Sriwijaya Journal of Environment
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Antibacterial Activity of Lactic Acid Bacteria Isolated from Cincalok Against *Escherichia coli* and *Listeria monocytogenes*

Zuwita Cahaya Rani Br Lubis[1], Yulia Oktavia[1*], Sri Novalina Amrizal[1], Wulandari[2]

[1] *Department of Fishery Product Technology, Maritim Raja Ali Haji University, Riau Islands, Indonesia*
[2] *Department of Fishery Product Technology, Animal Science Faculty, Jambi University, Jambi, Indonesia*
**Corresponding Author: yuliaoktavia@umrah.ac.id*

Article history

Received	Received in revised form	Accepted	Available online
04 October 2025	28 January 2026	28 January 2026	30 January 2026

Abstract: This study evaluated the antibacterial activity of lactic acid bacteria (LAB) isolates from *cincalok*, a traditional fermented shrimp product, against *Escherichia coli* and *Listeria monocytogenes*. Five LAB isolates (A1–A5) were screened for antagonistic activity following pre-incubation periods of 18, 21, and 24 hours. Only isolates A3 and A4 produced measurable inhibition zones after 24 hours and were selected for further antibacterial assessment using the disk diffusion method with their cell-free supernatants. Antagonistic assays showed that isolate A3 inhibited *E. coli* and *L. monocytogenes,* producing zones of 0.67 ± 0.29 mm and 1.33 ± 1.04 mm, respectively, while isolated A4 produced zones of 1.0 ± 0.5 mm and 1.33 ± 1.04 mm, respectively. In antibacterial assays, isolate A3 exhibited inhibition zones of 6.67 ± 2.08 mm (*E. coli*) and 4.67 ± 0.58 mm (*L. monocytogenes*), and isolate A4 showed 4.33 ± 0.58 mm and 3.67 ± 0.58 mm, respectively. Both isolates demonstrated weak to moderate activity, with efficacy influenced by the target species, incubation time, and environmental conditions. These results indicate that LAB isolate A3 has greater antibacterial activity, likely due to higher bioactive metabolite production. The findings highlight the potential of LAB from *cincalok* as a source of natural antibacterial compounds for food preservation or probiotic applications.
Keywords: Lactic acid bacteria, cincalok, antibacterial activity, Escherichia coli, Listeria monocytogenes

1. Introduction

Foodborne diseases constitute a global public health concern. They are defined as illnesses resulting from the ingestion of food or beverages contaminated with pathogenic microorganisms or their associated toxins. These diseases have gained substantial attention due to their wide-ranging clinical manifestations, spanning mild gastrointestinal disturbances to severe systemic complications that may lead to mortality. Although foodborne illness can result from contamination by molds, yeasts, and bacteria, bacterial pathogens remain the principal causes of food spoilage and foodborne outbreaks [1]. Among the most frequently implicated bacterial species are *Salmonella*, *Campylobacter*, *Listeria*, *Clostridium botulinum*, and *Escherichia coli* [2].

Pathogenic bacteria play a pivotal role in the epidemiology of foodborne disease, particularly *Escherichia coli* and *Listeria monocytogenes, which* are major etiological agents of severe foodborne infections. Pathogenic strains of *E. coli* produce potent exotoxins capable of inducing acute diarrhea, abdominal cramping, and life-threatening complications such as Hemolytic Uremic Syndrome (HUS). Contamination typically originates from undercooked meat, raw vegetables, or water of poor sanitary quality. In contrast, *L. monocytogenes* exhibits psychrotrophic properties, allowing growth at refrigeration temperatures and facilitating contamination of ready-to-eat foods, unpasteurized dairy products, and processed meats. Listeriosis represents a severe invasive disease that disproportionately affects pregnant women, older adults, and immunocompromised individuals.

Multiple foodborne outbreaks attributable to *E. coli* have been documented in Indonesia. For example, on March 2, 2017, a food poisoning incident in Kepek Village, Wonosari District, affected 79 individuals after consuming meals served during an aqiqah ceremony, with subsequent laboratory analyses confirming contamination by *E. coli* and *Salmonella* [3].

While no consumer-level listeriosis cases have been officially reported in Indonesia to date, the Fish Quarantine, Quality Control, and Safety Agency documented two rejections of Indonesian frozen shrimp exports to the United States in 2012–2013 due to *L. monocytogenes* contamination [5]. Such findings underscore the latent risk of emerging listeriosis cases within the national food system.

Considering the substantial hazard posed by foodborne pathogens, the development of safe, effective, and sustainable control measures has become increasingly imperative. In this context, lactic acid bacteria (LAB) have emerged as promising natural antimicrobial agents that can serve as alternatives to synthetic chemical preservatives. LAB produce a diverse array of antimicrobial metabolites—such as

lactic and acetic acids, hydrogen peroxide, diacetyl, and bacteriocins—that exhibit broad-spectrum inhibitory activities against foodborne pathogens through mechanisms including cell membrane disruption, intracellular acidification, and interference with metabolic homeostasis.

LAB are naturally present in numerous fermented foods, including traditional fermentation products widely consumed in Southeast Asia. One such product is cincalok, a fermented shrimp product originating from the Riau Islands, produced through spontaneous fermentation of *Acetes* sp. by endogenous microbiota. Recent studies have confirmed the presence of LAB in cincalok obtained from Pengujan Village [6]. LAB isolated from fermented foods are known to synthesize antimicrobial compounds that inhibit pathogens such as *Bacillus cereus*, *Clostridium botulinum*, and *Pseudomonas* spp. [7], highlighting their potential utility as natural biopreservatives.

This study builds upon previous work by Abiyyah *et al*. (2025), who successfully isolated LAB strains from cincalok. However, the inhibitory efficacy of these isolates against clinically significant pathogens such as *E. coli* and *L. monocytogenes* has not yet been elucidated. The present study aims to evaluate the antibacterial activity of these LAB isolates using in vitro inhibition zone assays. The results are expected to advance the understanding of LAB-mediated biopreservation and their potential application as natural antimicrobial agents to reduce the prevalence of foodborne pathogens.

2. Materials and Methods

2.1. Materials

This study used five Lactic Acid Bacteria (LAB) isolates (A1, A2, A3, A4, and A5) that had previously been studied and isolated from cincalok [6]. The test microorganisms *used were E. coli* and *L. monocytogenes* ATCC 14028. The growth media used included *De Man Rogosa Sharpe Broth* (MRSB) and *De Man Rogosa Sharpe Agar* (MRSA) for LAB, and *Nutrient Broth* (NB) and *Nutrient Agar* (NA) for test bacteria. *Mueller-Hinton Agar* (MHA) media was used to conduct an antibacterial activity assay. Other materials used were sterile disc paper, 0.3% chloramphenicol as a positive control, 70% ethanol, and aquabidest.

2.2. Methods

2.2.1. Lactic Acid Bacteria (LAB) Rejuvenation

Rejuvenation was carried out by transferring the LAB isolates into fresh growth media to restore their viability and metabolic activity. LAB isolates A1, A2, A3, A4, and A5 obtained from cincalok were used in this stage [6]. Each isolate was streaked aseptically onto MRSA plates from stock and incubated at 37 °C for 48 h [8].

2.2.2. Lactic Acid Bacteria (LAB) Reculture

Re-culturing of lactic acid bacteria (LAB) refers to the process of regrowing LAB cultures in fresh growth media. Rejuvenated LAB isolates (A1, A2, A3, A4, A5) previously maintained on MRSA medium were subsequently inoculated into MRSB medium using an inoculating loop. After inoculation, the MRSB medium was vortexed to ensure homogeneity and subsequently incubated at 37 °C [9]. Incubation was carried out at predetermined time intervals of 18, 21, and 24 hours to assess temporal effects on bacterial growthproducing antibacterial compounds.

2.2.3. Preparation of Test Bacteria

The test bacterial cultures were inoculated onto Nutrient Agar (NA) using a sterile inoculating loop and incubated at 37 °C for 24 h. Following incubation, a single loopful of bacterial growth was transferred into a test tube containing 10 mL of Nutrient Broth (NB), vortexed to achieve a homogeneous suspension, and incubated again at 37 °C for 24 h [10]. The resulting bacterial suspension was standardized by measuring its absorbance at 600 nm using a spectrophotometer, adjusting to match the McFarland standard 2.0, corresponding to approximately 6×10^8 CFU/mL and an absorbance of ~0.4 at the specified wavelength.

2.2.4. Antibacterial Extraction

The extraction was performed to obtain bioactive compounds from the LAB cultures for subsequent assessment of their antibacterial activity. The procedure involved centrifugation of the cultures in MRSB medium at 3,000 rpm for 30 minutes to separate the cells from the medium. The resulting cell-free supernatant was collected, filtered through sterile filter paper, and stored at 4 °C until further use for antibacterial assays [11].

2.3. Parameters for Antimicrobial Assay

2.3.1. Antagonistic Assay

The antagonistic activity of lactic acid bacteria (LAB) isolates was evaluated to determine their capacity to inhibit the growth of pathogenic bacteria. The assay was conducted using the disc diffusion method, a standard approach for assessing the antimicrobial potential of microbial metabolites [12]. A 50 µL aliquot of the test bacterial suspension was inoculated onto Nutrient Agar (NA) and evenly spread using a sterile L-shaped spreader. Sterile paper discs loaded with 20 µL of the LAB isolate were placed on the agar surface. The plates were incubated at 37 °C for 24 hours. The formation of a clear zone surrounding the discs was considered indicative of the antibacterial activity of the LAB isolates against the test bacteria. All antagonistic assays were performed in triplicate to ensure reproducibility. The diameter of the inhibition zone was determined using the

following formula:

Inhibition Zone Diameter = Total Diameter of Clear Zone – Disc Diameter

Results were interpreted based on the inhibition zone classification: weak (1–3 mm), moderate (3–6 mm), and strong (>6 mm) [13]. This test serves as an initial screening to determine whether the LAB isolates can inhibit the growth of the test pathogens. LAB isolates that are confirmed to form inhibition zones will subsequently undergo a cell-free supernatant antibacterial activity assay to confirm the activity of their extracellular metabolites.

2.3.2. Antibacterial Activity Assays

Antibacterial activity was evaluated using the disc diffusion method to assess the inhibitory potential of Lactic Acid Bacteria (LAB) extracts against *Escherichia coli* and *Listeria monocytogenes*. Sterile paper discs were loaded with 20 µL of LAB cell-free supernatant and placed on Mueller-Hinton Agar (MHA) plates previously inoculated with the test microorganisms. The assay included a positive control (0.3% chloramphenicol) and a negative control (MRSB medium). Plates were incubated at 37 °C for 24 hours, after which the diameter of the clear inhibition zones was measured to determine antibacterial activity. The inhibition zone (mm) was calculated using the following formula:

Inhibition Zone Diameter = Total Diameter of Clear Zone – Disc Diameter

The inhibition was classified as weak (<5 mm), moderate (5–10 mm), and very strong (>20 mm) [14].

2.4. Data Analysis

The antibacterial assay data were analyzed both descriptively and quantitatively by calculating the mean and standard deviation from three replicates for each treatment. Comparative analyses were performed by evaluating the results of this study against findings from previous antibacterial studies.

3. Results and Discussion

3.1. Antagonistic Activity of LAB Isolates Against Escherichia coli and Listeria monocytogenes

The assay was conducted to determine the ability of Lactic Acid Bacteria (LAB) isolated from cincalok to inhibit the growth of *E. coli* and *L. monocytogenes*. The results of the inhibition zone diameter measurement for the antagonist test are presented in Table 1.

Table 1. Inhibition zone diameter for the antagonistic activity assay of lactic acid bacteria

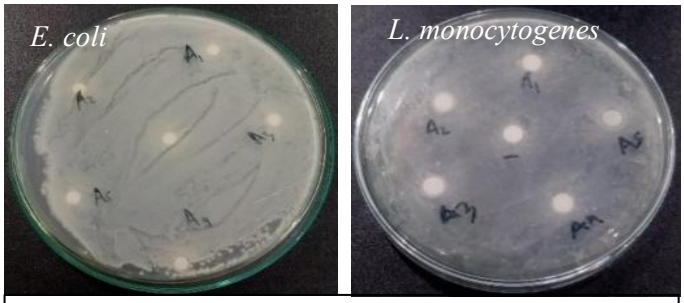
Isolates	Inhibition Zones Diameter (mm)					
	18 h		21 h		24 h	
	E	L	E	L	E	L
A1	0	0	0	0	0	0
A2	0	0	0	0	0	0
A3	0	0	0	0	0.67 ± 0.29	1.33 ± 1.04
A4	0	0	0	0	1.0 ± 0.5	1.33 ± 1.04
A5	0	0	0	0	0	0

Notes: E: *Escherichia coli,* L: *Listeria monocytogenes*

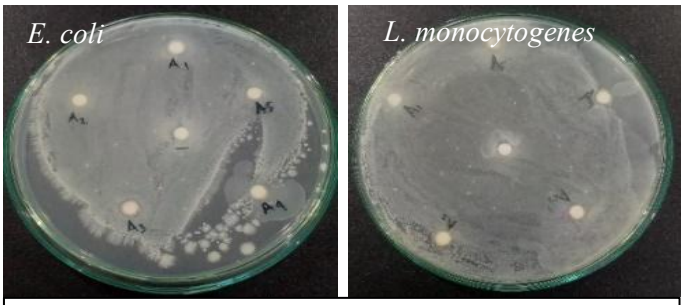
The data presented in Table 1 summarize the inhibition zone diameters of lactic acid bacteria (LAB) isolates against *Escherichia coli* and *Listeria monocytogenes* at incubation intervals of 18, 20, and 24 hours. Isolates A1, A2, and A5 exhibited no detectable antibacterial activity against either pathogen, as indicated by the absence of measurable inhibition zones (0 mm) throughout the observation period.

In contrast, isolates A3 and A4 showed antibacterial activity against both *E. coli* and *L. monocytogenes* at 24 hours. Isolate A3 produced inhibition zones of 0.67 ± 0.29 mm for *E. coli* and 1.33 ± 1.04 mm for *L. monocytogenes*, whereas isolate A4 produced inhibition zones of 1.0 ± 0.5 mm and 1.33 ± 0.33 mm, respectively. These observations suggest a time-dependent increase in antimicrobial activity, indicative of the accumulation or enhanced production of bioactive metabolites over the incubation period. The inhibition zones from the antagonistic assay of LAB isolates against *E. coli* and *Listeria* are shown in Figure 1.

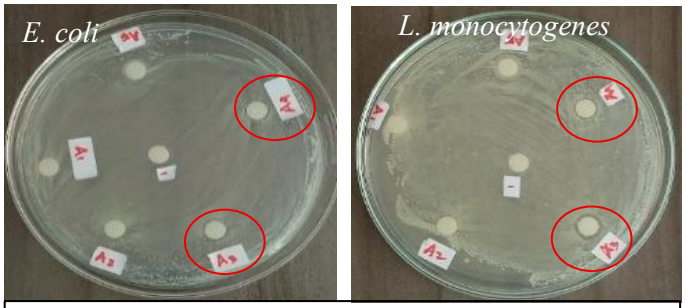
The results shown in Figure 1 indicate that the antagonistic activity of lactic acid bacteria (LAB) against pathogenic bacteria is evidenced by clear zones around the paper discs. In this study, clear zones were observed only after 24 hours of incubation, while no inhibition was detected at 18 or 21 hours, highlighting that the incubation period is critical for the expression of antagonistic activity in these isolates. Among the isolates, A3 and A4 produced the largest inhibition zones against *L. monocytogenes*, whereas A1 and A2 showed moderate activity, demonstrating isolate-specific differences in inhibitory potential. These observations suggest that the production of inhibitory compounds, such as organic acids, hydrogen peroxide, or bacteriocins, is closely linked to the growth phase of the LAB isolates. Consistent with previous studies [15,16], it is likely that the LAB cells entered the stationary phase around 24 hours, triggering the biosynthesis of secondary metabolites responsible for growth inhibition. Variation in inhibition zone diameters among the isolates may reflect differences in the timing or amount of antimicrobial metabolite production, suggesting that each isolate exhibits a unique antagonistic profile.



18 h incubation



21 h incubation



24 h incubation

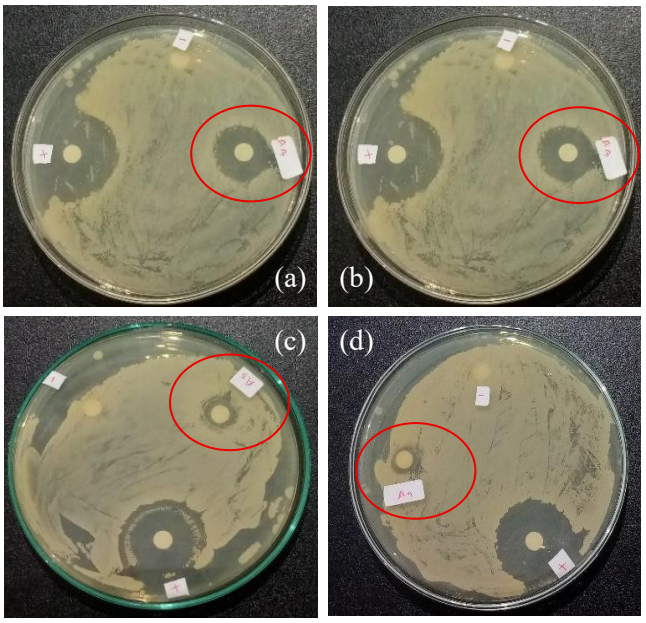
Figure 1. Antagonistic assay result of BAL isolates (A1, A2, A3, A4, and A5) against the test microorganism with different incubation times of LAB isolates

Under stress conditions, bacteria commonly synthesize secondary metabolites, including organic acids and bacteriocins, which function as defensive compounds and contribute to their antimicrobial activity. [17]. The inhibition zone at 24 hours indicates bacteriocin production activity when the isolate is in the stationary phase, and the concentration of the antibacterial compound is sufficient to inhibit the growth of *E. coli* and *L. monocytogenes* [18]. An inhibition zone was observed only for isolates incubated for 24 hours, likely because the antimicrobial metabolites had not yet reached the minimum inhibitory concentration (MIC) required to suppress pathogen growth.

During the exponential phase, LAB prioritize cellular division over the production of antagonistic compounds that serve as defensive metabolites. Postbiotic production begins during the logarithmic (log) phase and reaches peak antimicrobial activity at approximately 24–36 hours [19].

3.2. *Antibacterial Activity of BAL Isolates against E. coli and L. monocytogenes*

Antibacterial activity was assayed using supernatants of isolates A3 and A4 as test samples. Isolates A3 and A4 were chosen because they had the highest antagonistic activity in the previous assay. The inhibition zone of A3 and A4 supernatants against *E. coli* and *L. monocytogenes* is shown in Figure 2.



Note: (a) supernatant A3 against *E. coli*; (b) supernatant A4 against *E. coli*; (c) supernatant A3 against *L. monocytogenes*; (A3); (d) supernatant A4 against *L. monocytogenes*

Figure 2. Inhibition zone of antibacterial activity assay of A3 and A4 Isolates against the test microorganism

The antibacterial activity assay results revealed that A3 and A4, possess the ability to produce antimicrobial metabolites that effectively inhibit the growth of *Escherichia coli* and *Listeria monocytogenes*. This inhibitory effect was indicated by clear zones of inhibition surrounding the paper discs. Quantitative measurements of the inhibition zone diameters are presented in Table 2.

Table 2. Inhibition zone diameter in the antibacterial activity assay of lactic acid bacteria

	Inhibition Zones (mm)	
Isolates	*E. coli*	*L. monocytogenes*
A3	6.67 ± 2.08	4.67 ± 0.58
Control +	19.00 ± 3.00	17.33 ± 4.73
Control -	0	0
A4	4.33 ± 0.58	3.67 ± 0.58
Control +	19.00 ± 4.36	22.33 ± 4.16
Control -	0	0

Note:
control + (positive): chloramphenicol
control – (negative): MRSB

Isolate A3 showed higher inhibitory activity than A4, producing inhibition zones of 6.67 ± 2.08 mm against *E. coli* and 4.67 ± 0.58 mm against *L. monocytogenes*. In comparison, isolate A4 produced inhibition zones measuring 4.33 ± 0.58 mm and 3.67 ± 0.58 mm against the same test bacteria, respectively. Compared with other studies, some *L. plantarum* strains did not even form an inhibition zone against *E. coli* [20], whereas the isolates in this study produced larger inhibition zones against *L. monocytogenes* than those from Brazilian cheese. The growth of *L. monocytogenes* can be inhibited by the lactic acid bacteria strains *Lactiplantibacillus plantarum* and *Lactococcus lactis,* with inhibition zones of 4-5 mm [21].

In comparison with the positive control (chloramphenicol), which produced inhibition zones ranging from 17.33 to 22.33 mm, both isolates A3 and A4 exhibited lower antibacterial activity. According to the general classification of antibacterial activity, inhibition zones measuring 5–10 mm are considered moderate, while those measuring less than 5 mm are considered weak. Therefore, isolate A3 exhibits moderate antibacterial activity against E. coli and weak activity against L. monocytogenes, whereas isolate A4 shows weak activity against both test bacteria. The absence of inhibition zones in the negative control (MRSB) confirms that the observed antibacterial activity originates from metabolites produced by the lactic acid bacteria rather than components of the culture medium.

Isolate A3 exhibited higher antibacterial activity than A4 against *Escherichia coli* and *Listeria monocytogenes*, likely due to differences in the quantity and spectrum of antimicrobial metabolites produced. LAB can secrete various bioactive compounds, including bacteriocins, organic acids (such as lactic and acetic acids), and antimicrobial peptides, which can lower local pH and disrupt bacterial cell membrane integrity [22]. The inhibitory effect demonstrated by isolate A3 suggests a higher bacteriocinogenic potential or a higher concentration of secreted metabolites than in isolate A4. The bacteriocinogenic potential of LAB is crucial, as LAB produce peptides with antimicrobial activity via ribosomal pathways, which can form pores in the membranes of target bacterial cells [23].

Antimicrobial peptides synthesized by LAB can penetrate cell membranes and form pores; however, their effectiveness is influenced by the inherent compositional differences between Gram-negative and Gram-positive cell walls [25].

The outer membrane of Gram-negative bacteria can facilitate the uptake of small antimicrobial molecules. whereas the thick peptidoglycan layer in Gram-positive bacteria may impede diffusion and reduce efficacy. This differential barrier function has been documented in studies investigating the bacteriocin activity of lactic acid bacteria (LAB) against *Listeria monocytogenes* [26], and provides a mechanistic explanation for the generally smaller inhibition zones observed for *L. monocytogenes* compared to *E. coli.*

4. Conclusion

BAL isolates A3 and A4 from *cincalok* demonstrated antibacterial activity against *E. coli* and *L. monocytogenes*, although the inhibition was relatively weak (<5 mm). Despite this moderate activity, isolate A3 shows potential as a natural source of antibacterial metabolites for applications as food preservatives or probiotic agents. The antibacterial activity was influenced by factors such as the species of test microorganisms, incubation time, and environmental conditions, highlighting the need for optimization. Future studies should focus on testing these isolates against a wider range of pathogenic bacteria, optimizing growth and environmental conditions to enhance metabolite production, characterizing the specific bioactive compounds, and evaluating their safety, stability, and potential application in food products. These findings provide a foundation for using LAB from Cincalok to improve food safety and develop functional foods.

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