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by Sofie Sandi

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Acid Lactic Bacteria from Fermented Local Feed and it's Antibacterial Activity

Sofia Sandi, Miksusanti, Eli Sahara, Asep Indra Ali Munawar

***Abstract* – Lactic acid bacterial strains were isolated from fermented local feed (silage). The strain belonged to lactic acid that were referred to genus *Streptococcus lactis* and *Lactobacillus acidophilus*. The strains were identified to species by determination of morphological, cultural, physiological and chemical characteristics. The *Streptococcus lactis* strain has pH 3, 3–4 and *Lactobacillus acidophilus* has pH 5-6. These isolates were tested for inhibitory activity against feed-borne bacterial pathogens *E. coli*. Antibacterial effects of these lactic acid bacteria were determined by agar diffusion method. The inhibition zone were 0.634±0.81 mm^2 for *Streptococcus lactis* and 0.578±0.79 mm^2 for *lactobacillus acidophilus*. Optimum temperature for the growth of both lactic acid was 37°C. These lactis acid bacteria grow well at NaCl concentration 2%-4% w/v.**

***Keywords* – Streptococcus Lactis, Lactobacillus Acidophilus, Silage, Antibacterial, E. Coli.**

I. Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive, non spore forming, cocci or rod shaped, catalase –negative and fastidious organisms, as "Generally Recognized as Safe (GRAS) organisms. Mankind has exploited these bacteria for thousands of years for the production of fermented food because of their ability to produce desirable change in taste, flavor and texture. Different antimicrobial molecule such as lactic acid, acetic acid, hydrogen peroxide, carbon dioxide and bacteriocins produced by these bacteria are widely known to inhibit feed borne pathogens and spoilage microorganisms, thereby extending the shelf-life. [1]. Increasing consumer demand for natural and 'additive-free' products has led to greater interest in the application of natural inhibitory substances as feed preservatives, which could replace or reduce the use of chemical additives [2]. The antimicrobial compounds produced by lactic acid bacteria are natural preservatives as such and could be used as preparations for increasing the shelf-life and safety of minimally processed feeds. Bacteriocins of lactic acid bacteria are biologically active proteins or protein complexes that act as bactericidal against gram-positive bacteria usually closely related to the producer strain.

Properties such as: tolerance to bile salts, and low gastrointestinal pH, the colonization and adhesion capacity of the intestinal tract, the antimicrobial activity through lactic acid production, thermoresistance that could adapt itself to the technological condition and the processing the product as well as the complex visceral ecosystem , the survival rate during the starter culture storing and the feed product are only some of the biotechnological researchers preoccupation nowadays [3], [4].

The aim of this work is to isolate, identificate and to characterize the phenotype of lactic acid bacteria strain from fermented local feed (silage) from South Sumatra. The local feed consisted of variety vegetable waste (mustard green, cabbage, cesim, cassava starch, and waste of tofu).

II. Materials and Methods

Lactic acid bacteria were isolated form fermented local feed (silage). The isolation was performed by the routine microbiological procedure and inoculation on a solid medium. Selective media for lactic acid bacteria were used –MRS-agar. The cultivation was performed without shaking at the appropriate temperature (32°C) and 40°C) for five days. Once single colonies were obtained they were inoculated in MRS broth. After growing the cultures were diluted and tested for purity on the suitable agar medium.

A. Identification of the Bacterial Strains

The determination of the strains was performed according to their morphological, cultural, and physiological [5].

Purification colonies were again culture was confirmed by Gram staining, catalase assays, and pH determination of broth culture of LAB with pH meter. Pure colonies were again cultured on MRS agar and in MRS broth and store at 4°C in refrigerator until used.

B. Phenotypical Characterization Methods of the Lactic acid Bacteria Strains.

The production of catalase:

This assay was verified through the depositing of several drops of oxygenated water on the surface of colonies on agarised MRS.

C. The Optimal Temperature for Development:

The isolated strains have been inoculated on the MRS broth and incubated at 5°C, 15°C, 30°C, 37°C, 45°C, 48°C and 60°C. The analysis done spectrophotometrically – measuring the OD600 values at 12h and 24h, this indicates the degree of increase and growth.

D. The Effect of Sodium Chloride Upon the Increase and Growth:

This assay has been estimated through the inoculation of the isolated strains on specific medium which NaCl in variable concentration of 2, 4, 6, 8, 10% (w/v) is added. The tubes have been incubated at 37°C and OD580 at 12h and 24h has been determined in order to estimate the increase and growth of pure clones in the circumstances imposed by salinity.

E. Detection of Antagonistic Activity

Antagonistic activity screening was investigated against test bacteria by agar well diffusion assay [6]. Molten agar (48 °C) was first seeded with the indicator organism 200 mL of overnight culture per 25 mL of agar, 2 × 10^6 cells/mL). The inoculated medium was rapidly dispensed

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in sterile petri dishes and, after solidify cation, dried for 30 min under a laminar flow hood. Wells of uniform diameter (6 mm) were cork bored in the agar and sealed with 15 mL of tempered soft agar. The extracts of lactic cultures obtained from overnight cultures were applied to 2 different processes. (I): centrifuged at 2500 × *g* for 5 min (II). Each of the solutions was sterilized with Millipore membrane filter (0.22 mm pore diameter) before loading to wells. Aliquots (80 mL) of these solutions were dispensed in the wells, and plates were incubated overnight at 30 °C. Antagonistic activity was expressed as the area of inhibition surrounding each agar well. The antagonistic activities of samples were determined for each isolate by the well diffusion assay for persistence of the inhibition zone. In addition, 80 mL of filter sterilized lactic acid solutions adjusted to the same pH as (I) was spotted as control tests. The plates were incubated 14-16 h of 37 °C and checked for inhibition zones.

F. Observation with TEM

TEM observation for *E. coli* was determined by our previous method [7]. Nutrient broth containing *E. coli* (cultured for 24 h) was centrifugated 5000 rpm at 25°C for 10 min, and the precipitate was washed three times with PBS (0.1 M, pH 7.4). Glutaraldehyde (0.5%) was added to the precipitate and kept for 15 min at 4°C. The bacterial cells were collected by 20 min centrifugation (15000 rpm, 4°C). The cells were fixed with 3% glutaraldehyde for 2 h, post-fixed with 1% osmium tetroxide for 3 h, dehydrated with graded acetone solutions at 4°C for 20 min and embedded in epoxy. Thin sections cutted by a microtome (Ultracut-E, Reichert-Jung, Austria) were stained with 1% aqueous toluidine blue at 40°C, then observed by TEM .

G. Determination of organic acids

Determination of organic acids was carried out according to Shah and Ravula (2000). Briefly, 3 mL yoghurt samples were mixed with 50 µL of 15.5 M nitric and 1.0 mL of 0.01 M sulfuric acids. The resulting mixture was centrifuged at 14,000 × g for 30 min using an Eppendorf 5415C centrifuge for removal of proteins. The supernatant was filtered through a 0.20 µm membrane filter into an HPLC vial. The separation of organic acids was achieved using a Varian HPLC fitted with an Aminex HPX - 87H, 300 x 7.8 mm ion exchange column (Biorad Life Science Group, Hercules, USA) and a guard column maintained at 65 °C. The mobile phase was 0.01M H_2S0_4 with a flow rate of 0.6 mL/min. Quantification of acetic and lactic acids were performed from the standard curves obtained using

III. Result and Discussion

A. Isolation of Lactic Acid Bacteria From fermented Local Feed

The initial isolation and identification was based on morphological appearance and catalase test. Thus, the lactic acid bacteria show small, colonies, having 1 – 2mm diameter, slightly, lenticular, with a round perimeter, smooth, white-cream colored (fig.1), colonial characters that distinguish from the colonies of other microorganisms in the product's specific microbiota. Thus, the lactic acid bacteria show no gas when treated with 3% H_2O_2.

Fig.1. Colony of acid lactic bacteria

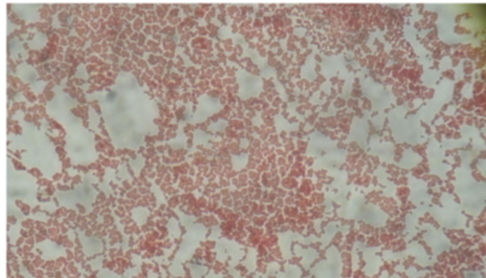

Fig.2. Chain of *Streptococcus Lactis*

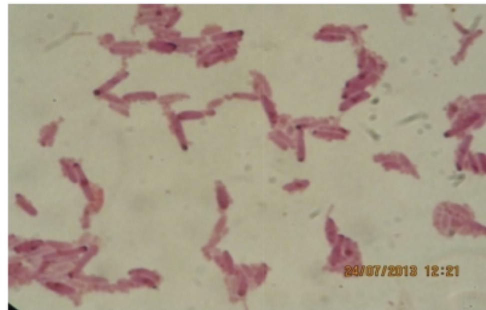

Fig. 3. Chain of *L. acidophilus*, Lentingular, with a round perimeter colony

Using pH meter, it showed that *Streptococcus lactis* have lower pH than *Lactobacillus acidophilus*. *Streptococcus lactis* has pH between 3,3-4, but *Lactobacillus acidophilus* has pH 5-6. The results show that all strains were Gram positive. Moreover the strain show negative catalase.

B. Phenotypical characterization of the lactic acid bacteria strains

All the strains of *Lactobacillus* sp. studied grow in a temperature interval of 28°C to 45°C the optimum temperature being 37°C at 12 h and 24 h. The strains record higher values of OD600 after 24 h and at 37°C, but it develops faster (at 12 h) at 40°C than at 37°C (fig.4).

All the strains of *Lactobacillus sp.* studied grow well at concentrations of 2 – 4% (w/v) NaCl in medium and not prefer a higher salinity (fig.5).

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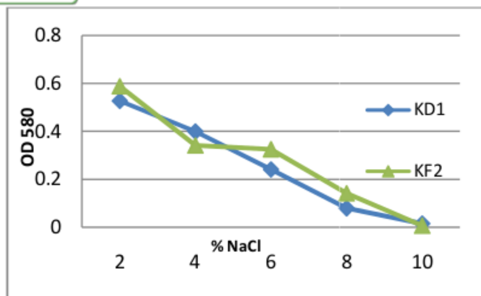

Fig.4. The Growth of LAB in Presence NaCL (KD1:*S.lactis*, KD2. *L. acidopillus*)

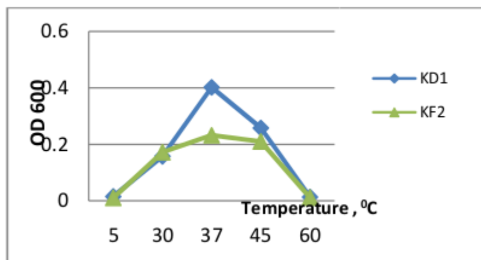

Fig.5. The Growth of LAB under variation of temperature

From Fig 4 showed that both LAB can not growth at the presence of NaCL 10%. Fig 5 significantly showed that both LAB have maximum temperature growth at 37°C (Fig 5).

C. Antibacteial Activity of Lactid Acid Bacteria Against E. coli.

Table 1: Inhibition Zone of Lactis Acid Bacteria

Pathogen Bacteria	Sample	Zone Inhibition (mm^2)
E. coli	*S. Lactis* (KD1)	0,634± 0,81
	L. acidophilus (KD2)	0.578±0.79

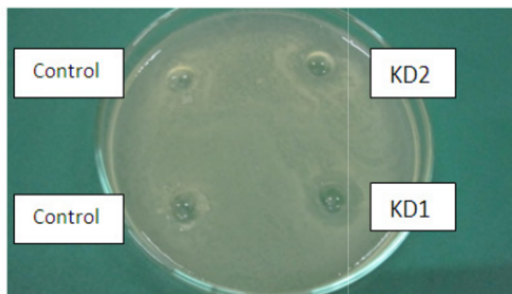

Fig.6. Inhibition Zone of Lactic Acid Bacteria against *E. coli*

Lactic acid bacteria used in the present study screened for antimicrobial activity in the well diffusion assay in Table 1. Under the well diffusion test, there are bacteria strains within the lactic acid bacteria isolates that exhibit significant inhibitory activity against test bacteria.

These strain exhibited antagonistic effects on agar medium against some Gram-negative indicator microorganisms, *E. coli*. The inhibition relatively low. The phenomena, against Gram-negative bacteria was already reported [8]. The outer membrane of Gram-negative bacteria may protect the cytoplasm membrane from the action of the antimicrobial compound of lactic acid bacteria. Membrane which is rich in lipopolysaccharide molecules presenting a barrier to the penetration of many antimicrobial components of acid. Additionally, the resistance is also associated with the enzymes in the periplasmic space, which can break down the antimicrobial components of lactis acid bacteria [9].

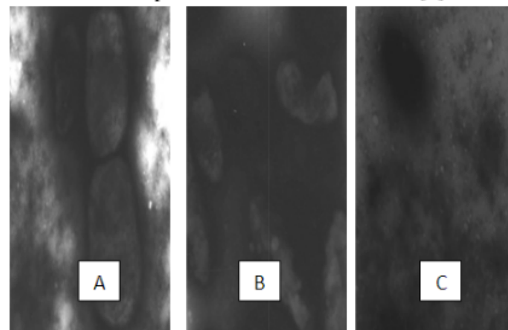

Fig.7. Transmission electron microscope diagram of *E. coli* in the absence (A) and presence (*L. acidophilus*: B, and *S. lactis*: C)

From TEM analysis showed that the cell that treated with *L. acidophilus* and *S. lactis* experiences damage. This type of cell usually was mentioned as ghost cell. Ghost cell usually only consists of cell skin but did not contain the contents of the cell. The formation of the cell ghost, because of the disturbance of the synthesis of the cell wall and the change of cell permeability and cause the material cell went out [10].

Bacterial cell that treated with *S. lactis* showed more damage than the cell that treated with *L. acidophilus*. This phenomena similar with the result of agar well diffusion method. Both of agar well diffusion method and TEM analysis showed that *S. lactis* has greater antibacterial activity against *E. coli* than *L. acidophilus*. One of the mayor fact that support this phenomena is the pH *of S. lactis* lower than pH of *L. acidophilus* which mean that *S. lactis* produce higher acid than *L. acidophilus.*

IV. Conclusion

The inhibitory action of LAB bacteria from these fermented local feed (silage) can be due to the accumulation of main primary metabolites such as lactic and acetic acids, ethanol and carbon dioxide. Additionally, LAB are also capable of producing antimicrobial compounds such as formic and benzoic acids, hydrogen peroxide, diacetyl, acetoin and bacteriocins such as nicin [9]. The production levels and the proportions among those compounds depend on the strain, medium compounds and physical parameters [11]. The antagonistic effects of LAB

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towards Gram negative pathogens could be related to the production of organic acids and hydrogen peroxide [12]. In conclusion, the results obtained from this study demonstrated the antimicrobial attributes of the isolated lactic acid bacteria from fermented local feed. According to previous studies, a large number of lactic acid bacteria strains with different bioactive potentials especially in the form of antimicrobial properties have been identified from a variety of plant sources mostly in the from of fermented vegetables [13] These scientific evidences have been a motivating factor to choose a plant based fermented product prepared from different vegetables which could further confirm the results of this study. On the other hand, such positive outcomes would be a leading point towards application of simple worthy traditional methods such as fermentation in producing natural healthy feed products and encouraging producer of feed to include such valuable feed items into the in eating habits of cattle. Hope these friendly feed groups would be added to daily diet of cattle (such as pegagan duck) to improve their body immunity hence, decreasing unnecessary intake of chemical antibiotics. However, further in vitro and in vivo studies are required according to selection criteria including adhesion to mucosal cells of the gastrointestinal tract, bile salt and acid tolerance, bile salt hydrolase activity, viability, resistance to antibiotics, safety and organoleptic properties to be applicable in different feed products such as starter culture in fermented local feed.

V. Acknowledgments

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Author's Profile

Sofia Sandi

was born at Jambi, 23 November 1970. She has got her bachelor degree from Jambi university, South Sumatra Indonesia, on 1995 In 2003, she has got her master degree from Bogor Agriculture Institute (IPB), West Java Indonesia. She got her Doctoral degree also from Bogor Agriculture Institute (IPB), West Java Indonesia in 2010. She is a lecturer at Nutrition and Animal Feed Department, Sriwijaya University (UNSRI). She had been presented and published her researches result in several Journal such as: 1).The nutrient quality of cassava by addition of cow rumen fluid enzyme, which was published at Journal Agriculture Science and Techology 2).Quality of Silage Nutrition Which Had been Given Enzyme from Cow Rumen and *Leuconostoc mesentreroides*, and was published at Animal Science Journal.

Miksusanti

was born at Bengkalis, Riau, middle Sumatra on 23 July 1968. She has got her bachelor degree from Chemistry department Andalas University (UNAND), West Sumatra Indonesia, on 1991. In 1995 she got her master degree on Chemistry from Institute Technology of Bandung (ITB), West Java Indonesia. She has got her doctor degree in Food Science on 2009 from Bogor Agriculture Institute (IPB), West Java Indonesia. She is a lecturer at Chemistry .Department, Sriwijaya University (UNSRI). She is co worker in biochemistry laboratory at Chemistry Department Faculty of Mathematic and Natural Science UNSRI. She had been presented and published her researches result in several seminars and journals e.i 1. Mode of Action Temu Kunci Essential Oil on *E. coli K1.1* Cell determined by Leakge of Material Cell and Salt Tolerance Assays, *Hayati Journal Bioscience* ,(IPB 2009); 2. Lymphocyte Proliferation by Temu lawak (*Curcuma xanthorrhiza* ROXB) Essential Oil, *Proceeding of, International Seminar on Chemical, Biological and Environmental engineering,* Singapore 2012; 3. Antibacterial and Antioxidant of *Kaemferia pandurata* Essential Oil and It's Application to Make Antibacterial and Antioxidant Edible Film From Sago Starch, *Proceeding National Conference of Essential Oil IV*, 2010 Bandung Indonesia.

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