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The possibility of a halal mix probiotic medium for the cultivation of *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae*

Yetti Marlida, Harnentis, Azizah, Yuliaty Shafan Nur, Frederick Adzitey, Norliza Julmohammad, Nurul Huda

ABSTRACT

This study aimed to determine the effects of interaction between media type (halal mix preparation) and culture mixtures of *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae* (probiotics). A completely randomised factorial design (CRFD) consisting of 2 factors and three replications was used, where factor A was a mixture of *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae* at a ratio of 1:1 (A1); 1:2 (A2) and 2:1 (A3) and factor B was the type of growth media, that is, control (B1), whey tofu, molasses, and fish waste flour (B2), and coconut water, onggok flour and shrimp waste flour (B3). The variables measured were viability, cell biomass, and pH. The results showed interactions between factors A and B, which were significantly different (p <0.05) in terms of viability, cell biomass, and pH. Based on the results of the study, it can be concluded that the mixture of *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae* at a ratio of 2:1 (A3), using coconut water, onggok flour, and shrimp waste flour (B3) as medium and incubated at 36 °C for 24 hours was the best medium. It had a 2.37 viability, 42.33 mg/ml biomass cell, and a pH of 2.37.

Keywords: Halal, *Lactobacillus plantarum*, *Saccharomyces cerevisiae*, viability, biomass cell

INTRODUCTION

Probiotics are microorganisms that harbour and maintain the digestive system of humans and animals. They are eaten by humans and given to livestock primarily as feed additives. Probiotics are live microorganisms supplied directly (direct-fed microbes) and might be a single culture or a blend. When given in adequate amounts, they provide health benefits to the host **[1]**. The benefits of probiotic bacteria for livestock include increasing the immune system and helping nutrient absorption **[2]**. Farmers use probiotics as feed additives because several countries have banned antibiotics as growth promoters and the tendency for pathogenic bacteria to develop resistance to certain antibiotics **[3]**. Lactic acid bacteria and *Saccharomyces cerevisiae* are two types of probiotics derived from bacteria and yeast that are extensively utilized in livestock. In recent years, lactic acid bacteria (LAB) and yeast have become more popular as probiotics in the industrial sector.

Lactobacillus plantarun N16 isolated from fermented buffalo milk called dadih is a probiotic due to its ability to survive at low pH, resistance to 0.03% bile, and ability to kill pathogenic bacteria such as pathogenic bacteria as *E. coli*, *S. aureus,* and *S. Enteritidis* **[4]**. *Saccharomyces cerevisiae* isolated from fermented fish or budu has also been reported to be a probiotic **[2]**. A combination of these two probiotics need to be considered because many commercial probiotics contain various types of microbes, for example, PoultryStar ME has *Enterococcus faecium*, *Lactobacillus reuteri*, *L. salivarius* and *Pediococcus acidilactici*) **[5]**, PrimaLac has Lactobacillus spp., *E. faecium* and *Bifidobacterium thermophilum* **[6]**, and Microguard contains various species of *Lactobacillus, Bacillus*, *Streptococcus*, *Bifidobacterium*, and *Saccharomyces* **[7]**. Lactic acid bacteria and yeast can be combined as probiotics to produce a symbiotic relationship. This was found in the research of Lara-Hidalgo et al. **[8]**, which reported that yeast could increase the number of lactic acid bacteria as probiotics for digestion and fat absorption in the digestive tract. This was supported by the findings of Paramithiotis et al. **[9]**. They reported that lactic acid bacteria produce lactic acid that can be used by yeast as a food source, and yeast produces catalase which can

eliminate H_2O_2 produced by lactic acid bacteria making yeast stimulate the growth of lactic acid bacteria. Rahman et al. **[7]** added that the number of *Lactobacillus* and *Saccharomyces cerevisiae* cells in a mixed culture growth medium was higher than in separate culture growth media.

Adequate nutrition is needed to ensure the survival of bacteria and yeast. Some of the nutrients required include carbon, nitrogen, and other minerals **[10]**. Commercial growth media such as MRS are specific media for the growth of lactic acid bacteria. However, its use on an industrial scale is still a challenge because it is relatively difficult and expensive to obtain. It is necessary to replace costly media with relatively cheaper media that support microbial growth in some communities - like Muslim and Vegetarian communities, where components in MRS broth/medium is an issue. Beef extract and peptone, nitrogen derived from animal sources used for MRS medium, should be avoided. For Muslims, all components of MRS must be halal (permissible for a follower of Islam)-certified, including its animal-derived parts. The primary media for *Saccharomyces cerevisiae* is YPD (bacto yeast extract, bacto peptone, D-glucose, and bacto agar), which must be changed to incorporate less expensive components and take into account the Muslim and Vegetarian communities.

The potency of waste as a natural growth medium for an economical source of carbon and nitrogen is expected to be an alternative solution to the problem of environmental pollution. This study explored natural growth media made from tofu liquid waste, molasses, fish waste flour, coconut water, onggok flour (tapioca waste flour), and shrimp waste flour to grow *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae*. So far, there have been no studies reporting on alternative media (mixed halal preparation) for the growth of *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae* as probiotics.

The research aimed to determine the viability, cell biomass, and pH of *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae* grown as culture mixture (halal mix preparations).

MATERIAL AND METHODOLOGY

Samples

Lactobacillus plantarum N16 and *Saccharomyces cerevisiae* were used as starter cultures. They were obtained from the Laboratory of Feed and Technology, Faculty of Animal Science, Universitas Andalas, Padang, Indonesia. The cultures were stored in a 10% skim milk mixture and 1% sucrose under -20 °C. Alternative materials such as whey tofu, molasses, fish waste flour, coconut water, onggok (tapioca waste flour), and shrimp waste flour were purchased from the local market.

Chemicals

Chemicals used in this study were MRS Broth medium (de Man Rogosa and Sharpe Broth), PDA (Potatoes Dextrose Agar), and PDB (Potatoes Dextrose Broth). All media used were also purchased from Merk, Germany. Tofu liquid waste, molasses, fish waste flour, coconut water, onggok flour, and shrimp waste flour were purchased from the local market.

Animals and Biological Material

Biological materials involved in this study were *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae* of our own collection isolated from the previous study.

Description of the experiment

The experiment consisted of 2 factors (A and B), where factor A was a mixture of *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae* at 1:1 (A1), 1:2 (A2), and 2:1 (A3), and factor B was the type of growth media, thus, control (B1), whey tofu, molasses, and fish waste flour (B2), and coconut water, onggok flour, and shrimp waste flour (B3). The variables measured were viability, cell biomass, and pH.

Laboratory Methods

Viability determination

Cell viability assay measures the number of live/metabolically active cells in a population. Viability was measured according to Pires et al. **[11]**. Viability tests were carried out before and after incorporating *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae* on natural media to ensure their growth using the plate count method. A total of 1 ml of the dilution was plated on a sterile petri dish, poured on MRS agar media, and shaken until evenly distributed. It was then incubated at 37 °C for 24 hours. Afterwhich viability was tested by measuring OD (Optical Density) using a spectrophotometer at an absorbance wavelength of 600nm.

Cell biomass determination

Cell biomass was measured based on the weight of the precipitate in the supernatant according to Pires et al. **[11]**. Centrifugation was carried out twice. Firstly, 10 ml of each sample was centrifuged at 1500 rpm for 10 minutes to remove media deposits. Secondly, 2 ml of each sample was centrifuged at 4,000 rpm for 10 minutes to separate bacteria from the media. The discarded supernatants and the remaining precipitates (pellets) were weighed to determine the wet weight. This research was conducted in three replications. The cell weight (X) was calculated using the following formula:

X (mg/ml) = weight of tube containing wet cells (mg) – weight of empty tube (mg) divided by sample volume (ml).

pH determination

pH was measured for each natural medium according to Matouskova et al. **[12]**. The natural medium was placed in a measuring cup and immersed in a calibrated pH meter. The pH value displayed on the pH screen was read when it was stable.

Sample preparation: There were two alternative media: 1) the media based on whey tofu consisted of whey tofu, molasses, and fish waste meal; 2) the media based on coconut water consisted of coconut water, cassava waste, and shrimp shell meal. The alternative media were prepared by a mixture of whey tofu or coconut water (90%), molasses or onggok flour (5%), and shrimp shell or fish waste meal (5%). The combination of *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae* (probiotic mixed) were based on the TPC (Total Plate Count) results and were divided into three ratios, namely 1:1, 1:2, and 2:1, cultured on MRS-B and incubated at 37 °C for 24 hours. The experiment was triplicated, and the total number of samples analysed was 18.

Statistical Analysis

The data from this research were entered into SPSS 26.0. (SPSS Analytics Partner). And was analysed using a two-way ANOVA (Analysis of Variance) at 0.05 to find the effects of viability, pH, and cell biomass from incorporating *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae* in the natural growth media. Tukey's test was applied to determine significant differences.

RESULTS AND DISCUSSION

Effect of culture and media type on the viability

Microbial growth curves are mathematical models that can aid in the study of microbial growth and behavior, as well as the selection of ideal growth circumstances. The turbidimetric method is an excellent alternative to study bacterial growth since optical density (OD) measurement gives real-time values of bacterial population and has practical significance when dealing with bacteria samples in high cell densities **[13]**, **[14]**. Compared to other techniques such as the standard viable count method, estimation of microbial growth characteristics based on absorbance measurement offers the advantages of being quick, non-destructive, affordable, and reasonably straightforward to automate **[14]**.

Table 1 shows the results for optical density (OD) measurements. There was significant interaction (p <0.05) between the cultures and media types, where A3 (culture with a ratio of 2:1 for *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae*) and B3 (containing coconut water, onggok flour, and shrimp waste flour) exhibited the highest viability value of 2.37; this value was not significantly different (p >0.05) from culture ratios A1:B3, A2:B3 and A3:B3, but significantly different (p <0.05) from other tested halal mix probiotic media.

Table 1 Viability of probiotics (*Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae*) on various culture and growth media.

Ratio of probiotics	Type of media			Mean
	B1 (Control)	B2 (Media 1)	B3 (Media 2)	
A1 (1:1)	1.32^{b}	1.94^{d}	2.24^{e}	1.83
A2 (1:2)	0.75^{a}	1.64^{cd}	2.27^{e}	1.55
A3 (2:1)	0.75^{a}	1.61^{bc}	2.37^{e}	1.58
Mean	0.94	1.73	2.29	

The growth of *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae* significantly affected the media because different media will support the growth of bacteria at different rates. This finding is consistent with other researchers **[2]**, **[12]**, **[15]**, **[16]**. The composition of the nutrients in media determines the growth rate, the product type, and the biomass yield. Acu et al. **[16]** reported that enrichment with fruit puree significantly affected *Lactobacillus paracasei* and *Bifidobacterium* spp. in terms of viability, colour, appearance, flavour, taste, and overall sensory scores of ice cream samples.

A medium must contain all the necessary nutrients or elements required to grow the microorganisms of interest. These elements, e.g., C, N, O, S, and P required by *Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae,* must be provided in a suitable form and ratios that are designed to achieve specific effects. The growing cells may require additional complex organic molecules (micronutrients) that they cannot synthesize but are essential for their growth **[17]**. The stability of the viability value of the probiotic mixture in B3 was influenced by its nutrient composition. B3 had abundant carbon due to the combination of coconut water and onggok flour. Meanwhile, B2 could have excess nitrogen (N) from the combination of tofu whey and fish meal waste. Agricultural wastes,

including woody materials, crop residues, and food by-products, are widely available and explored for LAB production because they offer potential environmental and economic benefits **[18]**. Low-cost nitrogen sources can be obtained from slaughtering by-products, fish processing by-products, agricultural waste, dairy industry by-products, and plant products. For example, the by-products of fish processing (chitinous, heads, viscera material, wastewater, etc.) are excellent nutrients for microbial growth **[19]**, **[20]**.

Effect of culture and media type on cell biomass

The highest biomass production was realized in the interaction between A3 (2:1) and B3 (90% coconut waste, 5% onggok flour, and 5% fish waste flour), which was significant (p <0.05) from other treatments (Table 2). The biomass for A3B3 was 42.33 mg/ml, while the lowest biomass production, 16.00 mg/ml, was observed for A2B3 interaction, with the same media but different culture ratios (Table 2). In this study, the higher the number of *Lactobacillus plantarum* N16 in the culture, the higher the biomass produced. Contrarily, the lower the ratio of *Lactobacillus plantarum* N16 in the culture, the lower the biomass produced. Stadie et al. **[21]** reported the symbiosis relationship between *S. cerevisiae* and *Zygotorulaspora florentina*, and *Lactobacillus nagelii* and *Lactobacillus hordei* led to an increased cell yield for all microorganisms. They also discovered that LAB's acidity of the medium helped *Z. florentina* to thrive, while the yeasts' synthesis of amino acids and vitamin B6 boosted Lactobacilli development. Liu et al. **[22]** experimented with improving the stability of *Lactobacillus rhannosus* in fermented milk using *Williopsis saturnus* var. *saturnus*. They found that *Williopsis saturnus* var. *saturnus* improved the stability of the milk for eight days in comparison to the control, which they attributed to the release of nutrients such as amino acids, peptides, and vitamins by the yeast.

Table 2 Biomass of probiotics (*Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae*) for various cultures and growth media (mg/ml).

Ratio of probiotics	Type of media			Mean
	B1 (MRSB)	B2 (Media 1)	B3 (Media 2)	
A1 (1:1)	20.00[a]	24.67[a]	17.33[a]	20.67
A2 (1:2)	22.00[a]	23.00[a]	16.00[a]	20.33
A3 (2:1)	23.00[a]	19.33[a]	42.33[b]	28.22
Mean	21.67	22.33	25.22	

Note: MRSB = de Man, Rogosa & Sharpe Broth.

In this research (Table 2), the novel and halal growth media biomass for *L. plantarum* N16 and *S. cerevisiae* were good quality compared to MRS broth. However, this commercial media has been optimized and used for five decades **[23]**. Nonetheless, coconut water, onggok flour, and shrimp waste flour in appropriate concentrations demonstrated the potency to be used to substitute MRS broth. Different researchers have reported that halal processed-peptone, yeast extract, and whey were preferable to MRS broths **[24]**, **[25]**, **[26]**.

Effect of culture and media type on change in pH

Probiotics (*Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae*) generated pH variations in the halal growing media, as shown in Table 3. Studies describing how changes in pH of the media affected the growth of bacteria or the production of some metabolites are widely available, however, few studies are available on the effects of pH of the medium during the growth of microorganisms. In this study, the initial pH was the same for the three media but differed at final growth.

Table 3 pH reduction caused by probiotics (*Lactobacillus plantarum* N16 and *Saccharomyces cerevisiae*) in the various culture and types of growth media.

Ratio of probiotics	Type of media			Mean
	B1 (MRSB)	B2 (Media 1)	B3 (Media 2)	
A1 (1:1)	0.95[a]	0.89[a]	2.37[e]	1.40
A2 (1:2)	1.21[b]	0.90[a]	2.21[d]	1.44
A3 (2:1)	1.51[c]	0.90[a]	2.38[e]	1.59
Mean	1.22	0.89	2.32	

Note: MRSB = de Man, Rogosa & Sharpe Broth.

Statistical analysis revealed significant differences (p <0.05) between factors A and B concerning the final pH of the medium. Based on the DMRT test, the highest pH reduction was A3B3 (2.38) and was not significantly

different (p >0.05) from treatment A1B3 (2.37) but significantly different (p <0.05) for other treatments. Nahariah et al. **[27]** reported that the decrease in pH is caused by fermentation activity which converts carbohydrates or sugars into acids. According to Maslami et al. **[28]**, the lowered pH was attributable to the formation of acetic and lactic acids by *L. plantarum* and *S. cerevisiae.* Both *L. plantarum* and *S. cerevisiae* ferment produced organic acid (malate acid) **[4]**.

Marlida et al. **[2]** and Younis et al. **[29]** reported that *S. cerevisiae* can inhibit the growth of pathogenic organisms by causing pH changes in the medium as a result of competition for nutrients, organic acid production, growth coupled with ion exchange, secretion of antibacterial compounds, production of high concentrations of ethanol, and release of antimicrobial compounds such as "mycocins" or killer toxins. *L. plantarum* also inhibits the growth of pathogenic bacteria by producing lactic acid and antimicrobial agents like bacteriocin **[4]**. Xie et al. **[30]** worked on improving the stability of *Lactobacillus rhamnosus* in fermented milk using *Williopsis saturnus* var. saturnus. Their work revealed that *Williopsis saturnus* var. *saturnus* enhanced the stability of *Lactobacillus rhamnosus* in the milk compared to the control. The enhanced stability was attributed to the excretion of peptides, amino acids, and vitamins by the yeast **[22]**. In addition, yeast metabolites have an important role in *L. rhamnosus* survival **[31]**.

CONCLUSION

Coconut water, onggok (tapioca waste flour), and shrimp waste flour (B3) were used to make a halal (permissible for a member of the faith of Islam) mixed probiotic medium for *L. plantarum* N16 and *S. cerevisiae* as an alternative media for MRSB, which was cultured for 24 hours at 36 °C. It had a viability of 2.37, a biomass cell concentration of 42.33 mg/ml, and a pH of 2.37.

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This article does not contain any studies that would require an ethical statement.

Contact Address:

Yetti Marlida, University of Andalas, Faculty of Animal Science, Department of Animal Nutrition and Feed Technology, Padang 25163, Indonesia,
E-mail: yettimarlida@ansci.unand.ac.id
ORCID: https://orcid.org/0000-0001-9134-3954

Harnentis, University of Andalas, Faculty of Animal Science, Department of Animal Nutrition and Feed Technology, Padang 25163, Indonesia,
E-mail: harnentis@ansci.unand.ac.id
ORCID: https://orcid.org/0000-0001-7580-5637

Azizah, University of Andalas, Faculty of Animal Science, Department of Animal Nutrition and Feed Technology, Padang 25163, Indonesia,
E-mail: nrlhd_usm@yahoo.com.my
ORCID: Not Available

Yuliaty Shafan Nur, University of Andalas, Faculty of Animal Science, Department of Animal Nutrition and Feed Technology, Padang 25163, Indonesia,
E-mail: yuliaty@ansci.unand.ac.id
ORCID: https://orcid.org/0000-0002-4689-9213

Frederick Adzitey, University for Development Studies, Faculty of Agriculture, Food and Consumer Sciences, Department of Animal Science, P.O. Box TL 1882, Tamale, Ghana,
E-mail: adzitey@yahoo.co.uk
ORCID: https://orcid.org/0000-0002-8814-0272

Norliza Julmohammad, Universiti Malaysia Sabah, Faculty of Food Science and Nutrition, Department of Food Science and Nutrition, 88400, Kota Kinabalu, Sabah, Malaysia,
E-mail: norliza@ums.edu.my
ORCID: https://orcid.org/0000-0003-1816-7127

*Nurul Huda, Universiti Malaysia Sabah, Faculty of Food Science and Nutrition, Department of Food Science and Nutrition, 88400, Kota Kinabalu, Sabah, Malaysia,
E-mail: drnurulhuda@ums.edu.my
ORCID: https://orcid.org/0000-0001-9867-6401

Corresponding author: *