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Optimizing Gram-Negative Bacteria Isolation from Children's Diarrheal Stool Samples in Jakarta: A Comparative Analysis of Six Culture Media

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Abstract

The prevalence of diarrheal illnesses in Indonesia remains a significant concern among children under the age of five, evident through diagnostic findings and symptomatic manifestations, leading to increased rates of mortality and morbidity. Correspondingly, this study aimed to identify, isolate, and culture gram-negative bacteria responsible for childhood diarrhea. Twenty stool samples were collected from children under five years old with acute diarrhea in Jakarta health centers and hospitals between October 2023 and January 2024. These samples were inoculated onto selective agars to facilitate the growth of gram-negative bacteria. After a 24-hour incubation period, colonies suspected to be *E. coli*, *K. pneumoniae*, *Salmonella*, *Shigella*, and *Vibrio* were identified utilizing the Vitek-2 compact system. The results revealed gram-negative bacteria in all the fecal samples from diarrheal children, with *Escherichia coli (E. coli)* and *Klebsiella pneumoniae (Klebsiella p.)* as the predominant species. In conclusion, utilizing various cultures and the Vitek-2 compact system, the present study elucidated the microbial landscape driving diarrheal morbidity in Indonesian children under five, with *Escherichia coli* and *Klebsiella pneumoniae* emerging as prevalent contributors to childhood diarrhea.

Keywords: Bacteria; Children; Culture; Diarrhea

Introduction

At the forefront of public health concerns in Indonesia lies the persistent threat of diarrheal illnesses among children under the age of five. Despite advancements in healthcare, this remains a significant concern, with implications for both mortality and morbidity rates. Research suggests that most cases occur in children between twelve and twenty-three months old, highlighting a critical window of vulnerability (Arifin et al., 2022). Diarrhea can be attributed to various factors, including infections, malnutrition, contaminated water, and food. In addition, numerous pathogens, encompassing bacteria and viruses, can potentially induce diarrhea (Hartman et al., 2023).

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It is alarming to note that a staggering 88% of diarrhea-related deaths are attributed to unsafe drinking water, inadequate sanitation, and poor hygiene practices. These conditions create fertile ground for spreading bacteria causing diarrhea, underscoring the urgent need for improved infrastructure and hygiene education (Bitew et al., 2017).

Gram-negative bacteria such as *Escherichia coli*, *Salmonella*, *Shigella*, and *Vibrio* are particularly concerning and pose significant public health risks, especially in developing nations like Indonesia. While *Salmonella* infections are often self-limiting, they can sometimes escalate to severe and life-threatening conditions, necessitating antibiotic intervention. Similarly, *Escherichia coli* infections have been associated with high fatality rates and growth issues in children, reflecting the complex interplay between hygiene, nutrition, and healthcare access (Oyofo et al., 2002; Kotloff, 2017).

Accurate identification of the causative agent is paramount for effective diagnosis and treatment. Variables such as changes in stool consistency, color, volume, and frequency offer valuable insights into the underlying etiology of diarrhea. Moreover, non-invasive tests like stool examination and culture are crucial in pinpointing the responsible pathogens and guiding targeted interventions and management strategies (Nemeth & Pfleghaar, 2024).

While previous studies have shed light on the prevalence of positive stool cultures and potential predictors, there remains a notable gap in research, particularly concerning young children with diarrhea in Indonesia. This underscores the significance of ongoing investigations aimed at culturing fecal samples from children below the age of two, specifically within health centers and hospitals in Jakarta. By expanding the understanding of the microbial landscape driving diarrheal illnesses in this vulnerable population, people can inform more targeted interventions and ultimately alleviate the burden of this preventable disease (Riaz et al., 2012).

Population and Sample

The population in this research included all children with diarrhea who visited the designated sampling site. The sample consisted of 5 children experiencing acute diarrhea who met specific inclusion criteria: being between the ages of 2 and 5 and providing informed consent as approved by the Research Ethics Committee at the Faculty of Medicine, University of Indonesia.

Sample collection

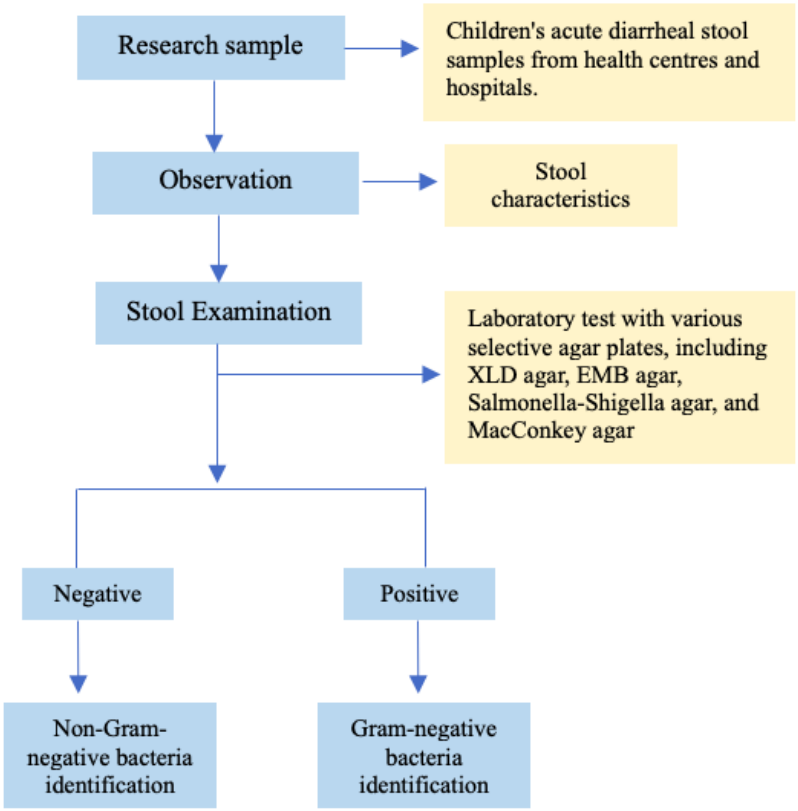
The research commenced with ethical approval from the Health Research Ethics Committee at the Faculty of Medicine, University of Indonesia (FMUI)-Cipto Mangunkusumo Hospital (CMH). This critical step ensures that the study adheres to ethical standards and protects the participants' rights and well-being. Employing the consecutive sampling technique, the researchers targeted children aged 2-5 years exhibiting clinical symptoms of acute diarrhea. The sampling occurred at various health centers and hospitals, including Senen Health Center and Cipto Mangunkusumo Hospital in Jakarta, Indonesia. Parents of eligible participants received a plastic bag, spoon/scoop, and a sterile container to collect their child's stool samples. This approach ensured the secure and hygienic collection of samples, minimizing the risk of contamination.

Subsequently, the collected stool samples were delivered to the FMUI Microbiology Laboratory for thorough analysis. Figure 1 provides a clear illustration of the research workflow.

Culturing Bacteria Isolation

The process of isolating and identifying bacteria from stool samples is a meticulous and multi-step procedure that requires precision and accuracy. Initially, all the stool samples were inoculated onto various selective agar plates. These plates included Xylose Lysine Deoxycholate (XLD) Agar, Eosin Methylene Blue (EMB) Agar, *Salmonella-Shigella* Agar, and MacConkey Agar.

Figure 1. *Schematic Flowchart of the Research Process*



These media were specifically designed to promote the growth of certain types of bacteria while inhibiting the others, thereby facilitating the isolation of the bacteria of interest. In parallel to the inoculation onto agar plates, a portion of each stool sample was added to selenite broth. It serves as an enrichment medium, promoting the growth of certain bacteria, particularly *Salmonella*. The use of selenite broth increases the likelihood of detecting these bacteria, even if they are present in small numbers. There were instances where no growth was observed on the agar plates, but the selenite broth exhibited turbidity, indicating bacterial growth. In such cases, a secondary inoculation was performed. The turbid broth was transferred onto the selective agar plates to encourage further growth and isolation of the bacteria. Stool samples were specifically inoculated onto Thiosulfate Citrate Bile Salts Sucrose (TCBS) Agar to detect the *Vibrio* bacteria. In this regard, the suspected *Vibrio* colonies, characterized by their yellowish

color on TCBS Agar, were subjected to further analysis.

Bacteria Identification

After a 24-hour incubation period, colonies suspected to be *E. coli*, *K. pneumoniae*, *Salmonella*, *Shigella*, and *Vibrio* were identified utilizing an automated machine, specifically the Vitek-2 compact system. This system uses biochemical characteristics for accurate species identification. For instance, suspected *Salmonella* colonies are confirmed by their characteristic black-centered appearance on *Salmonella-Shigella* Agar. Throughout this process, quality control measures are implemented to ensure the accuracy and reliability of results. These measures include checking each batch of reagents, discs, stains, antisera, and identification systems for positive, negative, and graded reactivity.

Data Presentation

Research data were analyzed descriptively by observing the parameter in the form of the number of positive samples of gram-negative bacteria.

Results

This research was conducted from October 2023 to January 2024 in public health centers and hospitals in Jakarta, including the Senen Health Center and Cipto Mangunkusumo Hospital. Five stool samples were collected from children under the age of five. The fecal samples were systematically examined utilizing various culture media, including Xylose Lysine Deoxycholate (XLD), Eosin Methylene Blue (EMB), Selenite broth, *Salmonella-Shigella*, MacConkey, and Thiosulfate Citrate Bile Salts Sucrose (TCBS), to determine the presence and semi-quantitative levels of bacterial growth. The semi-quantitative interpretations, ranging from +1 to +4, indicated the level of bacteria present in each quadrant of the streak plate. As shown in Table 1, Sample 1 exhibited robust growth of yellowish colonies in the 4th quadrant with a semi-quantitative interpretation of +4 on both XLD and EMB media, while Selenite broth showed clear growth. Sample 2 demonstrated similar results, with the addition of yellowish colonies in the 1st quadrant, rated at +1 on TCBS media. Sample 3 displayed limited growth in the 1st quadrant on XLD media, rated at +1, and clear growth in Selenite broth. Sample 4 exhibited growth of yellowish colonies in the 2nd quadrant, rated at +2 on XLD media, metallic green colonies in the 4th quadrant, rated at +4 on EMB media, and pink colonies in the 2nd quadrant, rated at +2 on *Salmonella-Shigella* media. Sample 5 showed growth of yellowish colonies in the 4th quadrant, rated at +4 on XLD media, metallic green colonies in the 4th quadrant, rated at +4 on EMB media, pink colonies in the 4th quadrant, rated at +4 on *Salmonella-Shigella* media, and yellowish colonies in the 1st quadrant, rated at +1 on TCBS media.

Table 1. *Culture results of the stool samples*

Sample Name	Media	Macroscopic/Colony Morphology	Zone of Growth	Semi-quantitative Interpretation
Sample 1	XLD	Yellowish	4th quadrant	+4
	EMB	Brownish	4th quadrant	+4

	Selenite Broth	Clear		
	Salmonella-Shigella	-	-	-
	MacConkey	-	-	-
	TCBS	-	-	-
Sample 2	XLD	Yellowish	4th quadrant	+4
	EMB	Brownish	4th quadrant	+4
	Selenite Broth	Clear	-	-
	Salmonella-Shigella	-	-	-
	MacConkey	-	-	-
	TCBS	Yellowish	1st quadrant	+1
Sample 3	XLD	Yellowish	1st quadrant	+1
	EMB	-	-	
	Selenite Broth	Clear	NA	NA
	Salmonella-Shigella	-	-	-
	MacConkey	-	-	-
	TCBS	-	1st quadrant	
Sample 4	XLD	Yellowish	2nd quadrant	+2
	EMB	Metallic Green	4th quadrant	+4
	Selenite Broth	Turbid	-	-
	Salmonella-Shigella	Pink	2nd quadrant	+2
	MacConkey	-	-	-
	TCBS	-	-	-
Sample 5	XLD	Yellowish	4th quadrant	+4
	EMB	Metallic Green	4th quadrant	+4
	Selenite Broth	Turbid		
	Salmonella-Shigella	Pink	4th quadrant	+4
	MacConkey	-		
	TCBS	Yellowish	1st quadrant	+1

Table 2 presents the results of the stool culture analysis. Bacterial identification using the Vitek-2 system confirmed the presence of three types of bacteria. *Klebsiella pneumoniae*, a member of the *Enterobacteriaceae* family, exhibited the highest prevalence, accounting for 60% of the analyzed samples (samples 1, 2, and 5). *Escherichia coli (E. coli)* (sample 4) and *Enterobacter cloacae* (sample 3)

were each found in 20% of the samples. These comprehensive findings could contribute significantly to the understanding of the microbial profiles present in the sampled environments, underscoring the importance of employing a combination of culture media and biochemical tests for accurate organism identification in microbial survey studies.

Table 2. *Stool Bacteria Identification Results*

Identified Bacterium	n	%
Escherichia coli	1	20%
Klebsiella pneumoniae	3	60%
Enterobacter cloacae	1	20%
Total	5	100%

Discussion

The stool samples were cultured on various selective and differential media tailored to identify gram-negative bacteria, as shown in Figure 2. The fecal samples were systematically examined using a variety of culture media, including Xylose Lysine Deoxycholate (XLD), Eosin Methylene Blue (EMB), Selenite broth, *Salmonella-Shigella* Agar (SS), MacConkey Agar, and Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS), as presented in Table 3. Samples 1, 2, and 5 exhibited robust growth of yellowish colonies in the 4th quadrant with a semi-quantitative interpretation of +4 on XLD media, while Selenite broth showed clear growth. Sample 3 displayed limited growth in the 1st quadrant on XLD media with a semi-quantitative interpretation of +1 and clear growth in Selenite broth. Sample 4 showed growth of yellowish colonies in the 2nd quadrant with a semi-quantitative interpretation of +2 on XLD media, metallic green colonies in the 4th quadrant with a semi-quantitative interpretation of +4 on EMB media, and pink colonies in the 2nd quadrant with a semi-quantitative interpretation of +2 on *Salmonella-Shigella* media. In this regard, the Selenite broth for this sample was turbid. Sample 5 exhibited yellowish colony growth in the 4th quadrant with a semi-quantitative interpretation of +4 on XLD media, metallic green colonies in the 4th quadrant with a semi-quantitative interpretation of +4 on EMB media, and pink colonies in the 4th quadrant with a semi-quantitative interpretation of +4 on *Salmonella-Shigella* media. No growth was observed for Sample 5 on MacConkey Agar. Growth on TCBS was observed only for Samples 2 and 5 with yellowish colonies in the 1st quadrant. No growth was observed for Samples 1 and 4, and the result for Sample 3 on TCBS showed colonies in the 1st quadrant but without any color formation.

The present study results resemble similar research in which the efficiency of two enrichment broths and four plating media was compared to detect enteric pathogens from 1,597 stool specimens. The comparison of plating media indicated that Xylose Lysine Deoxycholate (XLD) Agar and Hektoen Enteric Agar demonstrated equal efficacy in detecting both pathogens. Both were moderately more effective than *Salmonella-Shigella* Agar and superior to Eosin Methylene Blue Agar. Specifically, XLD identified 83% of *salmonella*, as determined by the composite of four media, and detected 90% of *shigella* (Taylor & Schelhart, 1971).

EMB Agar, a selective and differential medium, promoted the growth of gram-negative bacteria and differentiated between lactose fermenters and non-fermenters through color variations. In this study, *E. coli*, which typically forms metallic green colonies, was evident in Sample 4. *Klebsiella pneumoniae* and other non-fermenters, which appear brown or pink on EMB, were recorded in Samples 1 and 2, both in the 4th quadrant. These results could be compared to a similar study conducted by Virpari in 2013, where

59% of *E. coli* isolated from 100 stool samples exhibiting diarrheal symptoms produced a greenish metallic sheen on EMB Agar (Sparbier et al., 2012; Virpari et al., 2013). In the Selenite broth test, turbidity, which represents growth and suggests the presence of fecal pathogens such as *Salmonella* and *Shigella*, was observed in Samples 4 and 5. Conversely, Samples 1, 2, and 3 showed clear Selenite broth, indicating the absence of these pathogens. The Selenite broth medium enriched and isolated these pathogens by inhibiting competing flora.

The results of this study are comparable to those of similar research by Sparbier, where 4,847 samples were examined for the presence of *Salmonella* species. Out of these, 108 samples were tested positive for *Salmonella*. Among these, 66 were identified following the streaking of stool samples on Hektoen Agar, followed by MALDI Biotyper analysis of suspicious *Salmonella* colonies. On the first day of analysis, 34 samples were directly identified as *Salmonella*-positive from the Selenite enrichment broth (Sparbier et al., 2012).

Salmonella-Shigella (SS) Agar results are crucial for identifying *Salmonella* and *Shigella*. *Salmonella* is indicated by colorless colonies with black centers, while *Shigella* is represented by colorless colonies without black centers. In this study, Samples 4 and 5 demonstrated positive growth on *SS* Agar, with Sample 5 exhibiting robust growth, suggesting the presence of either *Salmonella* or *Shigella*. On the other hand, Samples 1, 2, and 3 indicated no growth on *SS* Agar, indicating the potential absence of these bacteria.

These results could be compared to a similar study conducted by Mardu in 2020 in Ethiopia, where out of the 59 samples cultured on *SS* Agar, two *Salmonella*-like and two *Shigella*-like species were presumably identified. These were further confirmed as *Salmonella* or *Shigella* species by inoculating them into selected biochemical tests (Mardu et al., 2020). TCBS Agar is a highly selective medium used for cultivating *Vibrio* species. On this medium, *Vibrio cholerae* colonies appear yellow due to sucrose fermentation, while *Vibrio parahaemolyticus* produces greenish colonies. In this study, Samples 2 and 5 showed a yellowish coloration in the 1st quadrant of TCBS Agar. These observations indicated different levels of growth and positive reactions for sucrose fermentation, suggesting the presence of *Vibrio* species. These findings align with a related research project conducted at FoodNet Sites between 1995 and 2000. In that study, microbiologists from 388 clinical laboratories were surveyed regarding their laboratory methods and policies for routine testing of stool specimens for *Salmonella*, *Shigella*, *Campylobacter*, *Vibrio*, *Yersinia enterocolitica*, and *Escherichia coli O157:H7*. The research revealed that out of the 388 participating laboratories, 276 (71%) reported testing stool specimens for *Vibrio* species. Among these, 212 (77%) conducted on-site testing, 113 (53%) utilized Thiosulfate Citrate Bile Salts Sucrose (TCBS) Agar, and 105 (50%) routinely tested all stool specimens for *Vibrio* species (Voetsch et al., 2004).

The comprehensive stool culture analysis provided a detailed understanding of the microbial composition within the examined fecal samples. The use of specialized culture media enabled the identification and characterization of bacterial growth, illuminating the diverse range of microorganisms present (see Figure 3). In all samples (100%), growth on Xylose Lysine Deoxycholate (XLD) was observed, indicative of lactose fermenters such as *Klebsiella pneumoniae* and *Escherichia coli*. Similarly, Eosin Methylene Blue (EMB) media showed growth in 80% of the samples.

The Vitek-2 bacterial identification system confirmed the prevalence of these enteric bacteria within the gastrointestinal tract. Contrary to previous reports, Selenite broth, utilized for the enrichment and isolation of *Salmonella* and *Shigella* species, exhibited no growth.

***Figure 2**. Stool culture results on different media: (a) EMB media, (b) TCBS media, (c) XLD media, (d) and (f) MacConkey media, and (e) Salmonella-Shigella media.*

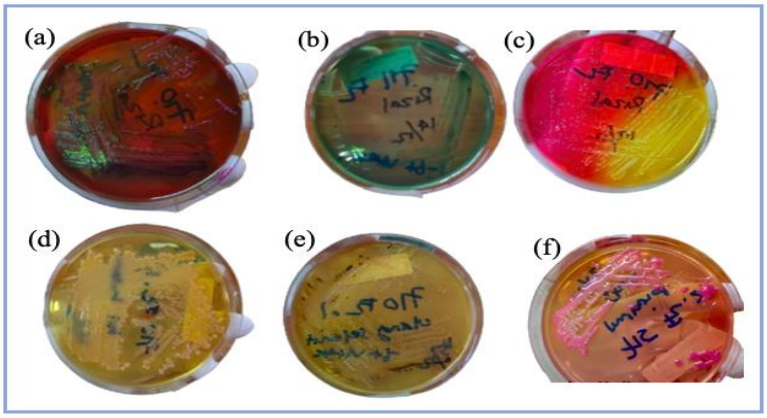


***Table 3**. Summary of Stool Culture Results in the 6 Different Media*

Media	n	%
XLD	5	100%
EMB	4	80%
Selenite Broth	0	0%
Salmonella-Shigella	2	40%
MacConkey	0	0%
TCBS	3	60%

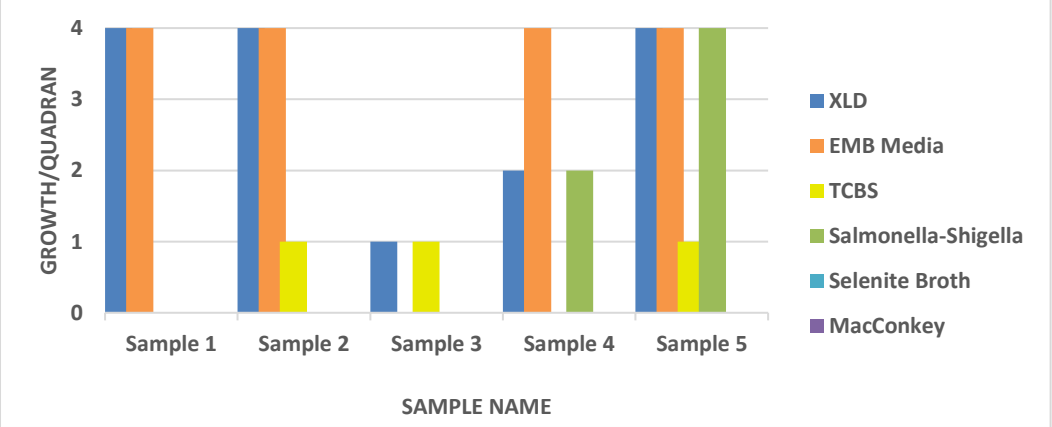
However, *Salmonella* and *Shigella* species were identified on selective media in 40% of the samples, emphasizing the importance of specific culture conditions for isolating these bacteria. MacConkey Agar, designed to cultivate gram-negative bacteria selectively, also revealed no growth. Thiosulfate Citrate Bile Salts Sucrose (TCBS) Agar, employed for isolating *Vibrio* species, demonstrated growth in 60% of the samples, indicating a potential presence of *Vibrio* organisms. These stool culture analysis results comprehensively portrayed the microbial landscape within the examined samples. The high prevalence of enteric bacteria and the identification of specific pathogenic species highlighted the complexity of the fecal microbiota. These findings advocate for an integrated approach, combining selective culture media and advanced identification methods, for thorough insights into research and clinical applications.

The prevalence of *Klebsiella pneumoniae*, identified in 60% of the samples, suggested a notable presence of this opportunistic pathogen known for its association with hospital-acquired infections; the elevated prevalence raised considerations for potential health implications within the studied population. Further investigations, including antimicrobial susceptibility testing, might provide insights into the clinical relevance of this finding. *Escherichia coli*, a common inhabitant of the human gastrointestinal tract, was found in 20% of the samples. This aligns with its expected presence in healthy individuals and underscores the utility of the stool culture analysis in capturing a diverse range of microbial species. *Enterobacter cloacae* was detected in 20%

of the total samples, indicating its presence within the fecal samples. *E. cloacae* is recognized as an environmental bacterium and a potential opportunistic pathogen. While its presence was limited in this dataset, its identification prompted considerations for its role in the broader context of gut microbiota dynamics. In conclusion, the stool culture analysis provided valuable insights into the distribution of specific bacterial species within the examined samples. The varying prevalence of *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* underscored the complexity of the gut microbiota.

These results could contribute to the foundation of knowledge necessary for comprehending microbial diversity in fecal samples. They might guide future investigations into the clinical implications of specific bacterial profiles within different populations. Furthermore, these results could be compared to a similar study conducted by Muziburrahman et al., where they found that the most represented bacteria in the study's fecal sample were *E. coli* (29.53%), *Klebsiella sp.* (25.50%), *Shigella sp.* (18.79%), *Staphylococcus aureus* (16.78%), *Salmonella Typhi* (8.73%), and *Proteus sp.* (0.67%). Males (61.76%) had more diarrhea than females (38.24%), with an age range of 1-3 years (53.92%), 0-1 years (37.26%), and 3 until <5 years (8.82%) (Muziburrahman et al., 2022). Hence, this research proved that *E. coli* bacteria was the leading cause of diarrhea in children under five years old.

***Figure 3**. Results of the 5 samples in the 6 different culture media*



Conclusion

This research presented preliminary findings regarding bacterial infections among children under the age of five who were experiencing acute diarrhea in Jakarta. The results highlighted *Escherichia coli* and *Klebsiella pneumoniae* as significant contributors to diarrhea among children under 5 years old. It underscored the significance of recognizing bacterial infections as potential factors in childhood diarrhea, particularly in developing nations.

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Indonesia Medical Educational Research Institute (IMERI).

Conflict of Interest

The authors/editors declare no conflicts of interest or financial concerns during the completion of this research.

Ethical Issues

This manuscript was approved by the Research Ethics Committee at the Faculty of Medicine, University of Indonesia.

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