Isolation And Characterization Of Lactic Acid Bacteria (Lab) From Small Intestine Content Of Duck (Anas Sp.) As A Probiotic Candidate

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Abstract. A group of lactic acid bacteria (LAB) as a probiotic agent have been known to have a beneficial effect on health chicken. The objectives of this study were to isolate, characterize and determine of the LAB which originated from the small intestine content of domestic duck (Anas sp.) for candidate of probiotic.

Isolation, selection and identification of bacteria was done using de Man Rogosa Sharpe (MRS) agar + 1% bromocresol purple. Characterization of bacteria based on morphological by Gram staining methods, and identification of biochemical characters using catalase activity, and Triple Sugar Iron Agar (TSIA). The data of isolate confirmed to Bergey’s Manual of Systematic Bacteriology. This research carried out on the Microbiology Laboratory, Microbiology Study Program, Yala Radjabhat University (YRU), Faculty of Science and Agriculture.

The results showed that according to physiological and biochemical characteristics in various media, the bacteria had Gram +, catalase -, coccus in shape, and were able to ferment glucose to be lactic acid. Based on the result, it is concluded that the LAB isolate shows a similar characteristic to the member of genus Lactobacillus sp. The isolated bacteria shows characteristic resistance to acidic pH and bile salts so the bacteria were fulfilled as a probiotic candidate.

Keywords: Isolation of LAB, duck (Anas sp.), probiotic candidate

1. Introduction

Naturally there are enormous bacteria which live in various environments such as soil, stale food, water, and even in the intestine pathways in humans or animals. They are many types of bacteria that can produce lactic acid, antimicrobials and other metabolic results that have a positive effect on the host. One type of the useful bacteria is lactic acid bacteria (LAB). They can be used as a probiotics which can improve lactose digestibility, control pathogenic bacteria in the digestive tract, produce antibacterial and inactivation of various toxic compounds. Åsa Ljungh & Torkel Wadström (2004) said that a number of Lactobacillus species, Bifidobacterium sp, Saccharomyces boulardii, and some other microbes have been proposed as and used as probiotic. Probiotics are defined as microbial dietary adjuvants that beneficially affect the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract [23]. The concept of probiotics has been defined by Fuller [9] which means "food supplement in the form of microbial life that has beneficial effects for the host animal by improving intestinal microbial balance". Several modes of probiotic action have
been proposed in the literature such as controlling the pathogenic bacteria, modulating immune responses, competing for adhesion receptors in the gut epithelium with toxin-producing bacteria [4, 5] and altering metabolism by increasing digestive enzyme activity [7]. Most of probiotic microorganisms belong to Lactic Acid Bacteria (LAB), such as Lactobacillus sp, Bifidobacterium sp and Enterococcus sp (Klein et al., 1998) [8]. Gabriela Perdigon et al., (2001) said that Lactic acid bacteria (LAB) are present in the intestine of most animals. Herich, and Levkut (2002), said that probiotic microorganisms including lactic acid bacteria (LAB) positively influence the composition of the gut microflora, they stimulate the production of secretory IgA, they affect the targeted transportation of the luminal antigens to Peyer’s patches and they increase the production of IFN-γ.

Lactic acid bacteria in this study isolate from the intestine contents of ducks (Anas sp.), because ducks are more resistant to poultry diseases. These scientific evidences have been a motivating factor to choose a domestic duck (Anas sp) which could further confirm the results of this study. The objectives of this research was to determine the LAB to isolate, characterize and determine of the LAB which originated from the small intestine content of domestic duck (Anas sp) for candidate of probiotic.

2. Research Methods
This research design was to isolate, characterize, and further select the most suitable strain of LAB from small intestine contents of local domestic duck (Anas sp). Preparation of isolation, selection, and characterization of LAB was carried out in the Microbiology Laboratory, Microbiology Study Program, Yala Rajabhat University Faculty of Science and Agriculture.

Materials and Chemicals: The main materials used in this study were duck intestinum. All chemicals used in this study were of analytical grade, mostly purchased from Sigma Chemical Co. (St. Louis, MO). De Man, Rogosa, and Sharpe (MRS) and nutrient broth media were purchased from Merck (Darmstadt, Germany) and Oxoid Ltd. (Basingstoke, UK), Bromocresol Purple, Paint Gram Kit, HCl, NaOH, NaCl, Bile Salt.

Methods to Isolation of Bacterial Strains
The sample used in this research is taken from the contents of the duck intestine (Anas sp) aged 8 week, body weigh 1 kg, and purchased from a local farm ducks are taken from a farm in Pattani Province, Yala, Thailand. The section of 10-cm yeyunum, ileum and caecum of each ducks was removed and aseptically minced with contents. The resulting homogenates were serially diluted in phosphate buffer (pH 6) and plated onto de Man Rogosa Sharpe (MRS) agar + Bromocresol purple (BP) and then incubated in anaerobic jars at 37°C for 48 hours. Based on the halo zone surrounding each colony appearance as a lactic acid bacteria.

Gram Staining
The isolates were characterized by standardized methods using Gram staining. One batch of lactic acid bacteria is taken and mixed with distilled water. The mixture was flattened so that the bacterial cell is not too dense, and is passed over the fire to dry. Violet crystals are added above the isolate, wait 1 minute then rinse with running water. Iodine was added to the isolate, awaited 1 minute and then rinsed with alcohol. Safranin coloring is added, awaited 30 seconds then rinsed with running water.

Screening for Catalase Activity
Methods for screening a number of bacteria as a include catalase activity was measured by using hydrogen peroxide (H2O2).

Screening for Fermentation Activity
The assay of the bacteria by fermentation and lactic acid test to know homo-fermentative bacteria or heterofermentative bacteria. Finally the lactic acid assays was done to all bacteria by measuring the lactic acid production. Lactic acid production activities of the selected bacteria were examined. One strains obtained from avian intestine origin was indentified.
Screening for TSIA Fermentation Activity
Profile of *Lactobacillus* were identified based on biochemical characters using Triple Sugar Iron Agar (TSIA). For fermentation activity, all strains were subcultured on modified MRS (MRS with 0.25% starch instead of whole glucose) broth.

Screening for Candidate of Probiotic
For screening probiotic activity, all strains were subcultured on modified MRS broth. For detection of acid activity, the strains were cultured on MRS broth and after anaerobic incubation for 24 h at 37°C, 30 μL of culture supernatant was transferred onto a disc placed over a medium consisting of HCl (1.5%).

For screening probiotic for bile salt activity, 1 ose of bacteria was isolated into a tube containing 5 ml MRS + Bile Salt 0.3; 0.5; 1. then isolated into an incubator at 37°C for 20 hours. Isolates that are able to grow in bile salt conditions can be seen from the turbidity of the media and the presence of deposits and then 1 ml of isolates after 20 hours incubation was isolated into 10 ml MRS + Bile Salt then 1 ml of isolates were tested for OD in a spectrophotometer each stages are used for measuring 0 hours, and tests for the 1st, 2nd and 3rd hours are made.

Inhibition test with *S. aureus* and *E. Coli*
A total of 100 μl of isolates were isolated into 5 ml MRSB. Isolates were incubated at an incubator at 37°C for 24 hours. The incubation results were centrifuged at 6000 rpm for 15 minutes. Then the suspension of pathogenic bacteria was made i.e, *S. aureus* and *E. coli* were isolated into 10 ml of 0.85% NaCl solution and matched with Mc Farland index 0.5. After that the pathogenic bacterial suspension was isolated on the MHA media by using cotton swabs to cover the entire surface of the MHA media. Holes with 1 cm diameter are made on MHA media. Then 100 μl of the supernatant from the centrifuge was isolated at the hole in the MHA media. Incubated in an incubator at 37°C for 1x24 hours.

Data Analysis
The data of research were statistically analyzed by one-way analysis of variant (ANOVA) using procedure of statistical analysis software (SAS). When significant difference present then data analysis continuously by *Duncan Multiple Range Test* (DMRT) was used for comparison for each group. A value of *p*<0.05 was considered statistically significant.

3. Results And Discussion
   A. Results
   1. Isolation of Lactic Acid Bacteria (LAB)
   Isolation of Lactic Acid Bacteria (LAB) from the small intestine contents of the Ducks (*Anas sp*) using De Man, Rogosa, and Sharpe agar (MRSA) media contains Bromocresol Purple (BP) and spread plate method was obtained some colonies of LAB are shown in the table 1 as follow:

   Table 1: Isolated colonies of LAB

   | Source               | Type of Incubation | Sample | Dilution | Colony | Subculture |
   |----------------------|--------------------|--------|----------|--------|------------|
   | Duck Intestine       | Aerobic (A)        | 3      | 10 (-6)  | 21     | 5          |
   |                      | Anaerobic (N)      | 3      | 10 (-7)  | (-)    | (-)        |
   | Total Colony Count   |                    |        |          | 24     | 8          |

   Notes: (-): Not growing, A: Aerobic Isolate, N: Anaerobic Isolates.
Isolation results have obtained 8 colonies of LAB based on the presence or absence of clear zones or discoloration in the MRSA media contains Bromocresol Purple (BP) its means that bacteria can produce acid or can make the environmental conditions acidic. Lactic acid bacteria (LAB) is a group of bacteria that produce lactic acid as one of the main fermentation products of carbohydrate metabolism [7].

2. Morphological Characterization of LAB
All isolates that had been subcultured were 8 isolates then morphological characterization of the colonies. Observations result on colony morphology include shape, edges, elevation and color of colonies are shown in the table 2 as follow:

| Karakteristik BAL | Kode Isolat Bakteri Asam Laktat |
|-------------------|----------------------------------|
|                   | A1 | A2 | A3 | A4 | A5 | N1 | N2 | N3 |
| Warna             | Putih | Putih | Putih | Putih | Putih | Putih | Putih | Putih |
| Configuration     | Round | Round | Round | Round | Round | Round | Round | Irregular |
| Margin            | Entire | Entire | Entire | Entire | Entire | Entire | Entire | Lobate |
| Elevation         | Flat | Flat | Flat | Flat | Flat | Flat | Covex | Flat |
| Morphologi Gram   | Gram + | Gram + | Gram + | Gram + | Gram + | Gram + | Gram + | Gram + |
| Sel Bentuk        | Coccus | Coccus | Coccus | Coccus | Coccus | Coccus | Bacil | Bacil |

Colonies obtained were white, had a round configuration and only N3 isolates had irregular configurations, the shape of the entire colony edge except for N3 lobate isolates and mostly flat elevations and only N2 isolates had convex elevation.

All subculture isolates were then characterized by Gram staining. Lactic Acid Bacteria generally have gram-positive characteristics (Seely et al., 2001: 145-146). As a result, all 8 isolates of LAB obtained from the duck intestine (Anas sp.) were gram-positive bacteria.

Figure 1. a) LAB Gram positif, bacillus, b) Gram positif, coccus

Gram-positive bacteria have a special element, namely teichoic acid as much as 50% of the dry weight of cell walls. This element has a function to maintain ion transport, cell wall integrity and others so that it is resistant to autolysis and maintains external permeability. This ability is the basis for choosing gram-positive bacteria as probiotics because morphology and biochemistry are better able to survive in the digestive tract. All isolates obtained showed gram positive so that they could be used as probiotics.
3. Biochemical Test of Isolates of Lactic Acid Bacteria

Catalase test

The results of the catalase test on 8 bacterial isolates showed a negative result indicated by the absence of gas bubbles containing oxygen when the isolates were dripped with H2O2 solution. This is in accordance with the research of Stamer et al., (1979) which said that lactic acid bacteria including bacteria with negative catalase. Lactic acid bacteria generally have negative catalase characteristics (Seely et al., 2001).

Temperature Resistance

From the results of the temperature resistance test, all LAB isolates from the duck intestine are a type of mesophilic bacteria. This can be seen in table 3, where there are no isolates which can grow in temperatures of 45°C and 50°C. These results are in accordance with Anastiawan (2014: 60) Lactic acid bacteria isolated from the intestines of ducks can grow at a temperature of 15°C, 37°C, and 45°C. But growth looks better or maximum at 37°C.

| Temp  | Code of Isolates |
|-------|------------------|
|       | A1   | A2   | A3   | A4   | A5   | N1   | N2   | N3   |
| 20°C  | ✓    | ✓    | ✓    | ✓    | ✓    | -    | -    | -    |
| 30°C  | ✓    | ✓    | ✓    | ✓    | ✓    | -    | -    | -    |
| 37°C  | ✓    | ✓    | ✓    | ✓    | ✓    | ✓    | ✓    | ✓    |
| 45°C  | -    | -    | -    | -    | -    | -    | -    | -    |
| 50°C  | -    | -    | -    | -    | -    | -    | -    | -    |

Notes:
✓ = growth
- = not growth

PH conditions

The pH test results in Broth MRS can be seen from the turbidity of the medium and the presence of deposits. The pH test results are in table 4 as follows:

| Ph    | Kode |
|-------|------|
|       | A1   | A2   | A3   | A4   | A5   | N1   | N2   | N3   |
| 3     | +    | +    | +    | +    | ++   | +    | ++   | ++   |
| 3.5   | +    | +    | +    | +    | ++   | +    | +    | +    |
| 4     | +    | +    | +    | +    | +    | -    | -    | +    |
| 5     | +++  | +++  | +++  | +++  | +++  | -    | -    | +    |
| 7     | +++  | +++  | +++  | +++  | +++  | ++   | ++   |
| 9     | ++   | ++   | ++   | ++   | ++   | -    | -    | -    |

Notes:
+ = little
++ = bit little
+++ = many sediments

The pH test results (Fig 5) showed that 8 isolates were able to survive at low pH conditions of 3 and 3.5. But the most effective growth is at a normal pH between 6-7. Resistance to low pH is one of the main characteristics for probiotic bacteria (Saarela et al., 2000).
value in the digestive tract of poultry in each part is: cached (4.5), proventriculus (4.4),
gizzard (2.6), duodenum (5.7- 6.0), jejunum (5.8), ileum (6.3), colon (6.3), ceca (5.7), and
bile (5.9) (Sun, 2004).

NaCl Test
Digestive conditions of chickens have different NaCl content, depending on the content of
NaCl contained in rations or chicken feed. NaCl (3, 4, 6.5, 10). The results of the NaCl test
in MRS Broth media with isolation for 2x24 hours in 37°C temperatures are found in table 5
below:

| Table 5. Result of LAB in various concentration of NaCl |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| NaCl   | Kode  |
| +     | A1    | A2    | A3    | A4    | A5    | N1    | N2    |
| 3      | +     | +     | +     | +     | ++    | +     | +     |
| 4      | +     | +     | +     | +     | +     | +     | +     |
| 6.5    | +     | +     | +     | +     | +     | +     | ++    |
| 10     | +     | +     | +     | +     | +     | +     | +     |
| K      | +++   | +++   | +++   | +++   | +++   | +++   | +++   |

Notes:
+ = little
++ = bit little
+++ = many sediments

From the results of the test of resistance to NaCl the results are positive if turbidity and the
presence of sediment arise. All lactic acid bacterial isolates from duck intestines (Anas sp)
(Fig 6) can still survive in NaCl levels from 3% to 10%. LAB will tolerate high salt levels to
start the process of metabolism to produce acids that can inhibit the growth of undesirable
bacteria.

According to Axelsson (2004), LAB which can live at a salt concentration of 6.5% if it is
rod-shaped is included in the genus Lactobacillus. Certain lactic acid bacteria such as
_Pediococcus acidilactici_ able to grow at 5-10% NaCl levels (Romadhon, _et al._, 2012: 5).
According to Buckle _et al._, (1987), in the fermentation process of fish using high salinity, it
is estimated that the type of LAB that is able to grow and develop is from the genera
_Lactobacillus sp, Pediococcus sp, and Leuconostoc sp._

a. Tolerance to Bile Salt

The results of the preliminary test of resistance in the various concentration of bile salt on
MRS Broth media for 24 hours in temperatures 37°C are found in table 6 below:

| Table 6. Result of Bile Salt Test |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Bile Salt | Kode  |
| +     | A1    | A2    | A3    | A4    | A5    | N1    | N2    |
| 0.3    | +     | +     | ++    | +     | +++   | -     | +++   |
| 0.5    | +     | +     | ++    | +     | +++   | -     | +++   |
| 1      | +     | +     | ++    | +     | +++   | -     | +++   |
| K      | +++   | +++   | +++   | +++   | +++   | +++   | +++   |
Notes:
+ = little
++ = bit little
+++ = many sediments

From the results of the preliminary test all isolates had 3 isolates which showed that the isolate was able to continue to grow effectively in 0.1 to 1% bile salt content (Figure 7) which was seen from the change in color of the MRS medium broth to become cloudy and there were many deposits. The isolates are isolates A1, N2, and N3. Bezkorovainy (2001: 401) states that bile in the small intestine inhibits the growth of existing microbes, therefore LAB, especially Streptococcus which will be used as a probiotic must be able to survive against bile salts in order to live and perform its role when in the chicken intestine. The concentration of bile salts in the digestive tract averages around 0.3%, and can range up to an extreme of 2.0% during the first hours of digestion (Gotcheva et al., 2002).

The three isolates were further tested by conducting a test using the plate count agar method. To ascertain whether within the first 3 hours after isolation in the MRS broth media added Bile Salt isolates still grow and live also to count the number of bacterial colonies. Further spectro test results of the 3 selected LAB isolates can be seen in table 7 as follows:

| Code | hour ke- | Bile salt (%) | K   | 0.3  | 0.5  | 1   |
|------|---------|---------------|-----|------|------|-----|
| A5   | 0       | 0.291         | 0.240| 0.197| 0.183|
|      | 1       | 0.495         | 0.462| 0.322| 0.162|
|      | 2       | 0.669         | 0.665| 0.552| 0.131|
|      | 3       | 0.130         | 0.129| 0.112| 0.103|
| N2   | 0       | 0.243         | 0.232| 0.197| 0.207|
|      | 1       | 0.824         | 0.668| 0.815| 0.706|
|      | 2       | 0.266         | 0.252| 0.271| 0.325|
|      | 3       | 0.290         | 0.255| 0.314| 0.365|
| N3   | 0       | 0.087         | 0.111| 0.072| 0.124|
|      | 1       | 0.082         | 0.414| 0.136| 0.518|
|      | 2       | 0.090         | 0.137| 0.431| 0.173|
|      | 3       | 0.154         | 0.179| 0.142| 0.174|

According to Siti Umniyati (2009: 171) Lactic acid bacteria genus Streptococcus when tested in a concentration of bile salt (bile salt) 0% to 1% for 20 hours still able to grow and live. Where in the growth phase there is a slow phase that occurs at the 0th hour to the 4th hour because the initial hours of incubation density have not increased significantly (P> 0.05). Density starts to increase significantly (P <0.05) from the 5th to the 11th hour, this phase is then called the exponential phase. The next phase is the stationary phase which lasts from the 12th hour to the 20th hour (end of observation). This can be proven in the results of further tests on 3 selected isolates (Table 7) where the spectro results show that until the third hour there is still growth of lactic acid bacteria. This growth does not increase significantly according to the statement of Siti Umniyati (2009: 171) where in the 3rd hour it is still included in the slow phase so that growth does not increase significantly. In addition, colony
counts are also carried out using the plate count agar method. The three isolates selected were isolated in 10ml MRS broth + Bile Salt media and isolated for 3 hours. 1ml is taken every 1 hour to see the OD values in the spectrophotometer and 1ml taken for serial dilution to 10-3. A total of 0.1 ml of 10-3 dilution results were isolated in Nutrient Agar medium with 2x repetitions and isolated for 1x24 hours at 37°C. Calculation of the number of colonies needs to be done because the results of the OD values in the spectrophotometer only show the turbidity level of the MRS broth media, without knowing the condition of the bacteria in the media is still alive or not. From the calculation of the number of colonies 3 isolates in all levels of bile salt cannot count the number of colonies, because the colonies that grow in all plates show a number of 300 < (Fig. 8). But these results still show that 3 selected LAB isolates are probiotic candidates because they can survive and grow in 1% bile salt content.

### Anti Pathogenic Bacteria

**Table 8. Diameter inhibition of pathogenic bacteria S. aureus and E. coli**

| Patogens | Sample | Code  |
|----------|--------|-------|
|          |        | A5    | N2  | N3  |
| S. aureus| 1      | 0.605 | 0.205 | 0.025 |
|          | 2      | 0.495 | 0.015 | 0.02  |
|          | 3      | 0.235 | 0.01  | 0.025 |
| average  |        | 0.445 | 0.076 | 0.023 |
| E. coli  | 1      | 0.434 | 0.105 | 0.05  |
|          | 2      | 0.38  | 0.075 | 0.015 |
|          | 3      | 0.33  | 0.115 | 0     |
| average  |        | 0.38  | 0.098 | 0.022 |

These isolates showed resistance to stomach pH (pH 3.0), tolerance against 0.3% bile concentration. Three of the isolates were observed as potential probiotic. Two of them are bacilli and the other is cocci. Two lactobacilli were identified as *Lactobacillus sp.* In the light of this study, it is observed that, small intestine of duck are a source of potential probiotic strains.

### 4. Conclusion

The results of this study suggest that the isolated bacteria have been isolated from small intestine of domestic duck (*Anas sp.*) having the character of Gram positive, catalase negative, rod form, respectively, and therefore the bacteria are genus *Lactobacillus spp.* Based on the result, that the bacteria could survive on the acidic pH and bile salts is prerequisite for choosing as a probiotic candidate.

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