Fate of Thai *Escherichia coli* O157:H7 and Non-O157 lineages in Pasteurized Milk

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Abstract: The contamination of *E. coli* O157:H7 in pasteurized milk is critical for the aspect of food safety. The detection of *E. coli* O157:H7 including non-O157 from bovine-origined samples have been documented in Thailand. These include the contamination of bacteria in milk. In this study, we examined the ability of Thai *E. coli* O157:H7 and non-O157 lineages to survive under the standard pasteurization condition, including the propagative behaviors in pasteurized milk either at ambient temperature or refrigerated condition. The results revealed that at starting inoculum size of 1.5×10^3 cfu/ml, all tested bacterial strains exhibited the viability of approx. 2.5 log cfu/ml at pasteurization temperature after 60 second of incubation period. At 120 second, all strains except *E. coli* non-O157 strain PSU1 and *E. coli* O157:H7 strain PSU2, displayed the viability at over 1 log cfu/ml. In the determination of propagative behaviors of the tested strains, the results revealed that small amounts of *E. coli* O157:H7 and non-O157 could be rapidly propagated in pasteurized milk. The starting inoculum size of approx. 2 log cfu/ml of tested bacteria rapidly increased to 9 log cfu/ml within 30 h of incubation at 25ºC while at refrigerated temperature most of the tested strains increased for approx. 100 folds within 54 h. These results demonstrated the ability of Thai *E. coli* O157 :H7 and non-O157 lineages in the tolerance of heat inactivation at pasteurization temperature. In addition, it revealed their capability of rapidly propagating in pasteurized milks. Thus, they are able to pose the health risk for the population in this area in the case of O157 and non-O157contamination.
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1. Introduction

Escherichia coli O157:H7 (hereinafter abbreviated as *E. coli* O157) including non-O157 are reported to be the human pathogens, conferring severe illnesses ranging from bloody diarrhea to hemolytic uremic syndrome (HUS) (Riley et al., 1983; Karmali et al., 1983), resulting in the death cases all around the world. These types of microorganisms frequently habour the pivotal virulence factors, Shiga toxins, Stx1 and Stx2. The *N*-glycosidase of A-subunit of Stx removes one alanine residue from 28S ribosomal RNA, resulting in the inhibition of protein expression and cell death (Nataro and Kaper, 1998). Children and elderly are more susceptible to *E. coli* O157 infection (Rangel et al., 2005). The children with HUS are shown to have the mortality rate of 3% to 5% (Siegler et al., 1994; Scheiring et al., 2008). Many routes of transmission have been documented for *E. coli* O157 infection. Foods were reported as the most important vehicles for *Escherichia coli* O157 Rangel et al., 2005). Unpasteurized apple cider (Besser et al., 1993), yogurt (Morgan et al., 1993), especially the bovine-origined foods such as ground beef and raw milk (Griffin and Tauxe, 1991; Bhat and Denny, 2007) have been implicated in O157 infections. Raw milk is one of the food vehicles associated with *E. coli* O157:H7 outbreaks (Anon, 1994; Bhat et al., 2007). Raw milk was first documented to be implicated in *E. coli* O157 transmission to human in 1986 in Wisconsin, USA (Martin et al., 1986). Since then, it was considered as one of the important vehicles for *E. coli* O157. Several outbreaks have been reported as the results of raw milk consumption. During the first week of December, 2005, public health officials in Clark county, Washington obtained the notification of four county residents which were confirmed to be infected by *E. coli* O157:H7 (Bhat and Denny, 2007). The residents were reported to consume raw milk. Another outbreak resulted from the consumption of raw milk was reported by Guh et al. (2010) in Connecticut, United States in July 2008. Seven confirmed cases out of 14 identified cases (50%) were documented in this outbreak. Ten of 14 (71%) were children aged less than 18 years, average age was 5 years. In addition, 1 (14%) was diagnosed as HUS.

Pasteurized milk has also been reported to be the cause of *E. coli* O157 outbreaks (Upton and Coia, 1994; Goh et al., 2002). One report from Goh et al.

(2002), they reported the outbreak from North Cumbria, England in March 1999. Eighty-eight infected people were laboratory confirmed *E. coli* O157. Twenty-eight of the confirmed cases were hospitalized. Three children suffered with HUS. Pasteurized milk from a local farm was revealed as an aetiologic agent. In Thailand, several reports described the detection of *E. coli* O157:H7 and non-O157 from different kind of samples in many areas. Especially, in south of Thailand, there have been reports of *E. coli* O157 and non-O157 detection from beef and bovine feces (Vuddhakul et al., 2000; Sukhumungoon et al., 2011a; Sukhumungoon et al., 2011b). Thus, there are also the high risks of O157 and non-O157 contamination in other kind of samples such as raw milk. In this study, we hypothesized that *E. coli* O157 and non-O157 that spread in Thai environment are able to be contaminated in milk. After pasteurization, they may survive and start propagating again even in refrigerated state. This study aimed to examine the ability in survival of Thai *E. coli* O157 and non-O157 lineages after pasteurization and simulate their propagative behaviors in pasteurized milk either in ambient temperature or in refrigerated state.

2. Material and Methods

Bacterial Strains

Two *E. coli* O157:H7, PSU2 and PSU132, and two *E. coli* non-O157, PSU1 and PSU17, were used in the experiment. *E. coli* O157 strain PSU2 exhibited genotype *stx*1$^-$, *stx*2$^+$, *eae*$^+$. This virulence gene pattern of O157 strain was the most frequently found in this area over the last decade. PSU132 displayed genotype *stx*1$^-$, *stx*2$^-$, *eae*$^-$, which was rarely found. This strain was also selected as a surrogate in this study. *E. coli* non-O157 strains PSU1 and PSU17 were *stx*1$^+$, *stx*2$^+$, *eae*$^-$ and *stx*1$^-$, *stx*2$^+$, *eae*$^-$, respectively. These strains were capable of producing Shiga toxin1 in high amount. *E. coli* O157 strains EDL933 which represented the high pathogenicity was included in this experiment (Table 1).

Table 1. Bacterial strains used in this study

Strain	Genotype	Origin	Reference
PSU1	*stx*1$^+$, *stx*2$^+$, *eae*$^-$	Beef	This study
PSU2	*stx*1$^-$, *stx*2$^+$, *eae*$^+$	Beef	This study
PSU17	*stx*1$^-$, *stx*2$^+$, *eae*$^-$	Beef	This study
PSU132	*stx*1$^-$, *stx*2$^-$, *eae*$^-$	Beef	This study
EDL933	*stx*1$^+$, *stx*2$^+$, *eae*$^+$	Human	O'Brien et al., 1983

Survival of *E. coli* O157 and Non-O157 at Pasteurization Temperature

In order to investigate the ability of bacteria to survive at pasteurization temperature, bacteria were spiked into pasteurized milk (9% fat) and the temperature was brought to 72ºC for eight time points (0, 30, 45, 60, 75, 90, 105, and 120 second). Briefly, an isolated colony of each strain was cultured in 3 ml of tryptic soy broth (TSB) with 150 rpm at 37ºC for 6 h to prepare the working culture. Bacterial concentration was adjusted to 1.5×10^8 cfu/ml using normal saline solution (0.85% NaCl solution, NSS) by McFarland Densitometer (Biosan, Latvia). Adjusted *E. coli* cells were spiked into a total of 8 tubes containing 9 ml of pasteurized milk (final concentration of bacterial cells equaled 1.5×10^3 cfu/ml). The temperature in waterbath was pre-heated to 72ºC. The temperature was observed to be constant at 72ºC for 10 min before performing the next step. Then, all 8 tubes were immersed in waterbath at 72ºC. Tubes were sequentially taken out and immediately immersed on ice when they reached their time points (first tube was taken out from waterbath when the time reached 15 second and the last tube was taken out when the time reached 120 second). Surface-plate count was performed on MacConkey agar and Sorbitol MacConkey agar for *E. coli* non-O157 and *E. coli* O157, respectively, to count the survived bacteria. The experiment was performed in duplicate.

Fate of *E. coli* O157 and Non-O157 in Pasteurized Milk

In order to monitor the growth of the bacteria in pasteurized milk, four strains of *E. coli* O157 and non-O157 were spiked into the pasteurized milk and monitor the fate of them at six time points (0, 6, 18, 30, 42, and 54 h). In brief, the bacterial culture was grown as stated above. Each bacterial strain was examined individually by diluting the bacterial culture in NSS and inoculation of 1 ml into 99 ml of pasteurized milk was carried out (a final bacterial concentration of 1.5×10^2 cfu/ml). The same set of experiment with the final bacterial concentration of 1.5×10^4 cfu/ml, was also performed. Milks were held at 10ºC and 25ºC to simulate the situation of milk refrigerated in the supermarket and the situation in processes before placed in the supermarket, respectively. Subsequently, the bacteria were enumerated by a viable count on Sorbitol MacConkey agar for O157 strains and on MacConkey agar for non-O157 strains. The experiment was performed in duplicate.

3. Results

Survival of *E. coli* O157 and Non-O157 at Pasteurization Temperature

In order to determine that Thai *E. coli* O157 and non-O157 lineages are capable of surviving the temperature for pasteurization. The temperature of 72ºC was applied in pasteurized milk spiked with bacteria at 8 time points. From the starting inoculum size of approx. 3 log cfu/ml, all strains were shown to be still viable at over 2.5 log cfu/ml at 30 second. Focusing at 60 second, all but one were still exhibited the viability at over 2.5 log cfu/ml (Figure 1). The decrease of viable cells was clearly detected at 75 second of incubation. At 120 second of incubation, all strains except PSU1 and PSU2 displayed the viability at over 1 log cfu/ml (>150 cfu/ml). This clearly represented that the tested bacteria could be survived under standard pasteurization condition.

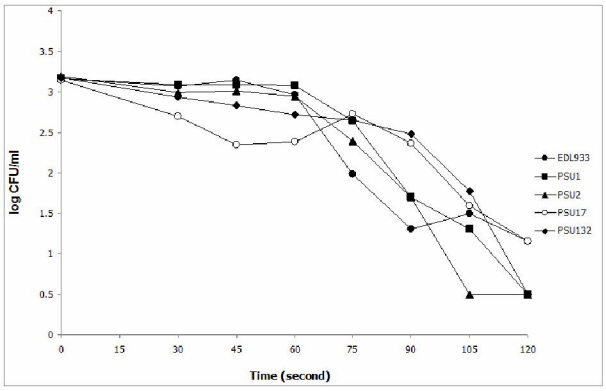

Figure 1. Survival of Thai lineage *E. coli* O157:H7 and non-O157 under pasteurization condition.

Fate of *E. coli* O157:H7 and Non-O157 in Pasteurized Milk

In order to investigate the propagative behaviors of *E. coli* O157 and non-O157 in pasteurized milk, in this experiment, two initial inoculum sizes of bacteria were spiked into the milk, 1.5×10^2 cfu/ml and 1.5×10^4 cfu/ml. Focusing on inoculum size of 1.5×10^2 cfu/ml at 25ºC, *E. coli* O157 strain EDL933 population increased rapidly from 2 log cfu/ml to 8 log cfu/ml within 18 h and further increased to 9 log cfu/ml within 30 h. *E. coli* O157 strain PSU2, PSU132 and non-O157 strain PSU1 exhibited the similar phenomena as strain EDL933. *E. coli* non-O157 strain PSU17 reached only 7 log cfu/ml at 18 h of incubation, however, it was also able to reach 9 log cfu/ml within 30 h. At 10ºC, a highly pathogenic strain EDL933 was found to be grown from 2 log cfu/ml to 4 log cfu/ml within 54 h. Similar phenomenon was found in the case of *E. coli* O157 PSU2 and non-O157 PSU17 (Figure 2).

When 1.5×10^4 cfu/ml of inoculum size in milk at 25ºC was analyzed, it revealed that the population of EDL933 was increased from a starting inoculum size to 9 log cfu/ml within 30 h. However, the rest of four strains reached 9 log cfu/ml within only 18 h. At 10ºC, despite the fact that *E. coli* non-O157 strain PSU17 and O157 strain PSU2 were kept at low temperature, they could be propagated from a starting inoculum size, 5 log cfu/ml, to 6 log cfu/ml and 7 log cfu/ml, respectively. *E. coli* non-O157 strain PSU1, *E. coli* O157 strain PSU132 and EDL933 were able to reach approx. 5.5 log cfu/ml within 54 h (Figure 3).

4. Discussion

The growing behaviors of *E. coli* O157 and non-O157 in pasteurized milk is important for food safety. During the last decade, several studies reported the detection of *E. coli* O157 and non-O157 serotype from bovine-origined samples in Thailand (Vuddhakul et al., 2000; Sukhumungoon et al., 2011a; Sukhumungoon et al., 2011b). Many strains of *E. coli* O157 and non-O157 were isolated. Some of them displayed the expression of Shiga toxins in high amounts. Therefore, it was clearly that bovine was the important source of *E. coli* O157 and non-O157 and these *E. coli* strains were probably also contaminated in raw milk as by several routes, direct contact of cow feces to the milk, bovine's udder mastitis, for instance. Adesiyun et al., (1997) has early shown that 17 (18.5%) of 94 Shiga toxin-producing *E. coli* (STEC) strains were isolated from bulk milk in Trinidad. Furthermore, Schneider et al. (2008) also reported the illness cases resulted from *E. coli* O157:H7 infection associated with raw milk and raw colostrums in California, United States. These informations emphasized that raw milk plays an important role as O157 vehicle. Thus, in this study, the assessment of heat inactivation tolerance of *E. coli* O157 and non-O157, including the propagative behaviors of them in milk were analyzed. Simulative circumstances of O157 in processed pasteurized milk prior to be sold in the supermarket and the situation in the refrigerated state in the supermarkets (25ºC and 10ºC, respectively) were examined to accomplish the critical informations.

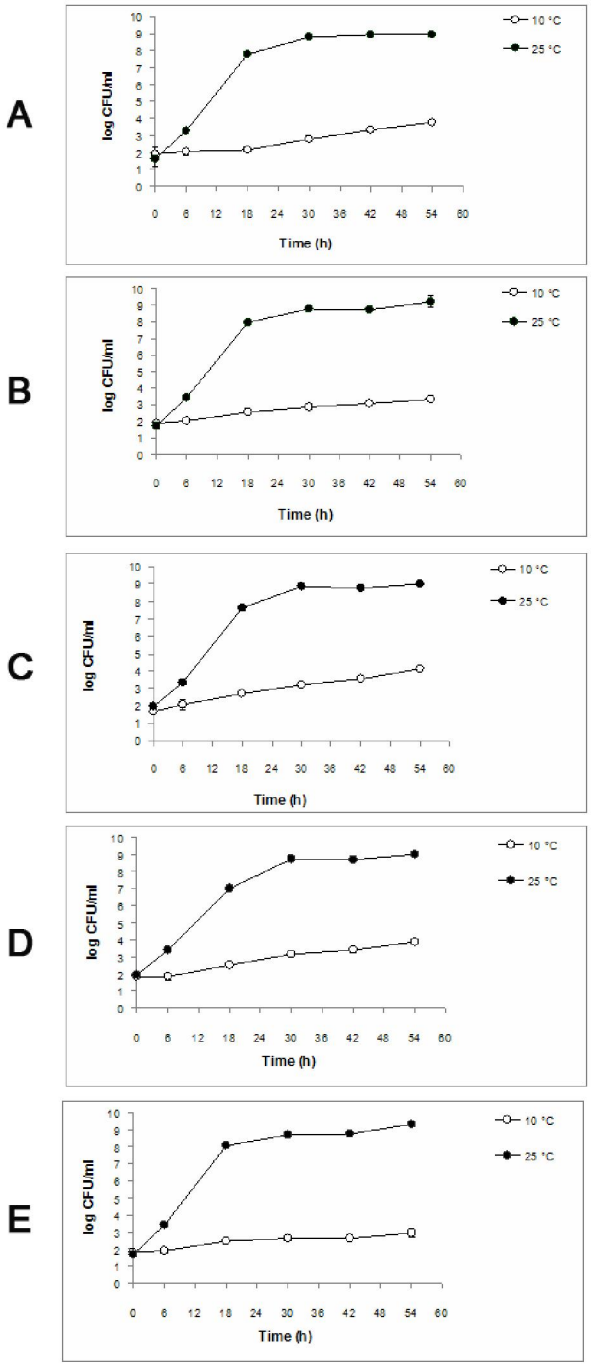

Figure 2. Propagative behaviors of *E. coli* O157:H7 and non-O157 in pasteurized milk at the inoculum size of 1.5×10^2 cfu/ml. A, *E. coli* O157:H7 strain EDL933; B, *E. coli* non-O157 strain PSU1; C, *E. coli* O157:H7 strain PSU2; D, *E. coli* non-O157 strain PSU17, E, *E. coli* O157:H7 strain PSU132.

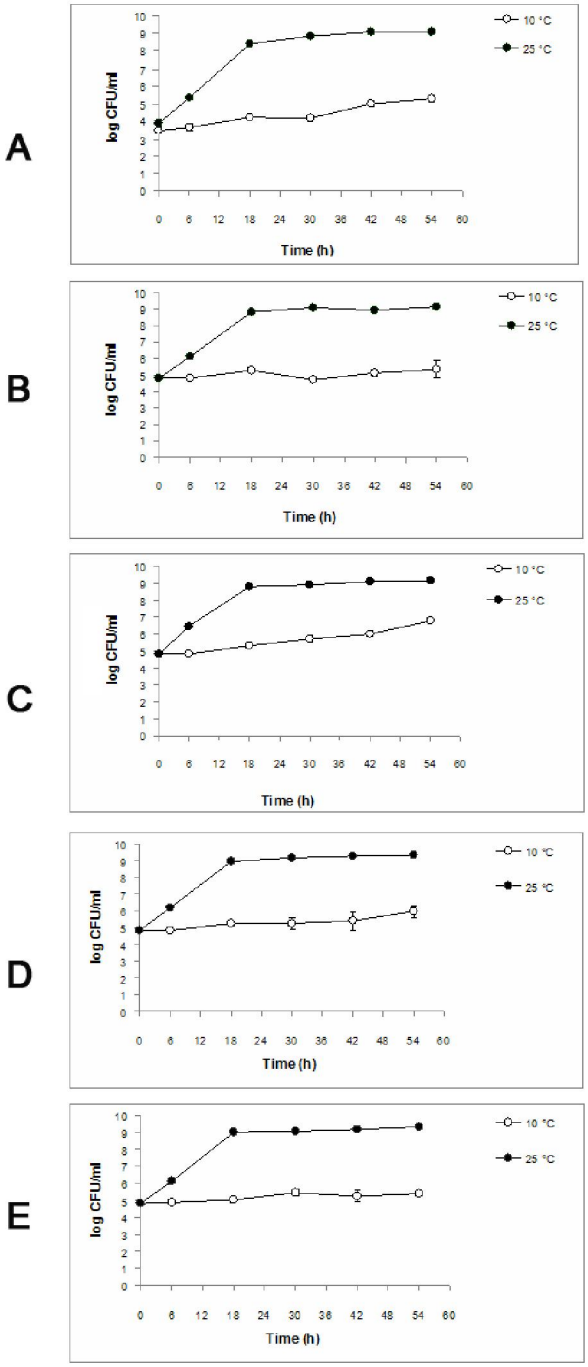

Figure 3. Propagative behaviors of *E. coli* O157:H7 and non-O157 in pasteurized milk at the inoculum size of 1.5×10^4 cfu/ml. A, *E. coli* O157:H7 strain EDL933; B, *E. coli* non-O157 strain PSU1; C, *E. coli* O157:H7 strain PSU2; D, *E. coli* non-O157 strain PSU17, E, *E. coli* O157:H7 strain PSU132.

Although the report from d'Aoust et al. (1988) confirm that the high temperature and short time processes (71.7ºC for 15 second), was sufficient to destroy approx. 1×10^5 cfu/ml of *E. coli* O157:H7. However, our results were different. The results clearly revealed that *E. coli* O157 and non-O157 were tolerated to a standard pasteurization condition for 60 second. Currently, the temperature at 71.7ºC for 15 second was used for pasteurization in milk industry. However, to obtain the concrete results, *E. coli* O157 and non-O157 were placed at 72ºC for 8 time points to monitor the viability of the tested bacteria. Focusing on the initial inoculum size of 1.5×10^3 cfu/ml, at 30 second, 2 folds of the general time for pasteurization, all strains were survived over 2.5 log cfu/ml. At 60 second, all strains were still survived over 2 log cfu/ml. Furthermore, at 120 second, the maximal time point, we observed that 0.5 log cfu/ml of *E. coli* O157 strain PSU1 and PSU2 were still survived and *E. coli* O157 strain EDL933 and non-O157 strain PSU17 survived at over 1 log cfu/ml (Figure 1). These informations were clearly shown that Thai *E. coli* O157 and non-O157 lineages were able to survive under the standard pasteurization condition. Usajewicz and Nalepa (2006) described the consistent observation. They demonstrated the destruction of *E. coli* O157:H7 strain 94 and strain 402 including the saprophytic *E. coli* strain 1 at 55ºC and 60ºC in two media, nutrient broth and skim milk. They found that at 60ºC, *E. coli* O157:H7 strain 402 and strain 94 were destroyed in 15 and 30 min, respectively, in nutrient broth. However, the amount of bacteria over 1 log cfu/ml were still observed at 30 min. Bacteria were discovered to be more tolerated to heat inactivation in skim milk rather than in the broth at both temperatures. This result also supported our observation.

After pasteurization, the bacterial survival was capable of propagating again in refrigerated pasteurized milks (Figure2 and Figure 3). We have demonstrated in Figure 2 that a small amount of bacteria, 2 log cfu/ml, could be increased for 10 to 100 folds within 54 h (approx. 2.5 days) at 10ºC. To assess the general temperature used for refrigerated pasteurized milks in this area, we surveyed many convenient stores including the supermarket in Hat-Yai city. The temperature used for refrigerated pasteurized milks in this area was ranged from 8ºC to 18.5ºC with average temperature equaled to 12.2 ºC. This range of temperature was higher than the temperature tested in this study, 10ºC. In the case of *E. coli* O157 contamination in this area, based upon 12.2ºC average temperature, it has high propensity in contribution of the propagation of bacteria in the milks, led to the high population of bacteria which was probably over 100 folds compared to the initial inoculum size in milk. Wang et al. (1997) demonstrated that at 5ºC, *E. coli* O157:H7 did not grow in raw milk. Moreover, the population of the bacteria decreased approx. 1.5 log cfu/ml within 28 days. Despite the fact that in the real circumstance, the consumers buy and drink the milk within few days after placing in the store, the temperature should be brought to be below 4ºC for preventing the propagation of bacteria.

In conclusion, Thai *E. coli* O157 including non-O157 lineages can be contaminated in unpasteurized milks obtained in this area. After pasteurization, a small amounts of bacteria were possibly survived and propagated in pasteurized milk again even in the refrigerated state. This will pose a health risk in the population in this area. A little prolongation of a standard pasteurization temperature is recommended to be applied in raw milk produced in this area, without the destruction of milk properties. In addition, the storage of pasteurized milks in the refrigerated shelves under 4ºC is also recommended to prevent the propagation of contaminated pathogens.

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