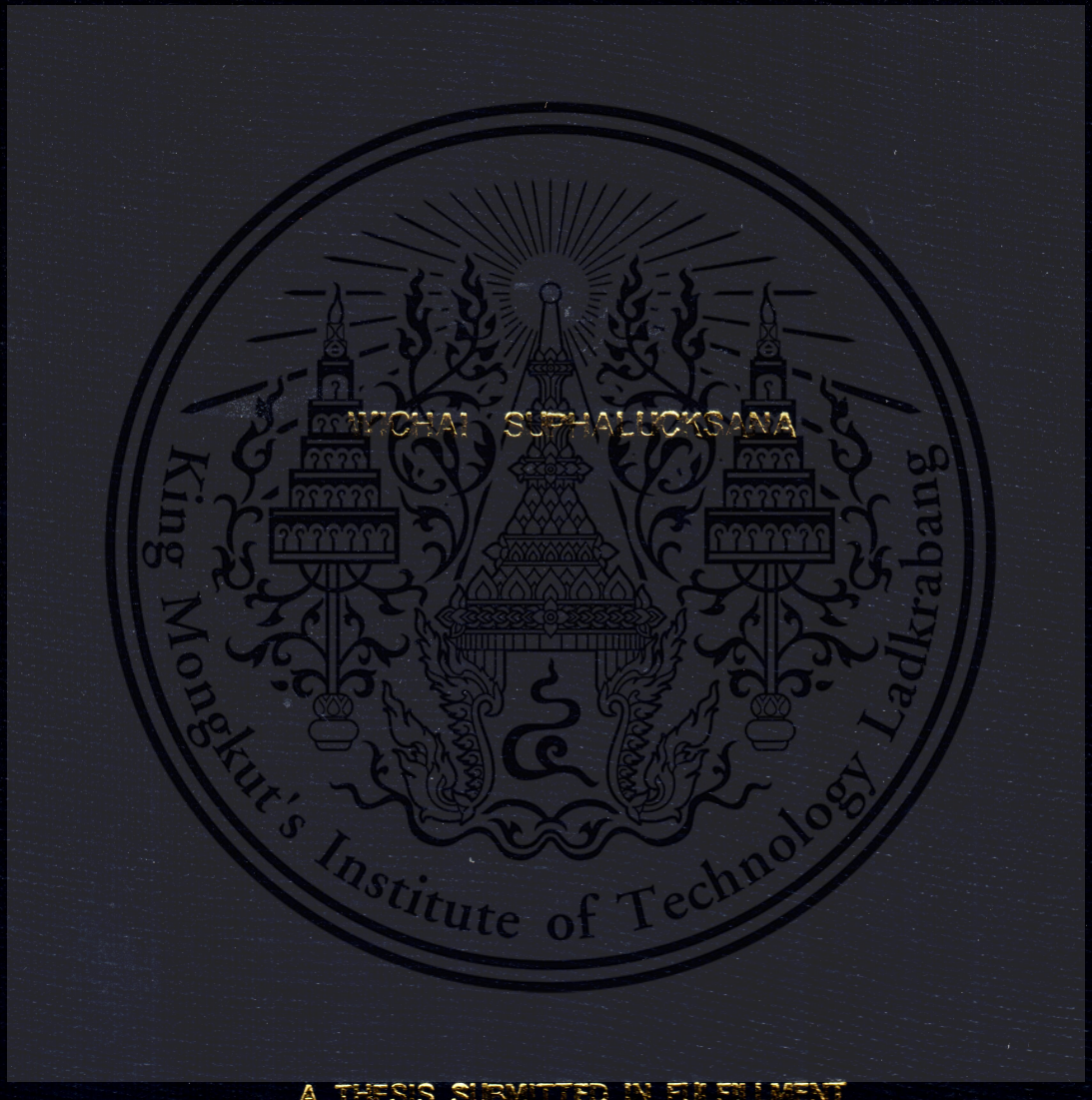
# RUMINANT NUTRITION IMPROVEMENT BY LACTIC ACID BACTERIA AND LEUCAENA APPLICATION




WICHAI SUPHALUCKSANA

A THESIS SUBMITTED IN FULFILLMENT
OF THE REQUIREMENT FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN AGRICULTURE
FACULTY OF AGRICULTURAL TECHNOLOGY
KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG
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|---|---|
| **หัวข้อวิทยานิพนธ์** | การปรับปรุงคุณภาพอาหารสัตว์เคี้ยวเอื้องโดยใช้ lactic acid bacteria และกระถิน |
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# บทคัดย่อ


การแยกแบคทีเรียกรดแลคติค 2ชนิดที่แตกต่างกันนั้น *Lactobacillus plantarum* H5-M13F และ *Pediococcus pentosaceus* Ac2-M13F ได้รับการยืนยันโดยการระบุลักษณะทางสัณฐานวิทยาและการจำแนกสายวิวัฒนาการ *Lactobacillus plantarum* H5-M13F มีรูปร่างเป็นท่อน, แกรมบวก ไม่สร้าง เอนไซม์ catalase การเกิดก๊าซที่มีวิถีการหมักแบบ homofermentative และสามารถเจริญเติบโตที่อุณหภูมิ 10 - 45° C ระดับ pH ที่ 3.5 - 9.6 และความเข้มข้นของ NaCl ที่ 1.0 - 10% *Pediococcus pentosaceus* Ac2-M13F มีลักษณะเป็นแบบกลม แกรมบวก ไม่สร้างเอนไซม์ catalase และการเกิดก๊าซที่มีวิถีการหมักแบบ heterofermentative สามารถเจริญเติบโตได้ที่อุณหภูมิ 10 – 45° C ระดับ pH 3.5 - 9.6 และความเข้มข้นของ $NaCl_2$ ที่ 1.0 - 6.5% Amplification ของ ITS จากแบคทีเรียกรดแลคติก H5-M13F และ Ac2-M13F ได้รับการยืนยันโดยใช้ Primer OPA-3 และ phylogenic tree จาก PAUP พบว่า H5-M13F อยู่ในกลุ่ม *Lactobacillus* sp. และAc2-M13F อยู่กลุ่ม *Pediococcus* sp. การวิเคราะห์สายวิวัฒนาการและสร้างเดนโดแกรม พบว่าไม่มีความสัมพันธ์ระหว่างสองกลุ่ม ผลการวิจัยพบว่า แบคทีเรียกรดแลคติก H5-M13F แยกได้จากหญ้าหมักส่วนใหญ่ (87.59%) ขณะที่แบคทีเรียกรดแลคติก Ac2-M13F พบว่ามีค่าต่ำในหญ้าหมัก (3.19%) *Lactobacillus plantarum* H5-M13F และ *Pediococcus pentosaceus* Ac2-M13F ที่แยกเชื้อจากหญ้ากินนีหมัก แสดงให้เห็นถึงความสามารถในการผลิตเอนไซม์ย่อยสลาย ซึ่งแบคทีเรีย *Pediococcus pentosaceus* Ac2-M13F ผลิตเอนไซม์ amylase และ protease ส่วนแบคทีเรีย *Lactobacillus plantarum* H5-M13F ผลิตเอนไซม์ protease

การศึกษาเพื่อปรับปรุงคุณภาพของสับปะรด โดยการเสริมใบกระถิน และใช้เป็นอาหารหยาบในระหว่างการขาดแคลนพืชอาหารสัตว์สด มี 5 treatment ดังนี้ 1. ต้นสับปะรด 100% 2. ต้นสับปะรดเสริมใบกระถิน 5% 3. ต้นสับปะรดเสริมใบกระถิน 10% 4. ต้นสับปะรดเสริมใบกระถิน 15% และ 5. หญ้า 100%. วางแผนการทดลองแบบบ completely randomized design (CRD) มี 4 ซ้ำ ทุก treatment ใส่ NaCl 1% ทำการปิดถังหมักให้สนิท เก็บรักษาที่อุณหภูมิห้องเป็นเวลา 21 วัน หลังจากนั้นทำการประเมินลักษณะทางกายภาพ องค์ประกอบทางเคมีและการวิเคราะห์เยื่อใย ลักษณะทางกายภาพของต้นสัปรดหมัก พบว่าสีของ ต้นสับปะรด 100% มีสีเขียวอมเหลืองเป็นลักษณะที่ดีในการหมัก สำหรับต้นสับปะรดเสริมด้วยกระถิน 5, 10 และ 15% มีสีน้ำตาลอ่อน ส่วนหญ้ากินนี 100% มีสีน้ำตาลเข้ม กลิ่นของการหมักทั้งหมดมีกลิ่นหอมและกลิ่นเปรี้ยวคล้ายผลไม้ดอง การประเมินองค์ประกอบทางเคมีมีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p < 0.01$) ต้นสับปะรดที่เสริมด้วยใบกระถิน 15% มีปริมาณโปรตีนและพลังงานเผาผลาญสูงสุดโดยมีค่าเฉลี่ย 8.19% และ 3,697.17 kcal / k ตามลำดับ เปอร์เซ็นต์ของเยื่อใย ลดลงเมื่อเพิ่มปริมาณกระถินลงในหญ้าหมัก หญ้ากินนี 100% มีค่าเยื่อใยสูงสุด ต้นสับปะรดหมักเสริมด้วยใบกระถิน สามารถปรับปรุงคุณภาพของพืชหมักสำหรับสัตว์เคี้ยวเอื้องได้

การวิจัยการย่อยได้ของอาหารสัตว์ครั้งนี้มีวัตถุประสงค์ เพื่อศึกษาประสิทธิภาพของแบคทีเรียแลคติคที่มีต่อการย่อยได้ของอาหารผสมสำเร็จรูปในหลอดทดลอง ที่มีส่วนผสมของต้นสับปะรดหมัก 55%, ฟางข้าว 15%, มันสำปะหลัง 5.6%, กากถั่วเหลือง 12.6% %, รำข้าว 5.6%, premixed 0.15% และกากน้ำตาล 2% การแยกและระบุเชื้อจุลินทรีย์จากการหมักโดยใช้หญ้ากินนี (*Panicum maximum*) วางแผนการทดลองแบบ completely randomized design (CRD) โดยทำการทดลอง 4 ซ้ำ มีการใช้ TMR ผสมด้วยแบคทีเรียกรดแลคติคดังนี้: - treatment 1 TMR ไม่ใส่เชื้อแบคทีเรียกรดแลคติก treatment ที่ 2 เป็น TMR ที่ใส่เชื้อ *Lactobacillus pantarum* (LP) treatment ที่ 3 ได้แก่ TMR ที่ใส่เชื้อ *Pediococcus pentosaceus* (PP) treatment ที่ 4 เป็น TMR ที่ใส่เชื้อ *Pediococcus acidilactici* (PA) และ treatment ที่ 5 คือ TMR ที่ใส่เชื้อ *L. pantarum* + *P. pentosaceus* + *P. acidilactici* (LPP) ทำการผสมแบคทีเรียกรดแลคติกลงใน TMR ใส่ถุงพลาสติกและเก็บไว้ที่อุณหภูมิห้องเป็นเวลา 7 วันก่อนการวิเคราะห์ การทดสอบการย่อยได้ของเอนไซม์ในหลอดทดลองใช้วิธี pepsin cellulase *in vitro* digestibility ผลการทดลองแสดงให้เห็นว่ามีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P < 0.01$) ของคุณค่าทางโภชนาการของ TMR โดยการวิเคราะห์

องค์ประกอบทางเคมี และการวิเคราะห์เยื่อใยในอาหารที่ใส่แบคทีเรียกรดแลคติก วัตถุแห้งเพิ่มจาก 68.28% เป็น 79.80%, 79.79%, 79.40%, และ 79.42% ใน LP, PP, PA และ LPP ตามลำดับ อาหาร TMR ที่ใส่เชื้อ PA ให้โปรตีนสูงขึ้นอย่างมีนัยสำคัญ (10.97%) มากกว่า PP (10.02%), LPP (9.77%) และ LP (9.65%) TMR ที่ใส่เชื้อ LP ให้ค่า NDF สูงขึ้นอย่างมีนัยสำคัญ (34.13%) รองลงมาคือ PA (32.61%), LPP (31.69%) และ PP (30.20%) TMR ที่ใส่เชื้อ PP มีค่า ADF ต่ำกว่ากลุ่ม LP อย่างมีนัยสำคัญ (20.29%) ต่ำกว่า LP (20.60%), PA (21.60%) และ LPP (22.59%) สำหรับการย่อยได้ พบว่า อาหาร TMR ที่ใส่ LAB โดยใช้เอนไซม์ pepsin cellulase ในหลอดทดลอง ที่ใส่แบคทีเรียกรดแลคติก LP, PP, PA และ LPP สามารถปรับปรุงการย่อยได้ของ TMR ซึ่งช่วยเพิ่มการย่อยได้ของ TMR ($P < 0.01$) TMR ที่ใส่เชื้อ PA มีการย่อยได้ดีกว่าอย่างมีนัยสำคัญ (66.21%) มากกว่า LPP (66.20%), PA (65.16%) และ LP (64.98%) อัตราการเพิ่มขึ้นและลดลงของการย่อยได้ และเยื่อใยในอาหาร TMR ที่ใส่ LAB พบว่า ค่าของวัตถุแห้งเพิ่มขึ้นและเยื่อใยมีค่าลดลง TMR ที่ใส่เชื้อ LP ให้วัตถุแห้งสูงขึ้น (+16.87%), มากกว่า PP (+16.86%), LPP (+16.31% และ PA (+15.76%) TMR ที่ใส่เชื้อ PP มีปริมาณเยื่อใยลดลง (-12.75%) มากกว่า PA (11.49%), LPP (-9.43%) และ LP (-8.23%) ดังนั้นจึงแนะนำว่า อาหาร TMR ที่ใส่แบคทีเรียกรดแลคติค อาจเป็นไปได้ในการพัฒนาเป็นอาหารสัตว์ชีวภาพสำหรับสัตว์เคี้ยวเอื้อง

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| **Degree** | Doctor of Philosophy |
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# ABSTRACT

Two different isolates of lactic acid bacteria, *Lactobacillus plantarum* H5-M13F and *Pediococcus pentosaceus* Ac2-M13F were confirmed by morphological and phylogenic identification. H5-M13F was rod shaped, gram positive, negative for gas production and catalase activity, homofermentative, and grew at 10 – 45 C, pH 3.5 – 9.6, and 1.0 -10 % NaCl. Isolate Ac2-M13F was coccus shaped, gram positive, negative for gas production and catalase activity, heterofermentative, and grew at 10 – 45 C, pH 3.5 – 9.6, and 1.0- 6.5 % NaCl. Amplification of the ITS regions from isolates H5-M13F and Ac2-M13F were confirmed by using Primer OPA-3. The phylogenetic tree from PAUP analysis indicated that isolate H5-M13F clustered with *Lactobacillus* sp. and isolate Ac2-M13F with *Pediococcus* sp. Phylogenetic analysis and dendrograms revealed no relationship between the two groups. It was found that isolate H5-M13F mostly isolated from silages (87.59%) while isolate Ac2-M13F was found at a low incidence in silages (3.19%). *Lactobacillus plantarum* H5-M13F and *Pediococcus pentosaceus* Ac2-M13F from silage of guinea grass showed the ability to produce extracellular degradative enzymes. *Pediococcus pentosaceus* Ac2-M13F produced amylase and protease and *Lactobacillus plantarum* H5-M13F produced protease.

The study was also to improve the quality of pineapple plant silage by leucaena supplementation and using it as roughage during the shortage of green forages. There were five treatments as follows: - pineapple plant 100%, pineapple plants mixed with leucaena 5, 10, 15% and guinea grass 100%. The completely randomized design (CRD) with four replications. All treatments were put in 1%NaCL and tightly sealed in plastic containers. They were stored at room

temperature for 21 days. The silage treatments were evaluated on physical characteristics, chemical composition and fiber analysis. The physical characteristic showed that the color of pineapple plant 100% was a yellow green color exhibited a good characteristic of silage. For pineapple plants mixed with leucaena at 5, 10, 15% respectively, there were a light brown color. Guinea grass 100%was dark brown in color. The aromas of all silage treatments were aromatic and acidic like pickled fruit. The evaluation of chemical composition showed a highly significant difference among the treatment groups ($p < 0.01$). Pineapple plants mixed with leucaena 15% was the highest in protein and metabolizable energy percentage with the averages of 8.19% and 3,697.17 kcal/k, respectively. The percentage of crude fiber was reduced with the increase of leucaena in the silage. Guinea grass 100% was the highest in crude fiber. The pineapple plant silage mixed with leucaena could improve the quality of silage for ruminants.

The research was to investigate the efficiency of lactic acid bacteria on *in vitro* digestibility of total mixed ration (TMR) containing pineapple plant silage 55%, rice straw 15%, cassava ship 5.6%, soybean meal 12.6%, palm kernel pulp 14.2%, rice bran 5.65%, premixed 0.15% and molasses 2%. The experiment was manipulated using completely randomized design (CRD) with four replications. Treatments were used as TMR treated with lactic acid bacteria as follows: - 1. TMR non-treated one, 2. TMR treated with *Lactobacillus pantarum* (LP), 3. TMR treated with *Pediococcus pentosaceus* (PP), 4. TMR treated with *Pediococcus acidilactici* (PA), and 5 TMR treated with *L. pantarum* + *P. pentosaceus* + *P. Acidilactici* (LPP). The treated TMR was mixed and put in polyethylene bags and stored at ambient temperature for 7 days before analysis. The *in vitro* digestibility test was used pepsin cellulase *in vitro* digestibility method. The results showed that there were significant differences ($P<0.01$) of the nutritive value of TMR by proximate analysis and fiber analysis which treated lactic acid bacteria. The dry matter was changed from 68.28% to 79.80%, 79.79%, 79.40%, and 79.42% for LP, PP, PA and LPP respectively. TMR treated with PA gave significantly higher protein (10.97%) than PP (10.02%), LPP (9.77%) and LP (9.65%). TMR treated with LP gave significantly higher NDF (34.13%) than PA (32.61%), LPP (31.69%) and PP (30.20%). TMR treated with PP gave significantly lower ADF (20.29%) than LP (20.60%), PA (21.60%) and LPP (22.59%). For the digestibility, results showed that the TMR treated with lactic acid bacteria using pepsin cellulase *in vitro* digestibility which applied LP, PP, PA and LPP could improve the digestibility of TMR, which significantly increased in digestibility of TMR ($P<0.01$). TMR treated with PA gave significantly higher digestibility (66.21%) than LPP (66.20%), PA (65.16%) and LP (64.98%). The rate of increase and decrease

of digestibility and fiber in TMR treated with LAB were increased in dry matter and decreased in fiber. TMR treated with LP gave higher increased dry matter (+16.87%) than PP (+16.86%), LPP (+16.31%) and PA (+15.76%). TMR treated with PP gave higher decreased in crude fiber (-12.75%) than PA (11.49%), LPP (-9.43%) and LP (-8.23%). Therefore, it was suggested that TMR treated with lactic acid bacteria of TMR may possible to develop as biological animal feed for ruminant.

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Wichai Suphalucksana

# CONTENTS

# CONTENTS (CONTINUED)

# LIST OF TABLES

# LIST OF FIGURES

# CHAPTER I

# INTRODUCTION

## 1.1 Statement and Significance of the Problem

Guinea grass (*Panicum maximum*) is a fiber resource generally used for ruminant feed in Thailand (Aganga and Tshwenyane. 2004). One problem is the lack of subsequent crops of good quality roughage which can be fed to dairy cows during the dry season. Therefore, implementation of quality roughage in the rainy season comes to storage in the form of fermented forage or silage that can solve the feed shortage problem. The main factors involved to produce silage more rapidly by using potent degradative microorganism especially lactic acid bacteria (LAB) (McDonald *et al.* 1991; Brookes and Buckle. 1992). The species diversity of bacteria may vary according to type of forage to be fermented (Cai *et al.* 1999a, 1999b; Ennahar *et al.* 2003; Wang *et al.* 2006; Parvin and Nishino. 2009, 2010). This research was focused on finding potent lactic acid bacteria to be used in silage for animal feed. Enzyme production activities in silage are also available to break down forage fiber. Wallace *et al.* (2001) reported that the relationship between enzyme activities and *in vitro* gas production were using grass and corn silage had significant positive correlations with cellulase activity and gas production from grass silage. The question is how to improve the nutrition of farmers' ruminant animals when each animal raiser keeps only one animal. In flood or summer, the major fodders available are grass or rice straw, together with limited quantities of hay and concentrated feeds. As a minimum, it is essential to provide a green fodder supplement to enhance rumen function for bovine animals. For animal raisers with limited production capacities, finding enough feed in the flood or summer months to maintain good production is always a problem. There were many forced to buy roughage, concentrates or silage just to keep their animals alive and are unable to benefit financially due to the higher prices paid for animal feed in the flood or summer months. The objective of this research was to identify and investigate effective lactic acid bacteria producing enzymes to degrade silage consisting of Guinea grass (*Panicum maximum*) for animal feed.

Pineapple is one of the economic fruits of Thailand. This product is mostly used for fresh fruit consumption and processing products within the country and exported in terms of canned fruits and frozen fruits. There are large amounts of pineapple plant which are harvested fresh for fruit consumption and processing products such as canned pineapple (Gowda *et al.* 2015). Thus, the

farmer must dispose of this large amount of pineapple plant waste to alleviates this problem for a green environment. Now they try to make a value-added aspect of pineapple and their by-products. Also, the use of pineapple plants contributes to useful material for the industrial sector such as textile, pineapple fibre cloth, enzyme, combustible material, etc. Pineapple is a component of the trunk. Farmers cut off the plants after harvesting. Pineapple leaves are waste or by – product of agriculture. There is almost all year long and will be much in range November – June because it is period when most farmers collect the yield delivered factory, which meet the drought season, where farmers shortage of fresh grass for ruminant. The analysis of pineapple leaves showed that the protein 8.47 %, fibre 17.89 %, ADF 25.87% and NDF 42.28 % (Warunee and Walaikhan. 1998). Pineapple leaves can be used as a component in total mixed ration for dairy feed, without any effect on milk production. The most important aspect of animal husbandry is to reduce the costs of production or the costs of animal feed in order to maintain the livestock business. The reduction in production costs that can be achieved is the feed efficiency. Ruminants are animals that use both concentrate and roughage. Roughage include forage crop such as grass and legume. But roughage is usually of low quality and insufficient for the needs of ruminants. Because the farmers have limited space in the preparation of forage crop or pasture and often lacking, especially during the dry season. There are various by – products in agriculture instead such as pineapple leaves, or pineapple plant can be used to feed dairy cattle (Prachya *et al.*2001). Pineapple leaves or pineapple plant can be a source of roughage for ruminants, but lack of knowledge and understanding of the proper use, the opportunity to be used to maximum benefit. Because of this, it is necessary to develop appropriate knowledge for farmer to increase farmers' incomes. Suchat *et al.* (2011) was to determine the effect of ensiled pineapple waste compared with pangola hay as roughage source on rumen fermentation and feed utilization of native cattle. The apparent digestibility of dry matter, organic matter, crude protein, neutral detergent fiber and acid detergent fiber in cattle fed only pineapple waste as roughage source was higher than in cattle fed only pangola hay. The pineapple waste has several benefits in terms of increasing caloric density, digestibility and feed utilization as compared to pangola hay. Moreover, it also enhances populations of dominant cellulolytic bacteria in the rumen. The chemical composition of the pineapple plant is high in fibre which makes it is a good source of fibre for ruminant feed. Pine apple plants could be used as ruminant feed in silage forms to preserves the quality of its nutrient (Sayan 2004). Furthermore, it can be kept for a long time. Pineapple plant silage can alleviate a malnutrition in ruminants during the dry season or flooding time. It is a high-quality

silage because it is good in digestibility and palatability. It is easy for animal raisers to make pineapple plant silage for their animals by themselves. This can help reduces animal feed cost and increase the quality of feed which is reflected in the high production performance of their animal. However, the quality of silage is depending on feed additive uses during the making processes.

## 1.2 Objectives

The general objective was to evaluate the lactic acid bacteria specifically, the study aimed to:

1.2.1 To identify of lactic acid bacteria in silage by polymerase chain reaction

1.2.2 To test of enzyme production of lactic acid bacteria

1.2.3 To screen lactic acid bacteria for ruminant nutrition improvement

1.2.4 To test leucaena to improve the quality of pineapple plant silage

## 1.3 Scope of the Study

This research work was covered for collection, isolation and identification of lactic acid bacteria and enzyme production from silage of guinea grass (*Panicum maximum*). Efficiency of lactic acid bacteria as the potent degradative microorganism to digest the total mixed ration by *in vitro*. Three lactic acid bacteria as *lactobacillus pantarum*, *Pediococcus pentosaceus* and *Pediococcus acidilactici* were treated with TMR and evaluation of Pepsin-Cellulase *in vitro* dry matter digestibility. Further research study was to test leucaena to improve the quality of pineapple plant silage.

## 1.4 Time and Place of Research Work

The study was conducted at the laboratory of the Department of Animal Production Technology and fishery, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL) Bangkok, Thailand. The Evaluation of goat production performance and nutrient digestibility was conducted at the Rus goat farm, Prachuap Khiri Khan Province. The research work of thesis was started from August 2014 until December 2016.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Lactic Acid Bacteria

Lactic acid bacteria are gram positive bacteria non-spore forming, non-pigmented, catalase-free and non-catalase and not moving but some stains rarely motile require little air to grow. Some microaerophiles can grow in strictly anaerobes. Lactic acid bacteria are characterized by ovoid cocci, short rod and long rod. (Brookes and Buckle. 1992; Axelsson 2004). Some species have both (Ennahar et al. 2003). Most of them are able to grow at temperatures ranging from 5 - 50 degrees Celsius (mesophilic). The optimum growth temperature is 25 - 40 degrees Celsius (McDonald *et al.* 1991; Axelsson 2004).

Lactic acid bacteria have outstanding properties highly resistant to lactic acid. The optimum pH for growth was 5.0-6.0. Some strains were able to grow at pH 4.0 and 3.5 with varying nutrient requirements, depending on species. Lactic acid bacteria found in silage are epiphytic lactic acid bacteria. The sugar components contained in the plant are available for growth (Woolford 1984; McDonald *et al.* 1991).

### 2.1.1 *Lactobacillus*

*Lactobacillus* sp. is the largest group of lactic acid bacteria. High diversity of phenotypes and biochemicals. The cells are short rod and long rod, or coccobacilli and aciduric. The ideal pH for growth is in the range of 5.5-6.2. It is a species that needs little air to grow need many nutrients, some strains could produce pseudo-catalase it was a bacteria fermented sugar both homofermentative and heterofermentative. The product is lactic acid with D-isomer L and DL (l, l and DL-lactic acid) depending on species. Moles of base guanine and cytosine (Mol% G + C) are between 32-53 percent (Axelsson 2004)

### 2.1.2 *Pediococcus*

*Pediococcus* is lactic acid bacteria that have cocci cells. There is a two-way divide on the same plane sorted in pairs or four adjacent cells. The cells are approximately 0.36-1.43 microns in diameter not moving non-spore forming and non - capsules. In general, the colonies are 1-3 mm in diameter. It can grow in both aerobic and facultative anaerobe conditions. It is a fermenting bacterium. homofermentative products are L and DL-lactic acid, depending on species (Axelsson.

2004). The molar percentages of base guanine and cytosine were 34-44 percent (Stiles and Holzapfel. 1997).

## 2.2 Identification of Lactic Acid Bacteria

### 2.2.1 Identification of lactic acid bacteria in the traditional way

It was a model used in the past as a media-dependent for isolation and identification culture-dependent approach that is separated by specific media such as MRS agar (de Man *et al.* 1960). Which this the formula was used to isolated lactic acid bacteria. Generally, the tamato juice agar (Kulp 1927) a formulation used for distinguishing the genus *Lactobacillus* sp. is the main or M17 agar (Terzaghi and Sandine. 1975), a formulation for *Lactococcus* species. These media provide the nutrients necessary for the growth of lactic acid bacteria (Kandler and Weiss. 1986). By isolation of lactic acid bacteria will be studied morphological that was based gram staining. Then check the shape and appearance of the cells under the microscope. Including some biochemical tests, catalase enzyme formation and oxidase. Generating gas or not generating gas from fermentation of glucose contained in liquid media. This is a typical detection (Kandler and Weiss. 1986; Temmerman *et al.* 2004). For detection to know the species level, it is a test that has a different growth condition such as the growth at the salt concentration pH, the bile that has been adjusted to the media used for the culture and the temperature of the media to cure at different levels. Measurement of the lactic acid form of the lactic acid product produced by lactic acid bacteria, including the biochemical studies of carbohydrate fermentation, used to help isolate strains of isolated lactic acid bacteria (Kandler and Weiss. 1986; Holt *et al.* 1994). However, the results provide the most accurate identification at the genus level (about 90 percent). While the accuracy of identification at the species level is not very high (about 38 percent). In addition, the identification of lactic acid bacteria in the traditional method will be able to study the types of lactic acid bacteria that can thrive under selected conditions.

Kamga and Kamga (1988) isolated and identified lactobacilli produced in Elephant grass and Guatemala grass silage using Rogosa agar for isolation. Then take a sample to check gram staining some biochemical and physical tests, as well as different carbohydrate fermentation, were found that the number of lactobacilli found in elephant grass and Guatemala grass silage was 11.8x104 and 11.3x105 CFU / g, respectively from randomized collection of lactobacilli 49 isolates by 43 isolates were Gram-positive bacteria rod shaped and not moving which the isolated isolates, 41 isolates did not produce catalytic enzymes non spore forming grow well in both

aerobic and facultative anaerobe conditions and can grow at 15 ° C slightly and only 2 isolates can grow at 45 ° C. And from 32 different carbohydrate fermentation forms, the isolates were identified as Lb. casei. Within 32 isolates, 22 isolates were identified as *Lactobacillus casei* subsp. Casei. The other 9 isolates were not lactobacilli. *Lactobacillus casei* was found to be a distinct species isolated from both types of grass silage.

Rooke (1990) reported that lactic acid and enterobacteria counts of silage samples in Durham and Northumberland farms between 1987 and 1988 was counted on MRS agar found that the average number of lactic acid bacteria in 1987 was significantly higher than in 1988 ($P < 0.01$), which was 5.46 and 4.85 log10 CFU / g, respectively. In 1988, Rogosa agar was used for counting lactic acid bacteria in addition to MRS agar. In 1988, the use of the Rogosa agar media for lactic acid bacteria counts was conducted in addition to MRS agar, by MRS agar there were more LAB detected. As for enterobacteria, there were no differences between the two years of detection at 4.76 log10 CFU / g.

**2.2.2 Identification of lactic acid bacteria by modern methods**

This is a method that is currently used identify in the media culture (traditional method) or non-media identification in culture. (culture-independent approach). Both methods will have some similar studies stages. That is, both methods can detect strains of lactic acid bacteria by reading the sequence nucleotide DNA or RNA of an organelle ribosomal (Temmerman *et al.* 2004; Ben Amor *et al.* 2007).

For identification of lactic acid bacteria in a modern method. By the method of identification that requires media in the culture must be able to grow the lactic acid bacteria before. If there are several types of lactic acid bacteria present in the natural habitat, biochemical and physical tests may be required, as well as various types of carbohydrate fermentation tests, to manage the lactic acid bacteria in groups. Then, the representatives of the group were extracted by genetic or DNA extraction prior to further analysis of the rRNA gene (Temmerman et al. 2004). If it is not possible to grow the bacteria, it will not be possible to analyze the bacterial species (Kopermsub 2008).

For non-media-dependent identifications in culture analyzed the type of lactic acid bacteria directly from natural sources that extract the genetic material of the bacteria contained in the sample directly and bring the genetic material to increase by polymerase chain reaction technique or polymerase chain reaction, PCR. This technique was used to increase the target DNA by using a primer for synthesis before analyzing the DNA pattern of samples by various techniques

(Handelsman 2004). Then take the sequence of nucleotides increase the number again by cloning into vector DNA and transferred to the host cell before analysis and comparison of the sequence of nucleotides and living organism's further information (Amann *et al.* 1995; Wang *et al.* 2006). For a molecular biometric method that was commonly used to identify LAB that occur in silage by using media in culture, the technique was used amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) to help the species identify or grouping. While, non-media identification in culture used the denaturing gradient gel electrophoresis technique (DGGE). single strand conformation polymorphism (SSCP) and temperature gradient gel electrophoresis (TGGE) for detection of strains or groups of bacteria (Kirk *et al.* 2004; Temmerman *et al.* 2004; Justé *et al.* 2008).

### 2.3 Characteristic of Guinea Grass (*Panicum maximum*)

The Guinea grass has the scientific name Panicum maximum, common names Guinea grass or buffalo grass. It is a perennial grass shortly rhizomatous rooting at the lower nodes. Leaf blades linear to narrow lanceolate. Leaves glabrous to hairy, 40-100 cm long, 1-3.5 cm wide, tapering to fine point. Panicle , 12-45 cm long, and 12-25 cm wide, spikelets 2.5-3 mm long. It is widely naturalized in the tropics. Grows naturally in open grasslands, resistant to drought and shade well but it was not resistant to floods. Planting seeds 1 - 2 kg / rai and it's can also be planted from root tillers or cutting with thick stemmed was 2 x 0.5 - 2 x 1 meter. Thailand yield 3,200 kg/rai crude protein 4 – 14 %, depending on the cutting life. *P. maximum* is well eaten by all classes of grazing livestock, with particularly high intakes of young leafy growth. Peiris and Ibrahim (1995) reported that the crude protein content in young Guinea grass and mature grass were 10.01 and 3.9 percentage. The organic matter digestibility of young grass (69%) and mature grass (62.5%).

### 2.4 Silage

Silage is the use of forage crops for the animals to fermentation using high initial moisture content (McDonald *et al.* 1991). Anaerobic fermentation process, which was based on lactic acid bacteria (LAB), that can use water soluble carbohydrates (WSCs) in raw materials. This produces high levels of lactic acid, resulting in a decrease in the pH value, which inhibits the growth of contaminated microorganisms that cause spoilage in forage crops fermentation (Brookes and Buckle. 1992). The good chemical composition of the silage is between 3.5-4.2. In addition,

lactic acid should not be less than 50 percent of all organic acids. Butyric acid content was not more than 0.5 percent and ammonia nitrogen ($NH_3$-N) content was less than 10-15 percent of total nitrogen (Somchit 2006; Church 1991; Seglar 2003)

Pasebani *et al.* (2010) investigated the number and types of lactic acid bacteria in grass, found that Guinea grass contained less lactic acid bacteria. In addition, the amount of lactic acid bacteria in the heterofermentative group more than Homofermentative groups and it is recommended that this grass fermentation should be added lactic acid bacteria to assist in the fermentation process after 2 days of incubation, the lactic acid bacteria in the water of fermented plants were 8 log $^{10}$ cfu / ml. When added to 1% w/w fermentation medium, 6.08 log$^{10}$ cfu Per gram of live plants, which is the appropriate amount for the initial culture of the fermentation process (McDonald *et al.* 1991; Weinberg and Muck. 1996).

McDonald et al. (1991) suggested that lactic acid content in good quality fermented plants it should be approximately 3 to13 percent of the dry matter content. It has been reported that acetic acid is the most common acid in non-supplemented fermented guinea grass (Niimi and Kawamura. 1998; Shao *et al.* 2004; Bureenok *et al.* 2005). Bustos *et al.* (2005) found that in the absence or absence of glucose *Lactobacillus pentosus* can produce lactic acid and acetic acid by using sugar pentose from hemicellulose. The ability to produce lactic acid and tolerance to low pH are important criteria to consider when selecting the strains of lactic acid bacteria for inoculants. The addition of lactic acid bacteria might improve silage quality and increase digestibility and voluntary intake (Ando *et.al.* 2006).

**2.5 Leucaena**

*Leucaena leucocephala* (Lam.) is a vigorous, rapidly growing, drought tolerant, palatable and high yielding tropical or subtropical legume (With annual rainfall of 500 to 3000 mm), enriched in protein (25-35% CP) and other nutritional components. It is considered a miracle tree for its protein rich foliage (20-30%), fast growing habit, drought tolerance, good high energy fuel, organic nitrogenous fertilizers and its charcoal gum etc. Multi-purpose uses in industries, drought and pest resistance capabilities, effectiveness to control soil erosion and properties to fix nitrogen in soil all made it something legendary. In wet tropics, yield of 20t dry matter/ha/year have been obtained with crude protein yields in excess of 3t/ha.

The chemical composition of *Leucaena leucocephala* depends on several factors. The major factors include location, variety and age of plant, soil type, season, drying methods. The nutrient

composition of *Leucaena* fodder exhibited considerable variations during different months of the year. The range of various nutrients are like, dry matter 24.98 to 36.39, crude protein 18.9 to 27.57, crude fiber 10.16 to 17.23, ether extract 2.59 to 5.88, nitrogen free extract 46.70 to 59.91 and total ash 7.49 to 10.90%. The range of nutrients reported by several other workers were : crude protein 15.22 to 31.43, crude fiber 7.33 to 16.65, ether extract 2.50 to 7.10, nitrogen free extract 38.62 to 57.6, neutral detergent fiber 27.3 to 46.30, acid detergent fiber 14.4 to 29.79, Cellulose 7.10 to 16.77, Hemicellulose 12.9 to 16.51, Lignin 4.4 to 12.81, total ash 6.8 to 12.5, Ca 0.70 to 2.70, P 0.17 to 0.35%; gross energy 19.0 to 20.1 MJ $kg^{-1}$ and $\beta$-carotene 227 to 246 mg $kg^{-1}$ (Monoj and Samiran. 2007).

*Leucaena leucocephala* (*Leucaena*) is a perennial tropical legume that can be directly grazed or harvested and offered to ruminants as hay, silage, or fresh. However, *Leucaena* contain phenolic compounds, which are considered anti-nutritional factors as these may reduce intake, digestibility and thus animal performance. The rumen microbial population, especially cellulolytic, proteolytic bacteria and fungal zoospores were enhanced in steers that received 60% of *Leucaena* silage. whereas the amylolytic bacteria population was not affected. Protozoal population was linearly decreased with increasing level of *Leucaena* silage. Moreover, N-balance and microbial protein synthesis were enhanced by *Leucaena* silage feeding and were the highest in 60% *Leucaena* silage. (Thien Truong *et al.* 2017).

**2.6 Pineapple**

Pineapple (*Ananas comosus*) is a commercial horticultural crop grown in some regions of India, East Asia, and Africa. In India, this crop is grown in about 90,000 ha of land, and about 35%fresh fruit is processed in industries for juice, jam, and canned products for human consumption. Out of the whole fruit, about 65 % is nonedible that includes spent pulp, peels, crown with leaves, and pomace and hence represents substantial residual biomass (Upadhyay *et al.* 2010). In India, an estimated quantity of 1.3 million tons of pineapple fruit residue is available annually and has no well documented usage except as a waste. Due to high moisture and sugar content, the keeping quality of pineapple fruit residue is low resulting in putrefaction. This residue is a serious problem for disposal and also is an environmental safety issue. There are few reports suggesting the use of pineapple fruit residue in livestock feeding (Das *et al.* 2013), but complete studies on assessing the nutritive value, improving the keeping quality and livestock performance fed on pineapple fruit residue, are lacking. Hence, the present investigation was

undertaken to develop an effective technique to preserve pineapple fruit residue and examine the possibility of using pineapple fruit residue in livestock feeding as a novel feed resource. Pineapple is a commercially important fruit crop grown in Asian and African countries. Pineapple fruit residue accounts for more than 65%of the processed fruits, and its disposal is a major problem due to its high moisture and sugar content predisposing it to fungal growth and spoilage. Silage technique was adopted to address this problem, and the pineapple fruit residue silage was evaluated for its feeding value. It was observed that on 15th day, the pH of pineapple fruit residue silage was 4.2–4.3 and lactic acid content was 6–8 % (DM basis). Combination of 4 parts leafy crown and 1-part peels/pomace was found very ideal to achieve moisture content of 65–70 % and produced a good quality silage with minimum fungal count (<3–4 colony forming units) on 15$^{th}$ day of ensiling. Nutritive value in terms of energy and minerals was superior to maize green fodder. Feeding trial in two groups of sheep with 10 numbers in each group fed total mixed ration (TMR) comprising 62 % pineapple fruit residue /maize silage and 48 % concentrate mixture (DM basis) for 75-day period did not show any adverse effects on nutrient utilization (DM, CP, NDF, ADF), serum biochemical (total protein, creatinine, urea nitrogen, SGOT, SGPT), and mineral profile (Ca, P, Mg, Cu, Zn, Mn) and supported a daily growth rate of 140 g. The overall performance was similar to those sheep fed total mixed ration with maize green fodder silage. Feeding pineapple fruit residue silage replacing hybrid napier green fodder in two groups of cows with eight in each group showed an improvement in average daily milk yield by 3.0 lit per cow and fat content by 0.6 U fed pineapple fruit residue silage-based total mixed ration as compared to cows fed hybrid napier green fodderbased total mixed ration. In both studies (sheep or cows), there was no evidence of metabolic or health-related disorders indicating that pineapple fruit residue silage was effectively utilized. Pineapple fruit residue that was hitherto wasted was successfully converted to silage and was found to be a valuable alternative to conventional green fodder. Ensiling of pineapple fruit residue not only improved the economics of feeding but also helped in overcoming the disposal problem (Gowda *et al.* 2015).

**2.7 The Total Mixed Ration: TMR**

The word "TMR" comes from total mixed ration or complete ration (CR) or mixed feed produced by mixing roughage and concentrate in a suitable ratio. The proportion of both types of food must be calculated from the dry matter to meet the requirements of ruminants then the beef cattle - dairy cattle instead of traditional farming. This will separate the roughage and concentrate

in the normal way that the dairy farmer will feed rough the whole day ad libitum and to concentrate food supplement 1-2 times a day while milking. For the convenience of transportation and storage, many companies produce total mix ration in the form of TMR pellets, TMR powder or TMR fermented feed (Chinda 1998; Muller 1990; Wachirapakorn 1997). Total mixed rations (TMR) have been widely used for dairy cattle because they combine forages, grains, protein feeds, minerals, vitamins and feed additives formulated into a single feed mix to satisfy the nutrient requirement of animal. TMR are highly deteriorative feedstuffs, needs to be prepared near to time of use. This rapid deterioration restricts its use on some farms due to labor shortage. Ensiling TMR avoids daily labor for TMR preparation, it also improves preservation and facilitate long distance transportation. Ensiling industrial and agricultural by-products with concentrate as TMR silage could provide year-round nutrition balance feed, and also could improve the palatability by altering odors and flavors from by-products through silage fermentation (Nishino *et al.*, 2003). Due to sufficient fermentable substrate and epiphytic LAB in TMR mixture, all the TMR silages showed good fermentation attributes. L. plantarum further enhanced the LA fermentation compared to control, even though the inoculant application rate was similar to the epiphytic LAB population. (Yuan, X., *et al.* 2015).

**2.8 The Role of TMR Feed**

The pH in the rumen is important to the digestive process of cattle. PH control in the rumen was fixed, it can increase digestion effectively. The pH range should be 6.0 - 6.5, which this pH was directly affected by feed. If cattle were fed separately between roughage and concentrate the pH in the rumen will change with the feed that was given. If feed cow with concentrate, which normally this feed will have high energy digestion. The condition in the rumen was decreased in pH. If the concentrate was large amount, the chances of the rumen become more acidic. If the pH was less than 5, the efficiency of the feed decreases. Dairy cows were low in milk and cattle show high acid in the stomach and when cows grazed or roughly fed, the acid in the rumen was higher. Because cattle were ruminating cause of salivation. It has an alkaline back flow into the rumen. It will help to improve the condition of the acid. So that, feeding roughage and concentrate simultaneously in the form of TMR diets. It is one way to control the level of acidity in the rumen, better to feed separately (Chinda 1998; Muller 1990).

**2.9 Ratio of Roughage to Concentrate**

In TMR diets, the proportion of roughage to concentrate was uncertain, depending on the quality of feed ingredients used in the formula feed. However, the higher proportion of roughage will result in a decrease in the dry matter intake of cattle. (Dhiman *et al.* 1995; Tessmann *et al.* 1991). As a result, the amount of milk and lactose in milk decreased but fat percentage in milk increased (Macleod *et al.* 1983), and when the cows get insufficient energy, the body pulls out the energy accumulated in the body to reduce the weight of cattle. Dhiman *et al.* (1995) studies on the proportion of roughage to concentrate that affects blood metabolism. It was found that the proportion of roughage increased but the concentration of ß-hydroxybutyrate increased as the proportion of roughage increased but the glucose content decreased. It was found that in the TMR formula should have a roughage of not more than 60 percent (Hermandez *et al.* 1976; Weiss and Shokey. 1990, 1991).

**2.10 Size of the Fiber in the TMR.**

In the TMR recipe, the need to reduce the size of the roughage to reduce feed spoilage can increase the dry matter intake and reduce the selection of foods. However, the reduction portion of roughage resulted in dairy cows reduced time to rumination, the secretion of saliva is reduced, the rate of flow of roughage from the rumen faster; the digestion of feed was low and the proportion of acetic acid to propionic decreased. It was also reduced the fat in the milk. Woodford *et al.* (1986) studied the length of the fiber at 0.26, 0.46, 0.64 and 0.90 cm, using the same neutral detergent fiber level of 27.4 percent. There were no statistically significant differences in feed intake, ruminating time, milk yield, digestibility and the flow rate of feed from the rumen. At the same time, the size of the fiber was longer than or equal to 0.64 cm, preventing the reduction of milk fat. Grant *et al.* (1990) studied the size of silage in 2.0, 2.6 and 3 mm. There was not difference in feed intake and milk yield. Milk fat was found to decrease when the fiber size decreased. In addition, the pH level and the proportion of acetic acid to propionic acid decreased when cattle fed a diet of reduced fiber size. The reduction in fat percentage was due to the fat production process in the milk, the volatile fatty acids were used as precursors, especially acetic. For this reason, the ruminants get a small fiber the rate of flow out of the rumen faster the amount of acetic acid produced was low and the milk fat was reduced.

**2.11 Utilization of TMR**

Mayed (2016) reported that TMR nutritional value consists of DM 83.98%, CP 13.37%, CF 15.49%, Ash 5.77%, NDF 45.96%, ADF 22.43% and ADL 10.60%. TMR diets containing 80% concentrate: silage 20% was most suitable for goats. There was feed intake (416.32 g/head/day), weight gain (5.16 kg/head), growth rate (61.43 g/head/day), feed conversion efficiency (9.76) and feed cost (2.64 baht/head/day).

# CHAPTER III

# RESEARCH METHODOLOGY

**This research work was divided into four parts.**

1. Isolation and identification of lactic acid bacteria.
2. Enzyme production analysis.
3. Application of Leucaena to improve quality of pineapple plant silage
4. Efficiency of lactic acid bacteria to digest the total mixed ration by *in vitro* technique

## 3.1 Isolation and Identification of Lactic Acid Bacteria

### 3.1.1 Isolation and identification of lactic acid bacteria from silage

#### 3.1.1.1 Silage preparation

Forty-five-day-old guinea grass (*Panicum maximum*) was cut into 5 cm pieces by sickle starting at the ground level. The harvested material samples were chopped to 2-3 cm by knife. The pre-silage material samples were mixed with 1% NaCl, put in polyethylene bags and stored at ambient temperature for 21 days. A total of 25 g per samples was dissolved in 100 ml sterile water and stirred for 10 min. The pH values were measured using a pH meter (Polan *et al.* 1998). After a 21 days fermentation, the color and aroma of the silages were described according to the score indices of Muhammad *et al.* (2008). For the color description, the silage was scored as 1 = dark or deep brown, 2 = light brown, 3 = pale yellow and 4 = yellowish green. For the aroma description, the silage was scored as 1 = putrid or rancid, 2 = pleasant, 3 = sweet and 4 = very sweet. The analysis of total count of lactic acid bacteria was done by dilution plate method.

#### 3.1.1.2 Isolation

The samples were collected from the silage in three parts of each silo, upper, middle and lower parts. The samples consisted of 100 g from each part which were mixed, and 10 g randomly collected sub samples were diluted with distilled water then mixed with 90 ml of 0.1 % peptone. The sample was serially diluted up to $10^{-6}$, then 0.1 ml was pipetted and spread plated on de Man,

Rogosa and Sharpe broth (MRS) mixed with calcium carbonate 1 % (de Man *et al.* 1960) and incubated at 30 ± 4°C for 48 hours the single colonies observed and isolated into pure culture.

**3.1.1.3 Morphological identification**

Gram stain technique was used to differentiate two large groups of bacteria between Gram positive and Gram negative which appeared violet or red, respectively. Those bacteria that hold on to the primary iodine dye complex and remain violet were characterized as called Gram positive and those which are decolorized and subsequently take up the counterstain (pink/red) that were characterized as gram negative. The morphology of isolated lactic acid bacteria was noted based on the methods of Kandler and Weiss (1986) (Gram's stain, Catalase test, and Gas production test). The resultant data were compared with the descriptions of Kandler and Weiss (1986) and Stiles and Holzapfel (1997). The catalase production test was done using a dropper or Pasteur pipette, placing 1 drop of 3% hydrogen peroxide onto the microorganism on the microscope slide, which were mixed. The microscope slide was immediately covered with a lid to limit aerosols and observe for immediate bubble formation ($O_2$ + water = bubbles). The formation of bubbles against a dark background enhanced readability. Positive reactions were indicated by immediate effervescence (bubble formation). The microscope slide placed over a dark background viewed with a magnifying glass or microscope (40 x) was used to observe weak positive reactions. No bubble formation (no catalase enzyme to hydrolyze the hydrogen peroxide) represents a catalase-negative reaction (South Bend Medical Foundation.2010). Gas production was tested by transferring pure cultures into MRS mixed with 1% NaCl. Durham tubes containing sterilized liquid media were incubated at 30 ± 4° C for 48 hours. Tubes filled with gas, indicated heterofermentation, tubes without gas, homofermentation (Hayward 1957). A growth test with different temperature regimes was done by transferring pure cultures into sterilized MRS media and incubating at 10, 30 and 45° C for 48 hours then observing for turbidity (Cai *et al.* 1998; Yang *et al.* 2010). Salt tolerance was tested at different concentrations of NaCl by transferring pure cultures into sterilized MRS mixed with sterilized NaCl at 1, 3, 6.5 and 10% w/v, and incubating at 30 ± 4°C for 48 hours and observing for turbidity (Cai *et al.* 1998; Yang *et al.* 2010). The growth test for different pH levels was done by transferring pure cultures into

sterilized MRS with pH adjusted to 3.5, 5.7 and 9.6, incubating at 30 ± 4°C for 48 hours and observing for turbidity (Kandler and Weiss. 1986; Yang *et al.* 2010). Morphological identification was done by following the methods of the Bergey Manual of Determinative Bacteriology (Holt *et al.* 1994).

**3.1.2 Phylogenetic identification**

The isolates of LAB were separately cultured on MRS mixed 1%calcium carbonate, incubated at room temperature (30 ± 4 °C) for 24-48 h; single colonies were collected for DNA extraction and observed under a compound microscope for morphological characteristics (Oneca *et al.* 2003). The OPA-3 primer (5'-AGTCAGCCAC-3') was used (Quere *et al.* 1997). The 25 µl PCR solution of consisted of 1X PCR buffer with $(NH_4)_2SO_4$ (Tris-HCl 75 mM, pH 8.8, 25°C, $(NH_4)_2SO_4$ 20 mM and Tween 20 0.01 %), $MgCl_2$ 2 mM, dNTP 400 µM, primer OPA-3 0.4 µM, Taq DNA polymerase 0.1 unit/ µl and the cultured colony to amplify DNA fragments. A cycle was consisted of an initial denaturation at 94 C for 5 minith and followed by 45 cycles of denaturation at 94° C for 30 secs, annealing at 36°C for 1 minith and extension at 72 °C for 1 minith. PCR products were sent to purify and sequence. The sequences were aligned and adjusted using Clustal X program for phylogenetic analysis. DNA sequences were edited and aligned with the Bio Edit program, version 7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were multiply aligned with Clustal X version 1.83 before performing the analysis using the maximum parsimony method with (PAUP) 4.0b (Swofford 1998). For maximum parsimony analyses, bootstraps of 1,000 replicates were performed to examine the relationships of each isolate. Maximum parsimony trees were calculated via fast stepwise addition with representative isolates from GenBank (http://www.ncbi.nlm.nih.gov) and *Enterococcus* spp. was used to analyze as the out-group. Genetic relationships among *Lactobacillus* spp. and *Pediococcus* spp. isolates were determined using the sequences of the ITS region of rDNA to construct phylogenetic trees.

**3.1.3 Proximate composition analysis of silage**

For each treatment, 1000 g of fresh material were random collected to determine nutrient composition. The samples were oven dried at 60°C for 48 hours prior to proximate analysis. Dry matter (DM), Ash, Crude fat (CF), Ether extract (EE), Crude protein (CP) and Crude fiber (CF)

were determined according to the methods of AOAC (1995). Neutral detergent fiber (NDF) and Acid detergent fiber (ADF) were determined according to the method of Van Soest and Robertson (1979). All analyses were conducted using the Fibertec System M6 (FOSS, USA).

## 3.2 Enzyme Production Analysis

The lactic acid bacteria were screened for their ability to produce extracellular degradative enzymes such as amylase, protease, lipase, cellulase and ligninase.

### 3.2.1 Enzyme production

The two species of LAB isolated from silage were selected for their ability to produce extra cellular degradative enzymes such as amylase, protease, lipase, cellulase and ligninase. Three replicates of each treatment were assayed, and non-transferred plates served as negative controls. Transferred plates were incubated at 25° C and checked at either 5 or 10 days depending on the growth rates. When the colonies grew over 50-60 % of the area of the plate, chemical indicators were added to assay enzyme activity and activity zones.

#### 3.2.1.1 Amylase

"Starch agar"

Composition of starch agar medium in g/l was prepared as follows: -

$KNO_3$ 0.5, $K_2HPO_4$ 1, $MgSO_4.7H_2O$ 0.2 $CaCl_2$ 0.1, $FeCl_3$ traces, potato starch 10 g, agar 15 g, $dH_2O$ 1000 ml. Mix, shake the pH (should be 7.2) and then autoclave. Lugol solution was consisted of 1 g crystalline iodine, 2 g KI, 300 ml dH2O, was used for staining lactic acid bacteria.

Pour 14 ml of sterile starch agar medium in to Petri dish, let the agar solidify, streak a drop of bacteria culture onto the starch agar plate, then was incubated at 30° C for 48 hours. When colonies were visible, it was flooded the plate with Lugol solution, then let the iodine react for at least 1 min. It was poured off the iodine from the plate, washed the plate with $dH_2O$ then observed the clear zone surrounding the colony. If Starch was presented in the agar, a blue – black color will appear: the test result is **negative** (i.e. hydrolysis of the starch did not take place). If the starch was hydrolyzed by the excreted amylase, a **clear zone** around the bacteria colony appears. A yellow zone around the colony in an otherwise blue medium was considered a positive test for starch hydrolysis as modified by Gessner (1980).

**3.2.1.2 Protease**

"Skim milk agar" was prepared by mixing: 1 g of agar suspended in 50 ml $dH_2O$ with 5 g skim milk powder suspended in 50 ml $dH_2O$ was made to 100 ml "Skim milk agar"; then was adjusted pH = 7.2 , then autoclaved and poured plates.

Pour 14 ml of sterile skim milk agar medium into a Petri dish was prepared and let the agar solidify, the plate was inoculated with one streak of inoculum, and incubated the plates at 30°C for 48 hours., then was observed the clear zone surrounding the colony. Clear zones surrounding visible colonies implies a positive caseinase (protease) reaction (Folasade and Joshua. 2005).

**3.2.1.3 Lipase**

Lipase activity test was performed by growing the isolates on trypticase soy agar. Autoclave 70 ml of trypticase soy agar growth medium was done and kept at 50° C. One liter of the agar was contained: 15 g Tryptone enzymatic digest of casein, 5 g Soytone - enzymatic digest of soybean meal, 5 g Sodium chloride, 15 g Agar. Final pH 7.3 ± 0.2 at 25°C were adjusted. The fat stained with Nile blue sulfate, add 3.5 ml of melted fat to 70 ml of trypticase soy agar medium was prepared and mixed in well and was dispensed into 5 Petr dishes. It was let for solidify and then streaked the plates with 3 pure cultures and 1 natural sample, then was kept one plate as un-inoculated. Fat with Nile blue sulfate (for fats which are liquid at room temperature) were stained. A saturated solution of Nile blue sulfate in $dH_2O$, add 1 N NaOH was prepared until complete precipitation, filtered and washed the precipitate. Nile blue sulfate oxazine base was used and dried the precipitate. A saturated solution of the Nile blue sulfate oxazine base was prepared and mixed. 10 ml of any fat (e.g., Tween 80, polyoxyethylene sorbitan monolete) with 1 ml of the Nile blue sulfate oxazine base solution, the fat was kept liquid, if necessary, in a heated water bath. 2 volumes of diethyl ether were added to the dye-fat mixture in a separator funnel and was separated the red ether-fat layer from the water layer and washed it several times with water, then separated the ether-water layer and evaporated the ether, autoclave for sterilization, then stored in refrigerator, then observed the clear zone surrounding the colony. A positive test was indicated by the occurrence of precipitated fatty acid crystals around the colony (Abdel-Rheem and Shearer. 2002).

## 3.3 Application of Leucaena to Improve Quality of Pineapple Plant Silage

### 3.3.1 Silage preparation

The harvested materials were 3 samples of para grass, leucaena and pineapple plants randomly taken and chopped to 2-3 cm. (Figure 1) The pre-silage material samples were 5 treatments as follows: - 1. Pineapple plant 100%, 2. pine apple plant mixed with leucaena 5%, 3. pineapple plant mixed with leucaena 10%, 4. pineapple plant mixed with leucaena 15% and 5. grass 100%. All treatments were put into 1%NaCL2 and tightly sealed in plastic containers. They were stored at room temperature for 21 days. A total of 25 g sample was dissolved in 100 ml sterile water and stirred for 10 min. The pH values were measured for acidity changes using the pH meter (Polan *et al.* 1998). After 21 days fermentation, the color and aroma of the silages were evaluated according to the indices score of Muhammad *et al.* (2008). For the color description the silage was scored as 1 = dark brown, 2 = light brown, 3 = pale yellow and 4 = yellowish green. For the aroma description the silage was scored as 1 = putrid or rancid, 2 = pleasant, 3 = sweet and 4 = very sweet.

### 3.3.2 Proximate composition analysis of silage

Fresh samples of 1,000 g were randomly collected to determine nutrient composition. The samples were done by oven drying at 60o C for 48 h prior to proximate analysis. Dry matter (DM), ash, crude protein (CP), crude fibre (CF), ether extract (EE), nitrogen free extract (NFE) and organic matter (OM) were determined according to the methods of AOAC (1995). Neutral detergent fibre (NDF) and Acid detergent fibre (ADF) were determined according to the method of Van Soest and Robertson (1979). Proximate analysis was done before and after fermentation. The experiment was repeated two times. Data were then computed using analysis of variance and treatment means were compared with Duncan's multiple range test (DMRT) at P = 0.05 and P = 0.01.

**Figure 3.1** The fresh samples of pineapple plant

## 3.4 Efficiency of Lactic Acid Bacteria as the Potent Degradative Microorganism to Digest the Total Mixed Ration by *in vitro*

### 3.4.1 Total Mixed Ration (TMR) preparation

The total mixed ration feed used in this study were pineapple plants silage and rice straw as the source of roughage mixed with concentrate. TMR was a recipe for goat meat (Table 3.1).

**Table 3.1** The ingredients of TMR

| Ingredients | kg/100kg |
|---|---|
| Pineapple plant silage | 55 |
| Rice straw | 15 |
| Cassava ship | 5.6 |
| Soybean meal | 12.6 |
| Palm kernel pulp | 4.2 |
| Rice bran | 5.6 |
| Premixed | 0.15 |
| Molasses | 2 |

The premixed consists per kilogram of DM: vitamin A 10,000 IU; vitamin D3 2,000 IU; vitamin E 20 IU; Cu 10 mg; Mn 80 mg; Zn 40 mg; Fe 50 mg; I 0.8 mg; Se 0.3mg; Co 0.3 mg

The experiment was manipulated using completely randomized design (CRD) with four replications. Treatments were used as total mixed ration (TMR) treated with lactic acid bacteria as follows: treatment 1 was TMR non-treated control, treatment 2 was TMR treated with *Lactobacillus pantarum,* treatment 3 was TMR treated with *Pediococcus pentosaceus,* treatment 4 was TMR treated with *Pediococcus acidilactici*, and treatment 5 was TMR treated with *L. pantarum* + *P. pentosaceus* + *P. acidilactici*. The treated TMR were mixed and put in polyethylene bags and stored at ambient temperature for 7 days before analysis.

**3.4.2 Proximate composition analysis of silage**

For each treatment, 1000 g of fresh material were randomly collected to determine nutrient composition. The samples were oven dried at 60°C for 48 h prior to proximate analysis. Dry matter (DM), Ash, Crude fat (CF), Ether extract (EE), Crude protein (CP) and Crude fiber (CF) were determined according to the methods of AOAC (1995). Neutral detergent fiber (NDF) and Acid detergent fiber (ADF) were determined according to the method of Van Soest and Robertson (1979). All analyses were conducted using the Fibertec System M6 (FOSS, USA). The experiments were designed as completely randomized design with 4 replications. The experiment was repeated two times. Data were computed using analysis of variance and treatment means were compared with Duncan's Multiple Range Test (DMRT) at P = 0.05 and P = 0.01.

**3.4.3 *in vitro* digestibility test**

Pepsin cellulase *in vitro* digestibility method was followed Mcleod and Minson (1978). The enzymatic digestion was determined by adding 0.5 g. of feed sample to test tube, size 2.5 cm. long 10 cm. with screw cap. A 500 ml. of acid – pepsin (0.2% pepsin with 0.1 N HCL) was prepared the day before and incubated at 39° C, then added and tubes were shaken and incubated at 39° C for 48 h. After incubation, feed sample were centrifuged for 10 min. Thereafter the tube was filtered by filter stick for suction water and the residues washed with distilled water and transferred to the respective test tube. A 50 ml cellulase acetate buffer solution (ONOZUKA 3S) was prepared the day before and incubated at 39° C, and tubes were shaken for 10 minutes, and incubated at 39° C for 48 h., then the tubes were filtered through pre – weighed sintered glass crucibles and washed with distilled water. Thereafter, the crucibles contained with residues were dried at 105° C for 12 h. and weighed to obtain analysis of dry matter. The crucibles with residues

were burnt at 500° C for 5 h. in furnace cooled to room temperature and weighed to obtain to analysis for dry matter digestibility.

# CHAPTER IV

# RESULTS

## 4.1 Isolation and Identification of Lactic Acid Bacteria

### 4.1.1 Silage preparation

After 21 days of ensilage, the plastic containers were opened and examined for gross characteristics. Overall, the silages were of good color, aromatic and acidic. The silages contained an average of 76.47 % moisture. The pH values of the silages averaged 5.53.

### 4.1.2 Morphological identification

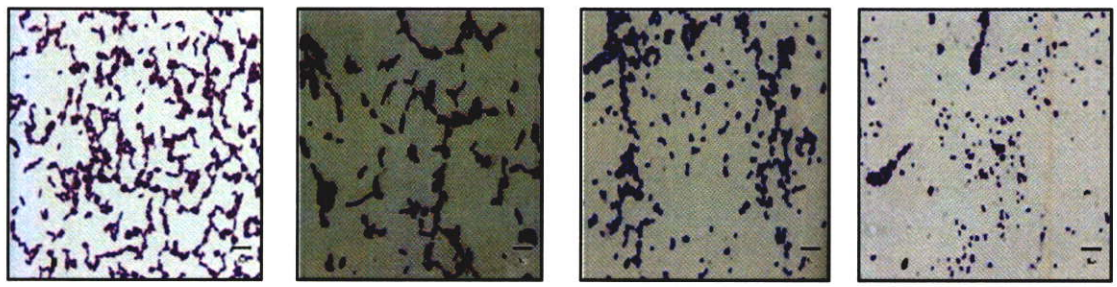
#### 4.1.2.1 Isolation

The results found that the characteristic of lactic acid bacteria (LAB) colonies selected on the recipe were different for the colonies. By the area the colonies, there was a clear zone around the colony. This was due to the addition of calcium carbonate to MRS prior to isolation of the bacteria, when LAB grows, lactic acid was produced. From the observation of colonies of LAB isolated from silage, it was found that colors of the colony were both white and slightly opaque. Colony appearance was quite opaque, the area around the colony was circular, entire, and the top of the colony is flat, convex and umbonate in the middle. The size of the colony was both large with a diameter of 2 – 3 millimeters and small with a diameter of 0.5 – 1.0 millimeters.

For the number of LAB found in silage was $5.99 \times 10^{6}$ cfu/g of silage. The number of different LAB could have several causes, for example the nutrient factors that were presented in plants silage. That was, the amount of sugar or soluble carbohydrates, which were important nutrients for the growth of LAB.

#### 4.1.2.2 Morphological identification

Sample of pure culture lactic acid bacteria (LAB) was studied the morphology by gram stain technique to identify the appearance of bacteria and the formation of the bacteria. The catalase production test, gas production from utilizing sugar, testing the ability of bacteria at temperature at concentration of sodium chloride and at various pH levels. It was found that all of isolates of the bacteria isolated were Gram positive. The shapes of cell were in the form of rod, cocci and somewhat similar to rounded oval. The cell as rod shape was arranged in a chain. The cocci shapes were arranged in chain, pairs and divided in four directions or tetrad (Figure 4.1).



A B C D

**Figure 4.1.** Morphology of lactic acid bacteria isolated from silage

A and B show characteristics of Lactobacilli

C and D show characteristics of Pediococci

Sample of isolated LAB almost of them could not produce catalytic enzymes. Because of its internal cell of LAB lacks such enzymes. By assaying the formation of catalytic enzymes of isolated bacteria samples, hydrogen peroxide solution was tested.

For gassing of glucose fermentation to determine the fermentation pathway of individual isolates, most LAB were fermented as homofermentative at 91.5 percent. For the LAB were fermented as heterofermentative accounted for approximately 8.5 percent. The samples of homofermentative were mostly rod shaped the heterofermentative were cocci. For the test of LAB growth ability at ambient temperature 10, 30 and 45 degrees Celsius at 1, 3, 6.5 and 10 percent sodium chloride concentrations and at pH 3.5, 5.7 and 9.6 for each isolate. They were able to grow at 10, 30 and 45 degrees Celsius, at sodium chloride concentrations of 1, 3, 6.5 percent and a pH of 3.5, 5.7 and 9.6 in each isolate that could be separated into 4 groups (A – D) as shown in Table 4.1. The 57 different isolates could be divided into two genera. *Lactobacillus plantarum* H5-M13F was commonly found in silages (an incidence of 87.59%), *Pediococcus pentosaceus* Ac2-M13F was found in silages at a lower incidence (3.19%).

**Table 4.1.** The characteristics of lactic acid bacteria isolated from silage

| Characteristic | Group | | | |
|---|---|---|---|---|
| | A | B | C | D |
| Morphology | Rods | Rods | Cocci | Cocci |
| Gram | + | + | + | + |
| Catalase test | - | - | - | - |
| Gas tast | - | - | - | - |
| Fermentation | Homo | Homo | Homo | Homo |
| Temperature | | | | |
| 10°C | + | + | - | - |
| 30°C | + | + | + | + |
| 45°C | + | + | + | + |
| $P^H$ | | | | |
| 3.5 | + | + | + | + |
| 5.7 | + | + | + | + |
| 9.6 | + | + | + | + |
| NaCL | | | | |
| 1.0% | + | + | + | + |
| 3.0% | + | + | + | + |
| 6.5% | + | + | + | + |
| 10.0% | ± | - | - | - |
| Identifided as | Lacto bacilli | Lacto bacilli | Ped. sp. | Ped. sp. |

**Note:** + = Positive reaction, - = Negative reaction, ± = Variable reaction

Homo = Homofermentative, Ped. = *Pediococcus* sp., Lacto bacilli = *Lactobacillus* sp.

**4.1.3 Phylogenetic identification**

Amplification of the ITS regions from isolates of *Lactobacillus* spp. and *Pediococcus* spp. used the Primer OPA-3. The phylogenetic tree from PAUP analysis placed the *Lactobacillus* spp. and *Pediococcus* spp. into two distinct groups. Phylogenetic analysis produced a dendrogram indicating that there was no relationship between the *Lactobacillus* spp. and *Pediococcus* spp. groups (Figure 4.2).

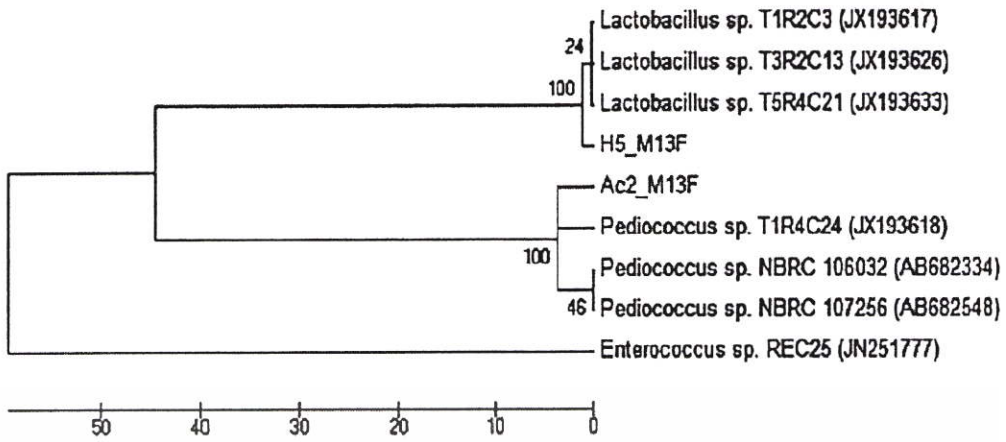



**Fig. 4.2.** Phylogenetic relationships of *Lactobacillus* spp. and *Pediococcus* spp. isolates based on the ITS regions of rDNA sequences. The phylogenetic tree was obtained by the maximum parsimony method using the PAUP4.0b program. *Enterococcus* sp. was used as the out group. The numbers above the lines represent the 1000 replicates parsimony bootstrap values.

### 4.1.4 Proximate analysis of silages

The proximate composition of the examined silages showed that dry matter changed from 20.9% before treatment to 23.5% after treatment. Ash content was 15.7 %, crude protein 7.9%, crude fiber 42.6%, ether extract 4.2%, nitrogen free extract 22.6%, organic matter 84.2%, neutral detergent fiber 56.5% and acid detergent fiber 37.8% (Table 4.2). The experiments showed that dry matter, ash, crude fiber and acid detergent fiber were increased after fermentation. With this, ether extract, nitrogen free extract, organic matter and neutral detergent fiber were decreased after fermentation.

**Table 4.2.** Composition of Guinea grass silage from proximate analysis

| Composition | Before fermentation (%) | After fermentation (%) |
|---|---|---|
| Dry matter (DM) | 20.9 | 23.5 |
| Ash | 11.1 | 15.7 |
| Crude protein (CP) | 7.9 | 7.9 |
| Crude fiber (CF) | 33.8 | 42.6 |
| Ether extract (EE) | 5.6 | 4.2 |
| Nitrogen free extract (NFE) | 35.9 | 22.6 |

*Pediococcus pentosaceus* produced amylase indicated by a yellow clear zone around the bacteria colonies (Figure 4.4).

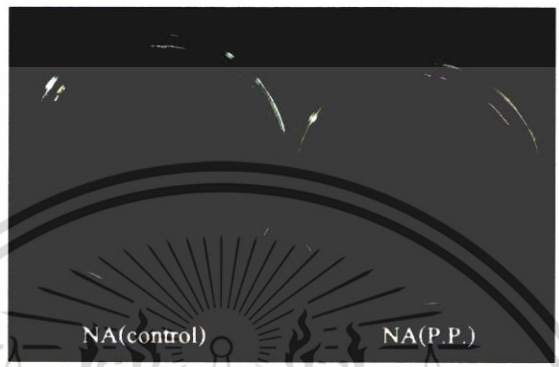



**Figure 4.4** Amylase production by *Pediococcus pentosaceus*

*Pediococcus pentosaceus* produced protease indicated by a clear zone around the bacteria colonies (Figure 4.5).

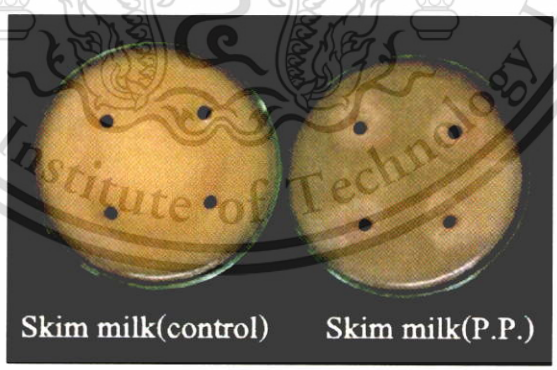



**Figure 4.5** Protease production by *Pediococcus pentosaceus*

*Lactobacillus plantarum* could not produce amylase, cellulase, ligninase and lipase as shown in Figure. 4.6.

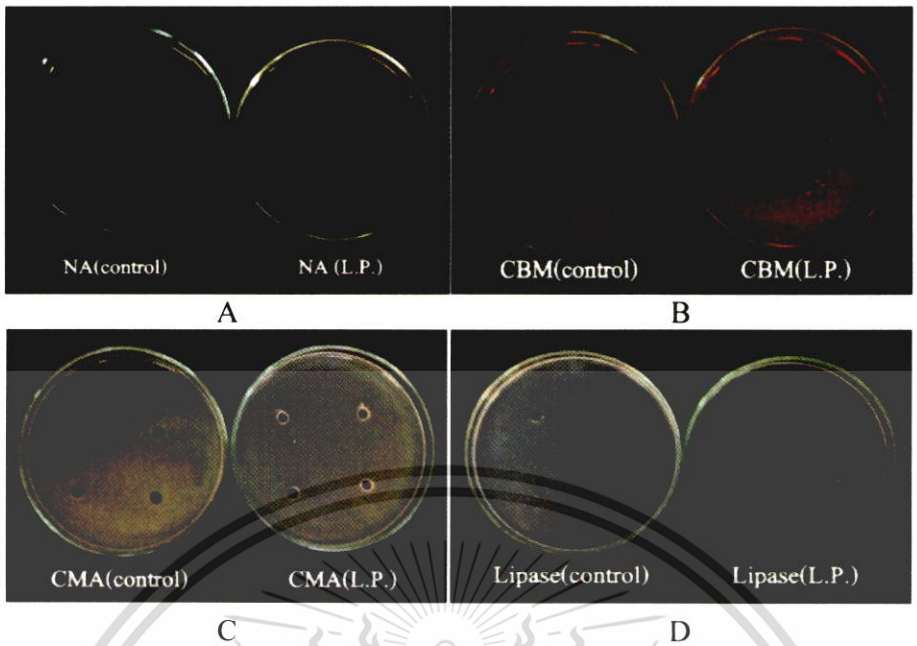



**Figure 4.6** The negative enzyme production by *Lactobacillus plantarum*: **A** = amylase **B** = cellulase **C** = ligninase D = lipase

*Pediococcus pentosaceus* could not produce cellulase, ligninase and lipase as shown in Figure. 4.7.

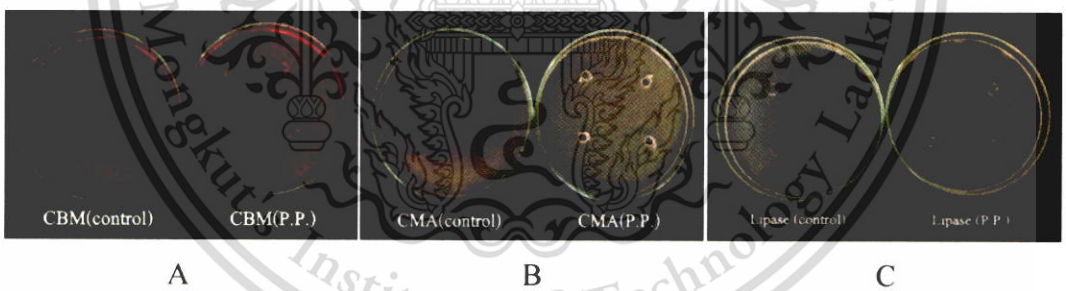



**Figure 4.7** The negative enzyme production by *Pediococcus pentosaceus*: **A** = cellulase **B** = ligninase **C** = lipase

## 4.3 Application of Leucaena to Improve Quality of Pineapple Plant Silage

### 4.3.1 Physical characteristic of silage

After 21 days of ensilage, the plastic containers were opened and examined for gross characteristics. The physical characteristic (Table 4.4) showed that the color of Pineapple plant 100%was a yellowish green color which was a good characteristic of silage (Figure 4.8).

For Pineapple plant mixed with leucaena 5, 10 and 15% they were light brown color. Grass silage 100% was a dark brown color, with quality being lower than Pineapple plant mixed with leucaena 5,10 and 15%. The aroma of silage, pineapple plant 100%, pineapple plant mixed with leucaena 5, 10 and 15% were aromatic and acidic like pickled fruit (sweet smell). For Grass silage 100% the aroma of silage was very sour. The pH values of silage pineapple plant 100%, pineapple plant mixed with leucaena 5, 10, 15% and grass silage 100% were 3.45, 3.43, 3.72, 3.52 and 4.27 respectively. Overall, treatments showed highly significant differences at a low level ($P < 0.01$). The moisture value of silage showed that highly significant differences ($P < 0.01$). Pineapple plant 100% gave significantly higher moisture (81.83%) than pineapple plant mixed with leucaena 15% (80.95%), pineapple plant mixed with leucaena 5% (79.77%), pineapple plant mixed with leucaena 10% (79.10%) and was lower in grass silage 100% (72.93%).

**Table 4.4** The Physical Characteristics of Silage

| | Silage | Character | |
|---|---|---|---|
| | | Color of silage | Aroma of silage |
| 1 | Pineapple plant 100% | Yellowish green | Aromatic and acidic |
| 2 | Pineapple plant mixed with leucaena 5% | Light brown | Aromatic and acidic |
| 3 | Pineapple plant mixed with leucaena 10% | Light brown | Aromatic and acidic |
| 4 | Pineapple plant mixed with leucaena 15% | Light brown | Aromatic and acidic |
| 5 | Grass silage 100% | Dark brown | Very sour |

Note: The color and aroma of silage were described according to the score indices of Muhammad *et al.* (2008)

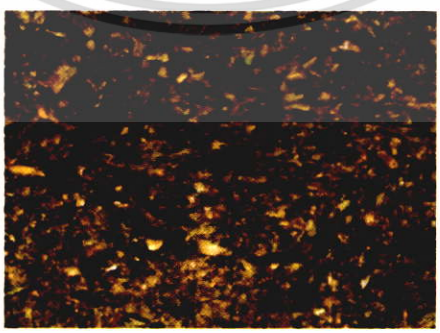


**Figure 4.8** The physical characteristics of pineapple plant silage

**Table 4.5.** Proximate composition analysis of pine apple plant silage

| Treatment | Nutritive value of pine apple plant silage | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | pH | DM | Ash | CP | CF | Ca | P | Energy |
| 1. P 100% | 3.45D | 18.17C | 9.38B | 6.13D | 24.18AB | 0.26C | 0.38A | 3,620.99A |
| 2. P+L 5% | 3.43C | 20.23BC | 9.05BC | 6.39CD | 21.76C | 0.32B | 0.29B | 3,471.06B |
| 3. P+L 10% | 3.72B | 20.90B | 9.71B | 6.99bC | 22.51C | 0.35B | 0.29B | 3,475.86B |
| 4. P+L 15% | 3.52C | 19.05BC | 8.14C | 8.19A | 23.15BC | 0.48A | 0.36A | 3,697.17A |
| 5. Grass 100% | 4.27A | 27.08A | 16.02A | 7.39B | 25.42AB | 0.34B | 0.19C | 3,029.80C |

Means followed by a common letter in each column are not significantly different ($p<0.01$) by DMRT. P= pineapple plant, L = leucaena, DM = dry matter, CP = crude protein, CF = crude fiber Ca = calcium, P = phosphorus

Results showed that fibre analysis by using Van Soest demonstrated the fibre digestion of pineapple plants mixed with leucaena silage could be degraded (Table 4.6). Pineapple plant mixed with leucaena 15% gave significantly lower neutral detergent fiber (42.19%) than pineapple plant mixed with leucaena 5% (43.30%), pineapple plant mixed with leucaena 10% (43.71%), pineapple plant 100% (47.10%) and grass silage 100% (48.76%), but it was not significantly different in pineapple plant mixed with leucaena. Pineapple plant mixed with leucaena 5% gave significantly lower acid detergent fiber (29.85) than grass silage 100% (29.88%), pineapple plant mixed with leucaena 15% (30.46%), pineapple plant mixed with leucaena 10% (30.90%) and pineapple plant 100% (31.94%) respectively. Grass silage 100% gave significantly lower acid detergent lignin (3.98%) than pineapple plant mixed with leucaena 15% (5.66%), pineapple plant mixed with leucaena 5% (5.99%), pineapple plant 100% (6.19%) and pineapple plant mixed with leucaena 10% (6.23%) respectively. It was not significantly different in acid detergent lignin of pineapple plant 100% and pineapple plant mixed with leucaena. In this study, pineapple plant mixed with leucaena 15% gave significantly higher metabolizable energy (3,697.17 kcal/k) than pineapple plant 100% (3,620.99 kcal/k), pineapple plant mixed with leucaena 10% (3,475.86 kcal/k), pineapple plant mixed with leucaena 5% (3,471.06 kcal/k) and grass silage 100% (3,029.80 kcal/k) respectively.

**Table 4.6.** Fibre analysis of pineapple plant silage

| Treatment | NDF | ADF | ADL |
|---|---|---|---|
| 1. P 100% | 47.10 A | 31.94 A | 6.19 A |
| 2. P+L 5% | 43.30 B | 29.85 B | 5.99 A |
| 3. P+L 10% | 43.71 B | 30.90 AB | 6.23 A |
| 4. P+L 15% | 42.91 B | 30.46 AB | 5.66 A |
| 5. Grass 100% | 48.76 A | 29.88 B | 3.98 B |

Means followed by a common letter in each column are not significantly different ($p<0.01$) by DMRT.

P = pineapple plant, L = leucaena, NDF = neutral detergent fiber, ADF = acid detergent fiber, ADL = acid detergent lignin

## 4.4 Efficiency of Lactic Acid Bacteria as the Potent Degradative Microorganism to Digest the Total Mixed Ration by *in vitro*

### 4.4.1 Chemical composition of total mixed ration (TMR)

The nutritive value of TMR revealed that dry matter 68.28%, crude protein 12.19%, crude fiber 15.06%, ASH 6.54%, neutral detergent fiber 32.25%, acid detergent fiber 23.74% and acid detergent lignin 5.71% respectively.

### 4.4.2 Changes in chemical composition of TMR treated with lactic acid bacteria

Results showed that the nutritive value of TMR by proximate analysis which treated *Lactobacillus pantarum* (LP), *Pediococcus pentosaceus* (PP), *Pediococcus acidilactici* (PA) and *L. pantarum* + *P. pentosaceus* + *P. acidilactici* (LPP) was indicated in Table 4.7. The dry matter was changed from 68.28 to 79.80, 79.79, 79.40, and 79.42% for *Lactobacillus pantarum*, *Pediococcus pentosaceus*, *Pediococcus acidilactici* and *L. pantarum* + *P. pentosaceus* + *P. acidilactici* respectively, which increased in dry matter. With this TMR treated with *Lactobacillus pantarum* gave significantly higher dry matter than *Pediococcus pentosaceus*, *Pediococcus acidilactici* and *L. pantarum* + *P. pentosaceus* + *P. acidilactici*. TMR treated with *Pediococcus acidilactici* gave significantly higher protein (10.97%) than *Pediococcus pentosaceus* (10.02%), *L. pantarum* + *P. pentosaceus* + *P. acidilactici* (9.77%) and *Lactobacillus pantarum* (9.65%), but it was not significantly different in *Lactobacillus pantarum*, *Pediococcus pentosaceus*, *Pediococcus acidilactici* and *L. pantarum* + *P. pentosaceus* + *P. acidilactici*. TMR treated with

*Pediococcus pentosaceus* gave significantly lower fiber (13.4%) than *Pediococcus acidilactici* (13.33%), *L. pantarum* + *P. pentosaceus* + *P. acidilactici* (13.64%) and *Lactobacillus pantarum* (13.82%), but it was not significantly difference between *Pediococcus pentosaceus* with *Pediococcus acidilactici* and *Lactobacillus pantarum* with *L. pantarum* + *P. pentosaceus* + *P. acidilactici*. TMR treated with *Pediococcus acidilactici* gave significantly higher ash (5.79%) than *Lactobacillus pantarum* (5.58%), *Pediococcus pentosaceus* (5.56%) and *L. pantarum* + *P. pentosaceus* + *P. acidilactici* (5.53%). TMR treated with *Pediococcus acidilactici* gave significantly lower AIA (1.29%) than *Pediococcus pentosaceus* (1.50%), *Lactobacillus pantarum* (1.55%) and *L. pantarum* + *P. pentosaceus* + *P. acidilactici* (1.56%), but it was not significantly difference in AIA between *Lactobacillus pantarum*, *Pediococcus pentosaceus* and *L. pantarum* + *P. pentosaceus* + *P. acidilactici*.

**Table 4.7.** Chemical composition of TMR treated with lactic acid bacteria

| Treatments | Chemical composition (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DM | CP | CF | Ash | Ca | P | AIA | Digest |
| 1. Control | 68.28 A | 12.19 A | 15.06 A | 6.54 A | 1.19 A | 0.33 A | 1.75 A | 60.31 C |
| 2. LP | 79.80 C | 9.65 C | 13.82 B | 5.58 C | 1.14 B | 0.28 B | 1.55 B | 64.98 B |
| 3. PP | 79.79 C | 10.02 C | 13.14 C | 5.56 C | 0.98 D | 0.25 C | 1.50 B | 65.16 B |
| 4. PA | 79.40 C | 10.97 B | 13.33 C | 5.79 B | 0.97 D | 0.25 C | 1.29 C | 66.21 A |
| 5. LPP | 79.42 B | 9.77 C | 13.64 B | 5.53 C | 1.04 C | 0.26BC | 1.56 B | 66.20 A |
| CV. | 0.55 | 5.50 | 0.93 | 1.03 | 2.70 | 5.70 | 3.47 | 0.43 |

Mean followed by common letter in each column are not significantly difference. ($P < 0.01$) by DMRT.
Control = non-treated, LP = *Lactobacillus pantarum*, PP = *Pediococcus pentosaceus*
PA = *Pediococcus acidilactici*, LPP = *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici*
DM = dry matter, CP = crude protein, CF = crude fiber, Ca = calcium, P = phosphorus, AIA = acid insoluble ash, Digest = digestibility.

Results showed that fiber analysis in TMR revealed that fiber digestion of TMR treated with *Lactobacillus pantarum* gave significantly higher neutral detergent fiber (34.13%)than *Pediococcus acidilactici* (32.61%), *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici* (31.69%) and *Pediococcus pentosaceus* (30.20%). It was not significantly differed in neutral detergent fiber

between *Pediococcus acidilactici* and *Pediococcus pentosaceus*. TMR treated with *Pediococcus pentosaceus* gave significantly lower acid detergent fiber (20.29%) than *Lactobacillus pantarum* (20.60%), *Pediococcus acidilactici* (21.60%) and *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici* (22.59%). TMR treated with *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici* gave significantly lower acid detergent lignin (4.88%) than *Pediococcus pentosaceus* (4.90%), *Pediococcus acidilactici* (5.08%) and *Lactobacillus pantarum* (5.24%). It was not significantly difference in *Lactobacillus pantarum*, *Pediococcus pentosaceus*, *Pediococcus acidilactici* and *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici* when compared with non-treated with lactic acid bacteria (Table 4.8).

**Table 4.8.** Fiber analysis of TMR treated with lactic acid bacteria

| Treatments | Fiber Analysis (%) | | |
|---|---|---|---|
| | NDF | ADF | ADL |
| 1. Control | 32.25 B | 23.74 A | 5.71 A |
| 2. LP | 34.13 A | 20.60 D | 5.24 AB |
| 3. PP | 30.29 C | 20.29 D | 4.90 B |
| 4. PA | 32.61 B | 21.60 BC | 5.08 B |
| 5. LPP | 31.69 B | 22.59 B | 4.88 B |
| CV. | 2.29 | 3.26 | 6.31 |

Mean followed by common letter in each column are not significantly difference. ($P < 0.01$) by DMRT.
Control = non-treated, LP = *Lactobacillus pantarum*, PP = *Pediococcus pentosaceus*
PA = *Pediococcus acidilactici*, LPP = *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici*
NDF = neutral detergent fiber, ADF = acid detergent fiber, ADL = acid detergent lignin

Results showed that the TMR treated with lactic acid bacteria using pepsin cellulase *in vitro* digestibility which applied *Lactobacillus pantarum*, *Pediococcus pentosaceus*, *Pediococcus acidilactici* and *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici* could improve the digestibility of TMR, which significantly increased in digestibility of TMR. With this TMR treated with *Pediococcus acidilactici* gave significantly higher digestibility (66.21%) than *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici* (66.20%), *Pediococcus pentosaceus* (65.16%) and *Lactobacillus pantarum* (64.98%). IT was not significantly different between *Pediococcus acidilactici*, *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici* and *Lactobacillus pantarum*, *Pediococcus*

*pentosaceus* (Table 4.8). The rate of increase and decrease of digestibility and fiber in TMR treated with lactic acid bacteria (Table 4.9) showed TMR treated with *Lactobacillus pantarum* gave higher increased dry matter (+16.87%) than *Pediococcus pentosaceus* (+16.86%), *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici* (+16.31%) and *Pediococcus acidilactici* (+15.76%). TMR treated with *Pediococcus pentosaceus* gave higher decreased in crude fiber (-12.75%) than *Pediococcus acidilactici* (11.49%), *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici* (-9.43%) and *Lactobacillus pantarum* (-8. 23%). For neutral detergent fiber in TMR treated with *Lactobacillus pantarum* and *Pediococcus acidilactici* were increased (+5.83% and +1.12%) but *Pediococcus pentosaceus* and *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici* the results showed that the value of neutral detergent fiber was decreased (-6.08% and -1.74%) respectively. TMR treated with *Pediococcus pentosaceus* gave higher decreased acid detergent fiber (-14.53%) than *Lactobacillus pantarum*, *Pediococcus acidilactici* and *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici* were -13.23%, -9.01% and -4.84% respectively. For acid detergent lignin also, the results showed that TMR treated with lactic acid bacteria were decreased. The digestibility of TMR treated with *Pediococcus acidilactici* gave higher increased than *L. Pantarum* + *P. Pentosaceus* + *P. acidilactici*, *Pediococcus pentosaceus* and *Lactobacillus pantarum* were +9.78%, +9.76, +8.41% and +7.74% respectively.

**Table 4.9.** The rate of increased and decreased digestibility and fiber in TMR treated with LAB

| Chemical composition | Before treated | After treated lactic acid bacteria (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | LP | +/- | PP | +/- | PA | +/- | LPP | +/- |
| Dry matter | 68.28 | 79.80 | +16.87 | 79.79 | +16.86 | 79.40 | +15.76 | 79.42 | +16.31 |
| Crude fiber | 15.06 | 13.82 | -8.23 | 13.14 | -12.75 | 13.33 | -11.49 | 13.64 | -9.43 |
| NDF | 32.25 | 34.13 | +5.83 | 30.29 | -6.08 | 32.61 | +1.12 | 31.69 | -1.74 |
| ADF | 23.74 | 20.60 | -13.23 | 20.29 | -14.53 | 21.60 | -9.01 | 22.59 | -4.84 |
| ADL | 5.71 | 5.24 | -8.23 | 4.90 | -14.19 | 5.08 | -11.03 | 4.88 | -14.54 |
| Digestibility | 60.31 | 69.98 | +7.74 | 65.16 | +8.41 | 66.21 | +9.78 | 66.20 | +9.76 |

LP = *Lactobacillus pantarum*, PP = *Pediococcus pentosaceus*, PA = *Pediococcus acidilactici*, LPP = *L. Pantarum* + *P. Pentosaceus* + *P. Acidilactici*, NDF = neutral detergent fiber, ADF = acid detergent fiber, ADL = acid detergent lignin

# CHAPTER V

# DISCUSSION

Evaluation results of the typical physical characteristics of the silage from Guinea grass were characteristics of colors and odors. Good color is yellowish green. The characteristics of the smell was sour aroma like pickle fruit. The smell is caused by the work of microorganisms that occur during the fermentation process. The substrate that was presented in the forage crops transformed into products by the microorganisms that occur on different fermented forage crops. It would be different products, caused the smell of silage. (McDonald *et al.* 1991; Brookes and Buckle. 1992; Woolford and Pahlow. 1998). The pH values indicated the anaerobic fermentation of the silages similar to the work of Schroeder (2004).

The samples were collected from the silage for isolation and identify by standard plate count on MRS (de Man *et al.* 1960) mixed with 1% calcium carbonate with spread plate technique. By the area the colonies, there was a clear zone around the colony. This was due to the addition of calcium carbonate to MRS prior to isolation of the bacteria, when lactic acid bacteria grown, lactic acid was produced. Normally, calcium carbonate does not dissolve, but if lactic acid is produced, it will convert calcium carbonate into calcium lactate with good water solubility (Frazier and Rupp, 1928). The added calcium carbonate acts as an indicator to enhance the appearance of the colonies (Sasiwimol and Adisorn. 2005 The characteristics of these lactic acid bacteria colonies have been reported to be common feature of lactic acid bacteria (Kandler and Weiss. 1986).

For the number of lactic acid bacteria present in silage. Parvin and Nishino (2009) found that the numbers ranged from $10^{7}$-$10^{13}$ CFU/g from the study of similar grass samples. The number of different lactic acid bacteria can be attributed to several factors, such as the nutrient factors present in silage, ie, the amount of sugars or carbohydrates that are soluble. It was an important nutrient for the growth of lactic acid bacteria. It was found that in tropical forage crops, these nutrients were lower than that of temperate forage crops (Catchpool and Henzell. 1971) and some nutrients such as manganese was presented in the plant to help maintain the survival of lactic acid

bacteria itself (Daeshel *et al.* 1987), or even the factor of ultraviolet light and temperature (Zhang *et al.* 2000). Including the number of such microorganisms that are presented in the forage crop prior to fermentation (Cai *et al.*1994). Normally, the number of lactic acid bacteria varies by different plant species. Epiphytic lactic acid bacteria are commonly found on the outer surface of plant parts such as stems, leaves and flowers, which were found on the leaves of the plant less than the structure of flowers and fruits. There was also an increased in the amount when the plant grows older (Greenhill 1964; Cai *et al.* 1994). Sample of pure lactic acid bacteria was preparied to study the morphology. Gravimetric staining was used to determine the morphology and the arrangement of the specimens. Catalase enzyme test, sugar production from sugar, testing of the ability to grow at temperature, at the concentration of sodium chloride and at various pH levels. Lactic acid bacteria can produce hydrogen peroxide by the process of metabolism that occurs during growth in the aerobic condition and hydrogen peroxide was created as an oxygen receptor and hydrogen peroxide was toxic to microorganisms that cannot produce a catalytic enzyme to destroy its activity. Because hydrogen peroxide acts as precursor of superoxide radical ($O^{-2}$) and hydroxyl radical ($OH^{\cdot}$) toxic to the cells (Caplice and Fitzgerald. 1999). Therefore, the assay for the formation of catalytic enzymes showed that LAB did not react with hydrogen peroxide. However, it was found 1 isolated to produce catalytic enzymes. It was possible that this sample could produce pseudo – catalase that were used to degraded peroxide (Kandler and Weiss. 1986).

Jolaosho *et al.* (2013) reported that the proximate composition and fiber fractions of silage from Guinea grass showed that DM was 21.03 %, CP 9.48 %, EE 6.5 %, ASH 8.13 %, NFE 15.58 % NDF 60.31 % and ADF 40.98 %. Iuchi *et al*, 2012 isolated *Pediococcus ethanolidurans* A4-27 from Japanese pickles (nuka-zuke) and found that it could produce a high molecular weight amylase. They indicated that the amylase enzyme from *P. ethanolidurans*is was useful for fermentation of rice bran-bed (nuka-doko) which contained more than 15% NaCl. Generally, the silage should have a darker color than fresh forage because the color of chlorophyll reacted with acid from fermentation. These changed them to become a magnesium free pigment phaeophytin. However, the carotene was a provitamin A which was suffered from oxidation at high temperature (Azim *et al.*2000; Wanapat 1986). The sweet smell was caused by lactic acid bacteria which utilized sugar in the forage to produce lactic acid and volatile acid (McDonald *et al.*1991; Merry *et al.* 2000). The pH values of silage were indicated that bacteria to produced lactic acid (Schroeder 2004). The pH of good quality silage was 3.5 – 4.5, if pH increases more than 5.1 it was low quality (church 1991). The silage making had high moisture and lowered dry matter

during raining season may have coursed risk for the silage to spoil. In cases where the silage had lower moisture and higher of dry matter it may have been more fibre difficult to compact the silage in the silo (Saranya and Jantakarn. 1997). For the good fermentation process and fast originate, dry matter had a loss of approximately 1 – 2 % from respiratory of forage during first stage (McDonald *et al.* 1991). The increased of ash occurred by the utilization of plant organic substance and change to inorganic substance by microorganism during fermentation (Frame 1994). McDonald *et al.* (1991) reported that usually decreases in protein was due to the initially digestion by microorganism, while the increased of protein may occurs by the influence of salt, which it prevents clostridium sp. to not destroy protein. However, fibre decreased during fermentation which may occurred have happened due to lactobacillus sp. which could have digested the cell wall (Mc Donald *et al.* 1991). For NDF the decreased in may be due to the part of cell wall and carbohydrate structure being utilized as an energy source for microorganism growth during fermentation (O'Kiely and Muck. 1998). The acid detergent fibre of silage trend were perhaps increased by the sugar structured in the plant cell with the microorganism being utilized and causing ADF increase (Campbell and Bruchanan-Smith. 1991). Generally, a good range of ADF in dairy cattle' feed should be around 40 – 60 % to produces butterfat in milk (Somjit 2006; Jantakarn 2009). The quantity of lignin, cellulose, and hemicellulose in feed are important for the forage crop of ruminants. A good quality of 850 forage crop should be low in lignin (Flores 1991). The leucaena was completed in nutrient and when mixed with the pineapple plant the metabolizable energy was increased (Piliwan *et al.* 1989). The reduced protein content is due to the utilization of protein in food in the diet by the microbial proteolysis process (Guo *et al.* 2013). CWC or NDF refers to group of chemicals that are components to the cell wall. Mostly cellulose hemicellulose lignin including cutin, silica and tannin. Normally, simple stomach animal those cannot be digested, while ruminant can have some microorganisms in the stomach, but can be more or less dependent on the amount of lignification, cutinization as well as the amount of silification in the feed. ADF the substances in the ADF are cellulose, lignin, cutin and acid insoluble ash (AIA) refers to ash that is insoluble in acid (Van Soest 1963; Van Soest 1964). Van Soest and Moore (1965) found that CWC digestibility was correlated with the amount or concentration of Lignin present in the ADF, especially when converted into logarithm. *Lactobacillus* sp, could produce protease and *Pediococcus* sp. was able to produce amylase and protease. These two species can possibly be used to improve the nutritional value of the silage, TMR or roughage for ruminant production (Suphalucksana and Soytong. 2017).

# CHAPTER VI

# CONCLUSION

Isolates of lactic acid bacteria identified based on their morphology were divided into two genera. *Lactobacillus plantarum* H5-M13F was found at a high incidence in silages (87.59%) and *Pediococcus Pentosaceus* Ac2-M13F was found at a low incidence (3.19%). The phylogenetic tree from PAUP analysis placed the recovered *Lactobacillus plantarum* and *Pediococcus Pentosaceus* into two distinct groups. Phylogenetic analysis revealed that there was no relationship between the *Lactobacillus* sp. and *Pediococcus* sp. groups. The proximate composition of the examined silages showed that silages contained an average of 76.47 % moisture. The pH values of the silages averaged 5.53, dry matter 23.5%, ash content 15.7 %, crude protein 7.9%, crude fiber 42.6%, ether extract 4.2%, nitrogen free extract 22.6%, organic matter 84.2%, neutral detergent fiber 56.5% and acid detergent fiber 37.8%. *Lactobacillus pantarum*, could produce protease and *Pediococcus Pentosaceus* was able to produce amylase and protease. These two species can possibly be used to improve the nutritional value of the silage, TMR or roughage for ruminant production.

The uses of leucaena to improve nutritive value in pineapple plant silage were determined. The physical characteristics, chemical composition and fibre analysis of pineapple plant silage were indicated that the color appearance of pineapple plant 100% was yellowish green color, pineapple plant mixed with leucaena 5, 10 and 15% were light brown color and grass 100% dark brown color. The aroma of the pineapple plant silage was aromatic and acidic like pickled fruit. The chemical composition analysis of silage found that highly significantly difference ($p< 0.01$). Which this pH value in rang 3.43 – 4.27, dry matter 18.17 – 27.08%, ash 8.14 – 16.02%, protein 6.13 -8.19%, fibre 21.76 – 25.42, neutral detergent fiber 42.91 – 48.76, acid detergent fiber 29.85 – 31.94, acid detergent lignin 3.98 – 6.23, calcium 0.26 – 0.48, phosphorus 0.20 – 0.38 and energy 3,029.80 – 3,697.17. Pineapple plant silage mixed with leucaena could improve the quality of nutritive value for ruminants.

Total mixed ration (TMR) treated with lactic acid bacteria may possible to develop as biological animal feed for ruminant. TMR were rapidly improving quality when using lactic acid bacteria. Feed composition analyses revealed that *Lactobacillus pantarum* gave the highest dry matter, calcium phosphorus. TMR treated with *Pediococcus acidilactici* gave significantly higher

protein, ash, acid insoluble ash, neutral detergent fiber.TMR treated with *Pediococcus pentosaceus* gave significantly lower fiber and acid detergent fiber. The TMR treated with lactic acid bacteria by using pepsin cellulase *in vitro* digestibility which applied *Lactobacillus pantarum*, *Pediococcus pentosaceus*, *Pediococcus acidilactici* and *L. pantarum* +*P. Pentosaceus* + *P. acidilactici* could improve the digestibility of TMR, which significantly increased in digestibility of TMR. With this TMR treated with PA gave significantly higher digestibility.

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# APPENDIX

*Bulgarian Journal of Agricultural Science, 23 (No 1) 2017, 86–91*
*Agricultural Academy*

# LACTIC ACID BACTERIA AND ENZYME PRODUCTION IN SILAGE OF GUINEA GRASS (*PANICUM MAXIMUM*)

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## Abstract

Wichai, S. and K. Soytong, 2017. Lactic acid bacteria and enzyme production in silage of guinea grass (*Panicum maximum*). *Bulg. J. Agric. Sci.*, 23 (1): 86–91

Two different isolates of lactic acid bacteria, *Lactobacillus plantarum* H5-M13F and *Pediococcus pentosaceus* Ac2-M13F were confirmed by morphological and phylogenic identification. H5-M13F was rod shaped, gram positive, negative for gas production and catalase activity, homofermentative, and grew at 10 – 45 C, pH 3.5 – 9.6, and 1.0 – 10 % $NaCl_2$. Isolate Ac2-M13F was coccus shaped, gram positive, negative for gas production and catalase activity, homofermentative, and grew at 10 – 45 C, pH 3.5 – 9.6, and 1.0 – 6.5 % $NaCl_2$. Amplification of the ITS regions from isolates H5-M13F and Ac2-M13F were confirmed by using Primer OPA-3. The phylogenetic tree from PAUP analysis indicated that isolate H5-M13F clustered with *Lactobacillus* sp. and isolate Ac2-M13F with *Pediococcus* sp. Phylogenetic analysis and dendrograms revealed no relationship between the two groups. It was found that isolate H5-M13F mostly isolated from silages (87.59%) while isolate Ac2-M13F was found at a low incidence in silages (3.19%). *Lactobacillus plantarum* H5-M13F and *Pediococcus pentosaceus* Ac2-M13F from silage of guinea grass showed the ability to produce extracellular degradative enzymes. *Pediococcus pentosaceus* Ac2-M13F produced amylase and protease and *Lactobacillus plantarum* H5-M13F produced protease.

*Key words:* lactic acid bacteria; enzyme; silage; guinea grass

## Introduction

Guinea grass (*Panicum maximum*) is a fiber resource generally used for ruminant feed in Thailand (Aganga and Tshwenyane, 2004). One problem is the lack of subsequent crops of good quality roughage which can be fed to dairy cows during the dry season. Therefore, implementation of quality roughage in the rainy season comes to storage in the form of fermented forage or silage that can solve the feed shortage problem. The main factors involved are to produce silage more rapidly by using potent degradative microorganism especially lactic acid bacteria (LAB) (McDonald et al., 1991; Brookes and Buckle, 1992). The species diversity of bacteria may vary according to type of forage to be fermented (Cai et al., 1999a, 1999b; Ennahar et al., 2003; Wang et al., 2006; Parvin and Nishino, 2009, 2010). This research was focused on finding potent LAB to be used in silage for animal feed. Enzyme production activities in silage are also available to break down forage fiber. Wallace et al. (2001) reported that the relationship between enzyme activities and *in vitro* gas production using grass and corn silage had significant positive correlations with cellulase activity and gas production from grass silage. The question is: how to improve the nutrition of farmers' ruminant animals when each animal raiser keeps only one animal? In flood or summer, the major fodders available are grass or rice straw, together with limited quantities of hay and concentrated feeds. As a minimum, it is essential to provide a green fodder supplement to enhance rumen function for bovine animals. For animal raisers with lim-

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ited production capacities, finding enough feed in the flood or summer months to maintain good production is always a problem. There are many forced to buy roughage, concentrates or silage just to keep their animals alive and are unable to benefit financially due to the higher prices paid for animal feed in the flood or summer months. The objective of this research was to identify and investigate effective LAB producing enzymes to degrade silage consisting of guinea grass (*Panicum maximum*) for animal feed.

## Materials and Methods

**Isolation and identification of lactic acid bacteria from silage**

*Silage preparation*

Forty-five day-old guinea grass (*Panicum maximum*) was cut into 5 cm pieces by sickle starting at the ground level. The harvested material samples were chopped to 2-3 cm by knife. The pre-silage material samples were mixed with 1% $NaCl_2$, put in polyethylene bags and stored at ambient temperature for 21 days. A total of 25 g per samples was dissolved in 100 ml sterile water and stirred for 10 min. The pH values were measured using a pH meter (Polan et al., 1998). After a 21-day fermentation, the color and aroma of the silages were described according to the score indices of Muhammad et al. (2008). For the color description, the silage was scored as 1 = dark or deep brown, 2 = light brown, 3 = pale yellow and 4 = yellowish green. For the aroma description, the silage was scored as 1 = putrid or rancid, 2 = pleasant, 3 = sweet and 4 = very sweet. The analysis of total count of lactic acid bacteria was done by dilution plate method.

*Isolation*

The samples were collected from the silage in three parts of each silo, upper, middle and lower parts. The samples consisted of 100 g from each part which were mixed and 10 g randomly collected sub samples were diluted with distilled water then mixed with 90 ml of 0.1 % peptone. The sample was serially diluted up to $10^{-6}$, then 0.1 ml was pipetted and spread plated on de Man, Rogosa and Sharpe broth (MRS) mixed with calcium carbonate 1 % (de Man et al., 1960) and incubated at 30 ± 4°C for 48 h The single colonies observed and isolated into pure culture.

*Morphological identification*

Gram stain technique was used to differentiate two large groups of bacteria between Gram positive and Gram negative which appeared violet or red, respectively. Those bacteria that hold on to the primary iodine dye complex and remain violet were characterized as called Gram positive and those which are decolorized and subsequently take up the counterstain (pink/red) that were characterized as gram negative. The morphology of isolated LAB were noted based on the methods of Kandler and Weiss (1986) (Gram's stain, Catalase test, and Gas production test). The resultant data were compared with the descriptions of Kandler and Weiss (1986) and Stiles and Holzapfel (1997). The catalase production test was done using a dropper or Pasteur pipette, placing 1 drop of 3% hydrogen peroxide onto the microorganism on the microscope slide, which were mixed. The microscope slide was immediately covered with a lid to limit aerosols and observe for immediate bubble formation ($O_2$ + water = bubbles). The formation of bubbles against a dark background enhanced readability. Positive reactions were indicated by immediate effervescence (bubble formation).The microscope slide placed over a dark background viewed with a magnifying glass or microscope (40x) was used to observe weak positive reactions. No bubble formation (no catalase enzyme to hydrolyze the hydrogen peroxide) represents a catalase-negative reaction (South Bend Medical Foundation, 2010). Gas production was tested by transferring pure cultures into MRS mixed with 1% $NaCl_2$. Durham tubes containing sterilized liquid media were incubated at 30 ± 4°C for 48 h. Tubes filled with gas, indicated heterofermentation, tubes without gas, homofermentation (Hayward, 1957). A growth test with different temperature regimes was done by transferring pure cultures into sterilized MRS media and incubating at 10, 30 and 45°C for 48 h, then observing for turbidity (Cai et al., 1998; Yang et al., 2010). Salt tolerance was tested at different concentrations of $NaCl_2$ by transferring pure cultures into sterilized MRS mixed with sterilized $NaCl_2$ at 1, 3, 6.5 and 10% w/v, and incubating at 30 ± 4°C for 48 h and observing for turbidity (Cai et al., 1998; Yang et al., 2010). The growth test for different pH levels was done by transferring pure cultures into sterilized MRS with pH adjusted to 3.5, 5.7 and 9.6, incubating at 30 ± 4°C for 48 h and observing for turbidity (Kandler and Weiss, 1986; Yang et al., 2010). Morphological identification was done by following the methods of the Bergey Manual of Determinative Bacteriology (1994).

*Phylogenetic identification*

The isolates of LAB were separately cultured on MRS mixed 1% calcium carbonate, incubated at room temperature (30 ± 4°C) for 24-48 h; single colonies were collected for DNA extraction and observed under a compound microscope for morphological characteristics (Oneca et al., 2003). The OPA-3 primer (5'-AGTCAGCCAC-3') was used (Quere et al., 1997). The 25 µl PCR solution of consisted of 1X PCR buffer with $(NH_4)_2SO_4$ (Tris-HCl 75 mM, pH 8.8, 25°C, $(NH_4)_2SO_4$ 20 mM and Tween 20 0.01%), $MgCl_2$ 2 mM, dNTP 400 µM, primer OPA-3 0.4 µM, Taq DNA polymerase 0.1 unit/ µl and the cultured colony to amplify DNA fragments. A cycle was consisted

of an initial denaturation at 94°C for 5 min and followed by 45 cycles of denaturation at 94°C for 30 sec, annealing at 36°C for 1 min and extension at 72°C for 1 min. PCR products were sent to purify and sequence. The sequences were aligned and adjusted using Clustal X program for phylogenetic analysis. DNA sequences were edited and aligned with the BioEdit program, version 7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were multiply aligned with Clustal X version 1.83 before performing the analysis using the maximum parsimony method with (PAUP) 4.0b (Swofford, 1998). For maximum parsimony analyses, bootstraps of 1000 replicates were performed to examine the relationships of each isolate. Maximum parsimony trees were calculated via fast stepwise addition with representative isolates from GenBank (http://www.ncbi.nlm.nih.gov) and *Enterococcus* spp. was used to analyze as the out-group. Genetic relationships among *Lactobacillus* spp. and *Pediococcus* spp. isolates were determined using the sequences of the ITS region of rDNA to construct phylogenetic trees.

**Proximate composition analysis of silage**

For each treatment, 1000 g of fresh material were random collected to determine nutrient composition. The samples were oven dried at 60°C for 48 h prior to proximate analysis. Dry matter (DM), Ash, Crude fat (CF), Ether extract (EE), Crude protein (CP) and Crude fiber (CF) were determined according to the methods of AOAC (1995). Neutral detergent fiber (NDF) and Acid detergent fiber (ADF) were determined according to the method of Van Soest and Robertson (1979). All analyses were conducted using the Fibertec System M6 (FOSS, USA).

*Enzyme production*

The two species of LAB isolated from silage were selected for their ability to produce extra cellular degradative enzymes such as amylase, protease, lipase, cellulase and ligninase. Three replicates of each treatment were assayed and non-transferred plates served as negative controls. Transferred plates were incubated at 25°C, and checked at either 5 or 10 days depending on the growth rates. When the colonies grew over 50-60% of the area of the plate, chemical indicators were added to assay enzyme activity and activity zones.

*Amylase* – A drop of the bacterial culture was streaked onto a starch agar plate, then incubated at 30°C for 48 h. When colonies were visible, the plate was flooded with Lugol's solution, then the clear zone surrounding the colony was monitored. If the starch was hydrolyzed by the excreted amylase, a clear zone around the bacterial colony appeared. A yellow zone around the colony in an otherwise blue medium was considered a positive test for starch hydrolysis as modified by Gessner (1980).

*Protease* – Skim milk agar plates were streaked with bacterial colonies, then incubated at 30°C for 48 h, and the clear zone surrounding the colony was monitored. Clear zones surrounding visible colonies implies a positive caseinase (protease) reaction (Folasade and Joshua, 2005).

*Lipase* – Lipase activity test was performed by growing the isolates on trypticase soy agar. A volume of 3.5 ml of melted fat stained with Nile blue sulfate was added to 70 ml of trypticase soy agar medium the plates were streaked with pure cultures and one non-inoculated plate served as the negative control. A positive test was indicated by the occurrence of precipitated fatty acid crystals around the colony (Abdel-Rheem and Shearer, 2002).

*Cellulase* – Cellulase activity was assessed using cellulose azure agar. The tested LAB were assayed by growing on Peptone Yeast Glucose (PYG) medium which consisted of peptone 1.25 g, yeast extract 1.25 g, glucose 3 g, agar 18 g in 1000 ml of sterilized water. Thereafter, the bacteria transferred to Cellulolytic Basal Medium (CBM) which consisted of $NH_4H_2P0_4$ 0.1g, KCl 0.2g, $CaCl_2$ 0.2g, $MgSO_4.7H_2O$ 0.2g, 4% of CMC 250 ml, agar 18g in 750 ml of sterilized water. The plates were incubation for 7-10 d before observation for the colony grow over 50% of the Petri dish. Then 2% of congo red solution was poured on the control and treated plates, and after 15 min was washed by sterilized water. Then 1M $NaCl_2$ solution was added and after 15 min a clear zone surrounding the colony was monitored. If a clear zone appeared, it implied to the production of cellulase (Paterson and Bridge, 1994).

*Ligninase* – Ligninase activity was determined by the peroxidase test in PYG and then transferred to corn meal agar (CMA) which consisted of corn meal agar 20 g per 1000 ml and incubated for 5-10 d. The colonies were observed and then using a sterilized cork borer (0.5 cm diameter) wells were made at the growing edges of colonies. One drop of solution was dropped into a well which consisted of 1% w/v of perogallic acid and 4% hydrogen peroxide. A positive test was indicated by the formation of a golden yellow to brown color around the colonies (Abdel-Rheem and Shearer, 2002).

## Results and Discussion

**Silage preparation**

After 21 d of ensilage, the plastic containers were opened and examined for gross characteristics. Overall, the silages were of good color, aromatic and acidic. The silages contained an average of 76.47% moisture. The pH values of the silages averaged 5.53. The pH values indicated the anaerobic fermentation of the silages similar to the work of Schroeder (2004).

**Isolation and Identification of lactic acid bacteria**

*Isolation*

The colonies of LAB grown on MRS had different char-

acteristics and clear zones were observed around colonies due to the production of lactic acid which causes the conversion of calcium carbonate to calcium lactate (Frazier and Rupp, 1928). The colonies of LAB were white, opaque around colony circular and entire, tops of colonies were flat, raised, convex or umbonate. These characteristics conformed to the general characteristics of LAB (Kandler and Weiss, 1986). The average total count of LAB after fermentation was $5.99 \times 10^6$ cfu/g.

***Morphological identification***

• The morphology of the isolated lactic acid bacteria after staining is shown in Figure 1. All bacteria isolated were Gram positive and rod or cocci shaped. The tested bacteria were unable to produced catalase as indicated by the catalase enzyme test (Caplice and Fitzgerald, 1999) and were determined to be LAB. The gas production test for fermentation pathway in each of the samples found that the LAB were homofermentative. The samples of homofermentative were mostly rod shaped the rest were cocci.

• They were able to grow at 10, 30 and 45 degree Celsius, at NaCl concentrations of 1, 3, 6.5 and 10% and a pH of 3.5, 5.7 and 9.6 in each isolate that could be separated into 4 groups (A-D) as shown in Table 1. When compared with the characteristics described by Kandler and Weiss (1986) and Stiles and Holzapfel (1997). The 57 different isolates could be divided into two genera. *Lactobacillus* sp.H5-M13F was commonly found in silages (an incidence of 87.59%) Ennahar et al. (2003), Parvin and Nishino (2009) reported that this bacterium can be commonly detected in silage. *Pediococcus* sp. Ac2-M13F was found in silages at a lower incidence (3.19%).

***Phylogenetic Identification***

Amplification of the ITS regions from isolates of *Lactobacillus* spp. and *Pediococcus* spp. used the Primer OPA-3. The phylogenetic tree from PAUP analysis placed the *Lactobacillus* spp. and *Pediococcus* spp. into two distinct groups. Phylogenetic analysis produced a dendrogram indicating that there was no relationship between the *Lactobacillus* spp. and *Pediococcus* spp. groups (Figure 2).

**Table 1**
**The characteristics of lactic acid bacteria isolated from silage**

| Characteristic | Group | | | |
|---|---|---|---|---|
| | A | B | C | D |
| Morphology | Rods | Rods | Cocci | Cocci |
| Gram | + | + | + | + |
| Catalase test | - | - | - | - |
| Gas test | - | - | - | - |
| Fermentation | Homo | Homo | Homo | homo |
| Temp | | | | |
| 10°C | + | + | - | - |
| 30°C | + | + | + | + |
| 45°C | + | + | + | + |
| pH | | | | |
| 3,5 | + | + | + | + |
| 5,7 | + | + | + | + |
| 9,6 | + | + | + | + |
| NaCL | | | | |
| 1,00% | + | + | + | + |
| 3,00% | + | + | + | + |
| 6,50% | + | + | + | + |
| 10,00% | ± | - | - | - |
| Identifided as | *Lactobacillus* sp. | *Lactobacillus* sp. | *Pedococcus* sp. | *Pedococcus* sp. |

*Note:* + = Positive reaction, – = Negative reaction, ± = Variable reaction; Homo = Homofermenttive

**Proximate analysis of silages**

The proximate composition of the examined silages (Table 2) showed that dry matter changed from 20.9% before treatment to 23.5% after treatment. Ash content was 15.7 %, crude protein 7.9%, crude fiber 42.6%, ether extract 4.2%, nitrogen free extract 22.6%, organic matter 84.2%, neutral detergent fiber 56.5% and acid detergent fiber 37.8%. Jolaosho et al. (2013)

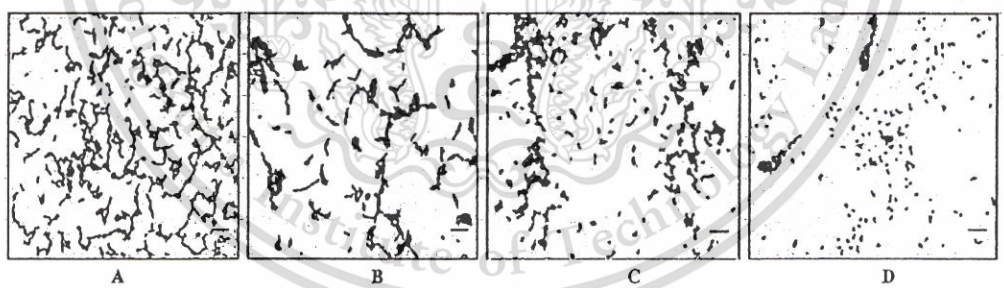


Fig. 1. Morphology of lactic acid bacteria isolated from silage
A and B showed characteristics of Lactobacilli
C and D showed characteristics of Pediococci

**Table 2**
**Composition of Guinea grass silage from Proximate analysis**

| Composition | Before fermentation | After fermentation |
|---|---|---|
| Dry matter (DM) | 20.9 | 23.5 |
| Ash | 11.1 | 15.7 |
| Curde protein (CP) | 7.9 | 7.9 |
| Crude fiber (CF) | 33.8 | 42.6 |
| Ether extract (EE) | 5.6 | 4.2 |
| Nitrogen free extract (NFE) | 35.9 | 22.6 |
| Organic matter (OM) | 88.8 | 84.2 |
| Nutral detergen fiber (NDF) | 58.6 | 56.5 |
| Acid detergen fiber (ADF) | 31 | 37.8 |

reported that the proximate composition and fiber fractions of silage from Guinea grass showed that DM was 21.03%, CP 9.48%, EE 6.5%, ASH 8.13%, NFE 15.58% NDF 60.31% and ADF 40.98%.

**Enzyme production properties**

The efficacy of *Lactobacillus* spp. and *Pediococcus* spp. from silage to produce extracellular degradative enzymes was examined. The results showed that the two genera were able to degrade, to some degree, starch and protein (Table 3). Lactic acid bacteria identified as *Pediococcus* sp. had able to produced amylase and protease. Iuchi et al, 2012 isolated *Pediococcus ethanolidurans* A4-27 from Japanese pickles (nuka-zuke) and found that it could produce a high molecular weight amylase. They indicated that the amylase enzyme from *P. ethanolidurans* was useful for fermentation of rice bran-bed (nuka-doko) which contained more than 15% NaCl.

*Lactobacillus* sp. produced protease indicated by clear zones around and below colonies.

*Pediococcus* sp. produced amylase indicated by a yellow clear zone around the bacteria colonies.

## Conclusion

Isolates of LAB were identified which based on their morphology were divided into two genera. *Lactobacillus* sp.H5-M13F was found at a high incidence in silages (87.59%) and *Pediococcus* sp. Ac2-M13F was found at a low incidence (3.19%). The phylogenetic tree from PAUP analysis placed the recovered *Lactobacillus* sp. and *Pediococcus* sp. into two distinct groups. Phylogenetic analysis revealed that there was no relationship between of the *Lactobacillus* sp. and *Pediococcus* sp. groups. The proximate composition of the examined silages showed that silages contained an average of 76.47% moisture. The pH values of the silages averaged 5.53, DM 23.5%, Ash content 15.7%, CP 7.9%, CF 42.6%, EE 4.2%, NFE 22.6%, OM 84.2%, NDF 56.5% and ADF 37.8%. *Lactobacillus* sp. could produce protease and *Pediococcus* sp. was able to produce amylase and protease. These two species can possibly be used to improve the nutritional value of the silage, TMR or roughage for ruminant production.

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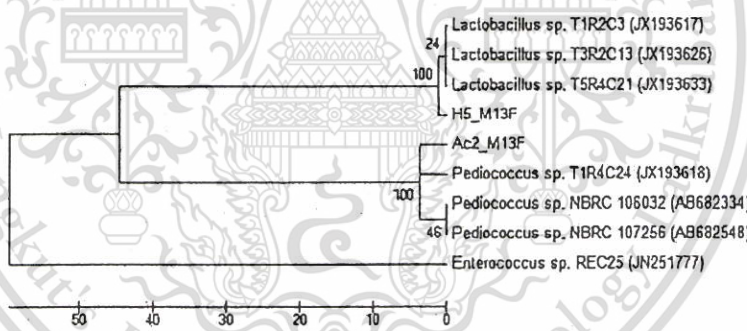


**Fig. 2. Phylogenetic relationships of *Lactobacillus* spp. and *Pediococcus* spp. isolated, based on the ITS regions of rDNA sequences**

**The phylogenetic tree was obtained by the maximum parsimony method using the PAUP4.0b program. *Enterococcus* sp. was used as the out group. The numbers above the lines represent the 1000 replicates parsimony bootstrap values**

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847

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# Using Leucaena to Improve the Quality of Pineapple Plant Silage

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The objective of this study was to improve the quality of pineapple plant silage by leucaena supplementation and using it as roughage during the shortage of green forages. There were 5 treatments as follows :- 1. Pineapple plant 100%, 2. pineapple plants mixed with leucaena 5%, 3. pineapple plants mixed with leucaena 10%, 4. Pineapple plants mixed with leucaena 15% and 5. grass 100%. The Completely Randomized Design (CRD) with four replications each was used in this experiment. All treatments were put in 1%$NaCL_2$ and tightly sealed in plastic containers. They were stored at room temperature for 21 days. The silage treatments were evaluated on physical characteristics, chemical composition and fibre analysis. The physical characteristic showed that the color of treatment 1 was a yellow green color, a good characteristic of silage. For treatments 2, 3 and 4 there was a light brown color. Treatment 5 was a dark brown in color. The aromas of all silage treatments were aromatic and acidic like pickled fruit. The evaluation of chemical composition showed a highly significant difference among the treatment groups ($p < 0.01$). Treatment 4 was the highest in protein and metabolizable energy percentage with the averages of 8.19% and 3,697.17 kcal/k, respectively. The percentage of crude fibre was reduced with the increase of leucaena in the silage. Treatment 5 was the highest in crude fibre. The pineapple plant silage mixed with leucaena could improve the quality of silage for ruminants.
Keywords: pineapple plant, leucaena, pineapple plant silage, ruminant feed

## 1. Introduction

Pineapple is one of the economic fruits of Thailand. This product is mostly used for fresh fruit consumption and processing products within the country and exported in terms of canned fruits and frozen fruits. There are large amounts of pineapple plant which are harvested fresh for fruit consumption and processing products such as canned pineapple (Gowda, 2015). Thus the farmer must dispose of this large amount of pineapple plant waste to alleviates this problem for a green environment. Now they try to make a value added aspect of pineapple and their by-products. Also, the use of pineapple plants contributes to useful material for the industrial sector such as textile, pineapple fibre cloth, enzyme, combustible material, etc.

Pineapple is a component of the trunk. Farmers cut off the plants after harvesting. Pineapple leaves are waste or by – product of agriculture. There are almost all year long and will be much in range November – June because it is period when most farmers collect the yield delivered factory, which meet the drought season, where farmers shortage of fresh grass for ruminant. The analysis of pineapple leaves showed that the protein 8.47 %, fibre 17.89 %, ADF 25.87% and NDF 42.28 % (Warunee and Walaikhan, 1998). Pineapple leaves can be used as a component in total mixed ration for dairy feed, without any effect on milk production.

The most important aspect of animal husbandry is to reduce the costs of production or the costs of animal feed in order to maintain the livestock business. The reduction in production costs that can be achieved is the feed efficiency. Ruminants are animals that use both concentrate and roughage. Roughage include forage crop such as grass and legume. But roughage is usually of low quality and insufficient for the needs of ruminants. Because the farmers have limited space in the preparation of forage crop or pasture and often lacking, especially during the dry season. There are various by – products in agriculture instead such as pineapple leaves or pineapple plant can be used to feed dairy cattle (Prachya et al., 2001). Pineapple leaves or pineapple plant can be a source of roughage for ruminants, but lack of knowledge and understanding of the proper use, the opportunity to be used to maximum benefit. Because of this, it is necessary to develop

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appropriate knowledge for farmer to increase farmers' incomes. Suchat et al. (2011) was to determine the effect of ensiled pineapple waste compared with pangola hay as roughage source on rumen fermentation and feed utilization of native cattle. The apparent digestibility of dry matter, organic matter, crude protein, NDF and ADF in cattle fed only pineapple waste as roughage source was higher than in cattle fed only pangola hay. The pineapple waste have several benefit in terms of increasing caloric density, digestibility and feed utilization as compared to pangola hay. Moreover, it also enhances populations of dominant cellulolytic bacteria in the rumen.

The chemical composition of the pineapple plant is high in fibre which makes it is a good source of fibre for ruminant feed. Pine apple plants could be used as ruminant feed in silage forms to preserves the quality of its nutrient (Sayan, 2004). Furthermore, it can be kept for a long time. Pineapple plant silage can alleviate a mal-nutrition in ruminants during the dry season or flooding time. It is a high quality silage because it is good in digestibility and palatability. It is easy for animal raisers to make pineapple plant silage for their animals by themselves. This can help reduces animal feed cost and increase the quality of feed which is reflected in the high production performance of their animal. However, the quality of silage is depends on feed additive uses during the making processes. This research is aimed at selecting the suitability of leucaena level to apply to pineapple plant silage making for ruminant feed.

## 2. Materials and Method

### 2.1 Silage preparation

The harvested material was 3 samples of para grass, leucaena and pineapple plants randomly taken and chopped to 2-3 cm. (Figure 1) The pre-silage material samples were 5 treatments as follows:- 1. pineapple plant 100%, 2. pine apple plant mixed with leucaena 5%, 3. pine apple plant mixed with leucaena 10%, 4. pine apple plant mixed with leucaena 15% and 5. grass 100%. All treatments were put into 1%$NaCL_2$ and tightly sealed in plastic containers. They were stored at room temperature for 21 days. A total of 25 g sample was dissolved in 100 ml sterile water and stirred for 10 min. The pH values were measured for acidity changes using the pH meter (Polan et al., 1998). After 21 days fermentation, the color and aroma of the silages were evaluated according to the indices score of Muhammad et al. (2008). For the color description the silage was scored as 1 = dark brown, 2 = light brown, 3 = pale yellow and 4 = yellowish green. For the aroma description the silage was scored as 1 = putrid or rancid, 2 = pleasant, 3 = sweet and 4 = very sweet.

*Figure 1 The Fresh samples of pineapple plant*

### 2.2 Proximate composition analysis of silage

Fresh samples of 1,000 g were randomly by collected to determine nutrient composition. The samples were done by oven drying at 60° C for 48 h prior to proximate analysis. Dry matter (DM), ash, crude protein (CP), crude fibre (CF), ether extract (EE), nitrogen free extract (NFE) and organic matter (OM) were determined according to the methods of AOAC (1995). Neutral detergent fibre (NDF) and Acid detergent fibre (ADF) were determined according to the method of Van Soest and Robertson (1979). Proximate analysis was done before and after fermentation. The experiment was repeated two times. Data were then computed using analysis of variance and treatment means were compared with Duncan's Multiple Range Test (DMRT) at P = 0.05 and P = 0.01.

## 3. Results

### 3.1 Physical characteristic of silage

After 21 days of ensilage, the plastic containers were opened and examined for gross characteristics. The physical characteristic (Table 1.) showed that the color of treatment 1 was a yellowish green color which was a good characteristic of silage (Figure 2). For treatment 2,.3 and 4 they were light brown color. Treatment 5 was a dark brown color, with quality being lower than treatment 1, 2, 3 and 4 (Muhammad et al., 2008). Generally, the silage should have a darker color than fresh forage because the color of chlorophyll reacted with acid from fermentation. These changed them to become a magnesium free pigment phaeophytin. However, the carotene was a provitamin A which was suffered from oxidation at high temperature (Azim et al.,2000 ; Wanapat, 1986). The aroma of silage treatment 1, 2, 3 and 4 were aromatic and acidic like pickled fruit (sweet smell). The sweet smell was caused by lactic acid bacteria which utilized sugar in the forage to produce lactic acid and volatile acid (McDonald et al.,1991; Merry et al., 2000). For treatment 5 the aroma of silage was very sour and may have occurred by the activity of proteolytic bacteria change protein to ammonia, volatile acid, amine and amide which dissatisfied in silage (Sayan, 2004). The pH values of silage treatment 1, 2, 3, 4 and 5 were 3.45, 3.43, 3.72, 3.52 and 4.27 respectively. Overall, treatments showed highly significant differences at a low level ($P < 0.01$). The pH values of silage was indicated that bacteria to produced lactic acid (Schroeder, 2004). The pH of good quality silage was 3.5 – 4.5, if pH increases more than 5.1 it was low quality (church, 1991). The moisture value of silage showed that highly significant differences ($P < 0.01$). Treatment 1 gave significantly higher moisture (81.83%) than treatment 4 (80.95%), treatment 2 (79.77%), treatment 3 (79.10%) and was lower in treatment 5 (72.93%). The silage making had high moisture and lowered dry matter during raining season may have coursed risk for the silage to spoil. In cases where the silage had lower moisture and higher of dry matter it may have been more fibre difficult to compact the silage in the silo ( Saranya W. and Jantakarn, A., 1997).

*Table 1 The Physical Characteristics of Silage*

| silage | | character | |
|---|---|---|---|
| | | Colour of silage | Aroma of silage |
| 1 | Pineapple plant 100% | Yellowish green | Aromatic and acidic |
| 2 | Pineapple plant mixed with leucaena 5%, | Light brown | Aromatic and acidic |
| 3 | Pineapple plant mixed with leucaena 10%, | Light brown | Aromatic and acidic |
| 4 | Pineapple plant mixed with leucaena 15%, | Light brown | Aromatic and acidic |
| 5 | Grass silage 100% | Dark brown | Very sour |

*Figure 2 The physical characteristics of pineapple plant silage*

850

### 3.2 Nutritive values of silage

The proximate composition of the examined silage is shown in Table 2. Results revealed that highly significant differences ($P < 0.01$). Treatment 5 gave significantly higher dry matter (27.08%) than treatment 3 (20.90%), treatment 2 (20.23%), treatment 4 (19.05%) and was lower in treatment 1 (18.17%). For the good fermentation process and fast originate, dry matter had a loss of approximately 1 – 2 % from respiratory of forage during first stage (McDonald et al., 1991). The value of ash showed that Treatment 5 gave significantly higher ash (16.02%) than treatment 3 (9.71%), treatment 1 (9.38%), treatment 2 and lower ash in treatment 4 respectively. The increased of ash occurred by the utilization of plant organic substance and change to inorganic substance by microorganism during fermentation (Frame, 1994). The calcium value of silage showed that treatment 4 gave significantly higher calcium (0.48%) than treatment 3 (0.35%), treatment 5 (0.34%), Treatment 2 (0.32%) and lower calcium in treatment 1 (0.26%) respectively. For the phosphorus treatment 1 gave significantly higher phosphorus (0.38%) than treatment 4 (0.36%), treatment 2-3 (0.29%) and lower phosphorus in treatment 5 (0.19%) respectively. Treatment 4 gave significantly higher protein (8.19%) than treatment 5 (7.39%), treatment 3 (6.99%), treatment 2 (6.39%) and lower in treatment 1 (6.13%) respectively. Leucaena had condensed tannin 4 – 6 % of dry matter which could be caught protein and protect the digestion by microorganisms. McDonald et al. (1991) reported that usually decreases in protein was due to the initially digestion by microorganism, while the increased of protein may occurs by the influence of salt, which it prevents clostridium sp. to not destroy protein. There were not significantly differences in protein, ether extract, calcium, and phosphorus. Pineapple plant mixed with leucaena 5% gave significantly lower fibre (21.76%) than pineapple plant mixed with leucaena 10% (22.51%), pineapple plant mixed with leucaena 15% (23.15), pineapple plant 100% (24.18%) and grass silage 100% respectively, but it was not significantly different in the pineapple plant mixed with leucaena. However, fibre decreased during fermentation which may occurred have happened due to *lactobacillus* sp. which could have digested the cell wall (Mc Donald et al., 1991)

*Table 2 Proximate composition analysis of pine apple plant silage.*

| Treatment | Nutritive value of pine apple plant silage | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | pH | Moist. | DM | Ash | CP | CF | Ca | P | Energy |
| 1 | $3.45^{d}$ | $81.83^{a}$ | $18.17^{c}$ | $9.38^{b}$ | $6.13^{d}$ | $24.18^{ab}$ | $0.26^{c}$ | $0.38^{a}$ | $3,620.99^{a}$ |
| 2 | $3.43^{c}$ | $79.77^{ab}$ | $20.23^{bc}$ | $9.05^{bc}$ | $6.39^{cd}$ | $21.76^{c}$ | $0.32^{b}$ | $0.29^{b}$ | $3,471.06^{b}$ |
| 3 | $3.72^{b}$ | $79.10^{b}$ | $20.90^{b}$ | $9.71^{b}$ | $6.99b^{c}$ | $22.51^{c}$ | $0.35^{b}$ | 0.29b | $3,475.86^{b}$ |
| 4 | $3.52^{c}$ | $80.95^{ab}$ | $19.05^{bc}$ | $8.14^{c}$ | $8.19^{a}$ | $23.15^{bc}$ | $0.48^{a}$ | $0.36^{a}$ | $3,697.17^{a}$ |
| 5 | $4.27^{a}$ | $72.93^{c}$ | $27.08^{a}$ | $16.02^{a}$ | $7.39^{b}$ | $25.42^{ab}$ | $0.34^{b}$ | $0.19^{c}$ | $3,029.80^{c}$ |

Means followed by a common letter in each column are not significantly different ($p<0.01$)

Results showed that fibre analysis by using Van Soest demonstrated the fibre digestion of pineapple plants mixed with leucaena silage could be degraded (Table 3). Pineapple plant mixed with leucaena 15% gave significantly lower NDF (42.19%) than pineapple plant mixed with leucaena 5% (43.30%), pineapple plant mixed with leucaena 10% (43.71%), pineapple plant 100% (47.10%) and grass silage 100% (48.76%), but it was not significantly different in pineapple plant mixed with leucaena. For NDF the decreased in may be due to the part of cell wall and carbohydrate structure being utilized as an energy source for microorganism growth during fermentation (O'Kiely and Muck, 1998). Pineapple plant mixed with leucaena 5% gave significantly lower ADF (29.85) than grass silage 100% (29.88%), pineapple plant mixed with leucaena 15% (30.46%), pineapple plant mixed with leucaena 10% (30.90%) and pineapple plant 100% (31.94%) respectively. The acid detergent fibre of silage trend were perhaps increased by the sugar structured in the plant cell with the microorganism being utilized and causing ADF increase (Campbell and Bruchanan-Smith, 1991). Generally, a good range of ADF in dairy cattle' feed should be around 40 – 60 % to produces butterfat in milk (Somjit, 2006; Jantakarn, 2009). Grass silage 100% gave significantly lower ADL (3.98%) than pineapple plant mixed with leucaena 15% (5.66%), pineapple plant mixed with leucaena 5% (5.99%), pineapple plant 100% (6.19%) and pineapple plant mixed with leucaena 10% (6.23%) respectively. It was not significantly different in ADL of pineapple plant 100% and pineapple plant mixed with leucaena. The quantity of lignin, cellulose, and hemicellulose in feed are important for the forage crop of ruminants. A good quality of

forage crop should be low in lignin (Flores, 1991). In this study, pineapple plant mixed with leucaena 15% gave significantly higher metabolizable energy (3,697.17 kcal/k) than pineapple plant 100% (3,620.99 kcal/k), pineapple plant mixed with leucaena 10% (3,475.86 kcal/k), pineapple plant mixed with leucaena 5% (3,471.06 kcal/k) and grass silage 100% (3,029.80 kcal/k) respectively. The leucaena was completed in nutrient and when mixed with the pineapple plant the metabolizable energy was increased (Piliwan et al., 1989).

*Table 3 Fibre analysis of pineapple plant silage.*

| Treatment | NDF | ADF | ADL |
|---|---|---|---|
| 1 | 47.10[a] | 31.94[a] | 6.19[a] |
| 2 | 43.30[b] | 29.85[b] | 5.99[a] |
| 3 | 43.71[b] | 30.90[ab] | 6.23[a] |
| 4 | 42.91[b] | 30.46[ab] | 5.66[a] |
| 5 | 48.76[a] | 29.88[b] | 3.98[b] |

Means followed by a common letter in each column are not significantly different ($p<0.01$)

Treatment 1. = pineapple plant 100%
Treatment 2. = pine apple plant mixed with leucaena 5%
Treatment 3. = pine apple plant mixed with leucaena 10%
Treatment 4. = pine apple plant mixed with leucaena 15%
Treatment 5. = grass 100%.

## 4. Conclusion

The uses of leucaena to improve nutritive value in pineapple plant silage were determined. This study was to improve the quality of pineapple plant silage by leucaena supplementation for using as roughage during the shortage of green forages. There were 5 treatments as follows:- 1. pineapple plant 100%, 2. pineapple plant mixed with leucaena 5%, 3. pineapple plant mixed with leucaena 10%, 4. pineapple plant mixed with leucaena 15% and 5. grass 100%. The silage samples were mixed with $NaCl_2$ all formula and kept tightly sealed in plastic containers and stored at room temperature for 21 days. The results of physical characteristics, chemical composition and fibre analysis of pineapple plant silage were indicated that the color appearance of formula 1 was yellowish green color, formula 2,3,4 were light brown color and formula 5 dark brown color. The aroma of the pineapple plant silage was aromatic and acidic like pickled fruit. The chemical composition analysis of silage found that highly significantly difference ($p< 0.01$). Which this pH value in rang 3.43 – 4.27, Dry matter 18.17 – 27.08%, ash 8.14 – 16.02%, protein 6.13 -8.19%, fibre 21.76 – 25.42, NDF 42.91 – 48.76, ADF 29.85 – 31.94, lignin 3.98 – 6.23, Ca 0.26 – 0.48, P 0.20 – 0.38 and energy 3,029.80 – 3,697.17. Pineapple plant silage mixed with leucaena could improve the quality of nutritive value for ruminants.

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852

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