PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

Pakistan Journal of Nutrition 15 (6): 519-523, 2016
ISSN 1680-5194

Molecular Characteristics and Identification of Lactic Acid Bacteria of Pineapple Waste as Probiotics Candidates for Ruminants

Mardalena, S. Syarif and S. Erina
Faculty of Animal Husbandry, Jambi University, Jambi, Indonesia

Abstract: The current study aims to identify the potential of lactic acid bacteria (LAB) from pineapple waste as candidate probiotics for ruminants. LAB were isolated from the skin of ripe pineapple (*Ananas comosus* L. Merr). Pineapple skin was crushed in a chopper then placed in a sealed container and stored in an aerobic conditions for a week. LAB antimicrobial tests were conducted on four types of pathogenic bacteria *E. coli* (NBRC14 and 237), *Staphylococcus aureus* (NBRC13 and 267), *Bacillus cereus* and *Staphylococcus aureus*). Bile tolerance testing was performed using 0.3% (w/v) and 0.5 (w/v) oxgall bile solution LAB grown only in MRS medium were used as a control. Tolerance to bile salts was calculated based on the log difference in the number of bacterial colonies growing under control versus treatment condition. The smaller the difference, the more tolerant LAB were to bile salts. Sequence analysis was performed using DNAstar software. Sequence alignment was analyzed by comparing the queried PCR sequences with those the NCBI Gene Bank data base using BLAST and then kinship examined with Clustal W. Lactobacillus plantarum isolates extracted from pineapple skin were able to live and thrive at room temperature for 6-h in storage and with stand a 0.3% (w/v) bile salts solution while maintaining optimum growth. These LAB were also able to inhibit the growth of pathogenic bacteria as Salmonella typhimurium, *Escherichia coli*, *Bacillus cereus* and Staphylococcus. The result suggest that LAB extracted from pineapple skin waste including *L. plantarum* and *L. pentosus* have the potential to be used as probiotics for ruminants.

Key words: Pineapple waste, lactic acid bacteria (LAB), probiotics

INTRODUCTION

Pineapple waste such as the skin contains vitamin C, flavonoids, saponins and phenols which have been shown improve milk quality (Mardalena *et al*., 2011) and body weight gain (Mardalena, 2012) in livestock by increasing the digestibility of feed and energy sources in the form of volatile fatty acid (Mardalena *et al*., 2014). Aside from being a source of antioxidants, pineapple skin waste also contains lactic acid bacteria (LAB). Mardalena *et al*. (2015) found that LAB isolated from fermented pineapple skin form yellowish-white shiny, round colonies.

The term "probiotics" comes from the Greek word meaning "to live". The term was first used by Lilley and Stillwell in 1965 and defined as a substance that was produced by microbes that can stimulate growth of other microbes (Sunaryanto *et al*., 2014). In fact probiotics are live microbial feed additive that can improve the balance of microbes in the digestive tract of host animals, thereby increasing health and productivity (Fuller, 1989). Recent trends have favored the use of natural feed additives to manipulate fermentation in the rumen as a replacement for additives such as antibiotics and chemicals that are ionophore increasing (Wallace *et al*., 2002; Hristov *et al*., 2003). Feed Additive are considered ideal if they meet certain requirements such as not being harmful to animals, humans, or the environment and do not leave residues in the body of animals products (Santoso *et al*., 2013).

Salminen and Wright (2004) stated that the main microorganisms contained in probiotics are fungi, such as *Aspergillus oryzae*, *Saccharomyces cerevisiae* and LAB including *L. plantarum* and *L. acidophilus*. Bacteria can be probiotic if they can survive the stomach and small intestine, being able to tolerant of acidic conditions and bile salts (Tuomola *et al*., 2001; Sunny-Roberts and Knoor, 2008). In addition, probiotic bacteria as a group should also be safe or generally recognized as safe. Results of some previous studies have indicated that use of *Saccharomyces cerevisiae* as a probiotic in ruminants can increase productivity (Newbold, 1995) and reduce methane emissions (Lila *et al*., 2004; Mwenya *et al*., 2004). Currently, there is no information about the potential of LAB derived from pineapple skin as probiotics in ruminants. Therefore, the present study aimed to isolate and identify LAB strains in pineapple skin waste and test their potential use as probiotics in ruminants.

MATERIALS AND METHODS

Lab and gram staining: LAB were isolated from the skin of ripe pineapple (*Ananas comosus* L. Merr). Pineapple

Corresponding Author: Mardalena, Faculty of Animal Husbandry, Jambi University, Jambi, Indonesia

skin was crushed in a chopper then placed in a sealed container and stored in an aerobic conditions for a week. After, LAB isolates were sup planted in liquid the Man Rogosa Sharp (the RMS) medium, then streaked onto solid medium in an anaerobic jar to verify their purity. The rejuvenated LAB isolates were grown for 24 h in liquid MRS medium in an anaerobic jar for better growth and incubated at room temperature. When cultures were ready for testing LAB colonies were Gram staining according to the method of Dwidjoseputro (1994).

LAB cultures: LAB bacteria were grown in MRS medium for 24 h at room temperature. A total of 1 lub of bacteria were inoculated in 50 ml of liquid MRS medium, placed in an anaerobic jar then incubated at room temperature for 24 h as a sub-culture. After a sub-culture 24 h, bacteria were cultured in 10% liquid MRS medium by pipetting 10 ml of the sub-culture in 90 ml of fresh liquid MRS medium and then spread by culture ages (0-8 h in 1 h intervals). Spreading was completed by serially diluting up to 100 µl and distributing them into petri dishes containing solid MRS medium and incubated at room temperature for 48 h. LAB culture production was then calculated bacterial growth curve created (Mustopa, 2009).

LAB isolates with antimicrobial activity: LAB antimicrobial tests were conducted on four types of pathogenic bacteria *E. coli* (NBRC14 and 237), *Staphylococcus aureus* (NBRC13 and 267), *Bacillus cereus* and *Staphylococcus aureus* (ATCC 25923, Lab Collection PAU IPB Bogor, Indonesia). LAB cultures (3 ml) were centrifuged at 8000 rpm for 5 min at 4°C and the supernatants used for antimicrobial testing. Bacteria (200 ml) already rejuvenated in Nutrient Broth were mixed with 20 ml of liquid Nutrient Pudding 40°C before being poured into a sterile petri dish and allowed to stand for 30 min to harden. Using sterile tweezers, paper discs were placed above, sterile tweezers Nutrient Pudding was then dropped into 20 ml of LAB supernatant with a micro pipette and placed into antibiotic penicillin testing of paper as a comparison, then incubated at 37°C anaerobically. The zone of inhibition surrounding each colony was measured after 24 h using a ruler (Mustopa, 2009).

LAB isolate tolerance to bile salts: Bile salt tolerance is a prerequisite for LAB colony formation and activity in host digestive system (Havenaar *et al*., 1992). Bile tolerance testing was performed using 0.3% (w/v) and 0.5 (w/v) oxgall bile solution LAB grown only in MRS medium were used as a control. Tolerance to bile salts was calculated based on the log difference in the number of bacterial colonies growing under control versus treatment condition. The smaller the difference, the more tolerant LAB were to bile salts.

Lab isolate identification polymerase chain reaction (PCR): Identification of LAB strains was completed with PCR amplication of 16S rRNA gene amplification by Polymerase Chain Reaction (PCR) and NCB (Basic Local Alignment Search BLAST) analysis of DNA sequences. http://www.ncbi.nlm.nih.gov.

Sequence analysis was performed using DNAstar software. Sequence alignment was analyzed by comparing the queried PCR sequences with those the NCBI Gene Bank data base using BLAST and then kinship examined with Clustal W (Mustopa, 2009).

RESULTS AND DISCUSSION

Rejuvenation of LAB and gram staining: LAB isolate rejuvenation produced pure cultures used for experimental assays such as for Gram staining (Fig. 1). Rejuvenation of bacteria was necessary to purify bacteria kept in refrigeration and produced more young bacteria. Unus (2005) showed that if bacteria are too old, they can absorb safranin (red color) and cause Gram-positive to be misidentified as Gram-negative.

Morphological examination revealed LAB isolates were rod-shaped, Gram-positive, catalase-negative and dark purple in color. Surono (2004) stated that variation in LAB characteristics is normal, but all are Gram-positive bacteria. Fardiaz (1992) classified of LAB and catalase-negative, as *Lactobacillus*, while spherical bacteria with an array of short and long chains belong to the genus, *Streptococcus*. These results indicate that the LAB isolates are potential candidate probiotics.

Culture of LAB isolates: Table 1 shows that optimal LAB growth was obtained after being stored for up to 6 h at room temperature but decreased drastically there after. After 6 h in storage, the average number of LAB obtained was 102 x 10^8 CFU/g. LAB isolates left at room temperature for up to 6 h could be -5°C to avoid bacterial death.

Figure 2 shows that LAB growth consisted of several phases of activity. According to Urnemi (2012) HB3.3 LAB growth is comprised four phases; lag, exponential growth, stationary growth and death. In the lag phase (0-3 h), the bacteria will acclimatize to environmental

Table 1: Growth of lactic acid bacteria time at room temperature

	---------- Bacteria No. (CFU/g) ----------		
Incubation time (h)	KLN1 Isolate	KLN2 Isolate	KLN3 Isolate
0	3.8 x 10^8	4.0 x 10^8	3.9 x 10^8
1	4.6 x 10^8	5.2 x 10^8	4.9 x 10^8
2	5.8 x 10^8	5.8 x 10^8	5.8 x 10^8
3	7.4 x 10^8	7.5 x 10^8	7.4 x 10^8
4	15.2 x 10^8	16.0 x 10^8	15.6 x 10^8
5	20.7 x 10^8	20.0 x 10^8	20.4 x 10^8
6	101 x 10^8	103 x 10^8	102 x 10^8
7	40 x 10^8	44 x 10^8	42 x 10^8
8	29 x 10^8	26 x 10^8	28 x 10^8

Table 2: Lactic acid bacteria antagonist test at pH 4.37 and 6

	Inhibition zone diameter (cm)							
	pH 4.37				pH 6			
	Well		Clear zone		Well		Clear zone	
Pathogenic bacteria type	1	2	1	2	1	2	1	2
B. cereus	0.75	0.75	1.45	1.45	0.75	0.75	1.3	1.35
E. coli	0.75	0.75	1.45	1.45	0.75	0.75	1.3	1.3
Staphylococcus aureus	0.75	0.75	1.45	1.45	0.75	0.75	1.3	1.3
S almonella typhimurium	0.75	0.75	1.25	1.25	0.75	1.15	1.15	1.15

Table 3: Results of BLAST analysis of lactic acid bacteria isolate DNA sequences

Accesion Number	BLAST result	Max score	Query coverage	E-value	Max. Identify
FJ 749374.1	*Lactobacillus plantarum* IMAU-4	2352	99%	0.0	99%
LC 071808.1	*Lactobacillus pentosus* JCM-1558	2351	99%	0.0	99%
KP 889230.1	*Lactobacillus plantarum*				
FJ-005	2351	99%	0.0	99%	
KT 327853.1	*Lactobacillus plantarum*				
S-27	2351	99%	0.0	99%	

Table 4: *Lactobacillus plantarum* resistance to bile salts

Treatment	Time incubation (h)	No. of cells (CFU/ml)
MRS	0	1.5×10^{-8}
	3	7.2×10^{-8}
MRS+0.3 GE	0	2.0×10^{-8}
	3	7.7×10^{-8}
MRS+0.5 GE	0	2.8×10^{-8}
	3	7.6×10^{-8}

GE: Bile salts. MRS: de Man Rogosa Sharp medium

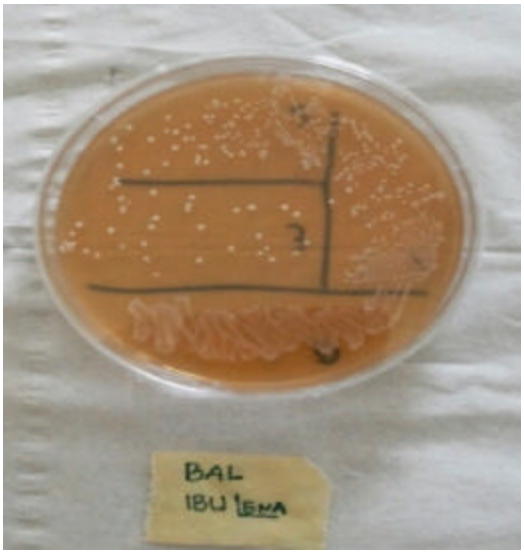

Fig. 1: Culture of pure lactic acid bacteria isolates

conditions (pH, temperature, nutrients, etc); this phase of bacterial growth is slow. In the exponential phase (4-6 h) the bacteria grows very quickly. In the stationary phase, there is neither an increase nor a decrease in the number of bacterial cells because the growth rate is equal to the death rate. Lastly the death phase (7-24 h) begins when the number of dead or drying cells exceeds that of growing cells.

Antimicrobial potential of LAB isolates: We found that pineapple skin LAB isolates were antagonistic toward four types of pathogenic bacteria including *Salmonella typhimurium* (ATCC 14028), *E. coli* (ATCC 25922), *B. cereus* and *Staphylococcus aureus* (ATCC 25923) (Table 2).

LAB isolates showed large zones of inhibition reaching between 1.15 to 1.45 cm in diameter. Zone of inhibition results of in the present study are larger than those reported by Yurleni *et al*. (2014), who showed that LAB isolates from fermented durian (DFY1) had a 1-1.3 cm zone of inhibition, while Urnemi (2012) reported that the zone of inhibition of LAB isolates from fermented Trinitario/hybrid cocoa varieties ranged from 27.00-32.50 mm. LAB isolates herein were found to have substantial antimicrobial activity as shown by their large zone of inhibition at pH 4.37 and pH 6. According to Surono (2004) most bacteriocins produced by probiotics are bactericidal causing loss of membrane potential. Pelczar *et al*. (1993) also suggested that the antimicrobial compounds produced by probiotics can be used to inhibit microbial growth altogether and/or kill microbes by damaging cell walls and membranes leading to lysis or inhibition of the synthesis components.

LAB isolates identification: BLAST results showed that LAB isolates had 99% query coverage with various strains of *L. plantarum* (Table 3).

The most commonly used probiotic bacteria are *Lactobacilli* because this group of bacteria possess almost all of the necessary characteristics of a probiotics. *Lactobacilli* can lower the pH of the intestinal environment by converting glucose into lactic acid, thereby inhibiting the growth of some types of pathogenic bacteria (Gotcheva *et al*., 2002). As a potential probiotic. LAB must be able to successfully compare with inhibit pathogenic bacteria to maintain the balance of intestinal microflora (Gildberg *et al*., 1997).

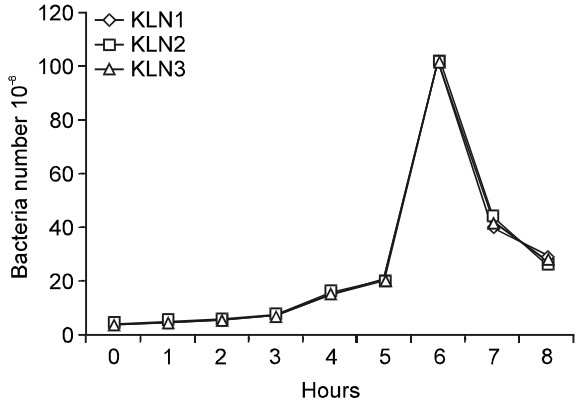

Fig. 2: Lactic acid bacteria culture curve

Gaggia *et al.* (2010) stated that LAB can be used as probiotics to improve gut microbiota because of their ability to produce various antimicrobial substances including lactic acid, alcohol, carbon dioxide, diacetyl, hydrogen peroxide, bacteriocin and other metabolites.

***L. plantarum* bile salt tolerance:** According to Bezkorovainy (2006), the most serious obstacle for probiotics in the intestines is bile salts and previous studies of probiotic bile salt tolerance *in vitro* can be divided into two types tolerance and growth. His study found *Lactobacilli* oxgall for up to 3 h and were generally more tolerant than *L. bifidobacterium*. The results of *L. plantarum* tolerance to bile salts are presented in Table 4 and indicate that *L. plantarum* isolates were quite tolerant of both 0.3% (w/v) and 0.5% (w/v) bile salts. However, tolerance was higher with 0.3% (w/v) bile salts. Du Toit *et al.* (1998) suggested that LAB tolerance to bile salts can be attributed to the enzyme bile salt hydrolase, which helps hydrolyze conjugated bile salts, reducing the toxic effects of bile on bacterial cells.

Conclusion: Herein, we found that LAB isolates could be left at room temperature for up to 6 h and still have viable growth. LAB isolates from the pineapple skin waste were identified as *L. plantarum*. *L. plantarum* was able to withstand bile salts at a concentration of 0.3% (w/v) and still maintain good growth. Further more, result showed that *L. plantarum* LAB isolates were able to inhibit growth of pathogenic bacteria. Including *Salmonella typhimurium*, *E. coli*, *B. cereus* and *Staphylococcus aureus*. Therefore *L. plantarum* has the potential to be used as a probiotic in ruminants.

ACKNOWLEDGMENTS

The authors thank the Directorate General of Research and Community Service, from the Ministry of Research and Technology (Indonesia) for providing research funds [competitive grants program to contract no.: 103 / UN21 / PL / 2015 (March 27, 2015)].

REFERENCES

Bezkorovainy, A., 2006. Probiotics: determinants of survival and growth in the gut. Am. J. Clin. Nutr., 72: 399-405.

Du Toit, M., C.M. Franz, L.M. Dicks, U. Schillinger, P. Harberer, B. Warlies, F. Ahrens and W.H. Holzapfel, 1998. Characterisation and selection of probiotic lactobacilli for a preliminary minipig feeding trial and their effect on serum cholesterol levels, faeces pH and faeces moisture content. Int. J. Food Microbiol., 40: 93-104.

Dwidjoseputro, D., 1994. Basics of Microbiology. Jakarta: Djambatan.

Fuller, R., 1989. Probiotics in man and animals. J. Appl. Bacteriol., 66: 365-378.

Fardiaz, S., 1992. Food Microbiology I. Gramedia Pustaka Utama, Jakarta.

Gaggia, F., P. Mattarelli and B. Biavati, 2010. Probiotic and prebiotics in animal feeding for safe food production. Int. J. Food Microbiol., 14: 515-528.

Gildberg, A., H. Mikkelsen, E. Sandaker and E. Ringo, 1997. Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (Gadus morhua) Hydrobiologia, 352: 279-285.

Gotcheva, V., E. Hristozova, T. Hristozova, M. Guo, Z. Roshkova and A. Angelov, 2002. Assessment of potential probiotic properties of lactic acid bacteria and yeast strains. Food Biotechnol., 16: 211-225.

Havenaar, R., B.T. Brink and J.H.J. Veld, 1992. Selection of strains for probiotic use. In: Probiotics. The Scientific Basis, R. Fuller Ed. Chapman and Hall, London, pp: 209-221.

Hristov, A.N., M. Ivan, L. Neill and T.A. McAllister, 2003. Evaluation of several potential bioactive agents for reducing protozoal activity *in vitro*. Anim. Feed Sci. Technol., 105: 163-184.

Lila, Z.A., N. Mohammed, T. Yasui, Y. Kurokawa, S. Kanda and H. Itabashi, 2004. Effects of a twin strain of *Saccharomyces cerevisiae* live cells on mixed ruminal microorganism fermentation *in vitro*. J. Anim. Sci., 82: 1847-1854.

Mardalena, L. Warly, E. Nurdin, R.W.R. Ningrat and Farizal, 2011. Milk quality of dairy goats by giving feed supplement as antioxidant source. J. Ind. Trop. Ani. Agric., 36: 205-211.

Mardalena, 2012. Feed supplements evaluation as antioxidants source and its effect on physiological response and productivity of dairy goats.. Dissertation. Andalas University.

Mardalena, Yurleni, S. Novianti and U. Amri, 2014. Evaluation of feed supplement as antioxidant source to rumen ecology. Pak. J. Nutr., 13: 381-385.

Mardalena, S. Syarif and S. Erina, 2015. The substitution effect grass with palm fronds fermented with prolinas against quality content of milk quality and production of dairy cattle. Report of Competitive Grant Research Year 1. Jambi University.

Mustopa, A., 2009. Collection Protocol for Molecular Virology Laboratory. Biotechnology Research Center. LIPI Indonesia, Bogor.

Mwenya, B., B. Santoso, C. Sar, Y.Gamo, T. Kobayashi, I. Arai and J. Takahashi, 2004. Effects of including β 1-4 galacto-oligosaccharides, lactic acid bacteria or yeast culture on methanogenesis, energy and nitrogen metabolism in sheep. Anim. Feed Sci. Technol., 115: 313-326.

Newbold, C.J., 1995. Probiotics for ruminants. Ann. Zootech., 45: 329-335.

Pelczar, M.J., E.C.S. Chan Jr and N.R. Krieg, 1993. Microbiology. 5th ed. New Delhi (India): Tata McGraw-Hill.

Salminen, S. and A.V. Wright, 2004. Lactic acid bacteria. microbiology and functional aspects. 2nd ed. New York NY (USA): Marcell Dekker, Inc.

Santoso, B., A. Maunatin, B.T. Hariadi and H. Abubakar, 2013. Isolation and identification of lactic acid bacteria of king grass (*Pennisetum purpureophoides*) as a candidate probiotic in animals. JITV, 18: 131-137.

Sunaryanto, R., E. Martius and B. Marwoto, 2014. Test Capabilities as agencia probiotic of *Lactobacillus casei*. J. Bioteknologi and Biosains Indonesia, 1: 9-15.

Sunny-Roberts, E.O. and D. Knoor, 2008. Evaluation of the response of Lactobacillus rhamnosus VTT E-97800 to sucrose induced osmotic stress. Food Microbiol., 25: 183-189.

Surono, I.S., 2004. Probiotics, Fermented Milk and Health. Cipta Karya, Jakarta.

Tuomola, E., R. Crienden, M. Playne, E. Isolauri and S. Salminen, 2001. Quality assurance criteria for probiotic bacteria. Am. J. Clin. Nutr., 73: 393S-398S.

Urnemi, 2012. Isolation, determination of antimicrobials and molecular characterization of lactic acid bacteria fermnetasi cocoa bean (*Theobroma cacao Lin*) from West Sumatra and its application to support public health. Dissertation andalas University.

Unus, S., 2005. Basic Microbiology. Papas Sinar Sinanti, Jakarta.

Wallace, R.J., N.R. McEwan, F.M. McIntosh, B. Teferedegne and C.J. Newbold, 2002. Natural products as manipulators of rumen fermentation. Asian-Aust. J. Anim. Sci., 15: 1458-1468.

Yurleni, Mardalena and U. Amri, 2014. Moleculer identification of lactic acid bacteria of durian and Its application to modifier rumen of ruminant. Reports of Competitive Research Grant. Jambi University.