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**ISOLATION, IDENTIFICATION AND ANTIBIOTIC SUSCEPTIBILITY OF
PROBIOTIC *LACTOBACILLUS PLANTARUM* NRC AM10**

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ABSTRACT

The *Lactobacillus* is a very important genus of lactic acid bacteria commonly found in nature in different fermented foods. Different species of this genus were used worldwide in food fermentations as probiotics. Therefore, the purpose of the present study was to isolate, identify probiotic *Lactobacillus* strain from fermented goat milk and determine the antibiotic susceptibility and carbohydrate fermentation patterns. According to the phenotypic characterization and carbohydrate fermentation profile (API[®]50 CHL strip), the tested strain will identified as *Lactobacillus plantarum* NRC AM10 with 99.9 %. The strain confirmed by both PCR and sequencing as the same strain with accession number CP023728.1. In addition, our results indicated that the isolated strain showed sensitivity toward nine different types of antibiotic discs (Penicillin G, Erythromycin, Ampicillin10 and 25 µg, Cephalocin10 and 30 µg, Clindamycin, Cortimoxazole and Nitrofurantion). On the other hand, the tested strain showed good resistance to the other types of antibiotic discs. The strain showed a good acidity development in skim milk and able to coagulate the milk after 16 h of incubation time. In conclusion, the natural fermented dairy products are good valuable sources for isolation of lactic acid bacteria with good probiotic and technological characteristics.

**Keywords: Lactic acid bacteria, *Lactobacillus plantarum*, probiotic dairy products,
antibiotic discs, technological characteristics**

INTRODUCTION

Nowadays, the consumption of dairy products supplemented with probiotics are receiving great deal of attention all over the world. These probiotics able to support and improvement the human health, enhance the microbial balance in the gut and use in fermented probiotic food productions [1, 2, 3, 4]. Lactic acid bacteria (LAB) have an essential role for extending the shelf life of fermented foods, like yoghurt, cheese, pickled olives, sauerkraut, meat products, vegetables, fish products and sourdough[5, 6]. *Lactobacillus plantarum* strains can use as biocontrol agents against potentially harmful microorganisms during food processing, storage and increase the shelf life, and safety of fermented foods [7]. Probiotics are live microorganisms which when administered in adequate amounts confer a health benefits on the host [8]. Different researches were done to isolate special probiotic lactic acid cultures for enhancing the quality and organoleptic properties of dairy products [9]. Traditional fermented dairy products are good valuable sources of LAB genetic biodiversity [10]. Recently, the use of genotypic characterization represented an advance in the taxonomy and identification of LAB. Several researches based only on the biochemical and phenotypic characterization to identify LAB could not

differentiate accurately between bacterial species. Thus, the use of molecular biology methods is very important and more accurate for identification of LAB [11, 12]. The *Lactobacillus* genus is a widespread lactic acid bacterium commonly found in a wide variety of fermented foods. About 183 species of *Lactobacillus* genus identified and used safely in fermented foods and dairy products [3, 13, 14]. *Lactobacillus plantarum* named firstly by Orla-Jennsen in 1919 as *Streptobacterium plantarum* then due to some biochemical and morphological characteristics done by Pederson in 1936 he renamed as *Lactobacillus plantarum*. It is widely used as a starter or adjuvant culture for the production of fermented foods such as milk, vegetables, coffee, meat and silage. In addition, it can found in numerous ecological niches, including the gastrointestinal tract of humans and animals [15, 16, 17]. The medical treatments by different antibiotics especially in children kill pathogens but in the same time, they also disrupt the beneficial microbes in the intestinal tract. This disruption in the microbial balance leads to antibiotic associated diarrhea AAD [18, 19]. Several studies reported that *Lactobacillus* species were able to antibiotic resistance like *Lb. acidophilus*,

Lb. delbrueckii subsp. *bulgaricus* [20], *Lb. johnsonii* [21], *Lb. reuteri* [22] and *Lb. plantarum* [23]. In fact, some probiotic strains with intrinsic antibiotic resistance could be useful for restoring the gut microbiota after antibiotic treatment [24]. Several meta-analyses that report a relative risk reduction of AAD with the use of probiotics during antibiotic administration [25]. In addition, many studies suggested that probiotics are able to reduce the risk of antibiotic-associated diarrhea [26, 27]. The minimum effective dose is 10^7 to 10^{10} CFU or 10^8 to 10^{10} CFU daily for efficacy across a wide range of indications [28]. Probiotics are one aspect in a comprehensive strategy to restore gut flora after antibiotic use and return a healthy gut ecosystem. This done by restoring the microbial balance in the gut and increasing the beneficial microbes. Therefore, the main goal of present work planned to study the phenotypic and genotypic characteristics of *Lactobacillus* strain isolated from goat's milk.

MATERIALS AND METHODS

Strain isolation and phenotypic characterization

Goat milk sample was homogenized and serially diluted up to 10^{-8} using sterile physiological saline (0.85 % NaCl w/v).

One milliliter was plated onto double sterile petri dishes and MRS agar was poured then plates were anaerobically (BBL Gas Pak Anaerobic System, USA) incubated at 37° C for 72 h. Pure colonies picked up and examined for gram staining, catalase production [2, 29]

Carbohydrate fermentation patterns

The purified strain was tested using API®50 CHL strips incubated at 37° C for 24 and 48 hours. These fermentation profiles were compared with the software APILAB plus V5.1 (BioMerieux, Lyon Company, France).

Genomic DNA extraction and polymerase chain reaction (PCR)

The Genomic DNA was extracted from overnight pure culture according to the procedure of MicroSeq® 500 DNA bacterial isolation kits protocol. The extracted DNA transferred into new micro centrifuge tube for using in the polymerase chain reaction to amplify the target DNA fragment. Twenty micro liter of extracted DNA was amplified using (veriti™ thermal cycler, applied biosystems, Singapore, Indonesia) according to the program presents in table (1). The PCR products were stored at - 20 °C until use in the following step.

Table 1: The program of polymerase chain reaction

Initial step	Each of 30 cycles			Final extension	Final step
	Cycle				
Hold	Melt	Anneal	Extend	Hold	Hold
95/10 min	95/30 Sec	60/30 Sec	72/45 Sec	72/10min	4 °C

Purification and sequencing the PCR product

Polymerase chain reaction product purified and automatically sequenced according to [30]. The obtained genome sequence was analyzed by using applied biosystems[®] 3500 genetic analyzer. The data were compared with the known data in basic alignment search tool (BLAST), NCBI GenBank, www.ncbi.nlm.nih.gov.

Acidifying ability

The acidifying ability of the tested strain was determined according to the methods described by [31, 32] with some modifications. The tested strain activated in 10 ml MRS broth at 37°C for 18 h until O.D at 620_{nm} reached to 1.45 using spectrophotometer (UV-VIS spectrophotometer PD-303 UV LTD Japan). After that a conical flask (200 ml) sterile reconstituted skim milk (12 % W/V) was inoculated with 2 % of the previously subculture. The change of pH was determined using a pH meter (JENWAY, Instrument Mode 3510) at zero, 4, 6, 12, 16 and 24 h of incubation at 37°C.

Antibiotic susceptibility test

The sensitivity of the tested strain to twenty types of multiantibiotic discs was determined using disc diffusion assay according to [33]. The plates were incubated anaerobically at 37 °C for 48-72 h. The sensitivity against antibiotics

evaluated by observation the diameter of inhibition zone around the antibiotic discs. The results were expressed as sensitive (S) or resistant (R).

RESULTS AND DISCUSSION

Phenotypic Characterization

The isolated strain was identified according to the microscopic examination as gram-positive rods, none sporulation, did not produce CO₂ from glucose and catalase negative. On the other hand, according to the carbohydrate fermentation pattern the data sheet illustrate in Figure 1. It was indicated that the isolate able to ferment about 26 type of carbohydrate in API[®]50 CHL strip after 48 h of incubation period and the result sheet was compared with the API[®] database, the isolated strain successfully identified as *Lactobacillus plantarum* with percent 99.9% table (2).

Genotypic Characterization of The Strain

The complete nucleotide sequence and electropherogram of *Lactobacillus plantarum* NRC AM10 were shows in figures (2 and 3). After obtaining, the nucleotide complete sequence of the tested strain it compared with the data deposited in the NCBI Gen-Bank by using the BLAST. After that, the molecular identification showed that the strains was identified as *Lactobacillus plantarum* with ID number CP023728.1 with high

similarity with the phenotypic result as shows in table (3). From our results, we confirmed that, the genotypic techniques are very rapid, accurate, more sensitive and reliable compared with phenotypic identification methods [12, 34].

Acidifying Ability

The acidifying ability of the tested strain expressed as pH decrease in skim milk medium shows in figure (4). The results showed that, the pH values of skim milk medium was gradually decrease in the first 8 hours of incubation at 37° C. by extending the incubation time a complete

coagulum of the milk medium was observed after 16 h . Our findings are in agreement with obtained results of [35, 36, 37]

Antibiotic Sensitivity

As presents in table (4) and figure (5) the tested strain showed high susceptibility and a wide range of resistance to 9 of antibiotic discs Penicillin G, Erythromycin, Ampicillin, Cephalocin, Clindamycin, Cortimoxazole and Nitrofurantion, Whereas the tested strain showed high resistance toward the other antibiotic discs [38].



Figure 1: Carbohydrate fermentation profile

0= Control 1= Glycerol 2= Erythritol 3= D-Arabinose 4= L- Arabinose 5= Ribose 6= D-Xylose 7= L-Xylose 8= Adonitol 9= Methyl-D-Xyloside 10= Galactose 11= Glucose 12= Fructose 13= Mannose 14= Sorbose 15= Rhamnose 16= Dulcitol 17= Inositol 18= Mannitol 19= Sorbitol 20= Methyl-D-Mannoside 21= Methyl-D-Glucoside 22= N-Acetyl-Glucosamine 23= Amygdalin 24= Arbutin 25= Esculin 26= Salicin 27= Cellobiose 28= Maltose 29= Lactose 30= Melibiose 31= Sucrose 32= Trehalose 33= Inulin 34= Melezitose 35= Raffinose 36= Starch 37= Glycogen 38= Xylitol 39= Gentiobiose 40= D-Turanose 41= D-Lyxose 42= D-Tagatose 43= D-Fucose 44= L-Fucose 45= D-Arabitol 46= L- Arabitol 47= Gluconate 48= 2-Keto-Gluconate 49= 5-Keto-Gluconate.

Table 2: Identification result of analytical profile index

EXCELLENT IDENTIFICATION			
Strip	API 50 CHL VS.1		
Profile	-----		
Note			
Significant taxa	% ID	T	Tests against
Lactobacillus plantarum 1	99.9	0.98	

Table 3: Similarity of genotypic and phenotypic identification

Isolate	identification	Strain	%
NRC AM10	Genotypic	Lactobacillus plantarum	100
	Phenotypic	Lactobacillus plantarum	99.9

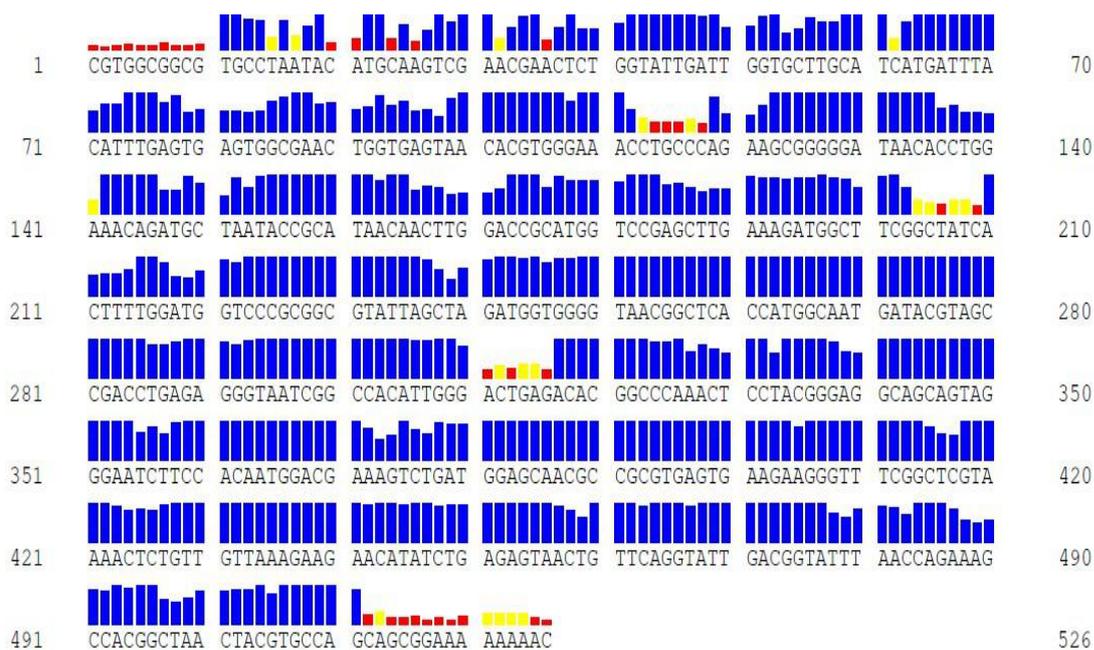


Figure 2: The complete genome sequence of *Lactobacillus plantarum* AM10

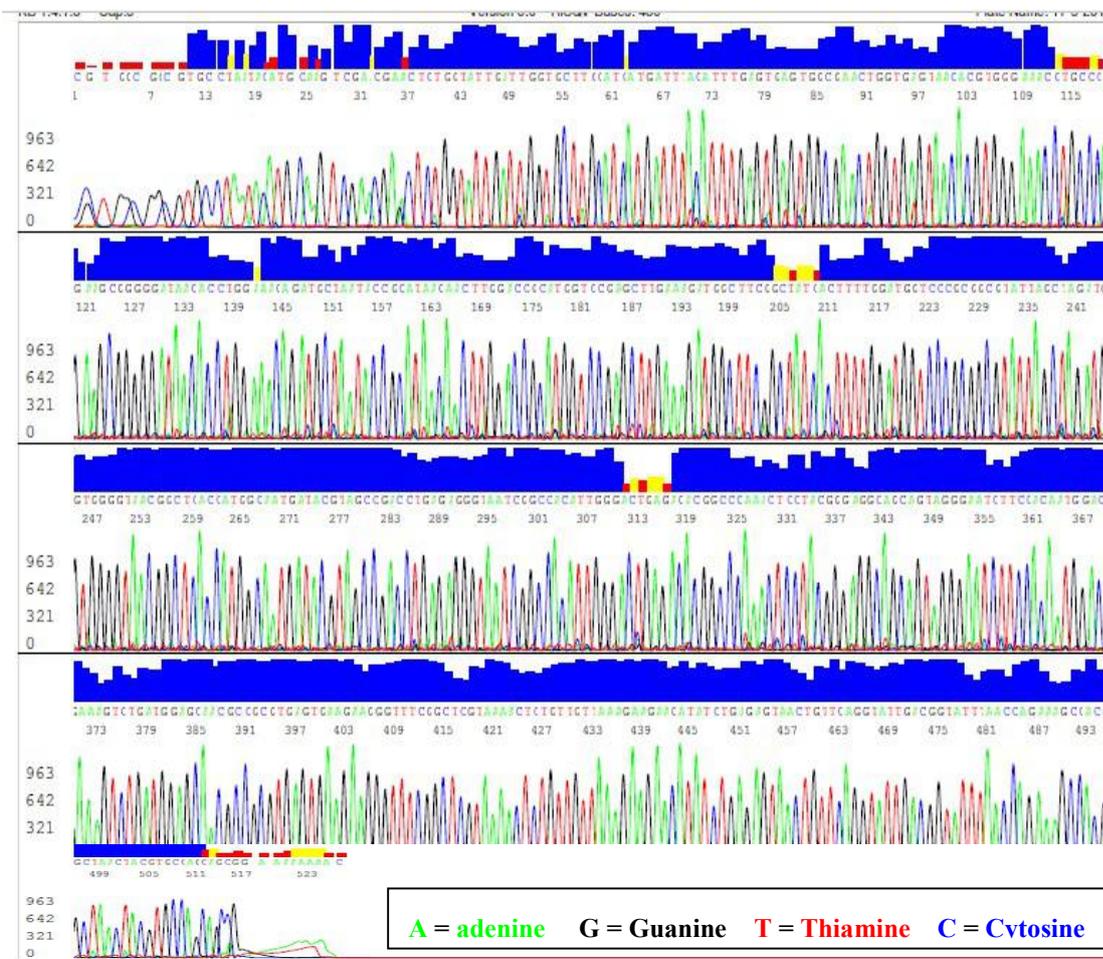


Figure 3: The electropherogram of genome sequence of *Lactobacillus plantarum* AM10

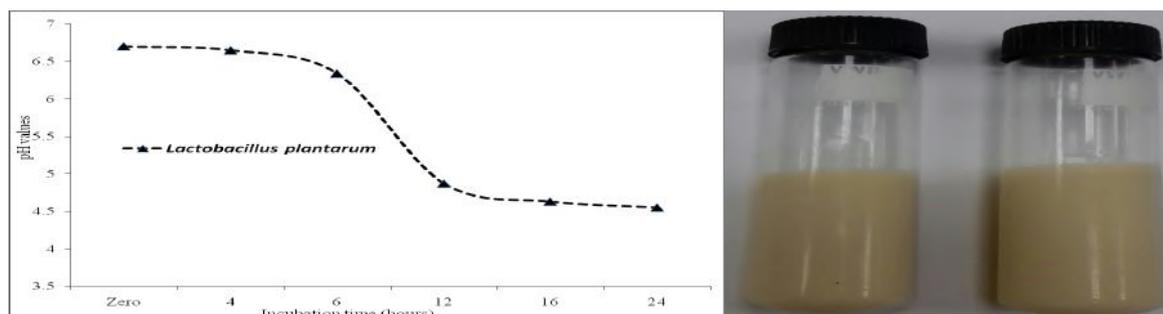


Figure 4: Coagulated sterilized skim milk because of acidity development

Table 4: Antibiotic resistance and sensitivity of *Lactobacillus plantarum*

Antibiotic	PG 10 units	E 15 µg	Ap 10 µg	KF 10 µg	CD 2 µg	TS 25 µg	Ap 25µg	KF 30 µg	TS 10 µg	NA 30 µg
Result	S	S	S	S	S	S	S	S	R	R
Antibiotic	NI 30 µg	NOR 10 µg	AK 30 µg	PY10 µg	CAZ 30 µg	CRO 30 µg	GM 10 µg	NET 30 µg	PRL 100 µg	TN 10 µg
Result	S	R	R	R	R	R	R	R	R	R

PG= Penicillin G, E= Erythromycin, AP= Ampicillin, KF= Cephalocin, CD= Clindamycin, TS= Cortimoxazole, NA= Nalidixic acid, NI= Nitrofurantion, NOR= Norfloxacin, AK= Amikacin, PY= Carbenicillin, CAZ= Ceftazidine, CRO= Ceftriaxone, GM= Gentamycin, NET= Netilmicin, PRL= Piperacillin, TN= Tobramycin, R= Resistance and S= Sensitive.



Figure 5: Antibiotic sensitivity of *Lactobacillus plantarum*

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