METABOLIC ACTIVITY OF *LUX*-MARKED *ESCHERICHIA COLI* O157:H7 DURING PRODUCTION AND STORAGE OF YOGURT

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ABSTRACT

The objective of this study is to estimate the metabolic activity and survival of bioluminescence *Escherichia coli* O157:H7 (a chromosomally lux-marked strain) during production and storage of yogurt.The effect of temperature on activity and survival of *E. coli* O157:H7 during the production of yogurt were studied by incubating inoculated milk at 43°C, 40 °C and 37 °C. The results showed that *E. coli* O157:H7 activity and cell counts increased immediately in all yogurt samples at the beginning stagesof fermentation then they declined to nearly of the initial levels at the end of fermentation. During storage yogurt at three different initial levels of pH (4.6, 4.3 and 4.0), significant differences (p<0.001) were found in cell activity and viable cellcount of *Escherichia coli* O157:H7, indicating that low pH provided ideal conditions to inhibit the activity of pathogens. The cell activity and survival of *E. coli* O157:H7 were significantly different during storage at three different temperatures (4 °C, 12 °C and 25 °C). At 25 °C, *E. coli* O157:H7 activity and cell counts were significantly lower (P<0.001) compared with other storage temperatures. Bioluminescence (metabolic activity) helps to track *E.coli* O157:H7 growth and survival during manufacture and storage of yogurt and interact with their acidic environments as a rapid assay method. Therefore, it can provide notice on the way of eliminating *E.coli* O157:H7 in the dairy products to avoid these pathogens.

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) is an important food borne pathogen which has been linked with a variety of illnesses in humans including haemolytic uraemic syndrome and the diarrhea, hemorrhagic colitis (1). *E. coli* O157 H7 have been isolated from different animal products like meat and dairy products, several infection outbreaks have shown that this pathogen can present in foods with low pH (2). An outbreak of

Escherichia coli O157:H7 infection has been linked to the consumption of yogurt (3). The survival of *E. coli* O157:H7 in fermented dairy products, specifically cheese and yoghurt for up to several days or weeks demonstrates the potential health risks linked with post-processing infection of even low levels of this pathogen in various dairy products (4). Although fermented milk products are considered safe due to their acidic environments and pH less than 4.6, a wide verity of acid food such as mayonnaise, apple cider and yogurt have been involved to be linked with some outbreaks of *E. coli* O157:H7 infection. (5 ,3). Persistence of this pathogen in yogurt demonstrations the ability of *E. coli* O157:H7 to tolerate the acidic environments(3). In addition, it has been found *E. coli* O157:H7 having the ability to adapt to acidic dairy foods during fermentation and enhance the survival of this pathogen in these products (6). The acid tolerance and adaptation of *E. coli*O157:H7 is believed to play a key role in foodborne infections by fermented milk(7). Survival of *E. coli* O157:H7 in fermented dairy products has been studied by many researchers over years*E. coli* O157:H7 was survived for up to 12 days in yoghurt at pH 4.0(4).Also, it was shown that *E. coli* O157:H7 to be survived in yoghurt for 7 days during storage at 4 °C (8). While other studies have foundthat *E. coli* O157:H7 survived for up to 21 days during refrigerator storage (5-7 °C) of kefir (9).Bioluminescent bacteria have been used as an indicator of survival of this pathogenic bacteriumin dairy products (10).The *lux*-marked genes, which encode bioluminescence, have been effectively used in studies of the behavior of microorganisms in their environment(11). *Lux*-marked *E. coli* O157:H7 strain could help us to determine the interactions between this pathogenic bacteria with starter culture in various fermented dairy products and under variety of storage conditions. Therefore, The purpose of this research was tostudy this bioluminescent construct to investigate the metabolic activity and survival of *E. coli* O157:H7 during production and storage conditions of yoghurt.

MATERIALS AND METHODS

Cultures

The chromosomally *lux*-marked (Tn*5 luxCDABE*) of *E. coli* O157:H7 has been used as a test strain in this study (11). Commercial Direct Set Lyophilized starter (Chr. Hansen)

including *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* have been used as the starter culture in yogurt production.

Production and storage of yogurt

Cow's milk was used to prepare yoghurt samples. Milk was heated to 85 °C, held there for 30 min and then cooled to 45 °Cthen added starter culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *Bulgaricus*)to milk at levels recommended by the company.Inoculated milk was divided into three parts.The first part of milk was contaminated with *Escherichia coli* O157:H7to obtain the desired final concentration of approximately 10^5 CFU/mL, then incubated at 37°C, 40°C, 43 °C until the desired acidity.The second part incubated at 43 °C until its acidity reached pH about 4.6, then contaminated with *Escherichia coli* O157:H7 *E. coli* O157 10^5 CFU/g, then stored at room temperature (25 °C), 12 °C and 4 °C for 14 days. The third part incubated at 43 °C and fermentation process continued until the desired acidity (pH 4.6, 4.3 and 4.0), then contaminated with *Escherichia coli* O157:H710^5 CFU/ gand stored at refrigerated temperature 4 °C for 14 days.

Preparation of *E. coli* O157:H7 inoculum

Bacterial inoculum was prepared from a fresh overnight culture (LB broth; Difco Ltd, Teddington, Surrey, UK; 18 h, 37 °C, 150 rpm) of bioluminescence *E. coli* O157:H7 in stationary growth phase. Cells were washed and concentrated by centrifugation.An inoculum (1 mL) of the mixture at the appropriate dilution was added to 99 mL of milk and mixed thoroughly in sterilised screw-cap bottles to obtain the desired final concentration of approximately 10^5 CFU/g.

Survival and metabolic activity of *E. coli*O157:H7

Metabolic activity of *E. coli* O157:H7 in milk was measured at 0 (immediately after inoculation of milk), 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420 min(pre-fermentation parts during production) and 0 (immediately after inoculation of yogurt), 1, 2, 4, 6, 8, 10, 12 and 14 days (post- fermentation during storage). At each time-point, a 1 mL aliquot from samples used for the enumeration study detailed above was placed into a plastic luminometer cuvette and its luminescence (RLU) was determined using a SystemSURE plus Pi-102 Luminometer (Hygiena International Ltd., UK).A parallel experiment was designed to assess variations in the survival of *E. coli* O157:H7 among the different yogurt types (pre-fermentation and post-fermentation).*E. coli* O157:H7 cells were enumerated at 0 (immediately after inoculation of milk), 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420 min until its acidity reached pH about 4.6 for pre-fermentation parts and 0 (immediately after inoculation of yogurt),1, 2, 4, 6, 8, 10, 12 and 14 days for post-fermentation parts. Yogurt samples were serially diluted in Ringer solution (Oxoid), and serial dilutions were plated onto CT-SMAC and incubated at 37 °C for 18 to 24 h. Non-sorbitol fermenting *E. coli* O157:H7 colonies were confirmed by agglutination with a latex test kit (Oxoid DR0620).

pH determined:

Yogurt samples pH values during fermentation and storage were measured with a standard pH meter (Hanna instruments pH 211). Calibration was performed using two standard buffer solutions at pH 4.0 and 7.0.

Statistical analysis

The changes in *E. coli* O157:H7 cell counts and cell activity (bioluminescence), and pH values during the 14 days incubation period were subjected to ANOVA tests and Tukey's test with significance at $p< 0.05$ using the statistical package SPSS 18.0 software (SSPS Inc, Chicago, Illinois, USA)

RESULTS AND DISCUSSION

Metabolic activity and cell count of *E. coli*O157 in yogurt samples during fermentation

Metabolic activity of*E. coli*O157 during fermentation at different temperatures (37°C, 40°Cand 43 °C) of yoghurt shown in Figure. 1. At 37 °C, *E. coli* O157:H7 cell activity increased from 3.09 to 5.68 $\log_{10}$ RLU after 240 min during the fermentation period, then the activity decreased to 2.67 $\log_{10}$ RLU at the end of fermentation period (pH about 4.6). At 40°C*E. coli* O157:H7 increased from to 3.17 to 5.79 $\log_{10}$ RLUduring 210 min of incubation, then decreased to 4.22 $\log_{10}$ RLU at the end of fermentation period. After 90 min of fermentation at 43°C*E. coli* O157:H7 activity increasedabout 2.0 log from initial activity of 3.31 to 5.33 l $\log_{10}$ RLU, then decreased to 2.56 $\log_{10}$ RLU at the end of fermentation period (210 min).The count of *E. coli*O157 during the fermentation of milk at 37, 40 and 43 °C are shown in Fig 2. The count *E. coli*O157 increased from 5.02, 5.21 and 5.13 log CFU/mLto 7.98, 7.89 7.12 during fermentation and reduced to4.22,4.88 and 4.35 log CFU/mLat end of fermentation period at incubation temperatures 37, 40 and 43 °C, respectively. The pH values decreased from 6.68,6.71 and 6.72 to 4.6 at the end of the fermentation period 410, 300 and 210 min during the incubation temperatures at 37, 40 and 43 °C, respectively. (Figure. 3)

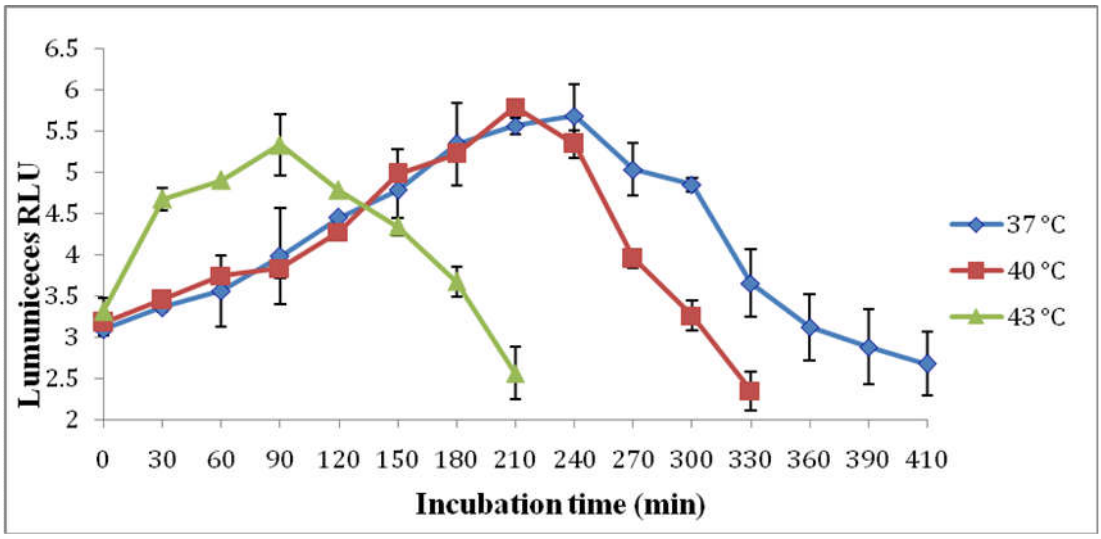

Figure: 1. Metabolic activity as measured by luminescence (RLU), of *E. coli* O157 in yogurt samples during fermentation Values represent mean ± standard error (n = 3).

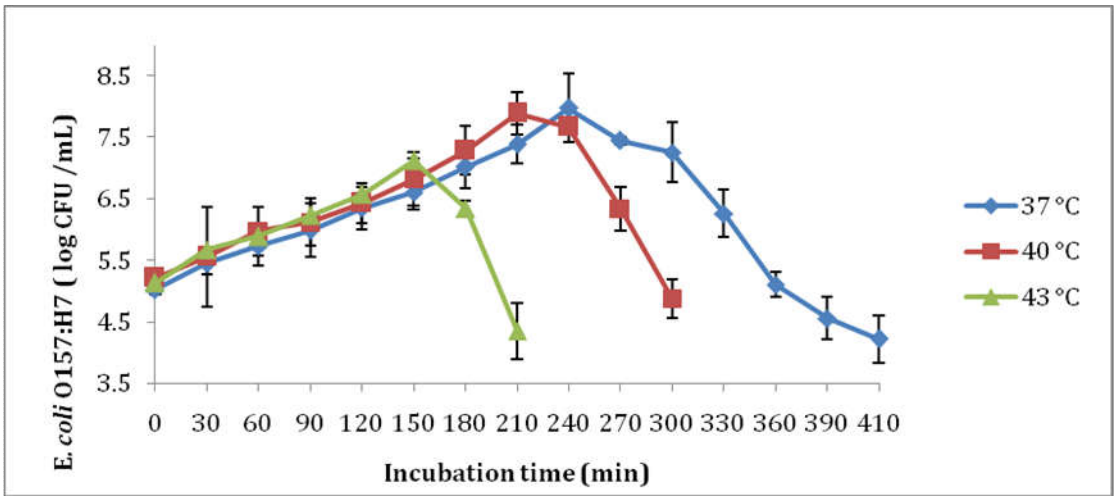

Figure:2. Cell counts (CFU/mL), of *E. coli* O157 in yogurt samples during fermentation. Values represent mean ± standard error (n = 3).

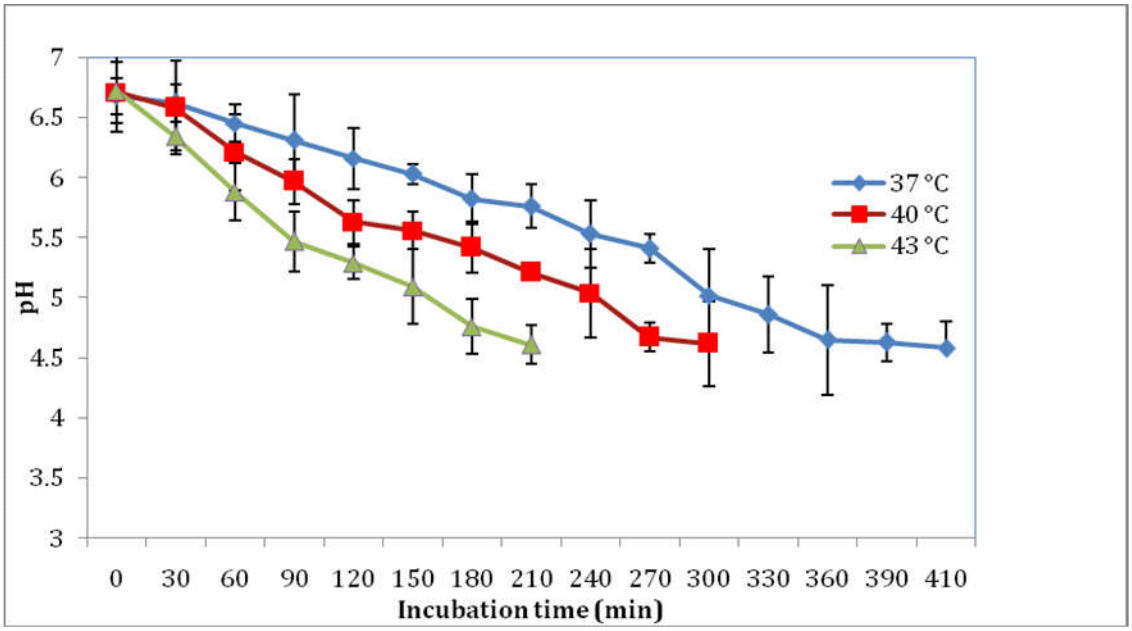

Figure:3pH of yogurt samples during fermentation. Values represent mean ± standard error (n = 3).

Luminescence directly reports on bacterial metabolic activity which represents a prerequisite for host infection (12,13,14). Our luminescence measurements show that, in general metabolic activity of *E. coli* O157 increase rapidly, then decreases rapidly during fermentation period at high temperature. The study has shown that *E. coli* O157 can grow

easily in the early stages of fermentation because pH and other antimicrobial substances produced by yogurt starters are limited (15). It was shown that *E. coli* O157:H7 survives the fermentation environments of yogurt at temperatures of 42°C up to 5 h (8).However, other researchers have found that *E. coli* O157:H7 cannot survive fermentationconditions of yogurt containing lactic acid bacteria starters at temperature of 47°C (16). According to (17), *E. coli* O157:H7 can survive and proliferate during fermentation of yogurt at temperatures 25 °C and 37 °C. In a study done by (18) *E. coli* O157:H7 does not grow well at 44°C to 45.5°C. In another study a *E. coli* O157:H7 grows best within a temperature range of 30°C to 42°C, the optimal temperature being 37°C (19). Whilst other studies have also demonstrated that *E. coli* O157:H7 can survive and proliferate during preparation and storage of yogurt, this study additionally revealed a corresponding increase in the pathogen's metabolic activity at different temperatures of fermentation.

Metabolic activity and cell count of *E. coli*O157 in yogurt samples during storage at different pH

Significant difference ($p< 0.001$) was shown among metabolic activity and cell counts of *E. coli*O157 during storage of yogurt samples at pH 4.6, 4.3 and 4.0. (Figure 4). The cell activity dropped afterwards in all yogurt samples, with that at pH 4.6 reaching zero on day14, that at pH 4.3 on day 12 and that at pH 4.0 samples on day 8. Viable cells number of *E. coli* O157:H7 in these yogurt samples decreased to undetectable level after 12 days and 14 days at pH 4.0 and 4.3, respectively. (Figure 5); whereas that at pH 4.6 the count of *E. coli* O157:H7 declined about 3-log cycle during storage periods. Although *E. coli* O157:H7 cells donot grow, it can survive at refrigeration temperature. The results clearly indicate a correlation between the drop in pH levels and the corresponding decline in metabolic activity and cell counts of *E. coli* O157:H7 in yogurt. In this study, pH values at which *E. coli* O157:H7 are eliminated are in agreement with previous studies, *E. coli* O157:H7 decreased from 0.8 to 1.76 $\log_{10}$ CFU/mL in yogurt (pH 4.4-4.5) made with yogurt starterafter 7 days of storage at temperature of 4°C (8). It was reported that *E. coli* O157:H7 (10^5 CFU/ g) survived storage conditionsof low pH levels of 4.0 , but did not

grow (20). The viable cells number of *E. coli* O157:H7 decreased from 10^5 CFU/mL toundetectable level in yogurt (pH 4.1) after 35 days of storage at temperature of 4 °C (21).

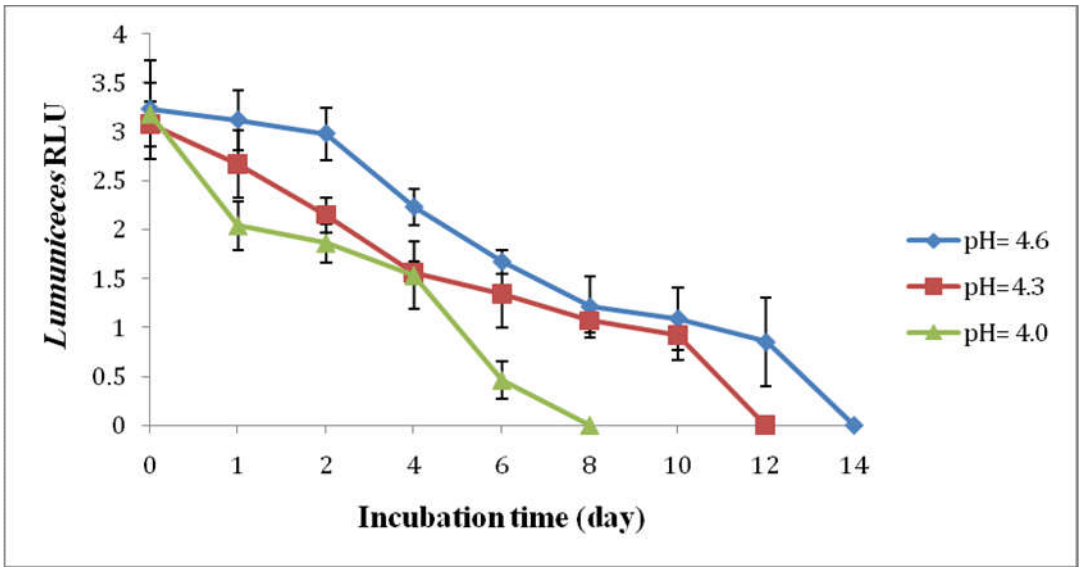

Figure:4. Metabolic activity as measured by luminescence (RLU), of *E. coli* O157 stored at pH 4.6,4.3, and 4.0. Values represent mean ± standard error (n = 3).

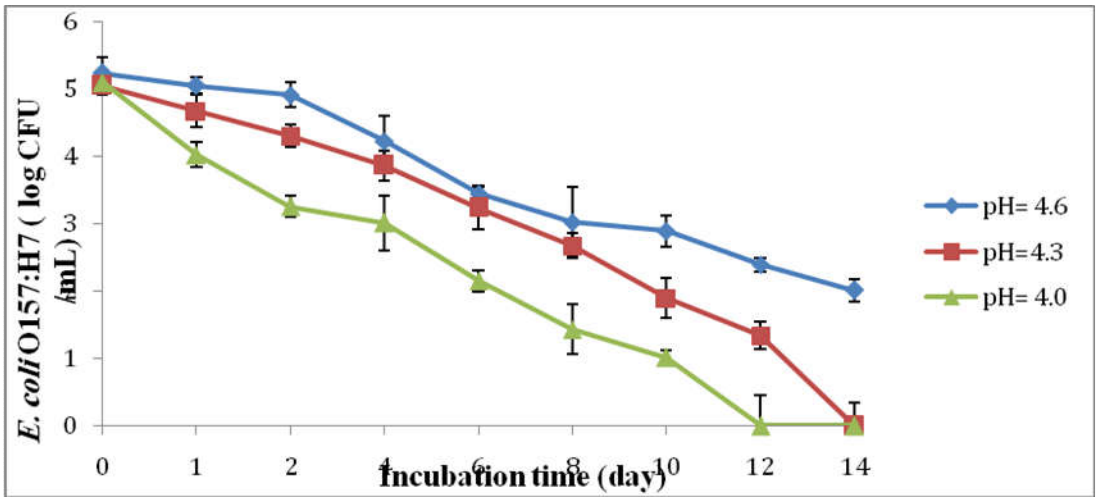

Figure:5. Cell counts (CFU/g), of *E. coli* O157 stored at pH 4.6,4.3, and 4.0. Values represent mean ± standard error (n = 3).

Metabolic activity and cell count of *E. coli*O157 in yogurt samples during storage

at different temperatures

The cell activity (luminescence) and survival of *E.coli* O157:H7 were significantly different ($p< 0.001$)during storage at 4°C, 12°C and 25 °C **(**Figure.6,7).Initial cell activity of *E. coli* O157:H7 were reduced from 3.14 $\log_{10}$ RLU to undetectable level after 14 days storage at 4 °C, whereas that at 12°C and 25 °C, *E. coli* O157:H7 cell activity reached to undetectable level after 8 and 4 days of storage periods, respectively.*E. coli* O157:H7 counts decreased from 5.11 CFU /gto 1.88 CFU /gat 4 °C during storage 14 days. Initial of *E. coli* O157:H7 count were reduced from 5.05CFU /gto undetectable level after 12 days, whereas that at 25 °C *E. coli* O157:H7 counts decreased sharply from 5.19 log CFU /gto zero after 8 days. The pH values decreased from 4.58,4.61 and 4.62 to 4.22, 3.56 and 3.04 at the end of the storage period (14 days) at 4°C, 12°C and 25°C, respectively. (Figure.8). The Results showed that storage temperature affected on metabolic activity and the survival of the pathogen in yogurt. When comparing the results of yoghurt stored at cold storage at 4 °C to those of yoghurt stored at 12°C at 25 °C it has found that *E.coli* O157 are inhibited more rapidly at 25 °C. Studies have indicated that *E. coli* O157:H7 die off faster at room temperatures (± 25 °C) than at 4°C storage. Although, at 25 °C *E. coli* O157:H7 failed to maintain its contamination level and decreased undetectable level after 5 days of storage (Figure.8). When comparing the results of yoghurt stored at cold storage at 4 °C to those of yoghurt stored at 12 °C at 25 °C it was found that *E.coli* O157 are inhibited more rapidly during storage at high temperatures. These observations are similar to that reported in previous studies. Many researchers were indicated that *E. coli* O157:H7 die off faster at room temperatures (± 25 °C) than at 4 °C storage. It was found that the survival of *E. coli* O157:H7 in traditional African yoghurt were significantly lower during storage at 25 °C compared with 4 °C (22)*E. coli* O157:H7 survived up to 17 d at both 4 °C and 10 °C in yogurt with pH 4.47 (10). It was found that *E. coli* O157:H7 cell activity reduced faster in different types milk during storage at 20 °Cthan at 4 °C storage (23). The fairly earlier decline of *E. coli* O157:H7 metabolic activity at 25 °C could be related with the low pH of the yogurt stored at this temperature. One of the reasons for the reduced survival of *E.coli* O157 at 25 °C can be of an increasing level of lactic acid generated by the starter culture. At 25 °C pH declines faster and this has an effect of inhibiting growth of *E. coli* O157:H7 at a much faster rate than at low

temperatures

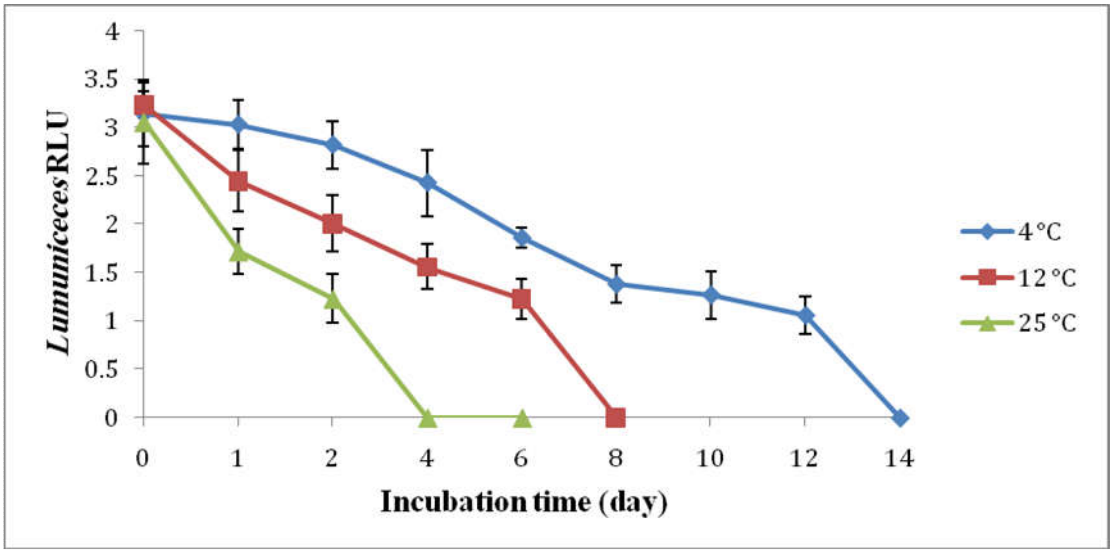

Figure:6. Metabolic activity as measured by luminescence (RLU) of *E. coli* O157 stored at 4°C, 12°C, and 25 °C. Values represent mean ± standard error (n = 3).

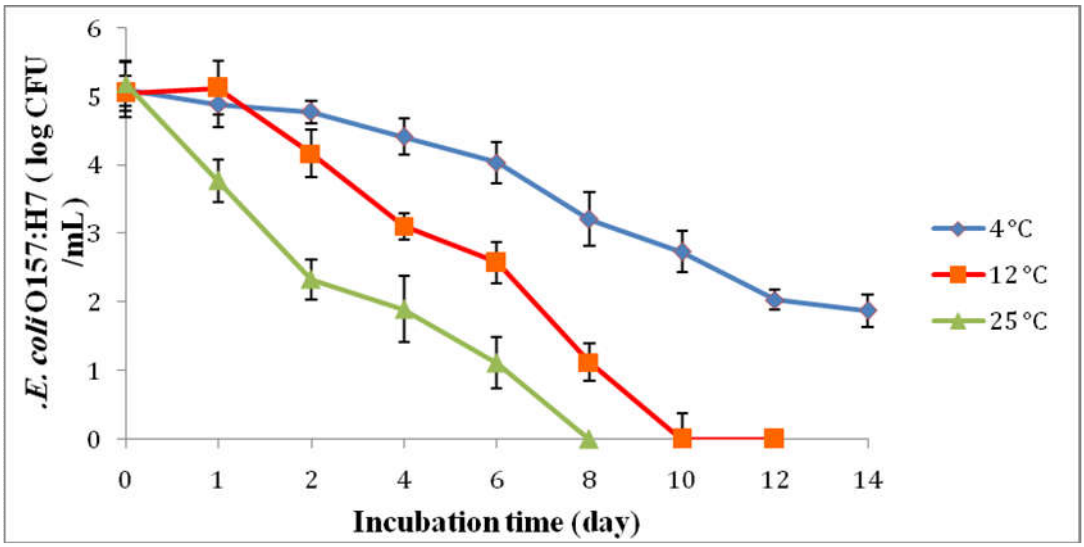

Figure:7. Cell counts (CFU/g), of *E. coli* O157 stored at 4°C, 12°C, and 25 °C. Values represent mean ± standard error (n = 3).

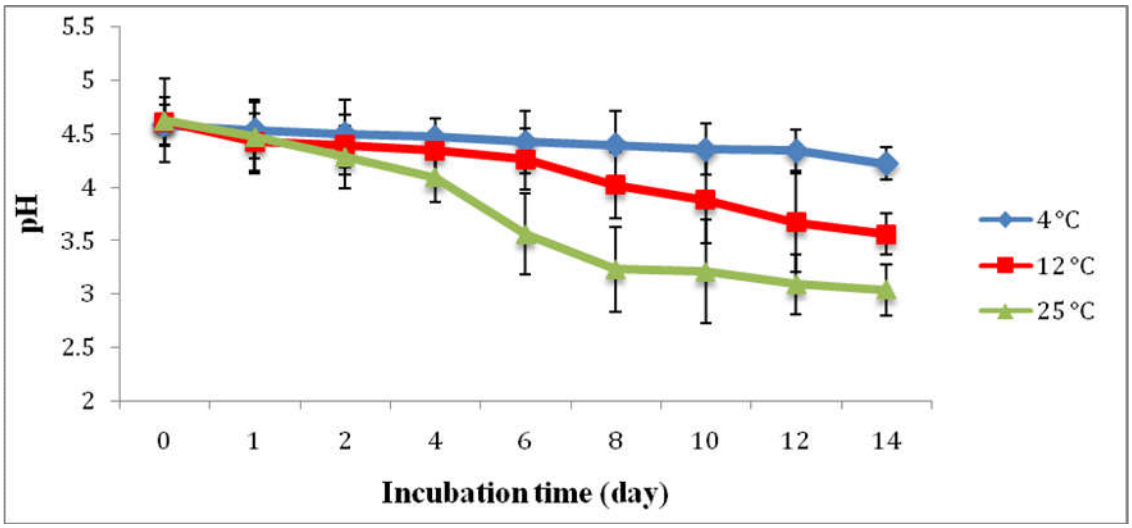

Figure: 8.pH values of yogurt samples stored at 4°C, 12°C, and 25 °C. Values represent mean ± standard error (n = 3)

CONCLUSIONS

In this study it has been found that the fermentation temperature used to produce yoghurt definitely affect the metabolic activity and survival *E. coli* O157:H7. The overall results demonstrate that *E. coli* O157:H7 has the ability to grow during first stages of milk fermentation and the pathogen can survive until the end period of fermentation. The faster fermentation, at a higher fermentation temperature of 43 °C, *E. coli* O157:H7 cell activity and count in milk sample increases rabidly and then progressively declines at the end of the' incubation. In contrast, fermentation at the lower temperatures of 37 °C or 40 °C was longer. The results of the present study showed that both the initial pH values and the temperatures during storage are significant (P<0.001) factors on the survival and metabolic activity of *E. coli* O157:H7. According to the results obtained from this study, It has been found that *E. coli* O157:H7 survives in yoghurt during fermentation and storage period even at low pH. Metabolic activity has helped us understand the details of bacterial ecology. This has permitted a detection of pathogen behavior during yogurt fermentation and storage and this can give the future studies to find biological control systems for pathogen.

الفعاليه الايضيه للسلاله المحوره جينيا Lux-marked *Escherichia coli* O157:H7 خلال تصنيع وخزن اللبن الرائب

نوفل عبدالامير حسين

قسم علوم الاغذيه -- كليه الزراعه - جامعه البصره

الخلاصه

هدفت هذه الدراسه الى تقدير الفعاليه الايضيه وعيوشيه السلاله المحوره جينيا Lux-marked *Escherichia coli* O157:H7خلال تصنيع وخزن اللبن الرائب.عند دراسه تاثير ثلاث درجات حراريه اثناء الحضن (٤٣ م°، ٤٠ م°، ٣٧ م)°على فعاليه الخلايا وعيوشيه البكتريا *E. coli* O157:H7 في الحليب الملقح اظهرت النتائج ان النشاط الايضي واعداد البكتريا ازدادت بشكل ملحوظ في المراحل الاولى من التخمر ثم بدأت بالانخفاض لتصل المستوى الاولي اثناء التلقيح عند نهايه فتره التخمر. وعند دراسه تأثير مستويات مختلفه من قيم الاس الهيدروجيني- 4.6)و 4.3 و(4.0 ودرجه الحراره 4 °C) و 12 °C و (25 °C اثناء اخزن منتوج اللبن الرائب الملقح ببكتريا *E. coli* O157:H7بعد الانتاج لمده٢٨ يوم اظهرت النتائج وجود انخفاض معنوي ($p<0.001$)عالي في فعاليه الخلايا والاعداد الحيه لبكتريا*E. coli* O157:H7 مع انخفاض الاس الهيدروجيني الاولي اثناء الخزن والذي يشير الى ان انخفاضالاس الهيدروجيني يوفر بيئه ملائمه لتثبيط نمو هذه البكتريا كما بينت نتائج الدراسه ان اعداد وفعاليه بكتريا *E. coli* O157:H7قد انخفض معنويا ($p<0.001$) عند خزنها على درجه حراره25 °C مقارنه مع درجتي الحراره الاخرى بالدراسه. نستنتج من هذه الدراسه ان قياس الفعاليه الايضيه لبكتريا *E. coli* O157:H7 يساعد في تتبع نمو وقابليه البكتريا على البقاء اثناء تصنيع وخزن اللبن الرائب سلوكها في البيئه الحامضيه للمنتوج كوسيله سريعه في التقدير الامر الذي يوفر معلومات للحد من نمو بكتريا *E. coli* O157:H7 ومنع الاصابه بالمرض.

REFERENCE

1. Griffin, P. M. and Tauxe, R. V. (1991). The epidemiology of infections caused by*Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associatedhemolytic syndrome. *Epidemiologic Reviews***13, 60– 97.**

2. Feng, P. (1995).*Escherichia coli* serotype O157:H7: novel vehicles of infection and emergence of phenotypic variants. *Emerging Infectious Diseases***1,16–21.**

3. Morgan,D.,Newman,C. P., Hutchinson, D. N., Walker, A. W., Rowe,B., and

Majid,F. (1993). Verotoxin producing *E. coli* O157 infections associated with the consumption of yogurt. *Epidemiology and Infection***111, 181–187.**

4. Dineen, S. S., Takeuchi, K., Soudah, J. E. and Boor, K. J. (1998). Persistence of*Escherichia coli* O157:H7 in dairy fermentation systems.*Journal of Food Protection***61, 1602 – 1608.**

5. Besser, R. E., Lett, S. M., Weber, J. T., Doyle, M. P., Barrett, T. J., Wells, J. G. and Griffin, P. M. (1993). An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. *Journal of the American Medical Association***269, 2217–2220.**

6. Gahan, G. M., O'driscol, B. and Hill, C. (1996). Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation. *Applied and Environmental Microbiology***62, 3128 – 3132**

7. Buchanan, R. L. and Edelson, S. G. (1996). Culturing enterohemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance of stationary-phase cells. *Applied and Environmental Microbiology***62, 4009–4013.**

8. Massa, S., Altieri, C., Quaranta, V. and De Pace, R. (1997). Survival of *E. coli* O157:H7 in yoghurt during preparation and storage at 4°C. *Letters in Applied Microbiology***24, 347– 350**.

9. Gulmez, M. and Guven, A. (2003). Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes* 4b and *Yersinia enterocolitica* O3 in ayran and modified kefir aspre- and post fermentation contaminant.*Veterinary Medicine – Czech***48, 126 –132.**

10. Hudson, L. M., Chen, J. Hill, A. R. and Griffiths,M. W. (1997). Bioluminescence: a rapid indicator of *Escherichia coli* O157:H7 in selected yogurt and cheese varieties. *Journal of Food Protection***60, 891–89.**

11. Ritchie, J. M., Campbell, G. R., Shepherd, J., Beaton, Y., Jones, D., Kilham, K.and Artz, R. R. E.(2003). A stable bioluminescent construct of *Escherichia coli* O157:H7 for hazard assessments of long–term survival in the environment. *Applied and Environmental Microbiology*, **69, 3359–3367**

12. Hale, T.L. and Bonventre, P. F. (1979). *Shigella* infection of Henle intestinal epithelial cells: role of the bacterium. *Infection and Immunity***24,879–886.**

13. Unge, A., Tombolini, R., Molbak, L. & Jansson, J. K. (1999). Simultaneous monitoring of cell number and metabolic activity of specific bacterial populations with a dual *gfp-lux AB*_marker system.*Applied and Environmental Microbiology***65, 813–821.**

14. Williams, A.P., McGregor, K.A., Killham, K. and Jones, D.L.(2008). Persistence and metabolic activity of *Escherichia coli* O157:H7 in farm animal faeces. *FEMSMicrobiology Letters***287, 168–173.**

15. Benkerroun, N., H. Ouben and SandineW.E. (2003). Effect of nisin on yoghurt starter and on growth and survival of *Listeria monocytogenes* during fermentation and storage of yoghurt.*International Journal of Food Microbiology* **1, 1 – 5**.

16. Soudah J., Boor K. , and Jeffers G. (1996). The persistence of *Escherichia coli* O157:H7 in dairy products fermented by lactic acid bacteria.*Journal of Dairy Science***79, 121–126.**

17. Ogawa, M., Shimizu, K., Tanaka, R., Hamabata, T., Yamasaki, S., Takeda. T., and Takeda,Y. (2001). Inhibition of in vitro growth of Shiga toxic-producing *Escherichia coli* O157:H7 by probiotic Lactobacillus strains due to production oflactic acid.*International Journal of Food Microbiology***68, 135–140.**

18. Raghubeer, E. F. and Matches, J. R.(1990). Temperature range for growth of *Escherichia coli*serotype O157:H7 and selected coliforms in *E. coli* medium. *Journal of*

*Clinical Microbiology***l28, 803– 805.**

19. Doyle, M. P. and Schoeni, J. L. (1987). Isolation of *Escherichia coli* O157:H7 from retail fresh meats and poultry.*Applied and Environmental Microbiology***53, 2394 – 2396.**

20. Conner, D.E. and Kotrola, J.S. 1(995). Growth and survival of *Escherichia coli* O157:H7 under acidic conditions. *Applied and Environmental Microbiology***61,382–385.**

21. Guraya, R., Frank, J.F. & Hassan, A.N.(1998). Effectiveness of salt, pH, and diacetyl as inhibitors for *Escherichia coli* O157:H7 in dairy foods stored at refrigerated temperatures. *Journal of Food Protection***61, 1098– 1102.**

22. Ogwaro, B. A., Gibson, H., Whitehead, M. and Hill, D. J. (2002). Survival of*Escherichia coli* O157:H7 in traditional African yoghurt fermentation. *International Journal of Food Microbiology***79,105– 112.**

23. Alhelfi, N. A., Lahmer, R. A., Jones, D. L.,and Williams, A. P. (2012). Survival and metabolic activity of lux-marked *Escherichia coli* O157:H7 in different types of milk. *Journal of Dairy Research***79, 257–261**.