Received: 26 October 2017 | Revised: 10 January 2018 | Accepted: 1 March 2018

DOI: 10.1111/jfs.12469

ORIGINAL ARTICLE

WILEY Journal of Food Safety

The inhibitory effect of traditional pomegranate molasses on *S. typhimurium* growth on parsley leaves and in mixed salad vegetables

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Funding information
Lebanese National Council for Scientific Research (CNRS), Grant/Award Number: 102598

Abstract

Pomegranate (PG) molasses is an essential condiment that is commonly used in Middle Eastern cuisine in local and international gastronomic markets. There is scarce information on the inhibitory effect of PG molasses on *Salmonella* growth mainly under *in situ* conditions and in a food matrix. PG molasses in different dilution ratios of 1:1 and 1:7, combined with sodium chloride (2%, v/v), vinegar (2.4%, v/v), and in a dressing mix was tested against *Salmonella enterica* serovar Typhimurium LT2 on parsley and salad vegetables. The results showed significant log reductions in *Salmonella* on parsley treated with PG solutions for 15, 30, and 60 min, reaching a level of 2.55 log cfu/g. The addition of sodium chloride (2%, v/v) and vinegar (2.4%, v/v) to PG molasses did not exert a synergistic or antagonistic effect on its antibacterial activity. Additionally, the application of PG molasses dressing on salads contaminated with low (3 log cfu/g) and high (6 log cfu/g) inoculum levels resulted in 2–3 log reduction independent of temperature ($p < .05$) compared with 0.5–1 log reduction for thorough washing alone which may damage the leaf surfaces. This study showed that PG molasses has a great efficacy against *S. typhimurium* and the potential to enhance the microbial safety of ready-to-eat salads and parsley leaves.

Practical applications

The implication of fresh-cut vegetables in food poisoning are well documented, at the same time, the washing methods in restaurants or home settings are reportedly not effective enough to mitigate the risks of pathogens found in vegetables. This study shows that Pomegranate (PG) molasses has a greater efficacy than chlorine (200 ppm) against *S. typhimurium* and achieved comparable to greater log reduction values than organic acids. The storage temperature and food matrix did not alter the potency of PG. The present work showed that promoting the use of PG as a natural additive and condiment to ready-to-eat (RTE) vegetables offers a great potential to effectively reduce the risk of *Salmonella* contamination and to improve their microbial safety. Furthermore, this study provided preliminary data that serve as additional inputs in quantitative microbial risk assessment models for estimating the risk of *Salmonella* infection associated with consuming RTE salads served in Mediterranean cuisines.

1 | INTRODUCTION

Pomegranate (PG), known by its botanical name *Punica granatum*, is an ancient fruit that is commonly cultivated in Iran, India, and the Mediterranean countries such as Turkey, Egypt, Tunisia, Spain, and Morocco (Ercisli, Gadze, Agar, Yildirim, & Hizarci, 2011) and its production reportedly reached around 2 million tons as a result of constant global demands (Yahia, 2011). In 2014, 42,000 tonnes pomegranates (imports minus exports) were added to the apparent consumption in the European Union and apparently, pomegranates-derived products are gaining popularity (CBI, 2016). Today, the PG molasses is widely available in the international gastronomic markets, whether in Middle Eastern supermarkets or in Middle Eastern cooking; this is driven by consumers appeal, the increasing interest in its nutritional value (Dhinesh & Ramasamy, 2016), and the recognized antioxidant, antitumoral, and anti-inflammatory properties from its bioactive compounds

such as anthocyanins, ellagic acid derivatives, hydrolyzable tannins, and other phenolic compounds (Longtin, 2003; Zarfeshany, Asgary, & Javanmard, 2014).

There is also interest in PG antimicrobial properties especially as there are many documented outbreaks associated with the consumption of fresh vegetables in recent decades caused by several pathogens including *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli* (EFSA/ECDC, 2015). A recent example is a salmonellosis outbreak in Australia traced to prepacked salad mixes from major supermarkets and that contained an unusually high level of *Salmonella* Anatum (FSANZ, 2016). One reason that salad items are frequently implicated in outbreaks of *Salmonella* is that the organism is capable of strongly attaching to damaged salad leaves in bagged salads (Koukkidis, Haigh, Allcock, Jordan, & Freestone, 2016). The risk of illness is present for consumers even if small quantities of the leaves are thus affected and it makes the sanitation process of bagged lettuce leaves a challenging task. This problem has driven researchers to examine a wide array of washing and disinfection methods to reduce microbial contamination on fruits and vegetables (Kim, Ryu, & Beuchat, 2006). Various chemical disinfecting agents were proven effective and have been routinely used such as chlorine, chlorine dioxide, hydrogen peroxide (EC Directorate, 2002); however, their limited efficacy in eliminating food pathogens and their potential adverse side effects on the health and quality of fresh produce (Ölmez & Kretzschmar, 2009) have promoted the search for better and safer alternatives. In this context, the decontamination effect of natural antimicrobials including organic acids such as acetic acid and citric acid have in fresh fruits and vegetables was documented (Rhee, Lee, Dougherty, & Kang, 2003). Additionally, over the past two decades, the antibacterial activity of pomegranate fruit, peels, and seeds extracts against foodborne pathogens such as *Staphylococcus aureus* and *E. coli*, fungal and parasitic infection was also examined by several studies using *in vitro* testing (Agourram et al., 2013; Al-Zoreky, 2009) and was found to be attributed to the presence of the active inhibitors, that is, phenolics, flavonoids, and tannins (Machado et al., 2003). Nevertheless, the antimicrobial impact of PG molasses in marinades and dressings for vegetable salads and its effectiveness in the presence of other natural antimicrobials and components of foods has not been sufficiently researched. PG molasses is commonly used for its intense flavor and taste that add a unique savor to Middle Eastern salads and meats and continues to be promoted as a product derived from the "Super fruit," branded for its high nutritional value and antioxidant capacity (Dhinesh & Ramasamy, 2016; Longtin, 2003). PG molasses is derived from the pomegranate juice obtained from arils, which contain the rich sources of phenolic and flavonoids, by boiling the juice to obtain a thick, dark red liquid without addition of sugars or other additives. The processing is typically characterized by cleaning the pomegranate fruits, crushing, extraction, filtration, clarification, and evaporation in open vessel or under vacuum (Vardin & Abbasoğlu, 2004)

Considering that the domestic washing methods of fresh vegetables commonly applied in the food service do not eliminate pathogens, that is, they achieve a bacterial reduction of 1–3 log cfu/g (Faour-Klingbeil, Kuri, & Todd, 2016; Pezzuto et al., 2016), there is the likelihood of such methods being inadequate (Faour-Klingbeil et al., 2016). Therefore, the understanding of the efficacy of PG molasses as a food additive that could exhibit an additional benefit in reducing the risk of bacterial foodborne illnesses associated with ready-to-eat (RTE) mixed salad vegetables is essential in risk analysis of biological hazards at the retails and food service end. The objective of this study is to investigate the antimicrobial effect of the traditional PG molasses in its pure form and combined with other antibacterial additives in a salad dressing mixture (SALD), against *Salmonella enterica* serovar Typhimurium LT2 (*S.* typhimurium) on parsley leaves and RTE salads.

2 | MATERIALS AND METHODS

2.1 | Determination of total phenolic content in traditional PG molasses

Total phenolic content (TPC) of the PG molasses was determined by Folin–Ciocalteu assay using gallic acid (GA) as the calibration standard (Singleton & Rossi, 1965). GA was used at the concentrations in the range of 10–2,500 mg/ml to construct a standard curve in order to calculate total phenol concentration of extracts as gallic acid equivalence (GAE). PG molasses were serially diluted in water to obtain a ratio of 1:1–1:16. Aliquots of 0.25 μl of each PG or GA standard solution was added to 625 μl Folin–Ciocalteu reagent, previously diluted with distilled water (1:1, v/v), then incubated at 50 °C for 5 min. Later, 0.5 ml of 7.5% sodium carbonate (Na_2CO_3) was added and absorbance was measured at 760 nm spectrometrically. TPC was standardized against GA and calculated in terms of GAE in mg/L.

2.2 | pH and titratable acidity

The pH of the working solutions and mix of PG molasses was measured using a digital pH-meter (Mettler Toledo MP220, OH) previously calibrated with pH 7.0 and 4.0 standard buffers. The titratable acidity was determined based on citric acid for PG and the acetic acid for the white vinegar using 0.1 M NaOH to the endpoint of pH 8.1 according to the AOAC method 942.15 (AOAC, 2000) and reported as % citric acid (w/v).

2.3 | Preparation of *S. typhimurium* cultures

Freeze-dried *S. typhimurium* LT2 (ATCC 700720) was obtained from Plymouth University, department of biological sciences. Cultures were grown from a stock kept at −80 °C, in brain heart infusion broth overnight at 37 °C, streaked on blood agar plates and incubated overnight at 37 °C. One to two colonies of the nalidixic acid adapted strains were transferred to 10 ml tryptone soya broth (TSB) (Oxoid, Basingstoke, Hampshire, England) supplemented with Nalidixic (TSBN) followed by incubation for 18 hr at 37 °C. Stock cultures on Plate Count Agar (Bio-rad laboratories, England) supplemented with 50 ppm nalidixic acid (PCAN) were stored at 4 °C.

2.4 | Preparation of the vegetables

Salad vegetables (parsley, tomato, cucumber, onions) were purchased fresh from the local market in Beirut on a daily basis for the direct use in

TABLE 1 Inhibitory effect of pomegranate molasses (PG) alone and in combination with NaCl and white vinegar on parsley inoculated with *S. typhimurium**

Molasses mix (stock solution)	Contact time (min.)	Mean ± *SD* (log cfu/g)	Log_{10} reduction log (N/N_0)	pH Values†
PG 1:1‡				
Control		**6.51[a] ± 0.90**		
	15	4.77[b] ± 0.76	−1.74	1.81
	30	4.36[b] ± 0.59	−2.15	1.80
	60	4.29[b] ± 1.02	−2.28	1.83
PG 1:7‡				
Control		**6.08[a] ± 0.18**		
	15	5.30[b] ± 0.62	−0.78	2.17
	30	4.90[b] ± 0.47	−1.18	2.20
	60	4.09[b] ± 0.74	−1.99	2.20
PGV‡				
Control		**5.98[a] ± 0.05**		
	15	3.82[b] ± 0.10	−2.16	2.35
	30	3.45[b] ± 0.13	−2.55	2.33
	60	3.66[b] ± 0.30	−2.32	2.27
PGS‡				
Control		**5.98[a] ± 0.05**		
	15	3.70[b] ± 0.28	−2.28	2.07
	30	3.52[b] ± 0.10	−2.46	2.07
	60	3.66[b] ± 0.16	−2.32	1.99

Different superscript letters in the same column within the same group indicate significant difference at $p < .05$.
*Inoculum size 10^6 cfu/ml ($n = 8$). †pH of full strength PG molasses was 1.03. ‡PG 1:1 [PG full strength diluted in an equal volume of sterile distilled water (SDW) at a ratio 1:1 (PG/SDW)]; PG1:7 [PG full strength serially diluted in sterile distilled water to a ratio 1:7 (PG/SDW)]; PGV = PG and white vinegar [PG 1:7 (PG full strength/SDW) and 2.4% acetic acid (v/v) white vinegar]; PGS= PG and NaCl [PG 1:7 (PG full strength/SDW) and 2% (v/v) NaCl].

the experiment. Damaged leaves of parsley and bruised produce were discarded. The selected vegetables were washed in a simple domestic method as observed in some foodservice facilities using running tap water for 10 s (Faour-Klingbeil et al., 2016) and drained in a salad spinner. Later, the parsley leaves were aseptically picked from the branches leaving about 5 cm of stalks, as typically used for making local salads.

2.5 | Procedure for inoculum suspension preparation and inoculation of parsley

Inoculum cell suspension of *S. Typhimurium* was prepared to achieve the concentrations of approximately 10^6 and 10^3 cfu/ml. A 100 g of parsley leaves were dipped into the 1 L culture suspension in sterile bags sufficient enough to cover all the sample and occasionally agitated to ensure even distribution of suspension for 30 min at RT (23 °C) to allow attachment (Lapidot, Romling, & Yaron, 2006). Parsley were decanted and placed on sterile paper for removing excess liquid for 60 min in a laminar flow cabinet. Afterwards, parsley was chopped in sterile conditions and stored for 22 hr at 4–5 °C until the application of PG molasses and its mixture,

2.6 | The experiment design

This study was conducted in two separate experimental stages. The experiment in stage 2 was constructed to resemble the preparation of typical vegetable salads with PG molasses.

2.6.1 | Stage 1: The effect of different concentrations of PG molasses alone and in combination with salt and vinegar on *S. typhimurium* on parsley leaves

Preparation of stock solutions of PG

The traditional PG molasses containing pure juice was purchased from a local producer and diluted with sterile distilled water to a workable dilution of 1:1 ratio for testing the efficacy of pomegranate in its highest concentration and 1:7 ratio, the latter is an estimated final dilution of PG molasses that maintains the unique intense flavor when it is mixed with the salad. PG solutions were also prepared by combining the PG molasses with white grape vinegar containing 6% (v/v) acetic acid and NaCl 10% (w/v) to yield final concentrations that would be used in the salad dressing as presented in Table 1. The traditional PG molasses (25 g) contained 16.5 g total carbohydrates, 12.9 g sugar, 1.5 g proteins, and 0.16 g total fat. All solutions were prepared immediately before use.

Application of PG molasses solutions

Inoculated samples (10 g) were transferred to sterile bags and mixed with 100 ml of PG solutions for 15, 30, and 60 min at room temperature (RT). After decanting, samples were rinsed once with deionized sterile water to remove the remaining PG solutions from the leaves. Samples of 10 g inoculated samples were not treated for control. All experiments were made at least four times in two replicates and plated in duplicates.

A similar experiment with the PG molasses diluted to 1:7 in sterile water was repeated using low inoculum levels to compare log reductions with the high inoculum levels

Enumeration

Ten grams of treated samples were suspended into 90 ml TSB in a stomacher bag and homogenized for 2 min at normal speed (230 rpm). The homogenate was serially diluted and duplicate 0.1 ml samples were spread plated onto Rapid *Salmonella* agar supplemented with 50 ppm nalidixic acid. To increase the limit of detection, 1 ml of the lowest dilution was distributed evenly over four plates (0.25 ml/plate).

A preliminary experiment was carried out to recover sublethally injured cells by direct plating after 4-hr pre-enrichment in buffered peptone water (BPW) (Oxoid, Basingstoke, Hampshire, England) (Sengun & Karapinar, 2004). There was an overgrowth of bacteria compared to inoculum levels (control), hence this method was eliminated. Alternatively, we used both selective and overlay methods for low inocula levels for resuscitation of acid-injured cells (Han, Linton, Nielsen, & Nelson, 2002), but we employed plating on selective agar as the method of choice for inocula with high numbers based on our preliminary trials and findings of Gündüz, Gönül, and Karapinar (2010a).

In the overlay plating method, duplicate 0.1 ml aliquots of inoculum were surface plated on PCAN medium and the plates were incubated at 37°C for 4 hr. Later, the plates were overlaid with selective agar, Rapid'*Salmonella* agar supplemented with 50 ppm nalidixic acid, and incubated for a further 20 hr at the same temperature.

The detection limit for this quantitative method was 10 cfu/g. Populations of endogenous *Salmonella* were determined in uninoculated controls by transferring 25 g samples to 225 ml BPW in a stomacher bag and homogenized for 2 min at medium speed. Homogenates of parsley were incubated for 1 hr at RT. Then, they were incubated for 24 hr at 37°C, and the following day 0.5 ml was transferred to 9.5 ml Rappapport (Bio-rad laboratories, England). After incubation for 18 hr at 45°C, cultures were streaked onto selective *Salmonella* agar plates and examined for *Salmonella* colonies after 24 hr incubation at 37°C. This method was also used for the detection of *Salmonella* in the event of no visual growth on treated samples. The microbial populations were converted to log cfu/g.

2.6.2 | Stage 2: the antibacterial efficacy of PG vinaigrette salad dressing on a raw salad mix prepared with contaminated parsley leaves

Preparation of solutions/salads

A salad mix weighing approximately 375 g was prepared according to the following recipe of a traditional mix (excluding other types of vegetable and spices to reduce the variables). More than one salad mix were prepared for the additional portions of samples needed in this experiment:

Salad Ingredients:

- 30 g parsley artificially contaminated leaves
- 3–4 radishes cut thin slices
- 2 medium size cucumbers sliced into small cubes
- 1 big tomato cut into medium size pieces
- 4 leaves of lettuce
- 1 small onion thinly sliced

PG vinaigrette salad dressing (PG SALD) ingredients:

- 70 ml pomegranate molasses (100% concentration)
- 35 ml extra virgin olive oil
- 15 ml white wine vinegar (6% acetic acid)
- 1 ½ tsp. Salt

Application of PG SALD to an artificially contaminated raw salad mix

The inoculated parsley was mixed with the cucumbers, onions, and tomatoes. The salad items were placed and mixed well in an autoclaved 2 L glass beaker using sterile spoons and spatulas. After 3–5 min of the mixing of ingredients, control samples were analyzed for enumerating *Salmonella* before the addition of the salad dressing (T_0 -no sauce). Later, samples of 75 g of the salad mix were individually placed into autoclaved glass beakers and treated accordingly:

1. 75 g kept for 1 hr at 5°C with no SALD to examine changes in Salmonella levels that may result from exudates of salad ingredients or from the pH (T1h mix no SALD at 5°C)
2. 75 g mixed with SALD and held for 1 hr at RT (T1h mix with SALD at RT)
3. 75 g mixed with SALD and held for 1 hr at 5°C (T1h mix with SALD at 5°C)
4. 75 g mixed with SALD without oil and stored for 1 hr at 5°C and RT to examine any changes in the antibacterial activity of PG as affected by the presence/absence of fats (T1h mix with SALD no oil at 5°C) and (T1h mix with SALD no oil at RT). This condition was only applied for samples with the high inoculum levels for a practical observation and detection purposes.

The 1-hr period is an estimated time for preparing and serving a bowl of salad for more than one person as an accompaniment to main courses as observed in our earlier work (Faour-Klingbeil et al., 2016). In each of the above conditions, the salads were mixed with the SALD using new sets of autoclaved utensils. During holding time, the beakers were partially covered to protect samples from external contact and contamination.

Enumeration

All strains enumeration was done before and after the treatment application. The initial determination of *Salmonella* was performed as described in the stage 1 experiment.

Regarding the salad mix, 25 g of samples were homogenized with 225 ml TSB considering the volume and weigh of the sliced salad ingredients. In this experiment, two plating methods were applied for the recovery of *S. Typhimurium*, selective and nonselective agar. Aliquots of 0.1 ml of homogenates were plated on Rapid'*Salmonella* agar and

nonselective agar (PCAN), and 1 ml equally pipetted onto four plates of each. Another 25 g of samples was macerated, and 0.1 ml of the exudates was plated. The plates were incubated at 37°C for 24 hr and then colonies were counted. The microbial populations were converted to log cfu/g.

2.7 | Statistical analysis

The effects of pomegranate and its mixtures on the survival of *Salmonella* on parsley were assessed by calculating the reduction of contamination levels in relation to the untreated samples of parsley, expressed in terms of log-cycles (i.e., log (N/N_0), that is, N_0 is the initial microbial load on untreated sample and N is the microbial load after treatment with PG). Microsoft Excel was used for this purpose. Descriptive analysis and a two-way ANOVA were used to present and compare mean counts of *Salmonella* spp. on parsley and salads among different groups of PG solutions and contact times. Assumption of normality was tested and Games-Howell's post hoc tests was performed. In all cases, the level of statistical significance was considered to be $p < .05$.

3 | RESULTS AND DISCUSSION

3.1 | Stage 1 experiment

3.1.1 | Titratable acidity and TPC

The titratable acidity of the PG was 6.6% (v/v) citric acid and falls in the range of 5.80 and 14.27, the values of % citric acid in pomegranate juice concentrate reported by Vardin, Tay, Ozen, and Mauer (2008). Similarly, the total acidity as citric acid of Turkish traditional pomegranate molasses ranged between 5.11 and 9.83; the large variation in acidity among the different PG products was ascribed to the variety of the pomegranate fruits and to methods of evaporation (İncedayi, Tamer, & Çopur, 2010).

The TPC in pomegranate molasses was determined spectrophotometrically using GA as the standard. The absorbance of different concentrations of the standard were taken to construct a calibration curve and the linearity range for this assay was determined as $y = 0.0018x$ mg GA/l ($R^2 = 0.9951$), giving an absorbance range of 0.14–4.50 AU (Figure 1). The amount of TPC obtained in our study was on average 3546 mg/L. This value is higher than the TPC amounts found in the pomegranate juice (1985.6–2948.7 mg/L) (Radunic et al., 2015), in the fresh pomegranate from Africa (1800–2100 mg/L) (Maria et al., 2010), and in pomegranates from Turkey (1245–3436 mg/L) (Ozgen, Durgac, Serce, & Kaya, 2008). It is substantiated that the content of TPC values in PG is variable and can significantly differ between cultivars (Radunic et al., 2015).

3.1.2 | Antibacterial effect of PG molasses

In general, the initial contamination levels of *S. typhimurium* on parsley leaves were significantly reduced after treatment with PG molasses solutions for 15, 30, and 60 min at RT (Table 1). The log reduction ranged from 0.78 to 2.28, 1.18 to 2.55, and 1.99 to 2.32 log cfu/g for 15, 30, and 60 min contact time periods, respectively.

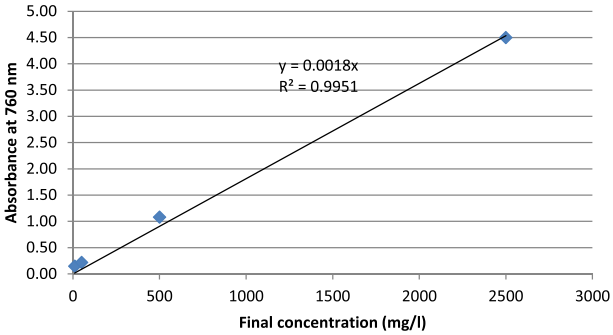

FIGURE 1 Calibration curve for GA standard to calculate TPC of extracts in terms of GAE. Data represent duplicated experiments in triplicates

Our results showed a lower range of bacterial reduction when compared to those reported by Karabiyikli and Kisla (2012), that is, 0.92–1.96 log cfu/g of *E. coli* O157 on parsley inoculated with 4 log cfu/g after 10 min treatment with sour pomegranates products. This variation is likely due to the different experimental method and to the use of diluted molasses in our study. It can also be explained by the fact that the antibacterial effectiveness can differ with fruits cultivars (Duman, Ozgen, Dayisoylu, Erbil, & Durgac, 2009).

Combining NaCl (2%, v/v) and white vinegar (2.4%, v/v) to a working concentration of PG molasses (which resulted in a final solution containing PG 1:7) did not exhibit neither antagonistic nor synergistic effects possibly due to bacterial stress adaptation and cross-adaptation (Begley & Hill, 2015; He et al., 2018). Nonetheless, the inhibitory effect of PG molasses with white grape vinegar (PGV), PG molasses with NaCl (PGS), and PG 1:1 was significantly greater than PG 1:7 at 15 and 30 min contact times.

Overall, the significant difference between PG 1:7 and the other three solutions, PG 1:1, PGV and PGS, did not exceed 1.6 log cfu/g reduction of *Salmonella* and varied between 0.96, 1.37, and 1.50 log cfu/g after 15 min and 0.97, 1.37, and 1.28 log cfu/g after 30 min, correspondingly (Figure 2).

With respect to contact time effect, there was no statistically significant difference between PGV, PGS and PG 1:1 and between the three contact time periods in the reduction of *S. typhimurium* ($p > .05$) (Figure 2). Additionally, the inhibitory effect of all the tested solutions showed a diminishing trend between the 30 and 60 min time period at RT (Figure 2) and a nonsignificant increase between 15 and 30 min treatment time. On the contrary, Karabiyikli and Kisla (2012) found that the inhibitory effect of PG sauces significantly increased with increasing application time. However, the comparison may not be precise given that the treatment time in their study was confined to a period of 15 min, hence the observed increase in PG activity was relative to the preceding early minutes of PG application. In this regard, studies have shown that the reduction of the microbiological contamination is achieved primarily in the first minute and additional reduction with increased contact time is not significant (Jongen, 2005). Besides, the antibacterial activity of natural compounds generally differs with the strains (Duman et al., 2009).

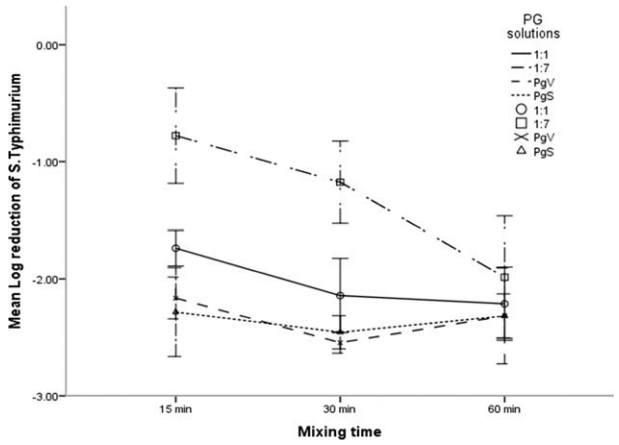

FIGURE 2 The log reduction trend of *S. typhimurium* after 15, 30, and 60 min of application of PG solutions (PG 1:1, PG 1:7, PGV, and PGS). The figure shows a diminishing trend in the antibacterial effect of the tested PG solutions (PG 1:1, PGV, and PGS) after 30 min treatment. Error Bars: ±2*SE*

We repeated the experiment with low inoculum levels using only the PG 1:7 solution to observe the antibacterial activity inhibitory effect of PG in scenarios of low initial contamination levels. The data obtained from the selective agar plating showed no significant reduction in mean counts of *S. typhimurium* on parsley compared to control group for the three-time periods ($p > .05$) (Table 2). The results obtained at low inoculum levels with the over-lay method were in line with those at high inoculum levels. The PG 1:7-treated samples had significantly lower *Salmonella* levels than the control group for the three different time periods ($p < .05$). It is important to note that the log reduction values with agar overlay plating were generated from the means of three replications since the other three replications had undetectable levels and negative results for *Salmonella* detection, hence were excluded for statistical purposes. Furthermore, the reduction in bacterial counts in the selective agar method decreased after 60 min and showed no significant difference from the samples treated for 15 and 30 min ($p > .05$), whereas the difference was significant with the overlay method ($p < .05$) (Table 2).

The overall results of this study demonstrated that time was not constantly a major factor that impacted the potency of PG molasses against *S. typhimurium*. For instance, the difference in bacterial counts between the 15 and 60 min treated samples ranged from 0.04 to 0.48 log cfu/g only and reached a max of 1.21 log cfu with PG1:7 (Table 1).

Overall, PG molasses exhibited an antibacterial effect on *S. typhimurium* that is comparable to other natural antimicrobials such as organic acids, oregano oil, sodium chloride as well to chlorine solutions (200 ppm) that achieved reduction values of 1–3 log cfu/g and in some cases <2 log cfu/g (Gündüz et al., 2010a,b). Additionally, a recent study by Pezzuto et al. (2016) showed that washing with sodium hypochlorite (200 mg/L) was only able to produce a significant 2 log reduction of *Salmonella* counts on raw rocket (*Eruca vesicaria*) with high initial contamination (7 log cfu/g). At the same time, our study showed that PG molasses has a greater efficacy than water in reducing *Salmonella* on parsley and other types of vegetables. Water wash regularly achieved between 0.5 and 1 log cfu/g reduction values (Faour-Klingbeil et al., 2016).

Based on our findings, we believe that the TPC amounts, classified as "medium" with the values between 1 and 5 g GAE/L (Rufino et al., 2010), is the major determinant for the antibacterial effect of the PG molasses. In contrast, the inhibitory effect of the tested pomegranate products on natural microflora, *S. aureus* and *E. coli* O157 was attributed to their acidic composition in the study of Karabiyikli and Kisla (2012). In this context, the pH of the four PG solutions in our study was measured during the different contact time periods (Table 1); the values showed no difference among the different PG solutions and treatment times in their acidity. Therefore, the preceding role of the PG acidity over the TPC amounts in determining the antibacterial activity does not hold.

3.2 | Stage 2 experiment

The PG molasses was prepared in a salad dressing mix and added to salad vegetables containing parsley leaves inoculated with *S. typhimurium*.

When the different salad ingredients were mixed with parsley without the addition of PG SALD, the mean counts of *S. typhimurium* were 3.34 ± 0.23 with low inoculum levels and 5.90 ± 0.10 with high levels. The onions and tomatoes did not exert any effect on microbial levels and the mean counts of *S. typhimurium* remained nearly constant,

TABLE 2 Inhibitory effect of pomegranate molasses (PG 1:7) on *S. typhimurium** inoculated on parsley at low levels

	Selective agar plating			Agar overlay plating		
Contact time (min)	Mean ± *SD*	cfu/g log reduction	NVG†/Repl	Mean ± *SD*	cfu/g log reduction	NVG†/Repl
Control	**3.75 ± 0.25**			**4.10[a] ± 0.13**		
15	2.42 ± 0.15	−1.32 ± 0.35	2/6	2.62[b] ± 0.14	−1.48 ± 0.13	3/6‡
30	2.06 ± 0.53	−1.80 ± 0.68	2/6*	2.54[bc] ± 0.24	−1.56 ± 0.11	3/6‡
60	2.13 ± 0.58	−1.61 ± 0.47	1/6	3.38[c] ± 0.10	−0.72 ± 0.23	3/6‡

Different superscript letters in the same column within the same group indicate significant difference at $p < .05$.
To avoid over or under estimation, the detection limit was set to 0.7 log cfu/g for samples with NVG and positive results for *Salmonella* detection.
*One replicate had negative results for *Salmonella*. †No visual growth. The numbers in the column indicate replicates with undetectable levels (NVG) out of a total of six replicates. The experiment was repeated six times to account for the variability and obtain validity at low detection levels. ‡The three replicates generated negative results for *Salmonella*.

TABLE 3 The inhibitory effect of pomegranate salad dressing on a salad containing parsley inoculated with low concentrations of *S. typhimurium*

Treatment conditions	Mean ± *SD*	Median
Initial bacterial level (control)	$3.54^{a} \pm 0.57$	3.48
T_0—no sauce*	$3.34^{a} \pm 0.23$	3.34
T_{1h}† mix no SALD at 5 °C	$3.02^{a} \pm 0.57$	3.00
T_{1h}† mix with SALD at 5 °C	$1.55^{b} \pm 0.84$	2.00
T_{1h}‡ mix with SALD at RT	$1.53^{b} \pm 0.80$	2.00

SALD = salad dressing. Same abbreviations apply to Table 4. Different superscript letters in the same column indicate significant difference at $p < .05$.
S. typhimurium levels after mixing contaminated parsley with other salad ingredients without PG SALD. †Salad with or without PG SALD held for 1 hr at 5 °C. ‡Salad with PG SALD held for 1 hr at RT (21–22 °C).

that is, 3.02 ± 0.57 and 5.45 ± 0.52 after 1 hr at 5 °C, correspondingly (Tables 3 and 4). Karabiyikli and Kisla (2012) found that the differences between the initial microflora of the different groups of kısır samples was a result of the ingredients added at the different steps, that is, herbs, salt, and lemon juice. Nonetheless, the decrease in the numbers of inoculated microflora was also nonsignificant.

The significant inhibitory effect of PG molasses was still noted when the contaminated salad vegetables were mixed with the PG SALD. The mean counts of *S. typhimurium* significantly dropped by 2 log cfu/g, from an initial level of 3.54 ± 0.57 to 1.55 ± 0.84 log cfu/g and 1.53 ± 0.80 log cfu/g after 1 hr at 5 °C and at RT, respectively (Table 3). With high initial contamination levels, the bacterial reduction levels reached 3 log cfu/g when the salads were mixed with the PG SALD, however, no significant difference was observed between the mean log number of *Salmonella* at 5 °C and RT (Table 4) indicating that temperature did not alter the potency of PG against *Salmonella*, in this case the TPC activity.

The information on the effect of cold temperature on the phenolic compounds and the activity of antioxidants is relatively scarce in literature. Our results corroborate with Van der Sluis, Dekker, Skrede, and Jongen (2002) who found that the phenolics content of juices were stable during storage at low temperatures, 4 °C or at 20 °C for 1 month and with the findings of Matthes and Schmitz-Eiberger (2009) on the stability of polyphenols in apples.

TABLE 4 The inhibitory effect of pomegranate salad dressing on a salad containing parsley inoculated with high concentrations of *S. typhimurium*

Treatment conditions	Mean ± *SD*	Median
Initial bacterial level (control)	$5.90^{a} \pm 0.10$	5.92
T_0—no sauce	$5.45^{a} \pm 0.52$	5.45
T_{1h} mix no SALD at 5 °C	$4.63^{a} \pm 0.55$	4.94
T_{1h} mix with SALD at 5 °C	$2.76^{b} \pm 0.30$	2.72
T_{1h} mix with SALD at RT	$2.78^{b} \pm 0.50$	2.69
T_{1h} mix with SALD no oil at 5 °C	$2.88^{b} \pm 0.09$	2.90
T_{1h} mix with SALD no oil at RT	$2.94^{b} \pm 0.17$	2.93

Different superscript letters in the same column within the same group indicate significant difference at $p < .05$.
Tukey HSD post hoc test in ANOVA was employed in comparing the means.

To exclude the possible protective effect of fats (olive oil) on *Salmonella* (Hiramatsu, Matsumoto, Sakae, & Miyazaki, 2005), PG dressing without olive oil was mixed with the mixed salad vegetables contaminated with high concentrations of *S. typhimurium* for an easier observation and detection of cells. The results showed no significant difference between PG dressing with and without oil in their inhibitory effect during cold holding and RT ($p > .05$) (Table 3) and were in line with Karabiyikli and Kisla (2012) who observed a decrease in the initial microflora of green vegetables by approximately 2–3 log after 10 min of contact time with the pomegranate products. Several factors including the food matrix are speculated to limit the availability of PG phenols to act as antimicrobials. For instance, the presence of lipids and low protein contents in food were shown to alter and reduce the potency of antioxidative activity of those compounds (Rico-Munoz & Davidson, 1984; Tassou & Nychas, 1994). On the other hand, it is suggested that the interactions of proteins with aldehydes, by hydrogen bonds between peptides and phenolic groups decrease the effective concentration of those solutions (Davidson, Sofos, & Brane, 2005).

4 | CONCLUSION

This study demonstrated that the traditional PG molasses had a significant antibacterial effect against *S.* typhimurium on parsley leaves and mixed salad vegetables compared with a straight water rinse, and that the increase in contact time does not necessarily promote further reductions in contamination levels. Additionally, PG molasses showed a comparable, and in some cases, greater efficacy against *Salmonella* than other natural antimicrobials and chlorine solutions which contain 200 ppm free chlorine and have an antibacterial activity that is often limited to 1–2 log cfu/g reduction.

The current results suggest that PG molasses have a great potential to enhance the microbial safety of parsley and salad vegetables as a food condiment. Disseminating its benefits as a safe and natural solution for bacterial reduction is important to maximize its use by chefs in restaurants and in households, particularly in Middle Eastern cuisine. Nevertheless, for an efficient reduction of the risks of foodborne illnesses associated with the consumption of RTE salads, the use of PG molasses should be ideally combined with proper disinfection methods and safe handling practices along the supply chain given that the infectious dose of some strains of *Salmonella* and other pathogens can be as few as 100 organisms or even lower.

This research was limited to determine the antibacterial effect of PG and the TPC values in parsley. There remains the need to conduct additional studies to discover the conditions for maximum inhibitory effect for PG molasses. Future work can consider different preparation and environmental conditions to evaluate both the antibacterial and

the anti-quorum sensing of PG molasses in order to provide a fundamental insight into the bacterial reduction mechanism.

ACKNOWLEDGMENTS

The authors are grateful for the support provided by the Department of Nutrition and Food Science, American University of Beirut, and for partial funding from the Lebanese National Council for Scientific Research (CNRS) [grant #102598].

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How to cite this article: Faour-Klingbeil D, Todd ECD. The inhibitory effect of traditional pomegranate molasses on *S. typhimurium* growth on parsley leaves and in mixed salad vegetables. *J Food Saf*. 2018;38:e12469. https://doi.org/10.1111/jfs.12469