhad et al. (2013) had confirmed that polyphenol oxidase (PPO) play an important role in oxidation and degradation of anthocyanins in pomegranate arils. Gil, Martínez, and Artes (1996) recommended an identification of genotypes which show less color change during storage. Experiments conducted by Ferrari, Marescaawa and

Ciccarone (2010) demonstrated that HPP at room temperature improves the quality of pomegranate juice, increases the intensity of the red color of the fresh juice and preserves the content of natural anthocyanins. Other authors (Fawale and Opara, 2013) reported significant decreases in fruit color intensity (C*) with increasing storage temperature and duration. However, Nanda, Sudhakar and Krishnamurthy (2001) did not observe significant changes in color of pomegranate fruit stored at 8°C, 15°C and 25°C o a 12-week period.

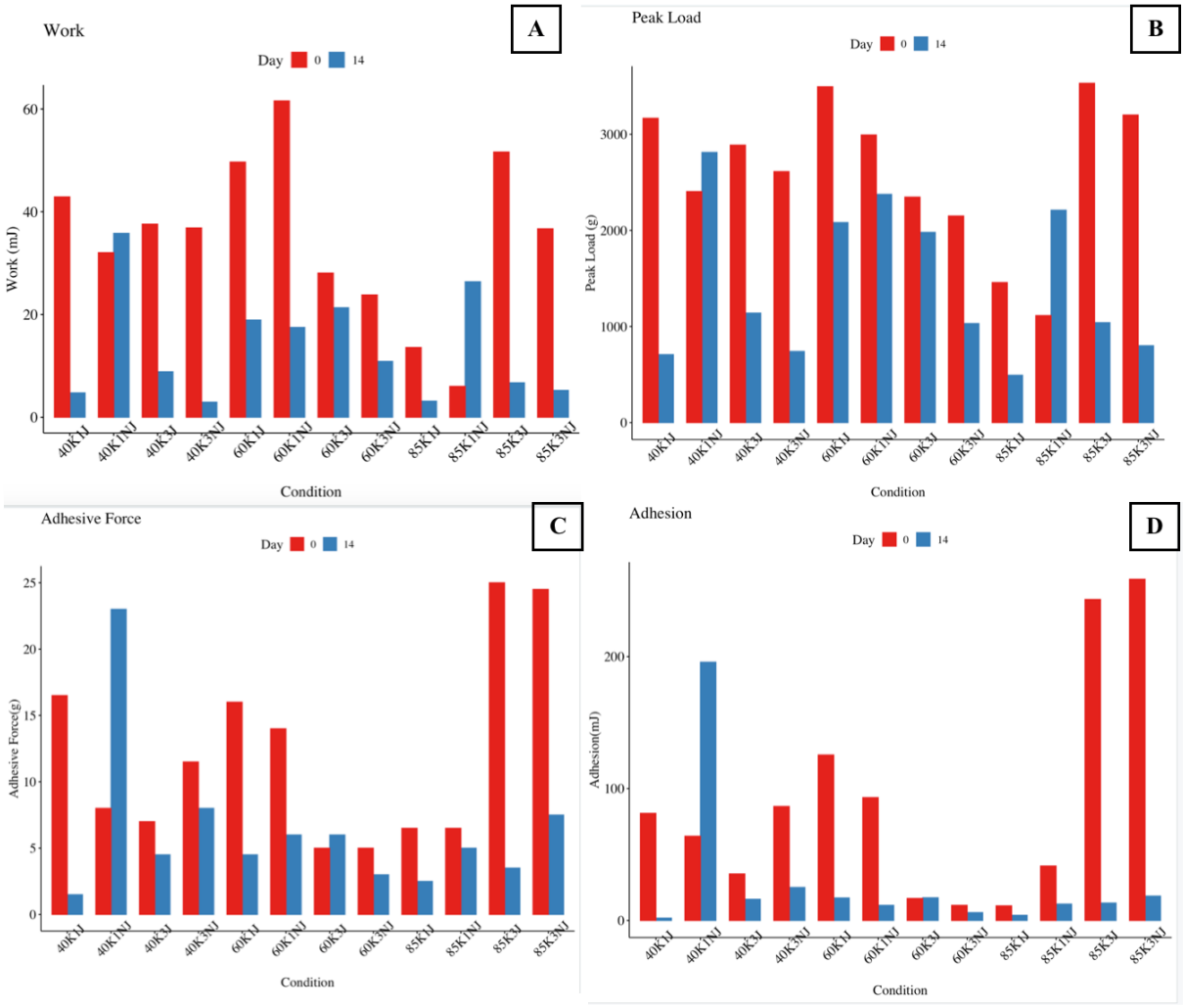
The ΔE values, which are an indicator of total color difference, showed some differences between treated samples without juice and the control. Higher pressures had the lowest ΔE values immediately after treatment. However, ΔE values increased, showing much difference in color, after 14-day storage. With pomegranate juice, treated samples did not show much color difference immediately after treatment and while in storage. In effect, higher L* values indicated that arils with pomegranate juice became brighter when treated at higher pressures (60k PSI, 85k PSI). These samples also had less browning and discoloration during storage.

Texture

The following parameters were measured during texture analysis:

1. Work (mJ) measures the energy required to deform the sample.
2. Final load (g) measures the load at maximum deformation, usually the same as peak load.
3. Deformation at peak (mm) is the distance to which the sample was compressed when the peak load occurred.
4. Adhesiveness or Adhesion (mJ) is the energy required to separate the probe from the sample on the return stroke.

5. Adhesive Force (g) is the force required to separate the probe from the sample on the return stroke.



Texture analysis results of HPP-treated aril samples, immediately after treatment (depicted in red) and after 14 days (depicted in blue)
Fig 9A - Work (mJ); Fig 9B - Peak Load (g); Fig 9C - Adhesive Force (g); Fig 9D - Adhesion (mJ)

Fig 9 shows the changes in texture of pomegranate arils immediately after treatment (day 0) and on day 14. Work for treated samples seemed to decrease after 14 and the highest difference was at 40K1J and the lowest was in samples treated at 40K1NJ. However, at 85K1NJ, an increase in “Work” was seen after 14 days and after treatment at 85K3J and 85K3NJ, there was a decrease

in Work. Addition of pomegranate juice seemed to render the arils softer after 14 days. Aleman et al. (1994) reveal that the texture of vegetables and fruits after pressure treatment render the product more pliable and increase their softness.

Adhesion and Adhesive force seemed to increase in samples with increasing pressure treatment immediately after treatment. However, at day 14, a decrease in Adhesion and Adhesive force was observed in all treated samples except at 40K1NJ where there was an increase. Samples showed the highest difference in adhesion and Adhesive forces after 14 days after treatment at 85K3J and 85K3NJ. The difference between Adhesion on day 0 and day 14 was greater in 85K3J. These observations suggest that arils become firmer after 14 days when treated at lower pressure of 40K. Higher pressures of 85K and addition of pomegranate juice makes pomegranate arils softer after 14 days.

CONCLUSION

High Pressure Processing (HPP) emerged in this study as an effective non-thermal processing method for inactivation of *Listeria monocytogenes* in pomegranate arils with minimal effect on pH, water activity, and Brix. The color of treated arils with juice appeared lighter and more appealing after storage for 14 days. HPP is an effective alternative for overcoming losses in nutritional and sensory properties caused by high temperatures used in conventional thermal processing. *L. monocytogenes* was inactivated at higher pressures. The inactivation rate increased with presence of pomegranate juice and longer holding time. The risk of *L. monocytogenes*, background microflora, yeasts and mold contamination can be safely reduced in pomegranate arils with treatments at 85,000PSI for 1 or 3 mins with/without pomegranate juice. However, the texture of the samples treated at 85,000 PSI with an addition of pomegranate juice seemed to be compromised after 14 days. Samples treated at lower pressure and without pomegranate juice

appeared firmer after 14 days. These treatments, nonetheless, achieved a significantly lower log reduction of *L. monocytogenes* and background microflora compared to treatments at high pressure. In order to achieve a high log reduction without losing the firmness of the arils, an HPP treatment at 85,000 PSI combined with pomegranate juice and a firming agent, such as calcium chloride, could be considered.

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