Inactivation of *Escherichia coli* O157:H7 by cinnamon extracts in carrot-kinnow mandarin blends

Moushumi Ghosh · Sarvnrinder Kaur · Abhijit Ganguli

Received: 13 October 2006 / Final revision: 1 March 2007 / Accepted: 12 October 2007

Abstract We investigated the antimicrobial efficacy of cinnamon extracts in laboratory prepared Kinnow-mandarin carrot blends challenged with *Escherichia coli* O157:H7. Freshly squeezed carrot and kinnow-mandarin juices were mixed to obtain a typical blend, inoculated with *E. coli* O157:H7 cultures at 10^2 CFU/mL with and without cinnamon extracts (0.3%) and stored at 4, 8 and 28°C for up to 10 hours. Counts on tryptic soy agar (TSA) selective medium (Mac conkey sorbitol agar) and thin agar layer (TAL) were determined at every 2 hour. The TAL method was used for recovery of sublethally injured cells. Inactivation of *E. coli* O157:H7 in blends containing 0.3% cinnamon extracts were observed, with killing effects being more pronounced at 28°C, similar trends were evident with blends stored under refrigeration conditions (4 and 8°C). The decrease in counts were attributed to several factors namely, pH, storage temperature and addition of cinnamon. The results of our study indicate that cinnamon extracts could be used as an effective, natural antimicrobial for assuring consumer safety at the point of preparation of carrot-kinnow mandarin blends, which is a popular, nutritional beverage consumed in India.

Keywords Unpasteurized · Carrot kinnow-mandarin blends · Cinnamon extracts Inactivation · *E. coli* O157:H7

M. Ghosh · S. Kaur · A. Ganguli (✉)
Department of Biotechnology and Environment Sciences
Thapar Institute of Engineering and Technology (Deemed University)
Patiala - 147 004,
Punjab, India

Tel: +91 / 175 / 2393387, Fax +91 / 175 / 2393738
E-mail: aganguli@tiet.ac.in

Introduction

The mounting apprehension for food borne disease outbreaks have prompted researchers across the globe to develop methods that guarantee food safety, one of which is the use of natural antimicrobials derived from both microbiological and plant sources. Several spices, condiments, and plant extracts have strong medicinal, preservative, and antioxidant properties¹. The antimicrobial activity of these ingredients is attributed to their essential oils, which are lipophilic and penetrate through the membrane to the interior of the cell and perform the inhibitory activity at the target site. Cinnamon effectively inhibits the growth of bacteria, although gram positive are more sensitive than gram negative¹. During the past 30 years *Escherichia coli* in addition to other enteric pathogens, has emerged as one of the most important human diarrheal pathogens; several outbreaks of food-borne infections due to *E. coli* O157:H7 have been reported in different parts of the world, following consumption of unpasteurized apple cider and orange juices^{2,3,4,5}. Developing countries, especially those from the tropics have temperatures that favor the proliferation of this bacteria, a fact reflected by the high prevalence of diarrheal diseases⁶. However, probably because of the recent emergence of this pathogen, there are few reports of its isolation in these countries.

In India, *E. coli* O157:H7 strains have been isolated from patients suffering from acute diarrhea, unpasteurized orange juice, healthy cattle and beef samples^{7,8,9,10,11}. Despite the fact that *E. coli* O157:H7 strains are increasingly being isolated in this part of the subcontinent, no studies have evaluated this pathogen in popular traditional beverages, consequently, no inactivation studies have addressed the utility of natural antimicrobials in popular beverages. Beverages, which com-

prise of freshly squeezed juices are consumed by a large section (all age groups and across the socio-economic spectrum) of the Indian population both in metropolitan and other big cities. Carrots and kinnow-mandarins (*Citrus nobilis* x *Citrus delicosa*) which are produced in considerable amounts during the months of October to March are preferred sources for fresh squeezed juices¹². Carrot kinnow-mandarin blends, prepared by combining portions of freshly squeezed carrot and kinnow-mandarin juices, enjoy a wide popularity. We recently isolated *E. coli* O157:H7 from carrot juices destined for preparation of such blends, the pathogen was shown to survive and grow at ambient temperatures, within periods of consumption in this blend. The objective of our work was therefore, to assess the potential of cinnamon extracts for inactivation of *E. coli* O157:H7 cultures at refrigeration and ambient storage temperatures in blends prepared from carrot and Kinnow-mandarins.

Materials and methods

Strains: *Escherichia coli* O157:H7 ATCC 43895 was used throughout this investigation. Stock cultures were maintained at 4°C with weekly transfer on tryptic soy agar (TSA, Difco, Detroit, USA). The organism was cultured in tryptic soy broth (TSB, Difco, Detroit, USA) at 37°C for 18h; inocula were transferred to TSB at 3 consecutive 24 h intervals before use in experiments.

Culture preparation: The bacteria were sedimented by centrifugation (4,000xg, 20min), washed three times in 0.1% peptone water and adjusted with 0.1% peptone water to obtain cell numbers of approximately 10^4 CFU/mL.

Sample preparation, inoculation and storage of carrot kinnow-mandarin blends: Undamaged, healthy carrots and kinnow-mandarins were surface sterilized with 70% ethanol and ground in a household juicer previously disinfected by repeated applications of antibacterial soap and boiling water. Each 10 mL blend comprised of 40 mL of carrot juice and 60 mL of kinnow-mandarin juice. Aliquots of 100 mL freshly prepared carrot kinnow-mandarin blends were placed into sterile flasks. Ground cinnamon (0.3% wt/vol) were added to the blends, controls consisted of blends lacking cinnamon. Aliquots of cultures (1mL) were inoculated into the blended juice samples to obtain approximately 10^4 cells per mL. Inoculated samples were incubated at 4°C, 8°C and 28°C for 10h.

Monitoring of *E. coli* O157:H7: Aliquots of juice samples were withdrawn after every 2 h, decimal dilutions from

each were prepared in 0.1% peptone water and spread plated onto tryptic soy agar plates and Macconkey sorbitol agar plates (in triplicates) and incubated at 37°C for 24 h were used to enumerate *E. coli* O157:H7. To recover sublethally injured cells, the thin agar layer method was used¹³.

Physicochemical and microbiological analysis: The pH in juice samples were measured potentiometrically with a pH meter combination electrode (Beckman Instruments, Fullerton, Calif.) and the AOAC official method of analysis was used to determine the total solid content of juice samples. For enumeration of aerobic mesophilic bacteria, yeasts and moulds, decimal dilutions of uninoculated juice samples were prepared in 0.1 peptone water. The samples were incubated at 4°C, 8°C and 28°C for 10h and aliquots (withdrawn after every 2 h) spread plated in duplicate onto tryptic soy and potato dextrose agar, incubated at 35°C for 24 h and at 30°C for 4 days respectively.

Statistical analysis: The experiment was performed twice. *Escherichia coli* O157:H7 counts were subjected to analysis of variance by the General linear model procedure of SAS. The statistical significance level was fixed at $P < 0.05$ for each variables (cinnamon dose, addition of cinnamon, medium and point of storage) differences among means were determined and the interactions among the four variables were tested.

Results

No pathogens or indicator microorganisms were detected in unchallenged laboratory prepared blends. The pH of freshly prepared carrot kinnow-mandarin blends was 3.18 ± 0.15 and increased slightly (3.8 after 8 h); the total solids content (11.5 ± 0.46) did not change significantly until a period of 8 h in juice samples held at 28°C. At 4°C *E. coli* O157:H7 counts (initially $4.8 \log \text{CFU/mL}$) in all samples decreased by 1.1 log CFU/mL until the termination of the experiment, whereas at 8°C a slightly higher decrease of 1.35 log CFU/mL was observed. *Escherichia coli* O157:H7 levels remained constant in untreated samples, throughout refrigeration temperatures of 4°C and 8°C. At 28°C, *E. coli* O157:H7 counts in all samples decreased by 1.5 log CFU/mL, in untreated samples held at 28°C, counts of *E. coli* O157:H7 increased ($P < 0.05$), and reached a maximum of 8.8 log CFU/mL (2-fold increase) after 6 hours, further increase in counts were not observed until the product was spoiled (10h)(Fig. 1). The thin layer agar method was as effective as TSA in recovering injured *E. coli* O157:H7, this method of-

fers both selectivity and resuscitation of injured cells thereby allowing the sublethally injured (through heat, organic acids, antimicrobials) population of specific pathogens to be enumerated. Mesophilic counts (Fig. 2) in uninoculated samples were $5.5 \log \text{CFU/mL}$ whereas that for yeasts and moulds was $\log 6.9 \text{CFU/mL}$ (Fig. 3). In treated samples, the mesophilic aerobes registered a decline of as much as $1.7 \log \text{CFU/mL}$, whereas only $1 \log \text{CFU/mL}$ decline was observed with yeast and molds.

Discussion

The carrot kinnow-mandarin blends present a unique combination of fresh squeezed vegetable and fruit juice and are gaining popularity both in the market and in bars because of increased nutritional health benefits, taste and cost. Our research was aimed at determining the efficacy of cinnamon in inactivating *E. coli* O157:H7 in this popular unpasteurized blend within a period of time they are actually consumed. Several authors have studied antimicrobial activity of plant essential oils and found cinnamon to be highly effective against pathogens such as *Salmonella* Sp and *E. coli*. The killing effect in our case was more pronounced at room temperature, this is not unexpected, since metabolism

Fig. 1 *Escherichia coli* O157:H7 counts in carrot kinnow-mandarin blends (with or without cinnamon extracts) at different storage temperatures (4,8 and 28°C) over a period of 10 hours. Symbols represent the means ($\pm$ S.E.) of three replications. Combinations with cinnamon are abbreviated as “Cin” followed by respective storage temperatures.

Fig. 2 Mesophilic bacterial count in uninoculated carrot kinnow-mandarin blends (with and without cinnamon extracts) at different storage temperatures (4,8 and 28°C) over a period of 10 hours. Symbols represent the means ($\pm$ S.E.) of three replications. Combinations with cinnamon are abbreviated as “Cin” followed by respective storage temperatures.

Fig. 3 Yeast-mold counts in uninoculated carrot kinnow-mandarin blends (with and without cinnamon extracts) at different storage temperatures (4,8 and 28°C) over a period of 10 hours. Symbols represent the means ($\pm$ S.E.) of three replications. Combinations with cinnamon are abbreviated as “Cin” followed by respective storage temperatures.

of microorganisms is active at that temperature, whereas at refrigeration temperatures these reactions become slower, therefore growth and death rates are lower. The ability of both survival and growth of *E. coli* O157:H7 in acidic foods and beverages at different storage temperatures have been documented¹⁴.

Fruits and vegetables can become contaminated with *E. coli* O157:H7 whilst growth in fields or during harvest, handling, washing/cleaning, processing, distribution, retail, preparation and final use^{15,16,17}. Contamination may be due to the use of improperly treated manure as fertilizer during pre-harvest of fruits and vegetables. Since *E. coli* O157:H7 has a ruminant reservoir and is resident much longer in manure than in live animals, improperly composted bovine manure could be important sources for transmission of this pathogen especially to produce such as radishes, carrots and leafy vegetables^{18,19}. At least two outbreaks of *E. coli* O157:H7 associated with lettuce were traced back to organic growers who contaminated the produce with manure containing *E. coli*²⁰. Additionally, recent studies on the fate of *E. coli* O157:H7 in cattle manure-compost amended soils on carrots indicate that high cell numbers of this pathogen could persist on carrots for more than 10 weeks²¹. In India, cattle feces are widely used as manure for agricultural practices (there are no regulations regarding safe composting practices for manures). The passage of *E. coli* O157:H7 into carrots through soils amended with cattle feces could be important for transmission of this pathogen in unpasteurized, fresh squeezed carrot juices or carrot kinnow-mandarin blends. Lack of screening and absence of hygienic practices during transport of carrots from farm to the street vendors may actually facilitate the entry of *E. coli* O157:H7 into the juices. Our results indicate that unpasteurized fresh squeezed, carrot kinnow-mandarin blends can support the survival of *E. coli* O157:H7 at refrigeration temperatures and growth at storage temperatures of 28°C, posing a risk to the consumers. Addition of cinnamon extracts at levels of 0.3% to such blends could offer substantial protection to the product, since actual levels of contamination expected, may be much lower than those simulated in this experiment. Besides this a general decrease in aerobic mesophiles possibly accounting for the inherent microflora was also observed, the yeast–mold count were also lowered, although not notably. Since both aerobic mesophiles altogether with yeast –mold can play an important role in spoilage, the contribution of cinnamon extracts in reducing their numbers and prolonging shelf life could be important, although more studies are warranted. Our initial studies are promising for further developing the carrot kinnow-mandarin beverage; however sensory acceptance is required for the cinnamon amended beverage before commercializing the product.

Acknowledgements The authors wish to thank the Director, Thapar Institute of Engineering and Technology, Patiala for providing the adequate infrastructure and financial support.

References

1. Zaika LL (1998) Spices and herbs: their antimicrobial activity and determination. *J Food Saf* 997–118
2. Cheng HY, Yang HY & Chou CC (2002) Influence of acid adaptation on the tolerance of *Escherichia coli* O157:H7 to some subsequent stresses. *J Food Prot* 65 (2):260–265
3. Besser RE, Let SM, Weber JT, Doyle MP & Barrett TJ et al (1993) An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. *J Am Med Assoc* 269:2217–2220
4. (CDC) Centers for Diseases Control and Prevention. Outbreak of *Escherichia coli* O157:H7 infections associated with drinking unpasteurized commercial apple Juice-British Columbia, California, Colorado, and Washington (1996) *Morbidity Mortal Wkly Rep*, 45:975
5. (CDC) Centers for Diseases Control and Prevention. Outbreak of *Escherichia coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider-Connecticut and New York, October, 1996 (1997) *Morbidity Mortal Wkly Rep*, 464–468
6. (WHO) World Health Organization. (2001). Global food safety Agenda and health. Report and draft of the secretariat, resolution: WHA 53.15, Geneva
7. Gupta S, Soni, NK, Kaur P & Sood DK (1992) Verocytotoxic activity of *Escherichia coli* O157 and other 'O' serogroups isolated from patients of diarrhoea. *Ind J Med Res* 95:71–76
8. Singh BR, Kulshreshta SB & Kapoor KN (1996) An orange juice outbreak due to enterotoxigenic *Escherichia coli*. *J Food Sci Technol* 32:504–506
9. Pal A, Ghosh S, Ramamurthy T, Yamasaki S & Tsukamoto T, et al, (1999) Shiga-toxin producing *Escherichia coli* from healthy cattle in a semi-urban community in Calcutta, India. *Ind J Med Res* 110:83–85
10. Dutta S, Deb A, Chattopadhyay UK & Tsukamoto T (2000) Isolation of Shiga toxin producing *Escherichia coli* including O157:H7 strains from dairy cattle and beef samples marketed in Calcutta, India. *J Med Microbiol* 49(8): 765–767
11. Khan A, Das, SC, Ramamurthy T, Sikdar A & Khanam J, et al (2002) Antibiotic Resistance, Virulence Gene and Molecular Profiles of Shiga Toxin-Producing *Escherichia coli* isolates from diverse sources in Calcutta. *Ind J Clin Microbiol* 40(6): 2009–2015
12. Mudgil S, Agarwal D & Ganguli A (2003) Microbiological analysis of street vended fresh squeezed carrot and kinnow-mandarin juices in Patiala city, India. *Internet J Food Saf*, 3:1–3
13. Kang DH & Fung DY (2000) Application of thin agar layer method for recovery of injured *Salmonella typhimurium*. *Int J Food Microbiol* 54:127–132
14. Chikthimmah N, Labrode LF & Beelman RB (2003) Critical factors affecting the destruction of *Escherichia coli* O157:H7 in apple cider treated with Fumaric acid and sodium benzoate. *J Food Saf* 6(4):1438–1442
15. Jaquette CB, Beuchat LR & Mahon BE (1996) Efficacy of chlorine and heat treatment in killing *Salmonella stanley* inoculated onto alfalfa seeds and growth and survival of the pathogen during sprouting and storage. *Appl Environ Microbiol* 62:2212–2215

16. Brackett RE (1999) Incidence, contributing factors, and control of bacterial pathogens in produce, Post Harvest Biol Technol 15:305–311
17. Kudva IT, Blanch K & Hovde CJ (1998) Analysis of *Escherichia coli* O157:H7 survival in ovine or bovine and manure slurry. Appl Environ Microbiol 64:3166–3174
18. Guan TY & Holley RA (2003) Pathogen survival in swine manure environments and transmission of human enteric illness, J Env Qual 32:383
19. Nelson H, (1997) The contamination of organic produce by human sources of human salmonellosis attributable to pork. In Proc. of the 3rd Int. Symp. On the Epidemiology and Control of Salmonella pathogens in animal manures. <http://eap.mcgill.ca/SFMC1.htm> (assessed: 5 Dec. 2004). Ecological Agriculture in Pork, Washington, DC. 5–7, Aug. 1999
20. Islam M, Morgan J, Doyle M & Jiang X (2004) Fate of *Escherichia coli* O157:H7 in manure compost amended soil on carrots and onions grown in an environmentally controlled growth chamber. J Food Prot 67(3):25–42