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Application of *Lactobacillus* inoculant from various rice paddy (*Oryza sativa*) to total mixed ration silage microbial composition

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Abstract. *Wahyudi A, Hendraningsih L, Sutawi S, Siskawardani DD. 2022. Application of* Lactobacillus *inoculant from various rice paddy (*Oryza sativa*) to total mixed ration silage microbial composition. Biodiversitas 23: xxxx.* Evaluation of microorganisms profile of total mixed ration (TMR) silage inoculated with lactic acid bacteria (LAB) from local rice paddy (*Oryza sativa* L.) in tropical climate was analyzed to solve the feed management system. This study aimed to compare the effect of local LAB inoculants with commercial strain *Lactobacillus plantarum* FCC 123 on microorganisms composition of TMR silage in small-scale silage preparation. LAB was isolated from whole crop rice (w) and rice straw (s). Local rice paddy was planted in field around the University of Muhammadiyah Malang, East Java Province, Indonesia. Four rice paddy varieties: Membramo (M), Ciherang (C), Rajalele (R), and Impari (I) were utilized for isolation of LAB. Then, LAB was selected and purified in lactobacilli deMan Rogosa sharp agar (MRSA) and partially stored in dimethyl sulfoxide (DMSO) at -30°C as microbial stock. These selected LAB was used as inoculants at room temperature for 30 days TMR silage incubation. Completely randomized design (CRD) with 6 treatments (T0: TMR without fermentation, T1: TMR silage without inoculants, T2: TMR silage with *L. plantarum* FCC 123, T3: TMR with Local LAB Cs, T4: TMR silage with local LAB Mw, T5: TMR silage with local LAB Rs) were applied. Microorganism composition of LAB, aerobic bacteria, coliforms, clostridia, and mold was measured as experiment parameters. This research showed that LAB could be isolated and identified from all local paddy rice, both whole crop and straw. The implementation of local LAB on ensilage TMR was not significantly different from commercial *L. plantarum* FCC 123. Both local and commercial strains increased LAB population and suppressed harmful microbes (coliform, aerobic bacteria, and mold). The LAB isolates from local paddy rice could well act as an inoculant for TMR silage preparation in tropical climate.

Keywords: Ciherang, *Clostridia*, *Coliform*, lactic acid bacteria, microorganism, mold, TMR silage

INTRODUCTION

Forage availability is abundant in rainy season, but it is very limited during dry season in Indonesia. Development of food industry produced a various types of by-products which have potential as environmental pollutants problem. Moreover, the by-products also had great potency as sources of new feedstuff. The most important objective of ensiling is to preserve forage accessibility for a whole year without any damages, thus improving the environmental sustainability of silage production (Soundharrajan et al. 2021). According to Ratnakomala et al. (2006), one way to overcome the shortage of forage during the rainy and dry seasons can be done by ensiling. Scientific opinion of the panel on Biological Hazards (2008) identified the issue of feed supply, ruminant livestock in wet tropical climates had a problem with quality, including contaminants exposure. Contaminants commonly found in the feedstuffs were pathogenic microorganisms that produce various toxic compounds such as coliform, clostridia, and fungi (Queiroz et al. 2018; Munoz et al. 2021). Therefore, feed management system is crucial to maintain the supply throughout the year. Several previous studies showed that in addition to preserving feed, using lactic acid bacteria (LAB) in silage preparation accelerates the process and ensures microbial detoxification of pathogens (Soundharrajan et al. 2021; Santos et al. 2013a). Some studies of silage preparation in sub-tropic climates showed that in addition to preserving feed, the use of LAB inoculants was able to improve silage quality (Yang et al. 2010; Huyen et al. 2020). Commercial lactic acid bacterial strains were used as inoculants in silage making more than 20 years ago (Muck et al. 2018; Puntillo et al. 2020), and *Lactobacillus plantarum* was a strain that was mostly used as bioinoculant for making silage (Oliveira et al. 2017).

Presently, one of the main challenges in the inoculants industry is the extent of variability in the effects of inoculant bacteria on the fermentation and preservation of silage, silage quality, and animal performance (Ávila and Carvalho 2020). Some LAB species were also selected as silage inoculants due to faster growth at high pH values (>5) compared to *L. plantarum* (Puntillo et al. 2020). The condition showed that not all commercial LAB strains were definitely suitable for use on all different substrates, as example, epiphytic LAB of corn plant might suitable for corn silage (Fabiszewska et al. 2019). Soundharrajan et al.

(2021) described that microflora of silages prepared from maize showed the most toxic molds such as *Arthrinium phaeospermum, Byssochlamys* sp., *Monascus ruber, Fusarium* sp., and *Penicillium* sp. The lack of inoculant may be related to ability of the LAB to grow rapidly in the forage mass and effectively compete with the epiphytic flora as well as the presence of adequate substrate, and it might also be related to specificities between the forage, the microorganisms present in the inoculant and the weather conditions (Agarussi et al. 2019). Muck et al. (2018) reported that the major international companies producing inoculants were based in Europe and North America. Therefore, these products had been developed for sub-tropic grasses, whole-crop corn, and alfalfa. The inoculants production might not be effectively utilized on tropical grasses or legumes, due to the environmental condition influencing the physiology and metabolism of the inoculated strains which affected the fermentation process (Agarussi et al. 2019). Recently, a heterofermentative LAB inoculant species, *Lactobacillus buchneri*, has become available commercially and produces high concentrations of acetic acid in silage that inhibit fungi and thus preserve silages susceptible to spoilage upon exposure to air (Weiss et al. 2016).

In various previous studies, the usage of local LAB in tropical condition could improve organic acid content and maintain the nutrient composition of TMR silage (Wahyudi et al. 2017; Oliveira et al. 2017; Muck et al. 2018). Another big challenge in silage preservation in the tropic is microbial contamination related to high temperature and humidity. Research related to feed microbial contamination is relatively rarely explored and has not received serious attention in the tropic. Further study is needed to analyze local LAB inoculant's role in inhibiting harmful microorganisms' growth on TMR in tropics.

Based on microbiological perspective, it is limited information available regarding the LAB profile of the enormous resource of rice paddy in tropical climate. Selected LAB from local rice paddy could be evaluated for the ability to improve rice straw containing TMR silage quality. Therefore, the purpose of the present study was to isolate and identify morphologically a tropical LAB that colonized rice paddy for ensiling TMR based on rice straw material. LAB was isolated from inhabited by several local rice paddy varieties and its straw. The ability to reduce fungi, coliform, and clostridia were compared with a commercial LAB i.e. *L. plantarum* FCC 123, which was commonly used as silage inoculant.

MATERIALS AND METHODS

Material Preparation

Whole crop rice (WCR) and rice straw were obtained from paddy field around Malang regency, East Java Province, Indonesia which had a temperature of 25-28°C during the day 21-23°C at night; and and relative humidity of61-83% and 83-88% respectively. Both WCR and rice straw have been cut at 10 cm over the ground. It was packed into plastic bags without sealing and chopped as soon as possible. Four varieties of rice paddy used as local LAB sources consisted of Membramo, Ciherang, Rajalele, and Impari 13. A commercial LAB (*L. plantarum* FCC 123) was used in silage production. *Lactobacillus plantarum* FCC 123 was obtained from culture collection Laboratory of PAU-Pangan Gizi Universitas Gajah Mada, Yogyakarta, Indonesia.

Isolation and morphological test

Isolation of LAB from several varieties of local rice paddy were conducted (Ni et al. 2015). Selection and purification of isolates were done onto deMan Rogosa Sharp agar medium *(*MRS; Difco Laboratories, Detroit*)*. After 24 h of incubation on MRS agar, Gram staining, catalase activity, and gas production from glucose of LAB were examined. Morphological colony and cell forming were identified under microscopic observation. Growth of LAB and pH value was observed in MRS broth after incubation at 30°C for 48 h. Each bacterial colony was purified twice by streaking on MRS agar plates. The purified bacterial strain was stored at -30°C in nutrient broth and dimethyl sulfoxide (DMSO) in ratio of 9:1 as microbial stock for further analysis. Pure bacterial strains were tested for effectiveness in the *in vitro* TMR silage preparation.

Total mixed ration silage preparation

Total mixed ration silages were prepared in a small-scale system of fermentation (Wahyudi et al. 2017). Approximately 100g portion of TMR material was packed into plastic film bags and sealed with a vacuum sealer machine (KRIS VS200 type, 22×500 cm). Dry matter (DM) content of TMR material was 64.77%, then it was adjusted to 45% for silage preparation. The bag silos were stored at room temperature (average 26°C) for 30 days of fermentation. Compositions of TMR were 16% rice straw, 20% rice bran, 34% cassava chip by-product, 6% molasses, 17.5% soybean meal, 4.5% fish meal, and 3% minerals. The composition was arranged consisted of 14% crude protein (CP) and 70% total digestible nutrients (TDN), and 2,000 k.cal metabolism energy (ME). The TMR was treated with *L. plantarum* FNCC 123 (10^{10}-10^{11} CFU/mL), local LAB Cs (Ciherang rice straw), local LAB Mw (Membramo whole crop rice), and local LAB Rs (Rajalele rice straw), and control.

Microbial analysis

Samples (10g) were blended with 90 mL of sterilized water and serially diluted in 10^{-1} to 10^{-5} in sterilized water. The total lactic acid bacteria (LAB have measured with plate count on lactobacilli deMan Rogosa Sharp agar (MRS; Difco) incubated at 30°C for 2 days under anaerobic condition in anaerobic jar. Coliform bacteria were counted on blue light broth agar (BLB; Nissui Ltd.) incubated at 30°C for 48 h. While, mold and yeast were counted on potato dextrose agar (PDA; P2182 Sigma-Aldrich) and incubated at 30°C for 24 h. Then, yeast was distinguished from molds or bacteria by colony appearance under microscopic observation. Bacilli were distinguished from aerobic bacteria by colony shape on incubation at 75°C for 15 min before incubation on nutrient agar at 30°C for 24 h

in aerobic condition. Clostridia were counted on Reinforced Clostridial Agar (RCA M154; Himedia) after incubating at an anaerobic box at 35-37°C for 40 to 48h. Moreover, colonies were counted as viable numbers of microorganisms in colony-forming unit per gram of fresh matter (FM) (Wang et al. 2022).

Research design

Completely randomized design (CRD) with 6 treatments and 3 replications was applied. The treatments consisted of T0: TMR with no fermentation, T1: TMR silage without inoculants, T2: TMR silage with *L. plantarum* FCC 123, T3: TMR silage with Local LAB Cs, T4: TMR silage with local LAB Mw, T5: TMR silage with local LAB Rs. The experiment parameters were evaluated with descriptive quantitative analysis.

RESULTS AND DISCUSSION

Isolation

Isolation of LAB on selective media from 4 rice paddy varieties both whole crop rice (WCR) and rice straw (RS) stems were obtained (Table 1). In general, the LAB could be isolated from all local rice paddy varieties from both WCR and RS. The optimal LAB count was obtained at 10^{-3} and 10^{-4} media dilution rates, whereas at dilutions of less than 10^{-3} or more than 10^{-4} produced undetected and uncalculated number of colonies. The data proved that LAB was an epiphytic microbe of a rice paddy in tropic. Furthermore, RS stem resulted more isolates than WCR.

Rice straws in Japan had less epiphytic LAB. The epiphytic LAB population on rice straw in China was less than 1.0×10^5 CFU/g FM, the number of aerobic bacteria was higher than that for molds and yeasts, which were more than 1.0×10^6CFU/g FM (Zhao et al. 2019). Natural populations of LAB in rice paddy were often heterofermentative and low in number (Ni et al. 2015). Recently, a heterofermentative LAB inoculant species, *L. buchneri* had become available commercially to produce high concentrations of acetic acid in silage to inhibit fungi and preserve silages susceptible to spoilage upon exposure to air (Pinto et al. 2020; Shah et al. 2020a; Shah et al. 2020b). Muck et al. (2018) reported that heterofermentative LAB species such as *L. brevis* and *L. buchneri* had been developed as silage additives to prevent aerobic deterioration of silage. A previous report had also suggested *L. buchneri* could strongly produce antimicrobial compounds (salicylic acid, benzoic acid, 3-phenyl lactic acid, catechol, 4 hydroxybenzoic acid, and hydrocinnamic acid) during silage fermentation (Guan et al. 2020). Thus, it's possible to develop the local LAB isolates from both WCR and RS as inoculants for silage preparation in tropical condition. Bernardes et al. (2017) stated that the higher ensiling temperatures typically lead to a shift from a homolactic to a heterolactic microbial population.

Morphological test

The criteria to use the LAB as an inoculant in TMR silage should be included rod-shaped, Gram (+)ve, low pH, and homofermentative. All strains were positive, homofermentative (Shah et al. 2017a). Some cell forming have been observed, including coccus, oval (coccus), and rod. The shape of the following strains, *HDASK* and *SK2A32*, are rod shapes and *SK3907, SK3A42* and *ASKDD* are Cocci shapes (Shah et al. 2017a.). The result indicated that the LAB type of WCR and RS were Gram(+)ve, heterofermentative and homofermentative (Table 2). A combination of numerous strains had been used as microbial inoculum. The production also varies using potential groups of *L. plantarum, Pediococcus acidilactici*, and *P. pentosaceus* (Reich and Kung 2010). *L. buchneri* is considered the best silage additive. These bacteria is able to improve aerobic stability, and synthesize antifungal components. Blajman et al. (2020) suggested that heterofermentative and homofermentative bacteria are powerful inoculants for improving silage quality, reducing contamination, and enhancing forage conservation for livestock.

Table 1. Isolates and number of lactic acid bacteria (CFU/g FM)

Isolation source		Dilution factors				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Whole crop rice	Membramo (M)	629	48	1	1	-
	Ciherang (C)	486	278	83	1	-
	Rajalele (R)	147	8	5	3	-
	Impari (I)	519	8	5	4	-
Rice straw	Membramo (M)	NC	NC	12	3	-
	Ciherang (C)	NC	104	52	13	-
	Rajalele (R)	NC	96	15	2	-
	Impari (I)	NC	NC	42	4	-

Table 2. Phenotypic characteristics of LAB, isolated from local rice paddy

Isolates	Shape	Stain type	Gas from glucose	pH	Fermentation type
$Mw1^{-3}$	Coccus	+	+	5.14	Heterofermentative
$Mw2^{-2}$	Coccus	+	+	5.12	Heterofermentative
$Mw1^{-3}$	Coccus	+	+	5.15	Heterofermentative
$Mw2^{-3}$	Coccus	+	+	5.33	Heterofermentative
$Mw3^{-3}$	Rod	+	-	3.99	Homofermentative
$Ms4^{-3}$	Coccus	+	+	5.40	Heterofermentative
$Cw1^{-3}$	Coccus	+	+	5.30	Heterofermentative
$Cw1^{-4}$	Coccus	+	+	4.99	Heterofermentative
$Cw2^{-3}$	Coccus	-	+	4.92	Heterofermentative
$Cs3^{-4}$	Coccus	+	+	4.92	Heterofermentative
$Cs4^{-4}$	Coccus	+	+	5.29	Heterofermentative
$Cs1^{-4}$	Rod	+	-	3.97	Homofermentative
$Rw2^{-3}$	Coccus	-	+	5.32	Heterofermentative
$Rw3^{-5}$	Coccus	-	+	5.34	Heterofermentative
$Rw1^{-5}$	Coccus	-	+	5.11	Heterofermentative
$Rs3^{-3}$	Coccus	+	+	5.14	Heterofermentative
$Rs1^{-4}$	Coccus	+	+	5.40	Heterofermentative
$Rs4^{-3}$	Coccus	+	+	5.25	Heterofermentative
$Rs2^{-4}$	Rod	+	-	3.93	Homofermentative
$Is1^{-3}$	Coccus	+	+	5.13	Heterofermentative

Mw: Membramo whole crop rice; Ms: Membramo straw; Cw: Ciherang whole crop rice; Cs: Ciherang straw; Rw: Rajalele whole crop rice; Rs: Rajalele straw; Is: Impari straw

In the present study, homofermentative types were selected as inoculants because it had a better ability to produce lactic acid. The encouraging effects of homofermentative microbes that were used in silage for improving the synthesis of metabolites and reducing dry matter losses (DML) (Kim et al. 2021). Lactic acid production was positively correlated with pH values, and almost 90% of products were resulted by LAB (Xu et al. 2017). Selection based on the morphological colonies and cells had obtained three bacterial isolates that had the potential as inoculants in TMR silage. One isolate, from Ciherang straw (Cs), one from Membramo WCR (Mw), and one from Rajalele straw (Rs). In the present study, there was inverted correlation between the number of isolates that have been obtained and the potential of LAB as inoculants. LAB which was isolated from Impari 13 did not show great potential as silage inoculants.

Ni et al. (2015) described that LAB strains was isolated from forage rice paddy silage were heterofermentative and the other strains showed homofermentative. Both homo- and hetero- LAB influenced the aerobic stability of forage, media composition, nutrition, and reducing undesirable bacteria (Blajman et al. 2020). Almost of the isolates were Gram-positive and catalase-negative rods or cocci. Yang et al. (2016) identified the isolates with Gram-positive and catalase-negative rods or cocci, produced lactic acid from glucose were LAB species. In the present study, all of isolates produced pH value from 3.93 to 5.40. The pH obtained from rice paddy silage, the range of values 3.5 - 7.0 (Ni et al. 2015). Furthermore, Ni et al. (2014) described, if isolated LAB strains unable to grow at 5°C and 50°C or pH 3, the strains were heterofermentative, and conversely if it could grow at pH less than 4 resulted homofermentative strains. Heterofermentative LAB used water-soluble carbohydrate (WSC) produced not only lactic acid but also acetic or propionic acid, ethanol, mannitol, and other compounds, whereas homofermentative LAB produced 90% lactic acid of all fermented products without energy loss (Alhaag et al. 2019).

Microbial composition of TMR silage

Silage is a forage plant utilized as feed preservation to maintain animal feed availability and quality. The main factors of silage damage were caused by unexpected microbes such as Coliform, aerobic bacteria, *Bacillus*, *Clostridia*, and molds (Wambacq et al. 2016; Santos et al. 2016; Santos et al. 2013b; Soundharrajan et al. 2021). In the present study, dry matter (DM) condition decreased from 64.77% in TMR become 43.34, 41.31, 42.93, 42.53, and 42.87% after incubation by LP, Cs, Mw, and Rs respectively in room temperature of tropical condition (Table 3). The pH value decreased from 6.83 in TMR become 5.08, 4.28, 4.55, 4.40, 4.63. The data showed that local LAB inoculants Cs, Mw, and Rs were significantly able to depress pH values equivalent to commercial LAB.

As observed in the present study, the effect of different LAB inoculates in King grass on different ensiling days, pH, acetic acid (AA) and butyric acid (BA) significantly ($P<0.05$) decreased (Shah et al. 2020c). Similar to present findings, Kim et al. (2015) and Yuan et al. (2015) reported that the addition of LAB, significantly ($P<0.05$) decreased pH, acetic acid, and butyric acid and the silage DM content was unaffected by bacterial application. As observed in this study, previous study on elephant grass (Shah et al. 2017b) and King grass (Shah et al. 2017c) identified that pH value and aerobic bacteria of control was higher than with LAB inoculants. Therefore, the adding LAB could improve the quality of product. According to Queiroz et al. (2018) some of the pathogenic bacteria that were frequently or occasionally associated with silage were *Enterobacteria*, *Listeria*, *Bacillus* spp., *Clostridium* spp., and *Salmonella*. The ensiling process in feed was linked to the fermentation of sugars that lead to a decrease in pH and the growth of undesirable microorganisms, such as yeasts and molds (Zhang et al. 2019; Shah et al. 2020a). Yeasts and filamentous mold were the main microorganisms involved in the aerobic deterioration process of silages (Santos et al. 2013b). When selecting a potent LAB for silage production that LAB should have characteristics of rapid growth (high cell density at ensiled silages), higher production of lactic acid with a marginal level of acetic acid, potent inhibition of pathogens (bacteria, yeast, and toxic metabolite producing mold), rapid reduction in pH, increased aerobic stability and dry matter content (DM), and long-term preservation with rich nutrients (Carvalho et al. 2021; Soundharrajan et al. 2017; Shah et al. 2021).

Table 3. Microbial Composition of TMR Silage

Item	TMR non fermentation	TMR non Inoculant	TMR with LAB inoculants			
			LP	Cs	Mw	Rs
pH	6.83±0.57	5.08±0.47	4.28±0.28	4.55±0.06	4.40±0.14	4.63±0.53
LAB (CFU/g FM)	1.87×10^4	3.4×10^5	5.1×10^5	4.9×10^5	4.7×10^5	4.8×10^5
Coliform (CFU/g FM)	1.74×10^4	7.6×10^4	1.63×10^3	2.46×10^3	2.05×10^3	2.14×10^3
Aerobic bacteria (CFU/g FM)	2.35×10^6	2.30×10^5	3.7×10^4	5.4×10^4	2.30×10^4	4.7×10^4
Bacilli (CFU/g FM)	2.89×10^4	2.05×10^4	1.35×10^4	1.48×10^4	1.35×10^4	1.29×10^4
Clostridia (CFU/g FM)	ND	ND	ND	ND	ND	ND
Molds (CFU/g FM)	2.19×10^6	2.35×10^5	4.6×10^4	4.2×10^4	2.19×10^4	4.7×10^5

Note: FM: fresh matter; LP: *Lactobacillus plantarum*; Cs: Ciherang straw; Mw: Membramo whole crop rice; Rs: Rajalele straw; ND: not detected

Lactic acid bacteria. Ensiling process in 30 days of incubation tends to increase LAB population. The LAB population was only 1.87×10^4 CFU/g FM in TMR without fermentation and elevated become 3.4×10^5 CFU/g FM after ensiling. This data showed that LAB was a bacterium inhabiting plant materials. Ensiling process grew up the LAB population in TMR silage. The essence of silages was LAB multiply in the anaerobic environment and produce lactic acid when the pH value lessen (Carvalho et al. 2021). The low pH restrained the growth of other infectious microbes (Hu et al. 2018). LAB population in TMR silage non-inoculant was lower than all fermented TMR with LAB inoculants LP, Cs, Mw, and Rs, respectively. This study strengthened that bacterial inoculants for silages could improve the feed quality. There were non-significant differences among LAB inoculants among treatments. The LAB population with commercial inoculant *L. plantarum* was 5.1×10^5 CFU/g FM then followed by local LAB Cs, Rs, and Mw inoculants that have populations of 4.9×10^5, 4.7×10^5, and 4.8×10^5 CFU/g FM respectively. The numbers of LAB in whole crop wheat silages in 30 days of fermentation was 1.25×10^4-5.1×10^5 CFU/g FM (Ni et al. 2015). Fabiszewska et al. (2019) defined that the most commercially available inoculants contain homofermentative LABs, which were fast and efficient to produce lactic acid, thus improving the silage fermentation while producing volatile fatty acids (e.g., acetic acid), heterofermentative species were included in silage starter cultures. According to Ellis et al. (2016), the most commonly used strains of LAB were *L. plantarum* and other Lactobacilli, and Enterococci (*E. faecium*) and some Pediococci. Homofermentatives LAB tended to be unable to maintain silage quality in aerobic conditions (Borreani et al. 2018; Wilkinson and Davies 2013; Peng et al. 2021). Some studies showed that although homofermentative LAB was more beneficial in anaerobic conditions, heterofermentative LAB actually increased the stability of silage quality in aerobic conditions (Muck et al. 2018; Peng et al. 2021). The present study proved that local LAB from local rice paddy was closest to *L. plantarum's* ability to encourage LAB populations in TMR silage. If LAB population was not available in silo, the silage condition could not suppress undesirable microbial growth, such as coliform, aerobic bacteria, clostridia, fungi, and resulting in poor silage quality. LAB promoted a rapid fermentation producing mainly lactic acid and decreased the pH to 4 rapidly, preventing further breakdown of the sugar and protein in the crop by other microorganisms (Yitbarek and Tamir 2014).

Coliform. Number of coliform in TMR without inoculants showed the highest amount, it was 7.6×10^4 CFU/g FM. The used of inoculants could reduce coliform population becomes 1.63×10^3, 2.46×10^3, 2.05×10^3, and 2.14×10^3 CFU /g FM respectively. The population coliform in TMR without fermentation was lower (1.74×104CFU/g FM) than in TMR silage without inoculant because TMR without fermentation contains more high dry matter. The dry matter content of TMR was 64.77%, whereas the TMR silages were 41.31- 43.34%. Bueno et al. (2020) stated that most TMR silages had been produced with DM content between 400 and 650 $g.kg^{-1}$ as fed, it was equivalent to 20-50% DM content. Coliform was enterobacteria, gram-negative, facultative anaerobic bacteria, some species could use nitrate as an electron acceptor in place of oxygen (Queiroz et al. 2018). Coliforms have been used as indicator organisms for nearly a century, first in evaluating water for fecal contamination and later in identifying unsanitary conditions in pasteurized dairy products and other foods (Martin et al. 2016). The presence of coliform in silage generally came from a slurry, manure, or soil. At the beginning of fermentation, coliform would catalyze water soluble carbohydrates (WSC) into acetic acid, ethanol, CO_2, and ammonia, causing energy loss (Xianjun et al. 2015). The coliform was eliminated within 3 d of ensiling with or without silage inoculation when the pH dropped below 4.0. In a similar study, *Escherichia coli* O157:H7 was eliminated from ensiled, artificially contaminated wheat and corn forages when the pH dropped below 4.0. A similar result was observed for *E. coli* O26, a different pathogenic strain of *E. coli*, in corn silages (Queiroz et al. 2018). The elimination of this pathogen was probably due to the low inhibitory pH, the enhanced antimicrobial activities of organic acids at low pH, or both (Zhang et al. 2021). In this present study, the pH value of TMR silage was more than 4, resulted the elimination process of coliform did not work well.

Aerobic bacteria. The population reduced from 2.35×10^6 become 2.30×10^5, 3.7×10^4, 5.4×10^4, 2.30×10^4, and 4.7×10^4 CFU/g FM after ensiling respectively. However, the population was still high. Total aerobic bacteria on plant prior to ensiling was more than 10^7 (Puntillo et al. 2020). After ensiling microbial composition was controlled by a combination of an anaerobic environment and a natural fermentation of sugars by lactic acid bacteria. In the present study, pH value could not decrease below 4. This indicated that aerobic bacteria were grown in silo. Aerobic bacteria use ethanol and produce acetic acid; if ethanol had been exhausted, it could grow on acetic acid, producing carbon dioxide and water. This would raise pH and permit other aerobic microorganisms to grow (Jin and Kirk 2018).

Bacilli. Number of TMR bacilli significantly reduced from 2.89×10^4 to 2.05×10^4 CFU/g FM after ensiling. Furthermore, the LAB inoculant utilization reduced the amount become and 1.35×10^4, 1.48×10^4, 1.35×10^4, and 1.29×10^4 CFU/g FM with administration of LP, Cs, Mw, and Rs, respectively. The facultative anaerobic bacilli used different sugars, which converted to organic acids, ethanol, 2,3-butanediol, and glycerol. Bacilli activity under anaerobic conditions was considered become relatively rare importance, in advancing the spoilage of silage when exposed to oxygen was more significant activity. Bacilli were one of the first groups of microorganisms to develop in silages after the aerobic spoilage process was initiated by yeasts. An earthy odor in silages was a sign of bacillus growth, and the silage likely presents a high pH (Kung et al. 2018).

Clostridia. In this study, clostridia were not detected (nd) for TMR and all TMR treatments. Clostridia was obligate anaerobic, affected on silage quality usually occur

long after the lactic acid bacteria had stopped actively growing in the silo. The presence of clostridia was an indicator of poor silage, therefore these TMR in this study were well prepared. Clostridial silage would reduce feed intake by livestock. Furthermore, Kung et al. (2018) and Li et al. (2020) stated there were two groups by the principal substrate fermented: *Saccharolytic clostridia* (*Clostridium tyrobutyricum* and *C. butyricum*) were usually found in silage and utilized soluble sugars or organic (lactic) acids to produce acetic acid and butyric acid, which had a strong, foul rancid-butter smell, and other species of clostridia (*C. sporogenes*) fermented both carbohydrates and proteins, converted to ammonia and amines matter. The excessive proteolysis gave a putrid, fishy or ammonia-like odor. Clostridial silages often had a slimy, olive-green appearance, and these silages had a low level of energy and high soluble protein, so feed intake would be low (Kung et al. 2018).

Molds. TMR non-fermentation contains 2.19×10^6 CFU/g FM of mold, whereas TMR silage without inoculants contain 2.35×10^5 CFU/g FM. The usage of inoculant both commercial or local LAB reduced mold content become 4.6×10^4, 4.2×10^5, 2.19×10^4, and 4.7×10^4 CFU/g FM respectively. Kung et al. (2018) analyzed that care should be taken when interpreting the numbers of yeasts and molds in silages, because numbers of yeasts and molds increased markedly from the time of sampling to arrival at the laboratory, especially in warm weather. These microorganisms were strictly aerobic, by comparison with other microorganisms in silage, which on the slowest growers. The total number of molds in silage should not be used as an indicator of mycotoxins (Gotlieb 2016). Mycotoxins were generally produced under stress conditions for the molds, and the environmental stressors that initiate mycotoxin production vary widely across species (Perincherry et al. 2019; Awuchi et al. 2022). The high numbers of mold in silage ($>10^6$ CFU/g of wet silage) were usually associated with aerobically spoiled silages, thus, a silage with a moderate amount of mold could still be relatively aerobically (Kung et al. 2018). Silages that were aerobically unstable might also present a mold smelly and have visible mold growth (Kung et al. 2018), but a silage with a moderate amount of yeasts could still be relatively aerobically stable.

In conclusion, Lactic acid bacteria were isolated from four rice varieties: Membramo, Ciherang, Rajalele, and Impari in tropical conditions and used to elevate the microbial composition of total mixed ration. The inoculation of local LAB in TMR silage increased the beneficial bacteria LAB, and reduced harmful microbes such coliform, and mold. Local LAB isolated had a potential equivalent to commercial LAB for TMR silage preparation in the tropical climate.

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