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Growth and Survival of *Eschericia coli* O157: H7 during the Manufacturing of *Ergo* and *Ayib*, Ethiopian Traditional Fermented Milk Products

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Research Article

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ABSTRACT

The behavior of *Escherichia coli O157:H7* was evaluated during the manufacturing of *Ergo* (Ethiopian naturally fermented milk) and *Ayib* (Ethiopian cottage cheese) using a challenge test. Four *E. coli* O157:H7 initial inoculum levels (high: ~$3x10^5$; medium: ~$3x10^4$; low: ~$3x10^3$; and very low: ~$3x10^2$ cfu mL^{-1}) were used together with a control milk with no lactic acid bacteria (LAB). *Ayib* was made by cooking defatted fermented milk after 72 h of fermentation at three cooking temperatures (50, 60 and 70°C). Samples were drawn at 0, 8, 24, 32, 48, 56 and 72 h of fermentation and at 0, 20, 40 and 60 min of cooking defatted fermented milk for bacterial enumeration and pH measurements. *E. coli* O157:H7 population decreased only by 1.2, 0.3 and 0.5 log cfu mL^{-1} after 72 h of fermentation at ambient temperature from initial counts of 5.7, 4.3 and 3.3 log cfu mL^{-1}, respectively. Counts increased from 2.6 and 3.0 log cfu mL^{-1} at the start of fermentation to 2.8 and 4.2 log cfu mL^{-1} at the end of fermentation when co-inoculated with LAB and in control milk with no LAB, respectively. *E. coli* O157:H7 survived up to 60 min of cooking defatted fermented milk during *Ayib*-making at 50 and 60°C with counts of 2.3-2.9 and 0.8-1.5, respectively. Complete inhibition of *E. coli* O157:H7 was achieved between 20 and 40 min of cooking at 70°C. The direct consumption of *Ergo* can represent an important source of *E. coli* O157:H7 infections. At the inoculum levels considered in the present study, the use of cooking temperature of 70°C for at least 40 min during *Ayib*-making is necessary to inactivate *E. coli* O157:H7.

INTRODUCTION

Milk and milk products are important in the diet of Ethiopians. Ergo is a naturally fermented milk at ambient temperature, which can be served directly as a refreshment or used as a raw material for the production of shelf stable milk products such as butter and Ayib (Ethiopian cottage cheese). Ergo is churned to produce butter using traditional churns such as clay pot and calabash. The defatted fermented milk, which is obtained as a byproduct of the butter-making process, is heated on a low fire (40-70°C) [1-3] for the manufacturing of Ayib. The curd (Ayib), separated from the whey, can be stored up to 7 days at 30°C [4]. Milk and milk products can be contaminated from sources such as diseased milking cows, milk handlers, contaminated equipment or cleaning water.

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Since its identification as an emerging pathogen in 1982 [5], *Escherichia coli* 0157:H7 continued to be an important food-borne pathogen of great public health concern. Health disorders caused by *E. coli* 0157:H7 range from self-limited, watery and bloody diarrhea to Hemolytic Uremic Syndrome (HUS) [6] and Thrombotic Thrombocytopenic Purpura (TTP) [7] that is more severe and life threatening.

A wide variety of foods, including beef [8], mayonnaise [9], apple cider [10] and apple juice [11] have been reported to be vehicles of *E. coli* 0157:H7 infections. Milk and milk products including raw milk [9,12], fresh cheese curds[13], yogurt [14] have been reported to be associated with several cases and outbreaks of *E. coli* 0157:H7 infections.

Although acid foods have generally been considered to be intrinsically safe due to their low pH and high acidity, diseases including *E. coli* 0157:H7 infections have been reported to be caused through the consumption of such foods. Survival of *E. coli* 0157:H7 in traditional African yogurt fermentation was reported [15]. The increased acid tolerance of *E. coli* 0157:H7 than other *E. coli* strains and its survival in many acid foods are documented [10]. Massa [16] also indicated that *Escherichia coli* 0157:H7 population did not change significantly after 24 h in traditional yogurt.

However, information on the fate of *Escherichia coli* 0157:H7 during Ergo-making is very limited and that on Ayib-making is lacking. The aim of this study was therefore to investigate the effect of fermentation of milk at ambient temperature and cooking of defatted fermented milk at different temperatures on the growth and survival of *Escherichia coli* 0157:H7.

MATERIALS AND METHODS

Materials

The milk sample used in the present study was commercial micro-filtered milk. The strain *Escherichia coli* 0157:H7 (Ref. N°. CIP 103571) used for the challenge test was purchased from Pasteur Institute (Paris, France). A mixture of cocci and rode shaped Lactic Acid Bacteria (LAB) isolated from naturally fermented milk that were Gram+, catalase– and oxydase– were used to initiate fermentation. Prior to carrying out the challenge test, this LAB mixture was inoculated in sterile milk and left to ferment at ambient temperature for around 36 h. This fermented milk was then examined for its organoleptic properties by a panel of 4 persons and confirmed to conform to the characteristics of naturally fermented milk.

Fermentation

Micro-filtered milk was aseptically distributed into sterile screw-capped bottles to get final volumes of approximately 100 mL in each bottle. Milk was then left to ferment at ambient temperature for about 72 h.

Ayib-making

In this experiment, Ayib was made according to the traditional procedure by cooking defatted fermented milk (72 h of fermentation at ambient temperature, 20 – 25°C) with *E. coli* 0157:H7 being inoculated at 0 h of fermentation. The cream layer was removed aseptically from the fermented milk before the start of the cooking. Three Ayib-making temperatures (50, 60 and 70°C) were used based on the practice in the study area. Products were kept in a water bath adjusted at 50, 60 and 70°C. Internal temperature of products was measured by inserting a thermometer inside the products and when the predefined internal temperatures were achieved, cooking continued for 60 min.

Inoculation of Test Organism and Initiation of Fermentation

Four initial inoculum levels of *E. coli* 0157:H7 were used: High (~$3x10^5$ cfu mL^{-1}), medium (~$3x10^4$ cfu mL^{-1}), low (~$3x10^3$ cfu mL^{-1}) and very low (~$3x10^2$ cfu mL^{-1}). A control milk (~$3x10^2$ cfu mL-1) with no LAB was also used. LAB was inoculated to obtain initial count of ~$3x10^5$ cfu mL^{-1} to initiate fermentation in all treatments except the control milk. Initial inoculum levels were estimated by adjusting to Mac Farland standard corresponding to concentration of microbial suspension using spectrophotometer (Spectronic 1202, Milton Roy). Suspensions were prepared in 0.1% peptone water (Oxoid, UK) from overnight grown *Escherichia coli* 0157:H7 culture on nutrient agar and in MRS broth from culture grown on MRS agar (48 h at 35°C).

Enumeration of Surviving Bacteria

The growth and survival of organisms were monitored by drawing 1 mL portion of the fermenting milk at 0, 8, 24, 32, 48, 56 and 72 h of fermentation. During Ayib-making, 1 mL or g of the product was drawn at 0, 20, 40 and 60 min of cooking from each treatment and the control groups. Test portions were directly mixed in test tubes containing 9 mL of 0.1% peptone water (Oxoid, UK) for *E. coli* 0157:H7 and MRS broth (Oxoid, UK) for LAB. Zero point one mL of appropriate dilutions were surface plated on duplicate Sorbitol MacConkey agar with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (SMAC-BCIG) (Oxoid, UK) plates for the enumeration of viable *E. coli* 0157:H7. LAB was enumerated after culturing duplicate surface plated MRS agar plates in an aerobic jar (Biorad, France) at 35°C for 48 h.

pH Measurement

The pH of the fermenting milk was measured at the time of sampling for microbial analysis by inserting the electrode of the digital pH meter (pH 330, Bioblock Scientific, Germany) into sample portions in a test tube.

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Statistical Analysis

Each experiment was replicated three times. Populations of *Escherichia coli* O157:H7 and LAB surviving fermentation during Ergo-making and heat treatment during Ayib-making were transformed to $\log_{10}$ values before subjecting to statistical analysis. Log transformed values (cfu mL^{-1} or g^{-1}) and pH determined at each sampling time of the test portion from the fermenting milk were analyzed using the General Linear Model (GLM) of the Statistical Analysis System. Least Significant Difference (LSD) test was used to separate means and differences were considered to be statistically significant when $P<0.05$.

RESULTS

Growth and survival of *E. coli* O157:H7 during Milk Fermentation (*Ergo*-Making)

Escherichia coli O157:H7 showed similar growth patterns at different initial inoculum levels with or without the presence of LAB in fermenting milk at ambient temperature for 72 h **(Figure 1).** *E. coli* O157:H7 population first increased ($p<0.05$) up to between 8 and 32 h of fermentation depending on initial inoculum levels then declined towards the end of fermentation at 72 h at which time counts decreased by 1.2, 0.3 and 0.5 log cfu mL^{-1} for high, medium and low initial inoculum levels, respectively from the initial counts. At very low initial inoculum level and in control milk with no LAB, however, *E. coli* O157:H7 count increased respectively by 0.2 and 1.2 log cfu mL^{-1}. LAB counts increased ($P<0.05$) up to 32 h of milk fermentation at ambient temperature and then declined towards the end of fermentation at 72 h at which time LAB counts increased by up to 0.6 log cfu mL^{-1} from the initial count. These trends were accompanied by a progressive decrease in pH from an average of 6.57 at the start of fermentation to an average of 4.4 and 5.04 when *E. coli* O157:H7 was co-inoculated with LAB and in the control milk with no LAB, respectively ($p<0.05$).

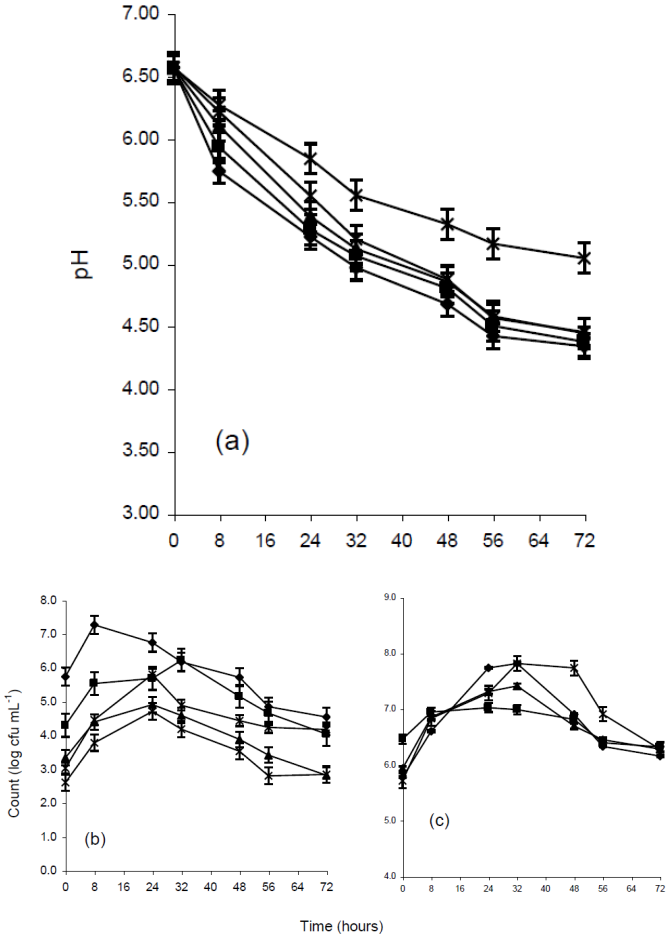

Figure 1. Changes in pH (a) and growth and survival of *E. coli* O157:H7 (b) and LAB (c) during *Ergo*-making (milk fermentation) at different initial inoculum levels of *E. coli* O157:H7: High: ~$3x10^5$ (◊); medium: ~$3x10^4$ (▪); low: ~$3x10^3$ (▲); very low: ~$3x10^2$ (X) and control milk with no LAB: ~$3x10^2$ (*).

Survival of *E. coli* O157:H7 during Cooking of Defatted Fermented Milk (*Ayib*-making)

The changes in the survival of *E. coli* O157:H7 and LAB during Ayib-making were different according to cooking temperatures **(Figure 2).** LAB survived the three cooking temperatures considered during Ayib-making. LAB numbers dropped from around 6.3 log cfu mL^{-1} of initial count to 3.1 - 4.8, 2.9 - 36 and 0 - 2 log cfu mL^{-1} ($p<0.05$) at the end of cooking at 50, 60 and 70°C cooking temperatures, respectively **(Figure 2)**. *E. coli* O157:H7 was inactivated below plating-detection limit (<10 cfu mL^{-1}) between 40 and 60 min of cooking at 50 and 60°C and between 0 and 20 min of cooking at 70°C when inoculated at low and very low initial

inoculum levels. At high and medium initial inoculum levels; and in the control milk with no LAB, however, *E. coli* O157:H7 survived 60 min of cooking with reduction in number respectively by 1.6, 1.7 and 1.8 log cfu mL^{-1} or g^{-1} at 50°C; and 3.7, 3.1 and 2.7 log cfu mL^{-1} or g^{-1} at 60°C cooking temperatures ($p<0.05$). When Ayib was made by cooking defatted fermented milk with high and medium initial inoculum levels and control milk with no LAB, *E. coli* O157:H7 count dropped by 2.5, 3 and 1.8 log cfu mL^{-1} or g^{-1}, respectively, and was not detected when determined at 40 min of cooking **(Figure 2).**

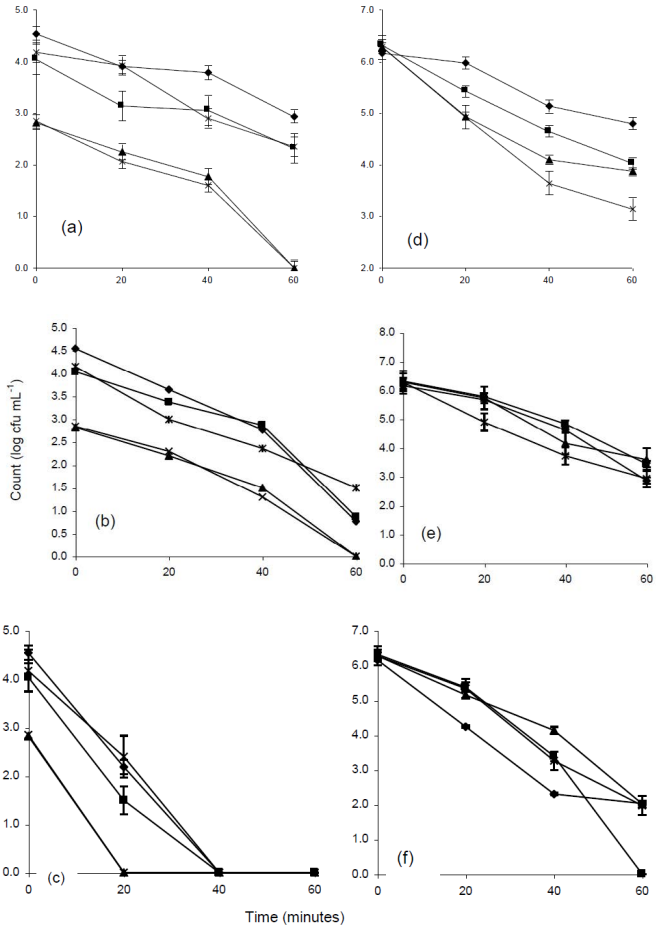

Figure 2. Survival of *Escherichia coli* O157:H7 at 50°C (a), 60°C (b) and 70°C (c) and LAB at 50°C (d), 60°C (e) and 70°C (f) during *Ayib*-making at high (◊), medium (▪), low (▲), very low (X) and control milk with no LAB (*) initial inoculum levels of *E. coli* O157:H7.

DISCUSSION

The current study focused on assessing the growth and survival of *Escherichia coli* O157:H7 during the fermentation of milk at ambient temperature for Ergo–making and heating of defatted fermented milk for the manufacturing of Ayib. Information on the growth and survival of *Escherichia coli* O157:H7 during traditional food manufacturing in general and milk processing in particular is generally scarce in Ethiopia.

A combination of factors including low pH and the production of bacteriocin, hydrogen peroxide, and ethanol by LAB were reported to have an inhibitory effect on *E. coli* O157:H7 in fermenting milk [17]. *E. coli* O157:H7 was reportedly more acid-resistant as compared to Salmonella Typhimurium DT104 and Listeria monocytogenes [18] and possess an inherently high permanent acid resistance [19]. Although, a wide pH range of 4.0–9.0 with optimum pH being 7 was reported for the survival of *E. coli* O157:H7 [20], survival of the organism at pH levels as low as 2.0 and as high as 11.0 was indicated [21]. The survival of *E. coli* O157:H7 at pH values of 3.7 in apple cider [21], 3.1 in mustard [22] and 3.65 in mayonnaise [23] was reported. In the present study, *Escherichia coli* O157:H7 survived in fermenting milk up to 72 h with cell counts ranged between 2.8 and 4.5 cfu mL^{-1} based on initial inoculum levels. *E. coli* O157:H7 counts increased by 2.1 log cfu mL^{-1} after 24 h and 0.2 log cfu mL^{-1} after 72 h of fermentation from initial count of 2.6 cfu mL^{-1}. Our result agrees with that of Kasimoglu and Akgun [24] who reported that *E. coli* O157:H7 cells increased from initial numbers of 10^2, 10^4 and 10^6 cfu mL^{-1} to 10^3, 10^5 and 10^7 cfu mL^{-1} respectively in traditional yogurt (pH 4.6) after 3 h of fermentation. Tsegaye and Ashenafi [25] also reported a similar trend in the same type of product.

Although acid–adaptation was reportedly increased the survival of pathogens such as *E. coli* O157:H7 in different food stress conditions [18,22,23], a higher survival population of non-acid-adapted than acid-adapted was noted in milk [26]. Most bacteria require an adaptation period to acid in order to exhibit an increased acid tolerance response; and acid adaptation was reported

to enhance survival of *Escherichia coli* O157:H7 [27]. As indicated by Archer [28] long-term survival of acid-adapted pathogens at low pH may trigger mutations of permanent acid stress resistance and of increased virulence. Because of the acid tolerance of *E. coli* O157:H7, the final pH of Ergo (on average 4.4 in this study) was insufficient to prevent the survival of the organism in the product. A similar observation was made by [29] for yogurt.

E. coli O157:H7 grows best within a temperature range of 30 to 42°C, the optimal temperature being 37°C [7]. Although, Raghubeer and Matches [30] reported that *E. coli* O157:H7 does not grow well at 44 to 45.5°C, in the present study viable *E. coli* O157:H7 cells were recovered after 60 and 20 min of cooking defatted fermented milk at 60 and 70°C, respectively. Spano [31] indicated that *E. coli* O157:H7 disappeared completely during stretching of curd for 5 min in hot water (80°C) during the manufacturing of Mozzarella. According to Buchanan and Edelson [32] and Murano and Pierson [33] the survival of acid-adapted pathogens increased against other stresses, such as heat.

In the present study, *E. coli* O157:H7 population decreased only by 1.2, 0.3 and 0.5 log cfu mL^{-1} after 72 h of fermentation at ambient temperature from initial counts of 5.7, 4.3 and 3.3 log cfu mL^{-1}, respectively. Counts increased from 2.6 and 3.0 log cfu mL^{-1} at the start of fermentation to 2.8 and 4.2 log cfu mL^{-1} at the end of fermentation when co-inoculated with LAB and in control milk with no LAB, respectively. At 24 h of fermentation *E. coli* O157:H7 number ranged from 4.7 to 7.3 log cfu mL^{-1} depending on initial inoculum levels at which time Ergo is directly consumed due to its preferred flavor [34]. The infective dose of *E. coli* O157:H7, on the other hand, is reportedly very low ranging from 2 to 100 *E. coli* O157:H7 viable cells depending on the food involved [35-39]. The direct consumption of Ergo, therefore, can represent an important source of *E. coli* O157:H7 infection. From the results of the current study it can be concluded that, at the inoculum levels studied, the use of cooking temperature of 70°C for at least 40 min during Ayib-making is necessary to inactivate *E. coli* O157:H7.

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