LACTIC ACID BACTERIAL SCREENING FROM GASTROINTESTINAL DIGESTIVE TRACT OF NATIVE AND BROILER CHICKEN FOR PROBIOTIC CANDIDATE PURPOSES

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ABSTRACT

The aim of his research was to obtain lactic acid bacteria (LAB) from gastrointestinal digestive tract (GIT) of chickens for probiotic candidate purposes. LAB was isolated from GIT of broiler and native chickens on selective medium (MRS+0.2% CaCO₃). Screening method based on microbiological and biochemical characteristics, antibacterial properties, growth on various temperature, aeration, and agitation, antibiotic sensitivity, and viability on acid pH, gastric juice and bile salt. Thirty nine of LAB isolates was selected from native chicken and 18 isolates from broiler chicken. The selected LAB inhibited Escherichia coli FNCC 0091 growth and grown on 30, 39 and 45°C of temperature, aerobic, anaerobic and agitation conditions. Biochemical identification using API 50 CHL kit revealed that I72 from native chicken ileum as Lactobacillus salivarius and Db9 from broiler chicken duodenum as Pediococcus pentosaceus. All LAB were resistant to Erythromycin, Penicillin G and Streptomycin. Based on characteristics, both the selected LAB have potentiality as chicken probiotic candidates.

Keywords : chicken, lactic acid bacteria, probiotic

INTRODUCTION

The history of the Indonesian poultry industry illustrates significant contributions to the national meat supply. Indonesian consumers preference for chicken meat creates a large domestic market (Helinna, 2001). Indonesian people consumption rates only 6.1 kg per capita per year, it is still lower than others leading countries (Bond et al., 2007). One of factor which
caused less production of chicken’s meat is the diseases problem (Patterson and Burgholder, 2003). Animal enteric pathogens are a direct source for food contamination. The prohibition of antibiotics as growth promoters (AGPs) use has been a challenge for animal nutrition therefore need to find alternative methods to control and prevent pathogenic bacterial colonization. The modulation of the gut microbiota with new feed additives such as probiotics against host-protecting functions to support animal health, is a topical issue in animal breeding and creates fascinating possibilities (Gaggia et al., 2010).

Currently, probiotics are used as health supplements in food and feeds and they are replacing the use of antibiotic growth promotors or chemical supplements (Kosin and Rakshit, 2006). Others, probiotic is a natural organic matter that could not leave the residual effect on animal product so it will not cause pathogen bacterial resistance effect. Fortunately, consumers are taking very serious attention on the food availability with the beneficial addition for their healthiness and also diseases prevention (de Lima and Filho, 2005).

Some of related research which contained lactic acid bacteria isolation and probiotic were L. reuteri, L. salivarius, or Lactobacillus spp use that could inhibited the pathogen bacterial such as Enterococcus faecalis, Enterococcus faecium, Listeria monocytogenes, and Salmonella spp. Some of Lactobacillus isolates could produce anti-microbe’s peptide or bacteriosin (Lima et al., 2007; Pilasombut et al., 2006). L. salivarius CTC2197 already know had inhibitory effect on S. enteritidis C-114 colonization by in vivo on GIT tract of chicken after single doses addition on feed mixture (Pascual et al., 1999).

The ideal requirements for probiotic agent purposes of microbes are animal host origin, non-pathogenic, withstand processing and storage, resist on gastric acid and bile, adhere to epithelium or mucus, persist in the intestinal tract, produce inhibitory compounds and modulate immune respns (Pattershon and Burgholder, 2003). The objectives of this research was to select LAB from native and broiler chicken which have ideal probiotic characteristics.

**MATERIALS AND METHODS**

**Lactic Acid Bacteria Isolation and Identification**

LAB isolated from chicken’s GIT tract of 5 month old of native chicken and 35 days old of broiler chicken (Cobb strain) using Torshizli et al. (2008) method. GIT sampling location was gizzard, duodenum, jejunum, ileum, and caecum. Samples were cutted, washed, and diluted in sterile peptone water (Oxoid) and made up to $10^5$ dilution. Each serials dilution was plated in de Man Rogosa Sharpe (MRS) Agar media (Oxoid) pH 6.2 then was added by 0.2% CaCO$_3$ (Merck) and incubated at 37°C for 24 h. The LAB colonies was detected by clearing zone appearance. LAB identification procedures consist of morphology, catalase, gas production, Gram staining, and motility tests. LAB isolates were maintained on microbank (Pro-lab) containing 15% glycerol.

**Antibacterial Activity Assay**

The selected LAB isolates grown on MRS Broth media at 37°C for 24 h. Cell free supernatant was obtained by centrifugation at 12,500 g for 20 min at 4°C. Supernatant was neutralized using 5 N NaOH (Merck), and sterilized using miliphore filter 0.20 µ. Antibacterial activities against E. coli FNCC 0091 in Nutrient Broth (NB) (Merck) medium were observed using turbidimetric method with incubation time for 48 h at 37°C. Sterile supernatants were mixed with double strength of NB about 1:1 (v/v) comparison and inoculated with 2% (v/v) of bacterial test. NB media without supernatant which had been inoculated with E. coli was used as a control. The optical density (OD) were observed at 0, 2, 4, 6, 8, 10, 12, 18, 24, 36 and 48 h incubation time using a spectrophotometer at $\lambda_{600 \text{ nm}}$ (Seeley et al., 2001).

**Optimization of the Growth Temperature and Condition**

A total of 1% (v/v) new cultures of the selected LAB inoculated on MRSB in Hungate tube then incubated at 30, 49 and 45°C, anerobic with CO$_2$ addition, aerobic without and aerobic with 100 rpm agitation. The control tube contained MRSB without LAB culture addition. The OD were measured using a spectrophotometer at $\lambda_{600 \text{ nm}}$ at 12 and 24 h incubation time.

**Biochemical Identification**

Biochemical identification of the selected LAB were observed by API 50 CHL kit (bioMērieux). The test procedure using the manual standard of API 50 CH kit. The
Bile Salt Tolerance

Bile salt tolerance was determined by modified method of Torshizi et al. (2008). A total of 1 ml LAB culture was centrifuged at 5000 g for 10 min at 4°C and washed in two times by using sterile PBS. The cells were diluted in 0.3 ml of PBS then mixed 0.2 ml of dilution and 1 ml PBS containing 0.3% (w/v) bile salt (Merck). The mixture was incubated at 37°C for 3 h and sampled after 0, 1 and 3 h. The cell viability was calculated using serial dilution and plated on MRS Agar media. Three replicates were used for each treatment.

Data Analysis

The quantitative data was analyzed by using One-way analysis of variance (ANOVA) with post hoc test (Duncan multiple F-test (P<0.05)) to distinguish the treatments means. The total of bacteria cell (cfu/ml) from viability test was converted to the logatimic value before statistically analysis.

RESULTS AND DISCUSSION

Lactic acid bacteria (LAB) which obtained from gastrointestinal digestive tract (GIT) were 39 isolates from native chicken and 18 isolates from broiler chicken. All LAB isolates had negative catalase, non gas production, non motile, Gram positive, rod and coccus shape characteristics. The LAB isolates characteristic and the optical density (OD) of E. coli on NB media containing extracellular metabolite of LAB isolates are shown in Table 1. The E. coli growth on media with extracellular metabolite of LAB isolates was lower than media without extracellular metabolite and they were significantly different (P<0.05) from control. Antibacterial activities of LAB isolates against E. coli was came from bacteriocins compound of metabolite extracellular during grow in media. Extracellular metabolite which have antibacterial activities such as antimicrobial peptide or bacteriosin of L. reuteri, L. salivarius, and Lactobacillus spp. were isolated from gizzard and caecum of poultry and inhibited Enterococcus, Listeria, and Salmonella (Lima et al., 2007). Alpha and beta bacteriosin Abp 118 produced by L. salivarius isolated from digestive tract of poultry and showed inhibition activities against B. coagulans ICM 2257T (Pilasombut et al., 2006). Bacteriocin from L. salivarius NRRL B-30514 could reduced Campilobacter jejuni population in digestive tracts of poultry (Stern et al., 2006). Lactobacillus sp. isolated from silage feed had antibacterial activities against E. coli and S. aureus (Damayanti et al., 2009). L. plantarum fed to broiler chicken showed therapeutec effect of bacteriocin against E. coli infection in broiler chickens (Ogunbanwo et al., 2004).

Inhibition mechanism of bacteriocin occured in two phases. First phase was bacteriocin absorption on specific and nonspecific receptor on target bacterial membrane cells. During this
phase, the bacteriocine became sensitive especially to proteolitic enzyme. Second phase was irreversible and involves lethal changes in the sensitive strains. The idea that bacteriocins act on the cell membrane has been well accepted (de Lima and Filho, 2005).

Based on data in Table 1, LAB isolates which had the highest inhibition against E. coli were Db9, D1, I72, Db2, Db5, Db1, D2, Ib1, T4 and D23, respectively. The differences of antibacterial activities in each isolates were based on the differences of LAB species and the ability to produce antibacterial compounds. Previous result from Torshizi et al. (2008) showed that P. pentosaceus TMU457 significantly had higher inhibition activity than L. fermentum TMU121 and L. rhamnosus TMU094 against E. coli and S. pullorum. Tatsadjieu et al. (2009) had reported that free cell supernatant from several strains of Lactobacillus had clear zone difference against E. coli.

Probiotic survival in agitation condition became essential factor because after entering the gastrointestinal of the host, the probiotic strains have to attach to the brush border of microvilli or adhere to the mucus layer to prevent sweep from the colon by peristalsis (Kim et al., 2007). All of LAB isolates had ability to growth at anaerobic and aerobic conditions whereas at aerobic condition with 100 rpm agitation, both I72 and Db9 isolates had higher growing ability than D1 and Db2 isolates. Differences in species level were effect on physiology and biochemical characteristic especially in growth optimum temperature which shown in Table 2. According to the growth curves of LAB during 24 h, the fourth of LAB isolates had the best growth at 39ºC. The lowest growth for I72, Db9 and D1 occurred at 45ºC, except for Db1 which occured at 30ºC.

Identification result by API 50 CHL kit are presented in Table 3. Two selected strains were identified as L. salivarius I72 and P. pentosaceus Db9. Both of LAB isolates had different ability to ferment carbohydrate, but they had similar ability to ferment monosaccharide such as glucose and fructose, and the other carbohydrate like N-acetylglucosamine and D-Trehalose. Several studies had found LAB isolate from digestive

| Isolates Code | Location       | Catalase Test | Motility | Gas Production | Gram Staining | Morphology | OD of E. coli |
|---------------|----------------|---------------|----------|----------------|---------------|------------|--------------|
| D1            | Duodenum of NC | -             | -        | +              | Coccus        | 0.508a     |
| D2            | Duodenum of NC | -             | -        | +              | Coccus        | 0.553b     |
| D23           | Duodenum of NC | -             | -        | +              | Coccus        | 0.642c     |
| I72           | Ileum of NC    | -             | -        | +              | Rod           | 0.546b     |
| T4            | Crop of NC    | -             | -        | +              | Rod           | 0.624c     |
| Db1           | Duodenum of BC | -             | -        | +              | Rod           | 0.550b     |
| Db2           | Duodenum of BC | -             | -        | +              | Rod           | 0.536b     |
| Db5           | Duodenum of BC | -             | -        | +              | Rod           | 0.546b     |
| Db9           | Duodenum of BC | -             | -        | +              | Coccus        | 0.465c     |
| Ib1           | Ileum of BC    | -             | -        | +              | Rod           | 0.556b     |
| K             | Control        | -             | -        | +              | Rod           | 0.694d     |

NC : Native chicken, BC : broiler chicken, OD : optical density at $\lambda_{660\text{ nm}}$

Means in the same column with different superscript indicates differ significantly $(P<0.05)$
Table 2. The Growth Parameter of Selected LAB in Different Temperature and Condition

| Isolate | Hours | Optical density (OD) λ<sub>600nm</sub> | Temperature (°C) | Condition |
|---------|-------|----------------------------------------|------------------|-----------|
|         |       |                                        | 30               | Agitation | Anaerobic | Aerobic |
|         |       |                                        | 39               |           |           |         |
|         |       |                                        | 45               |           |           |         |
| I72     | 12    | + + +                                 | + + + +         | + + +     | + + +     | + + +   |
|         | 24    | + + +                                 | + + + +         | + + + +    | + + +     | + + +   |
| D1      | 12    | + +                                   | + +             | +         | + + +     | +       |
|         | 24    | + + +                                 | + + + +         | + + +     | + + +     | + +     |
| DB1     | 12    | +                                    | + + + +         | + + +     | + + +     | + +     |
|         | 24    | +                                    | + + + +         | + + +     | + + +     | + +     |
| DB9     | 12    |                                      | + + + +         | + + +     | + + +     | + +     |
|         | 24    |                                      | + + + +         | + + +     | + + +     | + +     |

OD λ<sub>600nm</sub> = + : OD 0.5 – 1.0; + + : 1.0 – 1.5; + + + : 1.5 – 2.0; + + + + : >2

Table 3. Identification of LAB Isolates using API 50 CHL Kit

| No | Type of test | Db9 | I72 | No | Type of test | Db9 | I72 |
|----|--------------|-----|-----|----|--------------|-----|-----|
| 1  | Temoin       | -   | -   | 26 | Salicin      | +   | -   |
| 2  | Glycerol     | -   | -   | 27 | D-Cellibiose | -   | -   |
| 3  | Erythritol   | -   | -   | 28 | D-Maltose    | -   | +   |
| 4  | D-arabinose  | +   | -   | 29 | D-Lactose    | +   | -   |
| 5  | L-arabinose  | +   | -   | 30 | D-Melibiose  | -   | +   |
| 6  | D-ribose     | +   | -   | 31 | D-Sacharose  | -   | +   |
| 7  | D-xylose     | -   | -   | 32 | D-Trehalose  | +   | +   |
| 8  | L-xylose     | -   | -   | 33 | Inulin       | -   | -   |
| 9  | D-adonitel   | -   | -   | 34 | D-Melezitose | -   | -   |
| 10 | Methyl-βD-xylopyranoside | - | - | 35 | D-Raffinose | -   | +   |
| 11 | D-galactose  | -   | +   | 36 | Amidon       | -   | -   |
| 12 | D-glucose    | +   | +   | 37 | Glycogen     | -   | -   |
| 13 | D-fructose   | +   | +   | 38 | Xylitol      | -   | -   |
| 14 | D-mannose    | +   | -   | 39 | Gentibiose   | +   | -   |
| 15 | L-rhamnose   | -   | +   | 40 | D-Turanose   | -   | -   |
| 16 | Dulcitol     | -   | -   | 41 | D-Lyxose     | -   | -   |
| 17 | Inositol     | -   | -   | 42 | D-Tagatose   | +   | -   |
| 18 | D-mannitol   | -   | +   | 43 | D-Fucose     | -   | -   |
| 19 | D-sorbitol   | -   | -   | 44 | L-Fucose     | -   | -   |
| 20 | Methyl-αD-mannopyranoside | - | - | 45 | D-arabitol   | -   | +   |
| 21 | Methyl-αD-glucopyranoside | - | - | 46 | L-arabitol   | -   | -   |
| 22 | N-acetylglucosamine | + | + | 47 | Potassium gluconate | - | - |
| 23 | Amygdaline   | -   | -   | 48 | Potassium 2 ketogluconate | - | - |
| 24 | Arbutine     | +   | -   | 49 | Potassium 5 ketogluconate | - | - |
| 25 | Esculine     | +   | -   | 0  | Control      | -   | -   |

I72: *Lactobacillus salivarius* (99.9%); Db9:*Pediococcus pentosaceus* (85.1%)
Table 4. Antibiotic Sensitivity Test of LAB Isolates

| LAB isolates     | Erythromycin 15 µg (R) | Penicillin G 10 µg (R) | Streptomycin 10 µg (R) | Average (B) |
|------------------|------------------------|------------------------|------------------------|-------------|
| *P. pentosaceus* Db9 | 9.82                   | 3.50                   | 3.77                   | 5.69a(R)    |
| *L. salivarius* I72 | 11.83                  | 3.30                   | 4.15                   | 6.43a(R)    |
| Average (A)      | 10.83b(R)              | 3.40b(R)               | 3.96b(R)               |             |

R = resistance; Means in the same rows (A) and column (B) with different superscript indicate differ significantly (P<0.05)

The result of antibiotic sensitivity test showed that both of the selected strains had the same tolerance and not significantly different (P>0.05) to the other antibiotics (Table 4). Several poultry feeds contained some antibiotic in certain amount. The resistance characteristic of two LAB isolates to the broad spectrum of antibiotic (Erythromycin 15 µg), as well as Gram negative specific antibiotic (Penicillin G 10 µg) and Gram positive specific antibiotic (Streptomycin 10 µg) caused both of LAB isolates had possibility to survive in digestive tract of poultry which have exposed antibiotics. In a previous report, Torshizi et al. (2008) reported that all three selected LAB isolated from broiler chicken had some degree of antibiotic resistance against several of the tested antibiotic.

One of the essential characteristic of probiotic in order to give beneficial health for individual host was resistance to the effect of gastrointestinal environment such as acid and bile salt in digestive tract (Kosin and Rakshit, 2006). The result of pH acid, gastric juice and bile salt tolerance test of the two selected LAB isolates were shown on Table 5. This study showed that both of LAB isolates had ability to survive on pH 1, 2 and 3 after 1 hour incubation. Based on cell viability percentage, *L. salivarius* I72 and *P. pentosaceus* Db9 had the higher viability on the higher pH but not significantly different (P<0.05) to others. In the gastric juice tolerance test, both of LAB isolates also showed had viability after 1–2 hours of incubation. The cell viability of two LAB isolates were decreased at second hour of incubation but not significantly different to others (P<0.05). Similar to the result in gastric juice tolerance test, both of LAB had higher cell viability on 0.3% bile salt at one hour of incubation than three hours of incubation. However, both of LAB isolates were categorized had ability to survive in bile salt condition after 3 hours of incubation with 102.43-105.62% of cell viability.

LAB as an intestinal bacteria could experience a wide number of stresses in the intestinal tract including those caused by low pH and presence of bile. In this case, bile salt tolerances was thought to be an important aspect of survival for bacteria which inhabit the intestinal tract. Bile salt tolerance in intestinal lactobacilli associated with bile salt hydrolase (BSH) activity (O’Sullivan et al., 2009). BSH split the peptide linkage of bile acids, which results in removal of the amino acid group from the steroid core. The resulting unconjugated bile acids precipitate at low pH (Begley et al., 2006). On the basis of the results of molecular screening, both of selected LAB *L. salivarius* and *P. pentosaceus* had a genetic equipment for their survival at low pH (such as groEL gene for heat shock protein 60) and in the presence of bile salt (such as bsh gene for conjugated bile salt acid hydrolase) (Turpin et al., 2011). Based on the average of cell viability, both of the selected LAB had an equal viability on all treatments and had characteristic as probiotic bacteria.

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CONCLUSION

The selected LAB were *Lactobacillus salivarius* I72 found in ileum of native chicken and *Pediococcus pentosaceus* Db9 found in duodenum of broiler chicken. Both LAB isolates have antibacterial activities to *E. coli* FNCC 0091 and survive in anaerobic, aerobic and agitation conditions. They were also resistant to Erithromycin, Penicillin G and Streptomycin antibiotics. In generally, both of LAB isolates had tolerance in low pH (1, 2, and 3), gastric juice pH 2 and bile salt 0.3%. Based on the essential characteristics, it was concluded that *L. salivarius* I72 and *P. pentosaceus* Db9 were potential as chicken probiotic candidates.

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Table 5. The Cell Viability of Two LAB Isolates on Acid pH, Gastric Acid and Bile Salt

| LAB isolates          | Acid pH after 1 h | 3g/L Gastric Juice | 0.3% Bile Salt |
|-----------------------|-------------------|--------------------|---------------|
|                       | pH 1 | pH 2 | pH 3 | 1 h | 2 h | 1 h | 3 h |               |
| *P. pentosaceus* Db9  | 92.91| 100.5| 106.13| 86.62| 81.76| 99.12| 94.37| 94.48a        |
| *L. salivarius* I72  | 69.93| 85.97| 98.74| 89.81| 75.66| 112.12| 110.24| 91.78a        |
| Average (A)           | 81.42ab| 93.23ab| 102.43ab| 88.21ab| 78.71b| 105.62a| 102.43ab|               |

Means in the same rows (A) and column (B) with different superscript differ significantly (P<0.05)
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