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Enhancement of antibacterial compound production in lactic acid bacteria by co-cultivation

Villanueva Adrian Ray B*, Sepelagio Elma G, Besana Cyrelle M, Jumao-as Cromwel M, Tanabe Maria Elena N

Department of Biological Sciences, College of Science and Mathematics, University of Southern Mindanao, Kabacan North Cotabato, Philippines.

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ABSTRACT

The rising threat of antibiotic resistance necessitates the development of alternative antimicrobial strategies. Lactic Acid Bacteria (LAB), are GRAS or generally recognized as safe, produce antibacterial compounds with potential applications in food safety and preservation. This study aimed to optimize the production of antibacterial compounds by LAB isolates from goat's milk through co-cultivation. Three LAB isolates were co-cultivated under varying pH levels (5.5, 6.0, and 6.5) and incubation temperatures (25°C, 30°C, and 37°C). Their antimicrobial activity was evaluated against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* using the Kirby-Bauer disc diffusion method. Co-cultivation significantly enhanced antimicrobial activity compared to individual LAB isolates, producing inhibition zones of up to 22.76 mm against *B. subtilis* and 17.5 mm against *S. aureus*. In contrast, weaker inhibition was observed against *E. coli* and *P. aeruginosa*, with zones below 12 mm. Co-cultivation of LAB isolates from goat's milk enhances the yield and efficacy of antibacterial compounds, offering a promising approach for mitigating antibiotic-resistant pathogens and improving food safety applications.

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1. Introduction

The increasing global concern regarding antibiotic resistance has led to an urgent need to explore sustainable, natural alternatives to synthetic antimicrobials.

*Corresponding author. Tel.: 09660249110
E-mail address: adrianrayb.villanueva@gmail.com

Antibiotic-resistant pathogens are responsible for prolonged illnesses, increased healthcare costs, and higher mortality rates, particularly in developing countries (1,2). One promising group of microorganisms are Lactic Acid Bacteria (LAB). LAB are gram-positive, non-spore-forming, rod- or cocci-shaped bacteria that ferment carbohydrates to produce lactic acid. They are generally classified as GRAS

(Generally Recognized as Safe) and are commonly found in dairy products, fermented vegetables, and the gastrointestinal tract (3,4). LAB are notable for producing a broad spectrum of antibacterial compounds, including bacteriocins, organic acids, hydrogen peroxide, and diacetyl. These bioactive substances enable LAB to inhibit spoilage organisms and foodborne pathogens, positioning them as valuable agents in food safety and human health applications (1,5).

Milk, particularly goat's milk, serves as an excellent medium for isolating LAB due to its rich nutritional profile. Goat's milk contains essential macronutrients and micronutrients.

Contamination of milk can occur at multiple points, including during milking, handling, and storage. Microbial contaminants such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., and *Pseudomonas aeruginosa* are frequently isolated from raw milk and pose serious health risks. Furthermore, chemical contaminants, including residues of veterinary drugs, pesticides, and heavy metals, can accumulate in milk and compromise its safety (6,7). These issues highlight the need for natural antimicrobial systems that not only extend milk's shelf life but also reduce reliance on synthetic preservatives and antibiotics.

Previous studies (8) have explored the antimicrobial potential of LAB isolates, demonstrating their ability to produce various metabolites such as organic acids, hydrogen peroxide, and bacteriocins. These compounds are known to inhibit a broad spectrum of microorganisms, especially gram-positive bacteria. However, most of these studies have focused on individual strains such as *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus acidophilus*, which often exhibit limited inhibition against gram-negative bacteria due to the presence of an outer membrane that acts as a barrier to many antimicrobial compounds (6). Moreover, many studies lack optimization of culture conditions that could maximize antimicrobial compound production. The co-cultivation of LAB growing multiple LAB strains together has shown promise in enhancing bacteriocin output and expanding antimicrobial activity, yet it remains underexplored, particularly in LAB from goat's milk (9,10).

2. Materials and Methods

2.1. Source of LAB isolates

Putative isolates of LAB were obtained from prior studies at the Department of Biological Sciences, College of Science and Mathematics, University of Southern Mindanao, Kabacan, Cotabato, Philippines. All isolates were maintained in MRS (de Man, Rogosa, and Sharpe) broth at 4°C until further use.

2.2. Indicator bacterial strains

The indicator bacterial strains used in the study included *Bacillus subtilis* BIOTECH 1679, *Staphylococcus aureus* BIOTECH 1582, *Pseudomonas aeruginosa* BIOTECH 1582, and *Escherichia coli* BIOTECH 1634.

2.3. Reagents

MRS broth and MRS agar (HiMedia, India) were used for culturing LAB isolates. Mueller-Hinton Agar (MHA) (Condolab, Spain) was used for antimicrobial testing. Nystatin (ACME Laboratories, Bangladesh) was used to prevent fungal contamination. Sterile phosphate-buffered saline (PBS) (pH 7.4, UFC Bio, USA) was used for bacterial standardization. Ampicillin (Sandoz Gmbh, Austria) served as a positive

control, while sterile distilled water served as negative control.

2.4. Isolation and characterization of LAB

2.4.1. Selection and purification of LAB Isolates

LAB isolates were streaked onto MRS agar plates using the quadrant streak method and incubated at 37°C for 24–48 h. Well-isolated colonies were sub-cultured onto fresh MRS agar to obtain pure cultures. Morphological and microscopic analyses were performed using gram staining, to confirm LAB characteristics (11).

2.5. Antimicrobial activity assay

2.5.1. Kirby-Bauer disc diffusion method

Isolates were cultured in 15 mL MRS broth tubes, while the indicator strains were grown and standardized to a 0.5 McFarland standard (~1.5 × 10^8 cfu/mL) using phosphate-buffered saline (PBS, pH 7.4). A sterile cotton swab was used to evenly inoculate MHA plates with the indicator organism, and then sterile paper discs were impregnated with LAB cell-free supernatant before being placed onto inoculated MHA plates. Ampicillin was used as a positive control, and sterile distilled water as a negative control (12). Plates were incubated at 35°C for 18 h. Zones of inhibition were measured with vernier calipers and interpreted according to Klewicka & Libudzisz (13).

2.6. Co-cultivation and antimicrobial compound production

2.6.1. Co-cultivation of LAB Isolates

Selected LAB isolates were activated by three successive transfers in MRS broth, incubated at 37°C for 18–24 h under anaerobic conditions. For co-cultivation, 250 mL Erlenmeyer flasks containing MRS broth were inoculated with LAB pairs (0.01 µL each) standardized to a 0.5 McFarland standard. Parameters such as initial pH (5.5, 6.0, 6.5), incubation temperature (25°C, 30°C, 37°C), and incubation time (12, 24, 48 h) were systematically optimized (14).

2.6.2. Antimicrobial activity of co-culture supernatants

After incubation, co-cultures were centrifuged to obtain cell-free supernatants. The Kirby-Bauer disc diffusion assay was repeated as described above, using co-culture supernatants. Sensitivity to enzymatic degradation was assessed by treating supernatants with catalase (15,16).

2.7. Statistical analysis

All data were tested for normality using the Shapiro-Wilk test. As the data did not meet normality assumptions, Kruskal-Wallis test was used to analyze the differences in the antimicrobial inhibition zones between individual and co-cultivated LAB isolates. All statistical analyses were done using Jamovi 2.3.28 software, with the significance level set at $p<0.05$ (17,18).

3. Results

3.1. Characteristics of putative isolates of LAB isolated from goat milk

Seven bacterial isolates were morphologically characterized as gram-positive and exhibited uniform colony morphologies – circular in shape, with smooth margins and convex elevations. Their sizes ranged from small to large, and their coloration from white to milky white, with some appearing slightly translucent and with a morphology of rod in pairs and in short chains.

3.2. Antimicrobial activity of individual LAB putative isolates

LAB isolates 5, 6, and 7 demonstrated inhibitory activity against *S. aureus* and *B. subtilis*. However, none of the isolates showed inhibition against the gram-

negative test organisms. In contrast, the positive control, Ampicillin, exhibited inhibition against *E. coli, B. subtilis*, *S. aureus*, and *P. aeruginosa*.

According to the criteria established by Klewicka & Libudzisz (13) for assessing the antagonistic activity of LAB, isolates 5, 6, and 7 demonstrated a moderate inhibitory effect on *S. aureus* and weak inhibitory effect on *B. subtilis*. All three isolates showed no inhibitory activity against gram-negative indicator strains, as detailed in Table 1 and 2. Furthermore, based on CLSI (19) guidelines, *S. aureus, B. subtilis, E. coli, and P. aeruginosa* are susceptible to Ampicillin (according to Fig. 1, 2, 3).

3.3. Optimized conditions for the production of antimicrobial compounds

At pH 5.5, with a temperature of 30°C and an incubation period of 24 h, all LAB combination isolates showed weak and moderate inhibitory activity against *Escherichia coli* and *Pseudomonas aeruginosa,* while exhibiting strong inhibition against *Bacillus subtilis* and *Staphylococcus aureus*. At pH 6.0, with a temperature of 37°C and an incubation time of 12 h, all LAB combination isolates displayed weak inhibitory action on *P. aeruginosa*, moderate inhibition against *E. coli*, and strong inhibitory activity against *B. subtilis* and *S. aureus*. Similarly, at pH 6.5, with a temperature of 25°C and an incubation duration of 48 h, all LAB combination isolates demonstrated weak inhibitory activity against *E. coli* and *P. aeruginosa*, while maintaining strong inhibition against *B. subtilis* and *S. aureus*.

3.4. Antimicrobial activity of co-cultivated pairs of LAB isolates

Three pairs of co-cultivated lactic acid bacteria isolates exhibited significant inhibitory activity against *E. coli, P. aeruginosa, B. subtilis, and S. aureus.* The inclusion criteria as referenced to the study of Maldonado et al. (10), Hartmann et al. (20) and Calasso et al. (21) to combine LAB strains 5 & 6, 6 & 7, and 7 & 5 for antimicrobial testing arose from their demonstrated inhibitory activity of individual LAB isolates against *Staphylococcus aureus* and *Bacillus subtilis*, while showing no inhibitory effects against other test organisms such as *Escherichia coli* and *Pseudomonas aeruginosa*. This antimicrobial efficacy suggests that these specific combinations could potentially enhance the overall effectiveness of the LAB strains against the targeted pathogens. In this case, isolates LAB 5, 6, and 7 exhibited notable inhibitory activity against the gram-positive bacteria *S. aureus* and *B. subtilis*, indicating their potential as effective antimicrobial agents. The combinations of these strains may lead to synergistic effects, where the interaction between the strains enhances their individual antimicrobial capabilities.

According to the assessment criteria established by Klewicka & Libudzisz (13) for evaluating the antagonistic activity of co-cultivated pairs of LAB, the results indicate varying degrees of inhibitory effects against different pathogenic bacteria. Specifically, LAB Combination 5&6 and LAB Combination 7&5 exhibited a weak inhibitory effect on *Escherichia coli*, whereas LAB combination 6&7 demonstrated a moderate inhibitory effect against the same pathogen. In contrast, LAB combination 5&6, 6&7, and 7&5 showed a strong inhibitory effect on *Bacillus subtilis* and *Staphylococcus aureus*, indicating their potential efficacy against these common pathogens.

Table 1. Zone of inhibition of LAB isolates

Zone of inhibition in mm (mean) and inhibition response (IR)								
LAB isolates	**EC**	**IR**	**PA**	**IR**	**BS**	**IR**	**SA**	**IR**
5	6.0	-	6.0	-	11.13	+	15.14	++
6	6.0	-	6.0	-	10.14	+	14.08	++
7	6.0	-	6.0	-	11.11	+	12.17	++
+ control	15.19	S	21.48	S	37.28	S	29.89	S
- control	6.0	N/A	6.0	N/A	6.0	N/A	6.0	N/A

*Symbols stand for the following: +++ = strong; ++ = moderate; + = weak; and - = negative
*Letter codes stand for the following: EC = *Escherichia coli* BIOTECH 1634; PA = *Pseudomonas aeruginosa* BIOTECH 1335; BS= *Bacillus subtilis* BIOTECH 1679; and SA = *Staphylococcus aureus* BIOTECH 1582; S= Susceptible.
* Basis: CLSI (2023), Klewicka & Libudzisz (2004)

Table 2. Antimicrobial activity of co-cultivated pairs LAB putative isolates against different test organisms

LAB isolates combination	pH	Temperature (°C)	Incubation time (h)	Zone of inhibition (mm) & inhibition response (IR)							
				EC	**IR**	**PA**	**IR**	**BS**	**IR**	**SA**	**IR**
LAB 5 + LAB 6	5.5	30°C	24 h	8.61	+	6.6	+	19.84	+++	17.02	+++
	6.0	37°C	12 h	7.89	+	6.2	+	20.93	+++	17.18	+++
	6.5	25°C	48 h	***11.3**	+	***7.03**	+	***22.76**	+++	***17.2**	+++
LAB 6+ LAB 7	5.5	30°C	24 h	12.27	++	6.23	+	17.13	+++	17.26	+++
	6.0	37°C	12 h	12.18	++	6.4	+	17.23	+++	17.12	+++
	6.5	25°C	48 h	***12.33**	++	***7.13**	+	***17.26**	+++	***17.3**	+++
LAB 7 + LAB 5	5.5	30°C	24 h	6.51	+	6.7	+	18.64	+++	17.23	+++
	6.0	37°C	12 h	6.85	+	6.8	+	18.91	+++	17.39	+++
	6.5	25°C	48 h	***8.2**	+	***7.1**	+	***19.5**	+++	***17.5**	+++
+ control	N/A	N/A	N/A	15.19	S	21.48	S	37.28	S	29.89	S
- control	N/A	N/A	N/A	6.00	N/A	6.00	N/A	6.00	N/A	6.00	N/A

*Symbols stand for the following: +++ = strong; ++ = moderate; + = weak; and - = negative
*Letter codes stand for the following: EC = *Escherichia coli*; PA = *Pseudomonas aeruginosa*; BS = *Bacillus subtilis*; and SA = *Staphylococcus aureus*; S= Susceptible
* Basis: CLSI (2023), Klewicka & Libudzisz (2004)

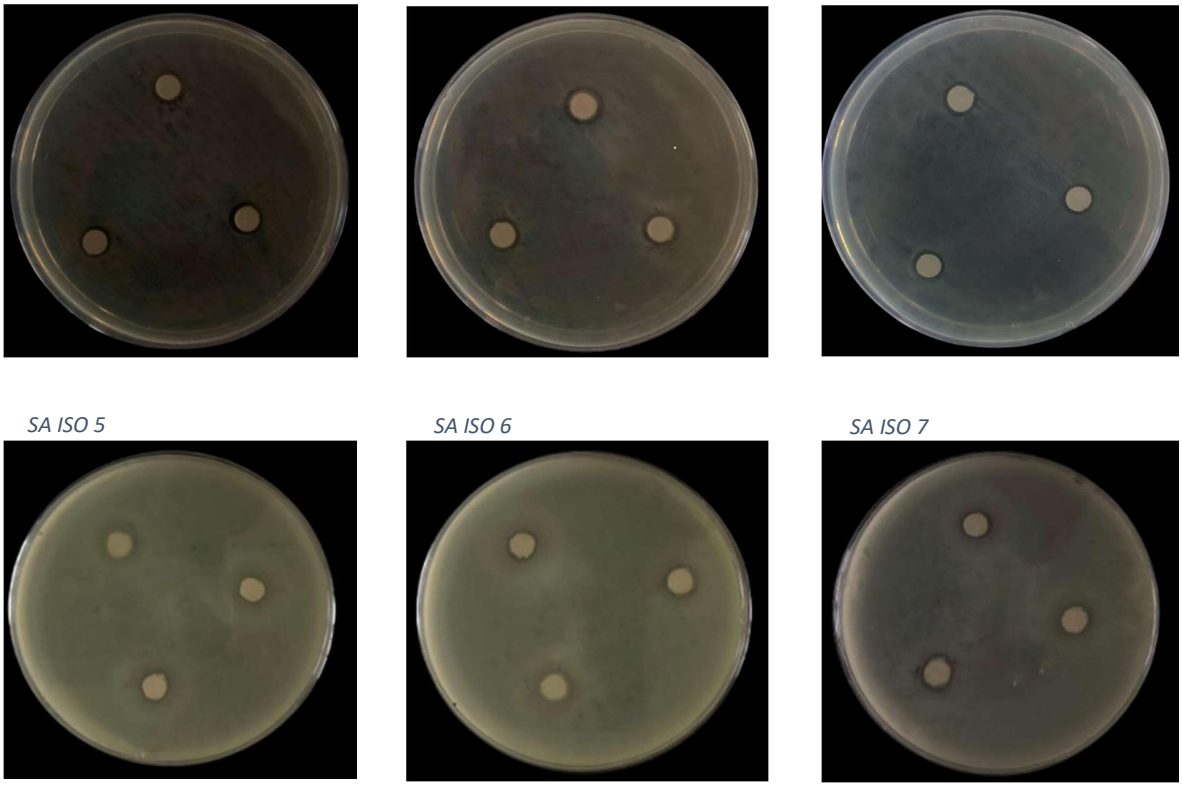

Figure 1. Antagonistic activity of isolate BS-ISO5, BS-ISO6, BS-ISO7, SA-ISO5, SA- ISO6, and SA-ISO7.

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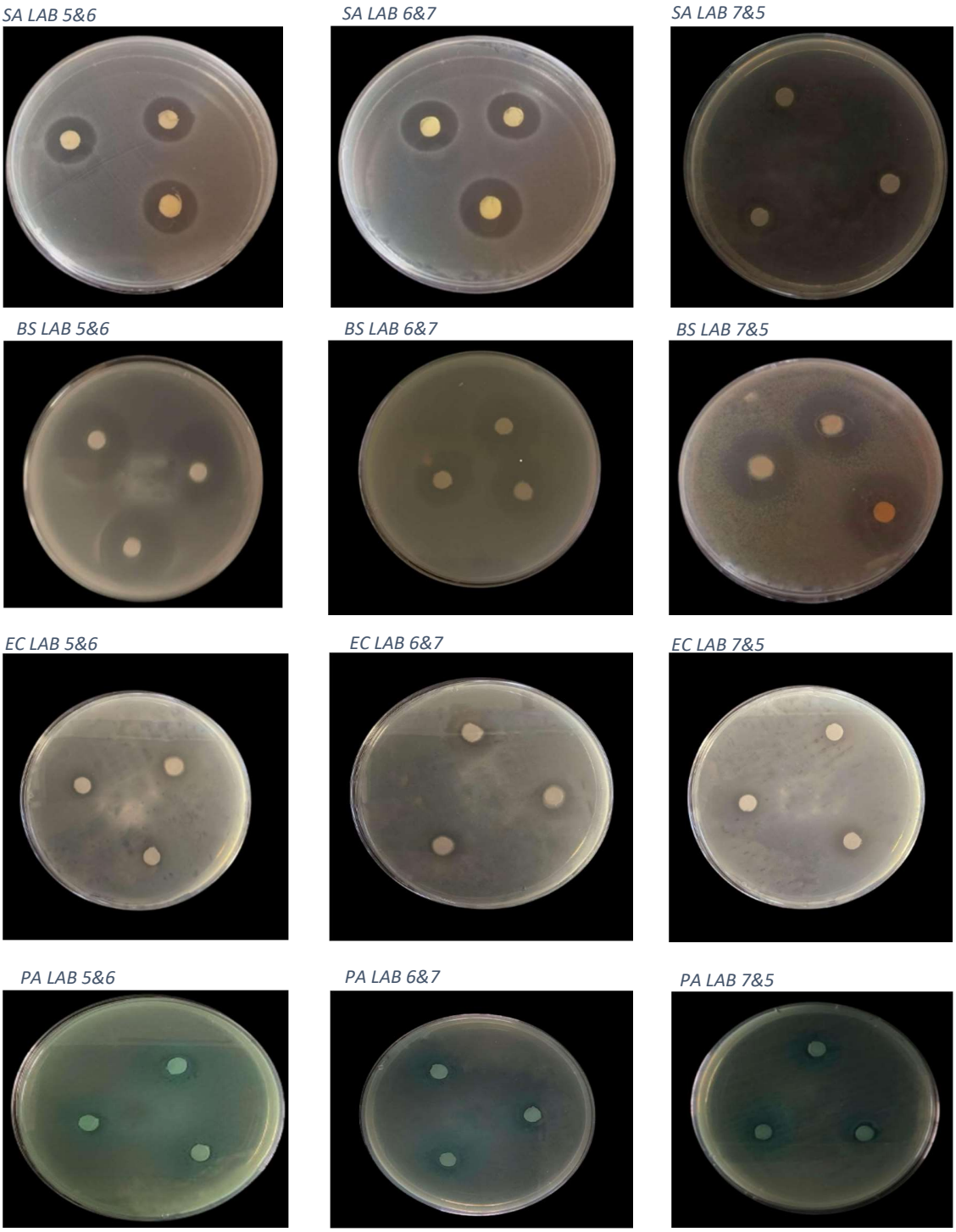

Figure 2. Antagonistic activity of LAB isolate combination 5&6, 6&7 and 7&5

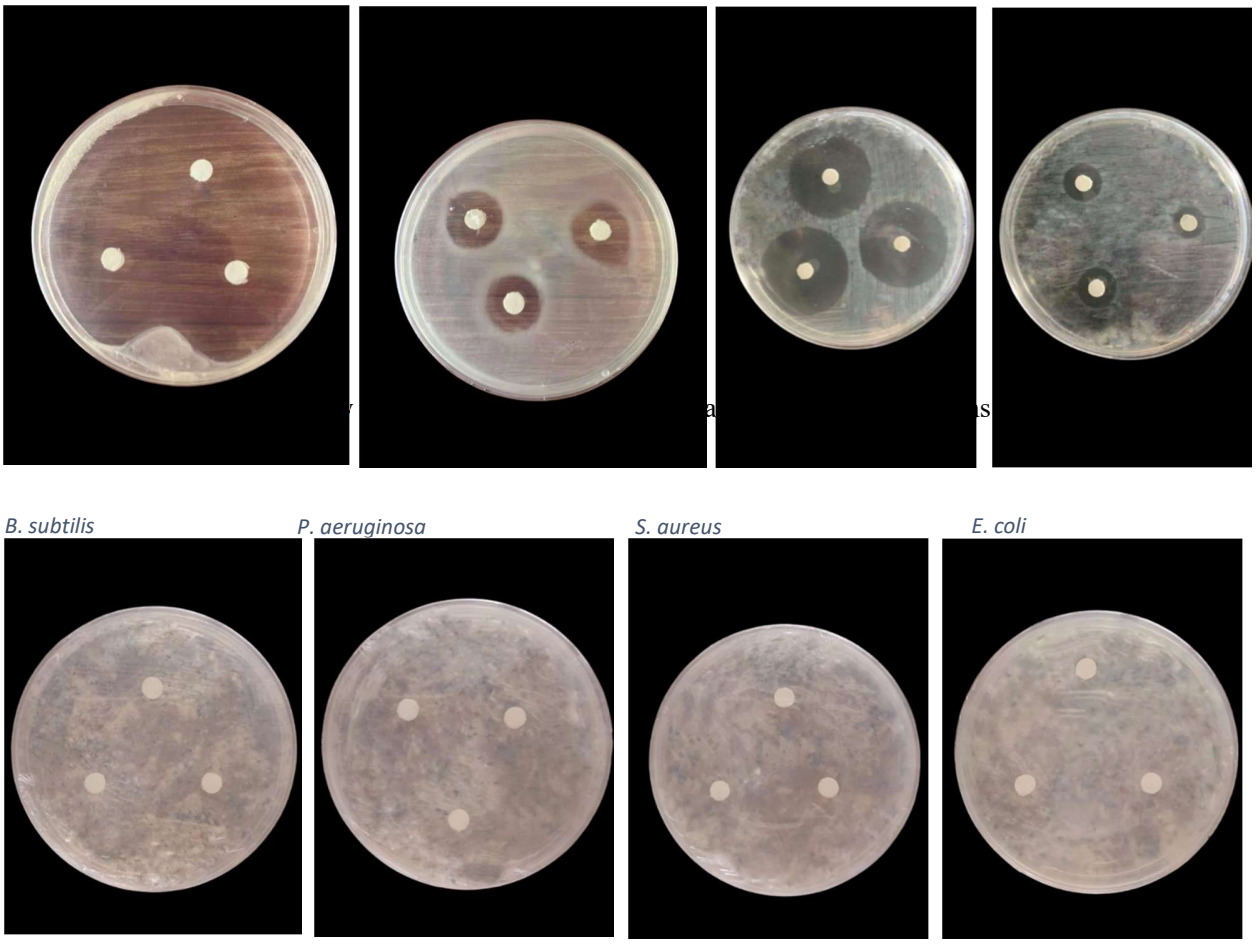

Figure 3. Antibacterial susceptibility testing of positive controls against the indicator strains.

However, all three pairs of LAB isolates showed weak inhibitory action on *Pseudomonas aeruginosa*, suggesting limited effectiveness against this particular strain. Notably, optimizing parameters on pH at 6.5, temperature at 25°C, and a 48h incubation period revealed greater production of antimicrobial compounds that demonstrated larger zones of inhibition on test organisms.

The antimicrobial activity of co-cultivated LAB isolates presents intriguing insights into their potential applications in food preservation and health. The results indicate that LAB combination 5&6 exhibits weak inhibitory action against *Escherichia coli* and *Pseudomonas aeruginosa*, while demonstrating strong inhibition of *Staphylococcus aureus* and strong inhibition against *Bacillus subtilis*. In contrast, LAB combination 6&7 shows moderate inhibition on *E. coli* and have strong inhibition against *B. subtilis, and S. aureus*, with weak action against *P. aeruginosa*. Lastly, LAB combination 7&5 reflects weak inhibitory action on both *E. coli* and *P. aeruginosa*, while having a strong inhibition on *B. subtilis* and *S. aureus*. These variations in antimicrobial activity suggest that specific combinations of LAB can influence the spectrum and strength of inhibition against different pathogens.

3.5. Comparison of antimicrobial activity of individual LAB and co-cultivated LAB isolates

Kruskal-Wallis test showed that there was a significant difference in the inhibition zones between individual and co-cultivated isolates across all four test organisms ($p>0.05$). The highest level of variation was observed against *Escherichia coli* ($\chi^2(5) = 15.8$, $p = 0.008$), indicating that co-cultivation had the strongest effect in enhancing inhibition against this gram-negative pathogen. Post hoc analysis (inferred through pairwise zone comparisons) showed that LAB combination 6&7 achieved the most notable inhibitory effect against *E. coli* with a mean zone of 12.33 mm, classified as moderate inhibition. This is particularly significant given that all individual LAB isolates (5, 6, and 7) demonstrated no activity against *E. coli* (6.00 mm), highlighting a synergistic effect through co-cultivation that overcame some resistance mechanisms typically presented by gram-negative outer membranes.

The second highest result was found for *Bacillus subtilis* ($\chi^2(5) = 15.3$, $p = 0.009$), where all co-cultivated combinations (5&6, 6&7, 7&5) displayed strong inhibitory activity (zones >17 mm), outperforming individual isolates which only achieved weak inhibition (10.14–11.13 mm). For *Staphylococcus aureus*, the Kruskal-Wallis test also confirmed a significant difference ($\chi^2(5) = 15.1$, $p = 0.010$). LAB combinations demonstrated strong inhibitory zones (17.02–17.5 mm), clearly surpassing the performance of individual isolates (12.17–15.14 mm, moderate inhibition). Notably, combination 7&5 yielded the largest inhibition zone (17.5 mm), indicating potent synergistic activity.

The lowest result was for *Pseudomonas aeruginosa* ($\chi^2(5) = 14.4$, $p = 0.013$). Despite the statistical significance, the actual inhibition remained weak across all treatments, with mean zones below 7.2 mm for both individual and co-cultivated LAB isolates.

4. Discussion

These findings are consistent with existing literature that describes LAB colonies as typically circular, with entire margins and smooth surfaces (22-24). Suryani et al. (25) reported similar findings where LAB colonies displayed a yellowish-white color and circular shape

with glistening convex elevations. LAB typically appear as rod-shaped bacteria that can occur in pairs or short chains (26). The variations in size and coloration among the colonies may indicate different strains or species within the LAB group, which could possess unique metabolic capabilities or functional properties.

Abachi et al. (27) recorded inhibition zones of 11–16 mm against *B. subtilis* in LAB isolated from fermented dairy, and Djadouni and Kihal (28) reported *S. aureus* inhibition between 13.5 mm and 18.3 mm in LAB from raw cow milk.

The findings indicated that the inhibitory activity of LAB isolates is limited. LAB are known to produce various antimicrobial substances such as bacteriocins, hydrogen peroxide, and organic acids, which contribute to their inhibitory effects (29). However, in the context of this study, the involvement of hydrogen peroxide is unlikely since all tested indicator strains are catalase-positive, suggesting they can neutralize hydrogen peroxide. Additionally, the potential impact of organic acids was mitigated by employing a phosphate buffer in the Mueller-Hinton agar (MHA), confirming that the observed antagonistic activity stems from other antimicrobial agents (30,31). LAB are known to produce a variety of antimicrobial substances, including bacteriocins, which specifically target related gram-positive species. Research has demonstrated that LAB isolates exhibit significant antibacterial activity against pathogens such as *Staphylococcus aureus* and *Bacillus cereus*, with inhibition zones indicating strong efficacy (32).

The observed lack of inhibition of lactic acid bacteria (LAB) isolates against gram-negative bacteria, particularly *Escherichia coli* and *Pseudomonas aeruginosa*, can be attributed to several intrinsic and extrinsic factors related to the structural characteristics of these pathogens and the nature of the antimicrobial compounds produced by LAB. Gram-negative bacteria possess a unique outer membrane composed of lipopolysaccharides (LPS), which acts as a formidable barrier against many antimicrobial agents, including those produced by LAB (33,34).

Maldonado et al. (10), Hartmann et al. (20) and Calasso et al. (21), which reported that slightly acidic pH levels enhance LAB metabolic activity while suppressing the growth of competing pathogens. The temperature ranges of 25°C, 30°C and 37°C is consistent with the physiological preferences of LAB, as observed by Maldonado et al. (10). The pH range not only supports LAB growth but also enhances their ability to produce bacteriocins and organic acids that inhibit pathogenic bacteria, while the moderate temperature range facilitates essential metabolic processes for antimicrobial production. Prolonged incubation times allow synergistic interactions among co-cultivated LAB strains, further increasing metabolite output; however, extended incubation beyond 48 h may lead to nutrient depletion or metabolite degradation, reducing antimicrobial efficacy.

Gänzle (35) highlighted that LAB produce various antimicrobial compounds, with their production being strongly influenced by environmental factors like pH and temperature. Similarly, Furtado et al. (36) reported that fermentation conditions, including an optimal pH of around 6.5 and temperatures near 25°C, are critical for maximizing antimicrobial metabolite production in microorganisms. Additionally, Liao and Nyachoti (37) emphasized that maintaining these specific conditions

enhances the production of inhibitory compounds by LAB, leading to greater zones of inhibition against pathogenic bacteria.

Co-cultivation of LAB isolates may enhance their antimicrobial properties through synergistic interactions. The presence of multiple LAB strains can lead to the production of a wider array of antimicrobial compounds which are known to contribute to the overall inhibitory effects observed (38,39). For instance, the stronger inhibitory action of combination 5&6, 6&7 and 7&5 against *B. subtilis* could be attributed to the synergistic effect of metabolites produced by the co-cultured strains, which may not be as effective when cultured individually. Moreover, the observed moderate inhibition of *E. coli* by combination 6&7 suggests that certain LAB strains possess specific antagonistic properties that can be enhanced through co-cultivation. Lactic acid bacteria can exert varying degrees of antimicrobial activity depending on their combinations (40,41). The antimicrobial compound produced by the co-cultivated lactic acid bacteria (LAB) isolates is catalase negative. This characteristic is significant because catalase is an enzyme that decomposes hydrogen peroxide, a reactive oxygen species that can be harmful to cells. The absence of catalase in these LAB isolates suggests that their antimicrobial activity may not rely on the production of hydrogen peroxide, which is often a common defense mechanism in bacteria. Instead, the antimicrobial effects might be attributed to other mechanisms such as the production of organic acids or bacteriocins, which do not involve catalase activity (42,43). Bacteriocins produced in co-culture exhibit broader and more potent activity due to inter-strain metabolic crosstalk and gene upregulation (9,35).

P. aeruginosa's robust outer membrane and efflux pump systems render it inherently less susceptible to bacteriocins and organic acids produced by LAB (43,44). Interestingly, although co-cultivation did not drastically enhance inhibition, slight increases in activity (e.g., 7.13 mm in 6&7) suggest a marginal improvement that may be optimized further through metabolic engineering or synergistic applications with other antimicrobial agents.

with other antimicrobial agents.

5. Conclusion

This study demonstrated that co-cultivation of LAB isolated from goat's milk significantly enhances the production and antimicrobial activity of antibacterial compounds, compared to individual LAB cultures. The optimized conditions—pH 6.0, 37°C, and 12–24 h of incubation—resulted in inhibition zones up to 22.76 mm against *Bacillus subtilis* and 17.5 mm against *Staphylococcus aureus*. These findings highlight the potential of co-cultivated LAB as natural bio preservatives for controlling gram-positive pathogens, contributing to food safety and potential applications in healthcare and agriculture. The antibacterial compounds produced by the LAB isolates were not purified or chemically characterized, limiting our understanding of the specific active components. Additionally, gram-negative bacteria exhibited limited susceptibility, and only four indicator organisms were tested, which may not represent the broader spectrum of pathogens.

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Author contributions

Co-authors' contributions have been organized according to Credit roles as follows: Elma G. Sepelagio: conceptualization, supervision, writing – revision and editing- original draft. Cyrelle M. Besana: supervision, conceptualization, writing - revision and editing. Cromwell M. Jumao-as: writing-revision, conceptualization, methodology. Maria Elena N. Tanabe: writing-revision, conceptualization, methodology.

Conflict of interests

The authors declare that they have no personal or financial interests to disclose in connection with this research. None of the authors has any financial or personal relationships with other individuals or organizations that could inappropriately influence or bias their work.

Data availability

Data generated and analyzed during this study are available on request from the authors. We encourage transparency and are willing to share data to support future research. Data will be provided subject to requests respecting the ethical and confidentiality conditions established during the study.

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