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Research Article

Acid Adaptation Effect on Survival of *Escherichia coli* O157:H7 in Fermented Milk Products

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Abstract: The growth and survival of acid-adapted and nonadapted *Escherichia coli* O157:H7 were determined in strained, set-type, and synbiotic yoghurt and kefir. Acid-adapted or nonadapted *E. coli* O157:H7 were inoculated into the synbiotic and set-type yoghurt before and after the fermentation process. Survival of the inoculated *E. coli* O157:H7 in these products was examined during storage at 4 °C.

Acid adaptation enhanced the survival of *E. coli* O157:H7 in kefir and strained yoghurt. Acid adaptation also enhanced the survival of *E. coli* O157:H7 in set-type yoghurt, which was inoculated after the fermentation process; however, acid-adapted cell inoculation before fermentation did not enhance survival.

These observations support the theory that acid adaptation is an important survival mechanism enabling *E. coli* O157: H7 to persist in fermented dairy products.

Key Words: *E. coli* O157:H7, fermented milk product, acid adaptation

Aside Adaptasyonun *Escherichia coli* O157:H7'nin Fermente Süt Ürünlerinde Canlılığına Etkisi

Özet: Bu çalışmada sinbiyotik yoğurt, set tipi yoğurt, süzme yoğurt ve kefirde, aside adapte edilen ve aside adapte edilmeyen *Escherichia coli* O157: H7 hücrelerinin gelişimleri incelenmiştir. Aside adapte edilen hücreler ve kontrol hücreleri sinbiyotik ve set tipi yoğurt örneklerine fermantasyondan önce ve sonra olmak üzere iki farklı aşamada ilave edilmiştir. Örneklere inokule edilen *E. coli* O157:H7'nin canlılığı 4 °C'de depolama sürecinde saptanmıştır.

Kefir ve süzme yoğurtta, aside adaptasyon *E. coli* O157: H7'nin canlılığını artırmıştır. Set tipi yoğurtta ise fermantasyon aşamasında ilave edilen örneklerde aside adaptasyon *E. coli* O157: H7'nin canlılığını artırmıştır. Bununla birlikte fermantasyondan önce inokule edilen örneklerde aside adaptasyonun *E. coli* O157: H7'nin canlılığına etkisi olmamıştır.

Bu sonuçlar aside adaptasyonun, *E. coli* O157: H7'nin fermente süt ürünlerinde canlı kalmasına olanak sağlayan önemli bir mekanizma olduğunu ifade eden teorileri desteklemektedir.

Anahtar Sözcükler: *E. coli* O157:H7, fermente süt ürünleri, aside adaptasyon

Introduction

Escherichia coli is a commonly occurring inhabitant of the colon of humans and other animals, but there are several pathogenic types of *E. coli*, which cause a variety of human diseases.

In 1982, *E. coli* O157:H7 was recognized as a human pathogen for the first time and food-borne illnesses caused by *E. coli* have been steadily increasing worldwide (1).

E. coli O157:H7 belongs to the group of enterohemorrhagic *E. coli* (EHEC) that produce toxins that cause diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in humans (2). Although the main reservoir of this pathogen appears to be cattle, the dynamics of *E. coli* O157:H7 in food-producing animals and the environment is not well understood. It is transmitted principally through consumption of contaminated foods, such as raw or under-cooked meat products and raw milk. During food preparation, fecal

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contamination of water and foods or cross contamination has been important routes of infection. A wide variety of foods has been implicated as vehicles of *E. coli* O157:H7 infection, including meat (3), milk (4), yoghurt (5), fruit juices (6), cheese (7), and vegetables (8).

These outbreaks, especially those involving acidic foods, have drawn attention to the acidic tolerance properties of *E. coli* O157:H7. Unlike most food-borne pathogens, *E. coli* O157:H7 is tolerant to acidic environments. Survival in apple cider (pH 3.6-4) (9) and mayonnaise (pH 3.6-3.9) has been reported (10). *E. coli* O157:H7 survived buttermilk fermentation (pH 4.4), and drying and storage of fermented sausage (pH 4.5) (11).

The resistance of *E. coli* O157:H7 to acidic conditions could be a result of an acid-induced acid tolerance response (ATR). ATR is a phenomenon by which microorganisms show an increased resistance to environmental stress after exposure to a moderate acid environment (12). Acid adaptation and increased resistance to acid stress have been observed in various organisms, including *Listeria* (13), *E. coli* O157:H7 (14), and *Salmonella* (12).

Several investigators have studied acid tolerance and survival of *E. coli* O157:H7 in milk products (15-17); however, in these studies acid tolerance was determined, but cells were not adapted by growing them at low pH, as required for the acid adaptation of cells.

The goal of this research, therefore, was to determine the effects of acid adaptation on the survival of *E. coli* O157:H7 in strained, set-type, and synbiotic yoghurt and kefir.

Materials and Methods

Bacterial strain, preparation of inocula, and growth conditions

E. coli O157:H7 932 (a clinical isolate) was obtained from Dr. M.P. Doyle (Center for Food Safety and Quality Enhancement, Department of Food Science and Technology, University of Georgia, USA). The organism was maintained on tryptic soy agar (TSA, PH 7.0: Difco) slants at 4 °C. Cultures were activated by transferring loop inocula into 10 ml of tryptic soy broth (TSB, PH 7.0: Difco) at 37 °C for 20 h. Following 2 consecutive 20-h culture transfers, a 50-ml TSB was inoculated with culture and incubated at 37 °C for 20 h.

Acid adaptation of test organism

To prepare the acid-adapted cells of *E. coli* O157:H7, the procedure described by Tosun (18) was adapted. Ten milliliters of *E. coli* O157:H7 cultured for 20 h at 37 °C in 50 ml of TSB was centrifuged (5000 rpm). The supernatant was discarded. The cell pellets were washed with E buffer (19) and were suspended either in 10 ml of pH 4.5 TSB (pH adjusted with 6 N HCl) for acid adaptation, or in 10 ml of pH 7.0 TSB for nonadapted cells. Cultures were incubated at 37 °C for 2 h. Then, acid-adapted and nonadapted cells were harvested by centrifugation. The supernatant was discarded and the cell pellets were used for survival studies.

Manufacture of synbiotic and set type yoghurt, and inoculation with *E. coli* O157:H7

A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve the health of the host. Probiotics can be defined as living microbial feed supplements, which beneficially affect the host animal by improving its intestine microbial balance (20). Using probiotic and prebiotic ingredients together is known as synbiotics. Inulin and oligofructose (prebiotics) were added to milk at a ratio of 1:1 for synbiotic yoghurt production. Milk samples were heated to 90 °C for 10 min and cooled to 45 °C. Then, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Lactobacillus bifidus* were added as starter cultures until the acidity reached pH 4.7. In the production of set-type yoghurt, milk samples were similarly heat-treated as in the production of synbiotic yoghurt, but 2% milk powder was used as an extra additive. After pasteurization, *S. thermophilus* and *L. bulgaricus* were added at 45 °C. Synbiotic or set-type yoghurt samples were stored at 42.5 °C until the acidity reached pH 4.7. The resulting yoghurt samples were stored at 4 °C.

Acid-adapted or nonadapted cell pellets were resuspended in 10 ml of pasteurized milk. Inoculations were done by using 2 different steps. In the first trial, 2 ml of acid-adapted and nonadapted cells of *E. coli* O157:H7 were inoculated into milk before fermentation at a level of 10^7 cfu/ml. In the second trial, 200 g of synbiotic and set-type yoghurt samples were inoculated with 0.2 ml of acid-adapted and nonadapted cells (10^7cfu/ml), and were then immediately incubated at 4

°C. Set-type and synbiotic yoghurts were produced 3 times and all analyses were performed in duplicate.

Manufacture of strained yoghurt and inoculation with *E. coli* O157:H7

Milk was heated to 90 °C, held there for 10 min and then cooled to 45 °C. *S. thermophilus* and *L. bulgaricus* were added to the milk as starter cultures and the milk was stored at 42.5 °C until its acidity reached pH 4.7. After fermentation, the coagulum was cooled to room temperature (21 °C) and refrigerated at 4 °C for 12 h. The yoghurt was then mixed thoroughly and transferred into a cotton-cloth bag. The cloth bag was hung to drain the whey (4 °C) for 12 h. Then, the strained yoghurt was divided into 2 equal parts; 0.1 ml of acid-adapted cells was inoculated into the first part at a level of 10^6 cfu/ml, whereas the other part was inoculated with 0.1 ml of nonadapted cells at the same level. Samples were incubated at 4 °C immediately after inoculation. Strained yoghurts were produced 3 times. The results were the average of production and all analyses were performed in duplicate.

Survival studies in Kefir and inoculation with *E. coli* O157:H7

Commercial kefir (pH 4.0) was purchased 3 times from Ege University Agriculture Faculty (İzmir, Turkey). The background flora of the kefir was determined before inoculation, after which time the colony morphologies were always easily distinguishable from *E. coli* O157:H7 on Sorbitol MacConkey Agar (SMA, pH 7.0: Difco).

Acid-adapted or nonadapted cell pellets were resuspended in 2 ml of kefir. Kefir samples (100 ml) were transferred into sterile 250-ml Erlenmayer flasks and 0.1 ml of acid-adapted or nonadapted *E. coli* O157:H7 was added at the same level (10^7 cfu/ml). Samples were incubated at 4 °C immediately after inoculation.

Enumeration of *E. coli* O157:H7

After *E. coli* O157:H7 had been inoculated into samples, viable cells were immediately enumerated. Samples inoculated with acid-adapted and nonadapted *E. coli* O157:H7 were stored at 4 °C. During the storage period, viable populations of *E. coli* O157:H7 were periodically determined.

For the enumeration of *E. coli* O157:H7, samples were serially diluted with E buffer and 0.1 ml of diluted samples was plated on SMA and incubated at 37 °C for 24 h. *E. coli* O157:H7 forms colorless colonies on SMA and these cells were confirmed by latex agglutination assay (21).

Determination of water activity

Water activity of the strained yoghurt was measured using a Durotherm Wert-Messer water activity meter (Germany)

Statistical analysis

Two replications of each experiment were done. Data were analyzed using SPSS (Version 6, 1993 SPSS Ltd, Surrey, UK). The least significant difference test was used to determine significant differences ($P \leq 0.05$) between populations of nonadapted and acid-adapted cells.

Results

Survival of *E. coli* O157:H7 in set type yoghurt

Figure 1 shows survival of acid-adapted and nonadapted *E. coli* O157:H7 in set-type yoghurt during storage at 4 °C. *E. coli* O157:H7 was inoculated into milk at 42.5 °C.

Populations of acid-adapted cells were less than those of nonadapted cells during the storage period, but differences were not significant ($P < 0.05$). After 11 days of incubation, the acid-adapted population was completely inactivated, but nonadapted cells were detected at 1.5 x 10^2 cfu/g. At 13 days, viable nonadapted *E. coli* O157:H7 organisms were no longer detected. The final pH at the last sampling was 4.4.

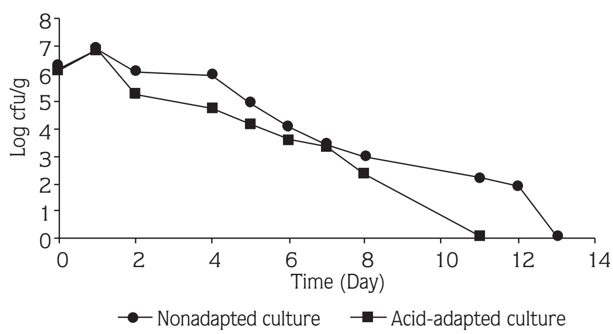

Figure 1. Survival of acid-adapted or nonadapted *E. coli* O157: H7 in set-type yoghurt during storage at 4 °C. *E. coli* O157: H7 was inoculated before fermentation.

Figure 2 shows the survival of acid-adapted and nonadapted *E. coli* O157:H7 inoculated after fermentation in set-type yoghurt during storage at 4 °C. Acid-adapted cells survived longer than the nonadapted cells in set-type yoghurt ($P \leq 0.05$), until 18 days. Nonadapted cells were not detected after 18 days of storage, but acid-adapted cells were detected at 1.5×10^3 cfu/g. Acid-adapted cells were also detected at 2.0×10^2 cfu/g after 22 days, but at the end of 23 days of incubation viable acid-adapted cells were not detected. pH was 4.3 at the end of storage.

Survival of *E. coli* O157:H7 in synbiotic yoghurt

Figure 3 shows the survival of acid-adapted and nonadapted *E. coli* O157:H7 in synbiotic yoghurt during storage at 4 °C. *E. coli* O157:H7 was inoculated into milk at 42.5 °C.

Acid-adapted and nonadapted cells of *E. coli* O157:H7 had similar acid tolerance and followed the same pattern of decline until day 22. Although acid-adapted cells showed a rapid decrease and could not be detected on day 26, 2.7×10^4 cfu/g were detected in the nonadapted population, but this was not statistically significant ($P \leq 0.05$).

Acid-adapted and nonadapted *E. coli* O157:H7 were inoculated after fermentation in synbiotic yoghurt. Populations of acid-adapted cells were smaller than those of nonadapted cells during 5 days of incubation; however, populations of acid-adapted cells after 5 days of incubation were significantly greater than respective populations of nonadapted cells; the differences were not significant. While 5.4×10^5 viable cells per gram were detected in the non-adapted population, 8.5×10^4 cells per gram were detected in the acid-adapted population at 26 days of incubation. The final pH at the end of storage was 4.5 for synbiotic yoghurt.

Survival of *E. coli* O157:H7 in strained yoghurt

Survival of acid-adapted or nonadapted cells in strained yoghurt (pH 3.7) stored at 4 °C was determined (Figure 4). A rapid decrease in the number of both acid-adapted and nonadapted cells was observed in strained yoghurt during the storage period; however, nonadapted *E. coli* O157:H7 died off more rapidly than acid-adapted cells ($P \leq 0.05$). After 5 h, few differences in survival were observed between the acid-adapted and nonadapted cells. After 24 h, nonadapted cells were completely inactivated and acid-adapted cells decreased by about 25-fold of their initial number; therefore, acid-adapted cells were not detected after 48 h.

Survival of *E. coli* O157:H7 in Kefir

Acid-adapted cells survived much better than nonadapted cells in kefir during storage at 4 °C ($P \leq 0.05$). After 8 days of incubation, the nonadapted population was completely inactivated, but acid-adapted cells were detected at 1.9×10^2 cfu/ml. At 12 days, viable acid-adapted *E. coli* O157:H7 were no longer detected (Figure 5). The pH of kefir was 4.0 and did not change during storage.

Discussion

Acid adaptation and increased resistance to acid stress have been reported in various food-borne pathogens, such as *Listeria* (22), *Salmonella* (12), and *E. coli* O157:H7 (23). It has also been reported that acid adaptation prolongs the survival of *E. coli* O157:H7 in various food systems, including cheese (24), apple cider,

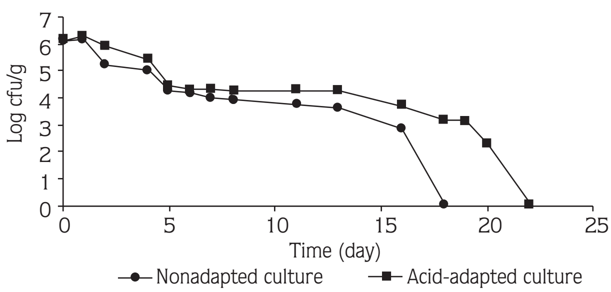

Figure 2. Survival of acid-adapted or nonadapted *E. coli* O157: H7 in set-type yoghurt during storage at 4 °C. *E. coli* O157: H7 was inoculated after yoghurt was made.

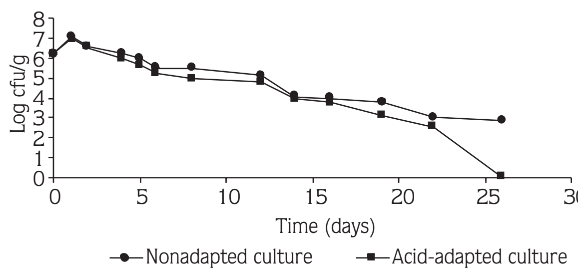

Figure 3. Survival of acid-adapted or nonadapted *E. coli* O157: H7 in symbiotic yoghurt during storage at 4 °C. *E. coli* O157: H7 was inoculated before fermentation.

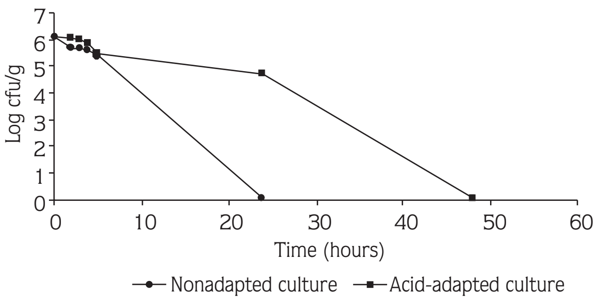

Figure 4. Survival of acid-adapted or nonadapted *E. coli* O157: H7 in strained yoghurt during storage at 4 °C.

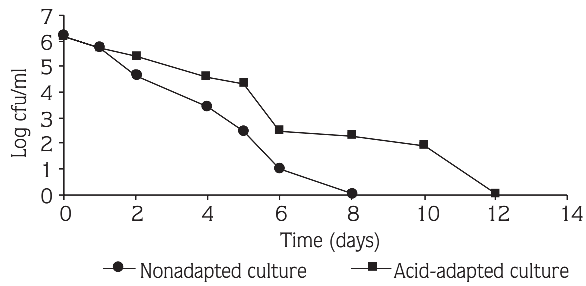

Figure 5. Survival of acid-adapted or nonadapted *E. coli* O157: H7 in kefir during storage at 4 °C.

sausage (11), and acid fruit juice (25). Several investigators have studied acid tolerance and survival of *E. coli* O157: H7 in yoghurt. For example, Hudson et al. (26) reported that *E. coli* O157: H7 inoculated into commercial plain yoghurt (pH 4.47) survived for 17 days. Guraya et al. (27) reported that *E. coli* O157: H7 survived in traditional yoghurt (pH 4.17- 4.65) for 7-35 days. In these studies, acid tolerance was determined, but cells were not adapted by growing them at low pH, as is required for acid adaptation of *E. coli* O157: H7.

Our results indicated that *E. coli* O157: H7 cells survived substantially longer in yoghurt when cells were inoculated after fermentation and that acid adaptation enhanced survival of *E. coli* O157: H7 in set-type yoghurt. Yet, when *E. coli* O157: H7 was inoculated before fermentation, acid adaptation did not enhance its survival in yoghurt. Cells inoculated into yoghurt before fermentation declined more rapidly than cells that were inoculated into yoghurt after fermentation. *E. coli* O157: H7 inoculated into milk was not exposed to an acidic environment, which is necessary for acid adaptation. Acid-adapted cells inoculated into pasteurized milk lost their acid tolerance before the pH dropped to 4.7; however, cells inoculated into set-type yoghurt may have enhanced survival because of acid shock. Acid shock may induce acid tolerance and increase the acid resistance of this pathogen. Leyer et al. (11) showed that *E. coli* O157: H7 had an acid-adaptive response and the expression of this system enhanced survival in the presence of lactic acid in acidified food products. Therefore, our results indicated that post-pasteurization contamination of *E. coli* O157: H7 may induce acid tolerance and increase resistance to acid in milk products.

Acid-adapted cells survived much better than nonadapted cells in kefir and in strained yoghurt, but *E. coli* O157: H7 died off very rapidly because of the low water activity of this product (a_w = 0.92).

In our study it was shown that adaptation to acid by *E. coli* O157: H7 may enhance its survival in kefir, strained yoghurt, and set-type yoghurt, provided that *E. coli* O157: H7 is inoculated into yoghurt after fermentation.

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