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Research Article
Lactobacillus plantarum IIA-1A5 Fermentation Patterns by Using whey, buttermilk and Whey Enriched by Skimmed Milk as Growth Media

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Abstract
Background and Objective: Whey is a byproduct of the cheese industry in Indonesia, while buttermilk is a byproduct from processing butter that has not been used in Indonesia. Whey and buttermilk residues have a very high nutrient value, especially for the growth of microbes. *Lactobacillus plantarum* (*L. plantarum*) IIA-1A5 is a lactic acid bacteria that was isolated from Indonesian local beef, Peranakan Ongole (PO). The availability of nutrients in whey and buttermilk provides an opportunity to utilize both as economically valuable growth media specifically for *L. plantarum* IIA-1A5. The objective of this research was to evaluate the *L. plantarum* IIA-1A5 fermentation pattern by using different growth media, whey but termilk and whey+skim (whey that was enriched by skimmed milk). **Materials and Methods:** *Lactobacillus plantarum* IIA-1A5 was grown in 3 different media: whey buttermilk and whey enriched by skim milk. The bacterial population and pH value were analyzed every 4 h during fermentation. The chemical composition of each media was determined. The best growth media was selected based on the growth rate and generation time of *L. plantarum* IIA-1A5 during fermentation. Crude antibacterial substances were collected from the best media and analyzed for antimicrobial activity against pathogenic bacteria. **Results:** Whey and buttermilk had a good nutritive value, including a high amino acid content and allowed complete growth of *L. plantarum* IIA-1A5. Whey+skim was the best growth medium for *L. plantarum* IIA-1A5 based on the bacterial generation time, which reached 1.96 h. *Lactobacillus plantarum* IIA-1A5 produced an antimicrobial substrate during a 20 h fermentation process. The cell-free supernatant, as an antimicrobial substrate, could inhibit pathogenic bacteria such as *Staphylococcus aureus* and *Escherichia coli*. **Conclusion:** The best medium for *L. plantarum* growth was whey+skim, based on the growth media composition, LAB population, growth rate, generation time and antimicrobial activities.

Key words: Fermentation, growth media, *Lactobacillus plantarum* IIA-1A5, whey buttermilk, whey+skim

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In Indonesia, milk consumption is approximately 11.8 L per capita per year and includes processed milk products[1]. Milk is consumed by people not only as fresh milk but also as processed milk products such as cheese. Cheese is a processed milk product that is created by coagulating casein and has a byproduct, whey; the yield of whey exceeds the yield of cheese itself. One kilogram of cheese is produced from approximately 10 L of fresh milk and the whey yield is approximately 8-9 L. Whey contains 4.7 g L^{-1} lactose, 5.6 g L^{-1} protein, 0.5 g L^{-1} fat, lactic acid and minor nutrients such as lactoferrin, lactoperoxidase, lysozyme, immunoglobulin, iron, iodine and vitamins[2].

Lactic acid bacteria (LAB) are generally found in milk, fresh meat, vegetables and their byproducts. The use of LAB as a starter culture in fermenting meat, milk, vegetables, fruits and their byproducts is the oldest method in food processing that results in a specific flavor[3]. One LAB is *Lactobacillus plantarum*. *Lactobacillus plantarum* IIA-1A5 is a LAB that was isolated from Indonesian local beef, Peranakan Ongole (PO). This LAB has characteristics of a probiotic and produces an antimicrobial substrate called plantaricin IIA-1A5[4,5].

Today, *Lactobacillus plantarum* (*L. plantarum*) IIA-1A5 is produced by using specific commercial media, de Mann Rogosa Sharpe broth (MRSb), which is relatively expensive and is not available at all times. Therefore, other alternative growth media that are affordable and easier to find, such as whey and buttermilk, is needed. Whey is a liquid that is obtained from processing cheese, casein or similar products by separating the liquid from the curd after the milk coagulation process by using a rennet enzyme[6]. Bylund[7] stated that buttermilk is a byproduct from butter processing. Pescuma *et al.*[8] stated that liquid whey contains lactose (5%), water (93%), protein (0.85%) and minerals (0.53%). Buttermilk is the leftover liquid after churning in the production of butter[9]. Buttermilk is a byproduct of butter production and contains 3.5% fat and lecithin[7]. The availability of nutrients in whey and buttermilk provides an opportunity to utilize it as growth media specifically for *L. plantarum* IIA-1A5 and for LAB in general. The objective of this research was to evaluate the *L. plantarum* IIA-1A5 fermentation pattern by using different growth media, whey buttermilk and whey+skim (whey that was enriched by skim).

MATERIALS AND METHODS

Buttermilk production: First, fresh cow milk was separated into skim and cream by using a cream separator (Milky FJ 130 ERR). Then, the cream was pasteurized at approximately 80-85°C for approximately 5 min. After that, the cream was cooled in a refrigerator to a temperature of approximately 4-80°C and the aging process lasted for approximately 12 h (overnight). Then, the cream was placed into the churner and the churning process was performed for approximately 30-45 min at 10°C. After the churning process was performed, the solid butter was separated from its buttermilk liquid. Then, the solid butter was placed into a kneader to undergo the kneading process. This process separated the butter and buttermilk liquid. The buttermilk liquid was collected for the next experiment.

Whey production: Milk was pasteurized at 62-65°C for approximately 30 min and then the milk was cooled to reach a temperature of approximately 30-40°C. After reaching 30-40°C, rennet was inoculated into the pasteurized milk at a concentration of 0.02 g per 1 L of milk. After approximately 30-60 min, the milk coagulated and curd was formed. This process was followed by a scalding process at 50°C until all the casein was transformed into curd and whey was left behind in the process. The curd was used to make cheese and the liquid was whey. The whey was collected and stored in a freezer for the next experiment.

Making whey+skim: First, whey and skim milk were solid non fat (SNF) standardized to buttermilk SNF. By using the Pearson square method, the composition of the whey and skim milk was formulated. After mixing whey with skim milk, the whey+skim was pasteurized at 62-65°C for approximately 30 min. Then, the whey+skim was collected and stored in a freezer for the next experiment.

Growth media chemical characteristic analysis: The chemical qualities of the growth media, including the fat, solid non fat, protein and lactose contents, density and freezing point, were analyzed using Lactoscan SP60 serial number 0129. Approximately 25 mL each of whey buttermilk and whey+skim was taken at and then placed into a cuvette (25 mL). The cuvette was put into the Lactoscan and the start button was pushed; the result came out automatically.

Amino acid composition of the growth media: The amino acid composition of whey and buttermilk was determined by using liquid chromatography on an Agilent 1200 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) according to Arief *et al.*[10]. Samples of growth media for whey and buttermilk were hydrolyzed with acid and then five microliters

of the sample was injected into the HPLC column. The amino acid separation was completed in approximately 30 min. The amino acids were separated using a ZORBAX SB RRHT C18 column (21×50 mm) with a 1.8 µm particle size (Agilent Technologies). Buffer A was 0.5 mM tridecafluoroheptanoic acid (TDFHA) in HPLC-grade water and buffer B was 100% acetonitrile. The initial ow rate was 2.4 mL min^{-1}. Separation was accomplished using a gradient recommended by the company (Agilent Technologies) and was maintained at 95°C under 550 bar. The chromatography was analyzed using the Agilent 1100 series diode-array detector at a data rate of 80 hz. The amino acid concentration in the sample was calculated according to the method described by Arief *et al.* [10].

$$\text{AA concentration}(\mu\text{mol}) = \frac{\text{Peak area sample}}{\text{Peak area standard}} \times \text{Standard concentration}$$

Lactic acid bacteria cultivation: *Lactobacillus plantarum* IIA-1A5 was refreshed from stock culture by using MRS (de Mann Rogosa Sharpe) broth. The bacteria were refreshed with 1 mL of sample inoculated in 9 mL of MRSB[10]. Then, the sample was incubated at 37°C for approximately 24 h. This refreshment process was conducted three times. The final refreshment was used in the next step of the study.

The growth curve of *L. plantarum* IIA-1A5 was analyzed by using three different growth media, whey buttermilk and whey+skim (whey that was enriched by skimmed milk), for 30 h and was observed every 5 h. Total lactic acid bacteria were calculated by using the pour plate method with buffered peptone Water as the dilution media at as much as 9 mL per dilution. The whey sample was diluted from 10-1 to 10-8. One mL of sample was taken from dilutions of 10-6,10-7 and 10-8 and placed into a sterile petri dish. Each dilution was repeated three times in duplicate. Approximately 15 mL of MRS agar was added to each of the petri dishes and each dish was homogenized by shaking 8 times so that the bacteria was spread around the petri dish. Agar was set aside until solid and then incubated upside down at 37°C for 24-48 h. The lactic acid bacteria that formed were calculated. The calculation based on the standard plate count (SPC)[11].

pH measurement during fermentation: The pH was measured using a pH meter that was calibrated to buffer pH 4 and 7 before use. Then, the electrode was washed with aquadest water before the pH meter was dipped into the samples. The pH value was read when it stabilized[12].

Preparation of cell-free supernatant as antimicrobial substrate: The preparation of cell-free supernatant (CFS) as an antimicrobial substrate was conducted according to Arief *et al.*[10]. Approximately 2000 mL of whey buttermilk and whey+skim was used. The supernatant was evaporated using a Heidolph VV micro evaporator until the volume reached half of the initial volume and the supernatant was concentrated for approximately 48 h. After that, the supernatant was centrifuged (Himac CR21G) at 15,000 rpm for approximately 20 min at 4°C. Next, the supernatant was filtered with a 0.20 µm filter and the pH was neutralized with the addition of 1 N NaOH until the pH reached 6.8. Then, CFS was obtained as an antimicrobial substrate and used for antimicrobial analysis.

Antimicrobial test against *Escherichia coli* and *Staphylococcus aureus*: An antimicrobial activity was determined using 0.85 Mc Farland standard (log 10 CFU mL^{-1}) according to the method described by Fatmarani *et al.*[13]. Mc Farland 0.85 used approximately 0.85% NaCl. The pathogen bacteria used were *Escherichia coli* (*E. coli*) ATCC 25922 and *Staphylococcus aureus* (*S. aureus*) ATCC 25923. Approximately 0.1 mL of pathogen bacteria indicator was added into the Mc Farland standard and then approximately 20 mL of MHA media was poured into the agar. A paper disc (Oxoid, United Kingdom) was soaked in approximately 100 µL of plantaricin IIA-1A5 using an Eppendorf tube for every sample. Paper discs that had already been soaked were placed in MHA media that had already been inoculated with the pathogen bacteria indicators. A petri dish that already contained a paper disc was closed using filter paper and then incubated at 37°C for approximately 24-48 h. The antimicrobial activity was determined by the formation of a transparent zone around the paper disc and was measured using calipers (Mitutoyo).

Data analysis: The experiment used a completely randomized design. The treatment was the different growth media, e.g., whey buttermilk and whey+skim. Each treatment was repeated three times. The microbiological quality data were transformed into log10 data. The data were analyzed using one-way analysis of variance (one-way ANOVA). Duncan's multiple range test was used to determine significant difference between means with a significance level of 5%[14].

RESULTS AND DISCUSSION

Nutrient composition of whey buttermilk and whey+skim milk: As shown in Table 1 buttermilk had the best nutrient composition among all the growth media, followed by whey+skim and whey. Buttermilk is a byproduct of butter

Table 1: Nutrient composition of media

	Buttermilk (%)	Whey (%)	Whey+skim (%)	Milk (%)	Skim (%)
Fat	3.025	1.665	nd	4.30	0.00
SNF	9.785	6.180	6.84	7.69	7.50
Protein	3.590	2.265	2.49	3.60	2.74
Lactose	5.380	3.395	3.75	4.30	4.13
Salt	0.790	0.495	0.57	0.70	0.63

Nd: Not detected by Lactoscan

processing from cream and has a composition similar to that of milk. Whey had the lowest quality nutrient composition compared to that of buttermilk and whey+skim because whey is a byproduct from cheese processing. The decrease in protein in whey might be caused by the loss of casein during cheese processing. The decrease in fat in whey might be caused by the loss of fat during cheese processing. The composition of whey influenced the growth of *L. plantarum* IIA 1-A5 and then, to enrich the whey nutrients, skim milk was added into the whey to produce the whey+skim medium (60:40%). There was a difference in nutrient composition for each of the growth media, which can be assumed to result in different growth patterns of *L. plantarum* IIA-1A5.

Amino acids are needed for *L. plantarum* IIA-1A5 to grow and produce plantaricin antimicrobial substances that consist of proteins. With different amino acids, the growth pattern was assumed to be different among the growth media. As shown in Table 2 buttermilk had a different amino acid composition than whey. However buttermilk and whey contained no serine, norvaline and sarcosine. Amino acids that had high concentrations (>100 ppm) in buttermilk and whey were glutamine, tyrosine, cysteine and valine. Whey had more than ten times the amount of histidine than did buttermilk.

Due to the amino acid composition of buttermilk and whey, they were good substrates for use in various biotechnological processes, such as the production of antimicrobial substrates from LAB. The nutritional requirements of LAB are specific, so culture media must be supplemented with various peptide sources and growth factors[15]. Panesar *et al.*[16] succeeded in growing *Lactobacillus casei* from whey to produce lactic acid. Hanoune *et al.*[15] performed research on the utilization of whey to optimize *Lactobacillus fermentum* DSM 20049 growth. Benaissa *et al.*[17] also succeeded in utilizing whey for the growth of *Lactobacillus* sp., resulting in sweet whey being used as an alternative substrate for LAB culture purposes and providing opportunities to make a new growth medium with a low cost. The proteolytic activity of *Lactobacillus* was also shown by *Lactobacillus* that was grown on whey media supplemented with tomato juice and yeast extract. This result showed that the growth of *Lactobacillus* on whey can support protein production, such as enzyme and antimicrobial activity.

Table 2: Amino acid composition of buttermilk and whey

Amino acid	Buttermilk (ppm kg^{-1})	Whey (ppm kg^{-1})
Aspartic acid	13.14	26.40
Glutamic acid	0.57	0.22
Asparagine	0.33	0.36
Serine	nd	nd
Glutamine	411.67	135.88
Histidine	25.71	252.39
Glycine	1.54	1.52
Threonine	1.05	1.09
Arginine	1.65	1.65
Alanine	15.94	16.07
Tyrosine	207.51	207.97
Cystine	563.03	559.85
Valine	115.58	109.45
Methionine	86.63	83.58
Norvaline	nd	nd
Tryptophane	0.56	24.89
Phenylalanine	207.09	174.10
Isoleucine	13.18	2.61
Leucine	73.29	49.94
Lysine	3.26	2.72
Hydroxyproline	44.92	71.77
Sarcosine	nd	nd
Proline	29.46	61.03

nd: Not detected

***Growth* characteristics of *L. plantarum* IIA-1A5 on different growth media:** *Lactobacillus plantarum* IIA-1A5 could grow in different media, such as buttermilk and whey+skim (Fig. 1). LAB require multiple nutrients for growth and they require numerous amino acids and other growth factors. Glutamic acid and histidine are essential amino acids that are needed for LAB growth[18]. In Fig. 1, the growth curve of *L. plantarum* IIA-1A5 in whey+skim was better compared to that of whey and buttermilk. The growth rate per 5 h is shown in Table 3. The growth rates of *L. plantarum* IIA-1A5 at the fermentation times of 0-5 h, 10-15 h and 15-20 h in whey buttermilk and whey+skim were significantly different ($p<0.05$). Based on these results, the growth of *L. plantarum* in whey was better than that in buttermilk. Pescuma *et al.*[19] stated that lactic acid bacteria in powdered whey had a higher growth rate than those in β-lactoglobulin (BLG) and α-lactalbumin (ALA), which

Table 3: Population change of *Lactobacillus plantarum* IIA-1A5 during fermentation

	LAB population change (log CFU mL^{-1})		
Fermentation time (h)	Whey	Buttermilk	Whey+skim
0-5	0.35±0.13^{a}	0.74±0.22^{b}	1.23±0.00^{c}
5-10	1.05±0.14	0.78±1.06	1.04±0.28
10-15	0.28±0.08^{a}	1.09±0.57^{b}	0.86±0.08^{b}
15-20	0.11±0.00^{a}	0.27±0.02^{c}	1.17±0.05^{b}
20-25	0.38±0.08^{a}	0.12±0.03^{b}	0.37±0.01^{a}
25-30	0.50±0.13^{a}	0.22±0.16^{b}	0.21±0.08^{b}

Different superscripts following values in the same column indicate significant difference ($p<0.05$)

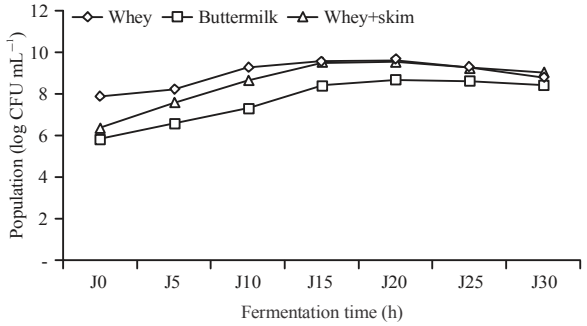

Fig. 1: Growth of *Lactobacillus plantarum* IIA-1A5 during fermentation in different media

was caused by whey fermentation for less than 4.5 h so that there was an increased amount of cells and protein hydrolysis. Whey is a byproduct from cheese production that contains lactose (5%), water (93%), protein (0.85%), minerals (0.53%) and minimal fat (0.36%). The major proteins in whey are β-lactoglobulin (BLG) (58%) and α-lactalbumin (ALA) (13%) and whey contains immunoglobulin, serum albumin and peptone protease in lower amounts[19].

The growth rate calculation showed that *L. plantarum* IIA-1A5 in whey+skim grew faster than that in buttermilk and whey and overall, whey was the worst growth media for *L. plantarum* IIA-1A5 (Table 3). This result was supported by the fact that the nutrient composition of whey+skim was better than that of whey and buttermilk. The generation time of *L. plantarum* IIA-1A5 in whey+skim media was approximately 1.96 h, followed by buttermilk and whey at approximately 2.44 and 3.58 h, respectively. The time needed for *L. plantarum* IIA-1A5 to grow exponentially by 2 fold in whey+skim media was 1.96 h. This result showed that *L. plantarum* IIA-1A5 grew faster in whey+skim media compared to in buttermilk and whey.

Lactobacillus plantarum IIA-1A5 in whey+skim at 0-5 h of fermentation had the highest population change compared to that in whey and buttermilk. The population change at 10-15 h of fermentation on buttermilk and whey+skim were not significantly different ($p>0.05$) but the population change in buttermilk was higher than that in whey (Table 4). At fermentation for 15-20 h, whey+skim had a higher population change than that of whey and buttermilk. Overall, whey+skim was a better quality growth media than buttermilk and whey for *L. plantarum* IIA-1A5. Whey+skim was chosen for the antimicrobial test in the next experiment.

Table 4: Growth rate and generation time of *Lactobacillus plantarum* IIA-1A5

Media	Growth rate	Generation time (h)
Buttermilk	1.44±0.13^{b}	2.44±0.71^{a}
Whey	1.21±0.01^{c}	3.58±0.14^{b}
Whey+skim	1.49±0.09^{a}	1.96±0.38^{a}

Different superscripts following values in the same column indicate significant difference ($p<0.05$)

pH value of *L. plantarum* IIA-1A5 during fermentation: During lactic acid fermentation, there was a decrease in pH. The use of whey as growth media for *L. plantarum* resulted in a higher pH, followed by whey+skim and buttermilk (Fig. 2). The pH of the whey was approximately 4.0-5.8, while that of buttermilk was approximately 4.0-4.8 and that of whey+skim was approximately 4.11-5.54. Marsh *et al.*[20] stated that the pH of a fermented product was influenced by the buffering capacity with different amounts and different types of protein. The growth of good lactic acid bacteria occurred at pH 6 and the growth rate decreased if the extracellular media became acidic[16]. The decrease in pH resulted from acid accumulation from lactic acid bacteria. Based on this research, whey, as a media, had a neutral pH, followed by whey+skim. There was a decrease in pH in whey and whey+skim. This decrease might have been caused by H^{+} ions that formed from lactic acid metabolism.

Antimicrobial activity of cell-free supernatant from *L. plantarum* IIA-1A5: The antimicrobial activity in buttermilk, whey and whey+skim was explored in this research. The antimicrobial activity was not significantly different in buttermilk, whey, whey+skim for approximately 15 and 25 h of incubation, while with *S. aureus* at 15, 20 and 25 h, there was a significant difference in antimicrobial activity

Table 5: Antimicrobial activity from *Lactobacillus plantarum* IIA-1A5 againts *Escherichia coli* and *Staphylococcus aureus*

	Growth media		
Fermentation time (h)	buttermilk	Whey	Whey+skim
Antimicrobial activity to *Escherichia coli*			
15	7.78±0.05	8.13±0.11	8.21±1.22
20	6.47±0.11[a]	7.03±0.10[b]	8.21±0.08[a]
25	7.38±0.07	7.02±0.09	7.75±0.54
Antimicrobial activity to *Staphylococcus aureus*			
15	6.96±0.03c	6.88±0.03[b]	6.57±0.06[a]
20	6.45±0.06[b]	6.18±0.00[a]	7.93±0.15[c]
25	6.53±0.08[b]	6.47±0.10[a]	7.33±0.06[c]

Different superscripts following values in the same rows indicate significant difference (p<0.05)

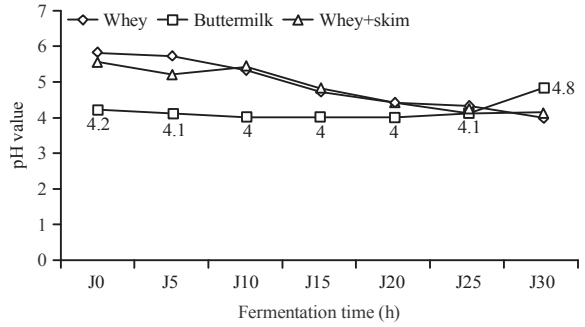

Fig. 2: pH value of *L. plantarum* IIA-1A5 during fermentation

among the media. The antimicrobial activity of the bacteriocin from *L. plantarum* IIA-1A5 against *S. aureus* was higher than that against *E. coli* (Table 5). The cell-free supernatant was free from lactic acid and contained bacteriocin as an antimicrobial substrate. Arief *et al.*[10] reported that *L. plantarum* IIA-1A5 grown in MRS (de Mann Rogosa Sharpe) broth could produce a bacteriocin called plantaricin. The plantaricin IIA-1A5 displayed remarkable antibacterial activity against *S. aureus* ATCC 25923. Plantaricin IIA-1A5 exhibited antibacterial activity through releasing cellular components of *S. aureus* ATCC 25923. Arief *et al.*[21] stated that the presence of bacteriocin from *L. plantarum* IIA-1A5 in sausages inhibited the growth of the pathogenic bacteria *S. aureus* and *E. coli* until day 6 of the experiment, which was better than that of nitrite.

Lactic acid bacteria (LAB) can produce bacteriocin as an antimicrobial substance. Bacteriocin can inhibit the development of pathogenic bacteria and spoilage microbes. The bacteriocins from LAB have attracted significant attention because of their potential use as safe additives for food preservation and the prevention of food spoilage by foodborne pathogenic bacteria[22-25]. Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins[26]. Cotter *et al.*[27] stated that bacteriocin is a ribosomal peptide produced by bacterial strains that has antagonistic properties against other bacterial strains. However, the bacteriocin-producing bacteria are protected against bacteriocin activity itself. Brandelli *et al.*[28] stated that whey is a peptide, so it can be a good source for antimicrobial substrates based on peptides.

The implication and application of this study is that waste from the cheese industry, such as whey, can be used as a beneficial growth medium for the bacteria *L. plantarum* IIA-1A5. Whey enriched by skim milk is a medium that contains enough nutrients for *L. plantarum* fermentation. Whey should be further processed and enriched by skim milk when used for fermented drink or antimicrobial bacteriocin production. The optimal fermentation time for *L. plantarum* IIA-1A5 in whey enriched by skim milk is 20 h.

CONCLUSION

Buttermilk, whey and whey+skim (whey that was enriched by skim) can be utilized as growth media for *Lactobacillus plantarum* IIA-1A5 and for media to produce plantaricin. The best media for *Lactobacillus plantarum* growth is whey+skim, based on the growth media composition and LAB population. *Lactobacillus plantarum* IIA-1A5 produced a bacteriocin, which had a good level of antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*, at 20 h of fermentation in whey+skim media.

SIGNIFICANCE STATEMENT

This study analyzed the use of whey and buttermilk as growth media for the lactic acid bacteria *Lactobacillus plantarum* IIA-1A5., The results could be beneficial for finding the fermentation patterns of *Lactobacillus plantarum* that could be used further in industry applications, such as in generating fermented drinks and bacteriocins for biopreservative production. Whey enriched by skim milk is the best media for fermentation. This study will help researchers

uncover the critical areas in the utilization of waste from the cheese industry to make highly valuable products that many have not been able to explore. Thus, a new method using the fermentation patterns, including the growth rate, generation time and antimicrobial production, of *Lactobacillus plantarum* IIA-1A5 in growth media based on whey may be developed.

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