# การหาสภาวะที่เหมาะสมในขั้นตอนการเพิ่มจำนวนเพื่อปรับปรุงการคัดเลือกเชื้อลิสทีเรีย

# MEDIA OPTIMIZATION OF ENRICHMENT PROTOCOL TO IMPROVE LISTERIA SELECTIVITY

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## บทคัดย่อ

งานวิจัยนี้เป็นการศึกษาการเจริญเติบโตและการคัดเลือกเชื้อลิสทีเรียอินโนคัวในขั้นตอนการเพิ่มจำนวนแบบคัดเลือก เพื่อสร้างความรู้พื้นฐานในการเพิ่มประสิทธิภาพให้กับขั้นตอนการตรวจเชื้อลิสทีเรีย ทั้งนี้ใช้เชื้อแอลอินโนคัวซึ่งเป็นเชื้อไม่ก่อโรคเป็นเชื้อทดสอบ เนื่องจากเชื้อแอลอินโนคัวมีลักษณะทางสรีรวิทยาใกล้เคียงกับเชื้อลิสทีเรียโมโนไซโตจีเนส สำหรับการทดลองได้ทำการเปรียบเทียบการเจริญของเชื้อแอลอินโนคัวบนอาหารเลี้ยงเชื้อเหลวที่ใช้กันอยู่ทั่วไป ได้แก่ ทีเอสบี โดยในขั้นตอนการเพิ่มจำนวนแบบไม่คัดเลือกของเชื้อแอลอินโนคัวร่วมกับการศึกษาผลของสารยับยั้งสำหรับการตรวจพบเชื้อแอลอินโนคัว การศึกษาผลของสารยับยั้งสำหรับการตรวจพบเชื้อลิสทีเรีย สารยับยั้งที่แนะนำให้ใช้ในการตรวจเชื้อตามวิธีมาตรฐานทั่วไป ได้แก่ เอ็นจีเอฟไอเอส ไอดีเอฟ ยูเอส เอฟดีเอ เอ็นเอ็มเคแอล ไอเอสโอ เอโอเอซี และยูเอสดีเอ-เอฟเอสไอเอส ทำการทดลองโดยเติมสารคัดเลือกสำหรับเชื้อลิสทีเรียที่ใช้ทั่วไปในอาหารเลี้ยงเชื้อเหลว ทีเอสบี เพื่อศึกษาการเจริญและการคัดเลือกเชื้อลิสทีเรีย ผลการวิจัยจากกราฟการเจริญของเชื้อพบว่า อคริฟลาวีน มีผลต่อการเจริญของเชื้อแบคทีเรียแกรมบวกอย่างมีนัยสำคัญ เชื้อแอลอินโนคัว สามารถทนต่ออคริฟลาวีน ได้น้อยกว่าเชื้อสแต็ปฟีโลคอกคัสออเรียส ในทางตรงกันข้าม โพลีมัยซิน บี มีประสิทธิภาพในการยับยั้งเชื้อแบคทีเรียได้ทั้งแกรมลบและแกรมบวก แต่ยับยั้งเชื้อแบคทีเรียแกรมบวกได้น้อยกว่า โดยโพลีมัยซิน บี ที่ความเข้มข้น 10 มิลลิกรัม/ลิตร

ซึ่งเป็นความเข้มข้นต่ำที่สุดในการศึกษา มีประสิทธิภาพในการยับยั้งเชื้อ อี โคไล จาก 6 ล็อก ซีเอฟยู/มิลลิลิตร ให้ลดลงเหลือน้อยกว่า 2 ล็อก ซีเอฟยู/มิลลิลิตร ภายในระยะเวลาการบ่ม 2 ชั่วโมง กรดนาลิซิดิกมีประสิทธิภาพในการยับยั้งแบคทีเรียแกรมลบเท่านั้น และมีประสิทธิภาพน้อยกว่าโพลีมัยซิน บี ในกรณีของลิเธียมคลอไรด์ ถึงแม้จะไม่มีผลในการยับยั้งเชื้อจุลชีพเป้าหมาย แต่ยังคงใช้ลิเธียมคลอไรด์ร่วมในการทดลองด้วย ในส่วนของการหาสภาวะที่เหมาะสมของส่วนผสมสารคัดเลือกทำการทดลองโดยการวิเคราะห์พื้นที่ผิวตอบสนองและสรุปได้ว่าส่วนผสมของสารคัดเลือกที่เหมาะสมสำหรับการแยกเชื้อ อี โคไล และเชื้อเอสออเรียส ออกจากเชื้อแอลอินโนคัว คือ การใช้อคริฟลาวีน 5.7 มิลลิกรัม/ลิตร โพลีมัยซิน บี 10 มิลลิกรัม/ลิตร ลิเธียมคลอไรด์ 20.7 มิลลิกรัม/ลิตร

**คำสำคัญ:** การคัดเลือกเชื้อลิสทีเรีย ลิสทีเรีย อินโนคัว การเพิ่มจำนวน สารยับยั้ง

## Abstract

*Listeria innocua* growth and selectivity during selective enrichment step were studied to formulate fundamental knowledge to enhance *Listeria* spp. detection protocol. *L. innocua* was used in this study as a non-pathogenic *Listeria* model because it shares similar physiological traits with *Listeria monocytogenes*. TSB, an effective non-selective enrichment medium, was selected to enhance the growth of *L. innocua* in the selective enrichment step. The effects of conventional inhibitors were studied by using common selective inhibitors suggested in most global microbiological detection standards (i.e., NGFIS, IDF, USFDA, NMKL, ISO, AOAC, and USDA-FSIS). The TSB broth base was modified by adding some conventional selective agents to study the growth and selectivity of *Listeria* spp. The growth profiles showed that acriflavine significantly affected the growth of Gram-positive bacteria. Polymyxin B inhibited Gram-negative bacteria as well as Gram-positive bacteria but to a lesser extent. As low as 10 mg/L of Polymyxin enabled effective reduction of *E. coli* from 6 to less than 2 log CFU/mL within 2 h of incubation. Nalidixic acid only suppressed Gram-negative bacteria and was not as effective as Polymyxin B. Although lithium chloride did not significantly affect the inhibition of any target microorganisms. Response surface analysis indicated that the optimum selective agents included 5.7 mg/L of acriflavine, 10.0 mg/L of Polymyxin B and 20.7 g/L of lithium chloride. This new media formula was proved to isolate *E. coli* and *S. aureus* from *L. innocua*.

**Keywords:** Listeria Selectivity, *Listeria innocua,* Enrichment Media, Selective Agents

## Introduction

There are numerous worldwide incidences of food borne illnesses. All of these numbers combined were caused from more than two hundred fifty different food borne pathogens [1]. For bacteria, there are five main species that contribute to all of these food borne outbreaks, starting with *Salmonella* spp., *Clostridium perfringens*, *Campylobacter*, *Escherichia coli*, and *Listeria monocytogenes* [2]. In all of these bacteria, *Listeria* spp. inflicted the highest mortality, more than sixteen percent of hospitalized cases resulted in death. The reported listeria outbreaks showed that the main causes were from delay of detection and false negativity [3-5]. These outbreaks can bring about serious economic and health damages. So to prevent these unfortunate situations, the isolation and detection of *L. monocytogenes* contamination were investigated. The idea is to identify the target microbe at the earlier stage of production as possible. The same conventional method to isolate *Listeria* spp. that recommended by international organization is composed of primary and secondary selective enrichment and selective agar plating. For the selective enrichment step, different organizations used very types and sequences of selective media to select *Listeria* spp. [6-8].

The selective agents in *Listeria* selective media collectively are acriflavine, nalidixic acid, lithium chloride, Polymyxin B, ceftazidime, colistin sulphate, cefotetan, fosfomycin, and amphotericin B. Polymyxin B, nalidixic acid, colistin sulphate and ceftazidime mostly inhibited Gram negative bacteria. However, there was a report that colistin sulphate partially suppressed the growth of *Listeria* [9]. Acriflavine and fosfomycin mainly suppress the growth of Gram positive bacteria and also affected the growth of *Listeria* [9-8]. Beumer and others [10] revealed that acriflavine had an impact on *Listeria* growth by increasing lag time of *Listeria.* Cefotetan is a broad spectrum antibiotic. It mostly inhibits cell wall synthesis of Gram negative bacteria [11]. Amphotericin B prevents the growth of yeast and mold by inhibit protein synthesis [10]. The multiplicity and redundancy of available standard media for isolation suggest a lack of consensus for an adequate, effective substrate [12]. It is important to refine the *Listeria* selective media because of the intrinsic probability of false negative results and the increasing frequency of human listeriosis outbreaks reported all over the world [13]. In this study, *L. innocua* was used as a *Listeria* model because it provides similar physiological traits with *L. monocytogenes* and under the digital microscope, colony size of *L. monocytogenes* and *L. innocua* on the selective agar were similar [14]. In addition, the initial cell population of *L. monocytogenes* and *L. innocua* on the three selective media with a regular (100%) inhibitor concentration had to exceed 105 CFU/mL to be detectable in 24 h. The growth of these two *Listeria* species at 109 CFU/ml on selective media (ALOA, PALCAM, and Oxford) was not significantly different from their growth on a non-selective medium (TSA) [15].

## Objectives

This research also emphasized on critical compositions of inhibitors in the conventional selective media. Improper concentration of selective agents would be able to affect the growth of *Listeria* spp. in food samples. Interestingly, different selective media composed with unique combination of selective agents. So the interaction of each selective agent on the growth and selectivity of *Listeria* strain was investigated to optimize the combination of selective enrichment medium.

## Methods

### Strain Preparation

*L. innocua* DMST 9011 and other competing bacteria (*E. coli* DMST 4609 and *S. aureus* TISTR 808) were obtained from either the Department of Medical Sciences Thailand (DMST, Bangkok, Thailand) and Thailand Institute of Scientific and Technological Research (TISTR, Bangkok, Thailand). In this study, the experiment used *L. innocua* DMST 9011 instead of *L. monocytogenes,* due to its non-pathogenic properties. Moreover, this strain has the most related physiological properties to *L. monocytogenes*, and both strains can be found in the same food products [16]. All pure cultures were multiplied on tryptic soy agar (TSA, Lab M, UK). One loopful of each strain was transferred into 100 ml of tryptic soy broth (TSB, Lab M, UK) and incubated at 35°C (200 rpm, 24 h). All strains were incubated to reach around $10^9$-$10^{10}$ CFU/mL. This initial cell was used to examine the interaction of conventional inhibitors on growth and selectivity of *Listeria* spp. For the investigation of an efficient of selective enrichment protocol ,all strains were diluted to the proper dilution around $10^2$-$10^3$ CFU/mL and use as the initial cell.

### Examination of the Interaction of Conventional Inhibitors on Growth and Selectivity of Listeria spp.

The broth base TSB was added the conventional selective agar by varying type and concentration if inhibitor. One hundred twenty microliter of desired *L. innocua*, *E. coli,* and *S. aureus* concentration was transferred into 1.2 mL of different treatment medium from each condition to 96 deep well plate then they were mixed by auto pipette. These samples were analyzed 10 times: 0, 2, 4, 6, 8, 10, 12, 16, 20, and 24 h.

### Optimization the Combination of the Effective Inhibitors

*L. innocua*, *E. coli,* and *S. aureus* were used to investigate the optimum combination of inhibitors. They were prepared in TSB to reach 9 log CFU/mL in shake flasks. One hundred twenty microliters of each culture was inoculated into 1.2 mL of media that contained different formulas according to Table 1 in 96 deep well plate. Selective agents that were chosen consisted acriflavine (5 and 10 mg/L), Polymyxin B (10 and 25 mg/L) and lithium chloride (20 and 40 g/L). The experimental design and analysis of obtained data which was the initial and the final cell count of microorganisms were performed using Minitab software. The test samples was incubated at 35°C for 24 h to study the growth kinetics.

Total colony forming units were detected on the media surface of 96-micro well lids.

Box-hehnken design was used to design the experiment to reduce the number of experiment from 20 experiments of Central Composite Design to 15 experiments of Box-behnken design. The initial and final cell of *L. innocua*, *E. coli,* and *S. aureus* which incubated in TSB that contained different formulas of inhibitors of each experiment was used to compare the inhibitory effect. The responses were analyzed by Minitab software to determine the optimum combination of the effective inhibitors. Table 1 showed media that contained different formulas and inhibitory effect of *L. innocua*, *E. coli,* and *S. aureus* from experimental design.

**Table 1** The media that contained different formulas and inhibitory effect of *L. innocua*, *E. coli*, and *S. aureus* from Box-behnken design

| Run Order | Acriflavine (mg/L) | Polymyxin B (mg/L) | Lithium Chloride (g/L) | Percent of Inhibition | | |
|---|---|---|---|---|---|---|
| | | | | *L. innocua* | *E. coli* | *S. aureus* |
| 1 | 7.5 | 17.5 | 20 | -3.03 | 100 | -2.48 |
| 2 | 7.5 | 17.5 | 30 | 1.44 | 100 | -1.48 |
| 3 | 5 | 25 | 30 | 4.32 | 100 | -0.56 |
| 4 | 5 | 17.5 | 20 | 1.71 | 100 | 3.17 |
| 5 | 5 | 17.5 | 40 | 1.13 | 100 | -2.39 |
| 6 | 10 | 25 | 30 | -0.23 | 100 | -5.70 |
| 7 | 7.5 | 10 | 20 | -1.47 | 100 | -0.09 |
| 8 | 10 | 10 | 30 | 4.40 | 100 | 4.46 |
| 9 | 7.5 | 25 | 20 | 0.98 | 100 | 1.97 |
| 10 | 7.5 | 10 | 40 | 0.64 | 100 | 3.70 |
| 11 | 5 | 10 | 30 | 0.43 | 100 | 2.11 |
| 12 | 7.5 | 25 | 40 | 1.06 | 100 | -0.84 |
| 13 | 7.5 | 17.5 | 30 | -3.62 | 100 | -6.40 |
| 14 | 10 | 17.5 | 40 | 3.35 | 100 | 1.78 |
| 15 | 10 | 17.5 | 20 | 4.01 | 100 | -12.38 |

The inhibitory effects were explored by using the number of initial cell count minus the number of final cell count and transforming it into the percent inhibitory effect.

Percent of inhibition = (Initial cell count (log CFU/ml) – (Final cell count (log CFU/ml))/ (Initial cell count (log CFU/ml)) * 100

**Cell Enumeration**

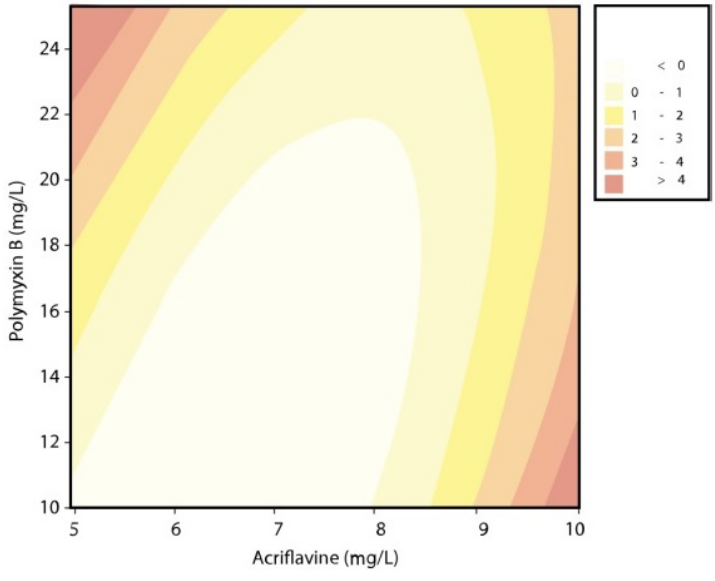
Initial cell number of each lot of recovered inoculums had been determined. Enumeration method was followed standard plate count (SPC), spread plate method. Diluted inoculums at dilution $10^{-2}$ and $10^{-3}$ were mixed and transferred 10 µL aliquots onto surface of separated; duplicate Tryticase Soy Agar (TSA) on 96-microwell plate. Then the samples were incubated at 35°C for 24 h.

**Statistical Analysis**

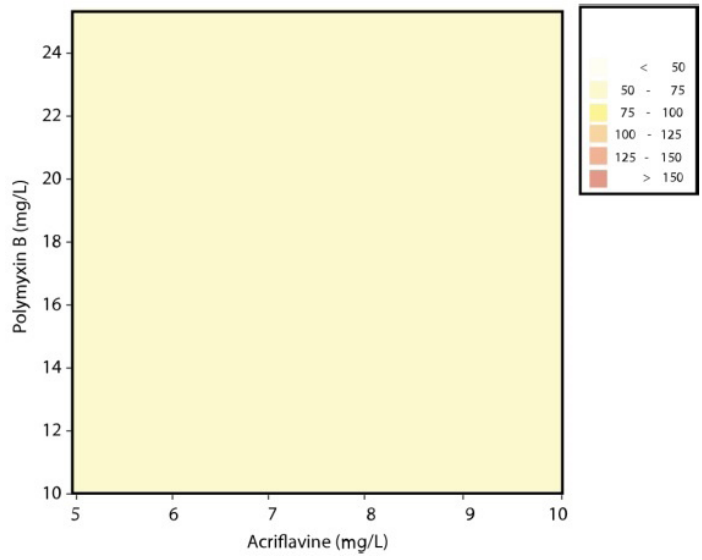
ANOVA was also used to determine the difference in inhibitory effect of Listeria detection on the three selective media at 24 h incubation. All statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Significant difference was designated at $P < 0.05$.

## Results

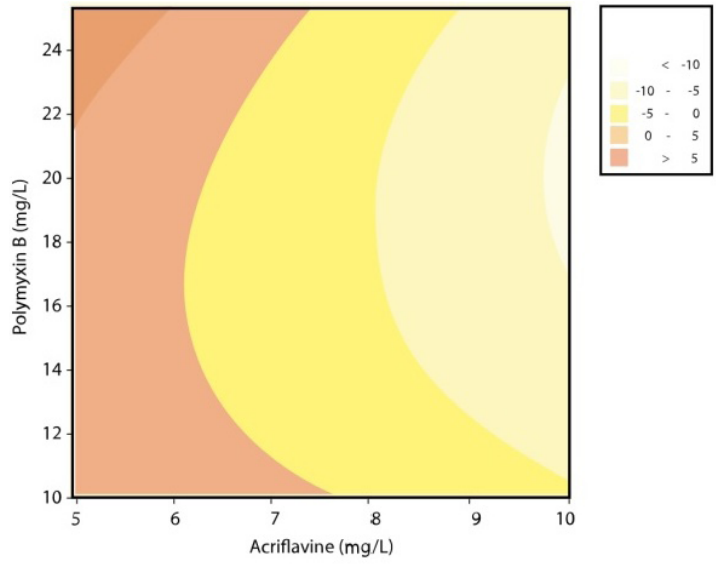
The results from experiment were presented in a contour plot as shown in Figure 1 – 3. Figure 1a showed inhibitory effect of acriflavine and Polymyxin B on *L. innocua* when the concentration of lithium chloride was stabilized. *L. innocua* was able to survive at the concentration of 8 mg/L acriflavine and 21 mg/L Polymyxin B. Interestingly *L. innocua* was inhibited when the concentration of each selective agent was at the high level. *E. coli* was totally suppressed in all concentrations at the 24 h. For *S. aureus*, the high inhibitory effect occurred at the high level of Polymyxin B and low level of acriflavine.




(a) *L. innocua*



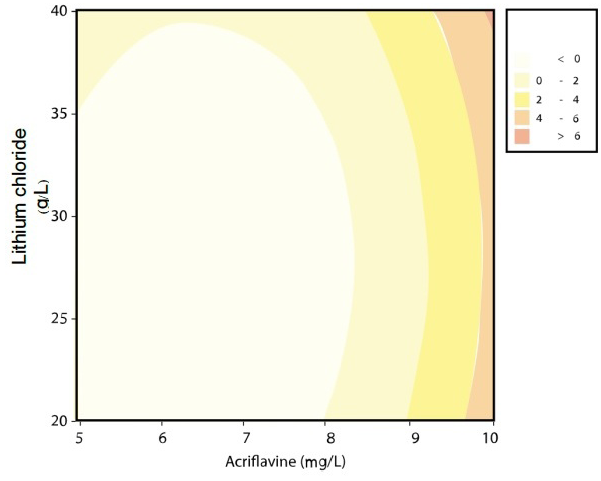

(b) *E. coli*



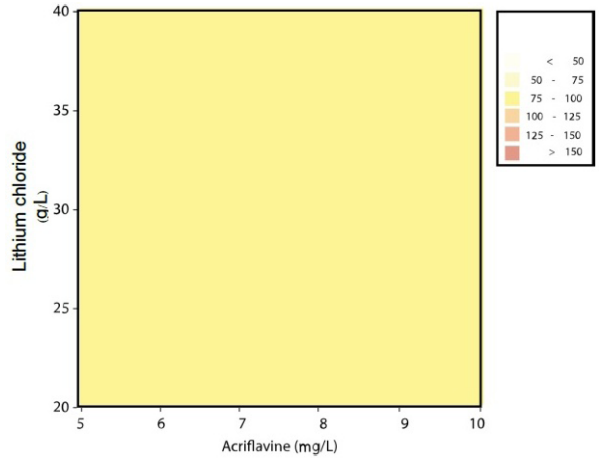

(c) *S. aureus*

**Figure 1** Inhibitory effects of acriflavine and Polymyxin B on a) *L. innocua*, b) *E. coli* and c) *S. aureus*

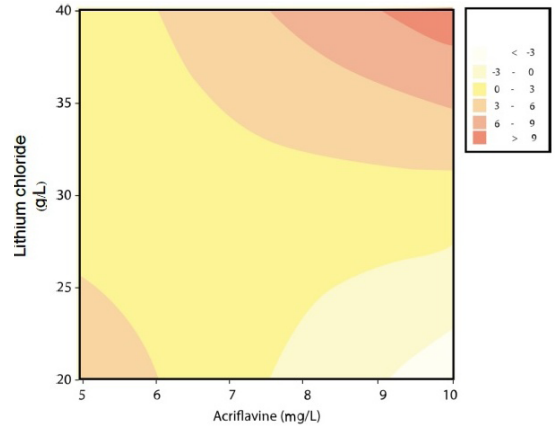
At the lowest concentration of Polymyxin B, the effect of combination using different levels of acriflavine and lithium chloride on the growth of target microoraganisms was studied (Figure 2).




(a) *L. innocua*



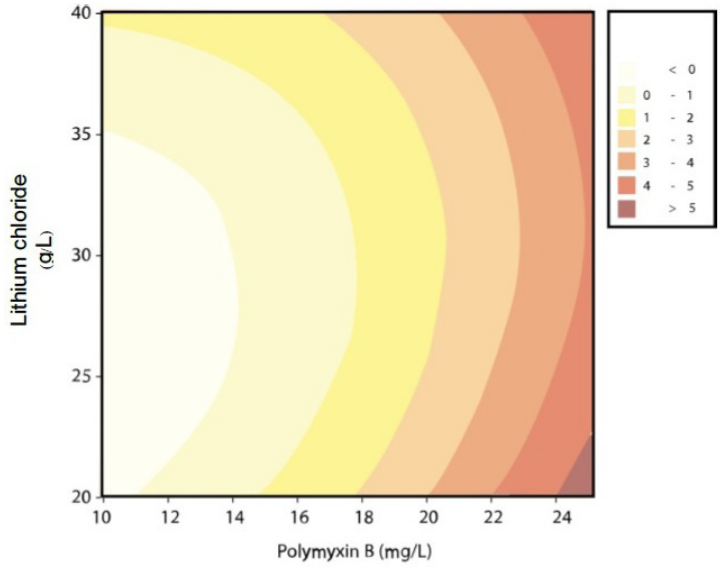

(b) *E. coli*



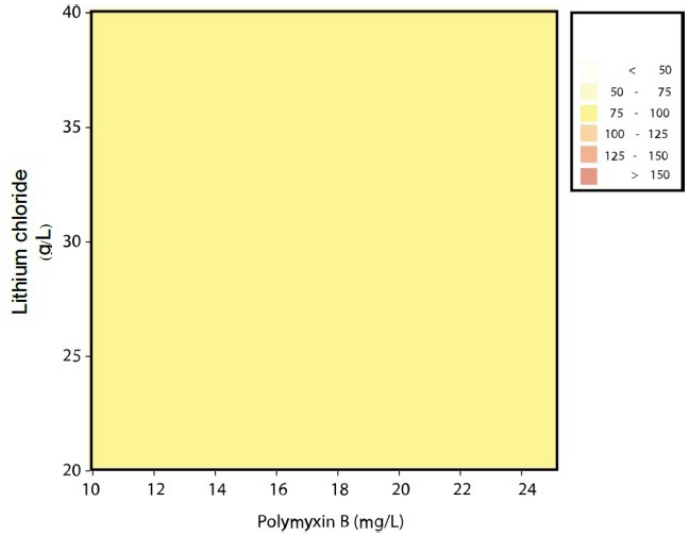

(c) *S. aureus*

**Figure 2** Inhibitory effects of acriflavine and lithium chloride on a) *L. innocua*, b) *E. coli* and c) *S. aureus*

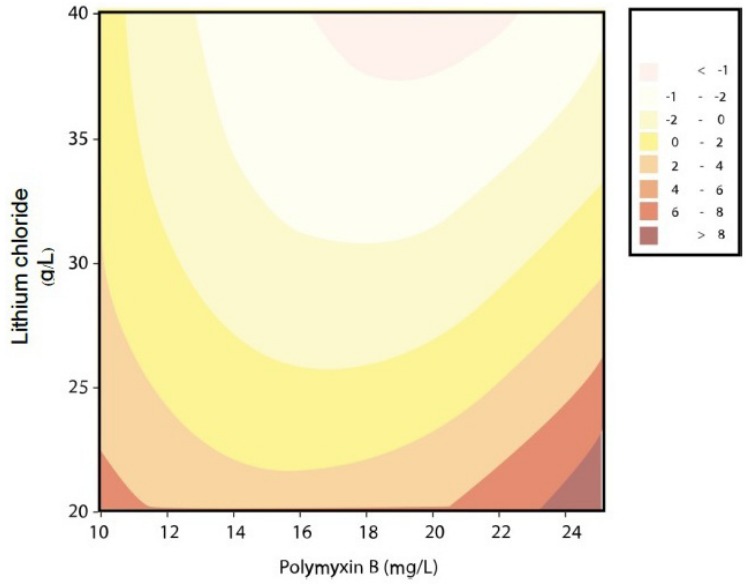
*L. innocua* was not affected by the increase of lithium chloride quantity. The inhibitory effect of *L. innocua* was increased with using more concentration of acriflavine. Although *E. coli* was cultured in the lowest Polymyxin B, the bacteria was totally repressed at 24 h. At low level of lithium chloride and high level of acriflavine, the inhibitory effect of *S. aureus* could not detect. The inhibitory effect of *S. aureus* was higher than 9 log CFU/mL when the cells were applied to the media contained the high levels of both lithium chloride and acriflavine. At the lowest acriflavine concentration, different levels of lithium chloride and Polymyxin B acted on the growth of each microorganism in different ways (Figure 3).




(a) *L. innocua*




(b) *E. coli*




(c) *S. aureus*

**Figure 3** Inhibitory effects of Polymyxin B and lithium chloride on a) *L. innocua*, b) *E. coli* and c) *S. aureus*.

Polymyxin B was effective in restraining the expansion of *L. inoccua*, while increasing of lithium chloride hardly effected on *L. innocua*. Again, *E. coli* was terminated in all treatments in these experiments. Interestingly, the quantity of lithium chloride added in the treatment was very effective when considered the use with combination of Polymyxin B. The increase of the concentration of lithium chloride decreased the inhibitory level of *S. aureus*. In addition, the highest level of inhibitory effect was at the highest concentration of Polymyxin B and lowest level of lithium chloride.

## Conclusions and Discussion

These results in Figure 1a-c indicated that the use of acriflavine together with Polymyxin B resulted in the decrease of inhibitory effect. In these media samples, acriflavine was able to bind to protein and Polymyxin B, which is a highly-active polypeptide and course the reduction of Polymyxin B inhibitory effect [10].

The contour plot revealed that when the inhibitors were mixed together, the concentration of each inhibitor affected the inhibitory activity of each target microorganism. Thus, response optimizer tool in Minitab software was used to investigate the optimum inhibitor combination. The optimization was carried out by defining minimum inhibitory effect for *L. innocua*, maximum inhibitory effect for *E. coli* and *S. aureus*. The analysis revealed that the optimum combination including 5.7 mg/L acriflavine, 10 mg/L Polymyxin B and 20.7 g/L lithium chloride. The predicted

inhibitory effect of microorganisms was as follows; *L. innocua* = -1.00, *E. coli* = 100.00 and *S. aureus* = 3.50. The composite desirability was 0.52. This combination was similar to the formula of Palcam Broth that contained 5 mg/L acriflavine and 10 mg/L Polymyxin B except for 10 g/L lithium chloride. The optimization of the selective agents combination indicated that the use of each inhibitor affected the growth and selectivity of each microorganism differently. The concentration of Polymyxin B influenced the efficiency of acriflavine. Besides, lithium chloride concentration affected the growth of *S. aureus* when used in conjunction with Polymyxin B. Finally, the optimum combination that obtained from the experimental design is similar to the combination of Palcam broth.

The use of alternative supplement agents in the selective enrichment step by modifying the TSB with the optimum selective agents included 5.7 mg/L of acriflavine, 10.0 mg/L of Polymyxin B and 20.7 g/L of lithium chloride, instead of imported commercial media could potentially reduce the cost of laboratory analysis. In addition, our protocol mostly eliminated false negative results by applying cultivation media with only mild usage of inhibitory agents. These are the highlights and beauty of our protocol to solve the industrial problem. This feature is a practical advantage when applying to a large volume of food samples.

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