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Potential Use of *Lactobacillus plantarum 1UHCC* as a Bio-hydrolyzer in the Development of the Sustainable Food Industry in Indonesia

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Abstract
The purpose of this study aimed to evaluate the potential and performance of *Lactobacillus plantarum 1 UHCC* (*L.plantarum 1UHCC*) bacteria as bio-hydrolyzer of the food industry in Indonesia. As much as 100g of fresh hide from Balinese cattle were used as a substrate. The fermentation time was carried out at 3 time levels, namely: 24 hours (T-24); 48 hours (T-48) and 72 hours (T-72). The bacteria were used *L.plantarum* which was isolated from milkfish extract. The results showed that in the solution of *L.plantarum* bacteria using collagen substrate of cattle hide, pH values were increased and the lactic acid levels were decreased significantly. The pH value and the lactic acid level of the solution were respectively around 5.77±0.03-6.13±0.02 and 1.65±0.26-2.02±0.05%. The total bacteria has increased and dissolved protein has decreased for fermentation time, but in general it is not significant. Total bacteria and dissolved proteins respectively 4.5±0.98-5.5±2.29 Log_{10}CFU/mL and 36.98±3.37-40.25±1.54 mg/mL. The application of 24 hours (T-24) fermentation time to *L.plantarum* bacteria using collagen substrate of cattle hide skin was considered as the optimum time to be applied in the fermentation process. Bacteria *L.plantarum 1UHCC* has the ability to hydrolyze collagen protein components, especially collagen extracts from cattle hide. Bacteria *L.plantarum 1UHCC* has the potential to be developed as a bio-hydrolyzer organism. Bacteria *L.plantarum* 1UHCC plays a very important role in the development of the food industry, especially in Indonesia.

Keywords: Bacteria, Cattle hide, Collagen, Fermentation, L.plantarum 1UHCC

1. Introduction

At present, the need for a bio-hydrolyzer in the food processing industry is increasing. This need increases with the development of human needs for food. The use of bio-hydrolyzer of microorganisms is increasingly needed. The use of bacteria as a bio-hydrolyzer is an option. in Indonesia, the need for bio-hydrolyzer is very much needed in food processing. The activity of each bacterium as a bio-hydrolyzer is different for each type and variant. The performance of each type of bacteria in the protein decomposition process differs from one another. The use of bacteria in breaking down proteins has been widely applied in the fields of food processing such as meat, milk, fish and eggs. To produce maximum performance, the bacteria must be able to grow well in the medium. Collagen protein extract from cattle hide is one of the potential growth media for bacteria. This is because collagen protein contains a number of essential amino

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acids needed for the growth process and a source of nutrition for bacterial microorganisms.

Cattle hide and bone are one of the livestock by-products that rich in protein compounds, especially collagen protein. Cattle hide is composed of several layers which are dominated by the dermis layer. This layer consists of 70-85% collagen protein (Sarkar, 1995; Said *et al*., 2015; Said *et al* 2019). In addition to producing collagen, Bali cattle in Indonesia are the main meat-producing (Abustam *et al*., 2018)

Collagen is one of the dominant types of structural proteins produced from animal body tissues (Zeugolis *et al*., 2008). Until now, collagen has been widely used in the food industry as gelling, stabilizing, foarming and emulsifying agents (Bhuimbar *et al*., 2019; Sato, 2019). Utilization of collagen extract in the food industry includes food supplements to prevent osteoporosis, osteoarthritis and premature aging (Rogart *et al*., 1999; Tian *et al*., 2011). The development of collagen extract has also directed as a health food containing natural antioxidants and texturing agents that will reduce the use of chemical preservatives (Pal and Suresh, 2016).

Utilization of collagen extract as an additive in functional food has not been widely developed. One effort that can be was to apply fermentation technology in the production process of collagen extract. The application of fermentation technology can be done was to obtain benefits as functional foods that are good for health, facilitate the absorption process in the digestive tract and extend the shelf life of the product (Solomons, 2002). Collagen extract can be added in to the processed meat products such as nuggets, meatballs and dairy products such as ice cream. Based on economic considerations, the fermentation process provides several advantages including: increasing production volume, reducing energy use and minimally produced waste so that it is more environmentally friendly (Haq *et al*., 2003).

The application of fermentation technology in the food industry by involving the role of microbial lactic acid bacteria (LAB) has been developed to produce functional food (Safari *et al*., 2012). The use of microbes from the type of *Lactobacillus plantarum* (*L.plantarum*) bacteria has been widely applied in the food processing industry. *L.plantarum* bacteria has been widely applied in food processing industries made from meat, milk, eggs and fish (Ummadi and Curic-Bawden, 2010; Fioramonti *et al*., 2003). The role of bacteria as decomposers of carbohydrates in the fermentation process, can also play a dual role as protein decomposers. Enzymes produced by bacteria are able to break down certain proteins that are specific in nature. Bacteria that produce enzymes to break down proteins can benefit from the proteins break down. One example is the use of *L.helveticus* bacteria which are used to break down meat and milk proteins to produce antihypertensive compounds (Fuglsang *et al*., 2003) and *Bacillus subtilis* FNCC 0059 for the broiler feather (Said *et al*., 2018).

Until now, the application of *L.plantarum* to break down collagen protein in cattle hide to produce a less known compound. To maximize the potential of *L.plantarum 1UHCC* in breaking down collagen protein, this study is very important and needs to specifically study the performance of bacteria during the fermentation process. This study was aims to evaluate the potential and performance of *Lactobacillus plantarum 1 UHCC* (*L.plantarum 1UHCC*) bacteria as bio-hydrolyzer of the food industry in Indonesia.

2. Materials and Methods

2.1 Research materials

The study was uses raw materials from fresh Balinese cattle hide, male, 3 years old. Cattle hide obtained from Tamangapa Slaughterhouse, Makassar City, South Sulawesi, Indonesia. The isolate of *L.plantarum 1UHCC* was result of isolation from milkfish extract media. *L.plantarum 1UHCC* isolate obtained from the Lab. Microbiology, Faculty of Mathematics and Natural Sciences, Hasanuddin University, Makassar. Bacterial growing media using MRS-broth (*Oxoid M0359*); nutrient broth (*HIMEDIA Ref M002-5006*); bacteriological agar (*Oxoid LP0011*); aquadest (*One Med/Water One*); alcohol 70% (*One-Med*). The equipment used includes: incubator (*Memmert BE 400*); electric oven (*Memmert 100-800*); laminar flow (*AIRTECH*) shaker (*IKA KS 260 basic*); autoclave (*YXQ.SG41.280*); hot plate (*Stuart SD162*); micropipette (*HUAWEI 14060806*); vortex (*Vortex IKA 3*); analytical scales (*Jc3-B scale/KERN ALS 220-4N*); pH meter (*Hanna HI 8424*); erlemenyer (*SCHOTT DURAN*); petri dish (*ANUMBRA*).

2.2 Research methods

2.2.1 Preparation of bacterial culture

One ampoule of *L.plantarum 1UHCC* bacterial isolate was propagated using nutrient broth + agar bacteriological + aquadest to make a culture with a concentration of *L.plantarum 1UHCC 5*%. A total 5.2 g of MRS-broth media were dissolved in 100 mL of aquadest and homogenized. A total 50 mL of bacterial growing media were made. Based on the amount of the solution, as much as 47.5 mL was then put into the erlenmeyer tube as a growing medium. The tube was covered with aluminum foil. The solution was sterilized using an autoclave at a temperature of 120-125ºC for 45 minutes. The solution was then cooled in laminar flow. The culture of *L.plantarum 1UHCC* bacteria was then inoculated into the media.

2.2.2 Cattle hide preparation

A number of one sheet of fresh cattle hide washed with running water. Cattle hide was heated at 90ºC in a container for 15 minutes. Hair on cattle hide was removed using special equipment. The hide was cut into cubes with a size of 1x1 cm. The hide was weighed and dried in an oven at 50ºC for 24 hours. Cattle hide samples were sterilized in 70% alcohol.

2.2.3 Research design and data analysis

Data were analyzed by variance based on a Completely Randomized Design (CRD) with the SPSS statistical program (one-way ANOVA). The treatment that showed a significant effect, then performed a significant difference test with Duncan's Multiple Range Test (DMRT) at the level of 5% (Steel & Torrie, 1991).

2.2.4 Parameter analysis

Total bacteria (Log_{10} CFU/mL) (Boczek *et al.*, 2014). The total plate count (TPC) method was used to calculate the total *L.plantarum 1UHCC* bacteria. A number of 1 mL of *L.plantarum 1UHCC* solution was diluted in 9 mL of sterile aquadest. The solution was homogenized with vortex. The dilution process was carried out from 10^{-1} to

10^{-12}. The dilution media was incubated at 37ºC for 24 hours. The basis for calculating the colony is 25-250, which is done 3 times (triploid).

pH value (León *et al*., 2008); (Rahman *et al*., 2008). The pH meter was calibrated using pH 4 and pH 7. The solution was heated at 70ºC and homogenized. The pH value of the solution was then measured at room temperature.

Dissolved protein (mg/mL) (AOAC, 1995). Determination of dissolved protein using the Lowry method. A total of 1.5 g of sample was inserted into a scale tube. A total of 7.5 mL of aquadest was added and then homogenized with vortex. The centrifugation process was carried out for 15 minutes. The supernatant was then boiled with a hotplate. A total of 2 mL of the supernatant was added with 1 mL of 10% TCA dilution. The mixture was centrifuged for 15 minutes. A total of 0.1 mL of TCA was added 1.9 mL of aquadest and 2.5 mL of Lowry reagent. The mixture was homogenized and stored at room temperature for 10 minutes. A total of 0.5 mL of folin reagent was added to the mixture and incubated at room temperature for 30 minutes to form a blue color. The absorbance was measured using a spectrophotometer with λ = 660 ηm. The results obtained were compared with a standard bovine serum albumin (BSA) solution. Soluble protein levels were determined using the regression equation y = ax + b, dissolved protein (mg/mL) (x) = (y-b)/a, where; a = 3.520; b = 0.058; y = absorbance; R^2 = 0.992.

Total acid (%) (AOAC, 1995). A total of 10 mL of suspension was added with three drops of PP indicator, and then titrated with 0.1N NaOH solution. Testing was done 3 times. The number of mL titrations can be determined through discoloration to the pink color. The test results are determined by the equation, total acid (TA) (%) = $(V_1)(N)(B)/(V_2)(1{,}000)$, where; V_1=volume of NaOH (mL); V_2=volume of suspension solution *L.plantarum* (mL); N=normality of NaOH (0.1); molecular weight of lactic acid (90).

3. Results and Discussions

3.1 Total Bacteria

Total bacteria is one of the parameters needed to evaluate the condition of bacterial growth on a collagen extract substrate from cattle hide at different fermentation processes. Comparison of the growth of *L.plantarum 1UHCC* bacteria on the substrate of cattle hide collagen extract was presented in Figure 1.

Based on Figure 1, the results show that during the 24-hour fermentation period (T-24) to 72 hours (T-72) there was a significant decrease in the total *L.plantarum* 1UHCC bacteria ($P<0.05$) respectively (5.5 ± 2.29 Log_{10} CFU/mL); (4.7 ± 0.99 Log_{10} CFU/mL) and (4.5 ± 0.98 Log_{10} CFU/mL). Statistically, the three treatments did not show significant differences ($P>0.05$). Fermentation time determines the difference in the number of microbes (Sun *et al*., 2010).

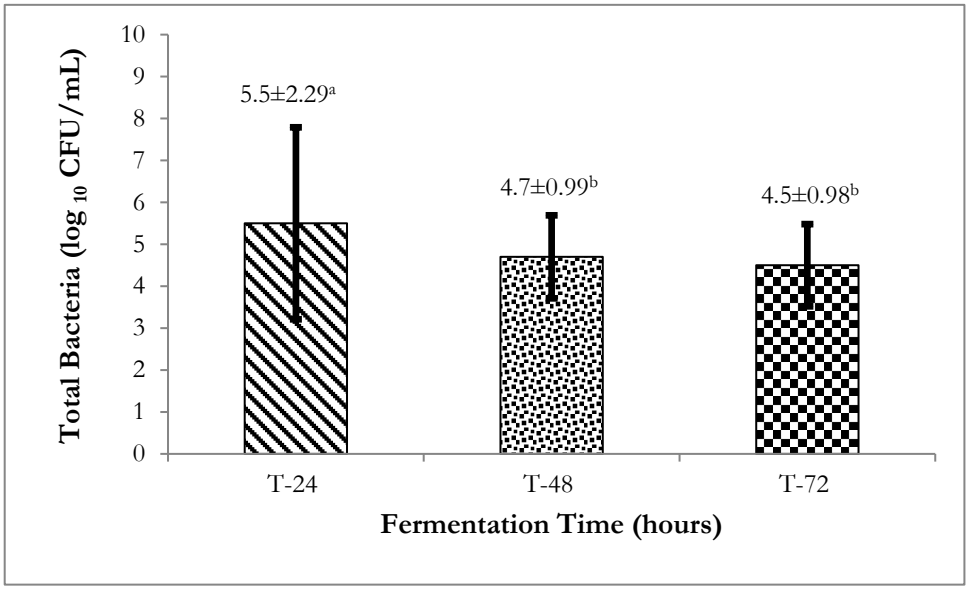

Figure 1. The total bacteria (Log$_{10}$ CFU/mL) of L.plantarum 1 UHCC bacteria on substrate collagen extract of cattle hide on the application of different fermentation times; [a,b]Different superscripts in each treatment showed significant differences (P<0.05); T-24=fermentation time for 24 hours; T-48=fermentation time for 48 hours; T-72=fermentation time for 72 hours

One of the factors that cause an increase in microbial population of bacteria *L.plantarum 1UHCC* is the availability of sufficient nutrients, including protein. Cattle hide is rich in collagen compound which is one of the important nutrients for microbial growth. Availability of sufficient nutrients will increase bacterial productivity. Productivity is described as a biomass output per unit of fermentation time (Stanbury *et al*., 2003). Lactic acid bacteria such as *L.plantarum* need a source of amino acids or peptides to grow (Savijoki *et al*., 2006). The results showed high population and speed of growth of *L.plantarum* bacteria during the fermentation process. This is because the nutrients needed by bacteria are available in very large quantities on the substrate. Collagen contained in cattle hide is rich in protein compounds, especially amino acids glycine, proline and hydroxyproline (Nagai *et al*., 2008).

3.2 pH Value

pH value is a parameter that is directly related to the fermentation process, especially lactic acid bacteria. A change in pH indicates a fermentation activity. The fermentation process is a process of changing carbohydrates into acids and water and several other products. The description of the change in pH of the solution containing *L.plantarum 1UHCC* bacteria during the fermentation process was presented in Figure 2. The results of the study (Figure 2) showed that the pH of the solution significantly increased during the process of fermentation. Statistically, the increase in fermentation time had a significant effect (P<0.05) on the increase in pH of the solution. At 24 hours fermentation time (T-24), the pH of the solution showed a value of 5.87±0.08. Furthermore, at 48 hours fermentation time (T-48) slightly decreased (5.77±0.03), but increased again at 72 hours fermentation time (T-72) (6.13±0.02). Changes in pH occur

due to fermentation activities. This process converts carbohydrates into acidic compounds. Related to this, an increase in pH up to 72 hours of fermentation time (T-72) was caused by the low carbohydrate content in cow skin substrate so that the amount of lactic acid formed was very small. The *L. plantarum 1UHCC* bacteria are able to metabolize these organic acids. Organic acids can then be utilized by *L. plantarum* as a carbon source (Filannino *et al.*, 2014; Lerena *et al.*, 2016).

Research has been reported by Ge *et al.*, (2019), that *L.plantarum 1UHCC* bacteria can inhibit protein oxidation during the process of fermentation on processed meat (sausage), increase protein degradation and provide comparative antioxidant effects as strains for commercial purposes. Changes in pH value can be caused by the production of organic acids during the fermentation process. This can affect antioxidant activity through changes in the content and structure of phenolic compounds (Mousavi and Mousavi, 2019). The *L.plantarum* bacteria can free phenolic compounds after being acidic and enzymatic from polymerized phenolic compounds during fermentation (Hur *et al.*, 2014).

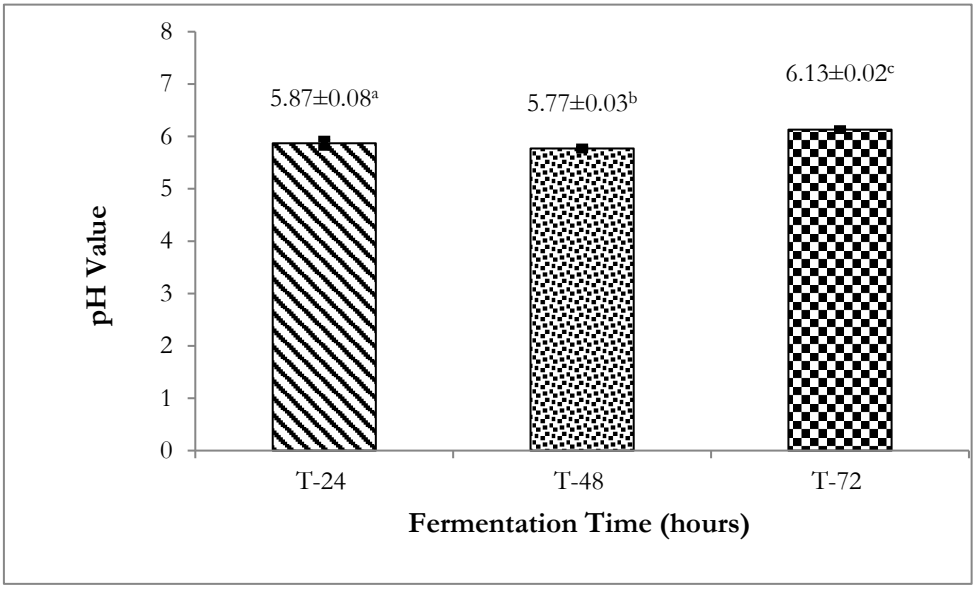

Figure 2. The pH value of L.plantarum 1 UHCC bacteria on substrate collagen extract of cattle hide on the application of different fermentation times; [a,b,c]Different superscripts in each treatment showed significant differences (P<0.05); T-24=fermentation time for 24 hours; T-48=fermentation time for 48 hours; T-72=fermentation time for 72 hours

3.3 Dissolved Protein

Soluble protein is a component that shows the amount of oligopeptides that are easily absorbed by the digestive tract. The use of mannan-oligosaccharide (MOS) has the greatest proliferative effect on *L.plantarum ATCC14917* in vitro (Cao *et al.*, 2019). Comparison of the amount of dissolved protein on the substrate of cattle hide collagen extract using *L.plantarum 1UHCC* bacteria was presented in Figure 3.

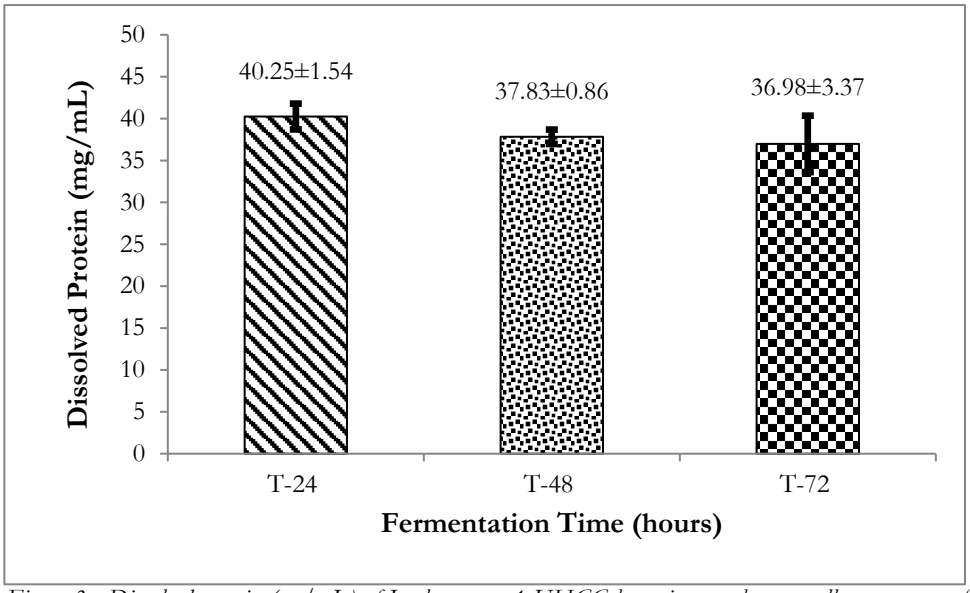

Figure 3. Dissolved protein (mg/mL) of L.plantarum 1 UHCC bacteria on substrate collagen extract of cattle hide on the application of different fermentation times; T-24=fermentation time for 24 hours; T-48=fermentation time for 48 hours; T-72=fermentation time for 72 hours

Based on the Figure 3, the results showed that the increase in fermentation time by *L.plantarum 1UHCC* bacteria on the substrate of cattle hide collagen extract reduced dissolved protein. However, statistically, the treatment was not significantly different ($P>0.05$). At 24 hours fermentation time (T-24), total dissolved protein was 40.25±1.54 mg/mL. Furthermore, at 48 hours and 72 hours of fermentation, both of them decreased by 37.83±0.86 mg/mL and 36.98±3.37 mg/mL respectively. The difference in the amount of dissolved protein was related to the condition of the substrate used. The substrate affects the maximum speed of the enzyme in breaking down the substrate. Protease enzymes play an important role in hydrolyzing proteins. This enzyme can break down protein bonds into peptides. Peptidase can break peptide bonds into amino acids. The amount of dissolved protein is an indication of the amount of protein undergoing the degradation process by the activity of the proteolytic enzyme *L.plantarum 1UHCC* bacteria.

Immunoreactivity of a protein was affected by changes in the primary structure. During the process, proteins can be degraded, aggregated, folded, and crossed. This will cause changes in immunoreactivity (Rahaman *et al*, 2016). Some metabolites such as antimicrobial peptides can play a role in the performance of lactic acid bacteria (LAB) and its metabolism. This can affect microbial safety, total population and the ecology of fermented products (Todorov *et al*., 2017)

3.4 Total Acid

The total acid content measured by the titration method was equivalent to lactic acid levels as a result of the fermentation process of *L.plantarum 1UHCC* bacteria. The

LAB were a type of bacteria that are widely distributed in Indonesia. The LAB can be produced from carbohydrate fermentation. The LAB plays an important role in maintaining normal conditions and maintaining the stability of the digestive tract from pathogen bacteria (Hooper and Gordon, 2001; Bäckhed *et al.*, 2005). Description of changes in lactic acid levels in a solution containing *L.plantarum 1UHCC* bacteria using collagen extract of cattle hide as a substrate was shown in Figure 4.

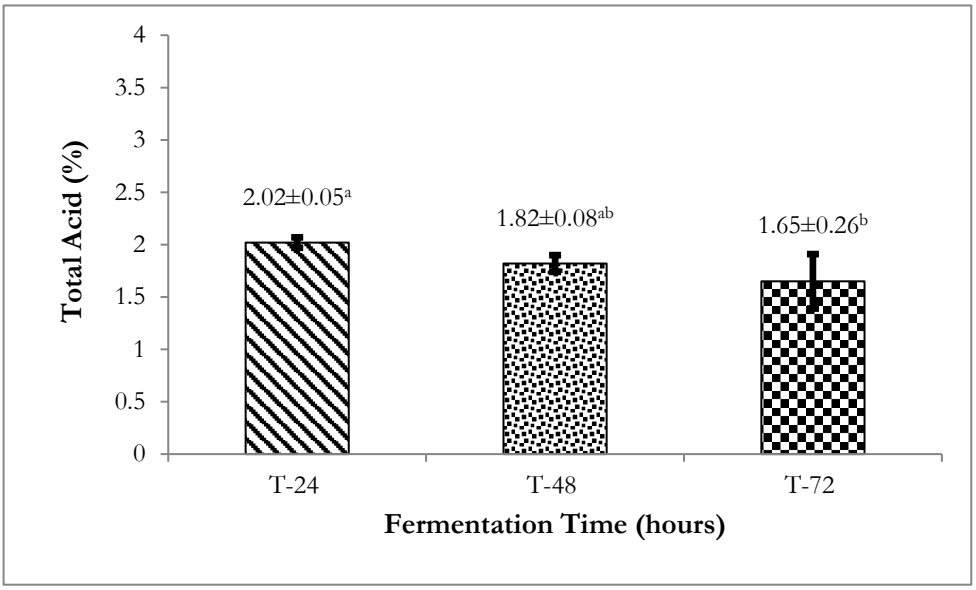

Figure 4. Total acid (%) of L.plantarum 1 UHCC bacteria on substrate collagen extract of cattle hide on the application of different fermentation times; [a,b]Different superscripts in each treatment showed significant differences (P<0.05); T-24=fermentation time for 24 hours; T-48=fermentation time for 48 hours; T-72=fermentation time for 72 hours

Based on Figure 4, it can be seen that the difference in fermentation time has a significant effect (P<0.05) on the acid content of the solution containing *L.plantarum 1UHCC* bacteria using a collagen extract of cattle hide. Increased fermentation time significantly reduces acid levels. Fermentation time for 24 hour (T-24); 48 hours (T-48) and 72 hours (T-72) produce acidic levels of 2.02±0.05%; 1.82±0.08% and 1.65±0.26% respectively.

The *L.plantarum* bacteria have good probiotic properties. This bacterium can tolerate the environment in the digestive tract. These bacteria can also metabolize and synthesize bacteriocin, which has a strong inhibitory effect on the growth of gram-positive and gram-negative bacteria. The *L.plantarum 1UHCC* bacteria are initially used to ferment milk and other dairy products (Spano and Massa, 2006). These bacteria are relatively resistant to acids and high fermentation temperatures because of their ability to produce bacteriocins (Gong *et al.*, 2010).

Conclusion

Application of *L.plantarum 1UHCC* as a hydrolyzing agent significantly decreases total bacteria, dissolve protein and total acid, but does not affect the pH value at different fermentation times. The 24 hour (T-24) fermentation time showed the most optimum fermentation time of *L.plantarum 1UHCC* to be applied to the collagen extract of cattle hide. The 24 hours (T-24) fermentation time process provides the best characteristics compared to other fermentation, especially in collagen extracts. Bacteria *L.plantarum 1UHCC* has the potential to be developed as a bio-hydrolyzer agent for the development of the food industry, especially those containing collagen extracts.

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