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Acid Resistance Test of Probiotic Isolated from Silage Forage Swamp on in Vitro Digestive Tract

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Abstract

Probiotics are living microorganisms able to reach the gastrointestinal tract and benefiting health, leaving no residue in the body. Probiotics can be obtained from lactic acid bacteria (LAB) which produced lactic acid and antimicrobial components. This study was conducted to determine the resistance of LAB isolates as probiotics from silage forage swamp at different pH distributed in vitro digestant. pH resistance testing of LAB was performed by introducing diluted bacteria into buffer solutions with different pH and a predetermined incubation time. The design used a complete randomized design consist of 3 treatments and 3 replications. The treatments were using isolate of Kumpai Tembaga silage (P1), isolate of 50% Kumpai Tembaga silage and 50% Kemon Air silage (P2), and isolate of Kemon Air silage (P3). The results showed that all isolates used were lactic acid bacteria, and the treatment significantly affected the LAB resistance test against low pH and high pH. Isolate from Kumpai Tembaga silage (P1) is a lactic acid bacterium with higher ability to survive in pH of in vitro digestive system.

Keywords: Probiotics, acid resistance, Kumpai Tembaga silage, digestive tract

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INTRODUCTION

Antibiotics use as poultry feed additive has decreased while some countries banned its used because of health threat against human due to drug residues and the emergence of bacterial strains that are resistant to antibiotics. The condition force efforts to find alternative source poultry feed additive such as probiotics.

Probiotics is a living microorganism able to reach digestive tract and provide health beneficial. The probiotics support useful bacteria, increase nutrient absorption and leave no residue within human body. The origin of probiotics is from bacteria. One of bacteria species possess the potential for probiotics use is lactic acids bacteria (LAB) which produce lactic acids and antimicrobial component. LAB can be obtained through fermentation process of animal feed product such as silage. Kumpai Tembaga silage was reported to produce average number of bacteria 8.24 (107 cfu/mL) while Kemon Air legumes 7.47 (107 cfu/mL) [1].

LAB requires suitable environment to survive i.e. pH condition. Bacterial resistance test against digestive tract pH is urgent and one of screening process to select bacteria as probiotics product. Hardiningsih et al. [2] described that bacteria can be use as probiotics product depend on its resistance against low pH, bile salt and its ability to live within digestive tract (from mouth to cecum). Research on bacterial resistance test require time and efforts as well as high cost if it conducted directly within animal. Here, we reported the LAB resistance test to

be use as probiotics isolated from swamp forage silage within in vitro digestive tract.

MATERIALS AND METHODS

Materials

This part should contain sufficient detail so that all procedures can be repeated. It can be divided into subsections if several methods are described. Heading and sub-headings can be used up to 3 levels. Chemicals grade and specification should be stated. Instruments used in the research should be also described its operational condition.

Methods

This research used completely randomized design consist of 3 treatments and 3 replicates. The treatment subjects were P1 (Kumpai Tembaga silage isolate), P2 (50% Kumpai Tembaga silage isolate and 50% Kemon Air silage isolate) and P3 (Kemon Air silage isolate). Data were analyzed using analysis of variance and if the result shows statistically significant difference, the result was further analyzed by smallest real difference (SRD) test [3].

pH Adjustment

Solution pH adjustment was made by adding into 150 mL distilled water certain amount of NaOH 0.1 N to raise pH and HCl 0.1 N to decrease it. The procedure is completed when the desired pH was achieved i.e. 3; 5.5; 6; 6.5; 7.5 and 8.

The Bacterial Resistance against Acid Condition

Testing done with grow 1% culture was about 24 hours into media MRS broth who had previously been by controlling the pH each pH 2.5 and pH 7.0. Next incubation for 24 hours at a temperature 37 °C, at the end of incubation done calculation the amount of bacteria with the methods the plates total (plate count) in a media agar MRS.

Lactic Acids Bacterial Resistance Test (Harahap, 2014)

The LAB resistance in the digestive tract was evaluated by set up several pH condition and incubation period. The in vitro system in resistance test was conducted by Lactic acids bacteria were placed in reaction tubes where it was diluted and mixed with solution using vortex device. The suspensions were incubated for several different duration. The solution (pH 3) which had been incubated for 90 minutes was vortexed and sampled as much as 1 mL.The procedure was repeated for other solution and incubation duration. The condition employed were pH 3 (90 minutes transit duration) in proventricular, pH 5.5 (50 minutes) in cache, pH 6 (8 minutes) in duodenum, pH 6.5 (30 minutes) in jejunum, pH 7.5 (70 minutes) in ileum and pH 8 (25 minutes) in rectum. Each of solution were placed on petri dish as much as 1 mL, added with MRS agar 15 mL, then homogenized, and incubated for 2x24 hours. The amount of LAB is calculated using following formula:

LAB population (cfu/g) = Number of colony x Dilution

RESULT AND DISCUSSION

The Bacterial Resistance against Acid Condition

Acid condition was necessary to carry out bacteria selection prior enter the intestine. Acid tolerance is one of important factor for LAB becomes a probiotic. This requirement is critical because LAB isolate meant to be probiotic must able to pass through acid condition within stomach before reaching out intestine [4].

Table 1. Isolate resistance against acids of several silage forage swamp

Isolate	Acid resistance	
	pH 2.5	pH 7
P1	++	++
P2	+	+
P3	+	+

Note: P1 (isolate kumpai tembaga silage), P2 (isolate combination of kumpai tembaga silage and kemon air silage), P3 (isolate kemon air silage). + (slightly cloudy), ++ (cloudy)

Table 1 reveals all isolate able to withstand at pH 2.5 and pH 7. This bacterial resistance can be observed from bacterial growth on the medium i.e. cloudy. P1 isolate from Kumpai Tembaga silage has higher pH resistance compare to P2 and P3 indicated by cloudy growth medium. High total acids within Kumpai Tembaga silage was suspected to increase total bacteria growth. Resistance test against acidity used 2 different pH i.e. 2.5 and 7 which according to Harimurti, isolate of LAB must be able to withstand in pH 2.5 for at least 2 hours to be used as probiotic [5]. Low pH condition within digestive tract can be found in gizzard that estimated as 2.5 [6]. Gurminarni reported microorganism with the ability to survive in low pH (< 3.0) generally forms spore as survival effort [7]. The microorganism begins to show life activity and start to colonize at pH 4.5 within small intestine following duodenum. Acid resistance bacteria can endure from membrane damage due to pH decrease extracellular compare to other bacteria. Lactic acid bacteria with high acid tolerance also has the ability to maintain cytoplasm pH in alkaline condition against extracellular pH decrease [8]. Probiotic used of LAB

therefore can be fulfilled with tolerance ability to acids.

Lactic Acid Bacterial Test against Low pH

The resistance of LAB against low pH was obtained by calculating LAB number grew on MRS agar media. The average amount of LAB at different pH for each treatment is shown on Table 2.

isolate must able to get through stomach acid condition before reaching small intestine [4]. Jannah explained further that LAB must able to adjust cytoplasm pH to maintain intracellular stays neutral even though its extracellular condition is acids [12].

All isolates used in this research able to grow optimally at pH 6. At more acidic pH, LAB amount is

Table 2. Isolate number LAB ($\log^8$ cfu/g) at low pH of silage forage swamp

Treatment	Acidity (pH)			
	3[1)]	5.5[2)]	6[3)]	6.5[4)]
P1	6.28±0.58 b	7.42±0.24b	7.68±0.58c	5.78±0.38c
P2	5.73±0.71a	6.54±0.33b	5.62±0.42b	3.37±0.55b
P3	4.78±0.45a	4.07±1.04a	4.13±0.12a	2.33±0.28a

Ket : [1)]incubated for 90 minutes, [2)]incubated for 50 minutes, [3)]incubated for 8 minutes, [4)]incubated for 30 minutes. P1 (isolate kumpai tembaga silage), P2 (isolate combination of kumpai tembaga silage and kemon air silage), P3 (isolate kemon air silage). Different superscript at the same column indicates statistically significant difference at test level 5%.

Analysis of variance suggested that the treatment gave significant difference ($P<0.05$) towards LAB number at low pH. Further test reveals at pH 3, average amount of LAB from P1 higher than other treatments while P2 and P3 does not statistically significant difference. At pH 5.5, LAB average amount of P3 lower than other treatments whereas P1 and P2 not significantly different. pH 6 and 6.5 provide LAB average number of P1 significantly different i.e. higher than other treatments while P3 isolate is significantly lower than other treatments.

The result of analysis also informed that P1 isolate produced more LAB compare to other silage at low pH. The P1 isolate was obtained from Kumpai Tembaga grass, which is, contain extra carbohydrate compare to other silage obtained from Kemon Air legume. Kumpai Tembaga grass has Nitrogen Free Extract (NFE) 46.76% and crude protein (CP) 17.42% [9]. Efendi suggested that carbohydrate contained within the fed provide a LAB growth support by supplying enough nutrient for the further regeneration process [10].

P1 isolate appears to growth more at pH 3 compare to other treatments. This result suggested P1 is able to survive within digestive tract particularly within proventricular and gizzard. Manin reported that lactic acid bacteria (Lactobacillus acidophilus and Lactobacillus fermentum) survive in pH condition of digestive tract of poultry i.e. pH as low as 3 such as within proventricular and ventricular [11]. Most of poultry digestive organ has acid condition with pH range 3-6, therefore bacterial resistance against low pH is required for isolate to be use as probiotics [5]. Salminen described that one of important criteria for probiotics development from LAB isolate i.e. the decreased. The lipopolysaccharide damage due to acid condition is responsible for the decrease of cell number and isolate resistance ability. The decrease of LAB colony number at lower pH due to stress created by pH different between upper digestive tract and lower digestive tract [13]. Sjofjan et al. (2015) added, microorganism able to survive at low pH by forming a spore and start to re-colonize at pH 4.5 [14]. Mojgani and Amirnia concluded that pH 5-6 could be considered as optimum pH of LAB growth on MRS agar media [15]. Harahap found out LAB stability (at pH base) was decrease drastically and cannot be detected at pH 8 (rectum) [16].

Lactic Acid Bacterial Test against High pH

LAB resistance test against high pH was obtained by calculating LAB number grew on MRS agar media. The average amount of LAB from each acid condition treatments (pH) is displayed on Table 3.

Analysis of variance result indicate that the treatment gave significant impact ($P<0.05$) towards LAB number at high pH.

Table 3. Isolate number of LAB ($\log^8$ cfu/g) at high pH of silage forage swamp

Treatment	Acidity (pH)	
	7,5[1)]	8[2)]
P1	4,51±0.06c	3,71±0.35c
P2	2,93±0.46b	2,57±0.12b
P3	0,00±0.00a	0,00±0.00a

Note: [1)]incubated for 70 minutes, [2)]incubated for 25 minutes. P1 (isolate of kumpai tembaga silage), P2 (isolated of kumpai tembaga silage and kemon air), P3 (isolated of kemon air silage). Different superscript at the same column indicates statistically significant difference at test level 5%.

Further test, which was conducted reveals at pH 7.5 and 8, average number of LAB P1 higher significantly, compare to other treatments while P3 lower than others. This result suggested low ability of P2 and P3 isolates to survive at high pH. P2 and P3 isolates were obtained from silage of Kemon Air legume, which has high protein content compare to grass hence the isolate amount is decrease at high pH. LAB ability is highly affected by the nature of bacteria, which is correlate to its origin [17]. Kemon Air contained crude protein as much as 28.02% (Ali et al., 2012). High protein content inhibits acidification process henceforth hinder the LAB growth [1].

The treatment we were conducted so far show that P1 can grow more at high pH compare to other isolates. P1 isolate also survive within digestive tract particularly at ileum and rectum. This ability was obtained by utilize carbohydrate and converts it into lactic acids which in turn lower surrounding pH hence it survives within digestive tract with high pH. LAB provides organic acids that can be used as acidification agent to lower pH of its surrounding [18]. Putri et al. reported that LAB has good amylolytic ability i.e. produce extracellular amylase and ferments starch directly into lactic acids by combining two different process i.e. enzymatic hydrolysis of carbohydrate (starch) and fermentation of sugar produced into lactic acids [17]. Nur suggested that the isolate has better enzymes, which can be used to produce acetate acids, LAB heterofermentative able to produce glucose-6-phosphate dehydrogenase enzyme and 6-phosphogluconate dehydrogenase enzyme whereas LAB facultative heterofermentative produced fructose diphosphate dehydrogenase in addition to two enzymes produced by the heterofermentative [18].

CONCLUSION

Isolate of Kumpai Tembaga silage is a lactic acid bacterium (LAB) with the ability to survive within pH condition of digestive tract in vitro.

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