Improving antibacterial activity and viability of *Lactobacillus plantarum* AKK30 as feed additive by addition of different oligosaccharides

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Abstract. Combination of lactic acid bacteria as probiotic with oligosaccharides substances has potentially improved nutrient utilization and poultry performance. A study was conducted to evaluate the addition of oligosaccharides inulin or mannan oligosaccharides (MOS) with different levels of oligosaccharides into growth medium on antibacterial activity and viability of *Lactobacillus plantarum* AKK30 in vitro. The experiment was arranged on the factorial design using two factors, the A factors were oligosaccharides types (inulin or MOS), and the B factors were increased level of oligosaccharides (0, 0.5, 1, 1.5 and 2%). Each treatment was conducted in 3 replications. Data were analyzed with analysis of variance (ANOVA) among the treatments that show significant differences (p<0.05) followed by Duncan post hoc test. The results showed that the combination of *L. plantarum* and oligosaccharides (P<0.05) influenced *L. plantarum* growth according to the value of OD (OD-590nm). However, a combination of *L. plantarum* and oligosaccharides was not significant (p>0.05) for antibacterial activity. Addition of mannan oligosaccharides (MOS) 0.5 - 2% could increase growth of *L. plantarum* growth with 0.5% MOS as optimum level.

1. Introduction

*Lactobacillus* and *Bacillus subtilis* have been increasingly tested as alternative growth promoters [1]. *Lactobacillus acidophilus* has antibacterial activity [2], improved intestinal health [3]. It affects intestinal morphology and thickness of broiler intestine [4] and resists lincomycin antibiotics, tetracycline and enrofloxacin [5]. *L. plantarum* has potential as antimicrobial [6] candidate as a probiotic.

Viability of Lactobacillus in chicken intestines is considered important because it affected the performance of broilers and digestibility [7] and accelerated the process of absorption and cell proliferation because a Lactobacillus could hydrolyze undigested carbohydrates into monosaccharides through anaerobic fermentation in the intestines [8]. Oligosaccharides are a source of nutrients for probiotic bacteria because the growth of probiotic bacteria can be increased by oligosaccharides [9]. Oligosaccharides are substrate that was used selectively and useful for certain microorganisms including Fructo Oligosaccharide (FOS), Mannan-Oligosaccharide (MOS) and inulin [10]. The use of oligosaccharides and probiotics could modulate antibacterial activity, and specificity [9] enhanced the growth of representative probiotic strains in vitro [11]. Combination of lactic acid bacteria as probiotic with oligosaccharides substances has potentially improved nutrient utilization and poultry performance.
A study was conducted to evaluate the addition of oligosaccharides (OS) consisting of inulin or mannan oligosaccharides (MOS) with different level of OS added into growth medium on in vitro antibacterial and growth activities of Lactobacillus plantarum AKK30.

2. Material and methods

2.1. Culture and media

The study was conducted at the Research Unit of Natural Product Technology (BPTBA)- Indonesian Institute of Sciences (LIPI) Microbiology Laboratory, from May to June 2018 using L. plantarum obtained from the AKK isolate belonging to the BPTBA-LIPI Microbiology Laboratory and the commercially obtained oligosaccharides consisting of Inulin [Orafti, Beneo-USA] and Mannan oligosaccharide (MOS) [Technomos, Biochem-Germany]. The factorial design experiment was arranged using two factors: the A factors were oligosaccharide types consisted of L. plantarum with inulin (Lp + inulin) and L. plantarum with MOS (Lp + MOS) and the B factors were increased level of oligosaccharides concentration (0, 0.5, 1, 1.5, and 2%; v/v) of which each treatment consisting of 3 replications. The parameters measured were optical density (OD), total lactic acid content, and antibacterial activity. Two mL of MRSB and 1% of L. plantarum were added into microtube and then were vortexed and incubated 24 hours at 37 ºC. A series of falcon tubes filled with 10 mL of sterile MRSB were added with either inulin or MOS oligosaccharides (0%, 0.5%, 1%, 1.5%, 2%) and 1% of L. plantarum culture (Table 1).

| Oligosaccharides | treatment | Inoculum (1% 0.1 mL/ 10 mL) |
|------------------|-----------|-----------------------------|
| MOS              | M0        | 0 %                         |
|                  | Ma        | 0.5%                        |
|                  | Mb        | 1.0%                        |
|                  | Mc        | 1.5%                        |
|                  | Md        | 2.0%                        |
| Inulin           | l0        | 0 %                         |
|                  | la        | 0.5%                        |
|                  | lb        | 1.0%                        |
|                  | lc        | 1.5%                        |
|                  | ld        | 2.0%                        |

2.2. Growth activity assay

Growth activity assay of Lactobacillus plantarum was measured by turbidimetric (optical density) using microplate reader (MultiSkan GO, Thermoscientific) at a wavelength of 590 nm [9]. Each 150 μL of inoculum (1% 0.1 mL / 10 mL) was poured in a wheel tube according to the scheme as seen in Table 1. The inoculum in the wheel tube was stored in a stock collection shutter and inserted on the plate reader. The absorbance value of the inoculum was recorded every 2 hours for 24 hours.

2.3. Analysis of lactic acid concentration

Analysis of lactic acid content was the principle of acid-base titration according to [12]. Two or three drops of 1% phenolphthalein (PP) C_{20}H_{14}O_{4} indicator was mixed with the samples and was titrated with 0.05 N NaOH solution until the color was pink. The total titrated solution was the total lactic acid in the sample. The formula was as follows:

\[
\text{Crude Lactic acid (%) } = \frac{(V_{Ts} - V_{to}) \times N \times MW \times Df}{V \times 1000} \times 100%
\]

\( V_{Ts} = \) titrant sample volume (mL) \hspace{1cm} \( MW = \) relative molecular of lactic acids 90.0 (g/mol)

\( V_{to} = \) blank (mL) \hspace{1cm} \( Df = \) dilution factor

\( N = \) normality of the titrant (NaOH 0.05 N) \hspace{1cm} \( V \) sample = sample volume
2.4. Antibacterial activity assay
Each isolate was grown in its media for 24 h at 37 °C and was centrifuged at 4136 x g for 15 min at 4 °C. Twenty-five ml of cell-free supernatant was incorporated into a blank paper disk (0.6 mm) [oxoid] and applied to an agar plate containing pathogenic bacteria as microbial indicator. Inhibition activities test against *Escherichia coli* FNCC 0194, *Staphylococcus aureus* FNCC 6049, *Pseudomonas aeruginosa* FNCC 0063, and *Salmonella pullorum* ATCC 13036 in nutrient agar (NA) media [Merck] were performed using agar diffusion method [13] with the incubation time and temperature of 24 h at 37 °C respectively. The parameters measured were diameter (mm) of the visible clear zone.

2.5. Data Analysis
Quantitative data from lactic acids content and antibacterial activity were analyzed by using analysis of variance (ANOVA) and followed by Duncan’s multiple range test to distinguish the effect of different treatment mean using CoSTAT statistical software [14].

3. Result and discussion
3.1. Growth activity
The growth curve of *Lactobacillus plantarum* AKK30 on different oligosaccharide media were shown in Figure 1. The average of optical density value on Lp + inulin (0.990) was significantly different (p <0.05) compared to Lp + MOS (0.703) and the addition of 0.5% oligosaccharides were the optimal level of growth of *L. plantarum* (1.009). This result indicated that the addition of inulin give better effect for the growth of *L. plantarum* in comparison with MOS as well as control and the optimal addition level was 0.5% which confirm the result of Setiarto [15] research who found that the addition of 0.5% inulin concentration to the MRSB media was the most effective modification in increasing *L. acidophilus* growth when compared to other treatments. It is probably because inulin is a good source of nutrition for the growth of *L. plantarum*. Oligosaccharides were prebiotic [16] and were a source of nutrition for lactic acid bacteria [17].

![Figure 1](image_url)

*Figure 1.* The growth curve of *L. plantarum* AKK30 which was supplemented with Inulin (a) and MOS (b)

From Figure 1, it can be seen that the combination of Lp + inulin started to enter and reached its stationary phase at 18 and 24 hours, respectively. Meanwhile, the optimum OD value could be found in the combination of Lp + 0.5% MOS which was significantly different in comparison with the control (p < 0.05) but not with other concentration treatments. The growth of the 2\textsuperscript{nd} to 12\textsuperscript{th}-hour incubation from all levels of MOS has entered the exponential phase but not for the control and from the 14th hour started to slow to the stationary phase 24 hours incubation. This result showed that the growth of *L. plantarum* was influenced by the addition of oligosaccharides even though the level of administration was not significantly different (p> 0.05). Oligosaccharides are carbohydrates that are digested into glucose [10], as a nutrient source for lactic acid bacteria growth [18], inulin and other oligosaccharides are appropriate for growth of
lactic acid bacteria including *L. plantarum* [19, 20] but not all oligosaccharides are able to assimilate the lactic acid bacteria fermentation process [9]. The rate at which oligosaccharides dissolved [18]. This indicates that inulin and MOS are needed as a source of carbohydrates in the *L. plantarum* fermentation process.

### 3.2. Analysis of lactic acid concentration

The effectiveness of lactic acid fermentation from the combination of *L. plantarum* and oligosaccharides were shown in Table 2.

| Oligosaccharides | Crude lactic acid concentration (%) |
|------------------|-----------------------------------|
|                  | 0.0%          | 0.5%          | 1.0%          | 1.5%          | 2.0%          | average |
| Inulin           | 19.44±0.63    | 22.32±1.81    | 19.83±3.73    | 20.07±2.44    | 20.10±3.01    | 20.35⁷ |
| MOS              | 18.15±1.53    | 19.20±2.76    | 20.82±1.68    | 19.02±1.05    | 22.65±1.15    | 19.97⁷ |
| Average          | 18.79⁷        | 20.76⁷        | 20.33⁷        | 19.55⁷        | 21.38⁷        |         |

MOS : Mannan oligosaccharides mean in the same column, and row differ significantly (P<0.05)

From Table 2, it can be seen that Lp + inulin from lactic acid concentration was not significantly different (p> 0.05) with Lp + MOS and but not with other concentration treatments. This result indicated that the possibility of oligosaccharides was not being used optimally by *L. plantarum* and because the type of BAL fermentation was different according to the type of BAL. *L. plantarum* is a homofermentative lactic acid bacteria that converts almost all the sugar used into lactic acid by the enzyme Lactate dehydrogenase through the reaction of glycolysis [18]. Lactic Acid Bacteria are gram-positive bacteria that cannot produce ATP so that ATP was only obtained from fermenting sugars [21] and producing lactic acid [8]. Other possibilities were the role of oligosaccharides such as inulin as prebiotics which regulate acidic conditions in lactic acid bacterial fermentation [22], prebiotics provides changes in intestinal microflora activity and regulate acidic conditions in the stomach and stimulate intestinal bacterial activity [10].

### 3.3. Antibacterial Activity

Antibacterial activity of Lp +inulin and Lp + MOS can be seen in Table 3.

| Oligosaccharides | Clear zone diameter (mm ±SD) |
|------------------|-----------------------------|
|                  | 0.0%          | 0.5%          | 1.0%          | 1.5%          | 2.0%          | average |
| *S. aerus*       |                            |              |              |               |               |         |
| Inulin           | 12.30±1.13      | 13.40±2.06    | 14.00±0.09    | 12.65±0.21    | 12.30±1.12    | 12.93⁷ |
| MOS              | 12.40±0.00      | 13.80±1.17    | 13.15±0.92    | 12.11±0.42    | 13.21±1.98    | 12.90⁷ |
| Average          | 12.35⁷          | 13.60⁷        | 13.56⁷        | 12.38⁷        | 12.76⁷        |         |
| *S. pullorum*    |                            |              |              |               |               |         |
| Inulin           | 16.35±2.05      | 15.7±1.69     | 18.95±5.44    | 16.95±1.77    | 16.35±2.05    | 20.35⁷ |
| MOS              | 17.10±0.00      | 17.55±3.89    | 16.50±1.69    | 16.55±3.46    | 16.00±4.22    | 19.97⁷ |
| Average          | 16.73⁷          | 16.53⁷        | 17.73⁷        | 16.75⁷        | 16.18⁷        |         |
| *E. coli*        |                            |              |              |               |               |         |
| Inulin           | 10.55±0.21      | 11.25±0.92    | 10.95±0.50    | 10.40±0.42    | 10.55±0.21    | 10.74⁷ |
| MOS              | 10.20±0.00      | 10.80±0.14    | 10.75±0.07    | 10.70±0.10    | 9.90±0.57     | 10.47⁷ |
| Average          | 10.38⁷          | 11.03⁷        | 10.85⁷        | 10.55⁷        | 10.23⁷        |         |
| *P. aeruginosa*  |                            |              |              |               |               |         |
| Inulin           | 9.20±0.71       | 9.90±0.71     | 9.20±2.12     | 11.40±3.25    | 9.20±0.71     | 10.57⁷ |
| MOS              | 11.1±0.00       | 10.10±1.28    | 11.15±2.33    | 10.65±2.90    | 9.85±0.92     | 9.78⁷ |
| Average          | 10.15⁷          | 10.00⁷        | 10.18⁷        | 11.03⁷        | 9.53⁷         |         |

MOS : Mannan oligosaccharides mean in the same column and row differ significantly (P<0.05)
Antibacterial activity of Lp + inulin and Lp + MOS were not significantly different (p>0.05) and but not with other concentration treatments. In Table 4, *L. plantarum* demonstrated antibacterial ability as evidenced by the inhibition zone on the growth of pathogenic bacteria, *Staphylococcus aureus* FNCC 6049, *Escherichia coli* FNCC 0194, *Salmonella pullorum* ATCC 13036 and *Pseudomonas aeruginosa* FNCC 0063, in the media but the anti-bacterial ability was not increased even though added oligosaccharides such as inulin and MOS. This indicated that oligosaccharides are nutrients for the growth of lactic acid bacteria including *Lactobacillus plantarum*, different from Baurhoo [23] that MOS has the ability to inhibit the growth of Gram-negative type 1 pathogenic bacteria in the digestive tract. Possibility not all types of oligosaccharides can synergize with BAL in accordance with Schrezenmeir [21] that probiotic Bifidobacteria can be synergized with oligofructose, but *Lactobacillus casei* cannot be in accordance with Figure 2.

![Figure 2](image_url)

**Figure 2.** Inhibitoty effect of the combination of *L. plantarum* with oligosaccharides against pathogenic bacteria

The treatment of Lp + inulin could be inhibited better than that of Lp + MOS for all tested pathogenic bacteria. The biggest inhibition was demonstrated in *Salmonella pullorum* (Figure 2). The result indicated that the combination of *L. plantarum* and inulin were effective to inhibit *Salmonella pullorum* in comparison with MOS as well as control, which confirm the result of Feng [24] research which found that the antibacterial activity of the *L. plantarum* was better to inhibit the growth of *Salmonella pullorum*. Antibacterial activity of *L. plantarum* was also caused by its ability to survive longer in the digestive tract even though it was acidic and could be produced more lactic acid [25]. Bacteriocin produced by probiotic bacteria had narrow and broad-spectrum activities against pathogenic bacteria [26] so that *L. plantarum* could be used as a probiotic for animals [24].

4. Conclusion

Addition of oligosaccharides (OS) consisted of inulin or mannan oligosaccharides (MOS) in medium growth did not significantly affect lactic acid production and antibacterial activity of *L. plantarum* AKK30 against to pathogenic bacteria (*S. aureus* FNCC 6049, *S. pullorum* ATCC 13036, *E. coli* FNCC 0194 and *P. aeruginosa* FNCC 0063). However, growth of *L. plantarum* AKK30 could be increased by addition of mannan oligosaccharide (MOS) at 0.5 – 2 % (w/v) in growth-medium.
5. Reference

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