Synergistic effect between *Lactobacillus plantarum* AKK30 and *Saccharomyces cerevisiae* B18 and the probiotic properties of microencapsulated cultures

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Abstract. The objectives of this study were to observe the microbial interactions between lactic acid bacteria (LAB - *Lactobacillus plantarum* AKK30) and yeast (*S. cerevisiae* B18) whether their effect is antagonistic or synergistic and to determine the potential of microencapsulated culture as probiotics for animal feed additive. The cell morphology of isolates were screened by macroscopic and microscopic examination. The synergistic effect of LAB and yeast was studied by disk diffusion and dilution method. The microencapsulated culture of LAB and yeast was prepared by spray drying method. Probiotic properties of microencapsulated cultures were examined for its viability on low pH, gastric juice and bile salt *in vitro*. The result of morphology cell showed that *L. plantarum* AKK30 had bacilli shaped with average length of cell was 1.35 µm at 48 h of incubation, whereas *S. cerevisiae* B18 had small budding characteristics with average length of cell was 3.19 µm at 96 h of incubation. The synergistic assay showed that *L. plantarum* AKK30 and *S. cerevisiae* B18 were alive and grew in a commensal relationship with diameter of clearing zone less than 5 mm and the population between two microorganisms was only 1 cycle log difference. Viability of microencapsulated culture was 97.25% for *L. plantarum* AKK30 and 91.66% for *S. cerevisiae* B18. The microencapsulated culture of *L. plantarum* AKK30 and *S. cerevisiae* B18 could be applied as probiotic for animal with good viability on pH 2 (95.18 and 87.95% respectively) and pH 3 (88.63 and 96.84% respectively) at 45 min of incubation, viability on gastric juice (66.58 and 92.70% respectively) at 45 min of incubation, and viability on 0.3% of bile salt (99.11 and 94.09% respectively) at 120 min of incubation. Thus, it could be concluded that LAB and yeast had synergistic effect and potentially use as a probiotic.
1. Introduction
Probiotics are defined as “live microorganisms (bacteria or yeasts), which when administered in adequate amount confer a health benefit on the host” [1]. Lactic acid bacteria (LAB) and yeast are potentially use as a probiotic agent due to its ability to survive in a variety of limiting conditions along the digestive tract, such as low pH of the stomach and the presence of bile salts in the duodenum [2,3], colonize in the digestive tract, and inhibit the growth of pathogenic bacteria. Several potential strains of LAB include Bifidobacterium, L. reuteri, and L. casei [4], while Sourabh et al. [5] reported that S. cerevisiae HM535662 had good tolerance at pH 2. This microbial survival at low pH may be due to various factors such as cell size, cell wall, pH composition, and variations in microbial strains [6-8]. The viable counts of probiotics for consumption are expected reach minimum acceptable concentration of 10^6 cfu/g or mL [9].

The antagonistic or synergistic effect is one of the most important properties in system that different microorganisms are living together [10]. The synergistic effect should be promoted, whereas the antagonistic effect is prevented to provide effective culture for probiotic purpose. Basically, different microorganisms between either different genera or species could be applied as a co-culture in the form of probiotics if they could live together and had a synergistic effect on each other. Commonly, microorganisms grown together in harsh conditions owing their resistivity and survival to the synergism between the microbial strains. Correlatively, the increase of microbial growth rate is determined in co-culture systems compared with the growth rate they experience alone in the medium [11].

Furthermore, both isolates were prepare in microencapsulated culture by spray drying method to maintain its viability for probiotic purpose. The viability was determine by comparing the cell colony before and after spray drying [12]. Viability assay of isolates was needed to determine the cell ability to grow and encapsulan ability to maintain the live cell of probiotic [13].

The objectives of this study were to determine the potential of LAB and yeast as probiotics candidate for animal feed additive and to observe the microbial interactions between LAB and yeast strains whether their effect is antagonistic or synergistic.

2. Materials and methods

2.1. Morphology observation of isolates
In this study, we used L. plantarum AKK30 and S. cerevisiae B18 with cholesterol-lowering activity that has been isolated from gastrointestinal tract (GIT) of Indonesian native chicken and Javanase duck [14,15]. Lactobacillus plantarum AKK30 was grown in de Man Rogosa Sharpe (MRS, Oxoid) at 37 °C for 24 h, whereas S. cerevisiae B18 was grown on Yeast Extract Glucose agar medium [(g/l) yeast extract, 5; glucose, 20; agar, 12.0; pH 7.0 ± 0.2] at 30 °C for 48 h. The cell morphology of isolates was screened by macroscopic and microscopic examination. Microstructure observation of L. plantarum AKK30 and S. cerevisiae B18 using scanning electron microscopy (SEM, Hitachi SU3500) were carried out by growing LAB on MRS Agar medium and incubated at 37 °C for 48 h, whereas yeast inoculated on YG Agar medium then incubated at 30 °C for 96 h. Each colony of LAB and yeast was taken with an osen needle then swab onto each carbon tape and dried, then coated with ion sputter with a setting of 10 mA for 60 seconds with magnification 20.000x and vacc (accelerating voltage) 10 kV for bacteria, whereas ion sputter with a setting of 10 mA for 50 seconds with magnification 10.000x and vacc. 5 kV for yeast.

2.2. Assay for antagonistic effect between LAB and yeast cultures
Antibacterial and antifungal effects were screened of L. plantarum AKK30 and S. cerevisiae B18 to each other. Experiments were performed by modified disk diffusion method according Çakır [16]. Briefly, S. cerevisiae B18 was inoculated into YG Agar by spread over the whole surface of the medium as a thin film. On the other hand, a sterilized 6 mm diameter blank disk (Oxoid) was put on the YG agar. Lactobacillus plantarum AKK30 was grown in MRS Broth then centrifuged at 5000 rpm for 10 min and supernatants were transferred to another sterile tubes. Supernatants were
treated into two groups. The first group was filtered using syringe filters (0.45 μm pore size), whereas the second group was not filtered. Blank disk that placed on the S. cerevisiae B18 film was loaded with 20 μL of filtered/non-filtered supernatants obtained from L. plantarum AKK30 then incubated at 37°C for 24 h. The inhibition of L. plantarum AKK30 growth was evaluated by measuring the diameter of the transparent inhibition zone around disk. This procedure was conducted for another group of petri dishes containing MRS agar spread with L. plantarum AKK30 by loading blank disks with filtered/non-filtered supernatants (20 μL) obtained from S. cerevisiae B18. Inhibition was scored positive if the width of the clear zone around the disc of the antimicrobial compound producer strain was 5.0 mm or larger.

2.3. Assay for synergistic effect between LAB and yeast cultures

The synergistic assay were performed for L. plantarum AKK30 and S. cerevisiae B18 according to modified dilution (the plate count) method on medium described by Korukluoglu et al. [10]. Two microorganism together (L. plantarum AKK30 and S. cerevisiae B18) were inoculated (2.5%, v/v respectively) into tube containing Tryptic Soy (TS) Broth. The tubes, including TS Broth and test microorganisms were incubated at 30 °C for 0 and 24 hours then after that, test microorganism was spread to the MRS and YG Agar by surface plate method to count the viable cell amounts. The counting was carried out to determine the synergistic effect between L. plantarum AKK30 and S. cerevisiae B18 cultures.

2.4. Microencapsulated culture preparation of LAB and yeast

Microencapsulated culture of L. plantarum AKK30 was prepared by encapsulation method using spray dryer (Buchi) according to Barbosa-Canovas [17]. Skim solution containing 20% of skim in distilled water (w/v) was prepared and sterilized (110 °C for 10 min). LAB isolate was inoculated in MRS Broth medium at 37 °C for 18 h. The culture was centrifuged at 4500 rpm for 10 min, then the biomass was mixed with skim solution. The solution was homogenized with a magnetic stirrer during spray drying process. Spray dryer operating conditions as follows: inlet air temperature 110 °C, outlet air temperature 63 °C, and the speed of pump 3. Colony of LAB microencapsulated culture obtained from spray dryer was count by the total plate count (TPC) method.

Encapsulation of S. cerevisiae B18 was performed using spray dryer (Buchi) refers to Chandrallekha et al. [18] method. Yeast isolate was inoculated in YG Broth medium at 30 °C for 24 h. The culture was centrifuged at 4500 rpm for 10 min, then the biomass was mixed with sterile maltodextrin solution containing 20% of maltodextrin in distilled water (w/v) and stirred with a magnetic stirrer during spray drying process. The operating conditions were: inlet air temperature 105 °C, outlet air temperature 66 °C, air pressure (2.5 psi), air flow rate (0.50 m³/min), flow rate (41 ml/min), and the speed of pump 3. Colony of yeast microencapsulated culture obtained from spray dryer was count by the total plate count (TPC) method.

2.5. Assay for probiotic properties of LAB and yeast

Microbial probiotic could applied in animal accordance to the feed retention time in each GIT. Taheri et al. [19] reported that retention time of feed were varied, start from crop (30 min; pH 4.5), proventriculus (15 min; pH 4.4), gizzard (90 min; pH 2.6), intestine (90 min; pH 6.2), and colon (15 min; pH 6.3). The microencapsulated culture of L. plantarum AKK30 and S. cerevisiae B18 were assayed as a probiotic candidate based on its ability to survive in acidic pH condition, gastric juice, and bile salt refers to Sabikhi et al. [20] method with slight modification on the incubation time according to retention time of feed in GIT.

Acid tolerance assay was observed by mix 0.5 g of the microencapsulated culture with 1 mL sterile Phosphate Buffer Saline (PBS) then 10 μL of the mixture was inoculated in 990 μL of PBS at pH 2 and 3 (pH adjusted with 1 M HCl) respectively then homogenized and incubated at 37 °C (L. plantarum AKK30) and 30 °C (S. cerevisiae B18) for 0 and 45 min. Serial dilutions of samples was performed in PBS then each culture inoculated on MRS/YG Agar and incubated for 48 h. Each cell colony was calculated by the total plate count (TPC) method.
Gastric juice tolerance was performed by prepare the artificial gastric juice from pepsin (Sigma, 3 g/L) solution at pH 2. Microencapsulated culture of *L. plantarum* AKK30 (0.5 g) was suspended with 1 mL sterile PBS. A total of 0.2 ml suspension was taken and mixed with 1 ml of sterile PBS containing artificial gastric juice. The mixture liquid was homogenized and incubated at 37 °C (*L. plantarum* AKK30) and 30 °C (*S. cerevisiae* B18) for 0 and 45 min. Serial dilutions of samples was made with sterile PBS and then each culture inoculated on MRS/YG Agar and incubated for 48 h for cell colony observation.

Bile salt tolerance was determined by mix 0.5 g of the microencapsulated culture with 1 mL sterile PBS then homogenized. A total of 0.2 ml each pellet suspension was suspended in 1 mL PBS containing 0.3% (w/v) bile salt (Merck). The mixture liquid was homogenized and incubated at 37 °C (*L. plantarum* AKK30) and 30 °C (*S. cerevisiae* B18) for 0 and 120 min. Serial dilutions of samples was performed then each culture inoculated on MRS/YG Agar and incubated for 48 h. Each cell colony was calculated by the total plate count (TPC) method.

2.6. Data analysis

The length of cell from SEM was analysed by ImageJ software [21]. The total of microbial cell (cfu/ml) from viability assay was converted to the logatimic value [22]. Percentage log viability = (log N / log N₀) x 100, where N – count (cfu/ml) after incubation, N₀ – count at time 0 (cfu/mL).

Statistical analyses were performed with Costat [23]. The quantitative data was subjected to analysis of variance (ANOVA) and the differences among mean treatments were analysed for significance using Duncan's Multiple Range Test [24].

3. Result and discussion

3.1. Morphology of LAB and yeast

Macroscopic morphology of *L. plantarum* AKK30 colonies were milky white, smooth slippery texture, shiny colony surfaces and flat edges, while macroscopic observations of *S. cerevisiae* B18 showed round, white, gray to brownish colonies, sparkling colony surfaces until slippery, dull, with soft texture [25].

Microscopic morphological observations of *L. plantarum* AKK30 cell using SEM with ion sputter coating (setting 10 mA for 60 seconds) and *S. cerevisiae* B18 cell with ion sputter coating (setting 10 mA for 50 seconds) are shown in Figure 1.

![Observation of *L. plantarum* AKK30 cell with magnification 20.000x and vac 10 kV (left) and *S. cerevisiae* B18 cell with magnification 10.000x and vac 5 kV](image-url)

Figure 1 showed that *L. plantarum* AKK30 had bacilli shaped and *S. cerevisiae* B18 had small budding characteristics. *Lactobacillus plantarum* tend to be short rod-shaped in suitable growth conditions and will tend to be longer under unfavourable conditions [26]. The microscopic appearance of these bacteria tends to be in the form of short rods with suitable growth conditions.
and tend to be longer in unfavourable conditions [27]. According to Saito et al. [28], the type of germination consists of no budding, small, medium, and large. The microscopic form of *S. cerevisiae* in the form of blastopores which are oval, cylindrical, oval, or ovoid short and long affected by strains. These yeast will divide and produce germinate budding multipolar at propagation time and the budding can form on the entire cell surface.

The analysis by using ImageJ software resulted that the average length of *L. plantarum* AKK30 was 1.35 µm at 48 h of incubation. Isolate could be seen with clear cell wall boundaries. The average length of *S. cerevisiae* B18 was 3.19 µm at 96 h of incubation. Isolates can be seen with clear and visible cell wall boundaries and also budding formation.

### 3.2. Antagonistic effect between LAB and yeast

Antifungal and antibacterial assay were carried out using the disc diffusion method. The overall results are summarized in the Table 1.

| Isolate                        | Diameter of clearing zone (mm) |
|--------------------------------|--------------------------------|
|                                | Filtered | Non-filtered |
| *L. plantarum* AKK30 against *S. cerevisiae* B18 | 0.53 ± 0.11<sup>b</sup> | 0.56 ± 0.18<sup>b</sup> |
| *S. cerevisiae* B18 against *L. plantarum* AKK30 | 0.95 ± 0.00<sup>a</sup> | 3.08 ± 0.46<sup>a</sup> |

Note: Average in the same column with different superscript shown significantly differ (P<0.05)

The results obtained showed that the diameter of clearing zone in the filtered supernatant of both isolates tested were ranged from 0.53 to 0.95 mm, whereas non-filtered supernatant resulted 0.56 to 3.08 mm. This data showed that non-filtered supernatant containing some viable cells resulted larger clearing zone (P<0.05) than filtered supernatant. Non-filtered supernatant obtained from *L. plantarum* AKK30 produced larger clearing zone (0.58 mm) against *S. cerevisiae* B18 compared to the filtered supernatant (0.53 mm), whereas diameter of clearing zone obtained from *S. cerevisiae* B18 against *L. plantarum* AKK30 also resulted the similar trend where non-filtered supernatant produced the larger clearing zone (3.08 mm) than filtered supernatant (0.95 mm).

Figure 2 and 3 showed the zone formation results. Korukluoglu et al. [10] observed that the non-filtered supernatant obtained from LAB containing some living cells and grown around the disk did not form a zone formation around the visible colonies of *S. cerevisiae* strain. These results indicated that the culture of *S. cerevisiae* and LAB isolated from kefir were alive and grew together in a commensal relationship.

![Disk which loaded with filtered supernatant of *L. plantarum* AKK30](image1.png)

![Disk which loaded with non-filtered supernatant of *L. plantarum* AKK30](image2.png)

**Figure 2.** The inhibition effect of *L. plantarum* AKK30 against *S. cerevisiae* B18
The clearing zone values in this study were less than 5 mm, therefore *L. plantarum* AKK30 showed no antifungal activity against *S. cerevisiae* B18 and *S. cerevisiae* B18 showed no antibacterial activity against *L. plantarum* AKK30. Lactic acid is one of the volatile organic acids; thus, the results showed that the accumulation of lactic acid was not sufficient to inhibit *S. cerevisiae* strains in this study. In contrast to our results, Durlu-Özkaya *et al.* [29] reported that except *L. plantarum* Lp19, all of the *Lactobacillus* strains they studied showed strong antifungal activity against *S. cerevisiae*.

### 3.3. Synergistic effect between LAB and yeast

The results of the synergy effect of *L. plantarum* AKK30 isolates and *S. cerevisiae* B18 by dilution (total plate count) method are shown in Table 2.

**Table 2. Population of LAB and yeast colonies**

| Isolate              | Population | (log cfu/mL) |
|----------------------|------------|--------------|
| *L. plantarum* AKK30 | 2.3 x 10^8 | 8.35 ± 0.06^a |
| *S. cerevisiae* B18  | 1.6 x 10^7 | 7.20 ± 0.13^b |

Note: Average in the same column with different superscript shown significantly differ (P<0.05)

Based on population data with the dilution method, it was found that both microbes (*L. plantarum* AKK30 and *S. cerevisiae*) could alive and grew together in the same medium and had a difference in population by 1 log cycle thus both isolates had a synergistic effect. A study by Soulides [30] reported that the viability of *Lactobacillus* and *Streptococcus* was increased as a result of their association with yeasts which, through synergism, either neutralized or assimilated lactic acid. This result was contrast with the research of Korukluoglu *et al.* [10] that according to the colony calculation in agar media (dilution method) showed that both non-volatile and volatile compounds produced by both LAB and *S. cerevisiae* had an inhibitory effect on each other. In addition, some of these microbes could increase the growth of other microorganisms as co-culture.

### 3.4. Viability of microencapsulated cultures

Population of *L. plantarum* AKK30 and *S. cerevisiae* B18 before and after spray drying were carried out to calculate the cell viability. The viability of microencapsulated cultures are shown in Table 3.
Table 3. Population of LAB and yeast cultures by spray drying

| Isolate                  | Population (log cfu/g) Before spray drying | Population (log cfu/g) After spray drying | Viability (%) |
|-------------------------|------------------------------------------|------------------------------------------|---------------|
| L. plantarum AKK30      | 9.28 ± 0.06<sup>a</sup>                  | 9.02 ± 0.33<sup>a</sup>                 | 97.25         |
| S. cerevisiae B18       | 7.37 ± 0.09<sup>a</sup>                  | 6.71 ± 0.13<sup>b</sup>                 | 91.16         |

Note: Average in the same row with different superscript shown significantly different (P<0.05)

The population of L. plantarum AKK30 before and after spray drying using skim solution as filler did not significantly differ (P>0.05) and the viability was 97.25%. The population of S. cerevisiae B18 colonies before spray drying was 7.37 log cfu/g and after spray drying using maltodextrin as a filler decreased (P<0.05) to 6.71 log cfu/g or the viability was 91.66%. These results indicated that microencapsulation process using a spray dryer could maintain the LAB and yeast's survivability.

Rizqiyati et al. [12] stated that bacterial resistance was determined by comparing the number of cells after and before spray drying. Viability of L. plantarum after spray drying for encapsulation treatment in the form of skim milk and arabic gum was relatively good around 89.36% where the number of bacteria before spray drying was 9.4 log cfu/g, after spray drying decreased to 8.4 log cfu/g, meaning that the population declined only 1 log cycle. Arslan et al. [31] stated that the population of S. cerevisiae var. boulardii with spray drying method using maltodextrin solution (20%, b/v) was 8.61 log<sub>10</sub> cfu/g or viability of 89.24%. Decreased viability during the spray drying process arises from dehydration and high temperatures. These two mechanisms occurred simultaneously, and had a negative effect on microbial survival [32]. The results of this study showed that the viability of both microorganism after the spray drying process was good. This good viability was the basis that the probiotic candidate microbes could be processed, packaged, and stored in dry culture. In order to provide health benefits, the number of bacteria must be a minimum of 10<sup>6</sup> cfu/gram or 10<sup>7</sup> cfu/gram in the product or be consumed in sufficient quantities to produce a daily intake of 10<sup>8</sup> cfu/mL [33].

3.5. Probiotic properties of LAB and yeast

Lactic acid bacteria isolated from the digestive tract of animals and humans are functional bacteria as a probiotic with the ability to survive in various limiting conditions along the digestive tract from the mouth to the intestine, such as low pH in the stomach and the presence of bile salts in the duodenum [19,20]. The results of the L. plantarum AKK30 and S. cerevisiae B18 tolerance tests on acid pH conditions (pH 2 and 3), gastric juice, and bile salts are shown in Figure 4.

![Figure 4. Viability of LAB and yeast in several assay condition](image-url)
Figure 4 showed that *L. plantarum* AKK30 had good viability on pH 2 and 3 (95.18 and 88.63%) at 45 min of incubation, simulated gastric juice (66.58%) on 45 min of incubation, and 0.3% of bile salts (99.11%) at 120 min of incubation. Several studies showed that LAB had potential as a probiotic. Sabikhi *et al.* [20] reported that microencapsulation of *L. acidophilus* LA1 in sodium alginate and starch provided better protection at simulated conditions of gastric pH (1.0, 1.5, and 2.0) and at high bile salt concentrations (1.0%, 1.5%, and 2.0%). Microencapsulation process resulted in better survival of cells at bile salt concentrations and low pH as compared to free cells. Microencapsulation of *L. acidophilus* using calcium chloride coating on sodium alginate capsules increased tolerance of the bacteria against harsh acidic (pH 2) and bile (1%) conditions [34]. The ability to survive in bile salts is likely because LAB is conjugated with bile salts so it is effective in reducing serum blood cholesterol levels [35]. The high activity of bile salts which are hydrolyzed in the intestinal lumen can reduce the ability of conjugated bile salts to break down fat [36]. Lin *et al.* [37] stated that the survival of LAB ≥ 50% at pH 2, this was assumed to be due to gastric fluid, while LAB survival in bile salt stress was considered good if the viability was ≥ 60%.

The survivability of *S. cerevisiae* B18 on pH 2, pH 3, gastric juice, and bile salt were 87.95, 96.84, 92.70, and 94.09% respectively. A study by Sourabh *et al.* [5] observed that *S. cerevisiae* (HM535662) tolerant at pH 2 (2.71 to 4.12 log cfu/ml), decreased population at pH 3 (0.88 to 3.06 log cfu/ml). Arslan *et al.* [31] stated that the survivability of microencapsulated *S. boulardii* using maltodextrin against simulated gastric solution was 63.62% at pH 2. The ability to tolerate gastric juice was one of an indicator, because good probiotics must be able to pass stresses in the gastrointestinal tract such as pH stress and gastric juice [6].

Microencapsulation technology ie spray drying was efficient method to provide protection against adverse conditions during gastrointestinal passage [9] and to maintain the viability of probiotic from harsh external environments [38].

4. Conclusion

The cell morphology showed that *L. plantarum* AKK30 had bacilli shaped and *S. cerevisiae* B18 had small budding characteristics with average length of cell was 1.35 μm and 3.19 μm respectively. Both microorganism were alive and grew in a commensal relationship with a clearing zone diameter of less than 5 mm and the population between two microorganisms differs only 1 log cycle. The microencapsulation process using spray dryer could maintain the viability of *L. plantarum* AKK30 (97.25%) and *S. cerevisiae* (91.66%). Microencapsulated cultures of *L. plantarum* AKK30 and *S. cerevisiae* B18 had good viability on pH 2 and 3, simulated gastric juice, and bile salts thus it could be used as a probiotic.

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