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ARTICLE

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Tofu Whey-Based Media for Probiotic *Lactiplantibacillus plantarum* D4 as a Halal Starter Culture

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Abstract: *In recent years, the development of probiotic-based fermented products with halal status has been a concern. The use of growth medium has relied on de Man Rogosa and Sharpe (MRS) as a relatively expensive standard medium, and its halal status is still uncertain. Extensive research has been carried out to investigate the development of low-cost halal alternative media for the cultivation of probiotic lactic acid bacteria (LAB). This study aimed to develop a probiotic halal and low-cost culture medium using a tofu whey-based medium. This study used three tofu whey-based media – A (tofu whey 100%), B (tofu whey 94.5%, molasses 3%, skim milk 2.5%), C (tofu whey 92.5%, molasses 3%, cheese whey 2.5%, tomato extract 2%), and MRS broth as a standard medium. Bacterial populations, total sugars utilized, total lactic acids produced, low pH (2.0) tolerance, and high bile salt concentration (oxgall 1.5%) were assayed. The highest bacterial population after 48 h of incubation was shown by medium B compared to medium MRS (12.34 ± 0.87 and 11.48 ± 0.3 log CFU/mL). Total sugars utilized by 48.28 ± 2.89, 38.89 ± 6.94, 39.14 ± 4.24, and 76.00 ± 1.41 %; on the other hand, total lactic acids produced by 0.16 ± 0.12, 0.03 ± 0.04, 0.31 ± 0.03, and 2.25 ± 1.48 % in A, B, C, and MRS, respectively. Probiotic tolerance at low pH and the presence of bile salts of Lactiplantibacillus plantarum D4 consistently showed a high survival rate in medium B compared to MRS. Based on these results, the components and proportions used in medium B were suitable for the growth of L. plantarum D4 as a halal probiotic starter candidate.*
Keywords: *halal medium, halal starter, Lactiplantibacillus plantarum, probiotic, tofu whey*

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1. Introduction

Lactic acid bacteria (LAB) as probiotics are fastidious microorganisms with nutritious requirements for growth and survival in challenging environments. LAB culture medium must contain carbon sources, nitrogen sources, minerals, vitamins, and buffering agents in a proper ratio (Ayad et al., 2020). The standard medium for culturing LAB is de Man Rogosa and Sharpe (MRS), containing essential nutrients to support LAB growth, including peptone, beef extract, yeast extract, dextrose, several minerals, and buffering agents (Hayek et al., 2019). The use of MRS on an industrial scale has some drawbacks, such as the high cost and the uncertain halal status of components obtained from animals. Halal food products are foods that have adhered to the required halal criteria and complied with Islamic law regulations (Irfan et al., 2023). The fermented product industry generally uses the LAB group as a starter culture. Some important aspects should be considered when producing microbial-based halal products; one is microbial growth medium (Kurniadi & Frediansyah, 2017). Various innovations must be made to develop alternative media with low-cost and halal status that allow LAB to grow and maintain its probiotic properties. Hence, the use of industrial food waste and agricultural waste products could be beneficial.

Indonesia is recognized for its numerous tofu manufacturing enterprises. Whey as a by-product can be used to develop alternative culture media (Hanoune et al., 2015; Manzoor et al., 2017). Whey is liquid waste from clumping processes, such as the production of tofu and cheese. Generally, these materials are disposed of as waste and potentially cause environmental problems (Jannah et al., 2021). However, waste can be a promising source of nutrition for LAB. Tofu whey contains organic compounds, including crude protein, carbohydrates, ash, and fats (Mujtahidah & Kusuma, 2019; Nostia & Kurniawan, 2023). The formula of tofu whey-based medium supplemented with carbon sources, nitrogen sources, and some vitamins and minerals is expected to be a good alternative halal culture medium for growing probiotic LAB. Molasses, cheese whey, skim milk, and tomato extract are recognized for their significant nutritional contents, which can potentially be used as medium components for LAB. Molasses is a by-product of sugar crystallization containing high sugars and minerals (Jain & Venkatasubramanian, 2017; Valli et al., 2012). Cheese whey also contains proteins, lactose, fats, and other minerals (Lievore et al., 2015; Lustrato et al., 2013; Panfilova et al., 2016), while skim milk contains lactose, peptides, and amino acids as nitrogen sources, and some vitamins and minerals (Kailasapathy, 2016; Magan et al., 2019; Mehta, 2015). Simple carbohydrates, vitamins, and essential minerals are found in tomato extract (Kurina et al., 2021).

Extensive research has been done to investigate the development of alternative media for cultivating probiotic LAB and is still focused on using chemical substances instead of food-related industrial wastes. The use of chemical substances requires a relatively high cost. Alternative media with carbon and nitrogen supplementations have been proven for growing LAB, namely sorghum malt extract (Byakika et al., 2020), sweet potato (Hayek et al., 2013), tofu-whey based medium (Jannah et al., 2021), dates-based medium (Keddari et al., 2021), tofu whey and coconut water-based medium (Marlida et al., 2022), and cheese whey (Kusmiyati et al., 2022). Additionally, research on the probiotic properties of the newly formulated media is still limited. Therefore, the objective of this study was to investigate the use of tofu whey as a basic component in developing a new halal and low-cost medium for the cultivation of probiotics and to evaluate the probiotic properties of *Lactiplantibacillus plantarum* D4 in the alternative media compared to MRS medium.

2. Materials and Methods

2.1. Bacterial Strain: Source and Culture Preparation

Lactiplantibacillus plantarum D4 used in this study was obtained from the Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, East Java. The starter was prepared by reviving 100 µL glycerol stock (-80°C) to 25 mL sterile MRS broth and incubated at 37°C for 24 hours. After overnight incubation, 1.5 mL of culture was transferred into micro-tubes for washing treatment three times using sterile phosphate-buffered saline (PBS) by centrifugation (4000 rpm for 5 minutes) to remove the residue of MRS medium. The cell density of the bacterial strain was adjusted to achieve 7 log CFU/mL and then stored at 4°C for further assays.

2.2. Media Preparation

The culture media used in this study included de Man Rogosa and Sharpe MRS (Merck, Germany) and tofu whey-based media supplemented with molasses, cheese whey, skim milk, and tomato extract. Some pre-treatments had been conducted before the medium was sterilized. Tofu whey, cheese whey, and blended tomato were centrifuged at 4000 rpm for 5 min to obtain the supernatant. Molasses was prepared

by diluting 15 g of sugarcane molasses into 1 L of distilled water to obtain 20 Brix. Skim milk preparation was conducted by diluting 50 g of skim milk into 1 L of distilled water. Skim milk used in this study was halal-certified New Zealand Milk Products (NZMP). The pH of the tofu whey-based medium was adjusted to 6.4–6.5 with NaOH or HCl prior to sterilization. The components of the medium were sterilized separately. The MRS, tofu whey, molasses, and tomato extract were autoclaved at 121°C, 1 atm for 15 min. Meanwhile, cheese whey and skim milk were sterilized by pasteurization (65°C for 30 min).

The medium formulations used were medium A which consisted of tofu whey (100%) as a control medium; medium B which consisted of tofu whey (94.5%), molasses (3%), skim milk (2.5%); medium C which consisted of tofu whey (92.5%), molasses (3%), cheese whey (2.5%), tomato extract (2%), and medium MRS broth as a standard medium. The composition and formula used for the tofu whey-based medium (A, B, and C) were slightly modified from Jannah et al. (2021). The probiotic strain was then inoculated into each medium (200 mL, v/v) and incubated at 37°C for 48 h.

2.3. Bacterial Population

The bacterial population in all tested media (A, B, C, and MRS) was determined during incubations of 0, 24, and 48 h. Samples were withdrawn for several serial dilutions using PBS solution and 100 µL from each dilution was spread into MRS agar containing 1% $CaCO_3$ and then incubated at 37°C for 48 h. The number of colonies formed was converted to log CFU/mL.

2.4. Determination of Total Sugars Utilized

The total sugar in the medium was determined before and after fermentation using the Anthrone method (AOAC International, 1995). In this method, a glucose solution is used as a standard solution to create a standard curve for measuring the total sugar content in the sample. The total sugars utilized are a percentage of the overall sample volume (Equation 1).

$$\text{Total Sugars Utilized (\%)} = \frac{\text{Difference total sugars before and after fermentation (\%)}}{\text{Total sugars before fermentation (\%)}} \times 100\% \quad (1)$$

2.5. Determination of Total Lactic Acids Produced

The total acid (TA) in the medium was determined by titration (AOAC International, 1995). The total lactic acids produced in the medium are expressed as a percentage of the total sample volume (Equation 2).

$$\text{Total Lactic Acids Produced (\%)} = \text{TA after fermentation (\%)} - \text{TA before fermentation (\%)} \quad (2)$$

2.6. Evaluation of Probiotic Properties

This study used a low pH and bile salts tolerance assay to screen for probiotic activity in LAB. The procedure was slightly modified from Adhawati and Jatmiko (2023). The tolerance of *L. plantarum* D4 to low pH and high bile salts conditions was determined through the survival rate (%) of the culture after exposure.

2.6.1. Low pH Tolerance Assay

Samples from all tested media (A, B, C, and MRS) were withdrawn and 100 µL of each medium was added into 900 µL sterile acidified PBS solution (pH 2.0). Incubation was set at 37°C for 2 h. The total of bacterial cells (CFU/mL) was calculated using the TPC method before and after incubation. *Lactiplantibacillus plantarum* D4 tolerance at a low pH was calculated as the survival rate (Mulaw et al., 2019) (Equation 3).

$$\text{Survival Rate (\%)} = \frac{\log \text{CFU } N_1}{\log \text{CFU } N_0} \times 100 \quad (3)$$

Note: N_1 is the cell number after 2 h of incubation, and N_0 is the initial cell count (before incubation).

2.6.2. Bile Salts Tolerance Assay

Samples from all tested media (A, B, C, and MRS) were withdrawn and 100 µL of each medium was added into 900 µL sterile PBS solution containing 1.5% oxgall solution. Incubation was set at 37^{O}C for 4 h. The total of bacterial cells (CFU/mL) was calculated using the TPC method before and after incubation. The same procedure (2.6.1) was also applied to determine the survival rate of *L. plantarum* D4 towards bile salts.

2.7. Statistical Analysis

All the statistical analyses were conducted using the SPSS 24.0 version. The experimental data was analyzed using various tests for each incubation period, including analysis of variance (ANOVA) with Tukey's HSD, Brown-Forsythe with Games-Howell, or Kruskall-Wallis with Mann-Whitney U tests with significance level (α) of 0.05. The different statistical analyses for each data package were according to normality and homogeneity test results.

3. Results and Discussion

3.1. Bacterial Growth

Three formulated tofu whey-based media (A, B, and C) were developed with different concentrations of carbon and nitrogen sources to evaluate the suitability of tofu whey as a basic component for the cultivation of probiotics compared to MRS as a standard medium. Figure 1 shows the bacterial populations (log CFU/mL) for *L. plantarum* D4 in all tested media during 48 h incubation at 37°C. Notably, no stationary phase was observed in all tested media, suggesting the presence of potential nutrients in the media that can be exploited for bacterial growth. Medium A consists of pure tofu whey, *L. plantarum* D4 showed the lowest growth at 0, 24, and 48 h of incubation compared to other media. There is only a slight difference among MRS, medium B, and medium C in the population of *L. plantarum* D4. The bacterial populations (log CFU/mL) after 48 h of incubation reached an average of 9.57 ± 0.44, 12.34 ± 0.87, 10.43 ± 0.02, and 11.48 ± 0.30 for A, B, C, and MRS, respectively. The population of *L. plantarum* D4 in medium A and C was 2 to 3 log CFU/mL less than medium MRS. Meanwhile, the population of *L. plantarum* D4 in medium B 1 log CFU/mL was higher than MRS after 48 h of incubation.

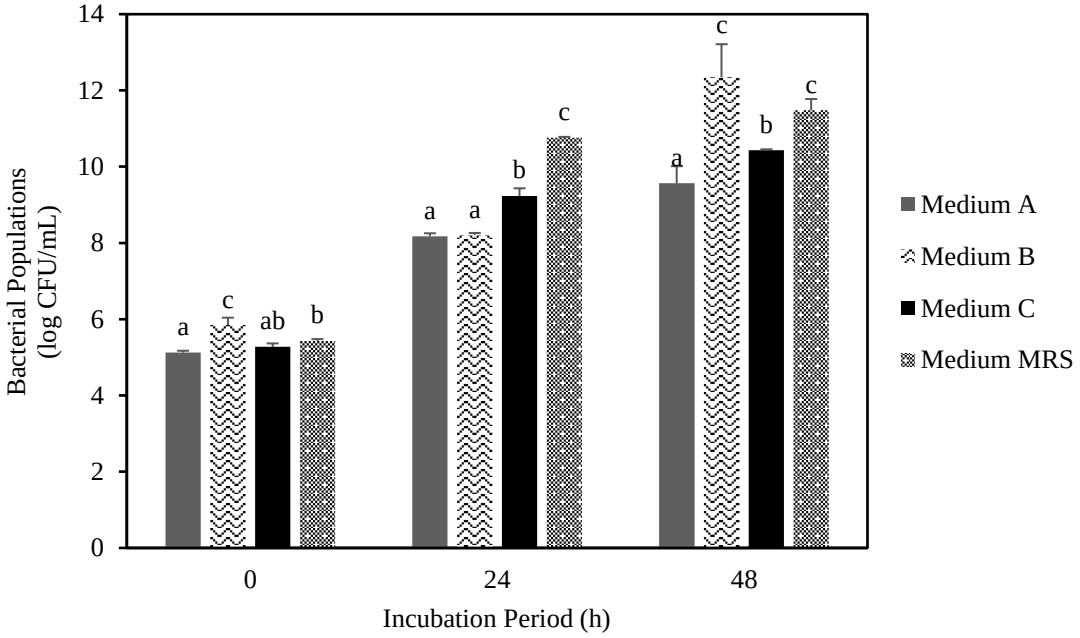

Figure 1. Bacterial growth of *L. plantarum* D4 in all tested media. Note: Different notations indicate a significant difference (p<0.05) between media at 0 h of incubation (Tukey's test), 24 h of incubation (Games-Howell test), and 48 h of incubation (Mann-Whitney test).

The results for bacterial populations signify that medium B was suitable for the growth of *L. plantarum* D4 compared to medium MRS. Previous research yielded comparable findings. Jannah et al. (2021) reported that LAB growth was enhanced in the formulated media, consisting of tofu whey, molasses, and skim milk, compared to MRS media. Another study reported that the optimal growth condition of *L. plantarum* RPR42 was achieved in a medium containing 22.5% sugarcane molasses (Papizadeh et al., 2020). The utilization of cheese whey as an alternative medium was also investigated by Kusmiyati et al.(2022), which demonstrated that all examined groups of *L. casei* could thrive in the cheese whey medium, with no significant difference observed compared to the MRS medium.

The LAB possesses the ability to not only ferment sugars but also hydrolyze proteins into amino acids and produce or convert vitamins and other constituents for growth (Wang et al., 2020). Microbial growth was enhanced in a sorghum malt extract medium by selectively including growth-promoting free

amino acid sources (Byakika et al., 2020). The production of amino acids can provide advantageous effects to the viability of LAB (Corsetti et al., 2016). Diverse sugars and nitrogen sources in the medium had distinct impacts on biomass output (Choi et al., 2021). In a previous study, the sweet potato base medium (SPM) developed by Hayek et al. (2013) showed only slight differences in the population of *Lactobacillus* strains observed between MRS, SPM2, and SPM3. The average population of *Lactobacillus* strains showed no significant differences (p>0,05) among the three media. SPM2 and SPM3, containing nitrogen sources from MRS, showed similar growth rates and slightly higher bacterial populations than MRS. Meanwhile, SPM1 was not supplemented with nitrogen sources and showed only 1–2 log CFU/mL, which was a lower bacterial population than MRS.

Tofu whey supplemented with molasses and skim milk can substitute the nutrient sources in standard medium MRS. Tofu whey is recognized for its nutritious content, functioning as a substantial provider of nitrogen and carbon (Mujtahidah & Kusuma, 2019; Nostia & Kurniawan, 2023). Molasses serves as a valuable carbon source for LAB, as it contains not only sugars but also amino acids and minerals, including manganese and magnesium provided in MRS medium (Jain & Venkatasubramanian, 2017; Palmonari et al., 2020). Skim milk has various beneficial contents, including lactose, which is a carbon source, peptides, amino acids, vitamins, and other essential minerals. The nitrogen sources in skim milk are present in higher quantities than nitrogen sources typically incorporated in LAB medium (Hayek et al., 2019). The result was in agreement with the previous studies (Hayek et al., 2013; Keddari et al., 2021; Śliżewska & Chlebicz-Wójcik, 2020), demonstrating that the alternative medium was comparable with the MRS medium in terms of bacterial growth. LAB growth and its metabolism depend on the medium properties, composition, and quality (Gunkova et al., 2021; Mendonça et al., 2023).

3.2. Utilization of Total Sugars

The average total sugars utilized from the three formulated media were significantly different when compared to the MRS medium. The highest total sugar utilization in all tested media used by *L. plantarum* D4 was observed in medium MRS (Table 1). The results were in agreement with the previous studies. Total sugars dropped by 95.2% in MRS broth and 5.6–9.1% in sorghum malt extract media (Byakika et al., 2020). According to Keddari et al. (2021), tested LAB strains exhibited superior sugar consumption in date powder medium. Strain Bb12 consumed 81.96% of sugars, whereas strain Bb443 consumed 80.58%. These yields were lower than at the end of fermentation of the two consuming strains, where almost all the sugar (95%) was in MRS. Although *Lactiplantibacillus plantarum* is recognized for its capacity to metabolize a wide range of carbon sources (Bosma et al., 2017), the LAB mostly undergoes fermentation of hexoses and disaccharides, with a limited ability to ferment pentose (Gunkova et al., 2021). A study conducted by Hongthong et al. (2019) found that *L. plantarum* can metabolize disaccharides like sucrose.

Table 1. Total Sugar Utilized and Total Lactic Acids Produced During *L. plantarum* D4 Fermentation in All Tested Media

Medium	Sugar Utilization (%)	Lactic Acid Production (%)
A	48.28 ± 2.89^{a}	0.16 ± 0.12^{b}
B	38.89 ± 6.94^{a}	0.03 ± 0.04^{a}
C	39.14 ± 4.24^{a}	0.31 ± 0.03^{b}
MRS	76.00 ± 1.41^{b}	2.25 ± 1.48^{c}

Note: Different notations indicate a significant difference between media (p<0.05), sugar utilization (Tukey's test), and lactic acids production (Mann-Whitney test).

3.3. Production of Total Lactic Acids

Similar findings were observed regarding total lactic acids produced (Table 1), with the MRS medium exhibiting the greatest increase in concentration, followed by medium C, A, and B, respectively. Previous studies have reported the same results, that the production of organic acids in alternative media was lower compared to MRS (Byakika et al., 2020; Hayek et al., 2013; Keddari et al., 2021). *Lactobacillus* requires sugars to be converted to glucose to produce lactic acids. MRS medium contained glucose, but media A, B, and C contained mono- and disaccharides, such as glucose, fructose, and sucrose from molasses and lactose from skim milk, which may take a longer time to produce lactic acid. Another reason may be due to metabolic pathways and by-product formation. *Lactiplantibacillus plantarum* is recognized as a hetero-lactic bacterium that automatically produces various metabolic products.

3.4. Probiotic Properties

3.4.1. Low pH Tolerance

During the incubation period, it was consistently observed that medium B showed the best survival rate in the low pH compared to other media (Figure 2). Under stress conditions, it is imperative to actively remove the accumulated hydrogen ions (H^+) from the cytoplasm and prevent their diffusion into the surrounding external environment (Mendonça et al., 2023). The LAB medium typically incorporates diverse nitrogen sources derived from proteins, peptides, or amino acids. Additional appropriate nitrogen sources improve fermentation performance (Barbosa et al., 2016). The most commonly utilized nitrogen sources of peptides and amino acids were skim milk powder and whey protein (Hayek et al., 2019). Tofu whey and skim milk in medium B were recognized as significant providers of specific important amino acids. Those amino acids are necessary for LAB survival during acidic conditions (Magan et al., 2019; Ali et al., 2020; Fathana et al., 2021). The study conducted by Senouci-rezkallah et al. (2011) provided evidence that the presence of certain amino acids enhanced the maintenance of pH homeostasis in probiotics. The amino acid deamination process produces NH_3, which causes an increase in pH levels in LAB cells (Mendonça et al., 2023). Molasses available in medium B also played a role as a buffering agent that maintained a stable pH in the medium (Razzaghi et al., 2020).

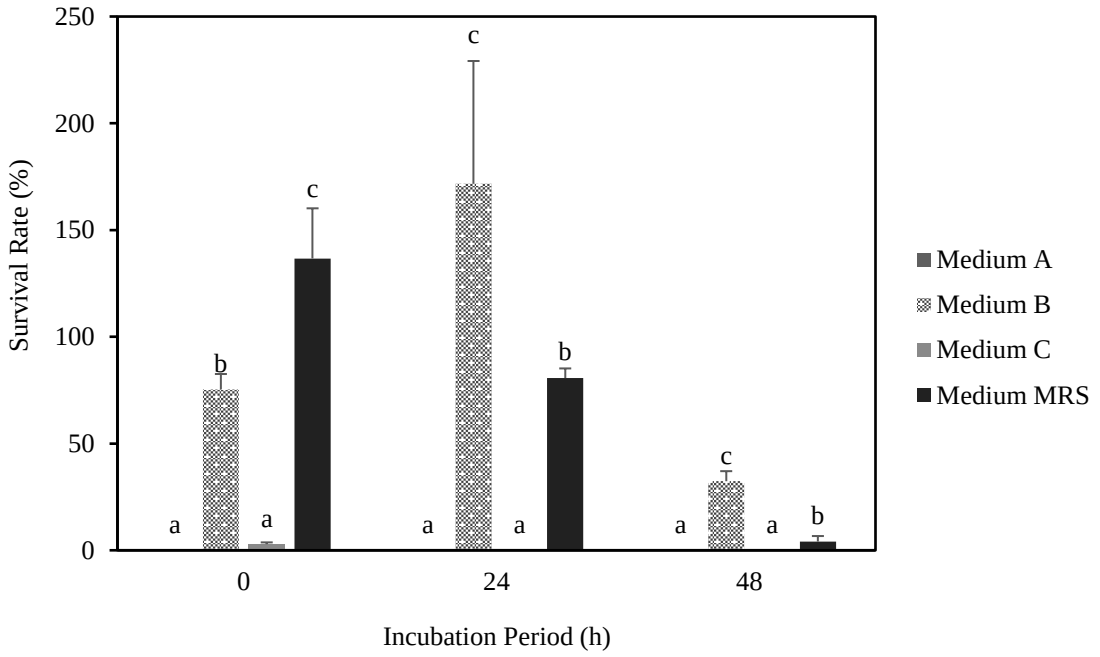

Figure 2. The survival rate of *L. plantarum* D4 during low pH in all media Note: Different notations indicate a significant difference (p<0.05) between media at 0 and 24 h of incubation (Tukey's test) and 48 h of incubation (Mann-Whitney test).

3.4.2. Bile Salt Tolerance

The survival rate of *L. plantarum* D4 to high concentrations of bile salts in media B and C showed high values as the incubation period increased, which was significantly different (p<0.05) from the media A and MRS (Figure 3). Media B and C consisted of various components recognized to possess higher nutritional contents compared to medium A, which comprised pure tofu whey. While tofu whey medium contains carbon and nitrogen sources, it is recommended that the addition of supplements was important to be considered. The survival of LAB in challenging environments was also facilitated by the presence of manganese, magnesium, B-complex vitamins, and gamma-aminobutyric acid (GABA) (Hayek et al., 2019; Mousavi et al., 2022).

Tomatoes and skim milk are known for their significant nutritional value. Tomatoes have substantial quantities of manganese, magnesium, B-complex vitamins, and fatty acids, including oleic acid (Kailasapathy, 2016; Ali et al., 2020; Mousavi et al., 2022). Oleic acid, like Tween 80 in MRS medium, functions as a surfactant agent (Hayek & Ibrahim, 2013; Reitermayer et al., 2018). The utilization of surfactants could enhance the absorption of nutrients. The use of Tween 80 has been found to have a notable impact on the LAB strains' recovery capacity and bile salt tolerance (Ibrahim et al., 2009; Li et al., 2011). Skim milk has also been proven to contain rich vitamins (including B-complex vitamins) and amino acid content, such as glutamate (Mehta, 2015; Kailasapathy, 2016). Glutamate is

involved in the biosynthesis of GABA. The production of GABA has been observed to enhance the ability of LAB to withstand challenging conditions, such as exposure to bile salts (Mousavi et al., 2022). *Lactiplantibacillus plantarum* is recognized as a potential probiotic candidate with the ability to synthesize substantial quantities of GABA (Gutiérrez et al., 2022; Kim et al., 2022; Mousavi et al., 2022). The medium MRS exhibited a comparatively reduced survival rate in terms of bile salt tolerance. The observed effects can be attributed to the presence of many by-products, which act as impediments for *L. plantarum* D4, hence interfering with its metabolic activities and defensive mechanisms in the presence of biliary stress (Lee et al., 2013).

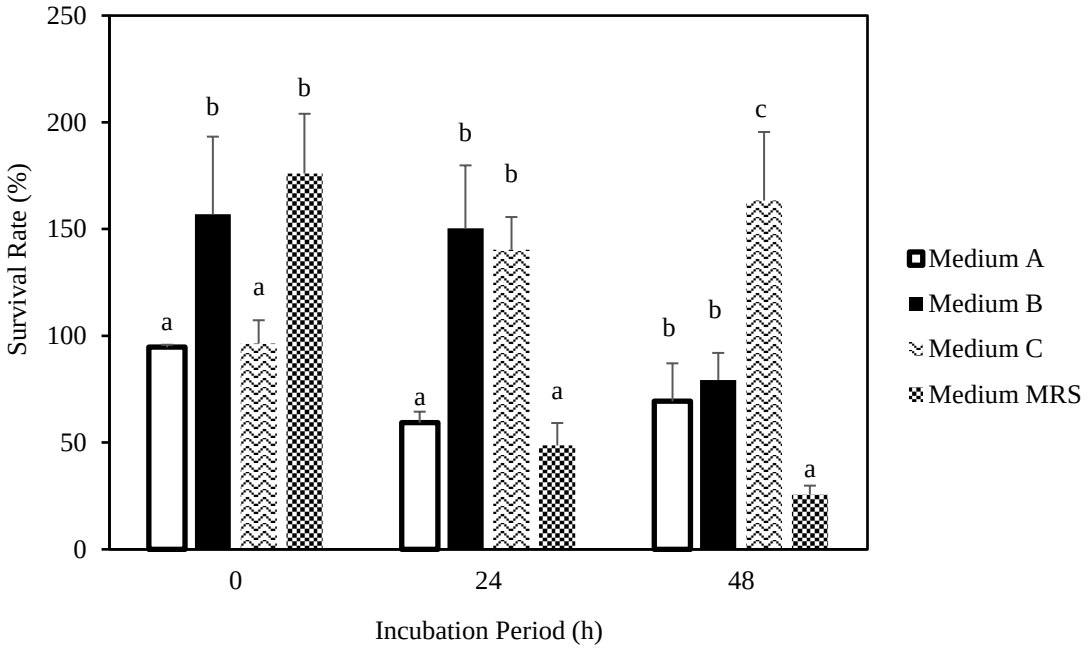

Figure 3. The survival rate of *L. plantarum* D4 in the presence of bile salts (oxgall 1.5%) in all media. Note: Different notations indicate a significant difference (p<0.05) between media at 0 and 48 h of incubation (Mann-Whitney test) and 24 h of incubation (Tukey's test).

4. Conclusion

Our results indicate that medium B is suitable for the growth of probiotic *Lactiplantibacillus plantarum* D4. The growth of the tested *probiotic* in medium B was higher than in MRS. In addition, the tolerance of the probiotic to acidic conditions and bile salt content grown in medium B consistently showed a higher survival rate than that of the standard medium (MRS). Based on the results of this research, medium B can be used as a potential alternative medium for halal probiotics starter. Further research should evaluate the potential of the selected alternative medium for the growth of probiotics from different genera, species, or strains. These findings lead to more interest in using agricultural waste products to develop halal and low-cost media for probiotics Lactobacilli.

CRediT Authorship Contribution Statement

Hikmah Maulidiyah: Conceptualization (equal), Data Curation (equal), Formal Analysis (lead), Investigation (lead), Methodology (equal), Project Administration (equal), Resources (lead), Software (lead), Visualization (lead), Writing – Original Draft (equal), Writing – Review & Editing (supporting). **Irfan Mustafa:** Conceptualization (equal), Methodology (supporting). **Yoga Dwi Jatmiko:** Conceptualization (equal), Data Curation (equal), Funding Acquisition (lead), Methodology (equal), Project Administration (equal), Resources (supporting), Supervision (lead), Validation (equal), Writing – Original Draft (equal), Writing – Review & Editing (supporting). **Salam A. Ibrahim:** Validation (equal), Writing – Review & Editing (lead).

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