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ARTICLE

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The Development of a Novel Halal Culture Medium Supports Lactiplantibacillus plantarum subsp. Plantarum Dad-13 Growth and Extracellular Metabolite Production

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Abstract: *The increasing demand for halal food products underscores the need for halal-compliant materials in food biotechnology, including culture media that support probiotic growth and extracellular metabolite production. This study evaluated the effects of a novel halal culture medium using meat peptone powder and yeast extract (YE) concentrations on the growth and extracellular metabolite production of Lactiplantibacillus plantarum subsp. plantarum Dad-13. Halal meat peptone powder was produced by hydrolyzing non-fat ground meat with crude bromelain, followed by spray drying at 155 °C. Fermentation media contained 1% (w/v) sucrose and varying concentrations of halal meat peptone and YE, followed by fermentation at 30 °C for 24 h, with commercial de Man, Rogosa and Sharpe medium (MRS) as the control. Cell growth, pH, and extracellular metabolites were monitored. Cell counts reached 10.14–10.44 log CFU/mL across the halal media, with no significant difference from MRS. At 1% (w/v) YE, gamma-aminobutyric acid (GABA) production reached 63.64 ± 4.53 mg/L, corresponding to 74.3% of that in MRS (85.68 ± 18.11 mg/L), while YE concentration significantly influenced GABA production. Meat peptone- and YE-based media were strongly positively associated with betaine, 2-pyridylacetylglycine, N-desmethyldiphenhydramine, and $C_{15}H_{33}NO$, whereas several volatile compounds were associated with specific formulations. The developed halal culture medium demonstrated potential as an alternative to MRS for high-density cultivation of L. plantarum Dad-13 and extracellular metabolite production, although further optimization is needed to enhance metabolite yields. This study provides a halal reformulation approach for microbial culture media, contributing to the development of halal biotechnology for probiotic cultivation.*

Keywords: *cell growth, extracellular metabolites, halal reformulation, meat peptone powder, yeast extract*

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1. Introduction

Probiotics are living bacteria that can provide health benefits when consumed in sufficient amounts (FAO/WHO, 2002). Among the well-known probiotics, *Lactobacillus plantarum* has been extensively studied for its potential benefits to human health. *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13, which is isolated from dadih, a traditional fermented buffalo milk from West Sumatra, Indonesia, has been found to have probiotic properties (Rahayu, Cahyanto, et al., 2016). It has been proven to be safe, does not translocate to the organs of *Sprague-Dawley* rats (Rahayu et al., 2019), and improved the microflora balance in the digestive tract (Rahayu, Yogeswara, et al., 2016). In addition, this culture can be a food fermentation starter for dairy (Luwidharto et al., 2022; Tologana et al., 2022; Wardani et al., 2017) and non-dairy (Alwi et al., 2023; Aziz et al., 2023; Leksono et al., 2021) products.

Lactic acid bacteria (LAB) are fastidious microorganisms that require many nutrients for growth; therefore, these must be provided in the growth media. In addition to a carbon source, bacteria require a nitrogen source for growth and metabolism. The raw material for protein hydrolysate production can be of animal- or plant-derived. The most common animal-derived proteins used in biotechnological applications are casein, whey, and meat obtained from different organs. Protein hydrolysate can be made by hydrolyzing protein sources with acid, alkaline, or enzymatic agents, which are the predominant hydrolysis agents.

A wide variety of proteases are commercially available from animal, plant, and microbial fermentations. But animal-derived enzymes can be halal or *haram* depending on their source. In Islam, halal means permissible and lawful, while *haram* means prohibited. Determining the halal status of enzymes, including the raw materials used in fermentation and probiotic production, is essential to support halal food production and ensure compliance with halal requirements. Enzymes derived from microorganisms during fermentation are considered halal if the raw materials and ingredients used in the growth media and final product are not from haram or doubtful sources (Ermis, 2017).

The global halal industry has expanded beyond food to encompass diverse sectors and is increasingly driven by demand for products that meet halal, ethical, and sustainability principles. Recent evidence from Indonesia demonstrates that halal certification can positively influence the operational and financial performance of micro, small, and medium enterprises (MSMEs), highlighting its growing importance for market access and business competitiveness (Nur Azizah et al., 2025). In Indonesia, halal certification has also become an increasingly important regulatory requirement, with the implementation of mandatory halal certification being phased across product categories under Halal Product Assurance and Government Regulation No. 42 of 2024, with mandatory certification for imported and MSME-originated food and beverage products in October 2026. Thus, ensuring halal compliance throughout the production chain—including materials and inputs used in food biotechnology—has become increasingly important. Producers must follow guidelines to guarantee the halal status of bioproducts. Consequently, the proportion of halal microbial products in the biotechnology sector is expected to increase (Karahalil, 2020).

Pratiwi et al. (2020) investigated several alternative sources of beef extract and peptone, as well as carbon sources, to develop halal media for the growth of *L. brevis*. However, the tested concentrations of these substances were insufficient to achieve bacterial growth comparable to that observed in de Man, Rogosa and Sharpe (MRS) medium broth. Our research team successfully developed media for probiotic bacterial production using locally sourced, halal-certified ingredients (Utami et al., 2019), which demonstrated growth support equivalent to the MRS media, making it suitable as a starter for large-scale fermented beverage production (Utami et al., 2020). However, this halal medium has drawbacks, including being time-consuming to prepare and requiring many additional ingredients besides liquid halal meat peptone, such as coconut water, sprout extract, and tomato extract. Its liquid form requires frozen storage and is bulky, making it less practical for preparation, storage, and handling in large-scale production of probiotics or starter cultures. Therefore, a simpler, halal, practical, and easily applicable medium for probiotic production needs to be developed to support large-scale probiotic and functional extracellular metabolite production.

Other studies have explored alternative halal culture media for LAB with cell growth comparable of commercial MRS growth media. Riftyan et al. (2022) developed a halal medium using catfish-derived peptone hydrolyzed with papain and demonstrated its suitability for the growth of *L. plantarum* TMW 1.1623 and *Streptococcus thermophilus*, with subsequent application in fermented milk production. Similarly, previous research developed a plant-based medium comprising soybean powder and sweet potato extract that supported the growth and maintenance of several LAB strains at levels comparable

to MRS (Ayu et al., 2023). However, previous studies focused mainly on alternative media's ability to support bacterial growth, viability, or use in fermented products, while the production of extracellular metabolites during cultivation received limited attention. In addition, existing formulations largely emphasized replacing conventional peptone sources with fish- or plant-derived alternatives. Thus, the potential of halal meat-derived peptone to support probiotic growth and extracellular metabolite production remains insufficiently explored. The present study addresses this gap by evaluating a halal meat peptone-based medium for *L. plantarum* Dad-13 growth and extracellular metabolite production, providing a baseline for further development and optimization of halal culture media for probiotic applications.

Probiotics are also known to produce functional compounds such as gamma-aminobutyric acid (GABA) (Diez-Gutiérrez et al., 2022). Other researchers reported that LAB produced extracellular metabolites during milk fermentation, including GABA, folic acid, bioactive peptides, and other functional compounds (Galli et al., 2022; Hosseini et al., 2024; Jayasree Joshi et al., 2024). Fermentation of milk and black soybean milk increased the antioxidant activity of the fermented product (Leksono et al., 2021, 2022; Luwidharto et al., 2022). During probiotic production in certain media, some extracellular metabolites with functional properties may also be generated (Chang et al., 2021; Ooi et al., 2021). However, the potential of halal culture media to support extracellular metabolite production alongside probiotic growth remains insufficiently explored. The novelty of the present study lies in the development of a halal meat peptone- and yeast extract (YE)-based medium and the simultaneous evaluation of its ability to support *L. plantarum* Dad-13 growth and extracellular metabolite production. This study therefore provides a baseline for understanding the relationship between halal medium composition, probiotic growth, and extracellular metabolite production, while offering a practical alternative to the conventional MRS medium for halal probiotic production.

2. Materials and Methods

2.1. Materials

Minced pineapples and halal beef were purchased from the local market in Yogyakarta, Indonesia. The NuCel YE with halal certification CICOT.HL. 99A6534560662, from NuCel, Procelys, France. Sucrose (PT Sugar Group Company, Indonesia), skim milk (PT Mirota KSM, Indonesia), and mineral water (PT Tirta Investama, Indonesia) were purchased from the local market and believed to be halal ingredients, as evidenced by the official halal logo (Halal Product Guarantee Agency, Ministry of Religion, Indonesia) on the packaging. The *L. plantarum* Dad-13 was obtained from the Food and Nutrition Culture Collection at the Centre for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. MRS agar (Merck, Germany), bacteriological agar (Oxoid, USA), and calcium carbonate ($CaCO_3$) (Merck, Germany) were used for microbial analyses.

2.2. Preparations of Meat Peptone Powder

The preparation of powdered meat peptone involved three stages: preparation of crude bromelain enzyme, hydrolysis of meat protein with crude bromelain enzyme, and production of powdered meat peptone. To prepare the crude bromelain enzyme, pineapples (500 g) were peeled and cut into small pieces. The extraction process was carried out using a juice extractor (Cosmos CJ 388). The crude bromelain liquid (280 mL) was automatically separated from the solid waste.

We performed crude bromelain hydrolysis of minced beef as described by Utami et al. (2019), with modifications. For meat protein hydrolysate production, halal minced beef (500 g) was added to an Erlenmeyer flask and mixed with 280 mL crude bromelain and mineral water until the volume reached 2 L. Hydrolysis was carried out at 60 °C for 2 hours using a water bath (Haake SWB), followed by cooling at room temperature. The hydrolysate was filtered through two layers of muslin. The liquid meat peptone was then spray dried (Buchi mini spray dryer B-290) at an inlet temperature of 155 °C, a pump rate of 25%, and an aspirator of 95%. The resulting meat peptone powder was then stored in a closed container at 4 °C for analysis of vitamins, minerals, and free amino acid content.

2.3. Inoculum Preparation

The stock culture of *L. plantarum* Dad-13 was stored in a cryoprotectant solution (20% sucrose and 2% skim milk) at -40 °C. A 0.1% mL (v/v) culture was recovered in 10 mL of sterile halal media consisting of 1% (w/v) meat peptone powder, 1% (w/v) YE, 3% (w/v) sucrose, and 95% (v/v) mineral water in a 15 mL conical flask, then incubated at 30 °C for 24 hours.

2.4. Fermentation of Halal Growth Media

To study the effects of halal growth media, 200 mL of sterile halal media at various concentrations of meat peptone powder (0.011% to 1%) and YE (0% to 1%), with 3% (w/v) sucrose, was inoculated with *L. plantarum* Dad-13 at 0.1% (v/v). Fermentation was carried out at 30 °C for 24 hours. Samples were collected at 12 and 24 hours of incubation and analysed for viable cell count of *L. plantarum* Dad-13, pH of the growth medium, and extracellular metabolite production.

2.4.1. Enumeration of Viable Cell Population

The population of *L. plantarum* Dad-13 was assessed by serial dilution of the collected fermentation sample in 0.85% (w/v) NaCl, followed by plating on MRS agar containing 1% (w/v) $CaCO_3$. After incubating at 30 °C for 48 hours, the bacterial colonies on the plates were counted and expressed as colony-forming units per milliliter (CFU/mL).

2.4.2. Measurement of pH

The pH of the culture media was measured directly at room temperature using a pH meter (Apera Instruments PH60S-Z).

2.4.3. Analysis of Vitamins

The vitamin B analysis was conducted using Ultra-Performance Liquid Chromatography (UPLC), following the method described by Antakli et al. (2015) with some modifications. 3 g of meat peptone powder was placed in a 25 mL volumetric flask. For general vitamin analysis, 0.05 M NaH_2PO_4 buffer at pH 6.3 was added halfway up the flask; for vitamin B2 analysis, deionized water was used as the solvent, followed by the addition of 200 µL of 1 N NaOH. The mixture was sonicated for 15 minutes, then homogenized in 0.05 M NaH_2PO_4 buffer at pH 6.3. For vitamin B2 analysis, deionized water was added before homogenization. The solution was transferred to a 2 mL tube, centrifuged at 14,000 rpm for 3 minutes, filtered through a 0.20 µm syringe filter, and stored in a 2 mL vial. 5 µL sample was injected into the UPLC system. Standard solutions were then prepared: thiamine hydrochloride (Supelco PHR 103) for vitamin B1, riboflavin (SIGMA PHR1054-1G) for vitamin B2, nicotinic acid (SIGMA PHR1276-1G) for vitamin B3, and pyridoxine hydrochloride (SIGMA PHR1036-500MG) for vitamin B6. Separation was performed on a C18 reverse-phase column, with detection by a Photodiode Array Detector at 245, 265, 265, and 283 nm for vitamins B1, B2, B3, and B6, respectively. The mobile phase comprised 0.1% (w/v) H_3PO_4 as eluent A and acetonitrile as eluent B. Chromatography was performed using a gradient elution at a column temperature of 40 °C, with a flow rate of 0.30 mL/min for 10 minutes.

2.4.4. Analysis of Minerals

Mineral analysis of meat peptone powder was performed using Inductively Coupled Plasma-Optical Emission Spectrometry (Agilent Technologies 700 Series ICP-OES, Santa Clara, CA, USA) following the AOAC (2012) guidelines for detecting calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), potassium (K), phosphorus (P), sodium (Na), and zinc (Zn) in fortified food products through microwave digestion. Approximately 0.5 g of culture media was placed in a vessel and digested with 10 mL of concentrated nitric acid (HNO_3). Microwave digestion was carried out at 150 °C with a 10-minute ramp and a 15-minute hold. After cooling, the sample was transferred to a 50 mL volumetric flask, and 100 mg·L−1 of yttrium (Y) was added as an internal standard. The solution was then diluted to volume with double-distilled water and filtered before analysis. ICP-OES was performed using a concentric glass nebulizer, with torch alignment intensities exceeding 1,000,000 for horizontal and vertical axes and torch positioning between −1 and 1. Absorbance for Fe, Zn, Mg, Mn, and Y was measured at wavelengths of 238, 213, 285, 257, and 371 nm, respectively. Mineral concentrations were determined using calibration curves from standard solutions of each element.

2.4.5. Analysis of Amino Acids

The amino acids were analyzed using a Waters Acquity UPLC H-Class system (Queenstown, Singapore) with the AccQ-Tag Ultra Kit (number 176001235), following the manufacturer's instructions. One gram of meat peptone powder was hydrolyzed with 6 N hydrochloric acid. The hydrolysate was transferred to a 50 mL volumetric flask and diluted to volume with double-distilled water. The solution was filtered through a 0.2 µm syringe filter (Sartorius Minisart, Germany) and mixed with a 2.5 mM internal standard, alpha-Aminobutyric acid (AABA). The protein hydrolysate was derivatized using the AccQ-Tag Ultra reagent mixture, which includes borate buffer, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and acetonitrile, following the kit instructions. The derivatized

sample was analyzed on a UPLC system featuring a Quaternary Solvent Manager, Sample Manager—Flow, column heater, an ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm), and a photodiode array detector from Waters. The chromatographic analysis employed four mobile phases: mobile phase A consisted of 100% AccQ·Tag Ultra A eluent; mobile phase B consisted of 10% (v/v) AccQ·Tag Ultra B eluent in HPLC-grade water (water-to-eluent B ratio of 90:10, v/v); mobile phase C consisted of 100% High-Performance Liquid Chromatography (HPLC)-grade water; and mobile phase D consisted of 100% AccQ·Tag Ultra B eluent. Samples of 1 μL were analyzed with the column maintained at 49 °C and the sample at 20 °C. The system pressure was approximately 13,000 psi, and detection was determined at 260 nm. The mobile phase flow rate and gradient are detailed in Table 1. Quantification was performed by comparing the areas of the sample or standard amino acids to the internal standard area.

2.4.6. Analysis of GABA

GABA analysis was conducted based on the method described by Zandy et al. (2017). The accumulation of GABA in the fermentation media was quantified using HPLC. First, mobile phases A and B were prepared. Mobile phase A consisted of methanol, 50 mM sodium acetate, and tetrahydrofuran in a ratio of 2:96:2, while mobile phase B consisted of 65% (v/v) methanol. Both mobile phases were vacuum-filtered and degassed before being used to condition the HPLC instrument. The GABA standard was prepared by dissolving GABA powder to 1000 ppm, then diluting to 100 ppm and further diluting to a series of concentration levels. For sample preparation, the solid sample was weighed and dissolved in a solution to a concentration of 1000 ppm. The sample solution was filtered through a 0.45 μm membrane filter and derivatized with o-phthaldialdehyde (OPA) at a 1:30 (sample:OPA) ratio. The OPA solution was prepared by dissolving 0.01 g of OPA in 1 mL of methanol, followed by the addition of 4 mL of borate buffer (pH 9) and 10 μL of 2-mercaptoethanol. The mixture was homogenized for 2 minutes prior to HPLC analysis.

2.4.7. Analysis of Untargeted Metabolites

The untargeted metabolites were analyzed using the method of Ao et al. (2023). A 200 mg sample, along with grinding beads, was placed in a centrifuge tube. Subsequently, 400 μL of an extraction solution consisting of methanol and water (4:1, v/v) containing 0.02 mg/mL of the internal standard (L-2-chlorophenylalanine) was added. Ultrasonic extraction was performed for 30 minutes at 5 °C and 40 kHz. The mixture was then allowed to stand at -20 °C for 30 minutes, followed by centrifugation at 13,000 rpm for 15 minutes at 4 °C. The resulting supernatant was injected into a liquid chromatography-mass spectrometry (LC-MS) system for data processing and analysis.

2.4.8. Analysis of Volatile Compounds

The analysis of volatile compounds in culture media followed a modified method from Caprioli et al. (2016). In a 22 mL SPME vial, 1 g of sample was placed and heated in a water bath before extraction with a 2 cm DVB/CAR/PDMS SPME fibre at 55 °C for 90 minutes. The sample was then injected into the gas chromatography-mass spectrometry (GC-MS) system (Agilent 7890A, Agilent 5975C XL EI/CI, USA) with the injector at 250 °C in splitless mode. The column used was DB-Wax (30 m x 250 m x 0.25 m), with an initial temperature of 40 °C held for 5 minutes, then ramped to 220 °C at 3 °C/min. Helium was used as the carrier gas at 1 mL/min, and volatile components were identified using the NIST 14 library.

2.5. Statistical Analysis

Results were provided as mean ± standard deviation (SD). Prior to analysis of variance, the normality and homogeneity of variance assumptions were assessed using the Shapiro–Wilk and Levene's tests, respectively. Data were subjected to two-way ANOVA, and mean differences were evaluated using Tukey's test ($p < 0.05$) with IBM SPSS Statistics v.23 (IBM, USA) ($n = 6$). The untargeted LC-MS metabolomics and GC-MS volatile compound analyses were conducted using a single sample ($n = 1$) for each analysis. Consequently, statistical analyses could not be performed, and the resulting data were evaluated descriptively based on the relative abundance or intensity of the detected metabolites and volatile compounds. Principal Component Analysis (PCA) of volatile compounds was carried out using XLSTAT 2024.4.1.1425. Data visualization was conducted using R (4.6.0) and RStudio (version 2025.05.1). The Venn diagram used ggplot22 and the ggVennDiagram package, while the heat plot was generated using ggplotify and the pheatmap package.

3. Results and Discussion

3.1. Characteristics of Meat Peptone Powder

Meat peptone was prepared by hydrolyzing halal minced meat with crude bromelain from pineapple juice. The use of a protease source from pineapple was due to availability and to fulfill halal requirements. Bromelain is a plant-based protease with broad industrial applications (Arshad et al., 2014). Meat peptone powder resulting from the spray drying process yielded 15.26 ± 2.71 grams, or 3.05 ± 0.54%, from 500 mL of liquid meat peptone. The results showed that spray drying at 155 °C yields a product with good consistency by converting the meat peptone solution into powder. Long and Mohan (2021) reported that an inlet temperature of 155 °C can increase the powder's dryness for spray-dried raw beef tongue.

LAB are fastidious microorganisms that require factors that they cannot synthesize, such as amino acids and vitamins, which must be provided in the media to support their growth and bioactivity (Utami et al., 2020). Vitamins, minerals, and free amino acid contents in meat peptone powder are presented in Tables 1, 2, and 3, respectively. Table 1 shows that, in meat peptone powder, vitamin B3 (niacin) had the highest level at 6.21 mg/100 g, followed by vitamin B5 (pantothenic acid) at 1.72 mg/100 g and vitamin B9 (folic acid) at 0.25 mg/100 g. However, B1 (thiamine), B2 (riboflavin), B6 (pyridoxine), and B12 (cobalamin) were not detected in the meat peptone powder. Table 2 shows that meat peptone powder had the highest K content (19,622.6 mg/kg), followed by P content (8,106.85 mg/kg). Meat peptone powder also contained other minerals, including Na, Ca, selenium (Se), Zn, Mn, Fe, and Cu, at lower levels. Table 3 shows amino acid compositions of meat peptone powder. L-cystine has the highest concentration (15,612.98 mg/kg), followed by L-leucine and L-arginine, each at around 11,000 mg/kg. Amino acids such as Glycine, L-histidine, L-tryptophan, L-aspartic acid, and L-proline were present at lower levels, below 2,000 mg/kg, making them the least abundant amino acids in meat peptone powder. This meat peptone powder provides not only a nitrogen source but also vitamins, minerals, and free amino acids that support the growth factor requirements of LAB.

Table 1. Vitamin Content of Meat Peptone Powder

Vitamin	Concentration (mg/100 g)
B1 (Thiamine)	ND
B2 (Riboflavin)	ND
B6 (Pyridoxine)	ND
B3 (Niacin)	6.21±0.04
B9 (Folic acid)	251.42±2.32
B12	ND
B5	1.72±0.02

ND = not detected

Table 2. Mineral Content of Meat Peptone Powder

Parameter	Unit	Concentration
Potassium (K)	mg/kg	1962.26±59.23
Manganese (Mn)	mg/kg	4.09±0.01
Sodium (Na)	mg/100 g	258.74±0.14
Phosphorus (P)	mg/kg	8106.85±0.56
Selenium (Se)	µg/100 g	11.29±1.44
Zinc (Zn)	mg/100 g	5.15±0.04
Iron (Fe)	mg/100 g	4.00±0.01
Calcium (Ca)	mg/100 g	61.87±0.40
Copper (Cu)	mg/kg	0.90±0.00

Table 3. Free Amino Acid Content of Meat Peptone Powder

Amino Acid	Concentration (mg/kg)
L-cystine	15,612.98±2.47
L-leucine	11,247.56±39.57
L-arginine	11,147.43±9.71
L-serine	7,720.77±9.91
L-alanine	4,830.91±18.19

Table 3. Free Amino Acid Content of Meat Peptone Powder (continued)

Amino Acid	Concentration (mg/kg)
L-methionine	4,539.00±1.68
L-lysine	4,371.54±4.43
L-phenylalanine	3,599.23±9.68
L-glutamic acid	3,374.38±12.75
L-tyrosine	3,066.48±6.56
L-isoleucine	2,522.71±1.70
L-valine	2,635.00±7.11
L-threonine	2,104.94±6.00
Glycine	1,608.98±6.81
L-histidine	1,154.32±1.15
L-tryptophan	953.64±2.28
L-aspartic acid	787.41±1.65
L-proline	660.06±0.57

3.2. Growth of Probiotic Lactiplantibacillus plantarum subsp. plantarum Dad-13

The growth medium consisted of 3% (w/v) sucrose and meat peptone, supplemented with various concentrations of YE. Table 4 shows the growth of *L. plantarum* Dad-13 cells in halal media based on meat peptone powder (1%, 0.5%, 0.1%, 0.05%, 0.01%, and 0%) and YE (1%, 0.5%, 0.01%, and 0%) at various concentrations. A 1% (w/v) concentration of meat peptone powder was equivalent to 25% liquid meat peptone, as reported by Utami et al. (2020). Overall, increasing the concentrations of meat peptone powder and YE tends to increase viable cell population. The highest cell growth was observed at a meat peptone powder concentration of 1% (w/v) and 1% (w/v) YE, with the number of cells being 7.54 CFU/mL, 10.35 CFU/mL, and 10.44 CFU/mL at 0, 12, and 24 hours, respectively, although these were not significantly different compared to another 1% (w/v) YE and meat peptone at various concentrations. Cell growth reached 10.14–10.43 log CFU/mL using 0.01% (w/v) to 0.1% (w/v) meat peptone mixed with YE of 0.01% (w/v) and 1% (w/v). Meanwhile, 0.5% (w/v) and 1% (w/v) meat peptone with only 0.01% (w/v) YE also yielded a cell count of 10 log CFU/mL. These results show better cell growth compared to our previous study of halal liquid meat peptone containing bean sprout extract, tomato extract, and young coconut water (Utami et al., 2020). YE contains vitamins, minerals, peptides, and amino acids that are important factors for the growth of LAB (Kevvai et al., 2014). MRS is a selective medium for LAB that contains meat peptone and YE (Corry et al., 2003). In this study, the growth medium contained only sucrose as a carbon source, meat peptone, and halal-certified Nucel YE from Procelys. Thus, meat peptone and YE play important roles in supporting the growth of LAB.

Changes in the pH of the growth media indicate cell growth and metabolic activity, as the metabolic activity of LAB produces acid, especially lactic acid (Ooi et al., 2021), leading to a decrease in pH in the fermentation media (Yang et al., 2018). Table 5 shows the pH measurements of halal media based on meat peptone powder during fermentation with varying concentrations of peptone and YE. The fermentation activity of probiotic bacteria was affected by media composition. The presence of YE significantly stimulated the fermentation process. This is in line with Mamun et al. (2023), in which the addition of YE to the growth medium resulted in greater acidification activity than other nitrogen sources, such as peptone, meat extract, and ammonium sulfate.

Table 4. The Growth of *L. plantarum* Dad-13 in Different Media Compositions

Meat peptone powder	Yeast extract	Viable cells (CFU/mL)		
		Initial	12 h	24 h
1%	1%	7.54 ± 0.35^{Ga}	10.35 ± 0.04^{Gb}	10.44 ± 0.01^{Gc}
	0.5%	7.32 ± 0.32^{EaFaGa}	10.00 ± 0.05^{EbFbGb}	10.34 ± 0.00^{EcFcGc}
	0.01%	7.72 ± 0.13^{EaFaGa}	9.73 ± 0.11^{EbFbGb}	10.15 ± 0.07^{EcFcGc}
	0%	7.59 ± 0.29^{DaEaFa}	9.34 ± 0.01^{DbEbFb}	9.94 ± 0.04^{DcEcFc}
0.5%	1%	7.54 ± 0.35^{FaGa}	10.21 ± 0.04^{FbGb}	10.42 ± 0.00^{FcGc}
	0.5%	7.32 ± 0.32^{EaFaGa}	9.99 ± 0.00^{EbFbGb}	10.20 ± 0.02^{EcFcGc}
	0.01%	7.72 ± 0.13^{EaFaGa}	9.79 ± 0.04^{EbFbGb}	10.03 ± 0.01^{EcFcGc}
	0%	7.59 ± 0.29^{CaDaEa}	8.99 ± 0.02^{C2D2E2}	9.87 ± 0.04^{CcDcEc}

Table 4. The Growth of *L. plantarum* Dad-13 in Different Media Compositions (continued)

Meat peptone powder	Yeast extract	Viable cells (CFU/mL)		
		Initial	12 h	24 h
0.1%	1%	7.54 ± 0.35^{FaGa}	10.13 ± 0.03^{FbGb}	10.31 ± 0.02^{FcGc}
	0.5%	7.32 ± 0.32^{BaCaDaEa}	8.88 ± 0.07^{BbCbDbEb}	$10.12 \pm 0.01^{BcCcDcEc}$
	0.01%	7.72 ± 0.13^{CaDaEa}	8.96 ± 0.08^{CbDbEb}	9.72 ± 0.13^{CcDcEc}
	0%	7.59 ± 0.29^{Aa}	8.08 ± 0.42^{Ab}	9.36 ± 0.01^{Ac}
0.05%	1%	7.54 ± 0.35^{FaGa}	10.11 ± 0.01^{FbGb}	10.29 ± 0.01^{FcGc}
	0.5%	7.32 ± 0.32^{DaEaFaGa}	9.86 ± 0.01^{DbEbFbGb}	$10.08 \pm 0.13^{DcEcFcGc}$
	0.01%	7.72 ± 0.13^{AaBaCaDa}	8.66 ± 0.29^{AbBbCbDb}	9.66 ± 0.09^{AcBcCcDc}
	0%	7.59 ± 0.29^{AaBaCaDa}	8.80 ± 0.01^{AaBaCaDa}	9.78 ± 0.04^{AaBaCaDa}
0.01%	1%	7.54 ± 0.35^{FaGa}	10.04 ± 0.01^{FbGb}	10.39 ± 0.02^{FcGc}
	0.5%	7.32 ± 0.32^{DaEaFaGa}	9.79 ± 0.04^{DbEbFbGb}	$10.14 \pm 0.06^{DcEcFcGc}$
	0.01%	7.72 ± 0.13^{AaBaCaDa}	8.78 ± 0.09^{AbB2CbDb}	9.53 ± 003^{AcBcCcDc}
	0%	7.59 ± 0.29^{AaBa}	7.87 ± 0.01^{AbBb}	9.61 ± 0.06^{AcBc}
0%	1%	7.54 ± 0.35^{EaFaGa}	9.82 ± 0.32^{EbFbGb}	10.31 ± 0.04^{EcFcGc}
	0.5%	7.32 ± 0.32^{DaEaFaGa}	9.89 ± 0.30^{DbEbFbGb}	$10.17 \pm 0.16^{DcEcFcGc}$
	0.01%	7.72 ± 0.13^{AaBaCa}	8.42 ± 0.56^{AbBbCb}	9.32 ± 0.33^{AcBcCc}

Description: Superscript uppercase letters (A–G) and lowercase letters (a–c) indicate significant differences according to Tukey's post hoc test ($p < 0.05$). Uppercase letters (A–G) indicate significant differences among treatments, whereas lowercase letters (a–c) indicate significant differences among fermentation times.

The initial pH was within the optimal range for LAB growth and decreased as the cells grew. At the end of the fermentation period, the entire medium reached pH ranging from 3.98 to 4.77. Media containing 1% (w/v) meat peptone, with or without YE, supported the growth of *L. plantarum* Dad-13, as shown by pH reduction when incubated at 30 °C for 24 hours, indicating that 3% (w/v) sucrose and 1% (w/v) halal meat peptone powder were sufficient for probiotic production, which simplified our previously reported liquid halal media fermentation process (Utami et al., 2020).

Table 5. pH of Different Fermented Media of *L. plantarum* Dad-13

Meat peptone powder	Yeast extract	Media pH		
		Initial	12 h	24 h
1%	1%	6.68 ± 0.23^{Aa}	4.59 ± 0.12^{Ab}	4.02 ± 0.09^{Ac}
	0.5%	6.68 ± 0.23^{Aa}	4.63 ± 0.11^{Ab}	4.02 ± 0.00^{Ac}
	0.01%	6.68 ± 0.23^{AaBaCa}	5.13 ± 0.13^{AbBbCb}	4.28 ± 0.15^{AcBcCc}
	0%	6.68 ± 0.23^{Aa}	5.04 ± 0.17^{Ab}	4.07 ± 0.07^{Ac}
0.5%	1%	6.68 ± 0.23^{Aa}	4.61 ± 0.01^{Ab}	3.98 ± 0.03^{Ac}
	0.5%	6.68 ± 0.23^{Aa}	4.61 ± 0.02^{Ab}	4.04 ± 0.04^{Ac}
	0.01%	6.68 ± 0.23^{AaBaCaDa}	5.23 ± 0.27^{AbBbCbDb}	$4.29 \pm 0.18A2^{BbCbDb}$
	0%	6.68 ± 0.23^{AaBa}	5.28 ± 0.24^{AbBb}	4.07 ± 0.11^{AcBc}
0.1%	1%	6.68 ± 0.23^{Aa}	4.51 ± 0.02^{Ab}	3.92 ± 0.01^{Ac}
	0.5%	6.68 ± 0.23^{Aa}	4.64 ± 0.00^{Ab}	3.94 ± 0.04^{Ac}
	0.01%	6.68 ± 0.23^{CaDaEaFa}	6.48 ± 0.00^{CbDbEbFb}	4.51 ± 0.19^{CcDcEcFc}
	0%	6.68 ± 0.23^{CaDaEaFa}	6.64 ± 0.05^{CbDbEbFb}	4.37 ± 0.01^{CcDcEcFc}
0.05%	1%	6.68 ± 0.23^{Aa}	4.68 ± 0.14^{Ab}	3.95 ± 0.04^{Ac}
	0.5%	$6.68 \pm 0.23^{AaBaCaDaEa}$	$5.69 \pm 1.42^{AbBbCbDbEb}$	$3.97 \pm 0.01^{AcBcCcDcEc}$
	0.01%	6.68 ± 0.23^{Fa}	6.66 ± 0.22^{Fb}	4.64 ± 0.27^{Fc}
	0%	$6.68 \pm 0.23^{BaCaDaEaFa}$	$6.35 \pm 0.01^{BbCbDbEbFb}$	$4.52 \pm 0.05^{BcCcDcEcFc}$

Table 5. pH of Different Fermented Media of *L. plantarum* Dad-13 (continued)

Meat peptone powder	Yeast extract	Media pH		
		Initial	12 h	24 h
0.01%	1%	6.68 ± 0.23^{Aa}	4.73 ± 0.01^{Ab}	3.93 ± 0.07^{Ac}
	0.5%	6.68 ± 0.23^{Aa}	4.72 ± 0.03^{Ab}	3.93 ± 0.07^{Ac}
	0.01%	$6.68 \pm 0.23^{BaCaDaEaFa}$	$6.67 \pm 0.40^{BbCbDbEbFb}$	$4.17 \pm 0.34^{BcCcDcEcFc}$
	0%	6.68 ± 0.23^{DaEaFa}	6.38 ± 0.03^{DbEbFb}	4.77 ± 0.30^{DcEcFc}
0%	1%	6.68 ± 0.23^{Aa}	4.93 ± 0.08^{Ab}	3.98 ± 0.00^{Ac}
	0.5%	6.68 ± 0.23^{Aa}	4.56 ± 0.02^{Ab}	3.99 ± 0.29^{Ac}
	0.01%	6.68 ± 0.23^{EaFa}	6.65 ± 0.42^{EbFb}	4.60 ± 0.08^{EcFc}

Description: Superscript uppercase letters (A–F) and lowercase letters (a–c) indicate significant differences according to Tukey's post hoc test ($p < 0.05$). Uppercase letters (A–F) indicate significant differences among treatments, whereas lowercase letters (a–c) indicate significant differences among fermentation times.

3.3. GABA Production by Lactiplantibacillus plantarum subsp. plantarum Dad-13

GABA analysis was performed using 1% (w/v) meat peptone, which was selected based on its ability to support the highest cell viability. Table 6 shows that the growth of *L. plantarum Dad-13* in media containing different combinations of halal meat peptone and YE was comparable to MRS media, with a maximum of 10.62±0.10 CFU/mL. This simple medium produced higher cell concentration than the alternative media for *Lactobacillus brevis*, as reported by Pratiwi et al. (2020). However, further study should be conducted to determine the effect of this simple alternative medium composition on the growth of other probiotic or LAB.

Table 6. Cell Growth and GABA Production by *L. plantarum* Dad-13 After Incubation

Yeast extract	Cell (Log CFU/mL)	pH	GABA (mg/L)
0%	10.12 ± 0.44^{A}	4.03 ± 0.10^{A}	28.74 ± 1.15^{A}
0.1%	10.25 ± 0.66^{A}	4.07 ± 0.12^{A}	32.02 ± 7.05^{A}
0.5%	10.39 ± 0.20^{A}	4.09 ± 0.04^{A}	53.87 ± 2.54^{B}
1%	10.62 ± 0.10^{A}	4.11 ± 0.05^{A}	63.64 ± 4.53^{B}
MRS	10.52 ± 0.13^{A}	3.79 ± 0.02^{B}	85.68 ± 18.11^{C}

Notes: MRS was used as a control. The growth medium contained 1% (w/v) sucrose, 1% (w/v) meat peptone, and various concentrations of yeast extract. The fermentation was conducted at 30 °C for 24 h. Different superscript letters (A-C) indicate significant differences according to Tukey's post hoc test ($p < 0.05$)

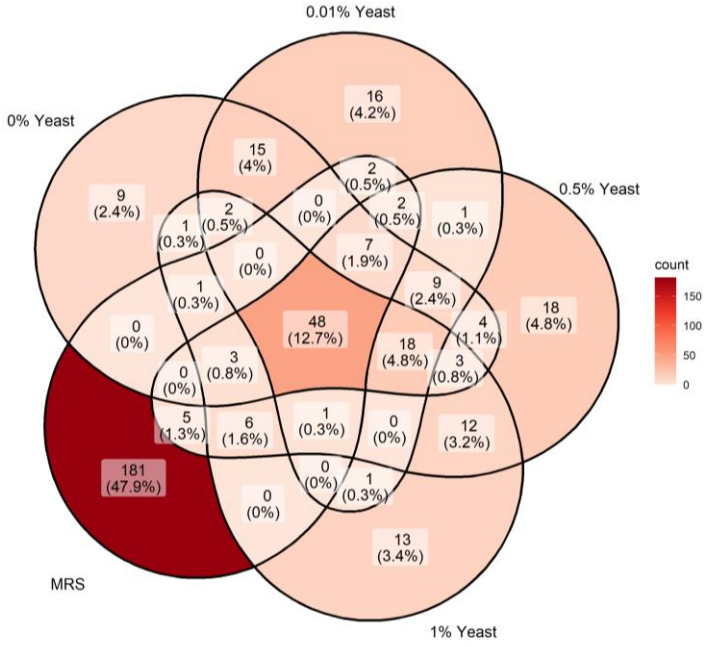
Probiotics are also known to produce functional compounds such as GABA (Diez-Gutiérrez et al., 2022). Other researchers reported that LAB produced extracellular metabolites during milk fermentation, including GABA, folic acid, bioactive peptides, and other functional compounds (Galli et al., 2022; Hosseini et al., 2024; Jayasree Joshi et al., 2024). This study examined extracellular GABA production by *L. plantarum* Dad-13 in media containing meat peptone and different concentrations of YE. The results showed the concentration of YE significantly affected extracellular GABA production. The higher the YE concentration, the higher the GABA concentration produced. However, the highest GABA concentration was produced from MRS medium. This result agrees with other findings, indicating that GABA production is affected greatly by media composition (Diana et al., 2014; Hsueh et al., 2017; Wu et al., 2017). The findings of this study provide important information on the production of probiotics using a simple halal growth medium and the simultaneous production of functional extracellular metabolites. Probiotic cells can be harvested by centrifugation to separate cells and free cell supernatant. The free cell supernatant can be further processed to develop functional extracellular metabolite products.

3.4. Extracellular Metabolites

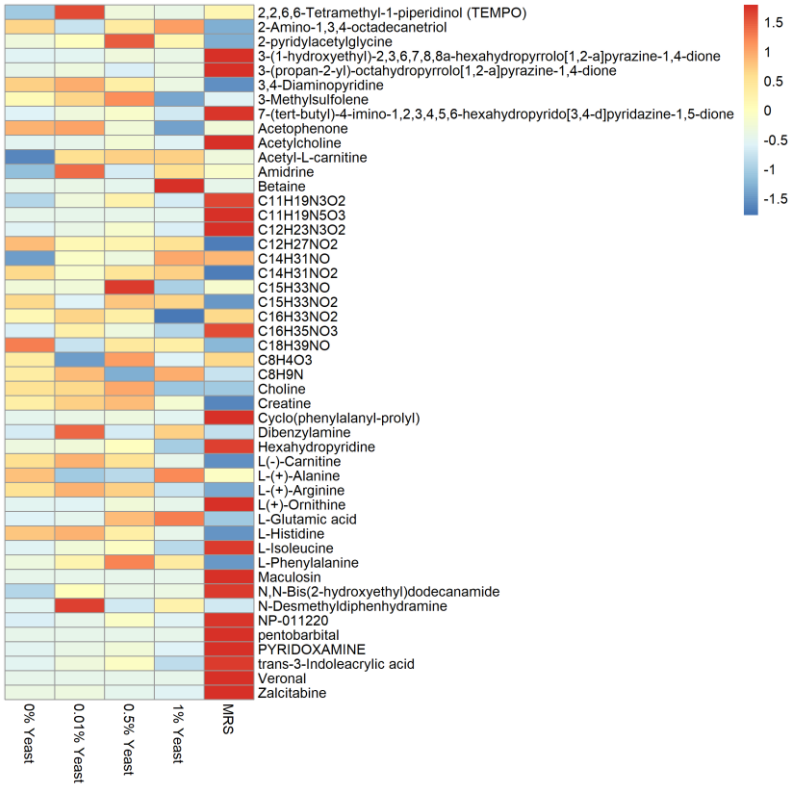
Extracellular metabolites are bioactive or metabolic compounds secreted by microbes into the fermentation media during growth. These include organic acids, bacteriocins, amino acid derivatives, exopolysaccharides, vitamins, peptides, and other soluble substances (Chang et al., 2021; Tang et al., 2023). They accumulate outside the cells and play important roles in microbial interactions, food preservation, flavor formation, and host health (Mora-Villalobos et al., 2020). The production of extracellular metabolites is affected by pH, nutrient levels, temperature, and fermentation duration (Pinu & Villas-Boas, 2017).

Extracellular metabolite analysis was performed using 1% (w/v) meat peptone, which was selected based on its ability to support the highest cell viability. Figure 1(a) shows the number of specific and shared metabolites among the media: YE-0%, YE-0.1%, YE-0.5%, YE-1%, and MRS. At the same time, the heatmap (Figure 1(b)) shows the production trend for each type of metabolite across the respective media. The Venn diagram revealed that the probiotic strain produced the most metabolites in MRS media (181 metabolites), whereas the number of metabolites produced in YE media ranged from 9 to 18. Furthermore, substantial overlap in metabolite production was noted, with 48 metabolites detected across all media, indicating a conserved core metabolic profile while highlighting the prominence of specialized metabolic responses, especially in MRS media.

The hierarchical clustering heatmap was generated to evaluate quantitative differences in metabolite production. Based on the heatmap, *L. plantarum* Dad-13 produced 48 metabolites, with an evident trend similarity across YE media, in contrast to the metabolite trend in MRS media. *L. plantarum* Dad-13 produced high yields of 20 specific metabolites from MRS, such as zalcitabine, veronal, trans-3-indoleacrylic acid, pyridoxamine, pentobarbital, maculosin, acetylcholine, and other compounds. In contrast, only four specific metabolites were highlighted in those grown in YE media: Betaine, 2-pyridylacetylglycine, N-desmethyldiphenhydramine, and $C_{15}H_{33}NO$. Extracellular metabolites $C_{18}H_{39}NO$ was prominent in YE-0%, 2,2,6,6-tetramethyl-1-piperidinol and N-desmethyldiphenhydramine in YE-0.01%; 2-pyridylacetylglycine and $C_{15}H_{33}NO$ in YE-0.5%; and betaine in YE-1%. Overall, the combined Venn and heatmap analyses indicated that, while a substantially conserved metabolic network exists across all five conditions, each medium also induced distinct metabolite production. According to Huang et al. (2025), substrate utilization and microbial metabolic capacity can influence the extracellular metabolite profile generated during fermentation. Extracellular metabolite profiling of the *L. plantarum* FRT4 supernatant followed fermentation in MRS medium, compared with non-inoculated MRS medium, showed substantial changes in metabolite composition, including increased levels of acetylcholine, nicotinamide adenine dinucleotide, and trans-3-coumarate, accompanied by decreased levels of uridine, inosine, and fructose-1-phosphate, suggested that *L. plantarum* FRT4 actively modulates pathways associated with neurotransmission, redox homeostasis, and purine metabolism.



(a)



(b)

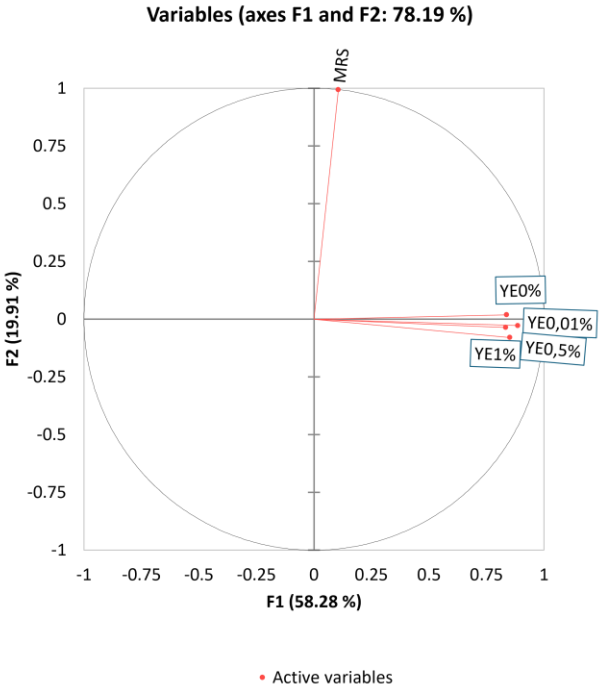
Figure 1. (a) Five-way Venn diagram, and (b) heatmap showing the extracellular metabolite profiles of *Lactiplantibacillus plantarum* subsp. *plantarum Dad-13* and their respective media.

3.5. Volatile Organic Compounds

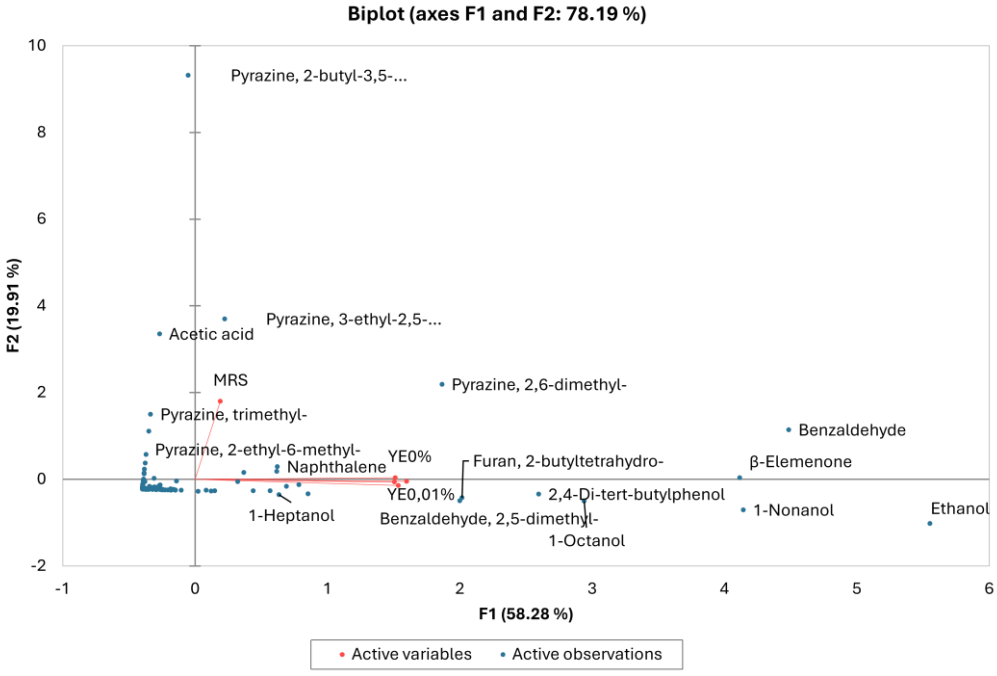
Volatile organic compounds (VOCs) are a subset of extracellular metabolites distinguished by low molecular weight and high vapor pressure, enabling them to diffuse easily through gases and liquids. In LAB and probiotic fermentations, VOCs are mainly produced of breakingdown of carbohydrates, amino acids, and lipids. They include alcohols, aldehydes, ketones, esters, sulfur compounds, organic acids, and terpenes. These substances significantly influence the aroma, flavor, and sensory profile of fermented foods and may be involved in microbial communication and ecological interactions (Mandha et al., 2022; Smid & Kleerebezem, 2014; Weisskopf et al., 2021; Zhou et al., 2026).

The PCA score plot (Figure 2(a)) shows the dimensionally reduced data for the active variables: the YE concentrations in the media (YE-0%, YE-0.1%, YE-0.5%, YE-1%, and MRS). These variables were reduced into two principal factors that account for 78.19% of the total variance. The loading plot illustrating the relationship between F1 and F2 shows the observed results for the variety of volatile compounds produced by *L. plantarum* Dad-13 in each medium. The loading plot can be interpreted using the inverse-relationship framework, which holds that properties on one side are negatively correlated with those on the other. Most variables were positively correlated with F1 (YE-0%, YE-0.01%, YE-0.5%, and YE-1%), whereas only MRS was positively correlated with F2.

Figure 2(b) shows that VOCs associated with the MRS treatment, particularly pyrazine, trimethyl-, pyrazine, 2-ethyl-6-methyl-, and acetic acid, were distributed in the negative F1 and positive F2 quadrants, reflecting their influence on the differentiation of VOC profiles between the MRS and YE media. In comparison, the YE treatments, regardless of YE concentration, were primarily positioned on the positive F1 side and near the F2 axis. Naphthalene, 1-heptanol, benzaldehyde, 2,5-dimethyl-, and 2-butyltetrahydrofuran were closely associated with this distribution and contributed to separating YE-based treatments from MRS. Among the detected compounds, ethanol, 1-nonanol, 1-octanol, 2,4-di-tert-butylphenol, β-elemenone, and benzaldehyde showed the strongest association with the positive F1 region, suggesting a major role in the variation between the VOC profiles of MRS and YE media. Rajendran et al. (2024) reported that variations in defined medium composition and LAB strains can substantially modulate the VOC profiles generated during fermentation. VOCs exhibited different results depending on amino acid supplementation of the different media and the LAB strain employed. In particular, the addition of threonine, methionine, and isoleucine resulted in pronounced changes in the relative concentrations of VOCs following fermentation with LAB.



(a)



(b)

Figure 2. (a) PCA score plot showing the variations among media types, and (b) PCA showing the associated volatile compounds detected in each medium (YE-0%, YE-0.01%, YE-0.5%, YE-1%, and MRS).

4. Conclusion

Halal meat peptone powder has a complex nutritional profile containing several B vitamins and minerals that supported the growth requirements of *L. plantarum* Dad-13 with or without the presence of YE. In addition, the developed halal meat peptone medium also supported the production of functional extracellular metabolites, such as GABA, by *L. plantarum* Dad-13. Hence, the developed halal meat peptone medium is comparable to MRS as a growth medium, although it produced less GABA. *L. plantarum* Dad-13 produced high yields of 20 specific metabolites from MRS, such as zalcitabine, veronal, trans-3-indoleacrylic acid, pyridoxamine, pentobarbital, maculosin, acetylcholine, and other compounds. In contrast, only four specific metabolites were highlighted in YE medium: betaine, 2-pyridylacetylglycine, N-desmethyldiphenhydramine, and $C_{15}H_{33}NO$. Different media compositions

resulted in distinct patterns of volatile organic compounds; the major compound takes a role in the strong separation between the VOC profiles of MRS and YE media were ethanol, 1-nonanol, 1-octanol, 2,4-di-tert-butylphenol, β-elemenone, and benzaldehyde. Overall, the developed halal meat peptone-based medium represents a promising alternative to conventional MRS medium for *L. plantarum* Dad-13 cultivation and extracellular metabolite production. Its ability to support high cell density using halal-derived components highlights its potential for application in industrial probiotic production and the development of halal probiotic products. Further optimization and validation using different bacterial strains or species are needed to validate the consistency of the medium in supporting optimal bacterial growth and extracellular metabolite production, followed by scale-up studies to evaluate its feasibility for commercial probiotic production.

CRediT Authorship Contribution Statement

Tyas Utami: Conceptualization, Validation, Formal analysis, Resources, Data curation, Writing—original draft, Writing—review and editing, Supervision, Funding acquisition. **Tumisih Septiani:** Methodology, Investigation, Visualization. **Ranto Jambi Subekti:** Methodology, Software, Data curation, Visualization. **Rachma Wikandari:** Validation, Writing—review and editing. **Imaduddin Priambudi:** Writing—review and editing, Visualization. **Putrika Citta Pramesi:** Software, Visualization. **Mariyatun Mariyatun:** Writing—review and editing, Project administration. **Dian Anggraini Suroto:** Conceptualization, Validation, Data curation, Writing—review and editing. **Hooi Ling Foo:** Writing – Review & Editing: critical review, commentary, and revision

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