Potential of Fermented Leek (*Allium porrum* L.) Cultured by *Lactobacillus plantarum* B1765 to Deliver Probiotics

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Abstract. The aim of this research is to study the probiotic properties of *L.plantarum* B1765 including the growth resistance at digestive tract pH, bile salts, antibiotics and invasion to pathogenic bacteria of *Staphylococcus aureus* and *Escherichia coli in vitro.* The potential of leek (*Allium porrum* L.) as a probiotic agent then were studied by inoculated the *L.plantarum* B1765 into pieces of leek and were fermented for 0, 6, 12, 18, 24 and 30 h at 37^0C. The growth, pH and total acid then were identified. The results showed that *L.plantarum* B1765 had probiotic characteristics, namely resistance to gastrointestinal pH and bile salts with viability of more than 90%, showed a strong bactericidal against *S. aureus* and moderate to *E. coli*. In addition, *L. plantarum* B1765 can survive at 50 ppm of amoxicillin. The *L.plantarum* B1765 probiotic growth well in leek as indicated by its optimum growth reach to 1.17 x 10^8 CFU / mL for 6 hours of fermentation, an increase of 1 log cycle from the initial conditions of inoculation. A decrease in pH to 3.24 and an increase in TAT of 0.393%. *L.plantarum* B1765 is a potentially probiotics candidate and fermented leek could be an agent to deliver the *L.plantarum* B1765.

Keywords: Uv-Vis Spectrophotometry, Antioxidant Activity, Phytochemical screening

1 Introduction

The current pattern of people's lives has shifted to the awareness that there is a relationship between food and health. Food products containing probiotics have been shown to provide health benefits if consumed as needed [1]. Probiotics can work well if they are able to colonize in the intestinal tract and are able to stick to the mucosal surface in the intestine. In order to reach the intestinal tract, probiotics must withstand various conditions such as the pH of the digestive tract, bile salts, invasion of pathogenic bacteria and the use of antibiotics.

The acid pH of gastric will affect the growth of bacteria where the bacteria are not acid-resistant, the intracellular pH conditions are not optimal (low), resulting in decreased activity of cell metabolic enzymes [2]. Bile salts will affect the permeability of cells which will react with the side of the cytoplasmic membrane of bacteria causing damage to the membrane structure and triggering lysis [3, 4, 5]. Probiotics can grow optimally at the pH of the intestinal tract with the average number of colony populations reaching 10^5 – 10^{10} CFU/g feces [6]. The role of probiotics to reduce pathogen like *E. coli* `and *S. aureus* in intestinal tract is very useful to prevent the spread of virulence and body tissue infection. Likewise, the viability of probiotics to certain antibiotics is also considered, so the viability in intestinal tract could be maintained to balance the intestinal tract microflora of the host [7]. *Lactobacillus plantarum* B1765 is an *bekasam*, a traditional Indonesian fermented food, isolate. In our previous research, this metabolism of the isolate showed an antihypertensive effect [8] and could managed the blood glucose of hyperglicemia rats, so is needed to study the probiotic properties of *L.plantarum* B1765 [9].

Leek (*Allium porrum* L.) is a foodstuff that has a limited function, as a condiment to flavoring foodstuff, even though leek has many health benefitly. The bioactive compounds in leek such as fructooligosacharide (FOS) and inulin which are known as prebiotic. Leek contains FOS of 5.2% [10] and inulin of 3 – 10% [11]. In our previous study, it was shown that *L.plantarum* B1765 growth well in yacon as a good source of FOS [12]. *L.plantarum* B1765 had also be studied has an inulinase activity [13]. Base on the research results, we need to study the potency of fermented leek, a good source of FOS and inulin, as a probiotic agent to deliver *L.plantarum* B1765. Leeks were inoculated *L.plantarum* B1765 on certain concentration, then fermented in various times at 37^0C. The total number of lactic acid bacteria, pH and acid total then was calculated. The results of this research will be base to develop a functional food product of fermented leek which has health benefit and safety [14, 15.

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2 Material and method

2.1 Preparation of inoculum of *L.plantarum* B1765

1 mL of isolate stock of *L.plantarum* B1765 was inoculated in 9 mL of *deMan Rogosa Sharpe Broth* (MRSB) then incubated for 20 hours at 37ºC The suspension then was subcultured into 9 ml of MRSB then reincubated for 24 h at 37°C before use as inoculum. Pellets then separated from suspension by centrifugation at 3500 ppm for 10 min, then resuspension into 10 mL of 0.85% NaCl. Suspension then was recentrifuged, pellet then separated and resuspension into 10 mL of 0.85% NaCl and was used as inoculum

2.2 Gastrointestinal pH tolerance of *L.plantarum* B1765

MRSB was conditioned at gastrointestinal pH (pH of 1, 2, 3, 7.5 and 8) by adding 10N HCl solution and 2N NH_4OH solution. Then sterilized at 121ºC for 15 minutes. A total of 1 ml starter culture of *L. plantarum* B1765 aged 20 hours was inoculated into these MRSB medium and MRSB without pH adjustment was used as a control, then incubated for 2 hours at 37ºC. A total of 1 mL of suspension was inoculated on the MRSB medium, mixed with agar powder 1.5% (w/v) and $CaCO_3$ 1% (w/v), incubated for 48 hours at 37ºC. Survival rate of the gastrointestinal pH tolerant of isolate was expressed as: Survival rate (%) = [cell number in gastrointestinal pH /cell number of control] × 100. Enumeration of viable cell number were done using total plate count (TPC).

2.3 Bile acid tolerance of *L.plantarum* B1765

A total of 1 mL starter culture of *L.plantarum* B1765 was inoculated into 9 mL of MRSB containing bile salt with various concentrations (1%, 0.7%, 0.5%, 0.3% and 0.1%) and incubated for 4 hours. MRSB without the addition of bile salt was used as a control. Subsequently, 1 mL of the suspension was inoculated into the MRS Agar medium containing of $CaCO_3$ 1% (w/v) and incubated for 48 hours at 37°C, then enumerated by TPC. Survival rate (%) = [cell number in bile salt concentration/cell number of control].

2.4 Antimicrobial activity against pathogenic bacteria of *L.plantarum* B1765

About 1mL subculture of pathogenic bacteria were poured in sterile plate and covered by Nutrient Agar (NA) medium. Disc paper were immersed in subculture *L. plantarum* B1765 suspension for 30 minutes, then placed on NA containing pathogenic bacteria and incubated for 24 h at 37^0C. The clear zone diameter that was resulted then measured and were classified the inhibition criteria including weak inhibition (0 – 3 mm of clear zone diameter), moderate (3 – 6 mm) and strong inhibition (>6 mm).

2.5 Resistance of *L.plantarum* B1765 toward antibiotic

About 1 mL of starter culture of *L.plantarum* B1765 was inoculated and covered with MRSA and incubated for 15 min at 37^0 C. The paper discs were immersed in antibiotic solution at various concentration (500 ppm, 250 ppm, 125 ppm and 50 ppm for 15 min, then were placed on MRSA containing the culture starter surfaces, and were incubated for 24 h at 37^0 C. The level of resistance were determined by measured the clear zone diameter and classified as resistance (0–11 in clear zone diameter), intermediate (11 – 18 mm), and sensitive (>18 mm) [16].

2.6 Leek fermentation

The leek was peeled and cut to a size of ± 1 cm^2 then weighed about 25 grams. The pieces of leek were soaked in a 1% (w/v) salt solution in a ratio of 1:3 in a glass bottle. About 2.5% (v/w) of the starter culture of *L. plantarum* B1765 2.5% (v/w) then inoculated into the cutting leek, then incubated at 0 h, 6 h, 12 h, 18 h, 24 h and 30 h at 37^0 C.

2.7 Enumeration of total lactic acid bacteria in fermented leek

Samples at each fermentation time were mashed and then 1 mL was taken and diluted using a sterile 0.85% NaCl solution starting with a 10^{-1} – 10^{-9} dilution. In the 10^{-5} -10^{-9} dilution, 1 mL was taken and put into a sterile petri dish, then was added a mixture of MRS broth, 1% $CaCO_3$, and 2.5% agar white plain powder. Then incubated upside down at 37^0C for 48 h. The growth of LAB colonies can be enumerated by the appearance of a clear zone.

3 Results and discussion

3.1 Gastrointestinal pH tolerance of *L.plantarum* B1765

L.plantarum B1765 survives at pH of 1, 2, 3, 7.5 and 8 with high survival rate between 92.5% – 97,4% (Table 1) and could be classify as resistant because has the survival rate of > 90%. pH of 1 – 2.5 and 7.2 – 8 representations of pH of stomach and intestine respectively [17,18], The bacteria that could survive in stomach, it will reach alive at the intestine. Some research showed that some of *L. plantarum* survive at low pH [19], [20], [21]. The survival of microorganism at gastrointestinal is the selection criteria of probiotics. The survival of lactobacilli at low pH was reported to be variable and strain dependent, with a survival rate of approximately 85% [22], [23]. Based on the high

resistance of *L.plantarum* B1765 at gastrointerstinal pH, the bacteria to be the candidate of prebiotics potentially.

Table 1. The survival rate of *Lactobacillus plantarum* B1765 at gastrointestinal pH

Initial population= 8,11 (log CFU/mL)		
pH	Final population*) (Log CFU/mL)	Survival Rate (%)
1	7,50	92,5
2	7,54	92,9
3	7,62	93,9
7,5	7,98	98,4
8	7,90	97,4

**incubation time : 120 minutes*

The survival of *L. plantarum* B1765 bacteria in gastric acid conditions is thought to be due to intrinsic factors, including (1) intrinsic homeostatic mechanisms that use cell transport systems including the Glutamate decarboxylase (GAD) system, the arginine deamination system (ADI), and the ATP-ase proton pump. which through this system is able to remove excess protons (H^+) from the cell cytoplasm so that the intracellular pH of the cell remains normal [24]. The cytoplasmic membrane of LAB consists of two layers of phospholipids (lipid bilayer) and its surface has protein and glycoprotein molecules so that it is semipermeable. These components limit the movement of compounds in and out specifically and prevent the penetration of proton (H^+) flow into the cytoplasm [25, 26].

Bacteria that are not resistant to gastric acidity are caused by the high HCl content in the stomach. HCl inhibits cell growth through denaturation of enzymes found on the cell surface, resulting in damage to lipopolysaccharides on the outer membrane and resulting in a decrease in cytoplasmic pH [26].

3.2 Bile salt tolerance

Bile salt tolerance is prerequisite for the metabolic activity and colonization of bacteria in the small intestine of the host. The survival rate of *L.plantarum* B1765 in various concentration of bile salt after incubated for 120 minutes was determined (Table 2).

Table 2. Survival rate in various bile salt concentration of *L.plantarum* B1765

Initial population= 8,17 (log CFU/mL)		
***Bile salt concentration* (% b/v)**	**Final population* (log CFU/mL)**	**Survival rate (%)**
0.1	8.13	99.5
0.3	7.96	97.4
0.5	7.74	94.7
0.7	7.67	91.8
1	54	90.3

L.plantarum B1765 showed bile salt tolerance ability with the high survival rate. The tolerance reduced as well as increasing of bile salt concentration. The survival rate at 0.1% bile salt reached of 99.5% with a total population of 13.4 x 10^7 CFU/mL (8.13 log CFU/mL). At the highest concentration of bile salts (1%), the survivability of *L. plantarum* B1765 is quite large (90.3%) and the number of LAB population was 3.5 x 10^7 CFU/mL (7.54 log CFU/mL)

Bacteria that are not resistant to the bactericidal activity of bile salts, it is thought to be due to changes in cellular permeability and leakage of intracellular material which causes cell lysis and metabolic activity in cells to stop, resulting in cell death [25, 26]. Several lactic acid bacteria from species isolated from the digestive system showed Bile Salt Hydrolase (BSH) enzyme activity. It is hypothesized that the BSH enzyme protects tissue cells from the toxicity of conjugated bile salts. The *L. plantarum* strain is known to have BSH enzyme activity based on research conducted [4] by proving that the BSH enzyme from *L. plantarum* acts to decompose conjugated bile acids into deconjugated bile acids. This deconjugated bile acid no longer has bactericidal activity and will not harm the integrity of the bacterial cell membrane so that bacteria can become resistant [24, 27].

3.3 Antagonistic to pathogen bacteria of *L.plantarum* B1765

The antagonistic nature of *L.plantarum* B1765 has been tested against *Escherichia coli* EPEC and *Staphilococcus aureus* by measured the diameter of inhibition zone surrounding the paper disc that was immersed in suspension of *L.plantarum* B1765 then places on NA medium inoculated with *E.coli* and *S.aureus* (Fig.1). *L.plantarum* B1765 showed antagonistic nature both to *E.coli* and *S.aureus* at moderate level (3.5 mm), and strong level (8.0 mm) respectively.

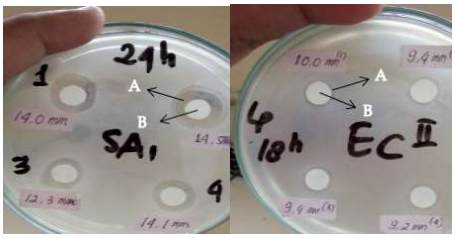

Fig. 1. The inhibition zones of L.plantarum B1765 against *S.aureus* (SA) and *E.coli* (EC)
A (inhibition zone): B (paper disc containing *L.plantarum* B1765)

LAB shows antagonistic properties against pathogenic microbes by producing several compounds that are antipathogenic, among others, organic acids, hydrogen peroxide (H_2O_2), diacetyl, lactoperoxidase and inhibitory substances in the form of specific protein complexes called bacteriocins. The bacteriocin of *L. plantarum* B1765 has been studied and showed activities inhibition of 1577,392 AU/mL toward *E.coli* dan 2062,259 AU/mL toward *S. aureus* respectively

[28]. LAB cell metabolism can produce organic acids such as lactic acid and acetic acid during the fermentation process [27, 29]. Lactic acid and acetic acid be able to weaken the permeability of Gram-negative bacterial cell membranes by damaging the lipopolysaccharide layer. The accumulated organic acids will completely dissociate to produce protons (H^+) so that excess proton ions tend to lower intracellular pH and stop cell metabolism [30].

Hydrogen peroxide (H_2O_2) is an oxidizing agent, bleaching agent and antimicrobial properties while diacetyl is able to act as a binder of arginine binding protein in Gram-negative bacteria and will affect all cell metabolism related to arginine being suboptimal [31]. LAB strains are also known to produce bacteriosin, which is an antimicrobial peptide or exotoxin protein that prevents the growth of pathogenic bacteria, both Gram positive and Gram negative bacteria [32, 33].

The results showed that *L. plantarum* B1765 had relatively stronger antagonistic properties against *S.aureus* from the Gram positive group than *E.coli* from the Gram negative group. This is possible because of differences in cell walls where Gram-negative bacteria have a more complex wall structure. This may be related to the presence of a lipopolysaccharide (LPS) layer on the cell wall of Gram-negative bacteria that protects the cell membrane, as a target site for bacteriocins. While the structure of the cell wall of Gram-positive bacteria is simpler, including 90% of the cell wall consisting of peptidoglycan, making it easier for antimicrobial substances to enter the cell cytoplasm [25, 27, 34]. Based on the above, it was concluded that *L. plantarum* B1765 was capable of being bactericidal against the growth of the *S. aureus* pathogen with an inhibition zone of 8.0 mm (strong inhibition category) and showed bacteriostatic inhibition against *E. coli* with an inhibition zone of 3.5 mm and included the criteria for moderate inhibition.

3.4 Resistance of *L. plantarum* B1765 towards antibiotic

The resistance of *L. plantarum* B1765 toward antibiotic has been tested to amoxicillin at various concentration. The result of resistance and sensitivities of *L. plantarum* B1765 toward the amoxicillin antibiotic were describes in Table 3.

Table 3. Resistance and sensitivity of *Lactobacillus plantarum* B1765 toward amoxicillin antibiotic

Amoxicillin concentration(ppm)	Inhibition zone diameter (mm)	Level*
50	0,0	Resistance
125	12,8	Intermediet
250	18,0	Intermediet
500	24,9	Sensitive

*resistance interpretation *standard of amocsisilen (NCCLS, 2005): ≤ 10 mm resistance, 11 –19 mm intermediet, dan ≥ 20 mm sensitive*.

L. plantarum B1765 is known to be only resistant to amoxicillin concentration of 50 ppm, showed both intermediate resistance at 125 and 250 ppm, and sensitive at 500 ppm of amoxicillin. The higher the concentration of amoxicillin, the lower the resistance properties of the bacteria *L. plantarum* B1765 and become non-resistant because the antimicrobial properties produced by antibiotics tend to be stronger. Antibiotics are divided into 2 types, namely killing bacteria (bactericidal) and inhibiting bacterial growth (bacteriostatic) [35]. The relatively high concentration of antibiotic substances causes greater bactericidal and bacteriostatic properties in suppressing bacterial growth [36]. Inhibition zones or areas that are not overgrown with bacteria due to the inhibitory activity of the components of antibiotic compounds can be shown in Figure 2.

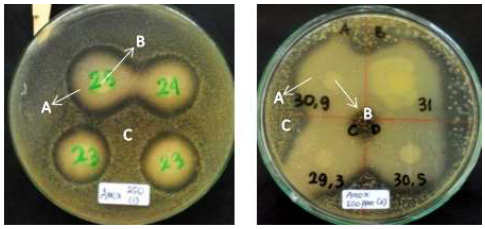

Fig. 2. Inhibition zone of amoxicillin toward L.plantarum. Inhibition zone of amoxicillin towards *L. plantarum* B1765; B paper dish that was immersed in amoxicillin at various concentration; C. growth zone of *L. plantarum* B1765

Amoxicillin is an antibiotic which kill the Gram positive and Gram negative bacteria potentially [37]. The high sensitivity of *L. plantarum* B1765 toward 500 ppm amoxicillin was predicted caused by lysis of cell wall of this bacteria.

Resistance is the ability of bacterial cells to neutralize and not be affected by the bacteriolytic properties of antibiotics because it is caused by several intrinsic factors such as: (1) bacteria produce inactivating enzymes; (2) the thickness of the outer cell wall of bacteria can inhibit the insertion of antibiotics into the cell; (3) changes in the work place (target site) of antibiotics in bacteria and; (4) antibiotics that enter the cell and accumulate in the cell will be released through an active transport mechanism to the outside of the cell [36, 38].

Amoxicillin contains a number of -lactam compounds as bactericidal components [39]. LAB strains are known to produce -lactamase enzymes, which are enzymes that function to degrade -lactam compounds and cause loss of bactericidal activity. However, in the pharmaceutical industry, the process of making amoxicillin antibiotics tends to be combined with clavulanic acid which functions as an inhibitor of the -lactamase enzyme, causing bacteria to become resistant [38]. Furthermore, the thickness of the peptidoglycan layer of bacteria determines the resistance of bacteria to antibiotics where the greater the thickness, the tighter the permeability system in

preventing the insertion of antibiotic components through the cell membrane, thus supporting the tendency of LAB to become resistant to antibiotics [40].

Antibiotic-resistant bacteria tend to develop an altered target site for antibiotic responsiveness. A change in the target site causes the antibiotic to not work against the bacteria that respond to it so that the bacteria avoid bactericidal activity and become resistant to the antibiotic [35].

Meanwhile, when the LAB defense mechanism is not able to work optimally, it can be overcome by using an active transport system. LAB strains are known to have a reversible transport system to maintain cell homeostasis. The mechanism of action is that components that are not needed by the cell are immediately excited outside the cell membrane so that the antibiotic components that have accumulated in the cell due to penetrating the cell membrane will not last long in the cytoplasm because it will be immediately pumped out to the outside of the cell membrane. Then the bactericidal activity of antibiotics can be reduced so as to avoid cell damage due to antibiotic activity and bacteria will become resistant [41].

3.5 Potential of leek as a probiotic agent to deliver *L.plantarum* B1765

The potensial of leek as a probiotic agent were determined from the growth ability in leek medium, pH and titration acid total of *L.plantarum* B1765 (Table 4).

Table 4. The total number of Lactic Acid Bacteria (LAB), pH and Acid Total

No	Fermentation time (h)	LAB Total (CFU/mL)	Log (CFU/mL)	pH	TAT (%)
1.	0	1,43 x 10^7	7,1553	4,34	0,076
2.	6	1,17 x 10^8	8,0682	3,58	0,312
3.	12	1,12 x 10^8	8,0492	3,52	0,342
4.	18	8,35 x 10^7	7,9217	3,44	0,362
5.	24	6,45 x 10^7	7,8096	3,26	0,385
6.	30	1,97 x 10^7	7,2944	3,24	0,393

The highest LAB growth was produced during the 6-h of fermentation, and reached 1.17 x 108 CFU/mL, an increase of 1 log cycle from the initial condition of inoculation, in which it was the logarithmic phase of *L.plantarum* B1765. During the fermentation time of 6 to 12 hours, the total LAB showed no significant difference, so that at this time *L.plantarum* B1765 entered the stationary phase. The stationary phase is a phase in which the number of bacteria does not increase significantly due to a reduction in the amount of nutrients and an accumulation of toxic amounts resulting from metabolism that causes disruption of the cell division process [42]. Furthermore, it experienced a death phase where the total LAB decreased from 12 hours of fermentation time to 30 hours.

Leeks contain FOS of 5.2g/100g [10] and inulin as much as 3 - 10% by weight [11]. It is suspected that the growth of *L.plantarum* B1765 in leeks is due to its ability to metabolize FOS and inulin [43]. Although not all LAB can utilize FOS and inulin, it were known that the *L.plantarum* strains can use FOS and inulin as growth media [44, 45, 46]. Another study stated that *L.plantarum* IS-10506 was able to grow on m-MRSB medium with 10% inulin. *L.plantarum* IS-10506 experienced the highest growth of 1.99 x 1010 CFU/mL at the 12th hour. In our previous study, L.plantarum B1765 growth well in yacon as a source of FOS and inulin and showed an inulase activity [12][13]. Furthermore, fermented leek inoculated with L.plantarum B1765 showed of 1.17 x 108 CFU/mL of total LAB at 6 hours so that leek is quite potential as a growth medium for *L. plantarum* B1765.

Fermentation time can affect the pH and TAT values due to the metabolic process of the *L. plantarum* B1765. In this research, the pH value decreased from the fermentation time of 0 hours to 30 hours (Table 5). *L.plantarum* is part of heterofermentative bacteria which was able to produce other than lactic acid but also acetic acid, ethanol, and CO_2 [47]. The pH values at 6 hours and 12 hours of fermentation did not show a significant difference. After 12 hours of fermentation, the pH value continued to decrease due to the hydrolysis of carbohydrates during the fermentation process which continued to cause the production of organic acids [48].

Inulin polyfructan from leek in -2,1 glycoside chain will be hydrolyzed into fructose monomer by *L. plantarum* B1765 due to the activity of inulinase enzyme [13]. The resulting fructose monomer is metabolized by the glycolytic pathway and produces Short Chain Fatty Acid (SCFA). The resulting SCFA will accumulate in the fermentation medium and undergo dissociation to form H+ and CH3CHOHCOO- ions. The high concentration of H+ ions in the fermentation medium will result in an increasingly acidic pH [48]. The accumulation of H^+ ions also affects the TAT value. The decreasing pH value indicates that more SCFA compounds are produced from bacterial. metabolic processes which then undergo dissociation to form H^+ ions. This will cause the TAT value to increase [49, 50]. TAT increased during the yakon tuber fermentation process from 0.044% to 0.452% [12].

4 Conclusion

L.plantarum B1765 is proven to have probiotic ability in terms of its resistance to gastrointestinal pH, bile salts, pathogenic bacteria and antibiotics. It was able to

survive at gastrointestinal pH of more than 90% resistance. resistant to bile salts reaching more than 90%. Regarding the invasion of pathogenic bacteria, *L.plantarum* B1765 is capable of being bactericidal against *S.aureus* with an inhibition zone of 8.0mm (strong inhibition) and *E. coli* with an inhibition zone of 3.5mm (moderate inhibition). In addition, *L.plantarum* B1765 was able to survive at a concentration of 50 ppm amoxicillin. Leeks proved capable of being a growth medium for *L.plantarum* B1765 as indicated by its optimum growth reaching 1.17 x 108 CFU/mL for 6 hours of fermentation, an increase of 1 log cycle from the initial condition of inoculation. This was followed by a decrease in the pH value to 3.24 and an increase in the TAT value to 0.393%. Thus, fermented leek is an agent to deliver probiotic potentially.

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