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Viability of Gram-Positive and Gram-Negative Bacteria in Liquid Storage Media

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Abstract
Bacteria can be cultivated and stored in media. In educational laboratories, liquid media are used to maintain stock cultures due to simplicity and cost-effectiveness. Maintaining bacterial viability is essential to ensure bacterial stability. Several factors influence bacterial survival, including temperature storage, duration, nutrient composition, osmotic pressure, pH, and sterility. Previous studies have applied glycerol supplementation in Tryptic Soy Broth as a preservative to improve bacterial viability. This study to observe bacterial survival in two types of liquid Bouillon and Tryptic Soy Broth (TSB)—with storage durations of one week, two weeks, and one month. The spread plate method used "L" glass rod on Nutrient Agar, with four treatment groups and replications. The results showed a significant increase numbers of bacteria in TSB after two weeks of storage, followed by a sharp decrease after one month. Showed that TSB was better at maintaining the viability of Gram-positive and Gram-negative bacteria.

Keywords:
Bouillon; Gram-positive; Gram-negative; Tryptic Soy Broth

INTRODUCTION

Bacterial culture preservation is essential for maintaining working stock cultures in educational laboratories to support practical bacteriology learning (1). Short-term and long-term preservation of bacterial cultures requires appropriate storage media (2). Storage media function to maintain bacterial viability, and storage conditions are influenced by the type of medium, bacterial age, population density, and physiological characteristics (3). In addition, bacterial viability is affected by metabolic by-products that may stimulate, inhibit, or even kill the bacteria(4).

Tryptic Soy Broth (TSB) and Bouillon are liquid media commonly used for storage because their nutrient composition supports the growth of both Gram-positive and Gram-negative bacteria (5). A study by Rahma (2024) demonstrated that *Escherichia coli* can form strong biofilms in TSB (6), indicating that TSB provides a supportive environment for the activity and survival of Gram-negative bacteria. At a temperature of 8–9°C (7). This medium may also influence the virulence factors of *Staphylococcus aureus* and affect its cytotoxicity (8). Tryptic Soy Broth is a complex medium containing glucose as a carbon source, dipotassium hydrogen phosphate, casein peptone, soybean meal peptone, and sodium chloride. According to Wijesinghe (2019), nutrient composition correlates with bacterial growth, metabolism, and the production of virulence factors (9).

In contrast, Bouillon has a simpler formulation and is considered suitable for Gram-negative bacteria, which may grow better under lower nutrient concentrations and at lower incubation temperatures (10). Storage temperature and duration affect the average total bacterial population (11), (12). Physiological adaptation and metabolism under dynamic substrate conditions indicate the presence of cellular mechanisms that enable bacteria to cope with stress and changes in substrate uptake, storage, and energy release, thereby helping compensate for declining microbial performance. (13).

Recent studies regarding the viability of Gram-positive and Gram-negative bacteria stored in Bouillon and TSB at 4–8°C with varying storage durations as liquid working stock media remain limited. Therefore, this study aimed to evaluate the ability of Bouillon and TSB to maintain the viability of Gram-positive bacteria

(*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*) in liquid storage media. Viability was assessed based on the number of surviving cells after the longest storage duration. The findings are expected to provide evidence-based practical recommendations for managing working stock bacterial cultures in educational laboratories.

METHODS

This was an experimental laboratory study using a posttest-only control-group design. Four treatment groups were used, and each group was performed four times. Ethical approval was obtained from the Ethics Committee of Poltekkes Kemenkes Surabaya (No. EA/4031/KEPK-Poltekkes_Sby/V/2025).

Samples

This study used Gram-negative bacteria (*Escherichia coli* ATCC) and Gram-positive bacteria (*Staphylococcus aureus* ATCC) standardized to 0.5 McFarland (equivalent to 1.5×10^8). The bacterial isolates were obtained from BBLKM Surabaya. Samples were divided into four groups with the following compositions: **Group 1:** Four tubes containing 4 mL TSB (Oxoid) + 20 µL *E. coli* ATCC. **Group 2:** Four tubes containing 4 mL Bouillon + 20 µL *E. coli* ATCC. **Group 3:** Four tubes containing 4 mL TSB (Oxoid) + 20 µL *S. aureus* ATCC. **Group 4:** Four tubes containing 4 mL Bouillon (Oxoid) + 20 µL *S. aureus* ATCC

All groups were incubated at 37°C for 24 hours using an incubator (Nuve). The bacterial suspensions were then stored in a refrigerator (Samsung) at 4–8°C for 1 week, 2 weeks, and 1 month.

Sample Dilution

Serial dilutions were performed for each storage duration. Five tubes were prepared, each containing 9 mL of sterile distilled water. In the first tube (10^{-1}), 1000 µL of the sample was added using a micropipette (Eppendorf) and homogenized using a vortex mixer. Subsequently, 1000 µL from tube 10^{-1} was transferred into tube 10^{-2} and vortexed. This procedure was continued until tube 10^{-5}. The same procedure was applied to each treatment group.

Colony Counting Procedure

From the 10^{-5} dilution, 20 µL was inoculated onto Nutrient Agar (Oxoid) using a micropipette (Eppendorf). The spread plate technique was performed using an "L"-shaped spreader on the agar surface. Plates were incubated at 37°C for 24 hours (Nuve incubator). Colonies grown on Nutrient Agar were counted using a colony counter. The colony count results were expressed in CFU.

Data Analysis

All data were analyzed using Two-Way ANOVA in SPSS version 22.0 for Windows. Data analysis was also presented descriptively.

RESULT AND DISCUSSION

This study evaluated the viability of *Escherichia coli* ATCC and *Staphylococcus aureus* ATCC stored in Bouillon and Tryptic Soy Broth (TSB) at 4–8°C with different storage durations.

Colony Count of Gram-Negative Bacteria (*Escherichia coli* ATCC).

The colony counts of *E. coli* ATCC in Bouillon and TSB with four replications are presented in Table 1.

Table 1. Colony counts of *Escherichia coli* ATCC in Bouillon and TSB at different storage durations

R	Number of Colonies (CFU)					
	1week		2weeks		1month	
	TSB	Bouil	TSB	Bouil	TSB	Bouil
EC1	7195	4020	92500	3000	7735	185
EC2	6435	9865	84000	3000	2525	200
EC3	5295	6035	92500	2500	3115	150
EC4	7125	4840	95000	15000	4635	315
Mean	6512.5	6190	91000	5875	4502.5	212.5

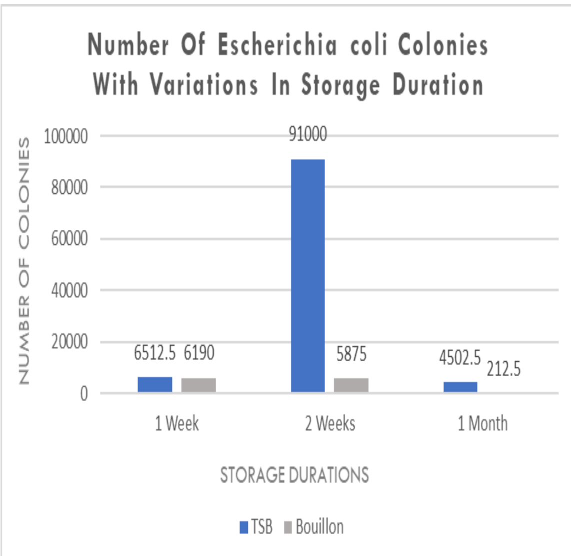
Description: R= Replikatio, TSB= *Tryptic Soy Broth media*, Bouil = Bouillon *media*

Two-Way ANOVA results showed significant differences ($p < 0.05$), indicating that media type, storage duration, and their interaction affected colony counts (Table 2).

Table 2. Two-Way ANOVA results for Bouillon and TSB with storage duration on *E. coli* colony counts

No	Faktor	Nilai P (Sig.)
1	Media type	0,000
2	Storage duration	0,000
3	Interaction (Media type & duration)	0,000

Interpretation: The type of media, storage duration, and their interaction affect the number of colonies.



Graph 1. The number of Escherichia coli colonies on the Bouillon and TSB media

A significant difference indicates that media type, storage duration, and their interaction influenced bacterial viability, as reflected by colony growth. Higher colony growth indicates higher bacterial viability. In this study, *E. coli* ATCC stored in TSB showed the highest colony count after 2 weeks, followed by a significant decline after 1 month. Meanwhile, *E. coli* ATCC stored in Bouillon showed the highest colony count at 1 week, a slight decrease at 2 weeks, and a drastic decline after 1 month. This depiction is reflected in graph 1, and the evidence of growth is visually evident in figure 1.

Colony Count of Gram-Positive Bacteria (*Staphylococcus aureus* ATCC)

Colony counts of *S. aureus* ATCC in Bouillon and TSB are presented in Table 3.

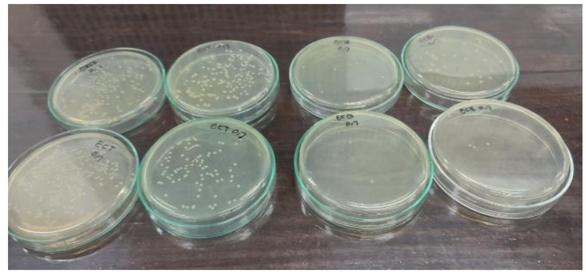


Figure 1. ECT growth of Escherichia coli ATCC bacteria in Tryptic Soy Broth (TSB) and ECB growth of Escherichia coli ATCC in Bouillon.

Two-Way ANOVA results showed significant effects ($p < 0.05$) of media type, storage duration, and their interaction on colony counts (Table 4).

Table 3. Colony counts of *Staphylococcus aureus* ATCC in Bouillon and TSB at different storage durations

R	Number of Colonies (CFU)					
	1week		2weeks		1month	
	TSB	Bouil	TSB	Bouil	TSB	Bouil
AU1	8740	5930	15000	1500	765	75
AU2	15175	9020	19000	500	625	135
AU3	12855	6035	39500	1500	615	90
AU4	21635	6995	34500	500	685	120
Mean	14601.25	6995	27000	1000	672.5	105

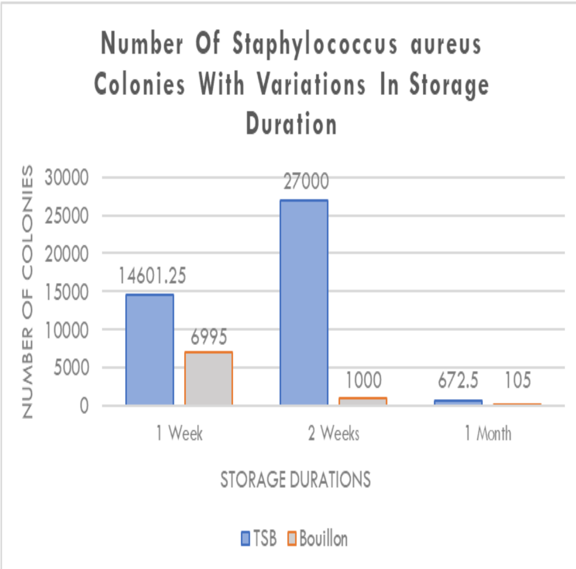
Description: R= Replikatio, TSB= *Tryptic Soy Broth media*, Bouil = Bouillon *media*

The significant p-values indicate that media type, storage duration, and their interaction affected colony growth. The colony count of *S. aureus* ATCC stored in Bouillon was highest at 1 week, decreased at 2 weeks, and showed a drastic reduction after 1 month. In contrast, *S. aureus* ATCC stored in TSB showed the highest colony count after 2 weeks, followed by a sharp decrease after 1 month. This description is reflected in Graph 2.

Table 4. Two-Way ANOVA results for Bouillon and TSB with storage duration on *S. aureus* colony counts

No	Faktor	Nilai P (Sig.)
1	Jenis Media	0,000
2	Lama Penyimpanan	0,000
3	Interaksi Jenis Media dan Lama Penyimpanan	0,000

Interpretation: The type of media, storage duration, and their interaction affect the number of colonies.



Graph 2. Graph of the number of Staphylococcus aureus colonies on Bouillon and TSB media.

The visual images of the differences in *Staphylococcus aureus* colony growth on the two storage media are clearly shown in Figure 2.

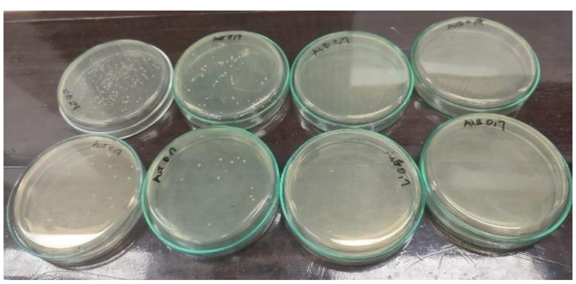


Figure 2. Growth of AUT of Staphylococcus aureus ATCC bacteria in Tryptic Soy Broth (TSB) and growth of AUB of Staphylococcus aureus ATCC bacteria in Bouillon.

Overall, the results indicate that TSB maintained the viability of both *E. coli* and *S. aureus* better than Bouillon. The significant p-value ($p < 0.05$) confirms a meaningful difference between the two media. These findings are consistent with Sunarno (2021), who reported that TSB is a nutrient-rich complex medium(14). The presence of casein peptone, soy peptone, glucose as a carbon source, buffering components, and pH stability contributes to bacterial growth and viability. Similar findings have been reported in previous studies evaluating *E. coli* survival in soil samples, which showed continued bacterial growth(15). Media containing high levels of casein may slow bacterial adaptation because bacterial proteases degrade casein into amino acids. After adaptation, bacterial cell numbers can increase rapidly(16). In addition, bacterial growth is influenced by physical environmental conditions(17). Bacteria are relatively robust and may survive at 4°C for up to 3 months; however, longer storage at low temperatures can reduce viability(18)

The highest colony counts for both *E. coli* and *S. aureus* in TSB occurred after 2 weeks of storage, followed by a significant decline after 1 month at 4–8°C. The behavior of E. coli bacteria, with patterns of increase and decrease, shows fluctuations in cell length. The cell length is determined by photomicrographs of the sample data, which depict that the average cell length is either maximal or minimal for each culture(19). This phenomenon shows the presence of adapttation, growth, and decline phases in microbial population dynamic(20). This pattern suggests an adaptation phase in the first week, followed by active growth during the second week due to sufficient nutrients, and then a decline after one month due to nutrient depletion and accumulation of inhibitory metabolites (21). Storage temperature plays a key role in determining viability during storage duration, as shown in previous studies on probiotic storage, where viability decreased significantly at temperatures above 30°C compared to lower temperatures (22)

Notably, *S. aureus* colony counts in TSB were higher than *E. coli* colony counts. Meanwhile, *E. coli* growth in Bouillon appeared more stable compared to TSB. This may be explained by the fact that TSB is considered a better recovery medium for Gram-positive bacteria (23). However, TSB may not be the optimal recovery medium for Gram-negative bacteria such as *E. coli*, which may grow better at lower nutrient concentrations and lower incubation temperatures (10). In line with Mahmmoud (2020), an optimal preservation method for *S. aureus* was saline, whereas distilled water was optimal for *E. coli* at storage temperatures of 4°C, 25°C, and −20°C (24).

To maintain bacterial viability in Tryptic Soy Broth (TSB), supplementation with glycerol as a preservative can be applied. This approach is supported by the study conducted by Abdurrahman *et al.*, which evaluated the viability of *Escherichia coli* in TSB supplemented with various concentrations of glycerol. The results indicated that a 10% glycerol concentration, combined with storage at −20°C for 42 days, yielded the most optimal preservation of bacterial viability(25). Bacterial viability is also strongly influenced by temperature. This is evidenced by research conducted by Deni Yanuarista *et al.*, which investigated the storage of *Staphylococcus aureus* in TSB at different temperatures. At 5°C, a viability reduction of approximately 0.95 log CFU/mL was observed from day 14 to day 84. In contrast, storage at −4°C resulted in a more pronounced decline of approximately 1.7 log CFU/mL, indicating that lower temperatures do not always correspond to improved viability preservation depending on cellular stress responses and storage conditions(26). In the present study, TSB media without the addition of preservatives stored at 4–8°C showed optimal bacterial viability during the first two weeks of storage. The colony counts reached 27,000 CFU for *Staphylococcus aureus* and 91,000 CFU for *Escherichia coli*. A critical decline phase for both bacterial populations was observed after one month of storage, indicating a significant reduction in viability beyond this period under non-preserved refrigerated conditions. This study was limited by the manual colony counting method.

CONCLUSION

This study concludes that Tryptic Soy Broth (TSB) has a higher capacity to maintain the viability of *Escherichia coli* and *Staphylococcus aureus* compared with Bouillon. The complex nutrient composition of TSB and storage duration significantly influenced colony counts. The highest bacterial colony counts were observed after two weeks of storage, while a significant decline in colony counts occurred after one month. The interaction between storage media and storage duration indicates that TSB is more effective in maintaining bacterial viability during storage.

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