Injury and Viability Loss of Escherichia coli O157:H7, Salmonella, Listeria Monocytogenes and Aerobic Mesophilic Bacteria in Apple Juice and Cider Amended with Nisin-Edta

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Abstract

For health reasons, people are consuming fresh juices or minimally processed fruit and vegetable juices, thereby, exposing themselves to the risk of foodborne illness if such juices are contaminated with bacteria pathogens. Behavior of aerobic mesophilic bacteria, Escherichia coli O157:H7, L. monocytogenes and Salmonella cells at 10^6 CFU/ml in apple cider (pH 3.9) and apple juice (pH 3.6), amended with nisin (500 IU/ml)+ethylene diaminetetraacetic acid (EDTA, 0.02 M) combination treatment and storage at 5°C and 10°C for 10 days as well as 22°C for 16 h was investigated. Populations of aerobic mesophilic bacteria increased in untreated apple cider stored at 5°C and 10°C for 10 days while E. coli O157:H7, L. monocytogenes and Salmonella slightly declined. A slight increase for E. coli O157:H7, L. monocytogenes and Salmonella in juices stored at room temperature (22°C) was observed. Treatment of juices with nisin+EDTA led to higher inactivation of bacterial populations including inoculated populations of E. coli O157:H7, L. monocytogenes and Salmonella. The surviving populations determined within 10 to 30 min of treatment include 18% of injured cells. And leakage of UV- absorbing materials were higher in samples containing the injured bacteria. The injured populations did not recover during storage at 5 or 22°C. Waiting up to 4 h before refrigeration of treated samples and leaving treated refrigerated samples at room temperature for up to 4 h did not cause significant changes in microbial populations. Addition of nisin+EDTA combination in unpasteurized apple cider or apple juice as a natural antimicrobial will improve the microbial safety of the juices. However, treatment of juices with nisin+EDTA combination is still subject to regulatory approval by the FDA.

Keyword: Apple juice; Cider; Nisin; EDTA; Combination; Injury; Storage

Introduction

Escherichia coli O157:H7, Listeria monocytogenes and Salmonella are all recognized foodborne pathogens [1-4]. The presence of any one of these pathogens in food is a safety hazard for both consumers and the juice industries as this has led to several food borne outbreaks [5-7] and costly recalls. These bacterial pathogens can persist in a variety of foods and particularly in low acid liquid foods. For example, Escherichia coli O157:H7 cells have been recovered in foods such as apple cider, potato, turkey roll, yogurt, raw milk, and raw fruits and vegetables [4,8]. Foodborne disease outbreaks involving E. coli O157:H7 in apple juices [6, 9,10] and Salmonella in orange juices [7] have raised concerns about the safety of consuming unpasteurized juices. Thermal processing is used by the juice industry to inactivate foodborne pathogens [11] and may lead to changes in off flavor characteristic in juices as a result of such treatment [12,13]. Consumers are becoming more health conscious and tend to opt for food classified as natural or those with minimal or no heat treatment. Unpasteurized apple cider can be classified as a natural or a minimally processed food, and therefore, is a potential vehicle for human bacterial pathogens that may lead to food borne illnesses. Hence, there is a need for alternative non-thermal processing treatments that can achieve a 5 log reductions of human bacterial pathogens [13].

Nisin is a pentacyclic heterodetic subtype a lanthibiotic peptide synthesized by Lactococcus lactis subsp. lactis [14-18]. It is an effective inhibitor of gram-positive bacteria [19-21] and bacterial spores [17]. There are several reports that nisin used in combination with a chelating agent, such as ethylenediaminetetraacetic acid (EDTA), exhibits a bactericidal effect towards both gram-positive and gram-negative bacteria [18,20-24]. Previously, we investigated the growth kinetics of Salmonella, E. coli O157:H7 and L. monocytogenes populations in apple cider amended with 300 IU nisin and the growth data were used to obtain the Lag Phase (LP), Growth Phase (GP) and the generation time (GT) [25]. In that study, effort was not made to investigate the behavior of injured populations in treated apple cider. In our current study, we increased the nisin concentration to 500 IU with the aim of enhancing maximum inactivation of bacteria while minimizing the possibility of generating higher populations of injured cells in treated apple and cider juices. In this study, the effect of nisin+EDTA combination treatment resulting to injury and the possibility of injured cells recovering in treated apple and cider juices during storage was investigated. Also, the effect of waiting period before storage of treated juices and or leaving treated refrigerated juices at room temperature for up to 4 h on microbial populations were investigated. Knowledge of the level of bacteria in freshly prepared and nisin+EDTA treated juice and the impact of waiting period before refrigeration and storage temperature should provide guidance to the juice industry and consumers alike in implementing HACCP plans and Good Manufacturing Practices (GMP’s).

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Materials and Methods

Bacterial strains, growth conditions, and preparation

*L. monocytogenes* (Scott A and LM-4), *Escherichia coli* O157:H7 strains SEA13888 and Oklahoma (apple juice cider-related outbreaks) as well as *Salmonella* Stanley H0585 and *Salmonella* Newport H1275 (all associated with alfalfa sprout-related outbreaks, obtained from Dr. Patricia Griffin, CDC) were maintained on Brain Heart Infusion Agar (BHIA, BBL/Difco, Sparks, MD) slants held at 4°C. All human bacterial pathogens were from the USDA-ARS-ERRC culture collection and were activated by two successive loop transfers at 18 h intervals (37°C) in 20 ml Trypticase Soy Broth (TSB, Difco, Detroit, MI) supplemented with 0.6% yeast extract (TSBY, Difco) for *L. monocytogenes*, and Brain Heart Infusion Broth (BHIB, BBL/Difco, Sparks, MD) for *Salmonella* spp., and *E. coli* O157:H7 cells. All bacterial cultures were individually harvested by centrifugation (10,000 g, 5 min) at 4°C and the cell pellets were washed in Phosphate Buffer Saline (PBS, pH 7.2, BBL/Difco). The cell pellets were used to prepare three different types of inoculum as stated below. Bacterial inoculum for *Escherichia coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* consisted of a mixture containing strains of individual genera (2 strains/genus) listed above at 10⁹ CFU/ml.

Bacterial inoculation

Apple juice and apple cider collected from a local juice processing plant were used in this study. On each day of collection, aerobic mesophilic bacteria of apple juice and apple cider including possible contamination by *L. monocytogenes*, *E. coli* O157:H7 bacteria and *Salmonella* spp. was assessed on selective and non-selective media. On the day of study, both the apple cider and juice were inoculated with a cocktail of *L. monocytogenes*, *E. coli* O157:H7 bacteria and *Salmonella* spp. prepared above at a final concentration of 4.3 log CFU/ml, respectively. Decimal dilutions of the inoculated juices were made with PBS (BBL/Difco), and aliquots (0.1 ml) were plated in duplicate on Modified Oxford agar (MOX, BBL/Difco) for *L. monocytogenes*, Sorbitol MacConkey Agar (SMAC) containing potassium tellurite 2.5 mg/l, cefixime 0.05 mg/l (TC-SMAC, BBL/Difco) for *L. monocytogenes*, BHIA for *E. coli* O157:H7 and Xylose Lysine Sulfide Tetracycline Tryptic Soy Agar (XT4 agar for *Salmonella* spp. and Plate Count Agar (PCA, BBL/Difco) with incubation at 36°C for 24 h to determine the number of colony forming units (CFU/ml).

Preparation of antimicrobial solutions

A stock solution of nisin at 100,000 IU was prepared from (10⁶ IU/g, Sigma, St. Louis, MO) by dissolving in 0.02 N HCl at pH 2.93. Similarly, a stock solution of 0.1 M disodium EDTA (Fisher Scientific Co., Pittsburgh, PA) was prepared in deionized distilled water (ddH₂O), and both nisin and the EDTA were autoclaved at 121°C for 15 min and then stored at room temperature until used. For test solutions, appropriate volumes of the nisin stock solution (125 µL and 5 mL of the EDTA were combined to 20 mL of apple juice or cider to get a final concentration of 500 IU/ml (nisin)+20 mM (EDTA) in the juices. The juices were then inoculated with a cocktail of *E. coli* O157:H7 bacteria, *L. monocytogenes* and *Salmonella* spp. at 4.3 log CFU/ml, respectively. Also, antimicrobial activity of nisin and EDTA alone at concentrations of 500 IU and 20 mM, respectively was tested against the inoculated human bacterial pathogens including the aerobic mesophilic bacteria of the juices. The juices containing each bacterium with or without antimicrobial amendment was vortexed and immediately stored at 5 and 10°C for 10 days, and at room temperature (~22°C) for 24 h. A second set of samples similarly treated was allowed to stay at room temperature immediately after treatments for 1, 2, 3 and 4 h before refrigeration. While a third set of samples were stored in the refrigerator immediately after preparation for 1, 2, 3 and 4 h and then were taken out and were left at room temperature for 24 h. Juices inoculated with human bacterial pathogens and those without the pathogens, and without antimicrobial treatments were used as the positive and negative controls.

Bacterial assays

Survival, inactivation and injured populations of native microflora and human bacterial pathogens in treated and untreated apple cider or juice stored as stated above were determined by plating 0.1 ml of each sample on TSA, MOX, TC-SMAC and XLT4 agar (BBL/Difco) at 0, 2, 4, 6, 8, 10 days for juices stored at 5°C and 10°C. While samples from juices stored at 22°C for 24 h were plated at 0, 2, 4, 6, 8, 10, 12, 14 and 16 h for similar determinations. In a third experiment, samples stored at 23°C for 1 h, were plated (0.1 ml aliquot) onto agar plate as listed above at 10, 20, 30, 40, 50 and 60 min. Decimal dilutions of the sample where appropriate depending on treatments and storage temperature were made before plating in duplicate on a range of agar media stated above. All plates were incubated at 36°C for 24 h to determine the number of colony forming unit that survived or were inactivated [26,27]. All pathogen determined were confirmed according to the FDA Bacteriological Analytical Manual following conventional biochemical methods for each pathogen [28]. Representative presumptive colonies of *L. monocytogenes* were subjected to analysis by use of API Listeria test kits (bioMerieux Marcy l’Etoile, France) for confirmation. Where colonies did not form on the plates, samples were subjected to enrichment method to monitor possible presence of the pathogens.

Microbial injury and viability loss

Surviving populations of human bacterial pathogens from non-selective-TSA and selective MOX, TC-SMAC and XLT4 agar plates were used to estimate microbial injury and viability loss. Injured bacterial populations were determined as follows [(number of viable cells determined on selective agar plates before treatment - number of viable cells determined on selective agar plates after treatment) / number of viable cells determined on selective agar plates before treatment] x 100%--------------------- [1]

The number of colony forming unit (CFU/ml) on nonselective and selective agar media was used to calculate the viability loss which is defined as the differences in log CFU/ml of bacteria between control and treated samples [27]. Leakage of bacteria intracellular ultraviolet (UV)-absorbing materials as a function of membrane damage was determined according to published reports [29,30]. Aliquots (1 ml) of treated and untreated juices were measured at 260 and 280nm for UV materials with a Spectrophotometer (DUR 530, Beckman Coulter, Fullerton, CA).

Finally, bacterial inactivation in treated juices was calculated as follows:

Log (No/N)------------------[2]


Data analysis

The experiments were repeated three times with duplicate determinations for each bacterium per juice, per treatment and per storage temperature and time. Microbial data determined from agar
plates were converted to log_{10} CFU/ml. Data were subjected to analysis of variance (ANOVA) using the Statistical Analysis System Program (SAS Institute, Cary, Version 9.12, NC, USA). Significant differences (p<0.05) between mean values of number of cells and treatment type were determined by the Bonferroni LSD method [31].

Results and Discussion

Effect of Nisin+EDTA on bacterial populations of treated Juice

The total aerobic mesophilic bacteria in apple juice and cider were determined immediately after collection from the producer to establish microbial base line for the study. Apple cider had the highest aerobic mesophilic bacteria than apple juice, and the populations determined in fresh apple juice and cider was 1.5 ± 0.4 and 4.3 ± 0.2 log CFU/ml, respectively. While the populations of aerobic mesophilic bacterial in control samples increased during storage at 22°C, the populations in nisin+EDTA treated samples decreased to approximately 1.0 log at 2 h and the surviving population did not change but slightly increase to approximately 1.5 log in apple cider (Figure 1). The nisin+EDTA treatment of apple juice and cider inactivated most of the aerobic mesophilic bacteria of apple juice and similar observation was seen in juices stored at 5°C and 10°C for 10 days. The populations of aerobic mesophilic bacteria increased in the control samples stored at 5°C and 10°C for 10 days (Data not shown) or at 22°C for 16 h. There were no colony forming units in treated apple juice plated on any of the selective or non-selective agar plates at day 2 and above suggesting total inactivation of the bacteria including yeast and mold.

After inoculation of the juices with E. coli O157:H7, Salmonella spp. and L. monocytogenes at approximately 4.2 log CFU/ml, and treatment with nisin+EDTA and storage at room temperature for 10 h, the efficacy of the treatment on surviving populations and viability loss of the human bacteria pathogens in apple juice is shown in Figure 2. Of all inoculated populations of human bacterial pathogens, only Listeria monocytogenes showed a slight increase in untreated juice during storage. Again, nisin+EDTA treatment inactivated all bacterial pathogens. The result of this study suggests that most of the bacterial inactivation and or the viability loss occurred in the treated juices within 2 h of storage at room temperature. Similar observation in bacterial inactivation was noted at day 2 in juices stored at 5°C or 10°C for 10 days. It is still recommended to store freshly prepared apple juice or cider at refrigeration temperature to slow down the growth of spoilage organisms.

Effect of treatment on bacterial injury

Figures 1 and 2 suggest that bacterial inactivation in treated apple juice and cider occurred within hours in samples stored at 22°C and at day 2 for juices stored at 5°C or 10°C. Figure 1 showed that 18% of the surviving populations of aerobic mesophilic bacteria determined at 2 h of storage were mostly injured cells. In juices inoculated with Salmonella spp., E. coli O157:H7 and L. monocytogenes, and then treated with nisin+EDTA combination, the populations of injured cells determined immediately for each pathogen in apple juice and cider is shown in Figures 3 and 4, respectively. The efficacy of nisin+EDTA treatment on bacterial injury varied in apple juice and cider with L. monocytogenes being more susceptible at a shorter time than any other pathogens tested within the 60 min of storage after treatments. A higher percentage of injured (99.9%) population for L. monocytogenes was determined within 10 min, while injured populations for E. coli O157:H7 and Salmonella spp. determined at 20 min was 85% and 87%, respectively (Figure 3). At 40 min of storage and above, the injured populations among the surviving pathogens could not be determined. No colony forming units was determined on appropriate selective or non-selective plates suggesting total inactivation in the juices. A similar observation was noted in cider juice and injured populations were below 20% at 40 min of storage (Figure 4). Unlike the apple cider, no injured populations were determined in apple juice at 40 min and above suggesting that the efficacy of the treatment was better in apple juice than the cider. The results of this study are in agreement with earlier studies that reported antimicrobial activity of nisin to be within minutes [32].

Bacterial inactivation by nisin-EDTA in treated juices

Inactivation of bacteria in apple juice and cider by the combined treatment of nisin+EDTA is shown Figures 5 and 6, respectively. Total inactivation of L. monocytogenes in apple juice occurred at 30 min for all pathogens (Figure 5). Nisin treatment alone was effective in killing...
Membrane damage and leakage of intracellular substances

Leakage of intracellular UV-materials from the injured bacteria in treated apple cider and juice was monitored and the result is shown in Figure 7. At 10 min after treatments and measurements, leakage of intracellular UV-substances determined from samples containing injured *L. monocytogenes* was significantly (p<0.05) different than values for *E. coli* O157:H7 and *Salmonella* spp., suggesting that nisin+EDTA treatment resulted to quicker and extensive damage to *L. monocytogenes* membrane than *E. coli* O157:H7 and *Salmonella* spp., respectively. This observation is consistent with earlier reports of faster inactivation of Gram positive bacteria than Gram negatives [17,32-35] and bacterial spores [17,34]. Among the three bacterial pathogens tested, only *L. monocytogenes* was inactivated by nisin treatment alone while the EDTA treatment did not cause significant changes in the bacterial populations (Data not shown). Therefore it is appropriate that *L. monocytogenes* being a Gram positive bacterium reacted differently to the treatment, and its susceptibility to nisin+EDTA was faster than *E. coli* O157:H7 and *Salmonella* spp which are Gram negative bacteria. When used in combination with a chelating agent nisin exhibits a bactericidal effect towards both gram-positive and gram-negative bacteria [19,20-23]. The efficacy of nisin+EDTA for bacterial inactivation in apple and cider juice is considered significant because of total inactivation in treated apple juice and low populations of surviving aerobic mesophilic bacteria in treated apple cider. Again, it would be wise to state that fresh cider had higher initial populations of aerobic mesophilic bacteria than apple juice.

There were no significant (p>0.05) changes in the surviving, injured or inactivated populations in treated samples left at room temperature for up to 4 h before refrigeration or in refrigerated samples that were taken out and left at room temperature for up to 4 h suggesting that the microbial safety of nisin+EDTA treated apple cider and juice will be enhanced during storage at room or refrigerated temperatures. The inability of nisin+EDTA to inactivate all the aerobic mesophilic bacteria in apple cider as opposed to the efficacy observed in the apple juice could be attributed to many factors. For example, the aerobic mesophilic bacteria in apple cider includes among other organism like lactic acid bacteria and pseudomonades that may be resistant to the treatment. There are several reports of antagonism of bacterial

$L. monocytogenes$ in apple juice and cider unlike the other pathogens where EDTA has to be added to get similar killing effect. Above 30 min of storage, no residual surviving populations were determined for all the human bacterial pathogens tested during storage. In cider juice similarly treated, bacterial inactivation followed the same trend observed in apple juice (Figure 6). A higher bacterial inactivation was achieved by the nisin+EDTA combination treatment of apple juice than cider however; results suggest that such treatments would improve the microbial safety of both juices. The injured populations decreased as the time of storage increased suggesting that these populations did not recover in treated juices during storage. At 8 h of storage, residual injured bacterial populations determined were not significantly (p>0.5) different than the populations determined at day 0 suggesting that the viable populations remaining in the samples were not susceptible to the treatments.
the concentration we tested for, our media pH was more acidic than what they used and was found to be more effective for inactivation of bacteria. In our preliminary study, we were able to established that a minimum nisin concentration at 12.5 µg/ml with or without 0.02 M EDTA was enough to inactivate L. monocytogenes while the addition of EDTA led to inactivation of Salmonella spp. and E. coli O157:H7 bacteria in the cider and apple juice. A maximum dose limits for nisin use is at 200 mg/kg in canned and plant protein foods and 500 mg/kg in dairy and meat products as set by the FDA and information on fruit juices is limited. The apple juice and cider studied had a pH range of 3.5 to 3.9, respectively. Therefore the use of lesser concentration of nisin at 12.5 µg/ml and 0.02 M EDTA in this study was deemed appropriate for this study.

In conclusion, treatment with nisin-EDTA, the acidity of the juice and the cold storage temperature enhanced the microbial safety of the treated juices by inhibiting recovery of any residual injured microbial populations during storage at 5°C. The results of this study indicate that addition of nisin+EDTA to a freshly prepared unpasteurized apple cider or juice will enhance its microbial safety even when the juice is left at an abusive temperature of 22°C for 24 h. Injured populations of human bacterial pathogens did not recover in nisin+EDTA treated juices during refrigerated or room temperature storage. Bacterial inactivation was faster in apple juice than apple cider and the efficacy was fast and most effective against L. monocytogenes than E. coli O157:H7 and Salmonella spp., respectively in all juices investigated. Also, the results will provide risk assessors and food safety managers a rapid means of estimating the likelihood that any of the pathogen, if present, would grow in response to the treatments and storage temperatures assessed in this study. However, the addition of nisin+EDTA combination treatment for juices is still subject to regulatory approval by the FDA.

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References


