Effect of *Psidium guajava* L. leaf extract on beef quality at different storage temperatures

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Abstract

Psidium guajava L., or guava, has been widely reported as having antimicrobial activities against foodborne pathogens. However, the efficacy of *P. guajava* leaf extract at different storage temperatures has not been extensively explored. Therefore, the present work investigated the effect of antibacterial activity of *P. guajava* leaf extract on beef quality at different storage temperatures. Disc diffusion assay was performed on selected foodborne pathogens (*Bacillus cereus* ATCC33019, *B. megaterium* ATCC14581, *B. pumilus* ATCC14884, *B. subtilis* ATCC6633, *Escherichia* coli ATCC43895, *Enterobacter aerogenes* ATCC13048, *Klebsiella pneumoniae* ATCC13773, *Pseudomonas aeruginosa* ATCC9027, *Salmonella* Typhimurium ATCC14028, and *Staphylococcus aureus* ATCC29737) to evaluate the antibacterial activity of the ethanolic extract of *P. guajava* leaves. The results revealed inhibition zones ranging from 7.00 ± 0.00 to 10.00 ± 0.00 mm. MIC and MBC assays were conducted to assess the bacteriostatic and bactericidal effects of the leaf extract at a concentration range of 0.08 to 2.50 mg/mL, and > 5.00 mg/mL, respectively. The stability of the leaf extract was also measured at different temperatures and pH conditions by disc diffusion assay with the minimum inhibition zone of 7.00 ± 0.00 mm. The application of *P. guajava* leaf extract (0.05, 0.50, and 5.00%) on beef samples resulted in a continuous decrease in Total Plate Count during 14-day storage at refrigerated (4.0 ± 2.0°C) and freezing (-18.0 ± 2.0°C) temperatures. The results revealed that *P. guajava* leaf extract can effectively serve as a natural meat preservative to prolong the shelf life of the treated beef up to 14 days.

Introduction

Beef is an essential part of the human diet which contains essential amino acids, high protein content, and beneficial micronutrients (Biesalski, 2005). In Malaysia, beef is the primary source of red meat for animal protein intake, and its annual per capita consumption was reported at 6.5 kg (DVS, 2018; Fazly Ann *et al.,* 2019). Similar to other protein foods, raw beef is also prone to microbial contamination and spoilage during storage (Iulietto *et al.,* 2015). According to the Centers for Disease Control and Prevention (CDC), 4,008 foodborne disease outbreaks implicated in a single food source have been reported from 1998 to 2015, and 641 cases (16%) were associated with the consumption of contaminated beef. The high loads of pathogens potentially affect the consumers after consuming cross-contaminated beef (Bersisa *et al.,* 2019).

Multiple studies have reported the presence of *Carnobacterium*, *Brochothrix thermosphacta*, *Campylobacter* spp., *Escherichia coli* O157:H7, *Clostridium perfringens*, *Lactobacillus*, *Pseudomonas* spp., *Leuconostoc*, *Yersinia enterocolitica*, *Salmonella* spp., *Listeria monocytogenes*, and *Staphylococcus aureus* in raw beef samples stored under different conditions (Zhao *et al.,* 2001; Bhargava *et al.,* 2011; Doulgeraki *et al.,* 2012; Bintsis, 2017). Centers for Disease Control and Prevention (CDC, 2017) has categorised six bacteria including *C. perfringens, Campylobacter* spp., *E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes*, and *S. aureus* as foodborne pathogens. Currently, chemical preservatives are used to prevent microbial spoilage of beef; but, these can adversely affect human health (Anand and Sati, 2013). Therefore, the consumer's concern about the safety of chemical preservatives is rising (Mepham, 2011; Shim *et al.,* 2011). This

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situation leads to the interest and focus on plant extracts for improving the microbiological safety and quality of meat products (Tayel *et al.*, 2012).

   Psidium guajava L., known as *'jambu batu'* in Malaysia, has been widely used in traditional medicine for generations (Gutiérrez *et al.*, 2008; Bijauliya *et al.*, 2018). The biologically active components of *P. guajava* are effective for treating cough, diarrhoea, stomach ache, ulcers, hypertension, and wounds (Ncube *et al.*, 2008). The antibacterial activity of *P. guajava* leaves against *Aeromonas hydrophila*, *Mycobacterium phlei*, *Clostridium maximum*, *Vibrio* spp., *Salmonella* spp., *S. aureus*, and *Shigella* spp. have been reported (Chah *et al.*, 2006; Rattanachaikunsopon and Phumkhachorn, 2010; Rishika and Sharma, 2017). The findings of previous studies have revealed that *P. guajava* leaves can be potentially developed as a natural sanitiser to replace the chemical ones.

   The antimicrobial activities of *P. guajava* have been reported in various studies (Gonçalves *et al.*, 2008; Esimone *et al.*, 2012). However, literature about the antibacterial activity of *P. guajava* leaf extract against foodborne pathogens is limited (Sanches *et al.*, 2005; Mahfuzul Hoque *et al.*, 2007; Rattanachaikunsopon and Phumkhachorn, 2010; Biswas *et al.*, 2013; Farhana *et al.*, 2017). The antibacterial properties of *P. guajava* provide an alternative to reduce the bacterial loads in raw beef, and counter the global issue of foodborne poisoning related to contaminated beef. Therefore, a detailed study was required to determine the optimum storage conditions for *P. guajava* leaf extract to effectively exhibit antibacterial characteristics. The present work thus evaluated (1) the antibacterial activity of *P. guajava* leaf extract against foodborne pathogens, (2) stability of the extract at different temperatures and pH conditions, and (3) its efficacy against bacterial populations in fresh beef at different concentrations and storage temperatures.

Materials and methods

Sample collection

   The *P. guajava* leaves were collected from Putra Agriculture Centre, Universiti Putra Malaysia (UPM), Selangor, oven-dried, and kept in sealed plastic bags at refrigerated temperature before extraction. Fresh beef samples were purchased from Pasar Awam Taman Seri Serdang, Selangor, transported in an icebox, and immediately processed within 1 h upon arrival at Food Safety and Quality Laboratory, Faculty of Food Science and Technology, UPM, Selangor.

Extraction of P. guajava leaves

   A slightly modified extraction procedure from Rukayadi *et al.* (2008) was carried out. Briefly, 100 g of dried *P. guajava* leaves were pulverised using a blender (Panasonic MX-GM1011, Panasonic Corporation, Osaka, Japan), and the fine powder was soaked overnight in 400 mL of 96.0% ethanol (Sigma-Aldrich, Missouri, USA) at room temperature. The soaked powder was vacuum-filtered through Whatman filter paper No. 1 (Whatman International Ltd, Middlesex, England) using EYELA aspirator pump (Tokyo Rikakikai Co., Tokyo, Japan). The filtrate was subsequently concentrated using a rotary vacuum evaporator (Heidolph VV2011, Heidolph Instruments, GmbH & Co. KG, Schwabach, Germany) at 150 rpm and 63.0°C for 30 to 40 min. The crude extract was stored at 4.0 ± 2.0°C until further use.

Preparation of P. guajava leaf extract and bacterial inoculum

   The stock solution of *P. guajava* leaf extract was prepared by adding 1 g of crude extract into 10 mL of 100% (100 mg/mL) dimethylsulfoxide (DMSO) (R & M Marketing, Essex, UK). To achieve a 1% (10 mg/mL) concentration of *P. guajava* leaf extract, 1 mL of stock solution was added into 99 mL of sterile distilled water. This solution (1%) was used to determine antibacterial activity by disc diffusion assay (DDA), minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). Different concentrations (0.00, 0.05, 0.50, and 5.00%) of *P. guajava leaf* extract were prepared for the bacterial enumeration in fresh beef samples. 5.00% concentration was prepared by adding 5.00 mL of the stock solution into 95.00 mL of 10% DMSO, whereas 0.50 and 0.05% concentrations were prepared by adding 0.50 and 0.05 mL of the stock solution into 99.50 and 99.95 mL of 10% DMSO, respectively. The treatment with sterile deionised water (DIW) (B. Braun Medical Industries, Penang, Malaysia) was considered as a 0.00% concentration.

   Different bacterial strains including *Bacillus cereus* ATCC33019, *B. megaterium* ATCC14581, *B. pumilus* ATCC14884, *B. subtilis* ATCC6633, *Escherichia coli* ATCC43895, *Enterobacter aerogenes* ATCC13048, *Klebsiella pneumoniae* ATCC13773, *Pseudomonas aeruginosa* ATCC9027, *Salmonella* Typhimurium ATCC14028, and *Staphylococcus aureus* ATCC29737 were purchased from American Type Culture Collection (Maryland, United States) and maintained at 4.0 ± 2.0°C on nutrient agar medium.

Disc diffusion assay (DDA)

Disc diffusion assay was performed by following the procedure of the Clinical and Laboratory Standards Institute (CLSI, 2012). Sterilised cotton wool swabs were used to spread the bacterial strains on Muller Hinton agar (MHA) using the streak-plating technique. Next, 6 mm sterile paper discs impregnated with 1% *P. guajava* leaf extract were placed in the agar plates previously streaked with the bacterial strains. Then, 0.1% chlorhexidine disc and 10% DMSO disc were placed in the agar plates as the positive and negative controls, respectively. The agar plates were incubated at $37.0 \pm 2.0°C$ for 24 h.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

The MIC of *P. guajava* leaf extract was determined by broth microdilution method using a sterile 96-wells round bottom microtiter plate (Greiner Bio-One GmbH, Frickenhausen, Germany). The inoculum suspensions were adjusted to $10^6 – 10^8$ CFU/mL against a McFarland standard. The wells in column 1 were filled with 200 µL of Mueller-Hinton broth (MHB), and served as negative control (without inoculum and *P. guajava* leaf extract). The wells in column 2 were filled with 200 µL of bacterial suspension (without *P. guajava* leaf extract), and served as positive control. The microdilution of the leaf extract was carried out to achieve different concentrations ranging from 5.000 mg/mL in column 12 to 0.009 mg/mL in column 3. The plates were aerobically incubated for 24 h at $37.0 \pm 2.0°C$. The MBC was determined by sub-culturing the suspension from each MIC well onto MHA plates. Next, 10 µL suspension from each well (column 1 to 12) was pipetted onto agar plates, and the plates were incubated at $37.0 \pm 2.0°C$ for 24 h.

DDA-based stability assessment of P. guajava leaf extract at different temperatures and pH conditions

Five sets of the extract were prepared by pipetting 1.0 mL of 1.00% *P. guajava* leaf extract solution into 1.5 mL sterile microcentrifuge tube (Eppendorf AG, Hamburg, Germany). Each tube was incubated at different temperatures (4.0 ± 2.0, 25.0 ± 2.0, 37.0 ± 2.0, 55.0 ± 2.0, and 80.0 ± 2.0°C) for 1 h before conducting DDA against foodborne pathogens. Similarly, four sets of the extract were separately prepared to evaluate the stability of *P. guajava* leaf extract at different pH conditions (pH 3.0, 5.0, 7.0, and 11.0). The inhibition zones were measured following incubation at $37.0 \pm 2.0°C$ for 24 h.

Enumeration of bacterial loads in the beef samples treated with P. guajava leaf extract

Fresh beef samples (5 g) were cut, placed in Universal bottles, and treated with different concentrations of *P. guajava* leaf extract (tap water, 0.00 (DIW), 0.05, 0.50, and 5.00%). The final volume of each bottle was 4.0 mL. The samples were stored at three different temperatures; freezer (-18.0 ± 2.0°C), refrigerator (4.0 ± 2.0°C), and room temperature (25.0 ± 2.0°C) for 14 d.

The bacterial enumeration of the treated beef samples was carried out at different intervals (0 min, 30 min, 1 h, 2 h, 4 h, 1 d, 7 d, and 14 d) of storage. The samples frozen for 7 and 14 d were thawed at 4.0 ± 2.0°C for 8 h before analysis. Serial dilution was performed by taking 10 µL of liquid sample from each treatment into 990 µL of 0.1% PBS to prepare 10^{-2} to 10^4 dilutions. Next, 10 µL from each dilution was spread onto Plate Count Agar (PCA) and Mannitol Salt Agar (MSA). All inoculated agar plates were incubated based on the requirement of each media. The logarithm numbers of colony-forming units per gram ($\log_{10}$ CFU/g) of samples were calculated by observing and enumerating the colonies after incubation at 37.0 ± 2.0°C for 24 h. All analyses were performed in duplicate for data verification.

Statistical analysis

Minitab® Statistical Software version 16 (Minitab Inc., Pennsylvania, USA) was used to perform statistical analysis. One-way ANOVA was carried out followed by *post-hoc* Tukey's test to analyse the significance among different treatments at a 95% confidence level ($p < 0.05$).

Results and discussions

P. guajava leaf extraction yield

The ethanolic extraction of *P. guajava* leaves by maceration produced 11.5 ± 0.6% recovery yield. Contrarily, Vongsak *et al.* (2013) achieved a higher yield (40.5%) of *Moringa oleifera* leaves by maceration using 70% ethanol. The maceration period is an important factor contributing to the recovery yield. The higher recovery yield might be due to the longer soaking period (72 h) of *M. oleifera* leaves before extraction as compared to the 24 h soaking period of *P. guajava* leaves in the present work.

Disc diffusion assay (DDA)

The antibacterial activity of ethanolic *P. guajava* leaf extract against the tested foodborne

pathogens by DDA produced inhibition zones of 7.00 ± 0.00 to 10.00 ± 0.00 mm. The largest inhibition zone of 10.00 ± 0.00 mm was observed on *P. aeruginosa* that was similar to positive control. Chah *et al.* (2006) have reported that methanolic leaf extract of *P. guajava* inhibited *P. aeruginosa* growth up to 14 mm diameter. The moderate antibacterial activity causing 8.50 ± 0.71 mm inhibition zones was observed on *B. subtilis* and *E. coli*, followed by 8.00 ± 0.00 mm on *B. megaterium, E. aerogenes, S.* Typhimurium, and *S. aureus*; and 7.50 ± 0.00 mm zone of inhibition on B. pumilus. The least inhibition zone of 7.00 ± 0.00 mm diameter was recorded on *B. cereus* and *K. pneumonia*. In the present work, the ethanolic extract of *P. guajava* leaves was found to be effective against *E. coli*; this contradicts the findings of Biswas *et al.* (2013). Abdelrahim *et al.* (2002) reported that methanolic extract of *P. guajava* bark exhibited high antibacterial activity against *E. coli, S. aureus, P. aeruginosa*, and *B. subtilis* by producing inhibition zones of 22, 20, 20, and 18 mm, respectively.

MIC and MBC values of P. guajava leaf extract

The antibacterial activity of *P. guajava* leaf extract was further confirmed by determining the MIC and MBC values. According to Talaro and Chess (2012), the MIC is the least concentration of the antimicrobial agent that visually inhibits the growth of microorganisms after 24 h of incubation. The MBC indicates the lowest concentration of antimicrobial agent that does not visually show any growth of microorganisms (Aamer *et al.*, 2014). In the present work, the MIC of *P. guajava* leaf extract ranged between 0.08 to 2.50 mg/mL against the tested foodborne pathogens, and the growth of *Staphylococcus aureus* was inhibited at the lowest concentration of 0.08 mg/mL. Henie *et al.* (2009) reported a comparatively higher MIC value of *S. aureus* against *P. guajava* methanolic extract concentration of 1.00 mg/mL. In the present work, the highest MIC was detected for *B. cereus* and *E. aerogenes* at 2.50 mg/mL, followed by *B. megaterium, E. coli*, and *S.* Typhimurium at 1.25 mg/mL, *K. pneumoniae* and *P. aeruginosa* at 0.63 mg/mL, and *B. pumilus* and *B. subtilis* at 0.16 mg/mL. The MBC values were recorded at > 5.00 mg/mL for all the tested pathogens. The MBC results indicated that higher than 5.00 mg/mL concentrations of *P. guajava* leaf extract was required to kill the tested pathogens.

Stability of P. guajava leaf extract at different incubation temperatures

The results of *P. guajava* leaf extract stability at different incubation temperatures is presented in Table 1. The results depicted growth inhibition of all the tested pathogens at an inhibition zone range of 7.00 ± 0.00 to 11.50 ± 0.71 mm diameter. Statistically, inhibition zones were not significantly different from each other at the refrigerator temperature (4.0 ± 2.0°C). The

Table 1. The effect of *P. guajava* leaf extract against tested foodborne pathogens at different temperatures.

Foodborne pathogen	Inhibition zone (mm)				
	4°C	25°C	37°C	55°C	80°C
Bacillus cereus ATCC33019	9.00 ± 0.00[Aa]	9.50 ± 0.71[ABa]	8.25 ± 0.35[Fa]	10.00 ± 0.00[Aa]	9.00 ± 0.71[Aa]
Bacillus megaterium ATCC14581	9.25 ± 0.35[Aa]	10.00 ± 0.00[ABa]	9.00 ± 0.00[Ea]	9.00 ± 0.71[Ca]	9.00 ± 0.00[Aa]
Bacillus pumilus ATCC14884	9.25 ± 0.35[Aa]	9.50 ± 0.00[ABa]	9.00 ± 0.00[Ea]	9.50 ± 0.00[Ba]	8.50 ± 0.71[Ba]
Bacillus subtilis ATCC6633	8.50 ± 0.00[Aab]	7.75 ± 0.35[Cb]	9.50 ± 0.71[Da]	8.25 ± 0.35[Eab]	9.00 ± 0.00[Aab]
Escherichia coli ATCC43895	9.00 ± 0.71[Aa]	9.75 ± 0.35[ABa]	10.00 ± 0.00[Ca]	9.00 ± 0.00[Ca]	9.00 ± 0.71[Aa]
Enterobacter aerogenes ATCC13048	9.50 ± 0.71[Aa]	10.00 ± 0.00[ABa]	9.25 ± 0.35[Ea]	9.00 ± 0.00[Ca]	8.50 ± 0.71[Ba]
Klebsiella pneumoniae ATCC13773	9.25 ± 0.35[Aa]	7.50 ± 0.71[Ca]	7.50 ± 0.71[Ga]	9.25 ± 0.35[Ca]	8.00 ± 0.71[Ca]
Pseudomonas aeruginosa ATCC9027	9.50 ± 0.71[Aab]	10.75 ± 0.35[Aa]	10.25 ± 0.35[Ba]	9.75 ± 0.35[Aa]	7.75 ± 0.35[Db]
Salmonella Typhimurium ATCC14028	9.00 ± 0.00[Aa]	8.50 ± 0.71[BCa]	9.00 ± 0.71[Ea]	8.50 ± 0.00[Da]	7.75 ± 0.35[Da]
Staphylococcus aureus ATCC2737	8.00 ± 0.71[Acd]	10.00 ± 0.00[ABab]	11.50 ± 0.71[Aa]	9.50 ± 0.00[Bbc]	7.00 ± 0.00[Ed]

Data are mean ± SD. Means with different uppercase superscripts indicate significantly different inhibition zones among foodborne pathogens at $p < 0.05$. Means with different lowercase superscripts indicate significantly different inhibition zones at different temperatures at $p < 0.05$.

inhibition zones ranged between 8.00 ± 0.71 to 9.50 ± 0.71 mm for all the tested pathogens. At room temperature (25.0 ± 2.0°C), the largest zone of inhibition was observed against *P. aeruginosa* (10.75 ± 0.35 mm), whereas *K. pneumoniae* showed the highest resistance towards the leaf extract with the smallest inhibition zone of 7.50 ± 0.71 mm. *P. guajava* leaf extract was found to be the most effective against *S. aureus* with an inhibition zone of 11.50 ± 0.71 mm at the incubation temperature of 37 ± 2.0°C. *P. guajava* leaf extract was noted to be the most effective against *B. cereus* at the heat treatment temperature of 55.0 ± 2.0°C, and created inhibition zone of 10.00 ± 0.00 mm. The lowest antibacterial activity was observed against *B. subtilis* with an 8.25 ± 0.35 mm clear zone. The leaf extract managed to retain its antibacterial activity at cooking temperature (80.0 ± 2.0°C) by producing inhibition zones of 7.00 ± 0.00 to 9.00 ± 0.00 mm. The largest inhibition zones were observed on *B. cereus, B. megaterium, B. subtilis*, and *E. coli*, whereas the smallest zone was measured against *S. aureus*.

The inhibition zones of *B. cereus, B. megaterium, B. pumilus, E. coli, E. aerogenes, K. pneumonia*, and *S.* Typhimurium were not significantly different at various temperatures. The increase in temperature from 4 to 25°C and 4 to 37°C significantly enhanced the inhibitory effects against *P. aeruginosa* and *S. aureus*, respectively. Contrarily, the increase in temperature from 4 to 25°C slightly reduced the inhibitory activity of

P. guajava leaf extract against *B. subtilis*. The incubation of *P. guajava* leaf extract at 25.0 ± 2.0 and 37.0 ± 2.0°C produced most of the larger inhibition zones in the tested pathogens. *P. guajava* leaf extract was less active at the cooking temperature (80.0 ± 2.0°C) as compared to other incubation temperatures. The slight loss of antibacterial compounds at this temperature might be responsible for the decreased activity. The flavonoid compounds and their derivatives have been reported to inhibit the growth of different bacteria (Naseer *et al.,* 2018). Similarly, terpinene and pinene in the aqueous extract of plant leaves exhibited antibacterial activity (Nair and Chanda, 2007). However, the present work has shown that *P. guajava* leaf extract retained the antibacterial compounds even after exposure to cooking temperature, thus exhibiting its application potential in food items.

Overall, the ethanolic extract of *P. guajava* leaves was stable at different temperatures with a slight decrease in the inhibitory activity. The results revealed that temperature plays an important role in maintaining the antibacterial activity of the leaf extract. Plant extracts should be stable at different temperatures for food applications. The stability of *P. guajava* leaf extract at a range of temperatures depicts its potential to serve as a natural food preservative.

Stability of P. guajava leaf extract at different pH conditions

Table 2. The effect of *P. guajava* leaf extract against tested foodborne pathogens at different pH conditions.

Foodborne pathogen	Inhibition zone (mm)			
	pH 3.0	pH 5.0	pH 7.0	pH 11.0
Bacillus cereus ATCC33019	9.00 ± 0.00[B]	8.00 ± 0.00[C]	7.50 ± 0.71[AB]	9.50 ± 0.00[B]
Bacillus megaterium ATCC14581	8.00 ± 0.00[B]	7.50 ± 0.00[C]	7.00 ± 0.00[B]	10.00 ± 0.00[B]
Bacillus pumilus ATCC14884	8.25 ± 0.35[B]	8.50 ± 0.71[BC]	8.00 ± 0.00[AB]	7.75 ± 0.35[C]
Bacillus subtilis ATCC6633	8.00 ± 0.00[B]	9.00 ± 0.00[ABC]	7.00 ± 0.00[B]	9.00 ± 0.71B[C]
Escherichia coli ATCC43895	8.75 ± 0.35[B]	10.00 ± 0.00[AB]	7.75 ± 0.35[AB]	10.50 ± 0.71[B]
Enterobacter aerogenes ATCC13048	8.50 ± 0.00[B]	10.50 ± 0.71[A]	8.75 ± 0.35[A]	9.25 ± 0.35[BC]
Klebsiella pneumoniae ATCC13773	8.00 ± 0.71[B]	8.50 ± 0.71[BC]	7.25 ± 0.35[AB]	10.00 ± 0.00[B]
Pseudomonas aeruginosa ATCC9027	8.00 ± 0.00[B]	9.25 ± 0.35A[BC]	7.25 ± 0.35[AB]	10.00 ± 0.00[B]
Salmonella Typhimurium ATCC14028	8.00 ± 0.00[B]	10.50 ± 0.71[A]	8.00 ± 0.00[AB]	10.50 ± 0.00[B]
Staphylococcus aureus ATCC2737	19.50 ± 0.00[A]	8.75 ± 0.35[BC]	8.50 ± 0.71[AB]	20.00 ± 0.00[A]

Data are mean ± SD. Means with different uppercase superscripts indicate significantly different inhibition zones among foodborne pathogens at $p < 0.05$.

The results of *P. guajava* leaf extract stability at different pH conditions is presented in Table 2. The results revealed that the growth of all the tested pathogens was inhibited with a range of 7.00 ± 0.00 to 20.00 ± 0.00 mm diameter inhibition zones. Statistically, the acidic condition (pH 3.0) inhibited the pathogens without significant difference, and inhibition zones ranged from 8.00 ± 0.00 to 9.00 ± 0.00 mm, except *S. aureus* where an inhibition zone of 19.50 ± 0.00 mm was observed. The leaf extract incubated at its original pH (pH 5.0) inhibited the growth of *E. aerogenes* and *S.* Typhimurium with the largest inhibition zone of 10.50 ± 0.71. The lowest inhibitory effect was observed against *B. megaterium* with an inhibition zone of 7.50 ± 0.00 mm diameter. At neutral pH condition (pH 7.0), *E. aerogenes* and *B. megaterium* developed the largest (8.75 ± 0.35 mm) and smallest (7.00 ± 0.00 mm) inhibition zones, respectively. The leaf extract incubated under alkaline condition (pH 11) produced inhibition zones in the range of 7.75 ± 0.00 to 20.00 ± 0.00 mm diameter. The strongest effect was observed against *S. aureus* whereas the weakest effect was observed against *B. pumilus*.

Generally, the ethanolic extract of *P. guajava* leaves was stable at different pH including acidic and alkaline conditions. Plant extracts should be stable under different pH conditions for food applications. The ability of *P. guajava* leaf extract to withstand various pH conditions can facilitate its development as a natural food preservative.

Effect of P. guajava leaf extract on beef bacterial population after storage at different concentrations and storage temperatures

The total plate count (TPC) of the beef samples treated with different concentrations of *P. guajava* leaf extract at room temperature for 14 d is illustrated in Figure 1. The results showed an increasing trend of TPC for untreated beef samples during storage, whereas the TPC was significantly reduced up to 24 h in the samples treated with *P. guajava* leaf extract. These findings indicated that the antibacterial activity of the leaf extract can inhibit the growth of pathogens for the first 24 h at room temperature. Depending on the leaf extract concentration, some surviving pathogens can replicate during further storage. The results revealed that the growth of all the pathogens was effectively inhibited after 1 h of storage at 5.00% concentration. Comparatively, the concentrations of 0.50 and 0.05% reduced the TPC in the beef samples from 5.8 to 0.9 and 1.8 $\log_{10}$ CFU/g, respectively, after 24 h of storage before increasing again afterward. *P. guajava* leaf extract successfully prolonged the beef shelf life. The results revealed that the beef treated with 0.05% reached 6.0 $\log_{10}$ CFU/g in 7 d, whereas at higher concentrations the growth of bacterial population took more than 14 d to spoil the beef.

The antibacterial effects of different *P. guajava* leaf extract concentrations were further tested at refrigerator temperature, and the results are presented in Figure 2. The findings depicted decreased TPC values with the storage time. Samples treated at 5.00% concentration completely inhibited the growth of pathogens after 2 h of storage. The antibacterial activity was noted to be higher at room temperature as compared to the refrigerator temperature. However, bacterial growth was not detected at refrigerator temperature up to 14 d of storage. Beef treatments with 0.05 and 0.50% of *P. guajava* leaf extract under refrigerated conditions
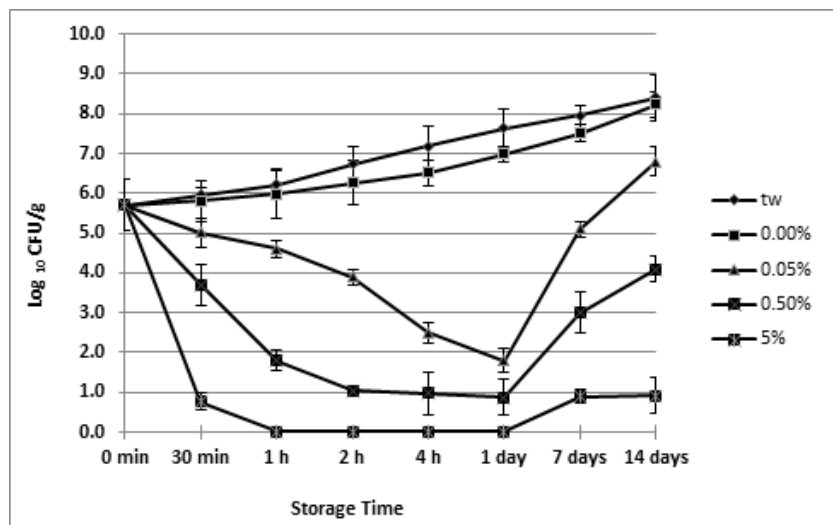

Figure 1. Total Plate Count of beef samples treated with 0.00, 0.05, 0.50, and 5.00% of *P. guajava* leaf extract, and stored at room temperature ($25.0 \pm 2.0°C$) for 14 d.
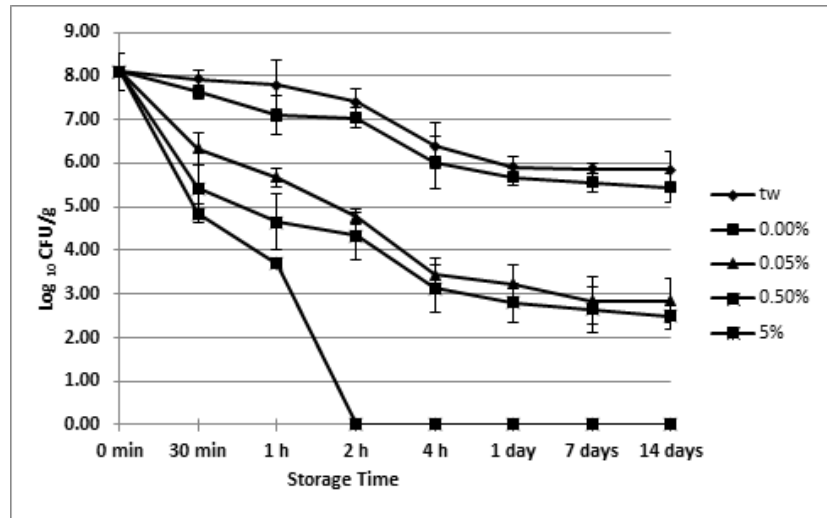
Figure 2. Total Plate Count of beef samples treated with 0.00, 0.05, 0.50, and 5.00% of
P. guajava leaf extract, and stored at refrigerator temperature (4.0 ± 2.0°C) for 14 d.

extended the shelf life up to 14 d. The antibacterial activity of *P. guajava* leaf extract against beef microflora was also stronger as compared to the non-commercial propolis stored at 2°C. Vargas-Sánchez *et al.* (2014) reported that the extract required at least 8 d to approximately achieve 4.0 $\log_{10}$ CFU/g, whereas 0.05% concentration of *P. guajava* leaf extract required less than 4 h of storage at 4°C.

The storage temperature is a crucial factor that determines the growth and distribution of microorganisms (Fazly Ann and Rukayadi, 2019). The leaf extract and storage of samples at freezing temperature synergistically reduced the growth of microorganisms through antibacterial effects and hostile storage conditions (Figure 3). The TPC was significantly reduced both in untreated and treated beef samples. The TPC value of the beef stored in tap water reduced from 8.0 to 6.0 $\log_{10}$ CFU/g after 4 h. The beef treatment with 5.00% concentration of *P. guajava* leaf extract completely inhibited microflora after 4 h of storage at freezing temperature. The total bacterial inhibition at the lower concentrations of 0.50 and 0.05% was noted after 7 and 14 d of storage, respectively. The present work demonstrated that in addition to the antibacterial effect of *P. guajava* leaf extract, the storage temperature also played a key role in the inhibition of bacterial growth. The lower storage temperature required lesser time for the complete bacterial inhibition. However, the shelf life of the treated samples was longer as the TPC remained below 6.0 $\log_{10}$ CFU/g even after 14 d of storage. According to the Fifteenth Schedule (Regulation 39, Table 1) of Food Regulation in 1985, the TPC level of unsafe meat for consumption is above 10^6 CFU/g.

The effect of *P. guajava* leaf extract on *S. aureus* Count was also observed in the present work. The results of the samples tested at different concentrations and stored at room temperature are presented in Figure 4. At room temperature, the population of *S. aureus* increased in untreated samples (tap water and 0.00% concentration) with the storage time. The samples treated with a 5.00% concentration of *P. guajava* leaf extract completely inhibited the growth of *S. aureus* in 30 min. At lower concentrations, a slow rate of inhibitory activities was observed against *S. aureus*. Samples treated with 0.50% concentration of *P. guajava* leaf extract required only 2 h, but the complete growth inhibition of *S. aureus* was noted after 24 h of storage. The inhibitory effects sustained up to 14 d of storage at room temperature.

The results of *S. aureus* Count at different *P. guajava* leaf extract concentrations along with the storage at refrigerator and freezer temperatures are presented in Figures 5 and 6, respectively. *S. aureus* population in the treated samples exhibited decreasing trends during 14 d of the incubation period. A similar trend was also observed in the untreated samples. The treatment of both samples at 5.00% concentration of *P. guajava* leaf extract coupled with the storage at the refrigerator and freezer temperatures completely inhibited the *S. aureus* growth after 2 h of storage. *S. aureus* population was not detected on the MSA plate after 7 d of storage at both temperatures and 0.50% concentration of *P. guajava* leaf extract. The freezing temperature and 0.05% concentration completely inhibited the *S. aureus* growth after 14 d of storage. However, at refrigerator temperature, the extract might require extended storage time to exhibit a
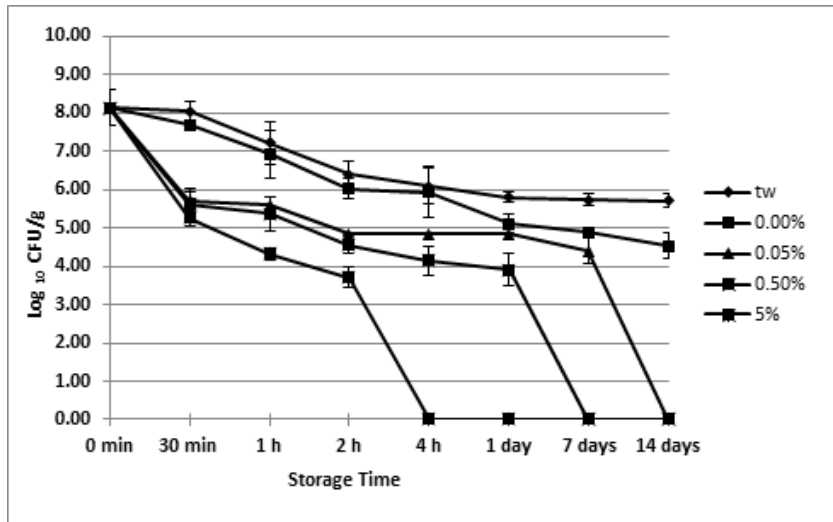
Figure 3. Total Plate Count of beef samples treated with 0.00, 0.05, 0.50, and 5.00% of *P. guajava* leaf extract, and stored at freezer temperature (-18.0 ± 2.0°C) for 14 d.
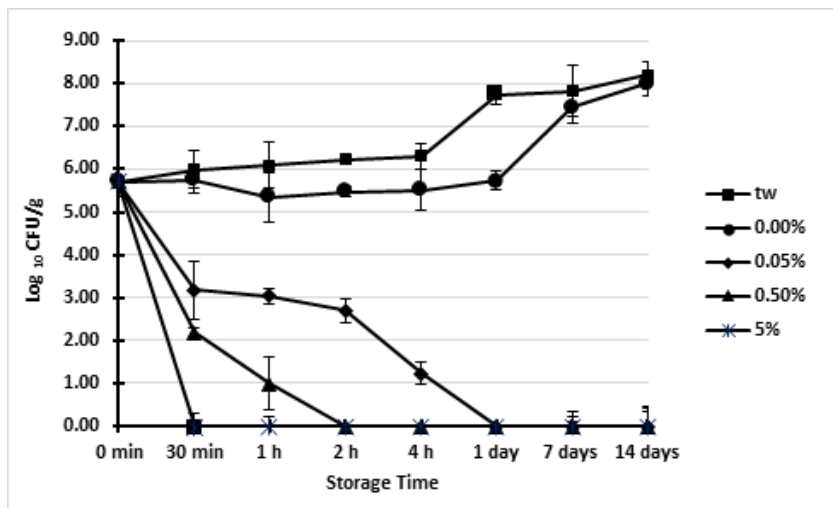

Figure 4. *Staphylococcus aureus* Count of beef samples treated with 0.00, 0.05, 0.50, and 5.00% of *P. guajava* leaf extract, and stored at room temperature (25.0 ± 2.0°C) for 14 d.

Figure 5. *Staphylococcus aureus* Count of beef samples treated with 0.00, 0.05, 0.50, and 5.00% of *P. guajava* leaf extract, and stored at refrigerator temperature (4.0 ± 2.0°C) for 14 d.
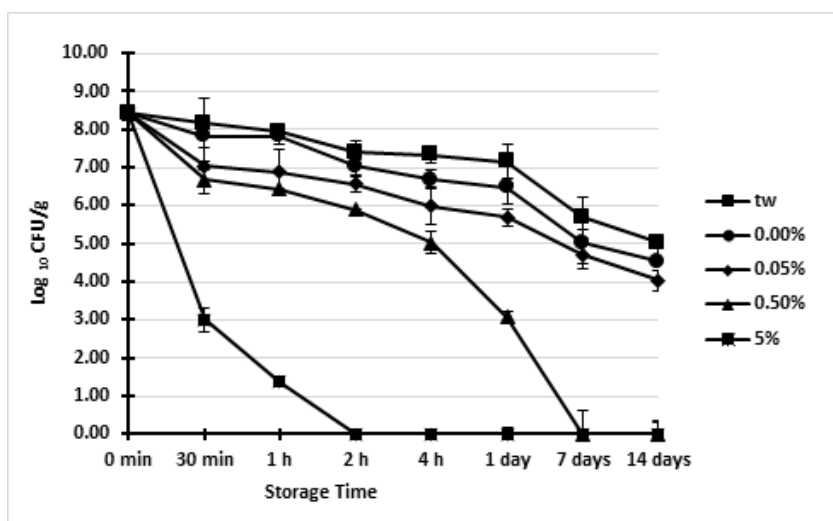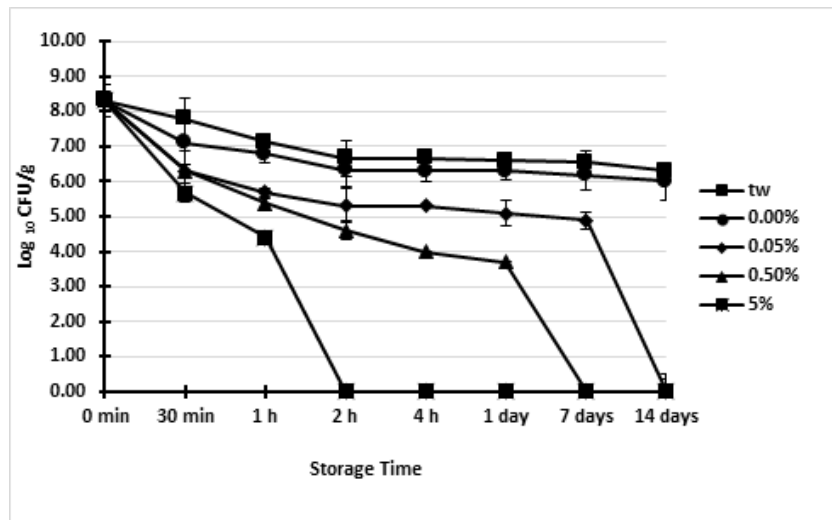
Figure 6. *Staphylococcus aureus* Count of beef samples treated with 0.00, 0.05, 0.50, and 5.00% of *P. guajava* leaf extract, and stored at freezer temperature ($-18.0 \pm 2.0°C$) for 14 d.

similar effect.

In short, antibacterial effects of *P. guajava* leaf extract and storage conditions jointly inhibited the growth of pathogens. Despite the fact that lower concentrations could not completely inhibit the bacterial growth after 14 d of storage, the TPC and *S. aureus* Count was still under the safety limit for consumption. The reduction in the bacterial counts during 14 d of beef storage could prolong the shelf life of the product. The findings revealed that *P. guajava* leaf extract can potentially be developed as a natural meat sanitiser.

Conclusion

In conclusion, *P. guajava* leaf extract exhibited antibacterial activity against all the tested foodborne pathogens. The leaf extract was also found to be stable at different temperatures and pH conditions. Therefore, the leaf extract can be developed into a natural food preservative as it could withstand various processing conditions. The leaf extract can also be used as meat sanitiser as it continuously decreased the bacterial loads during 14-day storage at refrigerator and freezer temperatures.

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