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USE OF Lactobacillus plantarum AND Propionibacterium freudenreichii ssp. Shermanii AS INOCULANTS IN CORN SILAGE FERMENTATION

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By

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Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfilment of the requirement for the Degree of Master of Science

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Bacterial inoculant as feed additives have been shown to be beneficial in silage fermentation in many parts of the world. However, there is still not much evidence of the use of bacterial additives in Malaysia and the impact of using them to produce quality feed for ruminant industry is rarely documented. Thus, this study was conducted to determine the effectiveness of using two bacterial additives for corn silage fermentation in Malaysia. The two bacterial species used in this study were *Lactobacillus plantarum* (B1) and *Propionibacterium freudenreichii* (B2). These bacteria were applied singly and as a mixture of both bacteria (B3).

Three experiments were conducted where the first experiment was to determine the suitable bacterial growth condition and inoculation size. The preferable growth media for B1 was found to be MRS agar (18-24 hours) while for B2, it was Reinforced Clostridium Difficle Agar (48-56 hours). The wavelength for OD measurement of B1 was 490 nm and 419 nm for B2. Suitable inoculation size for B1 and B2 applied individually was at 1 x 10^5 cfu/g, while suitable inoculation size for B3 is 0.5 x 10^5 cfu/g.

The second experiment was conducted to determine the effect of B1 and B2 applied singly or as a mixture to the fermentation characteristic and quality of corn silage. The physical characteristic of the silage measured were pH, mould and temperature while the quality measurement included crude protein (CP), dry matter (DM) and fibre content. The fermentation

products measured were water soluble carbohydrate (WSC) and fatty acid concentrations (lactic, propionic, acetic and butyric acids). A good silage quality should have a low pH, acetic acid, and butyric acid, but high lactic acid content.

Whole corn plant was harvested at dough stage (32 - 37% of dry matter) and cut into 2-3 cm particle size using a forage chopper. Two kilograms of chopped materials were placed on polyethylene sheet and sprayed with 10 ml of prepared bacteria solution. The forage were mixed thoroughly and ensiled in four replicates for each treatment in 4 L laboratory silo. Bacterial combination (B3) ended with the lowest pH (3.33), while the highest was B2 (pH 3.45). The pH of silage treated with bacteria showed a faster reduction compared to untreated silage. The use of *L. plantarum* alone rapidly reduced pH in the early stages of silage fermentation. Bacterial additives did not reduce mould infection and silage treated with B2 produced higher mould percentage than control (B0). Bacterial treatment did not reduce the fibre content as compared to control. Both bacteria in this research showed low activity on digesting fibre. Bacterial treatments gave a significant effect ($p<0.01$) on WSC, lactic acid (LA) and acetic acid (AA) while there was no significant effect on propionic acid and butyric acid. Fermentation with *L. plantarum* alone appeared to produce high LA concentration among all treatments. The LA production was 12 times higher than AA production showing that it promoted the homolactic fermentation to be dominant in the silage. However, when *L. plantarum* was mixed with *P. freudenreichii,* the fermentation tended to be dominated by *P. freudenreichii*.

The effect of bacterial additive was observed at 5 different ensiling period (3, 6, 9, 18 and 27 days) and the quality was determined in the third experiment. The time course study in fermentation indicated that the ensilation process was completed at day 6 even without using bacterial additives.

This research showed that silage produced with *L. plantarum* or *P. freudenreichii* either alone or mixed together produced a desirable silage property but it was not significantly better than control. It was speculated that naturally occurring bacteria was already adequate in the uninoculated corn silage because of the high amount of fermentable sugar in the corn itself which provided the essential source of energy for bacteria to carry out successful preservation during fermentation. Hence, the nutrient qualities of corn silage produced with the addition of the two selected bacteria strains were shown to be the same as uninoculated corn silage.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

PENGGUNAAN *Lactobacillus plantarum* DAN *Propionobacterium freudenreichii* ssp. *Shermanii* SEBAGAI INOKULAN DALAM FERMENTASI SILAJ JAGUNG

Oleh

NORAFIZAH BINTI HAJI ABDUL RAHMAN

Januari 2017

Pengerusi : Profesor Madya Mohd Ridzwan Bin Abd Halim, PhD

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Penggunaan bakteria inokulan sebagai bahan tambah makanan ternakan terbukti berfaedah membantu proses fermentasi silaj di kebanyakan negara luar. Namun begitu, maklumat-maklumat saintifik berkaitan kesan dan impak penggunaan bakteria sebagai aditif untuk industri ruminan di Malaysia masih lagi terhad dan perlu dikaji dengan lebih mendalam. Sehubungan itu, kajian ini dilaksanakan bertujuan untuk mengetahui keberkesanan penggunaan dua bakteria inokulan terpilih untuk fermentasi silaj jagung di Malaysia. Dua spesies bakteria yang digunakan di dalam kajian ini adalah *Lactobacillus plantarum* (B1) and *Propionibacterium freudenreichii* (B2). Kedua bakteria ini digunakan secara bersendirian dan juga digabungkan keduanya (B3).

Tiga ekperimen dilaksanakan dalam kajian ini. Eksperimen pertama adalah bertujuan untuk mengetahui kriteria asas untuk pertumbuhan bakteria dan kuantiti inokulasi yang bersesuaian untuk digunapakai untuk fermentasi silaj. Media yang sesuai untuk B1 adalah MRS (18-24 jam) manakala bagi B2 adalah media *Reinforced Clostridium Difficle* (48-56 jam). Gelombang OD 490 nm adalah bersesuaian untuk mengesan B1 manakala B2 dikesan pada 419 nm. Saiz inokulasi yang bersesuaian adalah 1 x 10^5 cfu/g bagi B1 dan B2 manakala kuantiti separuh daripadanya (0.5 x 10^5 cfu/g) adalah memadai bagi B3.

Dalam eksperimen kedua, kesan penggunaan bakteria B1 dan B2 secara individu atau gabungan keduanya (B3) dianalisis berdasarkan ciri-ciri fermentasi dan kandungan nutrisi silaj. Ciri-ciri fizikal fermentasi yang diukur adalah pH, peratusan kulapuk dan suhu manakala kualiti nutrisi yang ditentukan ialah kandungan protein kasar (CP), berat kering (DM), dan serat. Selain itu, produk yang terhasil daripada proses fermentasi seperti kandungan karbohidrat larut air (WSC) dan asid lemak (asid laktik, propionik, asetik dan butirik) turut dianalisis. Fermentasi yang baik akan menghasilkan silaj dengan pH yang rendah, kandungan asid laktik yang tinggi di samping mempunyai kandungan asid asetik dan butirik yang rendah.

Rumpun jagung telah dituai di peringkat *dough* (kandungan berat kering 32-37%) dan dikisar kecil (2-3 cm) dengan menggunakan pericik foder. Sebanyak 10 ml larutan bakteria yang telah disediakan disembur pada 2 kg foder yang telah dikisar dan kemudiannya digaul rata. Foder yang terawat kemudiannya disimpan untuk fermentasi di dalam balang (silo) berkapasiti 4 L. Di akhir proses fermentasi, B3 mencatatkan pH terendah (3.33), manakala B2 mencatatkan pH tertinggi (3.45). Silaj yang dirawat dengan bakteria menunjukkan penurunan pH yang lebih pantas berbanding silaj kawalan. Penggunaan *L. plantarum* secara individu menunjukkan kadar penurunan pH yang pantas semasa di awal tempoh fermentasi. Penggunaan bakteria didapati tidak mengurangkan pertumbuhan kulapuk malahan penggunaan B2 telah menunjukkan hasil kulapuk yang lebih banyak berbanding kawalan. Bakteria terpilih daripada kajian ini menunjukkan kadar serat yang setara berbanding kawalan (B0). Kedua bakteria yang digunakan di dalam kajian ini telah menunjukkan aktiviti penguraian serat yang rendah. Kombinasi bakteria (B3) tidak mengurangkan kadar serat manakala perlakuan dari bakteria lain tidak mengurangkan serat secara signifikan berbanding kawalan. Perlakuan bakteria memberikan kesan yang signifikan pada $p<0.01$ kepada karbohidrat larut air (WSC), asid laktik (LA) dan asid asetik manakala tiada perbezaan yang signifikan bagi asid propionik dan asid butirik. Penggunaan *L. plantarum* telah menghasilkan 12 kali kandungan laktik asid lebih tinggi berbanding asetik asid. Ini menunjukkan bahawa bakteria ini telah menyebabkan proses fermentasi menjadi homolaktik. Namun begitu, apabila digabungkan bersama *P. freudenreichii,* fermentasi telah beralih menjadi heterolaktik.

Kesan penggunaan bakteria sebagai aditif turut direkodkan pada 5 tempoh fermentasi yang berbeza di dalam eksperimen ketiga. Perbandingan kajian di antara tempoh fermentasi telah menunjukkan bahawa proses fermentasi telahpun lengkap pada hari ke 6 walaupun tanpa penggunaan bakteria sebagai aditif.

Oleh yang demikian, hasil daripada kajian ini telah membuktikan bahawa dengan menggunakan *L. plantarum* mahupun *P. freudenreichii* sebagai aditif sama ada secara bersendirian mahupun percampuran antara keduanya telah menghasilkan silaj yang berkualiti tetapi tidak mempunyai berbezaan yang signifikan berbanding kawalan. Disebabkan kandungan karbohidrat yang tinggi di dalam foder jagung, diandaikan bahawa bakteria semulajadi di dalam silaj telah mempunyai substrat yang mencukupi untuk mengendalikan fermentasi dengan sempurna tanpa perlu pertambahan bakteria lain sebagai aditif. Sehubungan itu, penggunaan dua bakteria terpilih ini telah menghasilkan kualiti silaj yang setara dengan silaj kawalan.

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APPROVAL

I certify that a Thesis Examination Committee has met on 12th January 2017 to conduct the final examination of **Norafizah Haji Abdul Rahman** on her thesis entitled **"USE OF *Lactobacillus plantarum* AND *Propionibacterium freudenreichii* ssp. *Shermanii* AS INOCULANTS IN CORN SILAGE FERMENTATION"** in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows;

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µ	micro
AA	acetic acid
ADF	acid detergent fibre
BA	butyric acid
cfu	colony forming unit
CP	crude protein
DM	dry matter
GC	gas chromatography
LA	lactic acid
LAB	lactic acid bacteria
M	molar
MJ/kg	megajoules per kilogram
MS	mass spectrometry
MW	molecular weight
NDF	neutral detergent fibre
nm	nanometer
nVFA	non volatile fatty acid
O. D.	optical density
PA	propionic acid
PAB	propionic acid bacteria
rpm	rotation per minute
v/v	volume per volume
VFA	volatile fatty acid
w/v	weight per volume
WSC	water soluble carbohydrate

CHAPTER 1

INTRODUCTION

Silages can be defined as forages, crop residues, or agricultural or industrial by products that have been preserved naturally for the use as animal feeds. It is conserved by reducing the pH through natural anaerobic fermentation or induced by additives and stored in closed containers. Success in silage-making depends on adequate levels of water soluble carbohydrate to be fermented to form lactic acid under anaerobic condition which eventually leads to a low pH environment that is required in preserving the forage (Henderson, 1993).

Silage is the product of a series of fermentation processes by which cut forage with high moisture content (60-75%) is allowed to ferment to preserve the quality usually by lactic acid bacteria (Kung, 2001). The acidic environment induced by lactic acid bacteria in anaerobic condition will resist further breakdown of plant material during storage (Woolford, 1984). The objective is to retain the nutrients present in the original forage and to be acceptable by livestock.

Most studies have shown that silage is more palatable than other conserved feed such as hay (Erdman et al., 2011; Holt et al., 2013; Arriola et al., 2015). Silage can be kept for months or years and can be used at any time as and when required, especially during periods of drought or floods. In a tropical country like Malaysia, silage production is preferred as it is less dependent on weather conditions for sun-drying to produce hay. Silage kept in trenches in Malaysia has been shown to be in good condition even after five years of storage (Chin and Idris, 1999). Later reports also showed that silage can be kept in a good conditions from 90 to 361 days (Kung et al., 2007a; Mohd-Setapar et al., 2012).

There are three basic conditions to make ensilation possible; i.e., fermentable sugars to support the bacterial activity, suitable conditions for the desirable bacteria to be active and suitable composition and correct numbers of the desirable microorganisms in the raw material (Aksu et al., 2006; Filya and Sucu, 2010; Queiroz et al., 2013). With a better knowledge on silage improvement nowadays, a technology to improve silage quality and recovery has been developed by using bacterial inoculant as additives.

Two different types of microorganism are involved in silage fermentation, one group is desirable while the other is undesirable. The example of desirable microorganisms are lactic acid producing bacteria (LAB), propionic acid producing bacteria (PAB) and some other fungal species that undergo homofermentative or heterofermentative process during fermentation. The desirable bacteria can improve the fermentation, shelf life, and quality of silages (Filya et al., 2007; Pedroso et al., 2010), while in the event of accidental soil incorporation, a long aerobic phase or slow acidification, the microbial communities in the silage will be dominated by *Clostridia*, yeasts, moulds and by chance incorporated pathogenic microorganisms such as *Listeria* spp. can cause anaerobic spoilage on silage (Dunière et al., 2013).

Lactic acid bacteria (LAB) are the most popular bacterial additives for silage fermentation. Studies on LAB showed that the bacteria can rapidly reduce pH and enhance silage quality (Kung et al., 2007b; Meeske and Basson, 1998; Ranjit and Kung, 2000). However, several microorganisms that are not LAB have been used as silage inoculants specifically for the purpose of improving aerobic stability (Kleinschmit and Kung, 2006; Kristensen et al., 2010). Propionic acid bacteria (PAB) was also being chosen for the ability to convert lactic acid and glucose to acetic and propionic acids that are more antifungal than lactic acid, thus it can stabilize silage longer after exposing to air and increase the aerobic stability. Flores-Galarza et al. (1985) and Dawson et al. (1998) reported that addition of *P. shermanii* prevented the growth of moulds and markedly reduced the initial population of yeast in high moisture corn where the final pH was greater than 4.5. Bolsen et al. (1996) reported more propionic acid, lower yeasts and moulds, and greater aerobic stability in corn silage (pH of 3.6) treated with *Propionibacteria*.

Studies on microbial additives for silage have been conducted in other countries and they have indicated significant benefits (Aksu et al., 2004; Huisden et al., 2009; Nkosi et al., 2010). Using bacteria as an additive enhanced nutrient recovery and improved fermentation quality. Research by Weinberg et al. (1995) proved the efficiency of using bacterial additives in silage production compared to non-treated silage (without bacterial additives) for corn and alfalfa silage.

Due to the lack of good quality feed and grazing area for ruminant production in Malaysia, intensive research on the use of indigenous feed resources are required to reduce the burden of feed imports. Furthermore, Malaysia also faces periods of feed limitation, usually due to drought or floods in certain parts of the country. The high humidity environment in Malaysia also causes the plant materials like corn to be easily contaminated by certain microorganisms like mould. This results in shortage of fresh feeds available to animals. Hence, strategies should be developed to sustain the animal production industry. Forage conservation is a practical consideration to ensure continuous supply of feeds throughout the year.

The use of microbial additives for silage making in Malaysia is not widely practised. Therefore, the information on the benefits and the effectiveness of microbial additives in silage making is needed to make appropriate recommendations for feed conservation under local conditions. Corn (*Zea mays*) was selected as the silage crop for this study as it is known to contain higher amounts of water soluble carbohydrates compared to tropical forage grasses. Soluble carbohydrates would be a source of nutrients for microbial fermentation for production of organic acids.

Thus, the aim of this study was to determine the nutrient quality of the corn silage produced by using hetero and homofermentative bacterial species applied singly or as a mixture in the corn silage fermentation. Specific objectives were:

a. To determine the optimum growth conditions for culture and multiplication of *L. plantarum and P. freudenrinchii* ssp. *Shermanii*.
b. To determine the effect of bacterial inoculants on corn silage characteristics and fermentation profiles after 4 weeks of ensilation.
c. To determine the effect of bacterial inoculants on corn silage characteristics and fermentation profiles at five duration of fermentation from 5 to 27 days of ensilation.

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