Enriched Nutrients of Napier grass using *Aspergillus sp.* through Solid State Fermentation

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DECLARATION

I hereby declare that the work embodied in here is the result of my own research except for the except as cited in the references.

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ABSTRACT

Napier grass *(Pennisetum purpureum)* is one of the popular tropical grasses. It is widely used to feed ruminant animals. However, the nutrient composition in Napier grass is still low and it is required to enhance the nutritive value of Napier grass using additives (e.g., molasses and fungi) that can fulfill the nutrient requirement by ruminants. Therefore, the objectives of this study were to evaluate the effects of *Aspergillus niger and Aspergillus awamori* on fermentation characteristics and nutritive values of Napier grass silage through solid-state fermentation. There were 3 treatments with 3 replications for each: i) Control (T1): Napier grass ensiled with 5% molasses, ii) T2: Napier grass ensiled with 5% molasses and *Aspergillus niger* (10 ml/kg silage; 10^6 spores/ml) and iii) T3: Napier grass ensiled with 5% molasses and *Aspergillus awamori* (10 ml/kg silage; 10^6 spores/ml). Napier grass was harvested at approximate 2 months of plant maturity, chopped manually at 2-3 cm in length, kept it air tight in plastic bag, and then stored it anaerobically for 21 days. The pH value, lactic acid, NH_3–N, dry matter (DM), crude protein (CP), ether extract (EE), crude fibre (CF), ash and nitrogen free extract (NFE) contents of silages were evaluated. There were significant ($p<0.05$) differences on pH value among the treatments. The pH value ranged from 4.08 to 4.20. The T1 silage showed lower ($p<0.05$) pH value (4.08) than T2 silage (4.20). No significant ($p>0.05$) differences were observed on lactic acid content in Napier grass silages among the treatments. The NH_3–N content in Napier grass silages were significantly ($p<0.05$) differed among the treatments. The DM, CP, EE, CF and NFE contents in Napier grass silages were significantly ($p<0.05$) varied among the treatments, while ash contents were not significantly ($p>0.05$) varied. The T2 silage showed the highest DM (24.04%), CP (11.42%), CF (29.00%) and ash (9.39%) contents, while T1 silage showed the lowest DM (21.04%), CP (9.16%), CF (23.29%) and ash (6.34%) contents. The T2 silage showed the highest EE content (7.65%), while T3 showed the lowest (0.58%). The T1 silage showed the highest NFE content (59.70%), while T2 silage showed the lowest (42.56%). The results of this current study suggest that the ensiled Napier grass with three weeks fermentation period contains the highest levels of CP. Hence, biological treatment of Napier grass using *Aspergillus niger* enhanced its nutritive value, which might be used for ruminant feed.

Keywords: Napier grass, *Aspergillus niger*, *Aspergillus awamori*, silage quality, nutritional composition.

Memperkayakan Nutrisi Rumput Napier menggunakan *Aspergillus sp.* melalui Penapaian Keadaan Pepejal

ABSTRAK

Rumput napier *(Pennisetum purpureum)* merupakan salah satu rumput tropika yang popular. Ia digunakan secara meluas untuk memberi makan haiwan ruminan. Walau bagaimanapun, komposisi nutrien dalam rumput Napier masih rendah dan perlu ditingkatkan nilai pemakanan rumput Napier menggunakan bahan tambahan (cth. molases dan kulat) yang boleh memenuhi keperluan nutrien oleh ruminan. Oleh itu, objektif kajian ini adalah untuk menilai kesan *Aspergillus niger* dan *Aspergillus awamori* terhadap ciri-ciri penapaian dan nilai pemakanan silaj rumput Napier melalui penapaian keadaan pepejal. Terdapat 3 rawatan dengan 3 ulangan untuk setiap satu: i) Kawalan (T1): Rumput napier diperam dengan 5% molases, ii) T2: Napier rumput diperam dengan 5% molases dan *Aspergillus niger* (10 ml/kg silaj; 10^6 spora/ml) dan iii) T3: Rumput napier diperam dengan molases 5% dan *Aspergillus awamori* (10 ml/kg silaj; 10^6 spora/ml). Rumput napier dituai pada kira-kira 2 bulan tanaman matang, dicincang secara manual dengan panjang 2-3 cm, disimpan kedap udara dalam beg plastik dan kemudian disimpan secara anaerobik selama 21 hari. Nilai pH, kandungan asid laktik, NH3–N, bahan kering (DM), protein kasar (CP), ekstrak eter (EE), gentian kasar (CF), abu dan ekstrak bebas nitrogen (NFE) silaj telah dinilai. Terdapat perbezaan yang ketara ($p<0.05$) dalam nilai pH antara rawatan. Nilai pH adalah antara 4.08 hingga 4.20. Silaj T1 menunjukkan nilai pH yang lebih rendah ($p<0.05$) (4.08) berbanding silaj T2 (4.20). Tiada perbezaan yang signifikan ($p>0.05$) diperhatikan dalam kandungan asid laktik dalam silaj rumput Napier antara rawatan. Kandungan NH3-N dalam silaj rumput Napier adalah ketara ($p<0.05$) berbeza antara rawatan. Kandungan DM, CP, EE, CF dan NFE dalam silaj rumput Napier adalah berbeza secara signifikan ($p<0.05$) antara rawatan, manakala kandungan abu tidak terjejas dengan ketara ($p>0.05$). Silaj T2 menunjukkan kandungan DM tertinggi (24.04%), CP (11.42%), CF (29.00%) dan abu (9.39%), manakala silaj T1 menunjukkan DM terendah (21.04%), CP (9.16%), CF (23.29%) dan kandungan abu (6.34%). Silaj T2 menunjukkan kandungan EE yang paling tinggi (7.65%), manakala T3 menunjukkan yang paling rendah (0.58%). Silaj T1 menunjukkan kandungan NFE tertinggi (59.70%), manakala silaj T2 menunjukkan paling rendah (42.56%). Keputusan kajian semasa ini mencadangkan bahawa rumput Napier diperam dengan tempoh penapaian selama tiga minggu mengandungi tahap CP tertinggi. Oleh itu, rawatan biologi rumput Napier menggunakan *Aspergillus niger* meningkatkan nilai pemakanannya, yang mungkin digunakan untuk makanan ruminan.

Kata kunci: Rumput Napier, *Aspergillus niger*, *Aspergillus awamori*, kualiti silaj, nilai pemakanan.

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LIST OF SYMBOLS AND ABBREVIATIONS

Reference No.

°C	Degree Celsius
ANOVA	One-way Analysis of Variance
CF	Crude fibre
cm	Centimetre
CP	Crude protein
DM	Dry matter
EE	Ether extract
g	Gram
H_2SO_4	Sulfuric acid
H_3BO_3	Boric acid
HCl	Hydrochloric acid
kg	kilogram
LAB	Lactic acid bacteria
M	Molarity
M	Molarity
ml	Millilitre
N	Normality
NaOH	Sodium hydroxide
NDF	Neutral detergent fibre
NFE	Nitrogen free extract
NH_3–N	Ammonia nitrogen
VFA	Volatile fatty acid
WSC	Water soluble carbohydrates

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Napier grass *(Pennisetum purpureum)* is one of the major tropical forage species used for ruminant animals. Napier grass is also known as Elephant grass and perennial tropical grass. Napier grass is widely used to feed ruminant animals and popular used in cut and carry system or made into silage or hay (Negawo et al., 2017). It is a highly adaptable plant that can be planted in a variety of state, including dry or wet climates, smallholder or large-scale agriculture [Elephant Grass (*Pennisetum Purpureum*) | Feedipedia, n.d.]. Napier grass is rapidly growing and has great annual yield productivity that is affected

by climatologic conditions such as temperature and rain season (Aroeira et al., 1999). Napier grass also has a high drought tolerance, provides good soil conditions, high photosynthetic and water-use competence (Dien & Peterson, 2008).

Aspergillus niger is a fungus known as "black mold" that can grow on exterior of specific vegetables and fruits. *Aspergillus niger* is an ascomycete fungus that is abundant in the surrounding and has the ability to infect in person (Perfect et al., 2001). *Aspergillus niger* is the commonly organisms applied in industrial invention such as fermented foods, organic acids, and enzymes (Ademark et al., 1998). *Aspergillus niger* acts as a global carbon cycle since this fungus can be noticed in soils. This fungus is an organism with an extensive range of hydrolytic and oxidative enzymes taking part in the classification of plant lignocellulose. A ton of these enzymes from *Aspergillus niger* are essentially used in the biotechnology application (Baker, 2006).

Solid-state fermentation can be identified as fermentation requiring no water or less amount. But should provide it with adequate moisture in assisting the microorganism growth and metabolism (Vandenberghe et al., 2000). Solid-state fermentation stimulates the growth of micro-organisms in nature on moist solids and has been credited to be responsible for the beginning of fermentation technique in ancient time (Mitchell et al., 1986). Back then, the fermentation

process was using solid-state fermentation principles. Solid-state fermentation caters large possibilities in agronomy scraps development (Pandey, 2003).

Silage is produced by the fermentation through the anaerobic condition of a crop, forage or agriculture derivative that typically more than 50% moisture composition. The main factors of ensiling are dry matter content, sugar content, the forages maturity and natural microbial substances. According to McDonald et al. (1995), there is variety of crop can be preserved as silage and commonly are grasses, legumes, whole cereal such as wheat and maize. Local farmers also can make their own silage rather than depending on imported feed (Jais et al., 2017).

1.2 Problem Statement

Napier grass needs minimum requirement of management for their growth. It produces a high biomass yield (de Morais et al., 2009), high potential re-growth (perennial grass), easy to propagate (Lee et al., 2016), in addition reducing soil erosion (Magcale-Macandog et al., 1998), reluctant to a variety of microorganisms (Van den Berg & Van Hamburg, 2015) and immerse capability

for cellulosic biofuel development (Tsai & Tsai, 2016). However, the nutritional content in Napier grass still low and not adequate for ruminant. Its nutritional values (7.0-9.0% crude protein and 7.5 MJ metabolizable energy/ kg dry matter) are considered too low. To implement Napier grass as the main consumption for ruminant animals, Gwayumba et al. (2002) suggested that a part of the Napier grass needs to be replaced with high protein or energy feed to constrain rumen microbial activity lessen and sunken digestibility.

Feedstuffs contribute almost 70% of total costs in animal production. In feedstuffs costs, protein sources present a massive amount because they are expensive and ruminant animal have to consume in large quantity. It is necessary that the new protein sources should be found for animal nutrition in order to reduce the pressure on the protein sources used animal nutrition made by increasing animal product demand in parallel with rising in human population. According to Oriol et al. (1988), *Aspergillus niger* is suitable for solid-state fermentation because they can rapidly grow in the low-water environment. They are extremely can be found in food variety such as grains, nuts and spices and more commonly in tropical and subtropical than in temperate climates (Pitt & Hocking, 2009).

Oyeleke et al. (2012) and Morgado et al. (2016) mentioned that *Aspergillus niger* can also boost up the digestibility of feed by generating enzymes lipase, cellulase, protease, amylase, and xylanase. Madamwar et al. (1989) reported that solid-state fermentation using *Aspergillus niger* presented 30% to 80% outstanding enzymatic activities than standard submerged fermentation. By the addition of *Aspergillus niger* through solid-state fermentation (ensiling) may aid in preserving effectively for a period and might help in enriched the nutritional value of Napier grass for animal feedstuff.

1.3 Objectives

a) To investigate the effects of *Aspergillus niger and Aspergillus awamori* on fermentation characteristics of Napier grass silage;
b) To evaluate the effects of *Aspergillus niger and Aspergillus awamori* on nutritive values of Napier grass silage.

1.4 Hypothesis

H_0: The usage of *Aspergillus niger* and *Aspergillus awamori* in Napier grass has no effect on the fermentation characteristics and nutritional values of Napier grass silage.

H_1: The usage of *Aspergillus niger* and *Aspergillus awamori* in Napier grass has beneficial effect on the fermentation characteristics and nutritional values of Napier grass silage.

1.5 Scope of Study

In this study, the fermentation characteristics of Napier grass and the nutritional value of Napier grass after treated with *Aspergillus niger* and *Aspergillus awamori* through solid-state fermentation were analyzed. The addition of the fungus *(Aspergillus niger and Aspergillus awamori)* may affect the quality and nutritional composition of the grass.

1.6 Limitation of Study

This study was intended to focus only on laboratory analysis. Hence, the effect of the enriched silage on animal consumption cannot be examined.

1.7 Significant of Study

Napier grass is an important forage in feed consumption for the ruminant animals. But the nutritional value in the Napier grass does not fulfill their nutrient requirements. By only feeding the forage to the ruminant animals without microbial properties are not effective and not economical for farmers.

Aspergillus niger may improve the nutritional value in Napier grass. According to research by Iyayi & Losel (2001) and Dei et al. (2008), *Aspergillus niger* can increase the nutrients compositions of feedstuffs and can lose its anti-nutritional components**.** In this study, it is proved that by utilizing

the fungus into the feed intake in Napier grass through fermentation process gave a good result in the nutritional value.

Solid-state fermentation is most qualified for filamentous fungi growth under minimal moisture conditions and is an economical technology for the bioproducts production (Salgado et al., 2015). Hence, this study provided the scientific proof of the effectiveness by adding *Aspergillus niger* that can enhance the nutritional value in Napier grass for study reference and may help to improve the economical of feed intake in ruminant animal for the farmers. Moreover, it was identified more information and nutritional values regarding the enriched Napier grass with *Aspergillus niger and Aspergillus. awamori.*

CHAPTER 2

LITERATURE REVIEW

2.1 Napier grass (*Pennisetum purpureum*)

2.1.1 Descriptions

Napier grass is a C_4 grass species that is more adaptable to warm and hot weather, dry and wet environments. Napier grass has high yielding grasses. Napier grass can grow usually up to 7.5m. Napier grass has bluish green hairy blades leaves with 30-120 cm long and 1-5 cm wide. Also known as Elephant

grass, it can be propagated by rhizome and stem (stolon). The inflorescence is yellow brown to purplish in color and have a stiff terminal bristly spike. The seeds are very rare to see. Seed production is very low and sometimes even no seed formation. According to Singh et al. (2013), the seeds cannot be used to propagate the Napier grass, since the grass has an open pollinated crop, the seedlings are also commonly heterozygous. As a result, propagation by stem cuttings is the best choice in breeding of Napier grass (Singh et al., 2013).

2.1.2 Adaptability

Back in the 1920s, Napier grass was first introduced to Malaysia (Halim et al., 2013). The Napier grass growth development is excellent and have a high adaptability with Malaysia's weather (Halim et al., 2013). As claimed by Singh et al. (2013), Napier grass is adapted to grow through a variety type of soil conditions and agro-ecologies from sea level to 2100 m. Singh et al. (2013) also stated that Napier grass has minor dry spells tolerance, even it grows best in annual rainfall which between 750 mm and 2500 mm. Napier grass has been widely used as a fodder crop since it has a high yield, easy to propagate and variety nutrients depending on the species, harvesting time, fertilization

management and weather condition (Kongkeitkajorn et al., 2020). Napier grass well grown on any soil variation but nevertheless greatly grown in deep, well drained friable loams with 4.5-8.2 pH (*Pennisetum Purpureum* Scientific Name Distribution, 2012). However, Napier grass is intolerant to flooding or waterlogging since it can grow range 25-40°C (Bogdan, 1977).

2.1.3 Nutrient Content

In the ruminant industry, Napier grass plays a big role since it has a high productivity. It has been used by smallholder farmers in cut-and-carry feeding management (Premaratne & Premalal, 2006). Indeed, Napier grass has a rather minimal amount of protein content which is about 10% dry matter (DM) however the young Napier grass can be highly nutritious. As mentioned by Sullivan et al. (2011), Napier grass is rich in fibre that depending on maturity stage, neutral detergent fibre (NDF) that have concentrations vary from 55 to 75% DM. It was supported by Kramberger & Klemenčič (2003) and Bayble et al. (2007), shows that as the grass matures, the DM production increases, while crude protein (CP) decreasing. As claimed by Jusoh et al. (2014), it is important to harvest at the selected maturity stage since it will affect the nutritional value

in the grass. Moreover, it is crucial to measure the quality of the Napier grass at a difference level of maturity since it will influence the silage making (Rambau et al., 2016).

Table 2.1.3.1: Taxonomic Position of Napier grass *(Pennisetum purpureum*) According to Anderson & Cronquist (1981).

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Liliopsida
Order:	Poales
Family:	Poaceae
Genus:	*Pennisetum*
Species:	*P. purpureum*

Reference: Mia (2017).

Table 2.1.3.2: Chemical Composition of Napier grass Cut at Different Heights.

Parameter	50 cm	75 cm	1 m	1.25 m	1.5 m
DM%	37.00	45.94	45.99	35.03	35.27
ASH%	6.00	7.48	8.60	11.90	12.63
CP%	13.29	10.33	8.85	6.67	4.79
NDF%	51.37	64.55	63.00	62.50	70.39
ADF%	37.00	33.50	33.00	36.50	40.50
Macro elements (%)					
P	0.07	0.05	0.06	0.05	0.04
Ca	0.11	0.10	0.11	0.11	0.14
Mg	0.05	0.05	0.03	0.07	0.06
Na	0.17	0.14	0.15	0.10	0.16
Minor elements (%)					
Fe	193.60	192.90	185.30	190.70	224.20
Mn	39.00	37.30	30.60	19.70	62.50
Zn	50.00	61.20	68.60	50.20	45.70
Cu	6.60	6.30	6.90	10.30	7.30

Reference: Aganga et al. (2005). DM, dry matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; P, phosphorus; Ca, calcium; Mg, magnesium; Na, sodium; Fe, iron; Mn, manganese; Zn, zinc; Cu, copper.

2.2 *Aspergillus sp.*

In 1729, *Aspergillus* was introduced by Pier Antonio Micheli in Micheli's Nova Plantarum Genera book publication. According to Plascencia-Jatomea et al. (2014), *Aspergillus* species are widely distributed in nature, especially in decaying organic matter. According to Palumbo et al. (2008), the Aspergillus genus comprises of various species including opportunistic pathogens, toxin producing and industrially important species, accounting for the majority of agricultural pollution. Palumbo et al. (2008) also mentioned that Aspergillus species could be useful in agricultural and might be helping in fungi strategies development. Several of Aspergillus species has been obtained by Generally Regarded as Safe (GRAS) also been used in feed and food production (De Vries & Visser, 2001). For industrial utilization, the black aspergili is the ideal organism used to produce a good fermentation with high protein productivity (Davies, 1994).

2.2.1 *Aspergillus niger*

Aspergillus niger is a common species from food with a broad usage in food production. *A. niger* is more superior in warm environment. As stated by Ziani et al. (2009), *A. niger* known as black molds on plant surfaces and powerful in seed decaying. *A. niger* is the most crucial in food decaying especially in fruit products such as vegetables and other crops (Plascencia-Jatomea et al., 2014). It was acknowledged by Pitt & Hocking (1997) that *Aspergillus niger* is responsible for post-harvest decay of fresh fruit but does not give too much impact.

2.2.2 Descriptions

As stated by Schuster et al. (2002), *Aspergillus niger* can grow at wide pH range which is 1.4–9.8 and has adaptability with variety temperature of 6–47°C but the appropriate temperature at 35–37°C. He mentioned that the water activity limit for growth is 0.88, thus high compared with other Aspergillus species. *Aspergillus niger* can distribute through the air with warm and humid places makes *Aspergillus niger* has the abilities to produce high conidiospores (Schuster et al., 2002). According to Schuster et al. (2002), *Aspergillus niger* became an industrially used organism when citric acid was first produced by fermentation in 1919. As stated by Roukas (2000), citric acid has been used in industries and significantly surpass other metabolites such as gluconic acid. *Aspergillus niger* is one of the most essential microorganisms used in biotechnology. *Aspergillus niger* was used to produce enzymes and citric acid (Schuster et al., 2002).

2.2.3 Nutrient Enrichment

Aspergillus niger is one of an organism of selective choices because they easily handled, have the capability to gained from cheap raw ingredients and high productions of citric acid (Mourya & Jauhri, 2000). The growth of citric acid is not a popular occurrence, but it plays a role in growing the organism under some particular limitations. Hence, incubation requirements act as an essential part and highly impact the citric acid production level (Grewal & Kalra, 1995; Mourya & Jauhri, 2000). Based on research by Schuster et al. (2002), *Aspergillus niger* and *Aspergillus wentii* are used in fermentation process to produce high number of citric acid since it is economical and cheap. Other than that, Frost GM (1987) mentioned that *Aspergillus niger* has a variety of enzymes such as amyloglucosidase, protease and pectinase that have been utilized and created in lab culture. Oboh et al. (2002) used *Aspergillus niger* in cassava fermentation and concluded that *A. niger* has potential in protein increasement in cassava flour also can decrease the tannin and cyanide composition in cassava products. Aydın et al. (2018) observed that *Aspergillus niger* could enhance the nutritional content in grape seed through solid-state fermentation.

2.3 *Aspergillus awamori*

2.3.1 Uses

Aspergillus awamori mainly used in alcoholic beverage industry known as Awamori in Japan (Hong et al., 2013). According to Pitt & Hocking (2009), *Aspergillus awamori* commonly used in food fermentations and is possibly a cultivated form of *Aspergillus niger*. Mohd-Razali et al. (2020) reported that *Aspergillus awamori* is formerly recognized in the awamori process that involves the conversion of starch to citric acid. *Aspergillus awamori* might give aid in successful protein digestibility enhancement, which is soluble protein composition with low phytic acid through solid-state fermentation from coconut residue (Mohd-Razali et al., 2020). Webb & Wang (1997) mentioned that the addition of *Aspergillus awamori* also could improve the nutrient composition in wheat.

2.4 Silage

Silage is one of the greatest ways to preserve forage crops such as Napier grass. Commonly, to get the best silage, the crop can be harvest at early age with 30% of dry matter. According to Ranjit & Kung (2000), ensiling is a process by lessening the pH value to increase the forage permanency by using an anaerobic and adequate fermentation process. The minimal level of oxygen and the addition of lactic acid reduce the microbial metabolism and conserve the nutrients composition in the crop (McDonald et al., 1995).

2.4.1 Silage Quality

Factors for good quality silage can be determined by the fresh crop with an efficient fermentation process. A good fermentation process will be resulting in more digestibility feed that might improve the animal performance. As mentioned by Da Silva et al. (2017), the most crucial elements in making silage are the amount of dry matter, sufficient water-soluble carbohydrates (WBC) and low buffering quality. Silage technology is important to produce a high-quality

silage. By using right process to stimulate the sufficient anaerobic condition, we can preserve many types of crops that are available for the animal by making it into silage (Da Silva et al., 2017). Da Silva et al. (2017) stated that crops should be harvested for ensiling because crops have a high nutritional content and there are between 30 and 35% in dry matter content. Plant maturity, nutrient composition and moisture content in the crop can be influenced in producing a good quality of silage. The parameters of silage quality are ammonia nitrogen content, pH value, lactic acid content and volatile fatty acids (VFAs) content.

According to Kung & Shaver (2004), silages that have higher amount of ammonia will show high acetic acid level because of the long-time taken to fermented. Clostridia can help in breaking down the proteins, but somehow it also will show significant loss of silage quality. With clostridia, it will help to produce high value of dry matter content, high level of ammonia nitrogen but it will make the silage poor palatability (Ward & Ondarza, 2008). Ammonia nitrogen content can be lower too with help of microbial activities, which is more than 60% from the previous studies (Muck & Kung Jr, 1997).

According to McDonald et al. (1991), Lactic acid bacteria (LAB) can tolerate in low moisture environment and capable to fermented of high dry

matter yield. LAB fermentation can help to slow down the clostridia and enterobacteria growth. LAB can be found on harvested crops and they ferment easily including sugars like glucose and fructose to lactic acid. As mentioned by McDonald et al. (1991), the produced LAB would increase the hydrogen ion concentration and undissociated acids until a point at one-point organism cannot grow. Moisture content and temperature can affect the growth of clostridia and enterobacteria at crucial pH (Yitbarek & Tamir, 2014). The higher the moisture value in material, the lower the pH value in silage end product. Muck & Kung Jr (1997) reported that microbial inoculation can lower down the pH value as well can improve the lactic: acetic ratio. As reported by Ward & Ondarza (2008), the higher the pH value drop, dry matter that is conserved in the fermentation process will increase too.

Fermentation of forage is aimed to get low pH level through the formation of Volatile Fatty Acids (VFA) to conserve the forage into silage. There are factors that impact the silage pH, which is soluble carbohydrate content of the conserved crop, crop's moisture content, the length of chopped crop, compaction, organism that inhibited the fermentation process, contamination of forage with foreign substances and the forages buffering capacity. The VFA includes propionic acid, lactic acid, acetic acid and butyric acid (Ward & Ondarza, 2008).

Table 2.4.1.1: Parameter in Common Fermentation End Products in Grass Silages.

Parameter	Grass silage with 30-35% dry matter (DM)
pH value	4.3-4.7
Ethanol (%)	0.5-1.0
Ammonia nitrogen (% of CP)	8-12c
Lactic acid	6-10
Volatile Fatty Acid (VFAs)	
Acetic acid (%)	1-3
Butyric acid (%)	0.5-1.0
Propionic acid (%)	<0.1

Reference: Kung & Shaver (2004).

2.4.2 Ensiling Process (Fermentation)

The process of silage making is called ensiling. Ensiling is a method to preserve the forage under anaerobic conditions. According to Da Silva et al. (2017), the main principle of silage is anaerobic condition and water-soluble carbohydrates fermentation by the epiphytic LAB and lactic acid production in the fresh crop. Due to the pH in acid production, the silage material reduces and spoilage microorganisms are constrained (Elferink et al., 2000).

2.4.2.1 Phase I - Aerobic Phase

This is the first phase in ensiling. This phase will take a few hours to ensiled. The existed oxygen in the plant particles will slightly reduce because of the respiration of the crop and facultative aerobic organisms such as yeasts and enterobacteria. As mentioned by Da Silva et al. (2017), this phase must be short due to the converting plant sugar, water and heat release will resulting in decreasing in dry matter. According to Elferink et al. (2000), plant enzymes

such as proteases and carbohydrases are active during this phase, hence must make sure the pH still between the standard range (pH 6.5-6.0).

2.4.2.2 Phase II - Fermentation phase

In Phase II, silage will become anaerobic and remains for several days or week according to material in silage crops and conditions. Lactic acid bacteria (LAB) will be successfully developed during this phase. The pH also will decrease to 3.8-5.0 due to lactic acid and other acid production (Elferink et al., 2000).

2.4.2.3 Phase III - Stable Phase

During stable phase, the microorganism activities will reduce significantly. However, some acid tolerant microorganism will endure this phase and maintains a gradual carbohydrates and protein hydrolysis (Da Silva et al., 2017).

2.4.2.4 Phase IV - Feed-Out Phase or Aerobic Spoilage Phase

Phase IV is the most crucial phase in fermentation process. Feed out phase will start when we open the silos and exposed it to the air. This phase is important because the unwanted microorganisms devour the mixtures that make the silage stable in the silos which is lactic acid, within the existent oxygen and can create numerous mixtures diminishing the silage quality (Da Silva et al., 2017).

There are two important stages in Phase IV. The first stage is the spoilage stage. It will deteriorate and degrade the preserve organic acid by acetic acid bacteria and making pH increment. Then following by the second stage, the temperature also will rise. The molds, yeasts, and acetic acid bacteria consume the acids, sugars, and protein for heat releasing can cause significant differences in the chemical content in pH and lead to massive spoilage. It is essential to carefully opening silos to prevent from the unwanted microorganism increment. The environmental condition and silage characteristics are crucial in producing a good silage when exposed the silos in the air (Elferink et al., 2000).

Table 2.4.2.4.1: Silage Fermentation Stages and Their Mechanisms.

Stages	Phases name	Time taken	Mechanisms
1	Aerobic	A few hours	Atmospheric oxygen present between the plant particle reduced due to respiration
2	Fermentation	Several days/weeks	LAB develop and become predominant
3	Stable	As long as air prevented	Most microorganisms decrease
4	Feed out/ Aerobic Spoilage	Starts as soon as silage exposed to air	Process of spoilage started by yeast, temperature change and activity of spoilage microorganisms

Reference: Mohd-Setapar et al. (2012).

Table 2.4.2.4.2: Silage Fermentation Stages and Their Mechanisms.

Stages	Phases name	Time taken	Mechanisms
1	Aerobic	2 days	Breakdown of plants protein and reduce to amino acids
2	Anaerobic fermentation	2-3 days	Growth and development of acetic acid
3	Brings Phase 2 to and end	3-4 days	Enhances the growth and development of anaerobic group of bacteria and produces lactic acid
4	Lactic acid formation	4-21 days	Lactic acid begin to increase until pH is low enough to inhibit growth of bacteria
5	Material Storage	21 days	Large population of bacteria may grow and produce butyric acid instead of lactic acid
6	Aerobic decomposition	Starts as soon as silage exposed to air	High population growth of yeast or mold. Proper management is vital

Reference: Mohd-Setapar et al. (2012).

2.5 Silage Additives

2.5.1 Molasses

Molasses well known as nutrient additive in forages. Molasses is commonly made of sugars and is beneficial in enriched the fermentation quality in low DM grasses and low water-soluble carbohydrates (WSC) legumes and forages (Bolsen et al., 1996). According to Castle & Watson (1985), they observed that molasses was as effective as the formic acid additions in ryegrass silages. The value of the end products also increases due to the fermentation of the existing sugars in molasses (Yokota et al., 1998). Based of experiment by Danso & Nartey (2018), they found out that more dry matter (DM) recovery with molasses in fermentation characteristics in Napier grass. They also mentioned, we can store the silages even in high temperature after ensiled with molasses. The presence of molasses in ensiled Napier grass was enhance the physical properties and chemical composition (Danso & Nartey, 2018). Bureenok et al. (2019) stated that supplementary of molasses can enhance the feed intake on animals, fermentation quality and digestibility of Napier grass.

2.6 Proximate Analysis to Determine Silage Quality

The nutrient contents of silage are crucial to be analyzed. The nutritive value may vary for different types of forages used to make silage. Also, the other ingredients added in the silage may affect the nutrient contents of the silage. Dry matter is important determinant of intake and preservation values. Most of the silage analysis parameters are expressed on a DM basis. The optimum DM for intake is 28-32%. High value of DM will reduce aerobic stability of silage.

Measuring the nitrogen is an indicator of CP content. The optimum value of CP ranges from 9-15%. Usually, CP content is higher in leafy and higher DM digestibility silages. However, it can be varied for different types of silages. High CP content in silage tends to be rapidly degradable, leading to poor utilization, if diet energy is lacking. If the value of CP is <10%, it may delay the rumen microbial growth.

Crude fibre (CF) is to identify the content of indigestible cellulose, hemicellulose and lignin existed in the fermented crop. According to Da Silva et al. (2017), to isolate the cell wall residue that represents indigestible portions of

the forage, the usage of alkali and acid treatments are very crucial in CF analysis.

2.6.1 pH value

The pH analysis in silage is essential since it will be used to evaluate the quality of fermentation. pH will be influenced by the buffering capacity of the fermented crop. According to Yitbarek & Tamir (2014), legume has the highest buffering capacity rather than corn or grass silage. When the low in pH the silage end product will contribute to the sour taste and more acidic. According to Elferink et al. (2000) and Yitbarek & Tamir (2014), the pH required for fermented crop at 150, 250, 350 and 450 g DM/kg, is 4.10, 4.35, 4.60 and 4.85 correspondingly. In the present studies, silages conserved with lactic acid bacteria developed the greatest value of organic acids and low pH value enhanced the qualitative traits of the fermented silage compared with the untreated crop.

2.6.2 Lactic Acid Content

The produced lactic acid is essentially reliable in conserving the fermented crop. The amount of lactic acid required are affected by the crop and the dry matter content. The common lactic acid contents are between 3 and 12 (Lallemand Animal Nutrition, 2021). According to Kung & Shaver (2004), they highlighted that lactic acid content need to involve 65% of the total VFA and the lactic acid content. Ward & Ondarza (2008) stated that lactic acid is very crucial to produce a remarkable silage. Higher content of lactic acid normally will be resulting the lowest dry matter losses.

2.6.3 Ammonia Nitrogen Content

During fermentation, protein will be deteriorated into ammonia, amino acids, dipeptides, volatile basis and organic acids. Ammonia nitrogen in fermented crop is primarily made from clostridial fermentation of amino acids. Silages that have low content of ammonia nitrogen shows that the

fermentation of the silage is great. Higher values silages with ammonia contents >12% for grasses signify a poor fermentation (Lallemand Animal Nutrition, 2021). Steen et al. (1998) finalized that ammonia is an essential parameter of silage dry matter intake.

2.6.4 Dry Matter Content

Dry matter related to the remaining material after the water is evacuated. Then the moisture content evaluates the water present content in the fermented crop. Dry matter is essential parameter in ensiling and preservation values. Most of the silage analysis parameters are recorded on a DM content. The optimum DM for silage is 28-32%. The high value of DM will consequently reduce the aerobic activity in the silage process.

Dry matter content affects the amount of substrate required by affecting the pH at which lactic acid bacterial activity ceases. The DM also influences the growth of LAB and thus the pH time course. Elferink et al. (2000) also

indicated that the drier the crop being ensiled the greater the DM filling rate necessary to prevent excessive heating from respiration.

2.6.5 Crude Protein Content

Crude protein is the amount of protein of grass silage. Crude protein relies on the nitrogen value of the feed proteins. Crude protein analysis commonly used in animal husbandry. Crude protein content also affects the quality of the grass at the harvesting time. Young grass will produce high protein silage while older grass produces low protein content. (Silage Analysis., 2017.) As stated by Burgess & Nicholson (1984), higher CP levels will reduce the efficiency of nitrogen utilization for milk production in cattle.

Zailan et al. (2018) determined that the mean of CP content was highest in Dwarf (10.42 %), followed by Silver (9.61%) and least in both Red (8.80%) and Common (8.67%) Napier. The CP content of Common Napier increased significantly ($p \leq 0.05$) from 8.13 to 9.26% after ensiling process. Increase of CP content ($p > 0.05$) in Napier cultivars after ensiled could be accompanied by the populated anaerobic microorganism during ensiling process (Zakaria, 2011).

2.6.6 Crude Fat (Ether Extract) Content

The lipids are substances can be found in animal tissues and plants including fermented crop. Lipids are insoluble in water but soluble in common organic solvents such as benzene, ether and chloroform. Therefore, ether extract (EE) is determined by extraction with petroleum ether for a defined period. After evaporation of the solvent, the remaining residue called EE. According to McDonald et al. (1991), the forage nutritional composition which is leaf is richer than the stem in calcium, CP, minerals and EE.

2.6.7 Crude Fibre

Crude fibre is to identify the content of indigestible cellulose, hemicellulose and lignin existed in the fermented crop. According to Da Silva et al. (2017), to isolate the cell wall residue that represents indigestible portions of the forage, the usage of alkali and acid treatments are very crucial in CF analysis.

2.6.8 Ash Content

Ash indicates the total mineral content in the fermented crop. Ash content in silage approximately must have less than 10% (Lallemand Animal Nutrition, 2021).

2.6.9 Nitrogen Free Extract (NFE)

Nitrogen-free extract is designed to provide an estimate of water-soluble polysaccharides such as sugars and starch Total NFE is determined as the addition of CP content, EE, CF and ash; then will be subtracted from the total of silage composition percentage.

CHAPTER 3

METHODOLOGY

3.1 Materials

Aspergillus niger (derived from ATCC® 6275™) and *Aspergillus awamori* (from Dr. Mst Laila Naher, Faculty of Agro Based Industry, Universiti Malaysia Kelantan) were cultured on Potato Dextrose Agar (PDA) and were incubated at the laboratory of Faculty of Agro Based Industry (FIAT), Universiti Malaysia Kelantan (UMK). Napier grass (approx. 10 kg) at approximate 2 months of plant maturity were harvested manually from Agro Techno Park (ATP), UMK. Molasses was obtained from local supplier.

3.2 Chemicals

The chemicals used in this experiment were 70 ethanol, lactophenol blue solution, 1% phenolphthalein, 0.1 N of NaOH (sodium hydroxide), Kjeltabs tablets, H_2SO_4 (sulfuric acid) solution, 40% of NaOH (sodium hydroxide), 4% of H_3BO_3 (boric acid), 0.1 M of HC1 (hydrogen chloride) and 80 ml petroleum ether.

3.3 Equipment

The equipment were used in this experiment were agar plate, parafilm, incubator, hemocytometer, biosafety cabinet, microscope, autoclave, pH meter, beaker, filter paper, analytic balance, digestion tube, digestion rack, digestion block of Gerhardt Kjeldatherm, rack holder, 105°C oven, alkali tank of Gerhardt Kjeldatherm, receiver flask, aluminium foil, desiccator, Soxtec extraction, thimble, defatted cotton, Erlenmeyer titration flask Fbibertectm 8000 Auto Fibre Analysis System, Carbolite Gero 30 – 3000°C muffle furnace and Protherm muffle furnace.

3.4 Methods

3.4.1 Experimental Design

The experimental design of this study is shown in Table 3.4.1.1. All treatments were prepared using Napier grass, molasses, *Aspergillus niger* and *Aspergillus awamori*.

Table 3.4.1.1: Treatment In Silage Making with The Replication.

Treatment		
T1	T2	T3
Napier grass with 5% molasses (control)	Napier grass with 5% molasses and *Aspergillus niger* (10 ml/kg silage; 10^6 spores/ml)	Napier grass with 5% molasses and *Aspergillus awamori* (10 ml/kg silage; 10^6 spores/ml)
Replication 1	Replication 1	Replication 1
Replication 2	Replication 2	Replication 2
Replication 3	Replication 3	Replication 3

3.4.2 Sample Preparation

Aspergillus niger and *Aspergillus awamori* were cultured on Potato Dextrose Agar (PDA). *Aspergillus niger* strain were obtained from Global Manufacturing Distribution Center located at 200 Cooper Avenue North, Saint Cloud, MN, 56303, cultured and derived from ATCC® 6275™ in KWIK-STIK form in a lyophilized pellet, a reservoir of hydrating fluid and inoculating swab. While, *Aspergillus awamori* were subcultured from previous agar plate that was obtained from Dr. Mst Laila Naher, FIAT, UMK. Then, both of fungus were incubated at the laboratory of FIAT, UMK at room temperature for 7 days according to agar plate technique (Güngör et al., 2017). Agar plate technique was done by taking a drop of the culture on the upper layer of nutrient agar, then using loop wire to make a streaking. Incubated another 7 days at 40°C for subculture to isolated the pure culture (organism). Another 7 days until the fungus reached the maturity (turn into green) (Naher et al., 2012). The spores were prepared for the treatment. The spores were prepared by using serial dilution method to estimate the concentration of fungus by counting the number of colonies cultured. Next, counted the spores using Hemocytometer according to the colony forming unit (CFU/ml) (Güngör et al., 2017). The CFU were used to count the approriate amount of the concentration of fungal cells in a test sample which is viable under the microscope. The concentration used was 10^6 spores ml^{-1} of the cultured fungus.

The stock solution were prepared. The *Aspergillus niger* (1×10^6 spores/ml) and *Aspergillus awamori* (1×10^6 spores/ml) were diluted with 10 ml sterile distilled water and shaken well to dissolve each other to make the stock solution (Altop, 2019). Then, the stock solutions were inoculated with Napier grass silage.

The Napier grass at approximate 2 months of plant maturity were harvested and chopped into about 2-3 cm in length. After chopping, Napier grass were mixed with 5% molasses (T1), mixed with 5% molasses and *Aspergillus niger* (10 ml/kg silage; 10^6 spores/ml) (T2), and mixed with 5% molasses and *Aspergillus awamori* (10 ml/kg silage; 10^6 spores/ml) (T3) based on the percentage combination as treatments. Then were weighed and placed into empty zip lock plastic with weights 500 gm as in each replication. Each treatment was divided into 3 replications (T1-1.5 kg; T2-1.5 kg and T3-1.5 kg). All ingredients were mixed properly. All silos were packed properly with the crop materials, molasses and fungus and were airtight as much as possible. Then it was sealed and stored properly under anaerobic condition to allow the fermentation process.

After 21 days of ensiling, the samples of T1, T2 and T3 were taken. The samples were analysed for fermentation characteristics and proximate components.

3.4.3 Silage Quality and Proximate Analysis

Proximate analyses were used to determine the nutritional composition of silage. There were seven components that were determined, which were pH value, lactic acid, ammonia nitrogen (NH_3–N), DM, CP, EE, CF, ash and nitrogen free extract (NFE) contents. All components were expressed as the percentage (%) except pH value.

3.4.3.1 pH value

The pH value of the silage was determined using pH meter. Approximately 10g of silage sample according to their treatment were placed in a beaker with 50ml of distilled water for 30 minutes at room temperature. After 30 minutes, read the pH value by placing the electrode pH meter in the silage sample. The results were recorded.

3.4.3.2 Lactic Acid Content

Lactic acid content of silage was determined using titratable acidity test. About 5 g of silage sample were weighed and placed in the beaker. Then, 50 ml of distilled water were added into the beaker. The sample were boiled and to be driven the carbon dioxide and let it to be cooled down. The needed sample were filtered by using filter paper and the filtrate were collected. Next, 5 drops of 1% phenolphthalein were added to the filtrate. The filtrates were titrated with 0.1N NaOH until it changed into light pink or pale pink in color. The volume of titrated 0.1 N NaOH were recorded. Total acidity content was expressed as % lactic acid. The percentage of lactic acid was calculated by using the formula below:

$$Lactic\ acid\ (\%) = \frac{V \times N \times 9}{W} \qquad (3.4.3.2)$$

Where;

V = volume of titrated 0.1N NaOH

N = normality of NaOH

9 = molecular weight of lactic acid

W = weight of fresh sample

3.4.3.3 Ammonia Nitrogen Content

Ammonia nitrogen (NH_3–N) content of silage was determined by using Kjeldahl method which included digestion, distillation and titration process.

3.4.3.3.1 Digestion

1 g dried silage sample about 1 g were weighted and were placed it in the digestion tube. The digestion tube was filled with 10 ml of distilled water and 1 piece of Kjeltabs tablet. Then, 12 ml of concentrated H_2SO_4 solution were added into the digestion tube and were placed inside the digestion rack in the fume chamber. The digestion block of Gerhardt Kjeldatherm was turned on and was

heated for 400˚C for pre-heating, before placing the digestion rack. After the digestion process, the rack was removed into rack holder and let it cool down.

3.4.3.3.2 Distillation

About 40% of NaOH were placed in alkali tank of Gerhardt Kjeldatherm distillation unit. Next, digested samples were diluted with 80 ml of distilled water and 50 ml of 45% NaOH. About 30ml of receiver solution were added to receiver flask. About 250 ml of Erlenmeyer titration flask were placed on receiving platform and were filled with 4% boric acid (H_3BO_3) along with indicator and placed into receiver solution tank. The samples were distilled for 5 minutes. Color change was observed.

3.4.3.3.3 Titration

Boric acid receiving solution were titrated using standard 0.1 M HCl until it turned pink in color. The volume of HCl used were recorded and ammonia nitrogen were calculated using the formula below:

$$NH_3 - N\ (\%) = \frac{[V - V\ (blank)] \times N\ \times 14.007}{W} \quad (3.4.3.3.3)$$

Where;

V = volume of acid neutralized sample (ml)

N = concentration of HCl

14.007 = atomic weight of nitrogen

W = Weight of sample (g) in DM

3.4.3.4 Dry Matter Content

Dry matter refers to material that remain once when water is evacuated, so the moisture content reflects the number of water present within the feed ingredient. The nutrients in feeds required by the animal for maintenance, growth, pregnancy and lactation are part of the feed's DM portion.

Dry matter was determined by weighing 2 g of silage sample and it was placed on aluminium foil (shaped like a bowl). W1 was noted as the weight of empty aluminium foil bowl-shaped and W2 was noted as weight of sample in DM. The sample was dried at 110˚C in an oven for 12 to 24 hours. After dried, let the sample cool down in the desiccator. W3 was noted as final weight after drying process. Lastly, the DM was calculated by following the formula below:

$$DM\% = \frac{W3 - W1}{W2} \times 100 \qquad (3.4.3.4)$$

Where;

% DM = Percentage of dry matter

W1 = Weight of empty aluminium foil bowl-shaped (g)

W2 = Weight of sample (g) in DM

W3 = Final weight of dried sample (g)

3.4.3.5 Crude Protein Content

Crude protein was determined by using Kjeldahl method which involved digestion, distillation and titration process.

3.4.3.5.1 Digestion

1 g of dried silage sample were weighted and placed it in the digestion tube. The digestion tube was filled with 10 ml of distilled water and 1 piece of Kjeltabs tablet. Next, 12 ml of concentrated H_2SO_4 solution were added into the digestion tube and placed inside the digestion rack in the fume chamber. The digestion block of Gerhardt Kjeldatherm was turned on and heated for 400˚C for pre-heating, before placing the digestion rack. After the digestion process, the rack was removed into rack holder and let it cool down.

3.4.3.5.2 Distillation

About 40% of NaOH were placed into the alkali tank of Gerhardt Kjeldatherm distillation unit. Next, digested samples were diluted with 80 ml of distilled water and 50 ml of 45% NaOH. About 30ml of receiver solution were added to receiver flask. About 250 ml of Erlenmeyer titration flask were placed on the receiving platform and filled with 4% boric acid (H_3BO_3) along with indicator

and placed into receiver solution tank. The samples were distilled for 5 minutes. Color changes were observed.

3.4.3.5.3 Titration

Boric acid receiving solution were titrated with standard 0.1 M HCl until it turned pink in color. The volume of HCl used were recorded and CP were calculated using formula below:

$$NH_3 - N\ (\%) = \frac{[V - V\ (blank)] \times N\ \times 14.007}{W}$$

$$CP\ (\%) = N\%\ \times 6.25$$

(3.4.3.5.3)

Where;

V = Volume of acid neutralized sample (ml)

N = concentration of HCl

14.007 = atomic weight of nitrogen

W = Weight of sample (g) in DM

6.25 = factor to convert nitrogen to protein

3.4.3.6 Ether Extract Content

Ether extract content of silage were determined by extracting the fat from the sample using a solvent, then determining the weight of the fat recovered. The equipment that were involved in this procedure is analytical balance (at least 1mg sensitivity). The determination of EE was done by undergo the standard operating procedure by used the Soxtec extraction.

Firstly, the aluminium cups were heated for 30 minutes at 103°C and were dried in the desiccators for 20 minutes to cool it down. The cups were weighted and the readings were recorded by 4 decimal places (W1). About 2 g of sample were weighted into the thimble and the weight of sample were recorded (W2). The thimbles were moved into the thimble stand and were put a layer of defatted cotton on top of the sample. Next, the thimbles were moved to the thimble

support. The thimbles were inserted into the extraction unit by attaching them to the magnets. The cups were filled with 80 ml petroleum ether each and the cups were inserted into the extraction unit with the cup holder. The machine was started after the button was pressed. After the extraction finished, the cups were removed and heated for 30 minutes at 103°C. The cups then were allowed to cool down in the desiccators for 20 minutes. The cups were weighted and the final weight were recorded and noted as W3. The EE % were calculated using the formula below:

$$EE\% = \frac{W3 - W1}{W2} \times 100 \qquad (3.4.3.6)$$

Where;

W1 = Weight of empty cup (g)

W2 = Weight of sample (g)

W3 = Weight of residue after extraction process (g)

3.4.3.7 Crude Fibre Content

In fibre analysis test, every batch of samples were running along with a blank through entire procedure. Firstly, the pre-dried crucibles were placed on a balance and tared. Each sample was weighed with a precision of 1000 ± 2mg. If sample homogeneity permitted, a smaller sample was used to further improved the filtration speed. If fat or carbonate content was high, Fibretec Cold Extraction Unit were used to remove prior the analysis.

Next, the crucibles were placed in hot filtration unit using the holder and were locked into position in front of the heater in the Fibretec 8000 which the safety handle engaged were ensured first. Then, the reflector was placed in front of the crucibles. After that, Acid tank and Alkali tank were refilled with 1.25% sulfuric acid and 1.25% KOH respectively and were placed in the drawer (left for acid tank and right for alkali tank) and all connections were fixed. The amber bottle with antifoaming agent n-Octanol inside side door was refilled. Water tap was opened (about 2 ½ minutes) for reflux system.

Next, the instrument was turned on and the default CF program was started by pressed the “START” button twice for double confirmations. At the end of extraction, the reflector was removed, and the safety handle also was

lifted up. After that, the crucibles were moved to the cold extraction unit for acetone soaks by used the crucible holder and then, the crucibles were refilled with 20-25 ml of Acetone and soaked within 3-5minutes and this procedure were repeated three times. Let it evaporate. Next, the crucibles were dried off in an oven at 105 ± 2°C for at least 5 hours or at 130 ± 2°C for 2 hours. The crucibles then were cooled to room temperature in a desiccator and were weighed accurately to 0.1 mg. Then, the sample in the crucibles were ashed at least 3 hours at 525 ± 15°C. The crucibles were heated and cooled with caution. Finally, the crucibles were cooled down slowly to room temperature in a desiccator and weighed accurately to 0.1 mg. Fibre were calculated by using the formula below:

$$Crude\ fiber\ \% = \frac{W2 - W3 - C}{W1} \times 100$$

(3.4.3.7)

Where;

Crude fibre % = Percentage of crude fibre

W1 = Sample weight

W2 = Crucible + Residue

W3 = Crucible + ash residue

C = Blank

3.4.3.8 Ash Content

Ash content of silage was measured by burning off the organic matter of silage sample at 600˚C for 8 hours in a muffle furnace. Empty crucibles were weighed and noted as W1 and 2 g of sample were weighed and noted as W2. The sample were incinerated in furnace at 600˚C for 8 hours. Then, the sample were cooled down inside the desiccator. The final residue inside the crucible were weighed and noted as W3. Ash content was calculated by using the formula below:

$$Ash\ \% = \frac{W3 - W1}{W2} \times 100$$

(3.4.3.8)

Where;

Ash % = Percentage of ash

W1 = Weight of empty crucible (g)

W2 = Weight of sample (g) in DM

W3 = Weight of crucible and ash (g)

3.4.3.9 Nitrogen Free Extract (NFE)

Nitrogen-free extract (NFE) were designed to provide an estimate of water-soluble polysaccharides (sugars, starch). Total NFE were determined by difference method rather than be analyzed directly. By using this method, other constituents in feed (CP, EE, CF and Ash) were determined individually then will be summed and subtracted from the total % of silage composition. Total NFE were determined by using the formula below:

$$Total\ NFE\ (\%) = 100 - (\%\ CP\ + \%\ EE + \%\ CF + \%\ Ash) \quad (3.4.3.9)$$

Where;

% CP = Percentage of crude protein

% EE = Percentage of ether extract

% CF = Percentage of crude fibre

% Ash = Percentage of ash

3.5 Statistical Analysis

All data were analyzed using one-way ANOVA by using SPSS software. All differences among the treatments were considered at $p<0.05$. Duncan Multiple Range Test (DMRT) was used to differentiate between treatments at $p<0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Silage Fermentation Characteristics

Table 4.1.1: pH value, Lactic Acid and Ammonia Nitrogen Contents of Napier grass Silages Treated with or without *Aspergillus niger* or *Aspergillus awamori*.

Parameter	Treatments (mean ± standard deviation)			p-value
	T1	T2	T3	
pH	4.08 ± 0.15[a]	4.20 ± 0.45[b]	4.17 ± 0.20[b]	0.008
Lactic acid (%)	5.77 ± 0.50	7.16 ± 1.22	5.75 ± 0.64	0.142
Ammonia nitrogen	1.46 ± 0.11[a]	1.82 ± 0.83[c]	1.64 ± 0.17[b]	0.006

[abc] Means with different superscripts in a row differ significantly ($p<0.05$)

T1 = Napier grass silage treated with 5% molasses; T2 = Napier grass silage treated with 5% molasses and *Aspergillus niger* (10 ml/kg silage; 10^6 spores/ml); T3 = Napier grass silage treated with 5% molasses and *Aspergillus awamori* (10 ml/kg silage; 10^6 spores/ml).

4.1.1.1 pH value

The pH values of experimental Napier grass silages are shown in Table 4.1.1. Results showed that the pH values of Napier grass silages were significantly ($p<0.05$) varied among the treatments. The mean value of pH values for T1, T2 and T3 were 4.08, 4.20 and 4.17 respectively. The results showed that

different treatment had a significance effect ($p<0.05$) on pH value of Napier grass silage as the p-value was 0.008. The pH value of T1 silage was significantly ($p<0.05$) lower (4.08) than the T2 (4.20) and T3 (4.17) silages. However, no significant ($p>0.05$) differences were observed on the pH value between T2 (4.20) and T3 (4.17) treatments. The average pH values of three Napier grass silage were ranged from 4.08 to 4.20.

According to Ranjit & Kung (2000), ensiling is a process by lessening the pH value to increase the forage permanency by using an anaerobic and adequate fermentation process. According to Quality Grass Silage for Dairy and Beef Production Systems (n.d.), a silage with 20%–24% DM should have pH value between 4.0–4.1. The results indicated that all the treatments can be assumed as a good silage as the pH value for all treatments were in range of 4.08~4.20. Hence, silage treated with *Aspergillus niger* (T2) and *Aspergillus awamori* (T3) were beneficial in balancing pH value in Napier grass silage.

4.1.1.2 Lactic Acid Content

The lactic acid content of experimental Napier grass silages is shown in Table 4.1.1. Results showed that there were no significant ($p>0.05$) differences on lactic acid content in Napier grass silages among the treatments. The mean values of lactic acid content for T1, T2 and T3 silages were 5.77%, 7.16% and 5.75% respectively. No significant ($p>0.05$) differences were observed on lactic acid content in Napier grass silages among the treatments as the p-value was 0.142.

The common lactic acid contents are between 3 and 12 (Lallemand Animal Nutrition, 2021). As stated by Ward & Ondarza (2008), lactic acid is very crucial to produce a remarkable silage. Higher content of lactic acid normally will be resulting the lowest dry matter losses. The result shows the range of lactic acid percentage for all treatments were between 5.75%–7.16%. All treatments can be considered as good silage as the lactic acid percentages are slightly between 3%–12%. By adding *Aspergillus niger* in the silage can increase the lactic acid content but, in the result shown it was not significantly higher differences than the control (T1). Effective microbes (EM) can be added in order to enhance the production of lactic acid content to achieve the required

percentage of lactic acid of good silage (Ramli, 2020). Somehow, in T3 shows that the addition of *Aspergillus awamori* did not affect positively on the Napier grass silages as the *Aspergillus niger* (T3) There is little information on lactic acid content in Napier grass silages those are treated with *Aspergillus sp*. It indicates that *Aspergillus sp.* does not influence on lactic acid production in silage.

4.1.1.3 Ammonia Nitrogen Content (NH_3–N)

Ammonia nitrogen content in experimental Napier grass silages were shown in Table 4.1.1. Results showed that the NH_3–N contents in Napier grass silages were significantly ($p<0.05$) differed among the treatments. T2 silage contained significantly ($p<0.05$) higher NH_3–N content (1.82%) followed by T3 (1.64%) and T1 (1.46%). The results showed that different treatments had a significance effect ($p<0.05$) on the percentage of NH_3–N contained in Napier grass silage as the p-value was 0.006.

According to Steen et al. (1998), they finalized that ammonia is an essential parameter of silage dry matter intake. Silages that have low content of ammonia nitrogen shows that the fermentation of the silage is great. Higher values silages with ammonia contents >12% for grasses signify a poor fermentation. (Lallemand Animal Nutrition, 2021). Based on the results shown in this study, the NH_3–N content for all treatments were lower than 12%, so it can be assumed as good quality of silage. Hence, the addition of *Aspergillus niger* and *Aspergillus awamori* were affected positively in the ammonia nitrogen percentage of Napier grass silage.

4.2 Proximate Analysis

Table 4.2.1: Chemical Composition of Napier grass Silages Treated with or without *Aspergillus niger* or *Aspergillus awamori*.

Parameter	Treatments (mean ± standard deviation)			p-value
	T1	T2	T3	
Dry matter (%)	21.04 ± 0.60[a]	24.04 ± 0.55[c]	22.96 ± 0.46[b]	0.001
Crude protein (%)	9.16 ± 0.72[a]	11.42 ± 0.51[b]	10.21 ± 0.27[a]	0.006
Ether extract (%)	1.52 ± 0.60[a]	7.65 ± 1.42[b]	0.58 ± 0.30[a]	0.000
Crude fibre (%)	23.29 ± 0.61[a]	29.00 ± 0.37[c]	25.27 ± 0.56[b]	0.000
Ash (%)	6.34 ± 0.90	9.39 ± 1.80	8.70 ± 1.37	0.083
Nitrogen free extract (%)	59.70 ± 2.26[b]	42.56 ± 3.02[a]	55.50 ± 1.34[b]	0.000

[abc] Means with different superscripts in a row differ significantly ($p<0.05$).

T1 = Napier grass silage treated with 5% molasses; T2 = Napier grass silage treated with 5% molasses and *Aspergillus niger* (10 ml/kg silage; 10^6 spores/ml); T3 = Napier grass silage treated with 5% molasses and *Aspergillus awamori* (10 ml/kg silage; 10^6 spores/ml).

4.2.1.1 Dry Matter

The DM contents of experimental Napier grass silages are shown in Table 4.2.1. Results showed that the DM percentage of Napier grass silages were significantly ($p<0.05$) varied among the treatments. The mean values of DM content for T1, T2 and T3 were 21.04%, 24.04% and 22.96% respectively. The results showed that different treatment had a significance effect ($p<0.05$) on DM percentage of Napier grass silage as the p-value was 0.001. The percentage of DM of T1 silage was significantly ($p<0.05$) lower (21.04%) than the T2 (24.04%) and T3 (22.96%) silages. It showed that DM content for T2 silage was the highest (24.04%) and T1 was the lowest (21.04%). The average DM content of three Napier grass silages treatments were ranged from 21.04% to 24.04%.

Dry matter is important determinant of intake and preservation values. The optimum DM for silage is 28-32% (Kung & Shaver, 2004). The high value of DM will consequently reduce the aerobic activity in the silage process. Based on the result, there were significant different ($p<0.05$) on DM content among the treatments. A study by Hong et al. (2004) reported that *Aspergillus awamori* shows an increasing DM content from 88.37% to 90.94% in fermented soybean and 89.64% to 91.21% in fermented soybean meal. Treated silage with

Aspergillus niger (T2) showed the higher DM content; 24.04%, followed by silage treated with *Aspergillus awamori* (T3); 22.96% and T1 which is untreated silage was the lowest with 21.04%. Somehow, the result in this study cannot be assumed as good silage since the DM percentage were lower than 28% for all treatments. These results might be contributed due to the several factors such as plant maturity, harvesting time and irrigation that may contribute high moisture in Napier grass.

4.2.1.2 Crude Protein

The CP contents of experimental Napier grass silages are shown in Table 4.2.1. Results showed that the CP contents of Napier grass silages were significantly ($p<0.05$) varied among the treatments. The mean values of CP content for T1, T2 and T3 silages were 9.16%, 11.42% and 10.21%, respectively. The results showed that different treatments had a significance effect ($p<0.05$) on CP content of Napier grass silages as the p-value was 0.006. The CP of T1 silage was significantly ($p<0.05$) lower (9.16%) than the T2 (11.42%) and T3 (10.21%) silages. However, no significant ($p>0.05$) differences were observed on the CP content between T1 (9.16%) and T3

(10.21%) treatments. The average CP of three Napier grass silages were ranged from 9.16% to 11.42%. Based on the CP content in Table 4.2.1, it showed that CP content for T2 silage, Napier grass treated with *Aspergillus niger* was the highest (11.42%) followed by T3 silage, Napier grass ensiled with *Aspergillus awamori* (10.21%) and T1 which untreated Napier grass was the lowest (9.16%).

The optimum value of CP ranges from 9-15% (Quality Grass Silage for Dairy and Beef Production Systems (n.d.). However, it can be varied for different types of silages. According to Zailan et al. (2018), they determined that the mean of crude CP was the highest in Dwarf (10.42 %) followed by Silver (9.61%) and least in both Red (8.80%) and Common (8.67%) Napier. The CP content of Common Napier increased significantly ($P \leq 0.05$) from 8.13% to 9.26% after ensiling process. Increase of CP content ($p > 0.05$) in Napier cultivars after ensiled could be accompanied by the populated anaerobic microorganism during ensiling process (Zakaria, 2011). Based on the results in this study, it was common Napier grass has been used. Hence, silage treated with *Aspergillus niger* (T2) contained significantly ($p < 0.05$) higher CP percentage than the control silage (T1) or silage treated with *Aspergillus awamori* (T3) and it could be assumed as good quality of silage. The results of this study were similar with the findings of Oboh et al. (2002). They used *Aspergillus niger* in cassava fermentation and concluded that *A. niger* has potential in protein increasement in cassava flour. Aydın et al. (2018) observed

that *Aspergillus niger* could enhance the CP content in grape seed through solid-state fermentation. Other than that, Li et al. (2019) reported that *Aspergillus awamori* significantly improved the CP content in soybean meal from 50.84% to 60.58%. Moreover, research by Webb & Wang (1997) mentioned that the addition of *Aspergillus awamori* also improved the CP content in wheat. However, CP content in Napier grass silage using *Aspergillus awamori* (T3) was increased numerically but it was statistically non-significant ($p>0.05$) in this study. Hence, further study is needed to understand the mechanism why *Aspergillus awamori* did not increase the CP content in Napier grass silage.

The CP requirements by ruminant animal to maintain their rumen environment continuation is must greater than 7% otherwise forage digestion will decrease (Harty & Olson, 2020) because of lack of nitrogen for microbial growth. This is in line with the result from this study. Hence, the silage can be assumed as good silage since the CP percentage of experimental Napier grass silage was between 9.16% to 11.42%.

4.2.1.3 Ether Extract

The EE contents of experimental Napier grass silages are shown in Table 4.2.1. Results showed that the EE contents of Napier grass silages were significantly ($p<0.05$) varied among the treatments. The mean values of EE for T1, T2 and T3 silages were 1.52%, 7.65% and 0.58% respectively. The results showed that different treatment had a significance effect ($p<0.05$) on EE content of Napier grass silage as the p-value was 0.000. The EE of T3 silage was significantly ($p<0.05$) lower (0.58%) than the T1 (1.52%) and T2 (7.65%) silages. However, no significant ($p>0.05$) differences were observed on the EE content between T1 (1.52%) and T3 (0.58%) silages. The average EE contents of three Napier grass silages were ranged from 0.58% to 7.65%. Based on the EE content in Table 4.2.1, it showed that CP content for T2 silage, Napier grass treated with *Aspergillus niger* was the highest (7.65%) followed by T1 silage which was untreated Napier grass (1.52%) and T3, Napier grass ensiled with *Aspergillus awamori* (0.58%) was the lowest.

The addition of *Aspergillus niger* (T2) affected the EE content in the silage. This is in line with the findings by Oboh et al. (2002). They concluded that fat content in cassava flour were significantly higher by the addition of

Aspergillus niger from 2.6% to 5.7%. Silage treated with *Aspergillus niger* (T2) showed significantly higher in EE content than silage treated with *Aspergillus awamori* and control silage. As mentioned by Hui et al. (2010), fungi have ability to produce microbial lipids in the substrate during fermentation. This is in line with the findings of Altop (2019) who reported that the EE content was increased significantly in olive leaves by the addition of *Aspergillus niger*. Somehow, the *Aspergillus awamori* (T3) showed the lowest EE content (0.58%). The result of this study is in line with findings of Li et al. (2019) who concluded that fermented soybean meal using *Aspergillus awamori* reduced the level of EE from 2.11% to 2.07%. In contrast, Hong et al. (2004) observed that fermented soybean using *Aspergillus awamori* enhance the EE content from 18.80% to 21.62%. These contradictory results might be contributed due to the several factors such as plant maturity, fertilizer application, weather, harvesting time, irrigation and etc. In this case, the mechanism of decreasing EE content in Napier grass silage using *Aspergillus awamori* was not clear. Therefore, further study is needed to understand the mechanism.

It is suggested the fat content in ruminant diet should not exceed more than 7% (Palmquist, 1994; Ruminants | Livestock Farming | Megalac, n.d.) otherwise DM intake may become depressed leading to reduce fibre intake and poor digestibility. This is in line with the result of this study. Therefore, it can

be concluded that the silage in this study is good silage since the EE content were between 0.58% to 7.65%.

4.2.1.4 Crude Fibre

The CF contents of experimental Napier grass silages are shown in Table 4.2.1. Results showed that the CF percentage of Napier grass silages were significantly ($p<0.05$) varied among the treatments. The average CF of three Napier grass silages were ranged from 23.29% to 29.00%. It showed that CF percentage for silage treated with *Aspergillus niger* (T2) was the highest and untreated silage (T1) was the lowest. The results showed that different treatment had a significance effect ($p<0.05$) on CF content of Napier grass silage as the p-value was 0.000. The CF contents for T1, T2 and T3 silages were 23.29%, 29.00% and 25.27%, respectively.

Based on the result, the CF percentage for all treatments for T1, T2 and T3 were 23.29%, 29.00% and 25.27%, respectively. The addition of *Aspergillus niger* and *Aspergillus awamori* affected the CF content in the silage. This result

is in line with findings of Okpako et al. (2008) and Güngör et al. (2017) who suggested that *Aspergillus niger* increased significantly in CF content in cassava peels and sour cherry kernels using solid state fermentation. In contradictory, a study by Altop (2019) concluded that content of CF in olive leaves were decreased after fermented with *Aspergillus niger*. Xie et al. (2016) reported that *Aspergillus niger* can generate cellulase in olive leaf. Hence, the decrease in CF may be due to this cellulase production. These contradictory results might be contributed due to the several factors such as plant maturity, harvesting time, irrigation, fertilizer application and etc.

4.2.1.5 Ash

The ash contents of experimental Napier grass silages are shown in Table 4.2.1. Results showed that there were no significant ($p>0.05$) differences on ash content in Napier grass silages among the treatments. The mean values of ash content for T1, T2 and T3 silages were 6.34%, 9.39% and 8.70% respectively. It showed that ash percentage for silage treated with *Aspergillus niger* (T2) was the highest and untreated silage (T1) was the lowest. No

significant ($p>0.05$) differences were observed on ash content in Napier grass silages among the treatments as the p-value was 0.083.

Ash content in silage approximately must have less than 10% (Lallemand Animal Nutrition, 2021). Based on the result in this study, the ash percentage for T1, T2 and T3 silages were 6.34%, 9.39% and 8.70% respectively. So, all treatments can be assumed as good quality of silage. The addition of *Aspergillus niger* and *Aspergillus awamori* affected the ash content in the silage. This is in line with the findings of Oboh et al. (2002) who concluded that *Aspergillus niger* has potential in protein increasement in terms of ash content from 2.1% to 4.5% in cassava flour. Furthermore, Aydın et al. (2018) observed that *Aspergillus niger* could enhance the ash content in grape seed from 3.88% to 8.84% through solid-state fermentation.

Table 4.2.1.5.1: Mineral Requirement Level for Lactating Cows.

Mineral	Lactating Cows	
	Units	
Calcium	%	0.28 – 0.58
Cobalt	ppm	0.10
Copper	ppm	10.00
Iodine	ppm	0.50
Iron	ppm	50.00
Magnesium	%	0.20
Manganese	ppm	40.00
Phosphorus	%	0.22 – 0.39
Potassium	%	0.60
Selenium	ppm	0.10
Sodium	%	0.10
Sulfur	%	0.15
Zinc	ppm	30.00

Data from National Research Council (NRC). Nutrient requirements of beef cattle. 7th (revised) edition. Washington (DC): National Academy Press; 1996. p. 54.

Reference: Olson (2007).

The practical minerals contents of diets for lactating cows are shown in Table 4.2.1.5.1. According to Olson (2007), the optimum mineral contents needed for lactating cow were calcium (0.28–0.58%) and phosphorus (0.22–0.39%). Deficiency or inadequacy of mineral elements may lead to lessened feed intake and production of milk. It is important to include adequate minerals in the diet. This is in line with the result of this study. Hence, the silage can be assumed as good silage since the ash percentage are between 6.34% to 9.39%.

4.2.1.6 Nitrogen Free Extract (NFE)

The NFE contents of experimental Napier grass silages are shown in Table 4.2.1. Results showed that the NFE percentage of Napier grass silages were significantly ($p<0.05$) varied among the treatments. The mean values of DM content for T1, T2 and T3 were 59.70%, 42.56% and 55.50% respectively. The results showed that different treatment had a significance effect ($p<0.05$) on NFE percentage of Napier grass silage as the p-value was 0.000. The percentage of NFE of T2 silage was significantly ($p<0.05$) lower (42.56%) than the T1 (59.70%) and T3 (55.50%) silages. It showed that NFE content for T1 silage was the highest (59.70%) and T2 was the lowest 42.56%). The average NFE

content of three Napier grass silages treatments were ranged from 42.56% to 59.70%.

According to Cherian (2019), NFE is the major element of the rations of animals feeding stuffs, representing 40-70% of the total DM. The NFE helps as source energy for body processing and fat deposition. As reported by Papagianni (2007), soluble carbohydrates are firstly preferred to other nutrients for carbon sources by fungi. Based on the result in this study, the NFE percentage for T1, T2 and T3 silages were 59.70%, 42.56% and 55.50% respectively. The addition of *Aspergillus niger* (T2) affected the NFE content in the silage. This is in line with the findings of Güngör et al. (2017) who reported that the NFE content was decreased significantly in sour cherry kernel from 38.30% to 18.05% after fermented with *Aspergillus niger*. Oboh (2006) reported, the decreasing mechanism in the NFE may be due to the degrading of sugars by the enzymes secreted by *Aspergillus niger* for use as a carbon source. In addition, Güngör et al. (2017) observed that nutritional composition of grape seed was improved by fermented with *Aspergillus niger* from 26.06% to 13.17%. Moreover, study by Altop (2019) concluded that solid state fermentation by *Aspergillus niger* cannot be affected NFE content of the olive leaves. He obtained the decreasing in NFE content from 53.58% to 53.13% in fermented olive leaves using *Aspergillus niger*. Hence, the decrease in NFE

may be due to the sugar reducing by the *Aspergillus niger* enzymes as energy source.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

Changes in fermentation characteristics and nutrient compositions of Napier grass silages are thus likely to be influenced by *Aspergillus sp*. The addition of *Aspergillus sp.* has affected the silage fermentation characteristics (pH value, lactic acid and NH_3–N contents) and nutritional compositions (DM, CP, EE, CF, ash and NFE contents) of Napier grass silage. The T2 silage showed higher CP and EE contents than the T3 or T1 silages.

Aspergillus niger provided enhancement in fermentation characteristics and nutrient compositions of Napier grass silages. However, the addition of *Aspergillus awamori* did not affect the CP and EE contents of the ensiled Napier grass. Based on

the results, the mechanism of decreasing EE content in Napier grass silage using *Aspergillus awamori* was not clear. The CP content in Napier grass silage using *Aspergillus awamori* was increased numerically but it was found statistically non-significant. Therefore, further study is needed to understand the mechanism.

The results indicated that by utilizing *Aspergillus niger* can be used efficiently to make silage with Napier grass. Moreover, more volume of silage and more replications are needed to determine the accurate results for silage fermentation characteristics and nutritional composition. Furthermore, to justify the results shown in this study, animal feeding trial should be performed to observe the animal performance. Thus, it is recommended to farmers to utilize the fungus since it provides better nutritional composition in Napier grass silage than Napier grass silage without treated fungus.

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APPENDICES

APPENDIX A

Data using One-Way ANOVA with SPSS

pH value

Descriptive

		N	Mean	Std. Deviation	Std. Error
pH value	Control	3	4.0833	0.01528	0.00882
	Aspergillus niger	3	4.1967	0.04509	0.02603
	Aspergillus awamori	3	4.1700	0.02000	0.01155
	Total	9	4.1500	0.05745	0.01915

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
pH value	Between Groups	0.021	2	0.011	11.850	0.008
	Within Groups	0.005	6	0.001		
	Total	0.026	8			

Homogeneous Subsets

pH value

Duncan[a]

Treatment	N	Subset for alpha = 0.05	
		1	2
Control	3	4.0833	
Aspergillus awamori	3		4.1700
Aspergillus niger	3		4.1967
Sig.		1.000	0.315

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Lactic Acid Content

Descriptive

		N	Mean	Std. Deviation	Std. Error
Lactic acid	Control	3	5.7667	0.49692	0.28690
	Aspergillus niger	3	7.1600	1.21873	0.70363
	Aspergillus awamori	3	5.7533	0.64010	0.36956
	Total	9	6.2267	1.01268	0.33756

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Lactic acid	Between Groups	3.920	2	1.960	2.745	0.142
	Within Groups	4.284	6	0.714		
	Total	8.204	8			

Homogeneous Subsets

Lactic acid

Duncan[a]

Treatment	N	Subset for alpha = 0.05 1
Aspergillus awamori	3	5.7533
Control	3	5.7667
Aspergillus niger	3	7.1600
Sig.		0.097

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Ammonia Nitrogen Content

Descriptive

		N	Mean	Std. Deviation	Std. Error
Ammonia nitrogen	Control	3	1.4633	0.11372	0.06566
	Aspergillus niger	3	1.8233	0.08327	0.04807
	Aspergillus awamori	3	1.6367	0.04509	0.02603
	Total	9	1.6411	0.17259	0.05753

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Ammonia nitrogen	Between Groups	0.194	2	0.097	13.321	0.006
	Within Groups	0.044	6	0.007		
	Total	0.238	8			

Homogeneous Subsets

Ammonia nitrogen

Duncan[a]

		Subset for alpha = 0.05		
Treatment	N	1	2	3
Control	3	1.4633		
Aspergillus awamori	3		1.6367	
Aspergillus niger	3			1.8233
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Dry Matter Content

Descriptive

		N	Mean	Std. Deviation	Std. Error
Dry matter	Control	3	21.0400	0.59733	0.34487
	Aspergillus niger	3	24.0433	0.54976	0.31740
	Aspergillus awamori	3	22.9633	0.45764	0.26422
	Total	9	22.6822	1.39743	0.46581

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Dry matter	Between Groups	13.886	2	6.943	23.983	0.001
	Within Groups	1.737	6	0.289		
	Total	15.623	8			

Homogeneous Subsets

Dry matter

Duncan[a]

		Subset for alpha = 0.05		
Treatment	N	1	2	3
Control	3	21.0400		
Aspergillus awamori	3		22.9633	
Aspergillus niger	3			24.0433
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Crude Protein Content

Descriptive

		N	Mean	Std. Deviation	Std. Error
Crude protein	Control	3	9.1600	0.71582	0.41328
	Aspergillus niger	3	11.4167	0.51481	0.29723
	Aspergillus awamori	3	10.2100	0.27055	0.15620
	Total	9	10.2622	1.08122	0.36041

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Crude protein	Between Groups	7.651	2	3.826	13.492	0.006
	Within Groups	1.701	6	0.284		
	Total	9.352	8			

Homogeneous Subsets

Crude protein

Duncan[a]

		Subset for alpha = 0.05	
Treatment	N	1	2
Control	3	9.1600	
Aspergillus awamori	3	10.2100	
Aspergillus niger	3		11.4167
Sig.		0.052	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Ether Extract Content

Descriptive

		N	Mean	Std. Deviation	Std. Error
Ether extract	Control	3	1.5200	0.59808	0.34530
	Aspergillus niger	3	7.6500	1.41743	0.81835
	Aspergillus awamori	3	0.5767	0.29771	0.17188
	Total	9	3.2489	3.41705	1.13902

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Ether extract	Between Groups	88.499	2	44.249	54.063	0.000
	Within Groups	4.911	6	0.818		
	Total	93.410	8			

Homogeneous Subsets

Ether extract

Duncan[a]

		Subset for alpha = 0.05	
Treatment	N	1	2
Aspergillus awamori	3	0.5767	
Control	3	1.5200	
Aspergillus niger	3		7.6500
Sig.		0.249	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Crude Fibre Content

Descriptive

		N	Mean	Std. Deviation	Std. Error
Crude fibre	Control	3	23.2867	0.60871	0.35144
	Aspergillus niger	3	28.9800	0.36592	0.21127
	Aspergillus awamori	3	25.2667	0.55591	0.32095
	Total	9	25.8444	2.54338	0.84779

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Crude fibre	Between Groups	50.123	2	25.062	92.425	0.000
	Within Groups	1.627	6	0.271		
	Total	51.750	8			

Homogeneous Subsets

Crude fibre

Duncan[a]

		Subset for alpha = 0.05		
Treatment	N	1	2	3
Control	3	23.2867		
Aspergillus awamori	3		25.2667	
Aspergillus niger	3			28.9800
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Ash Content

Descriptive

		N	Mean	Std. Deviation	Std. Error
Ash	Control	3	6.3433	0.90456	0.52225
	Aspergillus niger	3	9.3900	1.79424	1.03591
	Aspergillus awamori	3	8.6967	1.36606	0.78870
	Total	9	8.1433	1.84080	0.61360

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Ash	Between Groups	15.301	2	7.651	3.888	0.083
	Within Groups	11.807	6	1.968		
	Total	27.108	8			

Homogeneous Subsets

Ash

Duncan[a]

		Subset for alpha = 0.05	
Treatment	N	1	2
Control	3	6.3433	
Aspergillus awamori	3	8.6967	8.6967
Aspergillus niger	3		9.3900
Sig.		0.086	0.567

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Nitrogen Free Extract Content

Descriptive

		N	Mean	Std. Deviation	Std. Error
NFE	Control	3	59.6900	2.26046	1.30508
	Aspergillus niger	3	42.5633	3.02460	1.74626
	Aspergillus awamori	3	55.2500	1.33989	0.77358
	Total	9	52.5011	7.95373	2.65124

ANOVA

		Sum of Squares	df	Mean Square	F	Sig
NFE	Between Groups	473.988	2	236.994	44.289	0.000
	Within Groups	32.106	6	5.351		
	Total	506.094	8			

Homogeneous Subsets

NFE

Duncan[a]

		Subset for alpha = 0.05	
Treatment	N	1	2
Aspergillus niger	3	42.5633	
Aspergillus awamori	3		55.2500
Control	3		59.6900
Sig.		1.000	0.057

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

APPENDIX B

Figure B (1): Potato Dextrose Agar (PDA) preparation.

Figure B (2): *Aspergillus niger* derived from ATCC® 6275™.

Packing: KWIK-STIK 2 Pack/kit

Cat. No: 0500P

Figure B (3): *Aspergillus niger and Aspergillus awamori* plate culture.

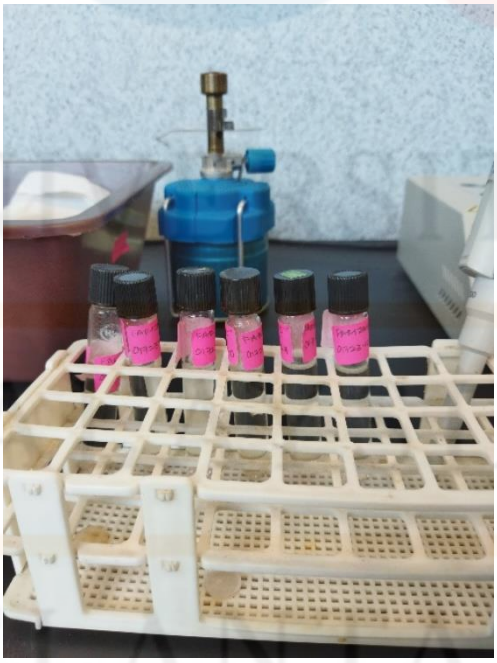

Figure B (4): Serial dilution of *Aspergillus niger* and *Aspergillus awamori.*

Figure B (5): Spore count using hemocytometer.

Figure B (6): Silage making.

Figure B (7): pH value analysis.

Figure B (8): Lactic acid content analysis.

Figure B (9): Dry matter analysis.

Figure B (10): Crude protein analysis.

Figure B (11): Ether extract analysis.

Figure B (12): Crude fibre analysis.

Figure B (13): Ash analysis.