Effect of Cinnamaldhyde on survival of *E. coli* O_{157}:H_7 in minced meat by using PMA- real time PCR

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Abstract

The antimicrobial effect of cinnamaldehyde on *Escherichia coli* O_{157}:H_7 in ground beef was investigated by inoculation of *Escherichia coli* O_{157}:H_7 into ground beef at 6 logs CFU/g, followed by addition of cinnamaldehyde (0% v/v, 0.3% v/v and 0.6% v/v). The inoculated ground beef was stored at 4˚C for 14 days, *Escherichia coli* O_{157}:H_7 count was determined by both culturing on Sorbitol Mackonkey agar (SMAC) and propidium Monoazide (PMA) real time PCR on days 0, 3, 5, 7 and 14. Cinnamaldehyde reduced the pathogen count by 3 log CFU/g in 14 days at 0.6% v/v and by 2.5 log CFU/g in 14 days at 0.3%. There were no significant differences between measuring of *Escherichia coli* O_{157}:H_7 count by both SMAC and PMA real time PCR. Cytotoxicity of Cinnamaldehyde were evaluated on BHK-21 cell line. Cinnamaldehyde can be best employed in the fight against *Escherichia coli* O_{157}:H_7 in meat products without causing any hazards to the consumer.

Introduction:-

Escherichia coli O_{157}:H_7 is one of the most notorious food-borne pathogens, with an infectious dose as low as a few hundred cells **(Karmali, 2004)**. Beef and dairy products, juices and fresh produce are foods that are often associated with *E. coli* O_{157}:H_7 outbreaks. *E. coli* O_{157}:H_7 infections can lead to nonspecific diarrhea, hemorrhagic colitis and even hemolytic uremic syndrome (HUS) **(Banatvala *et al.*, 2001)**. Majority of *E. coli* O_{157}:H_7 outbreaks have been associated with the consumption of undercooked ground beef and raw milk **(Armstrong *et al.*, 1996; Hancock *et al.*, 1997 and Mao *et al.*, 2001)**, Plant-derived essential oils represent a group of natural antimicrobials that have been traditionally used to preserve foods as well as enhance food flavor. Cinnamaldehyde is an aldehyde present as a major component of bark extract of cinnamon (Cinnamomum verum) **(Holley and Patel, 2005)**. Cinnamaldehyde is classified as a GRAS (generally regarded as safe) molecule by the United States Food and Drug Administration and is approved for use in foods (21 CFR 182.60) **(Adams *et al.*, 2004)**. Although Cinnamaldehyde has been reported to possess an antimicrobial property against food-borne pathogens **(Bilgrami *et al.*, 1992; Burt, 2004; Holley and Patel, 2005)**, its use for improving the safety of ground beef needs to be validated. The involvement of *E. coli* O157:H7 with the consumption of ground beef and beef products

necessitated further development of effective antimicrobial preservative systems for the meat industry in order to control such potential food poisoning organism.

Conventional culture-based methods, involving enrichment, isolation and confirmation steps, have been used for over a century due to their sensitivity, low cost, ease of use, and ability to monitor cell viability **(Murakami, 2012)**. However, they require four to five days to achieve confirmatory results. On the other hand, the polymerase chain reaction (PCR), a widely used nucleic acid-based technique, can identify target species within 3 h. However, conventional PCR cannot differentiate viable cells from dead cells **(Wang and Levin, 2006).** DNA from dead cells can lead to false-positive PCR results. Membrane integrity is considered the most important criterion for distinguishing between viable and irreversibly damaged cells **(Nocker *et al.*, 2006)**. Propidium monoazide (PMA), a DNA intercalating dye, can only penetrate dead or membrane-compromised cells and covalently bind to cellular DNA through photolysis. Consequently, the covalent link will render the DNA insoluble and inhibit PCR amplification of DNA from dead or membrane-compromised cells **(Nocker *et al.*, 2006)**. PMA is less likely to penetrate viable cells with intact membranes. **(Xiao *et al.*, 2013)** combined PMA staining with real-time PCR and successfully detected as low as 100 CFU/mL of *E. coli* O_{157}:H_7 with this assay and exclude those could die due to using of cinnmaldhyde. The aim of this study is to determine the effect of cinnamaldehyde against *E. coli* O_{157}:H_7 in ground beef and detection of count by both culturing on Sorbitol Maconkey agar (SMAC) and PMA- real time PCR.

Materials and methods

Bacterial culture and media

E. coli O_{157}:H_7 strain 505B was obtained from Food Microbiology Laboratory, University of Missouri, Columbia, MO. Strain was grown in tryptic soy broth supplemented with 0.5% yeast extract (TSBY; Difco Labs., BD Diagnostic Systems, Sparks, MD, USA) at 37 °C overnight (~109 CFU/mL). One milliliter of the strain was then harvested by centrifugation at 13,400 ×g for 5 min, and washed and serially diluted in 0.1% peptone water to yield cell suspensions ranging from 100 to 10^8 CFU/mL. The bacterial population in each culture was determined by plating 0.1-ml portions of appropriately diluted culture on duplicate Tryptic soy agar plates (TSA), with incubation at 37 °C for 24 h. 200 µl of the appropriately diluted suspension was used as the inoculum (7 log CFU).

Cinnamaldehyde

Cinnamaldehyde (catalog # 239968) was purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Cinnamaldehyde concentrations used in beef for this study included 0.3% and 0.6% v/v.

Sample preparation, inoculation and storage

Raw ground beef (20% fat) was obtained from local retail store and kept frozen until used. The meat was tested for the presence of *E. coli* O_{157}:H_7 by directly plating on Rainbow agar after enrichment on Tryptic soya broth (TSB) for 24hrs and immunomagnetic separation (IMS) Dynabeads® anti-*E. coli* O_{157} (Applied Biosystem Catalog nos. 71003, 71004). One ml of washed *E. coli* O_{157}:H_7 cell (10^9CFU/ml) was added to one kg of ground beef and mixed well using paddle mixer. The inoculated minced beef was then subdivided into 3 equal portions, followed by addition of one of the two concentrations of cinnamaldhyde to the 1st and 2nd portions respectively, 0.3%, 0.6% and the last portion was left as control positive. The 3 portions were held at 4ºC (chilling) for 14 days.

Microbiological analysis(Feng and Weagent 2002)

The population of *E. coli* O_{157}:H_7 in meat was enumerated on 0, 1, 3, 5, 7 and14 days of storage. Twenty-five grams of each stored ground beef sample was placed in sterile stomacher bags containing filter membranes (Filtra-Bag®, VWR International, Edmonton, AB, Canada). Samples were individually mixed with 225 mL of tryptic soya broth supplemented with 0.5% yeast extract (TSBY) and homogenized for 2 min by stomaching. One milliliter of homogenized suspension from each sample was removed for PMA treatment and plating directly or after serial dilutions (1:10 in PBS) on duplicate tryptic soya agar (TSA) and SMAC ((Oxoid CM 7) plates. Representative colonies (3–4 colonies on each plate) were confirmed as *E. coli* O_{157}:H_7 by streaking on Rainbow agar plates. Three samples for each treatment and control were included at each of the specified temperatures.

Treatment with PMA

Propidium monoazide (PMA) was purchased from Biotium Inc. (Hayward, CA, USA), and a 20 mM stock solution was prepared in sterile distilled water and stored in the dark at −20 °C. 2.5 μL of PMA were added to 1ml of the original solution of the meat samples. Following a 5-min incubation period in the dark at room temperature on a Lab quake Rotisserie (Barnstead International, Dubuque, IA, USA), samples were exposed to a 650-W halogen light for 10 min. The samples were placed on ice at a distance of 20 cm from the light source to avoid excessive heating. After the photo-induced cross linking, cells were collected by centrifugation at 13,400 ×g for 5 min, and washed in sterile distilled water under the same centrifugation conditions prior to DNA extraction **(Liu and Mustapha, 2014).**

DNA extraction

Cell pellets were resuspended in 100 μL of PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA), vortexed for 10 s and boiled for

25 min. Boiled cell suspension was centrifuged at 13,400 ×g for 3 min and 5 µL of the supernatant was used as template DNA for the real-time PCR assay.

Real-time PCR

Primers and probes targeting *E. coli* O_{157}:H_7 and pUC 19 were used as previously described by **Wang, *et al.* (2007 and 2009)** with minor modifications.The sequence of *E. coli* O_{157}:H_7 primer 1 is 5′-TTGACCCACACTTTGCCGTAA-3′, and that of *E. coli* O_{157}:H_7 primer 2 is 5′-GCGAAAACTGTGGAATTGGG-3′. The sequence of *E. coli* O_{157}:H_7 probe is 5′-5HEX-TGACCGCATCGAAACGCAGCT-3BHQ_1-3′. pUC 19 was used as an internal amplification control (IAC). The sequence of the IAC primer 1 is 5′-GCAGCCACTGGTAACAGGAT-3′ and that of the IAC primer 2 is 5′-GCAGAGCGCAGATACCAAAT-3′, and the sequence of the IAC probe is 5′-56FAM-AGAGCGAGGTATGTATGTAGGCGG-3BHQ_1-3′ **(Fricker *et al.*, 2007)**. A 7500 real-time PCR system (Applied Biosystems) was used. A PCR reaction of 50 µL contained 25 µL of 2× TaqMan™ Universal PCR Master Mix (Applied Biosystems), 0.5 µM of each *E. coli* O_{157}:H_7 primer, 0.4 µM of each IAC primer, 0.2 µM of *E. coli* and IAC probe, 0.25 pg of pUC19 (8.62 × 104 copies; Promega, Madison, WI, USA), and 3 µL DNA extract. Nuclease-free water (Promega) was used to adjust the reaction volume to 50 µL. The real-time PCR program consisted of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Cell culture

BHK-21 Baby Hamster Kidney-21 (ATCC origin) was grown in MEM (Minimal Essential Media), cat. No P01515-N50L and supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% v/v 10,000 units/mL penicillin-10,000 µg/mL streptomycin (Gibco) at the temperature of 37 °C and with a flow of 5% CO2.

Cytotoxicity determination

BHK-21 cells were cultured in 25 cm3 flasks. Final concentration of cinnamaldehyde was 0.6%. After 24 h incubation, medium was examined for cytopathic effects.

Statistical data analyses

The values of reduction in viable counts, $\log_{10}$ reduction of *E. coli* O_{157}:H_7 from each trial were plotted versus time of storage. Log_{10} reductions were analyzed using the regression procedure of the Statistical Analyses System (SAS) version 9.1 software for windows **(SAS Institute Inc., Cary, NC).** The model included the treatment concentrations and storage time as the major effects. Least significant difference test was used to determine significant differences (Pb<0.05) due to cinnamaldhyde concentrations and storage time on *E. coli* O_{157}:H_7 counts.

Results and discussion

Cinnamon is one of the world's oldest spices that have been used in food, beverage and cosmetic industry. The antimicrobial components of cinnamon essential oil are cinnamic aldhyde **(Beuchat and Golden 1989; Park *et al*., 2000b)** and eugenol **(Beuchat and Golden 1989 and Senhaji *et al*., 2007)**. Cinnamic aldehyde is an effective inhibitor of the growth of yeasts, bacteria and molds as well as toxin production by microorganisms. It can completely inhibit the growth of a number of bacteria such as *Staphylococcus* sp., *Micrococcus* sp., *Bacillus* sp. and *Enterobacter* sp. at 500 µg/ml **(Masuda *et al*., 1998)**. It has been reported that application of 1.5 mg disc of cinnamic aldehyde revealed potent antimicrobial effects against *Cl. perfringens*, *Bacteroides fragilis* and *Bifidobacterium bifidus* **(Lee and Ahn, 1998). Kim, *et al*. (2004)** recorded that *E. coli* O_{157}:H_7 counts decreased dramatically from $4.9x10^6$ to $1.0x10^2$ CFU/ml in the presence of 500 µg/ml of the purified from cinnamic aldehyde. **Moushumi, *et al.* (2007)** showed that 0.3% cinnamon extract reduce 2 $\log^{10}$ CFU/ml *E. coli* O_{157}:H_7 in carrot blend at 4ºC. **Senhaji, *et al.* (2007)** investigated the antimicrobial activity of 0.25% cinnamon essential oil against *E. coli* O_{157}:H_7 in Brain Heart Infusion broth, and reported that the bacterial count decreased by 4 $\log^{10}$ CFU/ml when incubated at 37ºC for 24 hours while at 0.05% of the oil, most of cells were killed after 30 minutes, they also mentioned that the maximum inhibitory concentration (MIC) of the cinnamon essential oil against *E. coli* O_{157}:H_7 was around 625 ppm.

Both culture-based and real time PCR-based tests confirmed that the ground beef was originally free of *E. coli* O_{157}:H_7.The effect of Cinnamaldehyde on *E. coli* O_{157}:H_7 in meat stored at 4 °C is shown in **Fig (1)**. The average population of *E. coli* O_{157}:H_7 in the treatment and control samples at 0 h was 6 log CFU/ml. Cinnamaldhyde concentrations at 0.3%v/v and 0.6%v/v reduced the *E. coli* O_{157}:H_7 count by 2.5 and 3 log CFU/g through the 14 days storage period respectively. However, *E. coli* O_{157}:H_7 population in the control samples decreased gradually then increased again at the fifth day of storage. Data on **table (1):** showed that both 0.3% and 0.6% cinnamaldehyde significantly reduced count of *E. coli* O_{157}:H_7 from the first day of storage tell the end of storage period. There were no significant differences between results obtained by both culture and real time PCR. However real time PCR could not detect count below 5 log CFU/g and the relatively high detection limit of viable *E. coli* O_{157}:H_7 cells in ground beef (10^5 cells/g) could be caused by: 1) the high counts of background microflora in the ground beef interfering with the detection of target *E. coli* O_{157}:H_7 cells; 2) the food samples which contain many organic and inorganic substances, such as phenolic compounds, fat, enzymes, polysaccharides, proteins and salts, all of which can either inhibit PCR amplification or lead to a reduction in amplification efficiency of PCR

reactions **(Španová *et al*.,2000)**; and 3) a small portion of viable cells that may not have recovered after addition of cinnemaldhyde and the intact membranes of some viable cells that might have been injured due to cinnemaldhyde and penetrated by PMA, leading to fewer DNA templates in the following PCR reactions. The high detection limit of real time PCR is not satisfactory and some papers tried to solve that as **Yang, *et al.*, (2013)** reported that 10^3 CFU/g of viable *E. coli* O_{157}:H_7 in ground beef could be detected by using magnetic nanobead-based immunomagnetic separation (IMS) combined with PMA multiplex PCR (mPCR). Although their IMS–PMA–mPCR assay achieved a better detection limit of *E. coli* O_{157}:H_7 in ground beef (10^3 cells/g) than this study (10^5 cells/g) did, it was still not sensitive enough to detect the pathogen at its infectious dose. An 8-h enrichment step, the PMA real-time PCR assay as described by **Liu and Mustapha, 2014** could detect as low as 1 CFU/g of viable *E. coli* O_{157}:H_7 in ground beef, which is even lower than its infectious dose. Moreover, a real-time PCR assay is more sensitive than traditional PCR and can be quantitative for pathogenic bacteria detection in food.

Cytotoxic effect of cinnamaldhyde on the cell culture used revealed no cytopathic effect of the essential oil in its higher concentration on the BHK-21 cell line.

Conclusion

The obtained results concluded that cinnamaldehyde can be best employed in the fight against *Escherichia coli* O_{157}:H_7 in meat products without causing either irritation to the cell line or the consumer.

Table (1): Effect of Cinnamaldehyde on *E.coli* O_{157}:H_7 in ground beef at 4 °C, count measured by both plating and rt-PCR. No significant difference (P<0.05) between cells contain same letter in the same row. No significant difference (P <0.05) between cells contain same Roman letter in the same column.

time by day	0.3% C		0.6%C		positive control	
	Palting	rt PCR	palting	rt PCR	palting	rt PCR
zero day	$_{a}6^{I}$	$_{a}6^{I}$	$_{a}6^{I}$	$_{a}6^{I}$	$_{b}6.2^{I}$	$_{b}6.2^{I}$
1st	$_{a}5.7^{II}$	$_{a}5.8^{II}$	$_{b}5.5^{II}$	$_{b}5.5^{II}$	$_{c}6.1^{I\,II}$	$_{c}6^{II\,III}$
3rd	$_{a}5.3^{III}$	$_{a}5.4^{III}$	$_{b}4.9^{III}$	$_{b}5^{III}$	$_{c}6^{II}$	$_{c}6^{II\,III}$
5th	$_{a}5^{III\,IV}$	$_{a}5^{IV}$	$_{b}4^{IV}$	N.D	$_{c}6.2^{I\,II\,III}$	$_{c}6.1^{III\,I}$
7th	$_{a}4.5^{V}$	N.D	$_{b}3.5^{V}$	N.D	$_{c}6.3^{III\,IV}$	$_{c}6.2^{I}$
14th	$_{a}3.5^{VI}$	N.D	$_{b}3^{VI}$	N.D	$_{c}6.4^{IV}$	$_{c}6.4^{IV}$

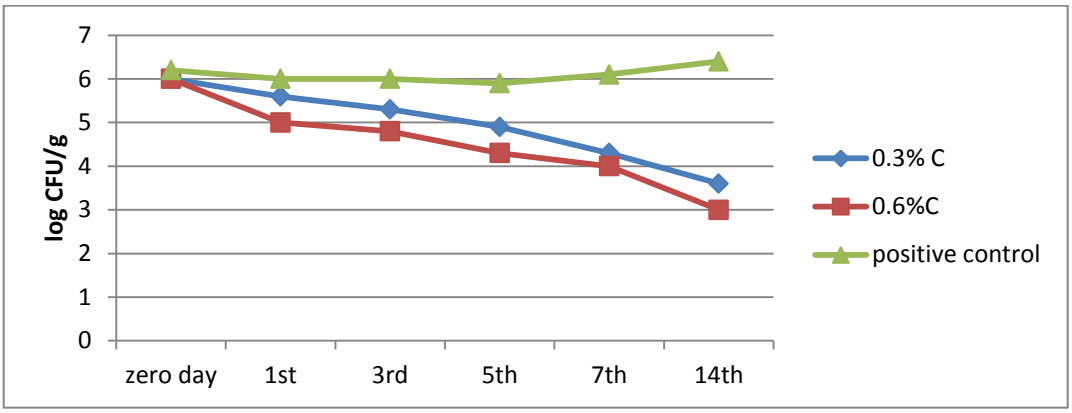

Fig. (1): Effect of Cinnamaldehyde on *Escherichia coli* O_{157}:H_7 in ground beef at 4 °C. Data are expressed as mean. Means were calculated from 3 experiments.

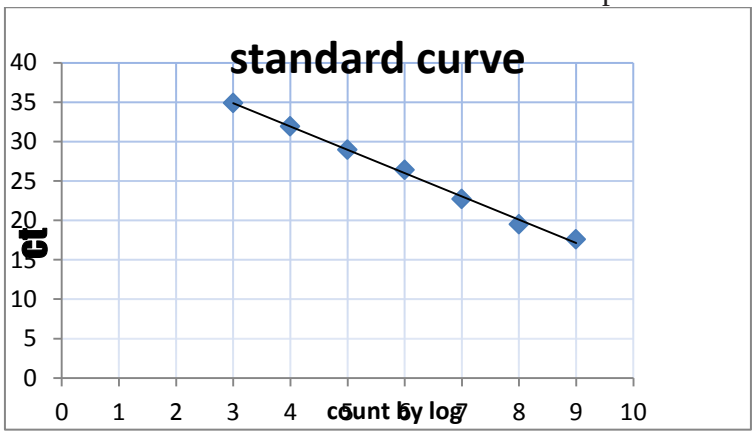

Fig. (2): Standard curve for detection of viable *E. coli coli* O_{157}:H_7 cells, by real-time PCR. Results are from two repeated experiments.

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