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DEVELOPING AN EFFECTIVE THIN AGAR LAYER METHOD FOR THE DETECTION OF INJURED CELLS OF *Escherichia coli* IN MILK

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ABSTRACT

Injured cells of foodborne bacteria are generated by exposure to sub-lethal doses of food preservation factors. These cells maintain viability but loss resistance to selective agents in differential media used for their detection in foodstuffs, and could thus lead to false negative results. Injured cells can be reliably recovered using a 3-stage method that requires at least 4 days of lengthy culture. This study was therefore designed to develop a time and effort-effective protocol for detecting injured cells of *Escherichia coli* in milk. Heat-injured cells of *E. coli* could be generated in reconstituted skim milk (RSM) by exposure to 55 ºC for 50 min. Heat-injured cells of *E. coli* could be recovered using direct plating on the nonselective media of tryptone soy agar (TSA), tryptone glucose extract (TGE), and plate count agar (PCA) with TSA supplemented with 1% sodium pyrurvate (NaPyr) showing the highest recovery efficiency. None of 3 selective media of MacConkey agar (Mac), eosin methylene blue agar (EMB) and violet red bile agar (VRB) was able to recover heat-injured cells of *E. coli* from RSM. However, supplementing these selective media with 1% NaPyr allowed the detection of heat-injured cells of *E. coli* with limited recovery rates. Overlaying a thin layer of a nonselective agar over another layer of a selective medium (the thin agar layer method) improved the recovery of heat-injured cells from RSM, compared to recovery by selective media only. Supplemented TSA (TSA+) combined with EMB showed the highest recovery of injured cells from RSM, compared to other combinations of nonselective and selective media. The TSA+/EMB combination could also successfully recover heat-injured *E. coli* from pasteurized buffalo's milk with different fat contents. These results presented the thin agar layer method involving a combination of TSA+/EMB, as a time and effort-effective protocol for the detection of injured *E. coli* in milk used for preparing different dairy products.

Keywords: injured cells, *Escherichia coli*, selective media, thin agar layer method, milk, dairy products.

INTRODUCTION

Escherichia coli is a frequent contaminant of dairy products that has been described as a causative agent of quality defects such as early gas formation and off-flavors in cheese (Sheehan, 2011). *E. coli* also involves pathogenic strains that have been associated with several foodborne outbreaks of disease worldwide (Kaper *et al*., 2004). The detection of *E. coli* in food products is challenged by the emergence of injured cells of the bacterium, following exposure to sub-lethal doses of food preservation factors including heating, salting, and fermentation (Wu, 2008; Wesche *et al*., 2009). When injured, *E. coli* cells loss their original resistance to selective agents in differential media used for their detection in foods, which leads to false

negative results (Fricker, 1987; Mackey, 2000, Wesche *et al*., 2009). However, injured cells can repair their cellular damage and regain their ability to grow and proliferate when surrounding conditions are improved. Developing a reliable protocol for detecting injured cells of *E. coli* is thus critical to ensure the quality and safety of final food products.

Previous work in our laboratory has compared the efficacy of direct plating on a selective, differential medium with that of a 3-stage method to detect *E. coli* in 69 samples of dairy and milk-related products (Yassin *et al*., 2011). The 3-stage method showed higher efficiency in detecting *E. coli* in these products than direct plating. This was attributed to the ability of this method to detect injured cells. The 3-stage method involved a pre-enrichment step in a nutritious, non-selective medium, which allowed the resuscitation of injured cells into intact, healthy cells that could resist selective agents. The pre-enrichment stage was followed by enrichment in a selective broth to limit the growth of competing microflora, while allowing the resuscitated *E. coli* cells to grow. The enrichment culture was then plated on a selective differential agar for the development of typical *E. coli* colonies. These 3 stages required 3 days of culturing in different media, in addition to another 24 h for biochemical confirmation using miniaturized identification systems. This raised the need for developing more time and effort-effective methods for detecting injured cells of *E. coli* in milk and dairy products. The present study was thus designed to fulfill this objective by examining the efficacy of different culture media and procedures as to finally devise an effective protocol for detecting injured cells of *E. coli* in milk.

MATERIALS AND METHODS

Cultures and growth conditions

Seven isolates of *E. coli* recovered from dairy and milk-related products by Yassin *et al*. (2011) were used in this study. *E. coli* isolates were grown on tryptone soy agar (TSA) (Oxoid, Basingstoke, UK). Prior to use, each strain was inoculated into tryptone soy broth (TSB) (Oxoid), followed by incubation at 37ºC for 24 h.

Generation of heat-injured *E. coli* cells

A 24 h culture of each *E. coli* isolate was inoculated in TSB to give an initial number of approximately 10^7 cfu/ml followed by incubation for 3 h at 37 ºC to the mid to late exponential phase (Baylis *et al*., 2000). The resultant culture was used to inoculate sterilized reconstituted skim milk (RSM) (10% TS), pre-heated to 50 °C, 55 °C, and 60 °C to give an initial viable count of approximately 10^6-10^7 cfu/ml. Inoculated RSM cultures were then incubated at 50 °C, 55 °C, and 60 °C in a water bath. Samples were taken at intervals to assess the viable numbers of *E. coli* by plating serial dilutions of each sample in saline solution (0.85% NaCl) onto TSA, and MacConkey (Mac) agar (Oxoid), followed by incubation at 37 °C for 24 h.

Assessment of the ability of different nonselective and selective agar media to detect heat-injured cells of *E. coli* in milk

Samples of heat-injured cells of *E. coli* in RSM were serially diluted in saline solution (0.85% NaCl) and plated onto 3 nonselective agar media: tryptone soya agar (TSA), tryptone glucose extract (TGE) agar, and plate count agar (PCA) and 3 selective agar media: MacConkey (Mac) agar, eosin methylene blue (EMB) agar, and violet red bile (VRB) agar. All media were produced by Oxoid and were used with or without supplementation with 1% sodium pyruvate (NaPyr). Inoculated plates were counted after incubation at 37 °C for 24 h (McDonald *et al*., 1983).

Assessment of the efficacy of the thin agar layer (TAL) method to detect heat-injured *E. coli* in milk

The thin agar layer (TAL) method described by Kang and Fung (1999 & 2000) and Wu *et al*. (2001) was carried out as follows. Thirteen ml of one of 3 selective media (Mac, EMB, and VRB) were poured into sterilized Petri dishes and left for approximately 15 min to solidify. Then, 7 ml of one of 3 non-selective agar media (TSA, TGE, and PCA) were overlaid onto the surface of the pre-poured and solidified selective agar medium. This produced 9 combinations of selective and nonselective agar media namely: TSA/Mac, TSA/EMB, TSA/VRB, TGE/Mac, TGE/EMB, TGE/VRB, PCA/Mac, PCA/EMB, and PCA/VRB. These agar medium combinations were prepared a day before use. In another series of experiments, nonselective agar media were supplemented with 1% sodium pyruvate (NaPyr) and then used in combination with the 3 selective media for recovering injured cells. Samples of heat-injured cells of *E. coli* in RSM were serially diluted in saline solution (0.85% NaCl) and plated onto these different combinations of selective and nonselective agar media, followed by incubation 37 °C for 24 h.

Statistical Analysis

Each experiment was repeated at least three times and data were analyzed using analysis of variance (ANOVA) and Duncan multiple range test ($P < 0.05$).

RESULTS AND DISCUSSION

Generation of heat-injured cells of *E. coli* in milk

To generate injured cells of *E. coli*, the organism was exposed to different heating temperatures of 50 °C, 55 °C, and 60 °C for 80 min, and samples were taken to assess the viable bacterial counts on TSA as a non-selective medium and Mac agar as a selective, differential medium. Due to the presence of selective agents in the Mac agar, it does not recover injured cells, whereas TSA is a nutritious, non-selective agar that could resuscitate injured cells (Mackey 2000; Wu 2008). Comparing the viable counts of the organism on both media during exposure to heat could thus enable detecting the stage(s), at which cells were injured. The seven *E. coli* isolates examined in this work showed the same following results.

Subjecting *E. coli* to 50 °C in reconstituted skim milk (RSM) resulted in a slow decline in its viable numbers over 80 min (Figure 1A). The viable

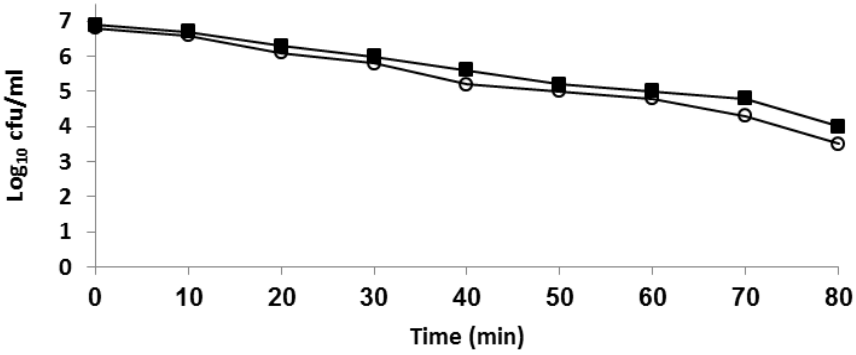
counts of the organism on TSA and Mac agar were very similar to each other at relevant observation times. This suggested that injured cells were not generated during this treatment. Heating *E. coli* at 55 °C was associated with a higher decline in viability, and different counts on TSA and Mac agar (Figure 1B). While the organism could be detected at viable counts of 5.2 log cfu/ml, 4.0 log cfu/ml and 3.1 log cfu/ml after 50 min, 60 min, and 70 min on TSA, respectively, no viable counts could be assessed on the Mac agar after the same corresponding times. Such differences in counts could reflect the generation of heat-injured cells after 50 min, 60 min, and 70 min of exposure to 55 °C. Heating at 60 °C was detrimental to the organism since it could not be detected on TSA or Mac agar after only 10 min of exposure to this temperature (Figure 1C). This did not allow the detection of any stages where *E. coli* was injured during this treatment.

These results showed that heat-injured cells of *E. coli* could be only generated by exposing cells to 55 °C for 50 min, 60 min or 70 min. Given that the organism had higher viable counts after 50 min of exposure to this treatment than 60 min or 70 min, this time was selected for generating injured cells in the next experiments.

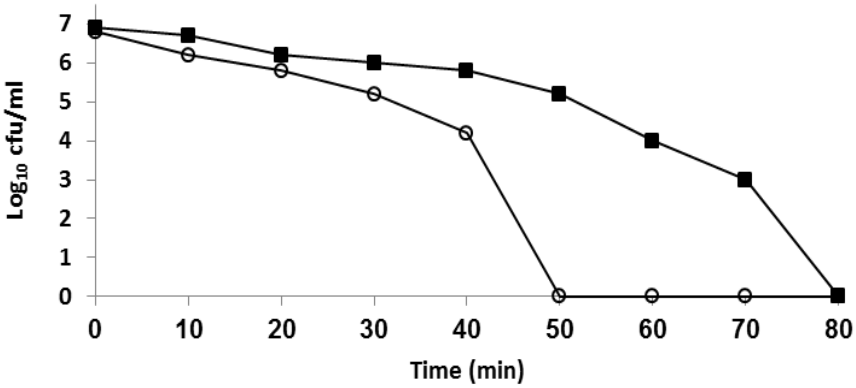
Detection of heat-injured *E. coli* in milk by different nonselective and selective agar media

The efficacy of different nonselective and selective agar media to recover heat-injured *E. coli* from milk was assessed. Heat-injured cells of *E. coli* were prepared in reconstituted skim milk (RSM) by exposure to 55 °C for 50 min. The resultant cells were then recovered by plating on 3 nonselective agar media; TSA, TGE and PCA and 3 selective agar media; Mac, EMB and VRB supplemented or not supplemented with 1% sodium pyruvate (NaPyr). Similar results were reported with the seven *E. coli* isolates examined in this study as follows.

It could be seen in Figure 2A that TSA with or without NaPyr supplementation had the highest ability to recover the injured cells of *E. coli* compared to TGE and PCA with or without 1% NaPyr ($P < 0.05$). However, it could be noted that the addition of 1% NaPyr resulted in improving the recovery of injured cells by each of the 3 nonselective media. This could be attributed to the involvement of sodium pyruvate in the degradation of hydrogen peroxide (H_2O_2), which is a metabolic by-product that inactivates injured cells and does not allow their detection on agar media (McDonald *et al*., 1983; Wu, 2008). Hydrogen peroxide is formed by incomplete reduction of oxygen during the respiration of bacterial cells, and can be toxic to injured cells since they may have reduced levels of catalase (Wu, 2008). Previous studies showed that sodium pyruvate increased the recovery of injured cells of other microorganisms including *Staphylococcus aureus*, *Salmonella* and *Cronobacter* (Baird-Parker & Davenport, 1965; Rayman *et al*., 1978; Al-Holy *et al*., 2008). The recovery of injured cells by the 3 nonselective media with or without NaPyr supplementation ranged from 3.5 log cfu/ml for PCA to 5.4 log cfu/ml for TSA + 1% NaPyr (Figure 2A). The order of these media according to their efficiency to recover injured cells was TSA > TGE > PCA. This applied to both the 1% NaPyr-supplemented or non-supplemented media.



A)



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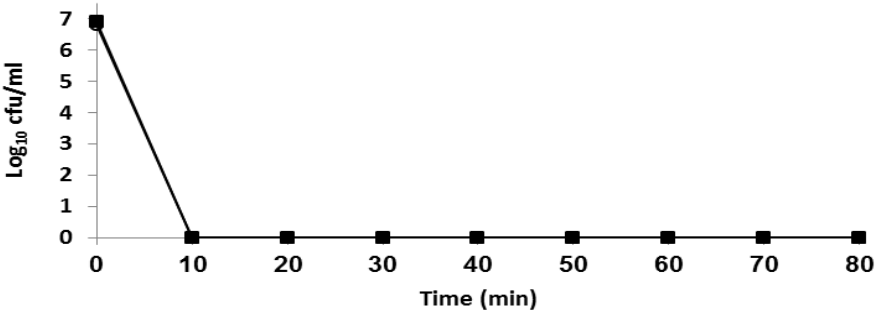


C)

Figure 1: Viability of *E. coli* during exposure to 50 °C (A), 55 °C (B) and 60 °C (C) for 80 min in reconstituted skim milk. Viable numbers were counted on TSA (■) and Mac agar (○).

Figure 2B presents the recovery of heat-injured *E. coli* in RSM using 3 selective agar media supplemented or not supplemented with 1% NaPyr. It could be seen that none of the examined non-supplemented, selective media could recover heat-injured cells. This could be attributed to the presence of selective agents in these media that are not tolerated by injured cells (Mackey, 2000; Wu, 2008). The addition of 1% NaPyr to these selective media could improve their ability to recover heat-injured cells. However, the recovery rates of these supplemented media were still low and ranged from 1.5 log cfu/ml for VRB + 1% NaPyr to 2.2 log cfu/ml for EMB + 1% NaPyr. These viable numbers were significantly lower than those recovered by the nonselective media described above ($P < 0.05$). The order of supplemented selective media according to their efficiency to recover injured cells was EMB > Mac > VRB.

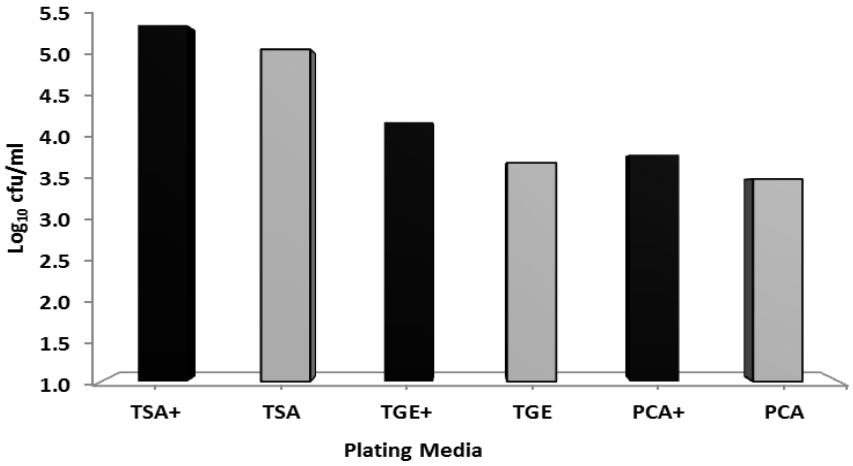
Detection of heat-injured *E. coli* in milk by the thin agar layer (TAL) method

The above results showed the high efficiency of nonselective agar media to recover heat-injured cells from milk. However, these media can not be relied on for detecting the organism in food samples since *E. coli* does not exist as pure cultures in food samples, but is normally mixed with other microorganisms. Plating food samples on nonselective media does not allow the inhibition of competing microorganisms, neither the differentiation of *E. coli* from other bacteria grown on the same plate. Therefore, further attempts have been made to develop a thin agar layer method (TAL) combining a selective and nonselective agar media for the detection of heat-injured *E. coli* in milk.

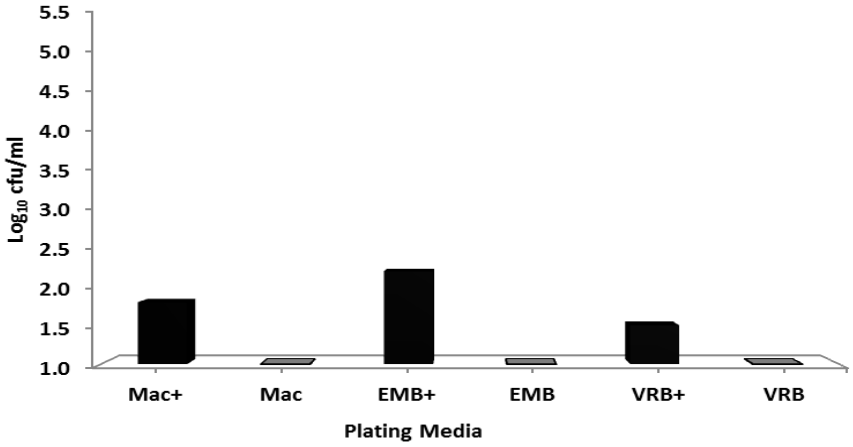
The TAL method involved overlaying a relatively thin layer of nonselective medium onto a pre-poured, selective agar (Wzu *et al*., 2001). The three nonselective agar media (TSA, TGE and PCA) supplemented or not supplemented with 1% NaPyr were overlaid in thin layers over each of the three selective agar media (Mac, EMB and VRB) pre-poured in Petri dishes. The resultant combinations of media were then evaluated for their ability to recover heat-injured cells of *E. coli* from reconstituted skim milk (RSM). Similar results were reported with the seven *E. coli* isolates examined in this study as follows.

As shown in Figure 3A, B and C, the use of the TAL method has generally improved the recovery of heat-injured cells of *E. coli* from RSM compared to the use of only selective media supplemented with 1% NaPyr without a thin layer of a non-selective agar (see Figure 2B). Figure 3A shows that the order of the efficiency of the different combinations of TSA with the 3 selective media was TSA/EMB > TSA/Mac > TSA/VRB. This applied to both the un-supplemented and supplemented form of the TSA medium. The same order of efficiency was also reported with the other 2 nonselective media; TGE and PCA in their combinations with the 3 selective media (Figure 3 B & C). These results agreed with the order of efficiency of the 3 selective media presented above in Figure 2B. The highest recovery of injured cells from RSM could be achieved using a combination of supplemented TSA (TSA+)

and EMB, where 4.5 log cfu/ml of heat-injured cells of *E. coli* could be detected (Figure 3A).

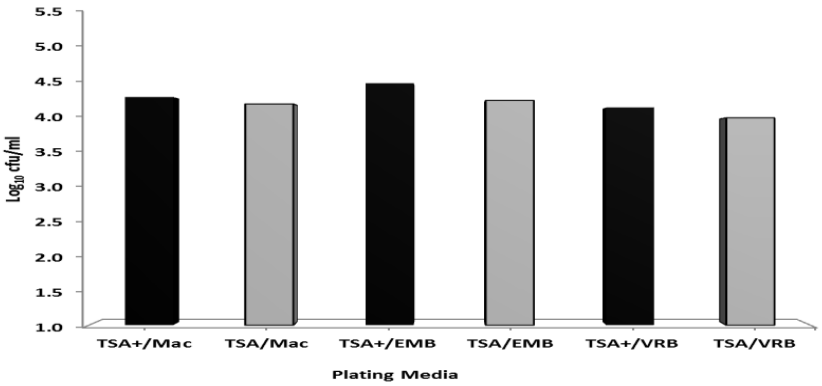


A)



B)

Figure 2: Recovery of heat-injured cells of *E. coli* from RSM using 3 nonselective agar media (A), and 3 selective agar media (B). Each medium was used with and without supplementation with 1% sodium pyruvate (NaPyr). The "+" sign refers to supplementation with 1% NaPyr.



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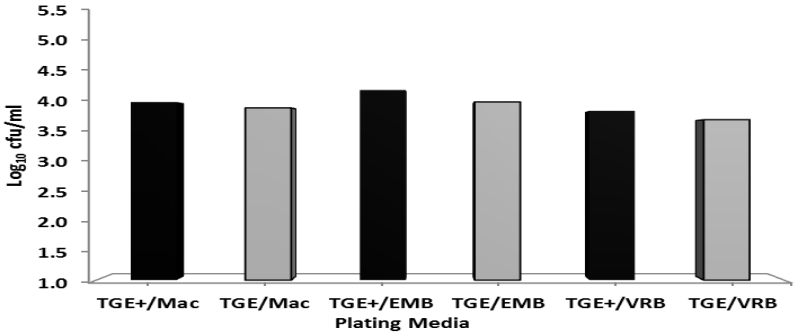

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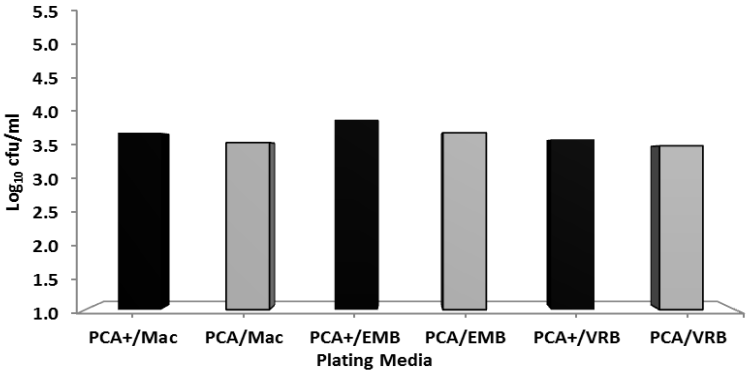

C)

Figure 3: Recovery of heat-injured cells of *E. coli* from RSM using the thin agar layer method involving different combinations between TSA (A), TGE (B), or PCA (C) and 3 selective agar media including Mac, EMB, and VRB. The "+" sign refers to supplementation with 1% NaPyr.

Whereas, the lowest recovery of 3.5 log cfu/ml of injured cells was reported with a combination between un-supplemented PCA and VRB (Figure 3C). However, this low recovery of injured cells was still higher than that observed with any of the supplemented selective media (see Figure 2B). The use of these supplemented selective media in combination with the non-selective media did not improve the recovery rates of the combinations presented in Figure 3 (*data not shown*).

Given the highest recovery of heat-injured cells from RSM by the TSA+/EMB combination, it could be recommended for use as an alternative to the lengthy 3-stage method for detecting injured cells of *E. coli* in milk. Using the TSA+/EMB combination of media prepared by the TAL method allows heat-injured cells to be resuscitated by the TSA nonselective thin layer within the first few hours of incubation (Wu, 2008). The EMB selective agar in the TAL combination inhibits other bacteria competing with *E. coli*, but does not affect the resuscitated cells of the targeted organism. The EMB medium also allows the differentiation of *E. coli* from other bacterial species that could grow on the TSA nonselective layer. In agreement with the present study, the TAL method was successfully used for the recovery of injured cells of *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Campylobacter coli* from food samples (Chang *et al*., 2003). Yuste and Fung (2003) also reported the efficiency of the TAL method to recover injured cells of *S*. Typhimurium, *Staphylococcus aureus* and *Yersinia enterocolitica* from apple juice.

Recovery of heat-injured *E. coli* from pasteurized milk with different fat contents using the TAL method

Given that the TSA+/EMB combination was successfully used to detect injured cells of *E. coli* in reconstituted skim milk, its efficacy was further examined to detect heat-injured cells of the microorganism in buffalo's milk containing different fat levels. This was to ensure the applicability of this method to dairy industry. It could be seen in Figure 4 that the TAL method involving the TSA+/EMB combination of media was successful in recovering heat-injured *E. coli* from pasteurized buffalo's milk containing 0.1%, 2%, 4%, and 6% fat.There were no significant differences in the recovery rates between milk samples with different fat contents. This suggested that the detection efficiency of the TSA+/EMB combination was not affected by the fat level of milk. Taken together, these results confirmed the applicability of the TAL method involving a combination of TSA+/EMB for detecting heat-injured cells in milk used for preparing different dairy products.

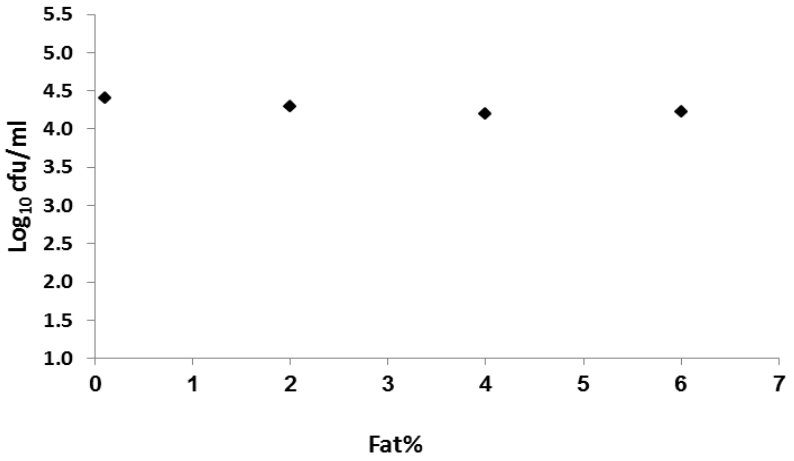

Figure 4: Recovery of heat-injured cells of *E. coli* from pasteurized buffalo's milk adjusted to different fat levels with the use of the TAL method employing a combination of the TSA+/EMB media.

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تطوير طريقة "طبقة أجار رقيقة" فعالة للكشف عن الخلايا المجروحة من الإيشريشيا كولاي في اللبن

وليد محمود الشارود* و محمد محمد زين الدين و مني أحمد يسن ***

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تنتج الخلايا المجروحة للميكروبات المرتبطة بالأغذية عند التعرض لجرعات تحت مميتة من عوامل حفظ الأغذية. وتستطيع هذه الخلايا الإحتفاظ بحيويتها ولكنها تفقد مقاومتها للعوامل الانتخابية الموجودة في البيئات التفريقية المستخدمة في الكشف عن الميكروبات في الأغذية، مما يؤدي إلي نتائج سلبية كاذبة. يمكن الكشف عن الخلايا المجروحة بدرجة موثوق بها وذلك بإستخدام طريقة مكونة من ثلاثة مراحل ولكنها تستغرق أربعة أيام علي الأقل من التنمية المزرعية الطويلة. ولذلك فقد تم تصميم هذه الدراسة لتطوير طريقة فعالة من حيث الوقت والجهد للكشف عن الخلايا المجروحة من ميكروب الإيشريشيا كولاي في اللبن. تم إنتاج خلايا مجروحة من الميكروب في اللبن الفرز المسترجع وذلك بالتسخين علي ٥٥ °م لمدة ٥٠ دقيقة. أمكن الكشف عن الخلايا المجروحة بالحرارة من الإيشريشيا كولاي بإستخدام طريقة التلقيح المباشر علي أطباق تحتوي علي بيئات غير متخصصة وهي TSA و TGE و PCA بحيث كان أفضل معدل لتقدير الخلايا المجروحة علي أطباق بيئة TSA المدعمة ببيروفات الصوديوم (١%). لم تستطع أي من البيئات الثلاثة المتخصصة والتي تم اختبارها في هذه الدراسة وهي Mac و EMB و VRB أن تقوم بالكشف عن خلايا الإيشريشيا كولاي المجروحة بواسطة الحرارة في اللبن الفرز المسترجع. إلا أن تدعيم هذه البيئات المتخصصة ببيروفات الصوديوم (١%) أدي إلي قدرتها علي الكشف عن الخلايا المجروحة ولكن بمعدلات محدودة. وُجد أن إضافة طبقة رقيقة من إحدي البيئات الغير متخصصة إلي طبقة أخري من بيئة متخصصة سبق صبها في طبق بتري – وهذا ما يعرف بطريقة "طبقة الأجار الرقيقة" – قد أدي إلي تحسن عملية الكشف عن خلايا الإيشريشيا كولاي المجروحة بالحرارة، وذلك بالمقارنة بقدرة البيئة المتخصصة بمفردها علي الكشف عن الخلايا المجرحة. أدي إستخدام بيئة TSA المدعمة ببيروفات الصوديوم (بيئة غير متخصصة) مع بيئة EMB (بيئة متخصصة) إلي الوصول إلي أعلي كفاءة ممكنة لطريقة "طبقة الأجار الرقيقة" وذلك للكشف عن الخلايا المجروحة من الايشريشيا كولاي بواسطة الحرارة. كذلك فقد أمكن بإستخدام هذه التوليفة من هاتين البيئتين الكشف عن الخلايا المجروحة من الميكروب في اللبن الجاموسي المبستر والمحتوي علي نسب مختلفة من الدهن. وبالتالي فإنه يمكن التوصية بإستخدام توليفة من بيئة TSA المدعمة ببيروفات الصوديوم مع بيئة EMB والتي يتم تحضيرها بطريقة طبقة الأجار الرقيقة كإختبار فعال من حيث الوقت والجهد للكشف عن الخلايا المجروحة من الإيشريشيا كولاي في اللبن المستخدم في صناعة المنتجات اللبنية المختلفة.