Persistence and survival of some food borne pathogens in neutralized unripe grape products

Nilgün Öncül, Şeniz Karabiyikli

Gaziosmanpaşa University, Tokat, Turkey

Keywords:

Grape
Neutralization
Antimicrobial
Survival
Foodborne
Pathogen

Article history:

Received 30.01.2016
Received in revised form 12.03.2016
Accepted 24.03.2016

Corresponding author:

Nilgün Öncül
E-mail:
nilgunoncul@hotmail.com

Abstract

Introduction. In this study, the persistence and survival of some food borne pathogens (*E. coli*, *L. monocytogenes, S.* Typhimurium and *S. aureus*) in neutralized unripe grape products (verjuice and sour grape sauce) which are particularly rich in antioxidants and organic acids were evaluated.

Materials and methods. The survival patterns of these pathogens in un-neutralized unripe grape products were determined previously. The test pathogens were inoculated in neutralized unripe grape products at two different inoculum doses (2 and 6 log CFU/mL) and all the samples were kept at room temperature (approximately 25°C) for 0, 5, 15 and 30 minutes after inoculation with pathogens, separately.

Results and discussion. The presence of initial microflora is important for food quality and safety. It was mentioned that the unripe grape products had no competitive microflora that could be affect the survival patterns of inoculated pathogens. The initial cell number of *E. coli*, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* were counted as to 2.50, 2.38, 2.52, and 2.21 log CFU/mL for low inoculation dose and 6.00, 6.49, 6.45, and 6.57 log CFU/mL for high inoculation dose, respectively. No viable cells were detected in negative controls. The decreasing numbers of tested pathogens were significant at low inoculation doses after 30 minutes ($p<0.05$), while there was no significant difference at high inoculum doses in the same treatment time ($p>0.05$). The unripe grape products have self-protection systems and they could be assumed as 'microbiologically safe products' when they were contaminated with pathogens at low levels, and it was associated with the phenolic content they have. However, food borne pathogens, at high contamination levels could survive in unripe grape products in case where the acidic environment was neutralized.

Conclusions.The inhibitory activity of unripe grape products generally based on phenolic compounds and organic acid contents, and the organic acids and phenolic compounds inhibit the pathogens in a synergistic way.

Introduction

Natural products are chemical compounds or substance obtained from a living organism or presented in nature which has pharmacological or biological activity [1]. Living organisms produce secondary metabolites that can be used as antimicrobial agent against to food borne pathogens [2, 3]. These secondary metabolites can be extracted from different origins as microorganisms, animals and plants [4]. These natural antimicrobials could yield better results than synthetic/chemical preservatives that especially have adverse effects on human health [5, 6]. The synthetic preservatives could be the reason of hives, itching, asthma, allergies, lung irritation, tumors, antibiotic resistance in human as well as mutagenic and carcinogenic effects on metabolism [7, 8, 9]. Therefore, natural antimicrobials, especially plants are been given more attention in the consumers due to their properties of ensuring the food safety by preventing the survival of pathogenic microorganisms [5, 6, 10].

In recent years there are lots of studies have been indicating the antimicrobial activity of plant based products. These studies have been mentioned that there are over 1340 plants with antimicrobials activities which are defined, and over 30.000 compounds have been isolated from plants that shown antimicrobial properties [10, 11]. The plants themselves (leaves, stems, buds, flowers, fruits, juices, seeds, bulbs and rhizomes) or the compounds held from plants (extracts, essential oils) have been used as plant based antimicrobials to ensure the food safety in these studies [10, 12, 13, 14]. The plant based products such as fruit and vegetables or their juices, herbs and spices, essential oils, extracts, and fermented products such as vinegar have been used to extend shelf life of foods with ensuring the food safety and quality [15, 16, 17].

The antimicrobial effect of fruit and vegetable juices as grape, pomegranate, noni, garlic, lemon, unripe papaya, raspberry, black currant, gooseberry, jostaberry, radish, leek, and onion were stated against *Bacillus* spp., *Bacteroides* spp., *Citrobacter* spp., *Clostridium* spp., *Micrococcus* spp., *Mycoplasma* spp., *Neisseria* spp., *Salmonella* spp., *Serratia* spp., *Shigella* spp., *Staplococcus* spp., *Aspergillus niger*, *Candida albicans*, *Corynebacterium xerosis*, *Cronobacter sakazakii, Enterococcus faecalis, Escherichia coli, Escherichia coli* O157:H7, *Klebsiella pneumoniae, Kluyveromyces marxianus, Listeria monocytogenes, Mucor indicus*, *Pseudomonas aeruginosa, Penicillium citrinum*, *Rhizopus oryzae*, *Rhodotorula rubra,* and *Trichoderma reesei* in various studies [18 – 31]. In addition, extensive researches have investigated the antimicrobial mechanism of these products against food borne pathogens. The mechanism is mainly attributed to organic acids, as well as, phenolic compounds [21, 31–33]. The several organic acids like benzoic, capric, fumaric, lactic, malic, tartaric, and acetic are found in foods [17]. The organic acids inhibit the microorganism by targeting their cell wall, membrane, metabolic enzymes, protein synthesis, and genetic material [34]. The phenolic compounds such cinnamic acid, caffeic acid, gallic acid play an important role in antimicrobial activity of fruit and vegetable juices [35, 36].The antimicrobial mechanisms of phenolic compounds associate with damaging the cytoplasmic membrane, collapsing the PMF (proton motive force), disruption of electron flow and depletion of active transport. In a result of these factors, the cell components become coagulated [5, 37].

Unripe grape products such verjuice and sour grape sauce are acidic juices with sour flavor [38]. Nikfardjam (2008) has studied with 7 verjuice samples obtained from different origins. The values of titratable acidity, sugar content, and total phenolic matter ranged between 19.6–39.6 g/L, 0.1–95.1 g/L, and 200–1330 mg/L, respectively [39]. In another research, the mean of pH, titratable acidity, and total phenolic matter were determined as 2.94, 2.74%, and 6900 mg/L [40]. The physicochemical and phytochemical properties, as well as antioxidant capacity of unripe grape products which are also used as material in this study have investigated previously. In that previous study, the mean values of pH, titratable acidity (%) and total phenol content

were 2.42, 3.84%, 473.97 mg/L, 1.036 μmol Trolox/mL (FRAP), and 0.421 μmol Trolox/mL (TEAC) [41]. The impact of unripe grape products on some food borne pathogens were also investigated and the minimum inhibitory concentrations (MICs) of five verjuice and five sour grape sauce samples on *Bacillus cereus, Escherichia coli, Listeria monocytogenes, Salmonella* Typhimurium, and *Staphylococcus aureus* were determined [42]. Analyze was performed for both un-neutralized and neutralized products to detect whether the inhibitory effect depends on the organic acid content of the samples or not. According to the results, the antimicrobial effect of these products is mostly related to their organic acid content. Nonetheless, the inhibitory effect is also dependent on their phenolic compounds.

As mentioned before, there are many studies on fruit or vegetable juice about their antimicrobial properties. The researches are mostly carried out on their original pH values to mention their inhibition mechanisms. However, there are limited studies on the antimicrobial effect of the neutralized juices on pathogenic microorganisms. Thus, this study was aimed to detect inhibition effect of neutralized unripe grape products on food borne pathogen due to their rich phenolic properties.

Materials and methods

Unripe grape products. Two kind of unripe grape products such as verjuice and unripe grape sauce were used in this study. Five verjuices and five unripe grape sauces were tested and the product details were represented in Table 1. The products were obtained by different production methods as traditional, laboratory scale, and industrial. The laboratory method was based on traditional one. The verjuice is produced by squeezing the berries, holding fresh juice by discarding the pomace. The unripe grape sauce has a heat treatment step after extraction of the mash. Some ingredients such as salt and/or olive oil could be added optionally before bottling in the production of the both products. The flow diagram of the production was shown in the Figure 1. The all of the samples were kept at -80°C until analyses and they were held at +4°C during a night for thawing before analyzing. The samples were aseptically neutralized to pH 7.00 (±0.20) with sterile NaOH solutions (106462, Merck, U.S.A).

Test cultures. Four different microorganisms were used for this research work as target pathogens and they were obtained from Gaziosmanpaşa University, Faculty of Engineering and Natural Science, Department of Food Engineering, Food Microbiology Laboratory. The target pathogens were *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 19115), *Salmonella* Typhimurium (ATCC 14028) and *Staphylococcus aureus* (ATCC 25923). Stock cultures were kept at -80°C in Brain Heart Infusion Broth (BHIB, Lab M, LAB049, UK) with 20% glycerol (1.04092.2500, Merck, Germany). The stock cultures were regenerated twice in BHIB at 37±2 °C for 18-24 hours.

Preparation test cultures for inoculation. High and low inoculum doses were performed in this research. The final inoculum dose in unripe grape products was approximately 6 log CFU for high dose, and 2 log CFU/mL for low dose for each pathogen. In the pre-treatment, the growth curves of target pathogens were detected for inoculating the pathogens while they were in the exponential (log) phases of the growth. According to these results, the regenerated cultures were incubated in BHI Broth at 37±2 °C for 1-8 hours. The bacterial cultures were diluted with 0.1% sterile peptone water (PW, Merck, 1.07224, Germany) accordingly to achieve 7 log CFU/mL for high dose and 3 log CFU/mL for low dose. The initial inoculum doses of pathogens were counted onto Brain Hearth Infusion Agar (BHIA, Lab M, LAB 048, UK) by spread plate method for detecting inoculation dose exactly. BHIA plates were incubated at 37 °C for 24-48 hours.

Table 1

Details about the unripe grape product samples tested

Samples		Region	Varieties	Ingredients	Production Method
VERJUICE	1	İzmir city	Yediveren	Verjuice, Salt (0.4%)	Laboratory production
	2	Antalya city	Margaz	Verjuice, Salt (0.4%), Olive oil (3%)	Traditional production
	3	Antalya city	Müşküle	Verjuice, Salt (0.4%), Olive oil (3%)	Traditional production
	4	Ankara city	Kalecik Karası	Verjuice	Laboratory production
	5	Tokat city	Narince	Verjuice, Olive oil (3%)	Laboratory production
SOUR GRAPE SAUCE	6	Aydın city	Yediveren	Verjuice, Salt (0.4%), Olive oil (3%)	Traditional production
	7	Aydın city	Yediveren	Verjuice, Salt (0.4%), Olive oil (3%)	Traditional production
	8	Tokat city	American Rootstock	Verjuice	Laboratory production
	9	Industrial product 1	Cabernet Sauvignon, Shiraz,Merlot	Verjuice	Industrial manufacturing
	10	Industrial product 2	Cabernet Sauvignon, Shiraz,Merlot	Verjuice, Salt (0.5%)	Industrial manufacturing

Inoculation of neutralized unripe grape samples. Firstly, nine milliliter of neutralized unripe grape samples were poured into sterile test tubes and then one milliliter of the test culture (at 7 log CFU/mL for high dose and at 3 log CFU/mL for low dose) was placed into the same tube, aseptically. The test tubes were homogenized (Velp, F202A0173, Europe) at 3000 rpm for 5 seconds. So, high (6.0 log CFU/mL) and low (2.0 log CFU/mL) inoculum doses have been achieved finally. For detecting the survival pattern of the test microorganisms, all treated samples were kept at room temperature (approximately 25 ^{o}C) during 0, 5, 15 and 30 minutes. The viable cell numbers were established by surface plating method on BHIA right after serial dilutions were prepared with 0.1% PW. Then, the BHIA plates were incubated at 37 °C for 24-48 hours. The pathogen cultures were assessed as positive control and the products without pathogens as negative control.

Statistical analyses. All experiments were carried out with two replicates and two parallels. The significant difference between the means was established by ANOVA variance analysis and Duncan tests. The results were analyzed with the SPSS statistical package program (SPSS 17.0 for Windows Evaluation Version, 17.0.3); SPSS Inc., Chicago, USA). Independent-Samples T-Test was used for comparing the means of verjuice and unripe grape sauce.

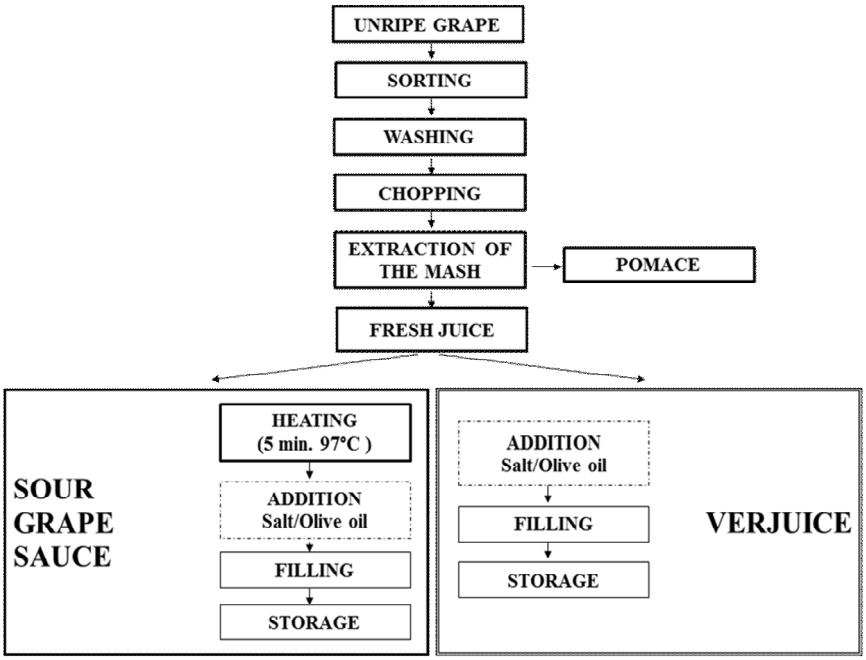

Fig. 1. Flow diagram for laboratory production of verjuice and sour grape sauce based on the traditional methods

Results and discussions

The microbiological properties of unripe grape products which were used in this research had been examined in the previous study. The unripe grape products were tested for enumeration analyses of total mesophilic aerobic bacteria, total psychrophilic aerobic bacteria, yeasts and molds, lactic acid bacteria, *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus*, total coliform bacteria and total fecal coliform bacteria. In the same time, the products were also tested for the presence of *Escherichia coli*, *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes*. The presence of initial microflora is important for food quality and safety. It was mentioned that the unripe grape products had no competitive microflora that could be affect the survival patterns of inoculated pathogens [42].

The initial cell number of *E. coli*, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* were counted as to 2.50, 2.38, 2.52, and 2.21 log CFU/mL for low inoculation dose and 6.00, 6.49, 6.45, and 6.57 log CFU/mL for high inoculation dose, respectively. No viable cells were detected in negative controls. There was significant differences between the positive control and treatment times in low inoculum doses ($p<0.05$) while there was no significant difference in high doses ($p>0.05$).These results possibly depend on increasing pathogens numbers while the antimicrobial ingredient amount is stable. Because of the effective component is constant, the increasing numbers of inoculated cells could not be inhibited effectively (tab. 2–5).

The neutralized products were produced a slight reduction on the number of *E. coli* in low dose ($p<0.05$), and this reduction was continued throughout the treatment period (tab. 2). The effect of samples 1, 2, and 4 were increased by treatment time. The differences between the

mean values of verjuices and sour grape sauces were not significant ($p>0.05$). However, in high inoculum doses the neutralized grape products had no inhibitory activity against the *E. coli* during the application times.

Results in Table 3 showed that, *S.* Typhimurium were inhibited significantly ($p<0.05$) at the beginning of treatment (0. min) by all the samples except samples 1 and 10 at low doses. The samples 1 and 10 also had inhibitory activity by increasing application time ($p>0.05$). On the other side, the neutralized products had no inhibitory activity against *S.* Typhimurium even after the 30 minutes of treatment time at high doses ($p>0.05$). The mean values of the verjuice and sour grape sauce were not significantly different ($p>0.05$).

The count results of *S. aureus* which were inoculated to neutralized products at low and high doses were shown in Table 4. The inhibitory activity of neutralized products on *S. aureus* at low doses were significant ($p<0.05$) when compared with the high doses ($p>0.05$).

The inhibitory activity of neutralized products against *L. monocytogenes* was indicated in Table 5. Some of the samples (1, 3, 5, 7, and 8) had inhibitory activity on *L. monocytogenes* at the beginning of the treatment in low doses. However, the sample 5 only had significant differences from the positive control during the application time ($p<0.05$). The inhibitory effects of verjuices and sour grape sauces were significant only at initial application time ($p<0.05$). At high doses, samples 1, 2, 8, and 9 were shown inhibitory activity at the beginning, and after 5 minutes of treatment ($p<0.05$), but the inhibition disappeared by increasing treatment time. The viable cell numbers were not significant compared to positive control after 30 minutes ($p>0.05$). Also, the inhibitory effects of verjuices and sour grape sauces were not significant ($p>0.05$).

The survival and growth patterns of *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogene*s in neutralized black carrot juice (pH 7.00) were investigated during incubation period at 4 °C and 37 °C for 7 days. The initial counts of *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogene*s were 6.25, 6.37, and 6.21 log CFU/mL at 37 °C and 6.20, 6.24, and 6.16 log CFU/mL at 4 °C, respectively. All the pathogens tested were counted as less than 1 log CFU/mL in neutralized black carrot juice samples stored at 37 °C for 7 days. However, all the pathogens could survive in the samples stored at 4 °C up to 7 days and the viable cell numbers were counted as 5.30, 4.13, and 3.12 log CFU/mL for *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogene*s at the end of 7^{th} day, respectively [43]. The survival and growth patterns of *S.* Typhimurium and *L. monocytogenes* were observed in neutralized sour orange juice during 4 °C and 37 °C for 7 days. *S.* Typhimurium and *L. monocytogenes* were separately inoculated in neutralized sour orange juice and the initial test cultures were counted as 6.26 and 6.11 log CFU/mL for both storage temperatures. The survivor numbers of *S.* Typhimurium and *L. monocytogenes* was not significant after 1 and 3 hours of application and was found as 5.29 and 5.76 log CFU/mL after 7 days at 4 °C. However, it was detected that *S.* Typhimurium and *L. monocytogenes* could survive – even grown – in neutralized juice sample during 1 and 2 days incubation at 37 °C. Conclusively, the numbers of pathogens was decreased to undetectable level after 7 days [44]. Survival of *S.* Typhimurium and *E. coli* O157:H7 in neutralized black mulberry juice was studied by Karabiyikli et al. (2012) [4]. The juice samples were inoculated with test pathogens (6 log CFU/mL) separately and were incubated at 4 °C and 37 °C for 7 days. The viable population of pathogens was increased up to day 2, and was not detected in the end of the treatment at 37 °C. However, population of both pathogens in neutralized black mulberry juice samples was decreased slowly over 7 days. The researchers were investigated survival pattern of *L. monocytogenes* in neutralized black mulberry juice in another study under the same conditions [46]. The juices inhibited approximately 1.5 log unit cells at 37 °C after 1 day incubation, and only approximately 1 log reduction was observed at 4 °C after 7 days.

Table 2

Inhibitory effect of neutralized unripe grape products on *E. coli* (log CFU/mL)

SAMPLES[1]		Low Inoculation Dose				High Inoculation Dose			
		0. min	5. min	15. min	30. min	0. min	5. min	15 min	30 min
VERJUICE	1	2.18 (±0.02) Ba[2]	1.98 (±0.10) Ca	1.90 (±0.00) Cc	1.90 (±0.03) Cbcd	5.86 (±0.10) Aa	5.81 (±0.06) Ab	5.96 (±0.08) Aabc	6.12 (±0.34) Aa
	2	2.13 (±0.04) Ba	2.11 (±0.10) Ba	1.78 (±0.07) Cd	1.84 (±0.04) Ccd	5.82 (±0.11) Aa	5.89 (±0.11) Aab	5.88 (±0.00) Ac	5.95 (±0.50 Aa
	3	2.09 (±0.19) Ba	2.15 (±0.09) Ba	2.20 (±0.02) Ba	2.15 (±0.09) Ba	5.90 (±0.04) Aa	5.86 (±0.16) Aab	5.99 (±0.03) Aabc	6.19 (±0.34) Aa
	4	2.10 (±0.12) Ba	1.99 (±0.02) Ba	1.75 (±0.01) Cd	1.80 (±0.00) Cd	5.91 (±0.25) Aa	5.95 (±0.03) Aab	5.88 (±0.12) Ac	6.21 (±0.07) Aa
	5	1.97 (±0.12) Ba	2.19 (±0.12) Ba	2.10 (±0.01) Bb	2.05 (±0.10) Babc	5.94 (±0.00) Aa	5.88 (±0.04) Aab	5.93 (±0.04) Abc	5.95 (±0.64) Aa
	Mean	2.09 (±0.11) x[3]	2.03 (±0.18) x	1.97 (±0.17) x	1.94 (±0.14) x	5.88 (±0.10) x	5.85 (±0.11) x	5.92 (±0.07) x	6.35 (±0.32) x
UNRIPE GRAPE SAUCE	6	2.18 (±0.05) Ba	2.03 (±0.21) Ba	2.10 (±0.05) Bb	2.04 (±0.08) Babc	5.88 (±0.08) Aa	5.91 (±0.08) Aab	5.98 (±0.02) Aabc	6.15 (±0.50) Aa
	7	2.07 (±0.16) Ba	1.94 (±0.09) Ba	1.98 (±0.01) Bc	2.03 (±0.03) Babc	5.90 (±0.19) Aa	5.93 (±0.22) Aab	6.15 (±0.04) Aa	6.29 (±0.50) Aa
	8	2.16 (±0.08) Ba	2.08 (±0.14) Ba	2.10 (±0.00) Bb	2.07 (±0.05) Bab	5.94 (±0.37) Aa	5.89 (±0.22) Aab	6.06 (±0.02) Aabc	6.11 (±0.45) Aa
	9	2.02 (±0.04) Ba	1.97 (±0.18) Ba	1.91 (±0.04) Bc	2.02 (±0.18) Babc	5.98 (±0.07) Aa	6.00 (±0.04) Aab	5.98 (±0.11) Aabc	6.08 (±0.85) Aa
	10	2.15 (±0.12) Ba	2.11 (±0.00) Ba	2.14 (±0.01) Bab	2.15 (±0.07) Ba	5.99 (±0.02) Aa	6.07 (±0.09) Aa	6.09 (±0.14) Aab	6.22 (±1.24) Aa
	Mean	2.11 (±0.10) x	2.02 (±0.12) x	1.98 (±0.20) x	2.06 (±0.08) x	5.93 (±0.15) x	5.96 (±0.13) x	6.65 (±0.09) y	6.42 (±0.60) x
Positive Control		2.50 (±0.07) Aa				6.00 (±0.21) Aa			

[1]All the samples were tested as negative control before inoculation, and initial microflora could not be detected (<1.00 log CFU/mL)

[2]n=4, (± standard deviation), different lowercase letters indicate differences between rows and different capital letters indicate differences between columns ($p<0.05$).

[3]n=20, mean values of the groups, x and y letters indicate differences between rows ($p<0.05$).

Table 3

Inhibitory effect of neutralized unripe grape products on *S.* Typhimurium (log CFU/mL)

SAMPLES[1]		Low Inoculum Dose				High Inoculum Dose			
		0 min	**5 min**	**15 min**	**30 min**	**0 min**	**5 min**	**15 min**	**30 min**
VERJUICE	1	2.13 (±0.10) Aba[2]	2.11 (±0.15) ABabcd	2.05 (±0.15) Babc	2.03 (±0.10) Bbc	5.38 (±0.80) Abc	5.82 (±0.10) Aabc	6.36 (±0.45) Aa	6.15 (±0.44) Aa
	2	2.09 (±0.14) Ba	1.81 (±0.07) Ccd	1.86 (±0.06) BCbcd	1.92 (±0.12) BCcde	5.91 (±0.47) Aabc	5.95 (±0.35) Ac	6.19 (±0.33) Aa	6.37 (±0.28) Aa
	3	2.08 (±0.18) Ba	2.17 (±0.05) ABab	2.15 (±0.04) Aba	2.16 (±0.06) ABb	5.75 (±0.80) Aabc	5.54 (±0.76) Aabc	6.60 (±0.48) Aa	6.60 (±0.77) Aa
	4	2.07 (±0.13) Ba	1.80 (±0.03) BCd	1.71 (±0.18) Cd	1.83 (±0.10) BCde	6.16 (±0.30) Aab	5.75 (±0.54) Aabc	6.21 (±0.57) Aa	6.51 (±0.70) Aa
	5	2.12 (±0.09) Aa	2.33 (±0.31)Aabcd	2.10 (±0.10) Aab	2.07 (±0.04) Abc	6.42 (±0.06) Aa	6.55 (±0.08) Aab	6.48 (±0.74)Aa	6.56 (±0.78) Aa
	Mean	2.10 (±0.10) x[3]	2.00 (±0.20) x	1.97 (±0.19) x	2.00 (±0.13) x	5.92† (±0.56) x	5.72 (±0.63) x	6.62 (±0.52) x	6.85 (±0.55) x
UNRIPE GRAPE SAUCE	6	2.05 (±0.03) Ba	1.99 (±0.18) Bbcd	2.02 (±0.15) Babc	1.99 (±0.08) Bbcd	6.50 (±0.19) Aa	6.48 (±0.23) Aab	6.35 (±0.94) Aa	6.39 (±0.88) Aa
	7	2.18 (±0.08) Ba	2.09 (±0.06)Babcd	2.12 (±0.06) Ba	2.11 (±0.00) Bbc	6.18 (±0.10) Aab	6.35 (±0.19) Aab	5.71 (±0.62) Aa	6.10 (±0.31) Aa
	8	2.16 (±0.06) Ba	2.00 (±0.06) Bbcd	1.80 (±0.00) Ccd	1.74 (±0.13) Ce	5.13 (±0.32) Cc	5.48 (±0.04) BCbc	6.36 (±0.50) Aba	6.51 (±0.72) Aa
	9	2.06 (±0.16) Ba	2.34 (±0.06) Aa	2.26 (±0.04) Aba	2.37 (±0.05) Aa	6.45 (±0.10) Aa	6.13 (±0.85) Aa	6.33 (±0.7) Aa	6.43 (±0.9) Aa
	10	2.28 (±0.06) ABa	2.14 (±0.06) Cabc	2.09 (±0.08) Cab	2.16 (±0.01) BCb	6.52 (±0.11) Aa	6.27 (±0.74) Aab	6.24 (±0.04) Aa	6.38 (±0.86) Aa
	Mean	2.14 (±0.10) x	2.10 (±0.15) x	2.05 (±0.17) x	2.07 (±0.22) x	6.18 (±0.59) x	6.26 (±0.59) x	6.43 (±0.68) x	6.84 (±0.69) x
Positive Control		2.38 (±0.03) Aa				6.49 (±0.25)A Ba			

[1]All the samples were tested as negative control before inoculation, and initial microflora could not be detected (<1.00 log CFU/mL)

[2]n=4, (± standard deviation), different lowercase letters indicate differences between rows and different capital letters indicate differences between columns ($p<0.05$).

[3]n=20, mean values of the groups, x and y letters indicate differences between rows ($p<0.05$).

Table 4

Inhibitory effect of neutralized unripe grape products on *St. aureus* (log CFU/mL)

SAMPLES[1]		Low Inoculum Dose				High Inoculum Dose			
		0 min	5 min	15 min	30 min	0 min	5 min	15 min	30 min
VERJUICE	1	1.92 (±0.04) Bc	1.92 (±0.04) Bbc	1.81 (±0.04) Cabc	1.77 (±0.00) Cb	5.87 (±0.69) Aa	5.81 (±0.90) Aa	5.79 (±0.48) Aa	5.65 (±0.33) Aa
	2	1.47 (±0.00) Bf	1.47 (±0.00) Bd	1.45 (±0.21) Bc	1.38 (±0.12) Bc	5.20 (±0.14) Ba	6.03 (±0.52) Aba	5.85 (±0.66) Aba	5.71 (±0.27) Aba
	3	1.65 (±0.07) Be	1.69 (±0.12) Bcd	1.73 (±0.05) Bbc	1.65 (±0.07) Bb	6.03 (±0.57) Aa	5.99 (±0.42) Aa	6.15 (±0.69) Aa	5.75 (±0.42) Aa
	4	1.77 (±0.10) Bd	1.53 (±0.08) BCd	1.45 (±0.21) BCc	1.38 (±0.12) Cc	5.78 (±0.85) Aa	5.70 (±0.77) Aa	5.78 (±0.54) Aa	5.69 (±0.60) Aa
	5	2.33 (±0.04) Aa	2.36 (±0.07) Aa	1.73 (±0.05) Bbc	1.80 (±0.14) Bb	6.20 (±0.28) Aa	6.30 (±0.32) Aa	6.01 (±0.61) Aa	5.87 (±0.59) Aa
	Mean	1.83 (±0.30) x[3]	1.78 (±0.32) x	1.63 (±0.19) x	1.60 (±0.20) x	5.81 (±0.55) x	5.96 (±0.51) x	5.91 (±0.47) x	5.73 (±0.35) x
UNRIPE GRAPE SAUCE	6	2.37 (±0.01) Ba	2.21 (±0.01) Cab	2.19 (±0.01) Ca	2.17 (±0.00) Ca	5.70 (±1.13) Aa	6.01 (±0.76) Aa	5.95 (±0.50) Aa	6.07 (±0.55) Aa
	7	2.38 (±0.04) ABa	2.17 (±0.14) Bab	2.17 (±0.08) Ba	2.14 (±0.15) Ba	6.43 (±0.12) Aa	6.14 (±0.52) Aa	6.02 (±0.35) Aa	5.81 (±0.41) Aa
	8	1.92 (±0.03) ABc	1.71 (±0.34) Bcd	1.66 (±0.42) Bc	1.38 (±0.12) Bc	5.40 (±1.05) Aa	5.70 (±0.59) Aa	5.60 (±0.50) Aa	5.67 (±0.68) Aa
	9	2.17 (±0.04) Bb	2.20 (±0.07) Bab	2.13 (±0.02) Bab	2.14 (±0.04) Ba	6.24 (±0.51) Aa	6.24 (±0.56) Aa	6.26 (±0.65) Aa	6.10 (±0.78) Aa
	10	2.30 (±0.03) Ba	2.22 (±0.10) Bab	2.21 (±0.01) Ba	2.17 (±0.14) Ba	6.45 (±0.00) Aa	6.26 (±0.35) Aa	6.32 (±0.50) Aa	6.05 (±0.42) Aa
	Mean	2.21 (±0.16) y	2.10 (±0.24) y	2.06 (±0.28) y	2.00 (±0.33) y	6.04 (±0.70) x	6.06 (±0.47) x	6.02 (±0.46) x	5.94 (±0.47) x
Positive Control		2.52 (±0.05) Aa				6.45 (±0.07) Aa			

[1]All the samples were tested as negative control before inoculation, and initial microflora could not be detected (<1.00 log CFU/mL)

[2]n=4, (± standard deviation), different lowercase letters indicate differences between rows and different capital letters indicate differences between columns ($p<0.05$).

[3]n=20, mean values of the groups, x and y letters indicate differences between rows ($p<0.05$).

Table 5

Inhibitory effect of neutralized unripe grape products on *L. monocytogenes* (log CFU/mL)

SAMPLES[1]		Low Inoculum Dose				High Inoculum Dose			
		0 min	**5 min**	**15 min**	**30 min**	**0 min**	**5 min**	**15 min**	**30 min**
VERJUICE	1	1.66 (±0.37) BCab	1.22 (±0.20) Cb	1.91 (±0.06) Aba	1.92 (±0.10) Aba	5.22 (±0.20) Cb	5.68 (±0.08) Dab	6.42 (±0.08) Aa	6.65 (±0.27) Aa
	2	1.61 (±0.29) ABab	1.72 (±0.12) ABab	2.05 (±0.01) Aa	1.26 (±0.49) Bb	5.80 (±0.54) Aab	6.27 (±0.33) Aab	6.02 (±0.99) Aa	6.60 (±1.17) Aa
	3	1.67 (±0.05) Bab	2.07 (±0.08) Aa	2.07 (±0.06) Aa	2.00 (±0.15) Aa	5.64 (±0.23) Cab	6.67 (±0.07) Aa	6.51 (±0.33) Aa	6.62 (±0.15) Aa
	4	1.72 (±0.14) ABab	1.73 (±0.39) ABab	1.67 (±0.41) Aba	1.24 (±0.39) Bb	6.11 (±0.14) Aab	6.33 (±0.24) Aab	6.32 (±0.41) Aa	6.61 (±0.50) Aa
	5	1.45 (±0.07) Bb	1.72 (±0.29) Bab	2.08 (±0.03) Aa	1.71 (±0.06) Bab	6.24 (±0.47) Aab	6.56 (±0.23) Aa	6.68 (±0.41) Aa	6.75 (±0.40) Aa
	Mean	1.61 (±0.19) x[3]	1.69 (±0.34) x	1.95 (±0.21) x	1.62 (±0.40) x	5.80 (±0.46) x	6.48 (±0.24) x	6.34 (±0.63) x	6.72 (±0.29) x
UNRIPE GRAPE SAUCE	6	1.87 (±0.00) Aab	1.97 (±0.21) Aa	1.79 (±0.29) Aa	1.99 (±0.18) Aa	6.11 (±0.50) Aab	5.93 (±1.06) Aab	4.86 (±2.69) Aa	5.34 (±2.35) Aa
	7	1.77 (±0.13) Bab	1.83 (±0.19) Aba	1.85 (±0.20) Aba	1.80 (±0.14) ABab	5.97 (±0.58) Aab	5.42 (±0.17) Ab	5.29 (±0.78) Aa	5.40 (±0.60) Aa
	8	1.69 (±0.07) Bab	2.16 (±0.16) Aa	1.97 (±0.06) Aa	2.08 (±0.08) Aa	5.52 (±0.73) Cab	6.28 (±0.30) ACab	6.65 (±0.33) Aa	6.77 (±0.20) Aa
	9	1.90 (±0.28) Aab	1.87 (±0.35) Aa	1.83 (±0.18) Aa	1.84 (±0.23) Aa	6.41 (±0.05) Ba	6.39 (±0.08) Bab	6.50 (±0.08) Aa	6.76 (±0.53) Aba
	10	2.00 (±0.08) Aa	1.72 (±0.03) Bab	2.00 (±0.08) Aa	2.04 (±0.18) Aa	6.53 (±0.24) Aa	6.47 (±0.56) Aa	6.77 (±0.01) Aa	6.92 (±0.29) Aa
	Mean	1.84 (±0.15) y	1.91 (±0.22) x	1.88 (±0.16) x	1.95 (±0.17) x	6.10 (±0.52) x	6.17 (±0.65) x	6.15 (±1.34) x	6.30 (±1.17) x
Positive Control		2.21 (±0.01) Aa				6.57 (0.03) ABa			

[1]All the samples were tested as negative control before inoculation, and initial microflora could not be detected (<1.00 log CFU/mL)

[2]n=4, (± standard deviation), different lowercase letters indicate differences between rows and different capital letters indicate differences between columns ($p<0.05$).

[3]n=20, mean values of the groups, x and y letters indicate differences between rows ($p<0.05$).

Conclusions

As a conclusion, unripe grape products have intrinsic characteristics as low pH values, high titratable acidities and rich phenolic content that create a hostile environment for bacterial growth and survival. Hereby, the present study is focused on evaluating the surviving of *E. coli*, *L. monocytogenes*, *S.* Typhimurium and *Staphylococcus aureus* in these neutralized products at room temperature for low and high inoculum doses. Although, statistically significant reductions were observed, the survived population is remarkable at high doses. The inhibition effect on the tested pathogens seems to be very limited or completely disappeared when the inoculation dose is increased. Even though, the phenolic composition of products may be varied due to their species, regions, harvesting time and ripening period, generally the inhibitive activity on target bacteria among the products was not significantly different ($p>0.05$). Therefore, the phenolic content of the samples indicate that these products could have antimicrobial effects on food borne pathogens – besides organic acid compositions.

References

1. Koehn F.E., Carter G.T. (2005),The evolving role of natural products in drug discovery, *Nature Reviews Drug Discovery*, 4, pp. 206.
2. Lv F., Liang H., Yuan Q., Li C. (2011), In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms, *Food Research International*, 44, pp. 3057.
3. Gyawali R., Ibrahim S.A. (2012), Impact of plant derivatives on the growth of foodborne pathogens and the functionality of probiotics, *Applied Microbiology and Biotechnology*, 1.
4. Tiwari B. K., Valdramidis V. P., O'Donnell C. P., Muthukumarappan K., Bourke P., Cullen P. J. (2009). Application of natural antimicrobials for food preservation, *Journal of agricultural and food chemistry*, 57 (14), pp. 5987–6000.
5. Burt S. (2004), Essential oils: their antibacterial properties and potential applications in foods—a review, *International Journal of Food Microbiology,* 94, pp. 223–253.
6. Negi P.S. (2012), Plant extracts for the control of bacterial growth: efficacy, stability and safety issues for food application, *International Journal of Food Microbiology*, 156 (1), pp. 7–17.
7. Rico D., Martin-Diana A.B., Barat J.M., Barry-Ryan C. (2007), Extending and measuring the quality of fresh-cut fruit and vegetables: a review, *Trends in Food Science & Technology*, 18 (7), pp. 373–386.
8. Rangan C., Barceloux D.G. (2009), Food additives and sensitivities. *Disease-a-Month*, 55 (5), pp. 292–311.
9. Oms-Oliu G., Rojas-Grau M.A., Gonzalez L.A., Varela P., Soliva-Fortuny R., Hernando M.I.H., Mnuera I.P., Fiszman S., Martín-Belloso O. (2010), Recent approaches using chemical treatments to preserve quality of fresh-cut fruit: A review, *Postharvest Biology and Technology* 57 (3), pp. 139–148.
10. Tajkarimi M., Ibrahim S., Cliver D. (2010). Antimicrobial herb and spice compounds in food, *Food Control*, 21, 1199.
11. Hayek S.A., Gyawali R., Ibrahim S.A. (2013), Antimicrobial natural products. *Microbial pathogens and strategies for combating them: Science, technology and education*, 2, pp. 910–921.

12. Soomro A.H., Masud T., Anwaar K. (2002), Role of lactic acid bacteria (LAB) in food preservation and human health – A Review, *Pakistan Journal of Nutrition*, 1(1), pp. 20–24.
13. Lopez A.S, Belloso O.M. (2008), Use of nisin and other bacteriocins for preservation of dairy products, *International Dairy Journal*, 18, pp. 329–343.
14. Gyawali R., Ibrahim S.A. (2014), Natural products as antimicrobial agents, *Food Control*, 46, pp. 412–429.
15. Entani, E., Asai, M., Tsujihata, S., Tsukamoto, Y., Ohta, M. (1997), Antibacterial actin of vinegar against food-borne pathogenic bacteria including *Escherichia coli* O157:H7 (Part 2), Effect of sodium chloride and temperature on bactericidal activity], *Kansenshogaku Zasshi,* 71 (5), pp. 451–458.
16. Tiwari R.P., Bharti S.K., Kaur H.D., Dikshit R.P., Hoondal G.S. (2005), Synergistic antimicrobial activity of tea & antibiotics, *Indian Journal of Medical Research*, 122(1), pp. 80-84.
17. Raybaudi-Massilia R.M., Mosqueda-Melgar J., Soliva-Fortuny R., Martin-Belloso O. (2009), Control of pathogenic microorganisms in fresh-cut fruits and fruit juices by traditional and alternative natural antimicrobials, *Comprehensive Reviews in Food Science and Food Safety,* 8, pp. 157–178.
18. Ryan T., Wilkinson J.M., Cavanagh H.M.A. (2001), Antibacterial activity of raspberry cordial in vitro, *Research in Veterinary Science,* 71 (3), pp. 155–159.
19. Onyeagba R.A., Ugbogu O.C., Okeke C.U., Iroakasi O. (2005), Studies on the antimicrobial effects of garlic (*Allium sativum Linn*), ginger (*Zingiber officinale Roscoe*) and lime (*Citrus aurantifolia Linn*), *African Journal of Biotechnology,* 3 (10), pp. 552–554.
20. Aibinu I., Adenipekun T., Adelowotan T., Ogunsanya T., Odugbemi T. (2007), Evaluation of the antimicrobial properties of different parts of *Citrus aurantifolia* (lime fruit) as used locally, *African Journal of Traditional, Complementary, and Alternative Medicines,* 4 (2), 185.
21. Saeed S., Tariq P. (2005), Antibacterial activities of Mentha piperita, Pisum sativum and Momordica charantia, *Pakistan Journal of Botany,* 37 (4), 997.
22. Rhodes, P.L., Mitchell, J.W., Melton, L.D. (2006), Antilisterial activity of grape juice and grape extract derivated from *Vitis vifera* variety Ribier, *International Journal of Food Microbiology*, 107, pp. 281–286.
23. Tomotake, H., Koga, T., Yamota, M., Kassu, A., Ota, F. (2006), Antibacterial activity of citrus fruit juices against Vibrio species, *Journal of Nutritional Science and Vitaminology*, 52, pp. 157–160.
24. Krisch J., Galgóczy L., Tölgyesi M., Papp T., Vágvölgyi C. (2008), Effect of fruit juices and pomace extracts on the growth of Gram-positive and Gram-negative bacteria, *Acta Biologica Szegediensis,* 52 (2), pp. 267–270.
25. Opara L.U., Al-Ani M.R., Al-Shuaibi Y.S. (2009), Physico-chemical properties, vitamin C content, and antimicrobial properties of pomegranate fruit (*Punica granatum* L.), *Food and Bioprocess Technology*, 2 (3), pp. 315–321.
26. Kim T.J., Silva J.L., Jung Y.S. (2009), Antibacterial activity of fresh and processed red muscadine juice and the role of their polar compounds on *Escherichia coli* O157:H7, *Journal of Applied Microbiology,* 107, pp. 533–539.
27. Duman A. D., Ozgen M., Dayisoylu K., Erbil S., N., Durgac C. (2009), Antimicrobial activity of six pomegranate (*punica granatum* l.) varieties and their relation to some of their pomological and phytonutrient characteristics*, Molecules*, 14, pp. 1808–1817

28. Kim T.J., Weng W.L., Silva J.L., Jung Y.S., Marshall D. (2010), Identification of natural antimicrobial substances in red muscadine juice against *Cronobacter sakazakii, Journal of Food Science,* 75 (3), pp. M150–M154.
29. Dahham S.S., Ali M.N., Tabassum H., Khan M. (2010), Studies on antibacterial and antifungal activity of pomegranate (*Punica granatum* L.), *American-Eurasian Journal of Agricultural & Environmental Sciences*, 9 (3), pp. 273–281.
30. Rivera A., Giono S., Gonzalez M., Rodríguez N., Cedillo L. (2011), Antibacterial effect of Morinda citrifolia fruit juice against mycoplasmas, *Annals of Biological Research,* 2 (3), pp. 491–497.
31. Lucera A., Costa C., Conte A, Del Nobile M.A. (2012), Food applications of natural antimicrobial compounds, *Frontiers in Microbiology,* 3.
32. Kıvanc M., Kunduhoğlu B. (1997), Antimicrobial activity of fresh plant juice on the growth of bacteria and yeasts, *Journal of Osmangazi University,* 1 (1), pp. 27–35.
33. Karapinar M., Sengun I.Y. (2007), Antimicrobial effect of koruk (unripe grape—*Vitis vinifera*) juice against *Salmonella* Typhimurium on salad vegetables, *Food Control,* 18, pp. 702–706.
34. Smid E.J, Gorris L.G.M. (2007), *Handbook of Food Preservation*, CRC Press, Newyork.
35. Cowan M.M. (1999), Plant products as antimicrobial agents, *Clinical Microbiology Reviews*, 12 (4), pp. 564–582.
36. Dorman H.J.D., Deans S.G. (2000), Antimicrobial agents from plants: antibacterial activity of plant volatile oils, *Journal of Applied Microbiology*, 88 (2), pp. 308–316.
37. Rasooli I. (2007), Food preservation–a biopreservative approach, *Food*, 1 (2), pp. 111-136.
38. Ahmadi L., Roney S. K. (2014), Pharmacological and Phytochemical Properties of Unripe Grape Juice (Verjuice): A Review, *Austin Journal of Nutrition & Metabolism,* 1 (2), 9.
39. Nikfardjam M.S.P. (2008), General and polyphenolic composition of unripe grape juice (verjus/verjuice) from various producer, *Mitteilungen Klosterneuburg*, 58, pp. 28–31.
40. Hayoğlu I., Kola O., Kaya C., Özer S., Turkoglu H. (2009), Chemical and sensory properties of verjuice, a traditional Turkish non-fermented beverage from Kabarcık and Yediveren grapes, *Journal of Food Processing and Preservation,* 33, pp. 252–263.
41. Öncül N., Karabıyıklı Ş. (2015), Factors affecting the quality attributes of unripe grape functional foods products, *Journal of Food Biochemistry*, 39, pp. 689–695.
42. Karabıyıklı,Ş., Öncül N. (2016), Inhibitory Effect of Unripe Grape Products on Foodborne Pathogens, *Journal of Food Processing and Preservation*, doi:10.1111/jfpp.12731.
43. Degirmenci H., Karapinar M., Karabiyikli S. (2012), The survival of *E. coli* O157: H7, *S.* Typhimurium and *L. monocytogenes* in black carrot (*Daucus carota*) juice, *International Journal of Food Microbiology*, 153 (1), pp. 212–215.
44. Karabıyıklı Ş., Değirmenci H., Karapınar M. (2014), Inhibitory effect of sour orange (Citrus aurantium) juice on Salmonella Typhimurium and Listeria monocytogenes, *LWT-Food Science and Technology*, 55 (2), pp. 421–425.
45. Karabiyikli S., Degirmenci,H., Karapinar M. (2012), The survival of *Escherichia coli* O157: H7 and *Salmonella* Typhimurium in black mulberry (*Morus nigra*) juice, *African Journal of Microbiology Research*, 6 (48), pp. 7464–7470.
46. Karabıyıklı Ş., Değirmenci H., Karapınar M. (2016), Inactivation of Listeria monocytogenes in Black Mullberry (*Morus nigra*) Juice, *Journal of Food Processing and Preservation*, doi:10.1111/jfpp.12840.