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# *Short communication:* Heat resistance of *Escherichia coli* strains in raw milk at different subpasteurization conditions

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## ABSTRACT

A commonly applied treatment of raw milk to reduce bacterial loads is the short-time application of heat at subpasteurization levels under continuous flow, generally referred to as thermization, because this method retains some of the beneficial properties of raw milk. In a previous study, *Escherichia coli* strains exhibiting increased thermotolerance were found, demanding investigations into their ability to survive thermization. Nine *E. coli* strains, including 4 Shiga toxin-producing *E. coli* (STEC) strains, were investigated for their reduction during a thermization treatment in raw milk using a pilot-plant pasteurizer to reflect typically applied commercial conditions. Six of the 9 *E. coli* strains, including the 4 STEC strains, were similarly inactivated at 60, 62.5, and 65°C, whereas increased thermotolerance was observed for 3 *E. coli* strains. All strains were reduced to <2 $\log_{10}$ at 60 and 62.5°C within 25 s. At 65°C, 6 of 9 *E. coli* strains were reduced by at least 5 $\log_{10}$ after 25 s, whereas at 67.5°C, such a reduction was observed for 8 strains. A much higher thermotolerance was found for *E. coli* strain FAM21805. For some *E. coli* strains, time–temperature combinations above 65°C were required to obtain a substantial reduction during a thermization treatment.

**Key words:** thermization, *Escherichia coli*, heat treatment, subpasteurization

## Short Communication

The use of raw milk in the production of cheese, for example, offers natural enzymes and microflora that give the final product a desired flavor of special quality (Bachmann et al., 2011; Van Hekken, 2012). However, the use of raw milk constitutes an increased risk to the consumer because of the survival or multiplication of pathogens during cheese making, including Shiga toxin-producing *Escherichia coli* (**STEC**; Deschênes et al., 1996; Espié et al., 2006; Peng et al., 2013).

Different methods are used to reduce bacterial loads in raw milk, of which thermization—heat treatment at subpasteurization temperatures for short periods—constitutes a relatively mild treatment that retains the desired features beneficial for the flavor of the product (Burbank and Qian, 2008; Van Hekken, 2012). Thermization reduces counts of bacteria that could produce proteases and lipases, which may negatively affect cheese ripening. In addition, thermization is frequently used by cheese manufacturers in Switzerland and other countries to improve the safety of the products. According to milk processing and quality management (Lewis and Deeth, 2009), a temperature of 57 to 68°C is applied for at least 15 s, and the milk shows activity of alkaline phosphatase, which distinguishes it from pasteurized milk.

Counts of *E. coli* rarely exceed $10^4$ cfu/mL in raw bulk tank milk or raw commingled silo milk (Van Kessel et al., 2004; Jackson et al., 2012). Therefore, a reduction of 5 $\log_{10}$ (5-D reduction) would inactivate at least the majority of *E. coli* that might occur in raw milk and this was used as a guidance value for estimation of thermization efficiency in this study.

To characterize the reduction of bacteria due to heat treatments, 2 parameters, *D*-value (the time taken at a certain temperature to reduce the cell numbers by 1 $\log_{10}$ cycle) and *z*-value (specifies the temperature increase required to obtain a decimal reduction of the *D*-value), were defined under the assumption that the reduction in bacterial count follows first-order kinetics. In this study, the *D*- and *z*-values of 9 *E. coli* strains isolated from raw milk cheese (Peng et al., 2012) were determined using a pilot-plant pasteurizer to apply thermization treatment, which reflects conditions used by cheese manufacturers. The pilot-plant pasteurizer was constructed to reflect a commercial pasteurization plant on a small scale (for details, see Hammer et al., 2002, 2005). Milk is heated in continuous flow, in which 2 plate heat exchanger sections (heater and cooler) are used. Basic technical data for the pasteurizer were as

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follows. A sample volume up to 30 L can be processed at holding times of 2 to 60 s (holding time depends on flow rate and holder volume). The flow rate can be varied between 15 and 80 L/h and the temperature range between 40 and 145°C can be covered, with accuracies of ±0.2 L/h and ±0.2°C, respectively. Reynolds numbers (dimensionless numbers for the turbulence of flow) are dependent on holding time and holding section. With the holder used in this study, Reynolds numbers were approximately 1,200 at a 25-s holding time and 2,000 at a 15-s holding time, which indicates flow at the transition between laminar and turbulent flow (Kessler, 1981). Alterations in the flow rate influence the residence time distribution but can be neglected if the curve of the residence time distribution is symmetrical. At a Bodenstein number (a dimensionless number to describe the transmission of heat in connection with the Reynolds number) of >640, a symmetrical residence time distribution can be proposed (Rao and Loncin, 1974), which was the case in all conditions applied. At low flow rates and low temperatures, as applied in this study, inactivation during heating and cooling within the plate heat exchangers is negligible compared with inactivation within the holding section.

Before each experiment, the entire pilot-plant pasteurizer was operated with water for 30 min at 98°C to clean and decontaminate it. During operation, the milk was continuously stirred. Temperature and flow rates were adjusted at the registration board. In consideration of the total volume of the pilot plant and the residence time distribution, after readjustments, at least 1 L of milk passed the plant before samples were collected to ensure that an appropriate amount of milk heated at the target temperature had passed the sampling valve. Sampling was carried out with sterile plastic syringes from a rubber-stoppered valve located downstream of the cooling section. After use, the pilot plant was cleaned and disinfected by circulation of an alkaline cleaner for 30 min at 80°C, an acidic cleaner for 20 min at 60°C, and a final rinse with water for 30 min at 98°C.

All *E. coli* strains used in this study (Table 1) were isolated from semi-hard raw milk cheeses, except for strain FAM21805, which was isolated from a soft raw milk cheese, and strain N09-1208, which was isolated from vat raw milk. Strains were maintained at −80°C in the Microbank system (Pro-Lab Diagnostics, Richmond Hill, ON, Canada). For precultivation, one bead was used to inoculate 10 mL of trypticase soy broth (Oxoid, Wesel, Germany) and incubated at 37°C for 24 h. Subsequently, 1 mL of this preculture was used to inoculate 500 mL of fresh trypticase soy broth in a total of 3 Erlenmeyer flasks for each experiment. The flasks were incubated on a shaker (60 rpm) for 24 h at 37°C.

For the experiments, bacteria were harvested by centrifugation for 10 min at 6,500 × *g* at 22°C. Pellets from 3 Erlenmeyer flasks were resuspended in a small amount of raw milk and added to 30 L of milk for the heating experiments. Before the experiment, the milk was stirred for at least 5 min to ensure proper homogenization and stirring was continued during the entire processing. The milk used for this study was whole raw milk, obtained from the Max Rubner-Institute research farm (Kiel, Germany). It was collected from selected cows with sterilized milking equipment after thorough teat cleaning to obtain a bacterial count as low as possible. Total colony counts <200 cfu/mL were achieved with this procedure.

**Table 1.** *D*-values (s) and *z*-values (°C) of 9 *Escherichia coli* strains[1]

| | | Temperature (°C) | | | | | |
|---|---|---|---|---|---|---|---|
| Strain | Serotype | 60 | 62.5 | 65 | 67.5 | 70 | *z*-value |
| FAM21846 | O16:H21 | 21.9 ± 4.9 | 12.7 ± 2.2 | 3.3 ± 0.1 | ND[2] | ND | 6.1 |
| K133[3] | O113:H4 | 48.4 ± 22.0 | 49.7 ± 19.9 | 4.6 ± 0.9 | ND | ND | 4.9 |
| N09–1208[3] | O26:H11 | 49.8 ± 16.4 | 31.9, 32.3[4] | 3.4 ± 0.6 | ND | ND | 4.3 |
| K303 | O9:[H21][5] | 53.8 ± 8.7 | 29.4 ± 7.1 | 2.9 ± 0.3 | ND | ND | 4.0 |
| K331/4[3] | O91:H21 | 71.7 ± 26.8 | 23.6 ± 12.4 | 3.2 ± 0.4 | ND | ND | 3.7 |
| K356[3] | O2:H27 | 80.5 ± 26.0 | 47.9 ± 7.7 | 3.0 ± 0.2 | ND | ND | 3.5 |
| FAM19195 | O8:H21 | —[6] | 131.4 ± 51.3 | 17.4 ± 3.9 | 4.5 ± 0.4 | ND | 3.4 |
| FAM21843 | O178:H12 | — | — | 27.1 ± 6.5 | 3.2 ± 0.3 | 2.8 ± 0.2 | 5.1 |
| FAM21805 | O68:H14 | — | — | 93.4 ± 47.0 | 47.1 ± 8.1 | 4.2 ± 0.4 | 3.7 |

[1]Mean ± SD of 3 experiments per strain and temperature for calculation of *D*-values (time needed at a certain temperature for 1 $\log_{10}$ reduction of the bacterial count); the *z*-value (temperature needed for 1 $\log_{10}$ reduction of the *D*-value) was calculated from the average *D*-values.

[2]Not determined.

[3]Shiga toxin-producing *E. coli* strain.

[4]Only 2 experiments due to low coefficient of determination.

[5]Phenotypically nonmotile.

[6]No significant reduction observed (reduction ≤0.25 $\log_{10}$).

To count the viable bacteria after heating, samples were immediately serially diluted 1:10 (vol/vol) with peptone saline solution [1 g of tryptone from caseine peptone (Oxoid) supplemented with 8.5 g/L sodium chloride (Merck, Darmstadt, Germany)]. From the appropriate dilutions, 0.1 mL, in duplicate, was spread onto Columbia blood agar plates supplemented with 5% (vol/vol) sheep blood (Oxoid) and incubated at 37°C for 72 h.

In pretrials, strains were tested at heating temperatures of 57.5, 60, 62.5, 65, 67.5, and 70°C, with a holding time of 20 s to determine the temperature region where considerable reduction can be expected (data not shown). For the experiments to determine the *D*-and z-values, the strains were treated at 3 successive preselected temperatures and at holding times of 15, 20, and 25 s, using 1 inoculated 30-L sample of milk for each run of the pilot plant. The holding times corresponded to flow rates of 20.3, 25.2, and 33.7 L/h, respectively. Temperature steps of 2.5°C and time gaps of 5 s were chosen. The activity of alkaline phosphatase in the heat-treated milk was analyzed according to ISO 11816-1:2006 (ISO, 2006). After a 25-s heat treatment at all temperatures applied, alkaline phosphatase activity in the milk was higher than the threshold level for pasteurized cow milk (350 mU/L; data not shown).

For the calculation of *D*-values, $\log_{10} N_t$ (count of surviving bacteria) divided by $\log_{10} N_0$ (initial count) was plotted against time. A log-linear regression curve was calculated utilizing Microsoft Excel for Mac 2011 (Microsoft Corp., Redmond, WA). The *D*-value was calculated as the divisor of 1 and the slope of the regression curve. Coefficient of determination ($R^2$) was calculated for analysis of the fit of the model. The *D*-values observed from 3 experiments were averaged and standard deviation was calculated. To determine *z*-values, the averaged *D*-values were $\log_{10}$ transformed and plotted against temperature, and the same procedure as described above was applied.

The heat resistance parameters (*D*- and *z*-values) of the 9 *E. coli* strains are summarized in Table 1. According to Martens (2003), the fit of the log-linear model was high, as the coefficient of determination ($R^2$) was at least 0.7 for 75 and 0.9 for 51 of the 81 calculated *D*-values. Five additional *D*-values ($R^2 \geq 0.6$) were used for further evaluation; data of one measurement were discarded because of its low $R^2$ value (0.3).

The measurement inaccuracy of the pilot-plant pasteurizer and especially the low slopes of regression curves for higher *D*-values are attributed to cause the larger standard deviations of the higher *D*-values. Therefore, the calculated *z*-values are a reference point that should be interpreted with caution. This is exemplified by the observation that the *z*-value of the least thermotolerant strain FAM21846 was higher than those of the other strains, including the nonpathogenic *E. coli* strains that exhibited increased thermotolerance. Of the 9 *E. coli* strains, an average *z*-value of 4.3 ± 0.9°C was observed, which is consistent with the data collected by Sörqvist (2003). Nevertheless, the literature data for *D*- and *z*-values of *E. coli* show considerable variation due to differences in the experimental design (e.g., food matrix, culture media; van Asselt and Zwietering, 2006).

Six of the 9 *E. coli* strains, including the 4 STEC strains, showed similar inactivation at 60, 62.5, and 65°C. At 60 and 62.5°C, the *D*-values ranged from 12.7 to 80.5 s, resulting in a decrease of, at most, 2 $\log_{10}$ due to thermization within 25 s. At 65°C, *D*-values were below 5 s, which corresponds to a reduction of more than 5 $\log_{10}$ with a 25-s thermization. This observation is in agreement with the findings of Schlesser et al. (2006), who proposed a subpasteurization heat treatment of milk at 64.4°C for 17.5 s before using the milk for Cheddar cheese production based on a demand of a 5-D reduction of *E. coli*.

For 3 *E. coli* strains, however, a much increased thermotolerance was observed. Unlike the other 6 *E. coli* strains, these 3 strains were not reduced more than 2 $\log_{10}$ within a 25-s thermization at 65°C. At 67.5°C, a 5-D reduction was observed within 25 s for the strains FAM19195 and FAM21843. Nevertheless, strain FAM21805 exhibited a *D*-value at 67.5°C of 47.1 ± 8.1 s, and therefore was barely reduced by thermization at 67.5°C. At 70°C, this strain finally exhibited a stronger reduction and the 5-D criterion was achieved within 25 s of thermization. This observation shows that some *E. coli* strains may survive a thermization treatment at 65 or 67.5°C. The mechanisms that increase the thermotolerance of these strains require further investigation. The 4 STEC strains that were used in this study did not show increased thermotolerance.

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