Formulation and optimization of alternative culture media for probiotic bacteria growth using tofu liquid waste

S N Jannah, S Pujiyanto, E Rosiana, S Purwantisari
Department of Biology, Diponegoro University. Jl. Prof Soedarto, Tembalang, Semarang, 50275, Central Java, Indonesia

Corresponding author: nurjannah.suroso@gmail.com

Abstract. Probiotics from lactic acid bacteria (LAB) are widely used in the livestock industry to replace the use of Antibiotics Growth Promoters (AGPs). LAB is a culture starter that is widely used in fermented foods. LAB has the ability to adapt to different substrates, resulting in its wide use in the fermentation of various food products. MRS (De Man Rogosa Sharpe) medium is a growth medium for LAB, but for the industrial-scale requires a high cost, therefore innovation is needed to find alternative culture media using local raw material. Tofu Liquid Waste (TLW) can be used as a growth medium for probiotic bacteria because it still contains enough nutrients source for growth. This study aims to determine the formulation and optimization of culture media for LAB isolates using tofu liquid waste. Six Lab isolates from chickens gastrointestinal tract were used in experiments to determine the ability to grow on TLW culture media. The growth assays are conducted by inoculating LAB isolates in the culture media formulation that divided into 4 groups, consisting of medium A (TLW+molasses 1.5%+skim milk 5%), B (TLW+molasses 1.5%+skim milk 2.5%), C (TLW+molasses 3%+skim milk 5%), and D (TLW+molasses 3%+skim milk 2.5%). The best LAB isolate to grow on TLW medium was Lactobacillus paracasei. The best TLW modification medium for Lactobacillus paracasei growth is D media. Modified media consisting of TLW, molasses and skim milk can be used as an alternative medium for LAB growth.

1. Introduction
The use of Antibiotics Growth Promoters (AGPs) for livestock products can cause the presence of antibiotic residue in the product and the development of resistant pathogenic microbes in the gastrointestinal tract (GIT) [1]. The use of antibiotics can be replaced with probiotics. Probiotics are supplements from live microbes that can change the composition and the metabolic activity of the natural intestinal microbiota or regulate the immune system reactivity beneficial to health. Probiotics can be used in the feed mixture that can improve feed quality and also able to reduce non-protein nitrogen in the blood, the concentration of uric acid, ammonia and urea in the blood [2]. Probiotics can improve health, feed efficiency ratio, immunity to disease, increase weight and production to the host [3].

Lactic acid bacteria (LAB) is one group of probiotics that well known for an important role in the food industry (fermentative capacities) and found normally in the Gastrointestinal tract of animal and human. LAB is a Gram-positive bacteria that has ability to lower the pH of the growing environment, and to produce antimicrobial agents and has many benefits for human health [4]. Attempts to propagate probiotic bacteria in the laboratory require a medium containing nutrients and a suitable growth environment for bacteria. Nutrition that needed bacteria for growth includes carbon, nitrogen, non-metal...
elements such as sulfur and phosphorus, metal elements such as Ca, Zn, Na, K, Cu, Mn, Mg, and Fe, vitamins, water, and energy [5].

The culture media are prepared to contain nutrients to support microbial growth such as de Mann Rogose and Sharpe (MRS) media used for the growth of LAB. The MRS medium is a suitable culture medium for cultivate and maintain LAB under laboratory conditions. On an industrial scale, the use of MRS as a medium requires high costs, so that an alternative replacement is needed by utilizing tofu liquid waste (TLW). Tofu is produced by grinding the soaked soybeans with water to obtain soy milk. The soymilk is then heated up to 80–90 °C and then coagulated by using calcium/magnesium salt or acid to form a gel-like structure, which becomes the tofu after pressing. Tofu liquid waste is the liquid produced from the remaining tofu clumping process in the tofu industry. The tofu processing industry, which usually is done by the small industries that do not carry out the waste treatment process properly. The soy whey contains the amount of nutrients and has high water content, it requires further treatment before it can be disposed of into the sewage. Discarding TLW as waste is a potential environmental problem because it is highly susceptible to putrefaction and can cause environmental problems. On the other hand, TLW is a relatively inexpensive source of protein that is widely recognized for its high nutritional and excellent functional properties [6].

TLW has been used as an alternative medium in lactic acid production by Streptococcus bovis [7]. Molasses is widely used as a substrate for bacterial growth. Lactobacillus delbrueckii subsp. delbrueckii can produce lactic acid by utilizing molasse as a carbon source. Molasse contains 40-60% sucrose, fructose, and glucose in lower concentrations [8]. Skim milk is non-fat milk or fat-free milk that has a high protein content, as a source of lactose, casein, and other nutrients needed for the growth of Lactobacilli. Skim milk and some of its proteins have proved to be very effective cryoprotectants agent for the LAB group [9]. With the combination of the three substrates, it is possible to be used as an alternative medium for LAB. The purpose of this study is to determine the best medium formulations and optimization for the growth of lactic acid bacteria in tofu liquid waste.

2. Material and methods

2.1. Bacterial culture
Six isolates of Lactic acid bacteria (LAB) that isolated from the gastrointestinal tract of Kampong Chicken, tofu liquid waste as basal medium, molasse, skim milk powder, MRS broth (Merck), Agar.

2.2. Morphological characterization of lactic acid bacteria isolates
For use, aliquots of these cultures were reactivated in de Man, Rogosa and Sharpe (MRS) broth (Merck), incubated at 37 °C for 24 h, then streaked on MRS agar, and incubated at 37 °C for 48 h. The colonies morphology were observed from colonies grown on MRSA media and the cells morphology were observed through Gram staining.

2.3. Modification Medium Preparation
For Tofu liquid waste (TLW) is used as a base growth medium of LAB with supplementation of molasses and skim milk. TLW that has been added molasses was sterilized at 121° C for 15 minutes. Skim milk that has been pasteurized at 85°C for 15 min added into modification medium. MRS broth medium used as growth control medium of LAB.

2.4. Selection of LAB isolates growth in tofu liquid waste medium
Six LAB isolates were transferred to MRS broth and incubated at 37 °C until they achieved a turbidity similar to 0,5 the MacFarland scale, which corresponds to approximately $10^8$ CFU/mL. Then, the isolates were grown in tofu liquid waste (TLW) added molasses 1.5% and skim milk 2.5% for 24 hours. The growth ability and counting the number of colonies that grew in TLW was seen from the growth of bacteria on MRS agar media which was incubated for 48 hours at 37°C using the SP-SDS (single plate-serial dilution spotting) method [10].
2.5. \textit{Experimental} model for the Formulation and Optimization of bacteria growth in various media

The growth of selected LAB isolate in various media groups was carried out using 5% of selected LAB isolate starter which had been incubated for 24 hours was inoculated into the modified medium and incubated using a rotary shaker 125 rpm at 37 °C. Sampling was carried out every 6 hours for 60 hours. Measurement of growth is done by calculating the colony in MRS agar by SP-SDS method [10]. The alternative media consist of tofu liquid waste (TLW), molasses, and skim milk. This media are divided into 4 different kinds of concentration materials. The formulation of modification medium are:

a. Medium A consists of TLW, Molasse 1.5%, and skim milk 5%.
b. Medium B consist of TLW, molasse 1.5%, and skim milk 2.5%.
c. Medium C consists of TLW, Molasse 3%, and skim milk 5%.
d. Medium D consists of TLW, Molasse 3%, and skim milk 2.5%.

The study used Completely Randomized Design with 4 treatments and repetitions by 3 times.

3. Results and discussion

3.1. Selection of LAB isolates growth in tofu liquid waste medium

All lactic acid bacteria isolates (Bub 1, Bub3, Bub 4, V52, C51, dan NN B) were grown on MRSA added with 0.5% CaCO$_3$ showed a clear zone around the colony, colonies are round shape with 1-2 mm in diameter, entire edge, convex and the cell morphology is rod-shaped and Gram-positive, negative catalase (Figure 1).

![Bub1, Bub3, Bub4, V52, C51, NN B](image1)

\textbf{Figure 1.} The cell morphology of lactic acid bacteria isolated from chickens gut.

Bacteria require appropriate nutrition and biophysical environment in order to grow. In this research, All LAB isolates could grow on tofu liquid waste (TLW) medium which was added with molasses 1.5% and skim milk 2.5%. Based on counting the number of bacteria using the SP-SDS method, the BUB3 isolate was the isolate that grows best on the modification media (Table 1). The BUB3 isolate was identified as \textit{Lactobacillus paracasei} (in another paper in review process).
Table 1. Total Number of Lactic acid bacteria isolates in TLW medium incubated for 24 hours

| LAB Isolate | Total Number (CFU/mL) |
|-------------|-----------------------|
| BUB1        | 5.0 \times 10^6       |
| BUB3        | 3.0 \times 10^7       |
| BUB4        | 7.4 \times 10^5       |
| VS2         | 7.0 \times 10^5       |
| VS3         | 5.0 \times 10^5       |
| CS1         | 2.0 \times 10^6       |

*L. paracasei* (Figure 2) produce antimicrobials against the growth of *Escherichia coli* and *Staphylococcus aureus*. Fermentation by lactic acid bacteria can cause a decrease in environmental pH so that it can inhibit the growth of pathogenic microbes [11]. *Lactobacillus paracasei* subsp. *paracasei* has been isolated from fermented sweet potato acid which naturally has the ability to hydrolyze raw starch to produce simple carbohydrates, including glucose and lactic acid. This ability can change the structural, physical, and chemical of starch grains. The ability of LAB to utilize carbohydrates is very important for intestinal colonization and increase carbohydrates so that it has the ability to compete in the intestinal microbiota [12].

![Figure 2. The colony morphology of L paracasei on MRS agar media with the addition of 1% CaCO3 (a) and cell morphology with Gram stain (b)](image)

The number of bacterial colonies growing on TLW media was calculated by measuring the number of colonies on MRS agar using the Single Plate-Serial Dilution Spotting method (Figure 3). The SP-SDS method showed satisfactory and acceptable results for counting the number of bacterial colonies at least one of the serial dilution. With SP-SDS can save equipment and media materials used in tests with conventional culture plating approaches. The TLW fermentation medium is very cloudy, so it is difficult to detect bacterial counts using a spectrophotometer. Using SP-SDS media makes it easy to count the number of bacteria and the count results only in living LAB colonies.
3.2. Growth of BUB3 isolates on various Modified TLW Media
Tofu Liquid Waste is a liquid waste product obtained during the production of tofu. The main component in TLW is protein. The protein content in TLW is 1-6 g/L. Under anaerobic conditions protein releases ammonia gas (NH₃) and hydrogen sulfide (H₂S) which can cause a foul-smelling aquatic environment [13]. The TLW modified media contains the appropriate nutritional composition needed for LAB isolates. LAB have the ability to adapt to different media, so it can be used to convert waste into useful LAB substrates. L. paracasei can grow well on various modified media after 24 hours of incubation (Figure 4)
Molasses is a kind of black syrup that is the residue from the sugar crystallization process. Molasses cannot be crystallized because it contains glucose and fructose which are difficult to crystallize. Molasses contains sugar which can be used as a culture medium for bacteria. *Lactobacillus delbrueckii* subsp. *delbrueckii* can produce lactic acid by utilizing molasses as a production medium [8]. Molasses is used as a culture medium because it contains a sufficient amount of carbon for the growth of lactic acid bacteria. Molasses contains 40-60% sucrose, fructose, and glucose in lower concentrations. The media formulation for the growth of *Lactobacillus sp* was carried out using a carbon source (C) in the form of molasses with a concentration of 3% and 5% and the optimum concentration of molasses for growth was 3% [14].

Skim milk is used as an additional source of nitrogen for LAB because of the content of lactose and casein. Casein is about 80% of the total protein content in milk, and the other is whey protein. Casein collects in colloidal structures in the form of casein micelles and makes up about 10% of the volume in skim milk [15]. LAB can take advantage of sugar derived from skim milk and sucrose [16]. Lactose in skim milk is converted by most of the LAB to produce lactic acid. LAB has proteolytic activity and it is very important to produce flavor compounds in the final product. LAB utilizes casein in milk to produce organoleptic properties [17]. Skim milk can maintain the viability of *L. salivarius* that microencapsulated using the freeze drying method [18].

Microbial growth curves can provide useful information for understanding microbial growth behavior and selecting optimal growth [5]. The growth assay of *L. paracasei* on modification medium of TLW that supplemented by molasses and skim milk resulting in a growth curve that consisting of exponential, stationary, and death phases. The growth of LAB in the modification medium and control have the same pattern of growth, but the control treatment showed a significant phase of death (Figure 5). The growth of *L. paracasei* on all alternative medium indicates that there has been no significant death phase. The number of LAB cells at 60 hours is still high when compared to the control medium.

There was no lag phase on *L. paracasei* growth in all treatments due to the use of a starter at the beginning of the inoculation of bacteria. The starter used for the growth of *L. paracasei* in modified media was 5%. The microbial cultures that have grown exponentially on starter media, and transferred to the medium with the same conditions, there will be no lag phase or adjustment phase in the new media, and an exponential phase can begin immediately. A microbial starter or inoculum is used in order to shorten the lag phase in microbial growth [5].
Figure 5. Growth of *Lactobacillus paracasei* in alternative medium. Medium A: TLW, Molasse 1.5%, and skim milk 5%; B: TLW, 1.5% molasse, and skim milk 2.5%; C: TLW, Molasse 3%, and skim milk 5%; D: TLW, Molasse 3% and skim milk 2.5%, K: MRS broth.

In the LAB growth diagram (Figure 5), it can be seen that the best growth of *L. paracasei* occurred in the formulation D media (TLW, 3% molasses, 2.5% skim milk) compared to other treatments (A, B, C, and Control). *L. paracasei* growth in all treatment media was better than isolates grown on control media (MRSB). These results may occur due to the composition of the modified TLW media containing nutrients suitable for *L. paracasei* so that the isolate growth becomes optimum. The hope is that the use of TLW formulation media can be used as a substitute for MRSB media, which is more expensive. The use of TLW as a growing medium is not optimal because TLW only contains a little sugar, while LAB requires sugar for growth. TLW contains nitrogen-organic (7.61%), total sugar (0.32%), reducing sugar (0.09%), and minerals [7]. The weakness of using TLW as a growing medium is that the total sugar content does not reach 1%, so that the need for source C is not fulfilled for LAB growth [19]. The addition of 3% molasses and 2% skim milk made the modified TLW medium a suitable medium for LAB growth.

4. Conclusion
Based on this study it is concluded that TLW can be used as an alternative bacterial growth media for Lactic acid bacteria. Growth media formulations can increase the viability of *Lactobacillus paracasei* when compare to the MRS medium. The best media formulation for *L. paracasei* growth is medium D that consist of TLW, Molasse 3%, and skim milk 2.5%.

Acknowledgement
This research is supported by the RPP research program from Diponegoro University.

Reference
[1] Gaggia F, Mattarelli P and Biavati B 2010 *Int. J of Food Microbiol.* 141 S15–S28.
[2] Bongaerts G, Severijnen R and Timmerman H 2005 *Med. Hypotheses* 64 64-68
[3] Guerra N P, Bernárdez P F, Méndez J, Cachaldora P and Castro P L 2007 *Anim. Feed Sci. Technol.*
[4] Salminen S and Wright A V 1993 *Lactic Acid Bacteria* (New York: Marcel Dekker, Inc) p 65
[5] Madigan M T and Martinko J M 2006 *Biology of Microorganisms* (Pearson Prentice Hall)
[6] Regenstein L Z J, Fei T and Yang L 2020 *Compr. Rev. Food Sci. F.*
[7] Ghofar A, Ogawa S and Kokugan T 2005 *J. Biosci. Bioeng* 6 606–612
[8] Dumbrepatil A, Mukund A, Shivani C, Jayant K and Digambar G 2008 *Appl. Environ. Microbiol.* **74** 333–35
[9] Montel M G M, Pasteris S E, Otero M C and Fatima N M E 2013 *Appl. Microbiol.*
[10] Thomas P, Sekhar A C, Upreti R, Mujawar M M and Pasha S S 2015 *Biotechnol. Rep.* **8** 45
[11] Jannah S N, Saraswati T R, Handayani D and Pujiyanto S 2018 *J. Phys.: Conf. Series* 1025
[12] Zhang L, Yu Y, Li X, Li X, Zhang H and Xu Y 2017 *Front. Microbiol.* **8** 1412
[13] Widyarani, Butar Butar E S, Dara F, Hamidah U, Sriwuryandari L, Hariyadi H R and Sintawardani N 2019 *IOP Conf. Series: Earth Env. Sci.* 277
[14] Sughra M G, Umar D M and Ahmed S A 2013 *J. Biotechnol.* **10** 63-73
[15] Corredig M, Nair P K, Li Y, Eshpari H and Zhao Z 2019 *J. Dairy Sci.* **102** 4772-4782
[16] Gille D, Walther, B, Badertscher R, Bosshart A, Brügger C, Brühlhart M, Gauch R, Noth P, Vergeres G, and Egger L F A O 2018 *Int. Dairy J.* **83** 17-19
[17] Moulay M, Benlancen K, and Aggad H 2013 *Adv. Environ. Biol.* **7** 999–1007
[18] Jannah S N, Dinoto A, Wiryawan K G and Rusmana I 2014 *Media Peternakan, J. Anim. Sci. Technol.* **37**(3) 182-189
[19] Cheng F, Chen H, Lei N, Zhang M and Wan H 2019 *Acta Univ. Cibiniensis Ser. E: Food Technol.* **23** 11