Isolation and selection of proteolytic lactic acid bacteria from colostrum of dairy cattle

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Abstract. Lactic Acid Bacteria (LAB) are bacteria that produce lactic acid and are very useful bacteria, one of which can act as a probiotic because it can produce bacteriocins and produce peptide compounds with high biological activity. Colostrum is the first fluid that comes out of the udder of the mother cattle after giving birth which is very good for newborns because it contains antibodies. This research aimed to isolate, selection and identify the types of LAB found in dairy cow colostrum, especially LAB which have superior proteolytic activity. LAB obtained is expected to have a positive effect on livestock productivity. This research was conducted In-vitro in a laboratory. The research procedure included the isolation of LAB from the colostrum of dairy cows, then the selection procedure by looking at the best protease enzyme activity qualitatively and quantitatively, and testing for resistance to low pH. Lactic acid isolation, selection, and identification were analyzed using descriptive analysis and quantitative analysis using one-way ANOVA for a completely randomized design. The results obtained 6 superior proteolytic LAB isolates based on the protease enzyme activity coded LAB pro 4, LAB pro 3, LAB pro 8, LAB pro 9, LAB pro 21, and LAB pro 20.

1. Introduction
Lactic acid bacteria (LAB) are bacteria that produce lactic acid. Lactic acid bacteria can be obtained through a process of isolation, selection, and identification, one of which comes from colostrum. Colostrum is a non-milk liquid that first comes out of the udder of cattle after the calf give birth. Colostrum is the milk liquid that comes out of its mother’s udder for the first time, at least up to three days after the animal gives birth [1]. Colostrum is very good for newborn livestock because it contains antibodies, especially immunoglobulin G and M. LAB obtained from colostrum, which is expected to have a positive effect on livestock immunity. Dairy cow colostrum is a yellow liquid released by lactating mother cows after giving birth for about 24 to 168 hours. Dairy cow colostrum (Bovine colostrum) contains active substances for immunity such as immunoglobulin and antimicrobial substances such as lactoferrin, lactoperoxide and lysozyme, as well as vitamins and minerals, contains little fat, and contains microbes [2].

One of the advantages of LAB is that besides being able to act as a probiotic because it is able to produce bacteriocins (a ribosomal protein), it can also produce peptide compounds with high biological activity both as antioxidants and as anti-bacteria. This can occur because some LAB is proteolytic bacteria. LAB proteolytic is a bacteria capable of secreting the protease enzyme. This means that the bacteria are able to produce and degrade complex proteins into simple compounds, both peptides and amino acids, which are then readily absorbed in the body. Naiola and Widhyastuti [3]
stated that protease is an enzyme that can hydrolyze protein into simpler compounds. Based on how to cut the bond, the protease enzyme can be divided into exopeptidase and endopeptidase.

The use of protease enzymes in various commercial products is increasingly widespread in line with advances in biotechnology. Protease is used in the industrial sectors, including in foods and foods processing, leather tanning, detergents, and liquid waste processing. In Indonesia, the need for protease enzymes is also increasing, but this need only depends on imported products. One way to anticipate dependence on imports requires efforts to produce protease enzymes. Protease enzymes produced by microbes have many advantages over synthetic proteases. Fuad et al. (2004) explained that the use of microorganisms for enzyme production through isolation, selection, and identification to purification has several advantages, including easy production on a large scale, relatively short production time and can be produced sustainably at a relatively low cost [4]. Protease-producing microorganisms can be bacteria, mold, or yeast. The current search or identification process with the rapid development of technology means that the identification of bacteria can be carried out on a molecular basis using the 16s rRNA gene. The research used dairy cow colostrum as a source of LAB microbes so that it is expected to be able to obtain superior proteolytic lactic acid bacteria isolates, and when applied for preservation and quality improvement of protein source feed ingredients can use simple technology, at low cost and can be scaled-up to produce more products.

2. Materials and methods

2.1. Tools and materials
The tools used in this research includes beaker glass, spatula, analytical scale, Petri dish, incubator (oven), water bath, hot pan, and magnetic stirrer, pH meter, measuring pipette, micropipette, blue tip, yellow tip, thermometer, refrigeration, vortex, autoclave, Laminar Air Flow (LAF), measuring cup, test tube, Erlenmeyer, bunsen, matches, tube rack, calipers, ring loop, Eppendorf, well diffusion molding equipment, filter paper Whatmann No. 1, aluminum foil, cotton, label paper, tissue, and spectrophotometer and other tools used in the isolation process to identification using the ribosomal gene 16s rRNA of lactic acid bacteria.

The materials used in this study include dairy cow colostrum obtained from dairy cattle in the Technical Implementation Unit of the Faculty of Animal Science UGM, MRS media (deMan Rogosa Sharpe), Agar (Americano Bacteriological), SMA media (Skim Milk Agar), pepsin, casein, buffer glycine-NaOH, Trichloroacetic acid (TCA), tyrosine, CaCO3, 70% alcohol, and distilled water and other materials used in the isolation process to identification using the ribosomal gene 16s rRNA of lactic acid bacteria.
2.2. *Isolat source enrichment*

The samples obtained were then taken to the Laboratory of Nutritional Biochemistry, Faculty of Animal Husbandry, Gadjah Mada University and prepared and enriched by growing on MRS broth medium. The MRS broth medium was made by weighing 2.55 g of MRS broth media and then put it in an Erlenmeyer. A total of 50 ml of distilled water as a solvent is added to the Erlenmeyer. The mixture was then homogenized with a magnetic stirrer at 120°C to boiling and was repeated 3 times so that the media was completely homogeneous. The homogeneous medium was then autoclaved. After the medium preparation is complete, then the medium obtained is added with a sample or source of bacteria, i.e., colostrum as much as 10% of the volume of the medium. This process is carried out in Laminar Air Flow (LAF) and takes into account the aseptic method. Erlenmeyer which has been filled with a medium and a source of bacteria is then covered with cotton and aluminum foil and incubated in an oven at 37°C for 24 hours.

2.3. *Isolation and purification of lactic acid bacteria*

LAB isolation was carried out using the *pour plate method* which referred to the research of Yusmarini et al [5] with a slight modification, starting with a serial dilution of the sample [5]. The multilevel dilution technique was carried out by taking 1 ml of colostrum bacterial enrichment sample as a source of LAB and put it in a tube containing 9 ml of sterile distilled water (tube no. 1) and homogenized. This means that the sample has been diluted 10<sup>1</sup>. The next step is to take 1 ml from tube No. 1 and put it in a tube containing 9 ml of sterile distilled water (tube no 2) and homogenize it well, this means that the sample has received a 10<sup>2</sup> dilution. The dilution step is carried out (as above) until the dilution is 10 times. The dilution process is carried out in laminar air flow by observing the aseptic method.

The next activity from each series of multilevel dilutions was taken as much as 1 mL and inoculated on the MRS medium that had been added with 0.5% CaCO<sub>3</sub>. Incubation is carried out in an oven at 37°C for 1 to 2 days. The bacterial colony used is in the 10<sup>5</sup> dilution serial Petri dish because in the Petri dish the growing colonies are clearly visible between lactic acid and non-lactic acid bacteria based on the clear zone and this can also be based on the growing colony requirements that have been described by Ulfiana et al (2012) between 25 and 250 in ALT/TPC calculations [6]. Isolates that grew and formed a clear zone on MRS agar and had different appearances were isolated and purified by means of a *spread plate method* on the same medium. The treatments were carried out and repeated 3 times in order to obtain pure isolates.

2.4. *Proteolytic LAB isolation and selection*

The previously obtained lactic acid bacteria (LAB) were then isolated and selected to obtain LAB which had proteolytic properties. Activities carried out refer to the Sugireng method (2016) with the pour method using a selective medium, namely SMA (Skim Milk Agar) [7]. SMA medium was made with a composition of 2% skim milk (Lactona) and 2% agar, then the bacteria were inoculated and incubated at 37°C for 24 to 48 hours. After the incubation period, an indication that the growing bacteria is proteolytic bacteria can be seen through isolates that grow well and have a clear zone around the colony. The proteolytic isolates obtained were purified by re-isolation to ensure the purity of the culture. Partially pure cultures are stored as stock and used for testing the protease enzyme activity both qualitatively and quantitatively.

2.5. *Qualitative proteolytic activity test*

Qualitative proteolytic activity or protease activity can be tested using the method of Afriani et al. [8] with a slight modification that is almost similar to the pathogenicity or inhibitory test method. The qualitative protease activity test was carried out by growing bacterial isolates on a 2% SMA (Skim Milk Agar) medium using the well diffusion method. First, SMA media was made which was then poured into each Petri dish. When the medium has become clotted or solid, then each Petri dish is perforated with a 7 mm diameter well molding tool as many as 4 parts. In each part of the well-formed, 50 µL of enzyme-producing bacteria were inoculated, positive control and negative control.
These activities are carried out in LAF and pay attention to aseptic methods. Then incubated for 24 to 48 hours at 37°C. A clear zone formed around the bacterial colony indicates a positive result producing the protease enzyme. The proteolytic activity and proteolytic index (IP) can be determined by the following formula.

Proteolytic activity (mm) = diameter clear zone – diameter colony bacteria

Proteolytic index (IP) = \frac{diameter clear zone – diameter colony bacteria}{diameter colony bacteria}

2.6. Quantitative proteolytic activity test

The quantitative proteolytic activity test was carried out by calculating the product resulting from the enzymatic activity then the results obtained would be entered into the standard tyrosine curve. The process stages carried out include the enzyme production process and the testing process itself. The enzyme production process is carried out by growing proteolytic LAB in a liquid MRS medium until it has an age of 12 hours. The 12-hour old bacteria were then transferred in a 1.5 mL microtube (Eppendorf). The process is carried out aseptically. The bacteria that have been transferred in the Eppendorf are then subjected to a centrifugation process at a speed of 10,000 rpm for 15 minutes at a cold temperature of 4°C. The supernatant produced from the centrifugation process (crude enzyme) is used as a sample or test material for quantitative prosthesis activity.

The next process is to test the protease enzyme activity by measuring the activity of the enzyme in terms of its action to degrade casein substrates. The process carried out includes the test material, among others, 1 mL of 2.5% casein is needed then added with 1.9 mL of glycine-sodium hydroxide buffer (0.1 M, pH 9.5) and 0.1 mL of enzyme source. was homogenized using a vortex. The mixture was then incubated at 42°C for 30 minutes. At the end of the incubation period, the reaction was stopped with the addition of 2 mL of 5% trichloroacetic acid (TCA). Then the empty enzyme tube is also given the addition of the TCA. The tube was left to remain at room temperature for 30 minutes, and the solution was filtered using Whatman filter paper no. 1. The last process that is carried out is the reading using a UV-Vis spectrophotometer with a wavelength of 280 nm. The results obtained were then entered and compared with the tyrosine standard. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 mg of tyrosine under test conditions [9].

2.7. Low pH resistance test

The low pH resistance test is carried out by inoculating bacteria in the MRS broth medium whose pH conditions have been adjusted in such a way that it has different conditions, namely (pH control of 6.5), pH 2, 3 and 4. This pH adjustment is carried out using a pH meter and the reagent material was 0.1 N HCl and 0.1 N NaOH. Bacterial culture was inoculated as much as 1% on the medium and incubated at 37°C. Observations were made for 3–5 hours at a wavelength of 600-660 nm. If the bacteria grows and the absorbance value (A) is <0.1 then the bacterial strain is considered not resistant to low pH, and if A≥0.1 then the LAB strain is resistant to low pH [10].

3. Results and discussion

3.1. Isolate source enrichment

Lactic Acid Bacteria (LAB) are included in good bacteria which are believed to be able to provide benefits and good (beneficial) effects for humans and livestock productivity. LAB has been known for its use in several fermentation processes and can be isolated from various sources, one of which is milk and milk products. Indriati (2010) states that lactic acid bacteria (LAB) can be isolated from milk because basically, the milk components contain the nutritional needs needed by bacteria to reproduce cholesterol levels in the blood, cancer antimutagen, antimicrobial, and immunity [11]. The enrichment was carried out during the research using colostrum where it was suspected that colostrum contained LAB and MRS medium as one of the selective LAB mediums. Elyza et al. (2015) explained that the
enrichment stage of bacterial isolates aims to provide opportunities for types of bacteria with small numbers and populations so that during isolation these types can have more opportunities to live [12]. The enrichment results show that the bacteria growth is the presence of bubbles that indicate bacterial activity. According to Sariadi (2013), it is explained that the bacteria enrichment medium continues to multiply by means of divided under conditions of abundant nutrition and a suitable environment [13]. The results of bacterial enrichment can be seen in figure 2.

3.2. Isolation and purification of lactic acid bacteria

Based on the results of bacterial isolation that has been carried out from the colostrum source of dairy cows, 87 isolates of bacteria as candidates for lactic acid or LAB were obtained (figure 3). The bacterial colonies suspected of LAB were based on the formation of a clear zone area around the growing bacterial colonies. Melliaawati et al (2015) stated that in the process of forming a clear zone around the bacterial colony inoculated on the medium, the addition of CaCO$_3$ 1% can be used as an indication that growing bacteria are able to secretion acid in media containing CaCO$_3$ [14]. This means that these bacteria are acid-producing bacteria.

The clear zone around the bacterial colony is thought to also represent the result of protein hydrolysis activity by proteases in the bacterial growth medium. Leory and De Vuyust (2004) stated that LAB is a bacteria that will quickly start the acidification process of the raw material by producing lactic acid [15]. Safitri et al. (2016) also explained that lactic acid bacteria (LAB) are usually grown on de Mann Rogose and Sharpe (MRS) media. MRS media is a specific medium for the growth of lactic acid bacteria [16].
3.3. Proteolytic LAB isolation and selection
Isolation was carried out by growing lactic acid bacteria on SMA (Skim Milk Agar) medium. The bacterial selection at the initial stage was followed by a qualitative proteolytic activity test. Munifah (2014) explains that the proteolytic selection medium that can be used is skim milk [17]. Milk is a suitable medium for growth because it is rich in nutrients. Skim milk has a high protein content. In milk, there is casein, which is a milk protein consisting of phosphoproteins that bind to calcium to form a calcium salt called calcium caseinate. These molecules are very large and do not dissolve in water and form colloids. This suspension is white and can be observed directly when suspended in solid media culture.

![Figure 4. Forms of bacterial colonies that form clear zones on SMA (Skim Milk Agar) media.](image)

A total of 25 bacterial isolates were isolated and during the selection process produced a clear zone around the colony (figure 4). The isolate is then given the code naming LAB pro 1 to LAB pro 25, which indicates that it has gone through a selection process as a candidate for lactic acid bacteria and is able to produce proteases. Lay (1994) states that the isolated bacterial isolates that have the proteolytic ability are indicated by the presence of a clear zone around the bacteria grown on agar skim media [18]. The clear or clear zone that is formed indicates that the bacterial isolates grown on the media are able to use the skim protein found in the media for growth so that the protein around the colony is used up, marked by the clear zone. Kabense et al (2019) added that the clear zone around the bacterial colonies that grew on the Skim Milk Agar (SMA) medium showed that the protein substrate contained in the skim milk medium had been broken down into simple peptides and amino acids by the protease enzyme [19].

3.4. Qualitative proteolytic activity test
Based on the results obtained from 25 bacterial isolates that were isolated using SMA medium, only 19 bacteria were obtained which when tested again qualitatively were able to produce clear zones and had proteolytic index (IP) values. The range of proteolytic index values produced by isolates with protease-producing potential ranged from 0.2 to 0.6. The results that have been obtained are presented in table 1.
Table 1. The proteolytic index of the colostrum source bacteria for dairy cows.

| Bacterial samples | Clear Zone |
|-------------------|------------|
| LAB pro 1         | ✓          |
| LAB pro 2         | ✓          |
| LAB pro 3         | ✓          |
| LAB pro 4         | ✓          |
| LAB pro 5         | -          |
| LAB pro 6         | -          |
| LAB pro 7         | ✓          |
| LAB pro 8         | ✓          |
| LAB pro 9         | ✓          |
| LAB pro 10        | ✓          |
| LAB pro 11        | ✓          |
| LAB pro 12        | ✓          |
| LAB pro 13        | -          |
| LAB pro 14        | -          |
| LAB pro 15        | -          |
| LAB pro 16        | ✓          |
| LAB pro 17        | -          |
| LAB pro 18        | ✓          |
| LAB pro 19        | ✓          |
| LAB pro 20        | ✓          |
| LAB pro 21        | ✓          |
| LAB pro 22        | ✓          |
| LAB pro 23        | ✓          |
| LAB pro 24        | ✓          |
| LAB pro 25        | ✓          |
| Control Medium    | -          |
| Control Pepsin    | ✓          |

Informations: ✓ produces a clear zone (has proteolytic activity).
- does not produce a clear zone (has no proteolytic activity).

3.5. Quantitative proteolytic activity test
The clear zone produced in the proteolytic activity test qualitatively shows the ability of the bacterial isolate to produce protease enzymes. The greater the clear zone produced is thought to have a positive correlation with the ability of these isolates to produce protease enzymes. Nakazawa and Hosono (1992), stated that the proteolytic activity of LAB was different for each strain even though it was in one species [20]. Quantitative protease or proteolytic activity testing was carried out based on the qualitative test results, meaning that this test was carried out only on the best 10 isolates that had the best activity qualitatively. The quantitative protease activity test results are presented in table 2 below.
Table 2. The results of the protease activity after regressing with the standard curve.

| Bacterial samples | Clear zone | Enzyme activity units (U/mL) |
|-------------------|------------|-----------------------------|
| LAB pro 3         | ✓          | 0.513                       |
| LAB pro 4         | ✓          | 0.849                       |
| LAB pro 8         | ✓          | 0.494                       |
| LAB pro 9         | ✓          | 0.434                       |
| LAB pro 11        | ✓          | 0.108                       |
| LAB pro 20        | ✓          | 0.346                       |
| LAB pro 21        | ✓          | 0.480                       |
| LAB pro 23        | ✓          | 0.208                       |
| LAB pro 24        | ✓          | 0.174                       |
| LAB pro 25        | ✓          | 0.270                       |

Table 2 shows that the isolate that had the highest enzyme activity was LAB pro 4 isolate with the enzyme activity unit value of 0.849 U/mL and the lowest was LAB pro 11 isolate, 0.108 U/mL. According to Agustien (2010), the specific activity of enzymes can be different from several types of lactic acid bacteria (LAB) [21]. The possibility of this is due to the different amounts of enzymes and protein enzyme amino acids produced by each isolate. Lehninger (1998) states that the activity of an enzyme is influenced by several factors, namely pH, substrate and enzyme concentration, temperature, and the presence of activators or inhibitors [22].

This shows that the 10 isolates are actually able to release tyrosine during the incubation period. Tyrosine is the result of the activity of the protease enzyme produced by bacteria in the breakdown of protein substrates contained in the growth medium. However, in this selection process, the protease activities of LAB pro 4, LAB pro 3, LAB pro 8, LAB pro 9, LAB pro 21, and LAB pro 20 were 6 isolates which produced the best results among the 10 bacteria. This again can be caused because each type of bacteria has different optimum conditions and is also influenced by different environmental factors such as temperature, pH, enzymes, and substrate concentration. Every bacterial bacteria has an optimum temperature and pH in its growth. The influence of growth temperature, media pH, and aeration are important parameters that affect bacterial growth and the production of protease enzymes [23]. Temperature and pH greatly affect enzyme activity when catalyzing a reaction. The increase in temperature and pH that exceeds the optimum conditions causes the structural weakness of the bonds in the enzyme [24]. Six proteolytic bacterial isolates that have the highest protease activity (figure 5), namely LAB pro 4, LAB pro 3, LAB pro 8, LAB pro 9, LAB pro 21, and LAB pro 20 will be used for further tests, namely low pH resistance as one of the requirements as probiotic candidates and tested for molecular identification.

Figure 5. The histogram of the results of the protease enzyme activity test (U/g) of the six best isolates.
3.6. Low pH resistance test

The results of the low pH resistance test showed that the 6 best isolates from the previous protease activity were resistant to low pH environments, namely pH 2, 3, and 4, although at low pH there was a decrease in the growth rate of these bacterial isolates than environmental conditions with higher pH. The results of the pH resistance test after incubation for 3 hours are presented in Table 3 below.

**Table 3. Test results of the resistance to low pH.**

| Isolate code | Optical density (OD) bacterial growth test results after 3 hours |
|--------------|---------------------------------------------------------------|
|              | pH 2  | pH 3  | pH 4  | pH 6.5 (control) |
| LAB pro 4    | 0.64  | 0.70  | 1.02  | 1.75             |
| LAB pro 3    | 0.51  | 0.62  | 0.92  | 1.67             |
| LAB pro 8    | 0.64  | 0.84  | 1.16  | 1.81             |
| LAB pro 9    | 0.65  | 0.86  | 1.12  | 1.83             |
| LAB pro 21   | 0.61  | 0.56  | 1.03  | 1.71             |
| LAB pro 20   | 0.60  | 0.79  | 1.08  | 1.84             |

Based on the table above, it can be seen that the 6 isolates obtained have a good growth rate in the low pH range of 2 to 4. The results can also be seen that the higher the pH value of bacterial growth tends to increase. Oozeer et al. (2006) stated that the 3 hour incubation time is a general description of the rate at which food passes through the stomach [25]. The stomach is the first barrier that must be passed before bacteria enter the intestine (bile salts). Generally, most of the microbes will die in the stomach which has environmental conditions with a low or very acidic pH [26]. One of the main requirements for strains that can be used as probiotic agents is to have resistance to acids and bile so that they can reach the intestines and have the ability to stick to the intestinal mucosa [25].

4. Conclusions

Based on the research results, it can be concluded that the samples from the colostrum of dairy cows at the UPT Faculty of Animal Science UGM have obtained candidates for lactic acid bacteria (LAB). Six bacterial isolates were obtained as superior proteolytic LAB candidates, i.e., LAB pro 4, LAB pro 3, LAB pro 8, LAB pro 9, LAB pro 21, and LAB pro 20. The proteolytic activity test results showed that the 6 isolates showed a clear zone around the media when tested qualitatively and had quantitative protease activity respectively LAB pro 4 0.849 U/mL, LAB pro 3 0.513 U/mL, LAB pro 8 0.494 U/mL, LAB pro 9 0.434 U/mL, LAB pro 21 0.480 U/mL, and LAB pro 20 0.346 U/mL. These isolates also showed a growth rate when inoculated at low environmental conditions of pH 2, 3, and 4, which is one of the main requirements as probiotic candidates.

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