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Growth and Survival of Lactic Acid Bacteria Isolated from Byproduct of Virgin Coconut Oil as Probiotic Candidate for Poultry

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Abstract: The main objective of this research is to investigate the potential of LABs isolated from processing byproduct of the VCO in *in vitro* conditions for use as probiotics in poultry. Forty eight LABs were isolated and four of them have been selected for further study i.e. Lh1, Lh2, Lh3 and Lh4. A series of tests carried out by studying the ability of bacteria to survive at 37 and 42°C, tolerance of LAB at pH 2, 0, 5, 7 and 7, 0 and tolerance to gastric juice as well as sensitivity to several antibiotics commonly was given to poultry. The survival of LABs was evaluated after 15, 30, 60, 90 and 120 and 300 min of incubation. The sensitivity test to antibiotics was performed by Muller Hinton's agar. All the bacteria showed tolerance and ability to grow at pH 5 and 7, but only Lh4 enabled to tolerate at pH 2. All of LAB can grow at gastric juice stimulated. Lh4 was not sensitive to all antibiotics (clear zones: 0.33 mm) but the other LABs were sensitive (clear zones: 5-12 mm). The conclusion of this research is the ability of LABs to grow in *in vitro* conditions varies. The Lh4 has demonstrated its ability to grow and the best survival with the OD (λ = 580) is 1.99 after 300 min of incubation at pH 2 and has shown the most resistant to all antibiotics tested with a wide clear zone 0.33 mm, hence potentially be used for probiotic in poultry.

Key words: Lactic acid bacteria, byproduct VCO, survival, growth, probiotic

INTRODUCTION

Probiotic has been defined as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance” (Fuller, 1989). Among the potential probiotics, Lactic Acid Bacteria (LAB) is reported to have important effects on animal performance (Chou and Weimer, 1999) and lactic acid bacteria as probiotic has been widely applied to improve poultry productivity. The claims made for probiotics mainly include improvement of growth rate and feed utilization (Denli *et al*., 2003; Patterson and Burkholder, 2003), disease resistance and reduction of gut shedding of enteropathogenic bacteria (Erkkila *et al*., 2000; Asahara *et al*., 2004).

Several studies have shown that the therapeutic value of live probiotic bacteria is more effective than unviable cells (Ouwehand and Salminen, 1988). Applications of probiotics, the microorganisms must pass through the gastrointestinal tract to their targets. Essentially, these microorganisms must survive in upper parts of the gastrointestinal tract to reach the hind gut in an active and functional form and to exert their beneficial properties (Sultana *et al*., 2000; Kim *et al*., 2006; Higgins *et al*., 2008; Higgins *et al*., 2010). The severe acidic conditions of the crop, proventriculus and gizzard could have an adverse effect on the bacteria. To remain and exert probiotic potential within their host, two factors are usually considered. First, probiotic strains must possess the ability to overcome the extremely low pH and the detergent effect of bile salts and arrive at the site of action in a viable physiological state (Chou and Weimar, 1999). Second, they should be capable of adhering to the intestinal mucosa and coaggregation.

Many lactobacillus strains isolated from various sources are being used as probiotic agents and it is unlikely that each species/strain possesses all of the desired characters that will make it a suitable probiotic. More investigations are required to study the survival of probiotics during pass on gastrointestinal tract into intestinal where the probiotic live. Some studies have been done to evaluate the tolerance of probiotics on gastrointestinal tract conditions (Gibson and Fuller, 2000; Holzapfel *et al*., 2001). The microbial cultures to be used as probiotics should be screened for their resistance to acidity (Conway *et al*., 1987; Havenarr, 1992; Charteris *et al*., 1998) and high temperature (Niamsup *et al*., 2003; Boonkumklao *et al*., 2006) due to the body temperature of chickens 42°C (Dawson and Whittow, 2000) and resistant to some antibiotics that commonly added to poultry ration (Gilliland *et al*., 1984; Niamsup *et al*., 2003).

LABs have been isolated from a variety of habitats. The variety of habitat of LAB explore have different activity. According to Okada (2003) has been cited by Surono (2004) that characteristics and properties of LAB isolated from plants and from milk are different to

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ferment various type of carbohydrates, for example *Lactobacillus delbrueckii bulgairicus* was isolated from milk cannot be grown on the plant because it cannot utilize maltose, while derived from fruit juice cannot be grown on milk because it cannot ferment lactose. The most common use of probiotic microorganisms is in fermented dairy products (Ouwehand *et al*., 2002). According to Leeson and Summers (2001), the major ingredients of poultry ration are corn and meals, which together to achieve 70-80% of the components of a feed. Therefore the using of probiotics while derived from dairy products is not optimal. It is therefore important to isolate the probiotic from the plant, one of the processing Virgin Coconut Oil (VCO).

Virgin Coconut Oil (VCO) or pure palm oil, represent especial product was made from coconut milk fresh with natural fermented method. VCO made without warm-up and treatment of chemistry. VCO was had enough recognized by Indonesian society widely. Purwati *et al*. (2006) reported that the byproduct of VCO processing contained LABs. Husmaini *et al*. (2007) reported that administration of blondo (byproduct of VCO processing) up to 15% in the diet can promote weight gains and feed efficiency in broiler chickens. This research aims to study the growth and survival of LABs isolated from processing byproduct of the VCO in *in vitro* conditions for use as probiotics in poultry.

MATERIALS AND METHODS

Isolation of lactic acid bacteria: Lactic Acid Bacteria (LAB) were isolated from byproduct VCO. The samples were serially diluted in to de Man Rogosa and Sharpe (MRS) broth (Mercks). Anaerobic cultivation was done following method of Hungate (1969) in MRS (Mercks) agar containing 0.5% $CaCO_3$. Incubation was carried out at 37°C for 3 days. Strain LABs were detected from those which produced clearing zone surrounding their colonies. Several characteristics of the strains was evaluated, including catalase reaction, gram stain and cell morphology. The LAB was stored in glycerol stock (15% glycerol + 85% MRS broth) on -20°C.

Starting inoculums preparation: For determining a starting inoculums, a loopful of each colony from glycerol stock were transferred into a universal bottle containing 10 ml MRS broth (Merck) and grown at 37°C under vigorous shaking. After 17-24 h, the culture was centrifuge (10.000 rpm, 5 min) followed by suspension of the pellet cells with 0.85% NaCl steril to the range absorbance (0.05) that proportional to the bacterial starting inoculum's population. At interval absorbance, samples were taken in which optical density (OD_{580}) was measured using a Spectrophotometer (Cary 50Bio UV Visible Spectrophotometer).

Growth and survival measurement: Forty eight LABs were isolated from byproduct of VCO and four of isolate were selected to further experiment (Lh1, Lh2, Lh3 and Lh4). The growth and survival rate of LABs were examined in both 37°C and 42°C temperature incubation. Tolerance of LABs to acidic pH was determined by growing of bacteria in acidic MRS broth at pH 2.0, 5.7 and 7.0. The pH was adjusted with 1 N HCl and 0.5 N NaOH. The survival of bacteria was evaluated after 15, 30, 60, 90 and 120 and 300 min of incubation. Tolerance of LABs to gastric juice transit was determined, as described by Dunne *et al*. (2001). For this purpose each isolated bacterial cultures were mixed with 3 ml of stimulated gastric juice and 1 ml of phosphate buffer saline at the starting inoculums. Bacterial survival was evaluated after 30, 60, 90 and 120 min of incubation.

Antibiotic sensitivity test (streptomycin, tetracycline, penicillin, chloramphenicol, erythromycin, ampicillin, oxytetracithin, sulphamethoxazole) was performed by Muller Hinton's agar. An inoculums of the test organism was prepared. Turbidity of the suspension was adjusted to 0.5 at 580 nm wavelength. Overnight incubation of this inoculums produced semi-confluent growth. Sterile cotton swabs were impregnated with the test and control organisms separately. These swabs were used to inoculate the specified areas of the petridishes. Antibiotic discs were applied with light pressure on the agar surface using flamed forceps after the inoculums had dried. The petridishes were incubated at 37°C for 24 h. The radial width of the zones outside the antibiotic discs was measured in mm. The results were interpreted based on the measurement of zone of inhibition (mm) in test organisms as follows: (a) sensitive: equal to, greater than or not less than 3-mm, (b) intermediate: $\geq$2-mm, (c) resistant: $\leq$2-mm.

RESULTS AND DISCUSSION

Isolated of LAB: In the present study, LAB species were isolated from byproduct of VCO processing. A total of forty eight strains of LAB which showed clearing zone surrounding their colonies were isolated. All bacterias were measured for their lactic acid production and detected as lactic acid bacteria. Four LABs have highest lactic acid production were chosen for further studies. There are Lh1, Lh2, Lh3 and Lh4. LABs candidate used as probiotics in poultry should be resistant to gastrointestinal stress conditions for their metabolic activity, as well as to colonize in the gastrointestinal tract. Therefore, it is necessary to evaluate the resistance ability of bacteria to gastrointestinal stress before their were used as probiotics. The isolated LABs were tested for resistance to temperature incubation, acidic pH, gastric juice transit and their resistant ability to some antibiotics.

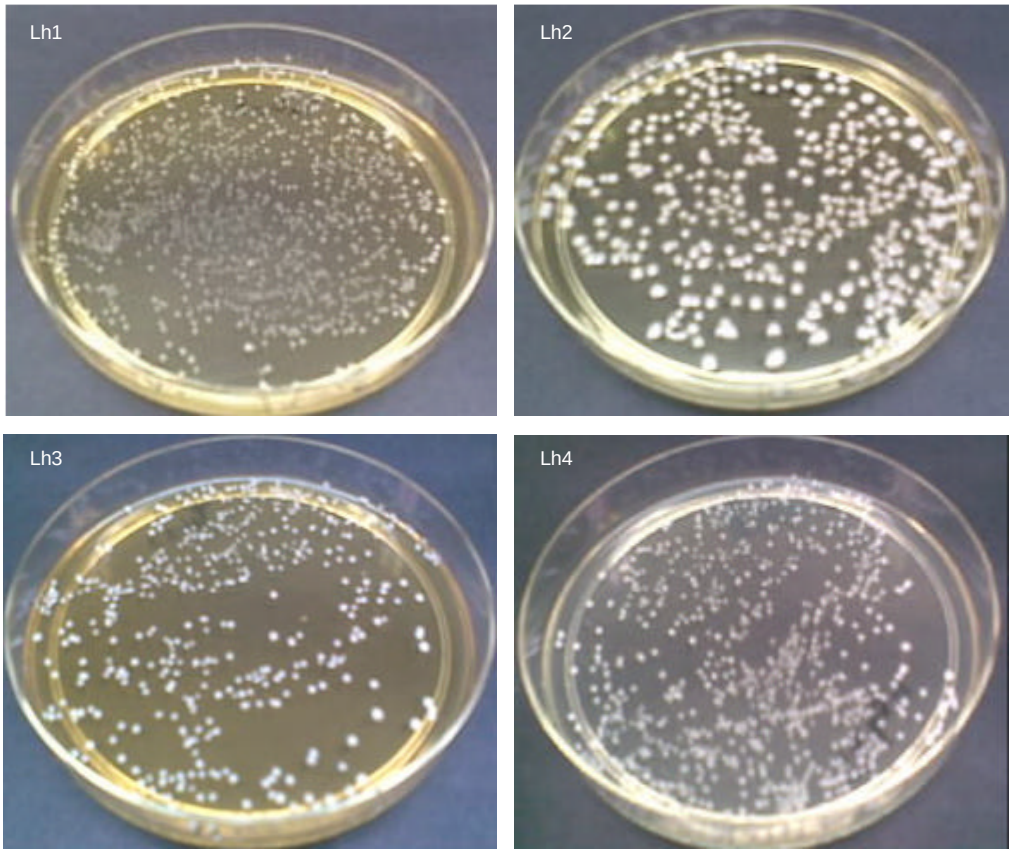

Fig. 1: The performance of isolated LABs after 48 h incubation on 37°C temperatures

Growth and survival: A temperature in the digestive tract of poultry is a 42°C (Dawson and Whittow, 2000), so that the candidate will be used as probiotic bacteria should be able to grow and survive at that temperature. The effects of temperature incubation on the LABs growth were shown in Fig. 1. All bacteria demonstrated growth at an incubation temperature of 37°C, but at 42°C only Lh1 and Lh4 bacteria can grow. Lh4 grow better than Lh1 at 42°C temperature, it mean Lh4 showed the highest tolerance among the four strains. Tolerance to temperature is vary in every strain. Based on the optimum temperature of growth, LAB are classified into 2 groups: the group mesophilic with an optimum growth temperature is 25°C, maximum temperature is 37-40°C and groups of thermophilic with optimum growth temperature is 37-40°C and the maximum temperature is 45-52°C. Some bacterias have the psychotropic characteristic (the ability to grow at 5°C or less like genus Leuconostoc and some facultative heterofermentative Lactobacillus species, especially *Lactobacillus sake* (Surono, 2004).

Tolerance level of all species to acidic environment was found significantly ($p<0.05$). Lh1, Lh2 and Lh3 could not survive at pH 2.0 and their OD values decreased. Lh4 was most resistant at acidic pH and its OD value increased at pH 2.0. There was no significant ($p>0.05$) difference among other species at pH 5.7 (Table 1). The ability of bacteria to survive at pH 7 varied. Observations on 15 min incubation showed only Lh4 that survived while 3 other bacteria showed low OD values, but after 180 min of incubation, OD values of LH2, Lh3 and Lh4 has increased. At 300 min incubation, all bacteria tested showed the ability to grow. According to Denbow (2000) the pH of digestive tract of chicken at proventriculus and gizzard are 2.5 and 4.8 respectively. Comparison to humans and domestic animals such as pigs and cattle, the alimentary tract of chicken is shorter. The time required for feed to pass through the entire alimentary canal is as short as 2.5 h (Duke, 1977). According to Ouwehand *et al*. (1999) the probiotic microorganisms are able to reach the Gastrointestinal Tract (GIT) and remain viable there for 4 h or more. Thus in this study only Lh 4 that has the ability to survive at pH 2 and pH 7. Tolerance level of all species to gastric juice environment was found no significantly ($p>0.05$). Lh1, Lh2, Lh3 and Lh4 were resistant at gastric juice. Tolerance of isolated LAB to gastric transit was evaluated after 30, 60, 90 and 120 min of incubation. Viable count of Lh1, Lh2, Lh3 and Lh-4 was found no significantly ($p>0.05$) Lh-3 were most sensitive to gastric juice but commonly the tolerance level was also no significantly ($p>0.05$) among all the species (Table 2).

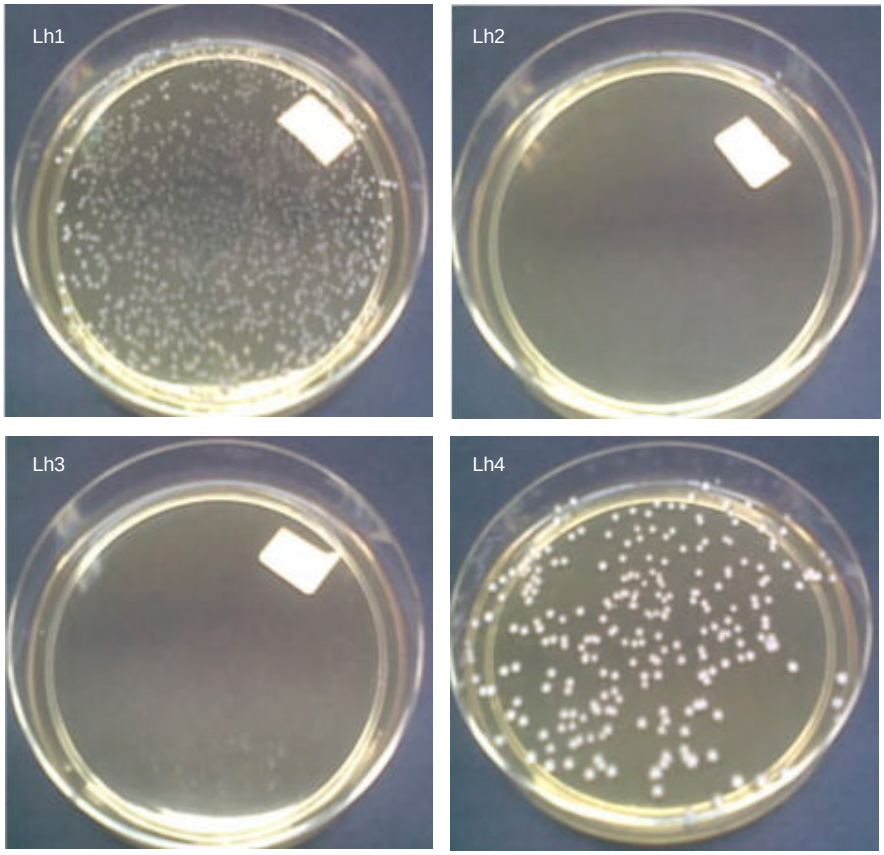

Fig. 2: The performance of isolated LABs after 48 h incubation on 42°C temperatures

Table 1: The Optical Density (OD) of LABs at different pH values

		Incubation time (minutes)				
		15	30	60	180	300
pH = 2	Lh1	0.091	0.029	0.080	0.032	0.034
	Lh2	0.030	0.019	0.029	0.185	0.068
	Lh3	0.623	0.042	0.054	0.023	0.066
	Lh4	0.631	0.941	1.213	1.610	1.890
pH = 5.7	Lh1	0.424	0.628	1.154	1.746	2.272
	Lh2	0.307	0.690	1.309	1.930	2.418
	Lh3	0.495	0.631	1.241	1.813	2.210
	Lh4	0.631	0.931	1.331	1.631	2.631
pH = 7.0	Lh1	0.044	0.089	0.236	0.387	1.166
	Lh2	0.094	0.193	0.418	1.168	1.445
	Lh3	0.057	0.269	0.487	1.067	1.546
	Lh4	0.487	1.067	1.467	1.974	2.532

Table 2: Mean values of viable count (Log_{10}) of LABs after gastric juice transit

Incubation time (minutes)	Lh1	Lh2	Lh3	Lh4
30	4.31	4.40	3.56	5.69
60	3.92	3.72	3.02	5.47
90	3.52	3.62	2.72	4.92
120	3.23	3.42	2.32	3.92

The sensitivities LAB to antibiotic test was show in Table 3. In this case, the strongest antimicrobial effect was shown by Lh4 while Lh1 and Lh2 only resistant to streptomycin and penicillin and Lh3 not resistant against all antibiotics tested (Table 3). The antimicrobial action due to the potential of Lh4 to produce lactic acid and bacteriocines. Strus *et al*. (2001) reported that these bacteria produce peptides having inhibitory properties. Lh4 was no sensitive to all antibiotic respectively (clear zones: 0.33 mm), however the other LABs were sensitive (clear zones: 5-12 mm) except Lh1 and Lh2 no sensitive to streptomycin (clear zones: 1.67 dan 1.00).

Table 3: Sensitivities of isolated LABs to some antibiotic

	Clear zones (mm)			
LAB	K	S	P	C
Lh1	4.67	1.67	2.00	5.67
Lh2	4.00	1.00	2.50	7.50
Lh3	2.00	2.50	10.00	10.50
Lh4	0.33	0.33	0.33	0.33
	Clear zones (mm)			
LAB	E	AMP	OT	TE
Lh1	5.00	4.00	3.33	6.67
Lh2	3.50	7.00	6.50	8.00
Lh3	12.00	9.50	8.00	8.00
Lh4	0.33	0.33	0.33	0.33

K: Kanamycin, P: Penicillin, E: Erythromycin, OT: Oxytetracithin, S: Streptomycin, C: Chloramphenicol, AMP: Amphycylin, TE: Tetracycline

Conclusion: The conclusion of this study is that lactic acid bacteria have been isolated from waste VCO have different growth and survive *in vitro*. Only Lh4 potentially be used as probiotics in poultry due to its ability to grow at 42°C incubation temperature, have an increased OD value at pH 2 and pH 7 and is resistant to all antibiotics tested, commonly added to poultry feed.

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