Probiotic properties of lactic acid bacteria isolated from faeces of *Pongo abelli* and *Macaca fascicularis*

I Jamilah*, A H Jhon, R F Siregar, and A Hartanto

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Jalan Bioteknologi No. 1 Kampus USU, Medan 20155, Indonesia

*Email: it@usu.ac.id

**Abstract.** Sumatran Orangutan (*Pongo abelli*) and Long-tailed Macaque (*Macaca fascicularis*) population are both facing a decline in the wild. Despite of the well-known cause of decline, which is habitat degradation, several issues like disease and infection are common in *ex-situ* conservation site. The study aimed to evaluate antimicrobial properties of lactic acid bacteria (LAB) isolated from faeces of *Pongo abelli* and *Macaca fascicularis*, as potential antagonist against common gut pathogens based on *in vitro* study. Following parameters were examined such as: antagonistic activity against *Salmonella typhii*, *Escherichia coli*, and *Shigella* sp. using disk-diffusion method; acid and bile-salt tolerance using simulated gastrointestinal fluids (SIF) and co-aggregation ability. The study obtained ten isolates in which six of them exhibited antagonist properties namely isolate OU1, OU2, OU5, MEP1, MEP3 and MEP5. Two isolates, namely OU5 and MEP5 showed good results in terms of acid and bile-salt tolerance and co-aggregation ability. The results then may imply the promising use of potential LAB as probiotics in the future study.

1. **Introduction**

Sumatran Orangutan (*Pongo abelli*) and Long-tailed Macaque (*Macaca fascicularis*) are two primate species from Indonesia which are facing a decline in the numbers of wild population [1]. Alteration of habitat, due to conservation effort by moving injured or quarantined primate into *ex-situ* conservation site, may lead to the inability of primates to adapt to environmental changes. These environmental changes may then support the emergence of certain health disorders and diseases during conservation effort. The disease posed a serious threat to primate’s survival in the new habitat [2].

Many infants of Orangutan and Macaque which were born in Zoo or Animal park, could not reach adulthood due to the lack of health monitoring and evaluation. Gastrointestinal disorder is the most common problem in captive primates. This disorder is characterized by symptoms of diarrhea caused by enteropathogenic bacteria. The degree of pathogenicity may be ranked from mild to severe diarrhea, according to the pathogen strains and virulences. The majority of infections were caused by *Shigella*, *Escherichia coli* and *Salmonella* [3]. Salmonellosis and Shigellosis are gut disorders caused by *Salmonella* and *Shigella* that were included into the list of 25 serious diseases of Orangutans. Salmonellosis is common to captive primate along with the less known amoebic dysentery. One of the *ex-situ* conservation efforts is by conducting a captive-breeding along with educational purpose and conservative promotion through animal park or zoo management. Taman Hewan Pematang Siantar (THPS), located in Siantar City, North Sumatera is one of ex-situ conservation...
agencies in North Sumatra [4]. There are currently held three captive Orangutans (one male and two females) and five Macaques (two males and three females). Based on information obtained from the officials, those primates are often experiencing diarrhea, as shown by defecating aqueous feces. Orangutans can be infected through contact with humans, food, dirty water, and from tourist activity.

The use of probiotics has been shown to improve the health of human digestive tract. Probiotics are non-pathogenic living microorganisms that positively affect the health of animals and humans by improving the balance of indigenous microbiota in the digestive tract. Probiotics are useful for health, by promoting other health benefits: increasing resistance to infectious diseases especially diarrhea, reducing the risk of lactose intolerance and improving the immune system. Studies on lactic acid bacteria (LAB) with probiotic properties for health has been done as much as the use of probiotics in the prevention of gastrointestinal infections. Some of the most common BAL species used as probiotics are Lactobacillus, Enterococcus and Lactococcus [5]. To improve the health of captive primates, integrating probiotics into their diet may be seen as promising way to combat against gastrointestinal disorders. The first step is to isolate compatible LABs from host samples and to evaluate their probiotic properties for safety use in the future.

2. Materials and Methods

2.1 Isolation of LABs from fecal samples
Fecal samples used in this study were obtained from captive Orangutan (P. abelii) and Long-tailed macaque (M. fascicularis) as permitted by officials of Taman Hewan Pematang Siantar, Siantar City, North Sumatera. The stool samples were collected in sterile bottle and stored in freeze condition prior laboratory use. Isolation step was carried out by inoculating an aliquot of sample into de Mann Rogosa Sharpe Agar (MRSA) supplemented with CaCO3 [6]. Colonies producing clear zones were picked and maintained in slant agar. Ten isolates namely OU1, OU2, OU5, OU7, OU9, MEP1, MEP3, MEP5, MEP8 and MEP10 were differentiated using standard morphological and biochemical characteristics. All isolates were found to be lactic acid bacteria.

2.2 Antagonism assay
Isolated LABs were tested for their inhibitory effects to the growth of pathogenic bacteria by using disc diffusion method [7]. Both LABs and pathogens (S. typhii, E. coli, Shigella) were grown in Nutrient Broth (NB) prior antagonism assay and incubated for 24 hr. Overnight culture LABs and pathogens were measured for their Optical Density (OD600) 0.5-0.6 = 10⁸ Colony Forming Unit (CFU) mL⁻¹. Sterile discs were immersed with LABs suspension and placed on top of Mueller Hinton Agar (MHA) previously swabbed with suspension of pathogens and incubated for 24 hr. The inhibitory activity depicted as clear zones from the discs were measured.

2.3 pH tolerance assay
The resistance of each LABs need to be evaluated for its ability to survive under gastrointestinal condition or acidic pH. LABs were cultured in on MRSB for 24 hr. MRSB media were then prepared with pH 2.5 and 7.2 using 0.1 N HCl and 0.1 N NaOH. One millilitre of each bacterial suspension 10⁸ CFU.mL⁻¹ was inoculated into MRSB medium and incubated for 90 min at temperature 28-32 °C. After incubation, dilution of each bacterial suspension using 0.9% NaCl to dilution 10⁸. One millilitre of each dilution was poured into sterile petri dish, then 15 mL MRSA were added into each petri dishes and incubated for 24 hour in room temperature. Colonies grown on MRSA representing the survived LABs were counted [8].

2.4 Bile-salt tolerance assay
Assay of LABs tolerance to bile salts were performed according to [9]. LABs were activated in MRSB and incubated for 24 hours at 37 °C. Overnight cultures were inoculated 2% into MRSB medium
containing 0.5% bile salts. The flasks were incubated at 37 °C for 4 hr. Controls were made without addition of bile salts in MRSB. After incubation, dilution of each bacterial suspension using 0.9% NaCl to dilution 10⁻⁸. One millilitre of each dilution was poured into sterile petri dish, then 15 mL MRSA were added into each petri dishes and incubated for 24 hour in room temperature. Colonies grown on MRSA representing the survived LABs were counted.

2.5 Co-aggregation assay

The assay was performed to simulate the interaction ability between LABs to stick together in the digestive tract, so it is not easily washed out due to intestinal movement and fluids. In this method, single and mixed cultures were used with a ratio of 1:1 (v/v) and co-aggregation was expressed in the decrease of OD between mixture and single isolate. Tests were performed using a spectrophotometer. Overnight cultures grown previously in MRS broth at 37 °C for 24 hours, were harvested by centrifugation (10,000 rpm, 10 min). Pellets were washed 2 times with sterile Phosphate Buffer Saline (PBS). The culture is then re-suspended to the same buffer, and measured for its decrease in OD until it reaches the final absorbance at 600 nm wavelength[10].

3. Results and Discussions

Isolated LABs were tested for its inhibitory effects against three bacterial pathogens, *S. typhii*, *E. coli* and *Shigella*. All LABs showed inhibitory effects to almost all tested pathogens with different results (Table 1). The diameter zones of inhibition were ranging between 9.30–3.68 across all isolates. Two isolates namely OU7 and MEP8 did not show any inhibitory effect against *E. coli* and were excluded for the next steps. Six LABs namely OU1, OU2, OU5, MEP1, MEP3 and MEP5 were then subjected to next parameters. Selection of potential LABs were made by considering the category of diameter zones of inhibition[11]. Highest antimicrobial activity was determined from diameter zones of inhibition larger than or > 6 mm, average activity between 3–6 mm and low activity between 0–3 mm.

| Isolates | *Salmonella typhii* (mm) | *Shigella* sp. (mm) | *Escherichia coli* (mm) |
|----------|-------------------------|-------------------|-----------------------|
| OU1      | 6.93                    | 7.06              | 3.68                  |
| OU2      | 6.56                    | 8.43              | 7.37                  |
| OU5      | 8.62                    | 6.12              | 8.43                  |
| OU7      | 5.36                    | 5.12              | 0                     |
| OU9      | 4.95                    | 6.87              | 1.87                  |
| MEP1     | 6.43                    | 6.15              | 8.12                  |
| MEP3     | 7.87                    | 6.93              | 6.49                  |
| MEP5     | 7.37                    | 5.62              | 6.27                  |
| MEP8     | 6.22                    | 9.30              | 0                     |
| MEP10    | 5.55                    | 6.46              | 3.98                  |

To determine the potential probiotic properties from LABs, tolerance to bile-salt and gastrointestinal pH need to be evaluated. Under physiological condition, bile-salt is normally excreted into intestinal environment and may act as antimicrobial substance. Six LABs were able to survive under bile-salt condition within 4 hour of incubation (Table 2). The surviving cells were represented in log CFU.mL⁻¹. Six LABs showed the same results by surviving 50% of their populations post-incubation. In application, probiotics must survive at least until 90 min within physiological condition and attached to epithelial cells before promoting health effects to their hosts [12].
Table 2. Percentage of surviving LABs population within bile-salt condition

| Isolates | Control (log CFU.mL⁻¹) | Simulation (log CFU.mL⁻¹) | Percentage of surviving population (%) |
|----------|------------------------|---------------------------|----------------------------------------|
| OU1      | 9.39                   | 5.35                      | 56.99                                  |
| OU2      | 9.38                   | 5.19                      | 55.42                                  |
| OU5      | 9.69                   | 5.15                      | 53.21                                  |
| MEP1     | 9.40                   | 5.23                      | 55.63                                  |
| MEP3     | 9.39                   | 5.19                      | 55.35                                  |
| MEP5     | 9.40                   | 5.12                      | 54.49                                  |

Figure 1. Percentages of surviving LABs population under pH condition

All LABs showed good endurance towards series of pH tested in acid tolerance assay. High survivorship was categorized as surviving LABs population no lower than 50% from its initial population [9]. No isolates were found to survive below 50% of their population although isolate OU1 and MEP3 were both the lowest in terms of percentage (84%) under acidic pH. Acid tolerance was one of important properties in determining potential strains of probiotics [13].

Co-aggregation assay is a simulation test to observe attachment between LABs to thrive within physiological condition. The assay was performed using spectrophotometer by measuring OD from combination of six strains compared with each OD from their pure or single strain solution. The result must show no apparent decrease of OD in multi strains solution compared to their single strain solution. The results showed that nine combinations failed to co-aggregate each other. Four combinations gave results in low percentage of co-aggregation (<50%) (Figure 2). Combination of OU5 + MEP5 showed the highest co-aggregativeness with percentage of 54% and therefore was assumed to be able to thrive or cooperate later in the physiological condition during attachment to intestinal lines. During co-aggregation, LABs may act as anti-competitor to pathogens by synthesizing antimicrobial compounds such as organic acids, antimicrobial proteins to compete in carbohydrate fermentation within host [14].
4. Conclusions

Fecal samples from Orangutan and Macaque is a valuable sources of LABs possessing probiotic properties. Six isolates namely OU1, OU2, OU5, MEP1, MEP3 and MEP5 have showed promising use as probiotics through antagonistic activity against gut pathogens and tolerance to acid and bile-salt with different degrees. Co-aggregation assay resulted in only one potential combination between OU5 and MEP5 to be assumed as effective probiotics later for concrete use. Therefore, we suggest future study regarding real time co-aggregation using human or animal cell lines for better understanding.

Acknowledgements

The authors would like to express highest gratitude to officials from Taman Hewan Pematang Siantar (THPS), Siantar, Sumatera Utara for research permit and ease of information exchange. The authors also would like to thank to Orangutan Information Center (OIC) for giving research funding for this study.

References

[1] Supriatna J and Wahyono E H 2000 Panduan Lapangan Primata Indonesia (Jakarta: Yayasan Obor Indonesia)
[2] Erdiansyah R, Agustina D and Jamin F 2014 Jurnal Medika Veterania 8 5
[3] Wahyuni T 1999 [Thesis] (Bogor: Institut Pertanian Bogor)
[4] Wich et al. 2011 Orangutan dan Ekonomi Pengelolaan Hutan Lestari di Sumatera (Jakarta: Barragraphia)
[5] Prasthani I, Padaga MC and Oktavianie DA 2010 [Tesis] (Malang: Universitas Brawijaya)
[6] Djide MN and Wahyuddin E 2008 Torani 18 211
[7] Kawai Y, Saito T, Uemura J and Itoh T 1997 Journal Biotechnology and Biochemistry 61 179
[8] Lin W, Hwang CF, Chen LW and Tsen HY 2006 Journal of Food Microbiology 23 74
[9] Vinderola CG and Reinheimer J A2003 Food Res Int 36 895
[10] Manin F 2010 Jurnal Ilmu-Ilmu Peternakan 13
[11] Pan X, Chen F, Wu T, Tang H and Zhao Z 2009 J. Food Control 20 598
[12] Chou LZ and Wemer B 1999 J Dairy Sci. 82 23
[13] Kimoto H, Kurisaki J, Tsuji NM, Ohmomo S and Okamoto T 1999 Appl Microbiol. 29 313
[14] Surono IS 2003 Asian Aust. Journal of Animal. Sci. 16 726