Effect of lactic acid bacterial starter KUB-G2 on grass silage quality and its microbial community performed using 140-ton plastic bag silos: a large-scale study

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Received: 25 December 2023; Accepted: 23 April 2024; Published: 7 May 2024

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RESEARCH ARTICLE

Abstract

This study investigated the effectiveness of lactic acid bacteria (LAB) starters in 140 tons of signal grass silage. We compared a locally developed starter, a combination of *Lactiplantibacillus plantarum* KUB-SP1-3 and *Pediococcus acidilactici* KUB-M6 (KUB-G2) with a commercial starter. We monitored the chemical and microbiological properties of silage at ensiling (day 0) and after 21 days. The results indicated that KUB-G2 produced high-quality silage. There were no significant differences in total viable microbes, lactic acid, or propionic acid contents between the starters. However, the silage produced with KUB-G2 had a lower acetic acid content than that produced with the commercial starter. KUB-G2 also resulted in a narrower microbiome profile dominated by Pediococci whereas the control group displayed a broader range of bacterial taxa with colonization by *Lactiplantibacillus* and *Lentilactobacillus*. These findings suggested that KUB-G2, with its distinct microbial profile, is a suitable replacement for imported commercial starters in large-scale signal grass silage production.

Keywords: silage; large-scale silage production; signal grass; lactic acid bacteria; organic acids; microbial community

Introduction

Ensiling, known for centuries, is a method to preserve forage. Lactic acid bacteria (LAB) ferment water-soluble carbohydrates under anaerobic conditions to produce lactic acid, which lowers the pH (Kung *et al.*, 2018). This inhibits the growth of undesirable microbes, causing animal and human health risks, such as mycotoxins (Li *et al.*, 2021) and silage deterioration by enterobacteria and yeasts (Carrizo *et al.*, 2021; Romero *et al.*, 2017). Additionally, qualified silage increases palatability of the feed for ruminants (Guo *et al.*, 2023).

Ensiling can be performed using various forage types (Okoye *et al.*, 2023). Grass silage has advantages, such as high productivity per area and harvest flexibility. However, compared to maize or sorghum silages, it has some unfavorable aspects, such as low soluble

carbohydrates, low dry matter (DM) content, high buffering capacity, low energy content, and low LAB concentrations (Jaipolsaen *et al.*, 2021; Keady *et al.*, 2008). Effective LAB additives can control the ensiling process and improve quality of grass silage in terms of palatability and inhibition of unwanted microorganisms.

Lactiplantibacillus plantarum KUB-SP1-3, isolated from Thai silage samples, produced a high level of lactic acid at various temperatures (30–45°C) (Ohmomo *et al.*, 2004, 2007). This strain showed high tolerance to heat and lactic acid, making it suitable for ensiling Napier grass in tropical regions. It achieved the highest lactate concentration of 1.35% (g/g of fresh silage) and maintained a low pH of 4 after ensiling, with a high number of viable cells (1×10^5 colony-forming unit (CFU)/g) even after 21 days. Nitisinprasert *et al.* (2000) identified *Pediococcus acidilactici* KUB-M6 as a promising LAB strain for production of silage. This strain produced significantly higher lactic acid (up to 14.11% g/g of fresh silage) in corn silage, compared to grass silage (up to 3.24% g/g of fresh silage) at various temperatures (37–50°C). Notably, ensiling without KUB-M6 resulted in significantly lower lactic acid content.

The use of homofermentative or heterofermentative LAB, such as *Lb. plantarum*, *Enterococcus faecium*, and *Pd. acidilactici*, is preferred to accelerate production of lactic acid, leading to a lower pH (Bao *et al.*, 2016). However, spoilage can occur during silo opening (Wilkinson and Davies, 2012). To reduce these risks, especially yeast contamination, heterofermentative LAB, such as *Lentilactobacillus buchneri*, is increasingly used as additional silage inoculants to increase content of acetic acid, consequently suppressing the growth of yeast cells (Nascimento *et al.*, 2022; Schmidt and Kung, 2010; Yin *et al.*, 2023). The benefits of *Lb. buchneri* may be distinct from bunker-made silage, in which a large surface area of the silo face is exposed to the air. However, it should be noted that a high content of acetic acid could affect the palatability and intake level of the feed for ruminants (Gerlach *et al.*, 2021).

Silage production in Thailand relies on imported commercial LAB starters. This presents difficulties in terms of regulations for importing biological materials and their high costs. Moreover, most imported starters are designed for silage production in continental and dry regions, which may not be suitable for tropical regions, such as Thailand. Therefore, the use of local starters may be a better option.

In this study, we ensiled signal grass (*Brachiaria brizantha*) using the conventional precision chopping method. The grass was stored in 20–25-ton bags with a narrow surface area. A mixture of the homofermentative LAB strains, *Lb. plantarum* KUB-SP1-3, and *Pd. acidilactici* KUB-M6, designated as KUB-G2, was used as an inoculant starter. We compared its effectiveness with that of a commercial starter containing *Lb. plantarum* CNCM MA18/5U and *Lb. buchneri* NCIMB 40788. Fermentation characteristics, chemical composition, and microbial communities of the silage produced with the two starter sources were evaluated after 21 days of ensiling. Our results showed that KUB-G2 is an effective LAB starter for large-scale silage production. Silage produced with KUB-G2 had similar levels of lactic acid production, total viable bacteria, LAB, and yeast as the control but lower production of acetic acid and a narrower microbial community.

Materials and Methods

Lactic acid bacterial inoculants

Two types of starters, KUB-G2 and the commercial product LALSIL Combo (Lallemand, France) in dry form were used as starters for making of silage. KUB-G2, consisting of *Lb. plantarum* KUB-SP1-3, and *Pd. acidilactici* KUB-M6 were prepared in de Man, Rogosa, and Sharpe (MRS) broth (Difco, France) at 37°C, a static fermentation for 16 h. Each culture was gently mixed with 15% (w/v) skimmed milk (Himedia, India) using a magnetic stirrer at 4°C for 1 h. The mixed solution was subsequently dried using a spray dryer (B-290; BUCHI, Switzerland). The spray-dry conditions were air inlet temperature at 170°C, air outlet temperature at 70°C, and feed rate of 0.2 mL/min to obtain a viable cell concentration of 1×10^{12} CFU/g. The powders of both strains were mixed at 1:1 ratio. Commercial starter cultures contained *Lb. plantarum* CNCM MA18/5U and *Lb. buchneri* NCIMB 40788 at a cell concentration of 10^{11} CFU/g as recommended by the company.

Ensiling

Two ensiling treatments of the control and the treatment KUB-G2 containing different starters of the commercial product LALSIL Combo and KUB-G2 were performed, respectively. Palisade signal grass (*Brachiaria brizantha*) planted at Pak Thong Chai Dairy Farm, Pak Chong District, Nakhon Rachasima Province (location: 14°39'51.8"N, 101°48'34.1"E) was used as a raw material in this study. About 45–50 days of regrowth was harvested using a precision chop harvester to obtain a size of approximately 3 cm with a moisture content of 50–60%. Freshly cut grass was transferred into plastic bag tunnels (Silotite Pro; AgFlex, Canada), and each starter suspended in 20 L of groundwater was directly sprayed into the respective chopped signal grass using an applicator to

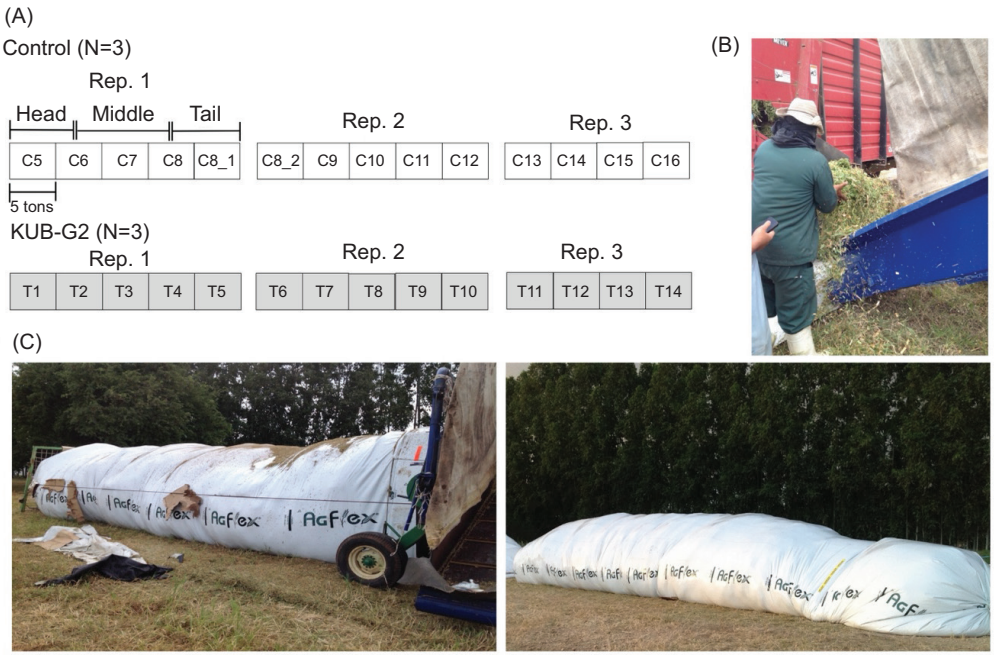

Figure 1. A large-scale silage production in 140-ton plastic bag silos. (A) Schematic diagram of silage in this study. (B) A worker loading signal grass supplemented with starters into a plastic bag silo. (C) Silos containing signal grass after closing and starting of fermentation.

obtain the final cell concentration of 1×10^5 CFU/g with the moisture content increasing by only 0.4%. Then, each silo was tightly pressed using an Ag-Bag model G6000 forage bagger (AG-Bag, WI, USA) to produce about 20–25 tons per bag of the treatment and control silage containing KUB-G2 and commercial starter LALSIL Combo, respectively.

Sample collection and transportation process

Each treatment consisted of three bags or three replicates (N = 3), which were sampled on days 0 and 21 from 20–25-m-long horizontal bag silo. Then, 250 g of each 5-ton silage sample, which was packed and positioned at head, middle, and tail (Figure 1), was randomly collected from each bag, transferred into a zipper storage bag, and kept in an ice box during transportation to the laboratory. Therefore, 14 samples from each sampling period and treatment were used as representatives of the silage to determine their quality by chemical and microbiological analyses.

Chemical analysis

The pH and moisture and fiber contents were determined using the methods described by Association of Official Analytical Chemists (AOAC; 2000). Nutritional values were determined using proximate analysis according to the method described by AOAC (2000).

Determination of viable cell concentration

The concentration of viable microbial cells was analyzed using the standard plate count method (AOAC, 2000). Then, 0.1 mL of suitable 10-fold serial dilution solution was transferred to plate count agar (Merck, Germany), potato dextrose agar (Merck, Germany) containing 10% (w/v) tartaric acid, and MRS containing 0.5% (w/v) $CaCO_3$ to determine mesophilic bacteria, yeast, and LAB and incubated at 37°C, 25°C, and 37°C for 1, 5 and 1 days, respectively. For LAB assay, only the colonies with clear zones were counted. Cell concentration was expressed as CFU/g of silage.

Determination of lactic acid and short-chain fatty acids

The concentrations of lactic, acetic, propionic, and butyric acids were measured using high-performance liquid chromatography (HPLC). Samples were prepared using the modified method described by Nakphaichit *et al.* (2014). Briefly, acids were eluted from 10 g of silage using 30 mL of distilled water at 4°C for 24 h. The supernatant was collected by centrifugation at 15,000×g for 10 min. Different concentrations of each acid (0.1, 0.2, 0.3, 0.4, and 0.5% v/v) were used to prepare standard curves. In all, 20 µL of mixture solution containing three volumes of analyzed sample or standard solution and one volume of internal standard (0.2% w/v tartaric acid) was filtered through 0.2-µm polyvinylidene difluoride (PVDF) syringe filters (Verical, Bangkok,

Thailand) and injected into a Breeze 2 HPLC system (Waters, MA, USA) with a Rezex ROR-organic acid column (Phenomenex, CA, USA). The mobile phase of 0.008-M H_2SO_4 was used at a flow rate of 0.6 mL/min. Signals were detected using an ultraviolet detector at a wavelength of 210 nm. All chemicals purchased were of HPLC analytical grade.

Bacterial DNA extraction

Bacterial DNA was extracted from 0.25 g of silage using a modified version of the method described by Yu and Morrison (2004). Each silage sample was transferred into a 2-mL sterile screw-cap tube containing 0.3 g of 0.1-mm zirconia beads (BioSpec, OK, USA) and 1-mL lysis buffer (500-mM NaCl, 50-mM Tris-HCl, pH 8.0, 50-mM etheylenediaminetetraacetic acid [EDTA], and 4% w/v $C_{12}H_{25}NaO_4S$) and homogenized for 3 min at maximum speed using a mini-beadheater (BioSpec, OK, USA). The lysate tube was incubated at 70°C for 15 min, with gentle shaking for every 5 min. The lysate was centrifuged at 16,000×g for 5 min at 4°C to collect supernatant. The supernatant was transferred to a sterile screw-cap tube containing 0.3 g of zirconia beads with 300-µL fresh lysis buffer, and homogenization was repeated. DNA was precipitated using one volume of isopropanol to obtain 100 µL of DNA solution in Tris-EDTA which was further purified using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's protocol and stored at –20°C until used.

16S rRNA gene amplicon sequencing and sequence data processing

The V3-V4 hypervariable region of *16S rRNA* gene was amplified using the primers Bakt_341F (5′-CGCTCTTCCGATCTCTGCCTACGGGNGGCWGCAG-3′) and Bakt_805R (5′-TGCTCTTCCGATCTGACGACTACHVGGGTATCTAATCC-3′) (Herlemann *et al.*, 2011) with barcodes. Polymerase chain reaction (PCR) was performed using the TaKaRa Ex Taq HS (Takara Bio, Shiga, Japan). PCR amplicons with index sequences were sequenced using a MiSeq reagent kit v3 (Illumina, San Diego, CA, USA). Amplicon sequence variants (ASVs) were assembled, trimmed, and denoised based on a quality score higher than 20 using the DADA2 pipeline (Callahan *et al.*, 2016) in QIIME2 (https://docs.qiime2.org/2021.2/tutorials/moving-pictures/; accessed: December 6, 2023). ASVs were taxonomically classified against SILVA 138.1 SSU Ref NR 99 using the QIIME2 feature-classifier classify-sklearn script (https://docs.qiime2.org/2022.8/data-resources/#taxonomy-classifiers-for-use-with-q2-feature-classifier; accessed: December 6, 2023) (Quast *et al.*, 2013).

Alpha and beta diversity analyses

Alpha and beta diversities were analyzed using QIIME2. Alpha-diversity indices, number of observed features, Shannon diversity index, and Faith's PD index were determined at a sequence depth of 5,740 reads per sample using the QIIME diversity alpha-group-significance script. Beta diversity, based on the Bray–Curtis dissimilarity, was determined using the QIIME diversity beta-group-significance script. Permutational multivariate analysis of variance (PERMANOVA) with pairwise adjustments was used to investigate statistical differences between KUB-G2 and control.

Linear discriminant analysis Effect Size (LEfSe)

Linear discriminant analysis Effect Size was calculated using online Galaxy, version 1.0 (http://galaxy.biobakery.org/: accessed: December 6, 2023) (Segata *et al.*, 2011). Bacterial composition data of all samples from phylum to species levels were used (Supplementary Table S1), in which the taxa were subjected to linear discriminant analysis (LDA) using a one-against-all strategy. Taxa showing an LDA score higher than 3.0 with $P < 0.05$ were selected as enriched taxa for each group.

Statistical analysis

Statistical analyses and graphics were performed using the RStudio software, version 2023.09.1+494 (https://posit.co/download/rstudio-desktop/; accessed: December 6, 2023) with the R software, version 4.3.1 (https://cran.rstudio.com/; accessed: December 6, 2023). Paired *t*-test, pairwise Wilcoxon rank-sum test, and one-way ANOVA with Tukey's multiple pairwise comparison were used to compare two groups and more than two groups, respectively, which were accomplished using generic functions in R and the multcomp package (Hothorn *et al.*, 2008). Box plots were constructed using the ggboxplot function of the ggplot package (Wickham, 2016).

Results and Discussion

Consistency of silage quality performed by starter supplementation

Two treatments with different starter types, including the control and KUB-G2 groups, were performed for 21-day silage making. Since the bag silo had a long bag length of up to 25 m, the fermentation quality of each position might vary because of oxygen interference and moisture content of raw material. This suggested that silage consistency must be investigated. Three positions, including

head, middle, and tail of each silage bag (Figure 1), were collected to determine pH and lactic acid production. On day 0, the pH of both treatments was in the range of 5.9–6.5, with no significant difference ($P > 0.05$) (Supplementary Figure S1). No lactic acid was detected. On day 21 fermentation, the pH dropped to 3.9–4.5, providing lactic acid concentrations of 2.28–3.54% (g/g DM). Both pH and lactic acid concentration at the three positions of each treatment showed no significant differences, as shown in Figures 2A and 2B, respectively. Lactic acid is the primary factor responsible for good silage quality. It is produced by LAB fermentation using water-soluble carbohydrates as a carbon source, resulting in rapid pH decrease under anaerobic conditions, which prevents silage from developing undesirable microbes, such as clostridia, yeast, and mold (Muck, 2010). Depending on the type of silage, lactic acid concentration is normally 4–7% (g/g DM), followed by acetic acid (2–3% g/g DM), propionic acid (<0.5% g/g DM), and butyric acid (<0.1% g/g DM) (Ward, 2009). However, unlike maize and other cereals, signal grass has low fermentative carbohydrates and high buffering capacity, causing lower lactic acid production in general (Heuzé *et al.*, 2021).

Effect of starters on pH and production of organic acid on days 0 and 21 silages

The determined pH and organic acid content produced by the microorganisms growing in the silage are shown in Table 1. On day 0, organic acids, including short-chain fatty acids (SCFA), were not detected in any of the groups, while the pH values of KUB-G2 were significantly lower than those of the control. After 21-day fermentation, the pH values of the silage treated with KUB-G2 and the control decreased and were significantly lower in KUB-G2, compared to the control. The amount of lactic and propionic acids produced by both treatments was not significantly different at $P > 0.05$ whereas the amount of acetic acid detected in KUB-G2 was significantly lower than that in the control ($P < 0.05$). Acetic and butyric acids played important roles in determining silage quality. Moderate concentration of acetic acid inhibits yeast and mold growth, thereby improving the stability of silage against aerobic deterioration (Danner *et al.*, 2003). However, previous studies reported that high concentration of acetic acid (>3% g/g DM) reduced palatability, causing lower feed intake in ruminants (Gerlach *et al.*, 2021; Krizsan and Randby, 2007; Manzocchi *et al.*, 2022). In this study, both treatments produced relatively lower acetic acid concentrations of 0.15–0.36% (g/g DM), which did not exceed the standard (1–3% g/g DM), as suggested by Ward (2009). It should be noted that further investigation on silage quality after exposing to air (aerobic stability) need to be performed. A high concentration of butyric acid leads to silage of poor quality, indicating that clostridial metabolism converting lactic acid to butyric acid is activated (Kung *et al.*, 2018). This leads to significant losses in DM and a decrease in energy recovery. In this study, butyric acid was not detected in any of the treatment groups. This implies that both treatments showed similar main chemical characteristics of pH and SCFA content, except for acetic acid concentration.

Effect of starters on viable microorganism changes on days 0 and 21 silages

The total viable cell counts of bacteria, yeast, and LAB were determined using microbiological methods, and the results are shown in Figure 3. No microbial group from these two treatments on days 0 and 21 ensiling showed a significant difference ($P > 0.05$). Total bacteria counts were 7.427 ± 2.39 log CFU/g and 6.500 ± 1.58 log CFU/g in both KUB-G2 and control groups, respectively (Figure 3A). The cell numbers in KUB-G2 and

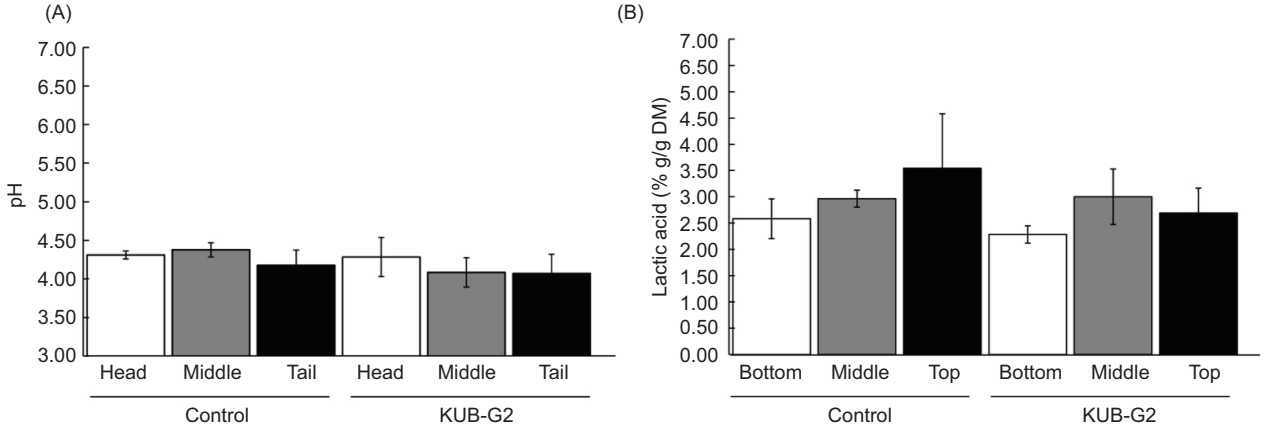

Figure 2. Acidic properties of silage on day 21 ensiling. (A) pH value. (B) Lactic acid (% g/g DM).

Table 1. Total pH and production of organic acids on days 0 and 21 silage supplemented with starters.

Parameters	Treatments					
	Day 0			Day 21		
	Control	KUB-G2	*P* value	Control	KUB-G2	*P* value
pH	6.42 ± 0.12	6.34 ± 0.24	0.013	4.29 ± 0.14	4.15 ± 0.23	0.036
Lactic acid (% g/g DM)	ND	ND	-	3.03 ± 0.66	2.66 ± 0.45	0.127
Acetic acid (% g/g DM)	ND	ND	-	0.36 ± 0.15	0.15 ± 0.08	0.037
Propionic acid (% g/g DM)	ND	ND	-	0.29 ± 0.23	0.30 ± 0.05	0.305
Butyric acid (% g/g DM)	ND	ND	-	ND	ND	-

P values were analyzed by paired *t*-test, comparing the starters on days 0 and 21.
ND: not detected; DM: dry matter.

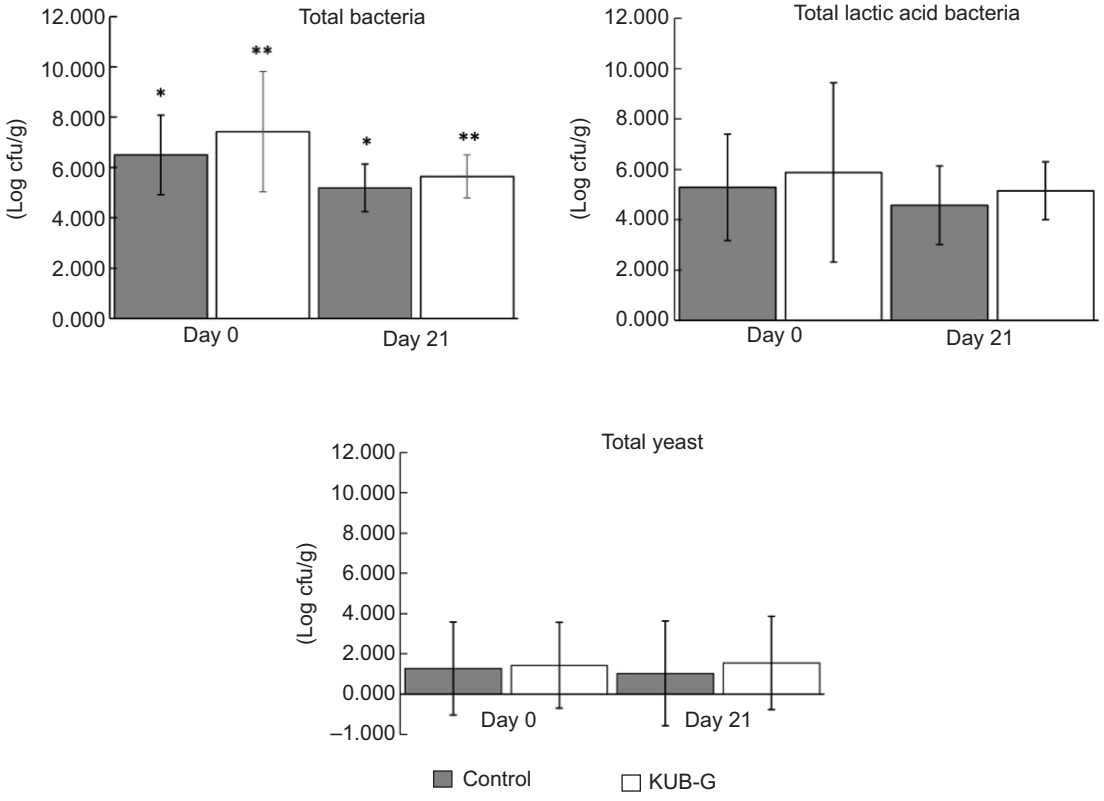

Figure 3. Viable cell counts of silage on days 0 and 21. (A) Total bacteria. (B) Total LAB. (C) Total yeast and mold. *, **Significant differences between ensiling days with the same treatment (*P* < 0.05, paired *t*-test).

control groups on day 21 ensiling decreased significantly to 5.647 ± 0.86 log CFU/g and 5.196 ± 0.95 log CFU/g, respectively ($P < 0.05$). These results implied that bacterial growth was suppressed in both treatments.

Considering LAB determination, the total viable cell concentration of LAB in KUB-G2 was 5.876 ± 3.56 log CFU/g, which was not significantly different from that of the control (5.286 ± 2.11 log CFU/g) on day 0 ensiling, while day 21 ensiling reduced cell numbers to 5.150 ± 1.15 log CFU/g and 4.575 ± 1.56 log CFU/g in KUB-G2 and control, respectively, with no significant difference between the two treatments ($P > 0.05$) (Figure 3B). It should be noted that KUB-G2 showed a wide range of standard deviations (SD) that were higher than those of the control. It can be presumed that poor KUB-G2 distribution occurred during starter application in early fermentation (day 0). However, after 21-day ensiling, LAB cells from the KUB-G2 treatment group displayed lower variation, indicating better dispersion of LAB growth.

Low concentrations of viable yeast cells were detected. The cell concentrations of 1.435 ± 2.13 and 1.275 ± 2.31 on day 0 ensiling changed to 1.552 ± 2.32 and 1.033 ± 2.60 on day 21 ensiling in the KUB-G2 treatment and the control, respectively (Figure 3C). However, no significant differences were observed between the two treatments ($P > 0.05$).

These results suggested that both microbial starters had no different effect in terms of microbiological property on large-scale silage production.

Effect of starter on microbial communities of silage post-ensiling

Deep sequencing of bacterial *16S rRNA* genes from silage samples was performed to explore post-ensiling microbiota diversity. Three indices were used to evaluate alpha diversity: observed features, Shannon diversity index, and Faith's PD index (Figure 4A). The results indicated that KUB-G2 had lower diversity than the control. However, there was no significant difference in diversity among treatments ($P > 0.05$). Considering beta diversity, principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity showed that both treatments had distinct differences in microbial communities ($P < 0.05$) (Figure 4B).

Bacterial compositions between silage treatments were investigated at phylum (p1–14), genus (g1–g84, only abundances >0.1% were included), and species (s1–s115, only abundances >0.1% were included) levels (Supplementary Tables S2–S4). Spearman's rank correlation was used to investigate correlation between the bacterial genera in silages and the organic acids produced (Supplementary Table S5). An investigation of the bacterial phyla was first carried out. The results indicated that common phyla are normally found in plants. Firmicutes (p1) and proteobacteria (p2) were the major phyla and were dominant in silages at abundances of 57.72% and 39.87%, respectively (Figure 5). However, these phyla showed no significant differences between the treatments ($P > 0.05$) (Supplementary Table S2). Firmicutes and proteobacteria are generally found as normal flora in plants according to their contributions to genus members (Bulgarelli *et al.*, 2013; Wu *et al.*, 2021). Moreover, they are common phyla of endophytic bacteria, such as *Rhizobium*, *Bacillus*, *Pseudomona*, *Burkholderia*, and *Enterobacter*, which play key roles in promotion of plant

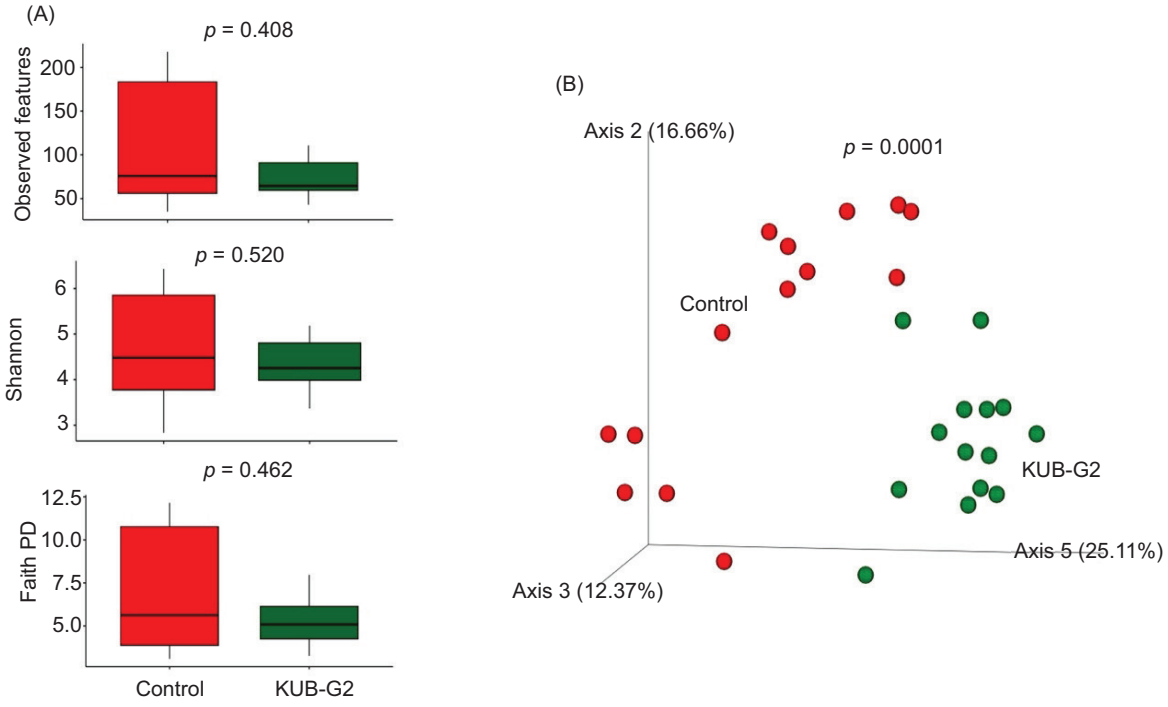

Figure 4. Microbial diversity in silages ensiled by LAB starters, the control (red), and KUB-G2 (green). (A) Box plots show alpha diversity indices across the treatments analyzed by observed features, Shannon diversity index, and Faith's PD index. The probability value (*P*) was determined using the Wilcoxon rank-sum test. (B) Principal coordinate analysis (PCoA) based on the Bray–Curtis distance matrix shows the separation of microbial diversities between two silage treatments. Statistical significance of different treatments was assessed using permutational multivariate analysis of variance (PERMANOVA; pairwise adonis).

growth (Vandana *et al.*, 2021). Actinobacteriota (p4) levels were significantly higher in the control group. In contrast, the abundance of verrucomicrobiota (p7) and patescibacteria (p8) was significantly higher in KUB-G2. It should be noted that all of these were minor phyla found in silages with abundances of only 1.29%, 0.02%, and 0.01%, respectively (Supplementary Table S2). Actinobacteriota, previously known as phylum actinobacteria, are normally found in grass silage (Lu *et al.*, 2021), and their members are organic acid producers (Boubekri *et al.*, 2022). Patescibacteria, a superphylum, is often found in groundwater environments (Tian *et al.*, 2020). This suggested that the patescibacteria found in silages must have originated in the groundwater used to mix starters before spraying them on grass.

The bacterial compositions of these two treatments were analyzed using LEfSe (LDA score > 3.0, and $P < 0.05$), as shown in cladogram (Figure 6), to investigate the overall microbiome profiles in silage on day 21 because of different starters supplemented at an early stage. The results indicated that a broad range of taxonomic groups, including common crop microbiome families Mycobacteriaceae (c), Microbacteriaceae (d), Lachnospiraceae (q), and Oxalobacteraceae (a2), were significantly dominant in the control, whereas KUB-G2 showed *Lacticaseibacillus* (f, g17 in Supplementary Table S3), *Limosilactobacillus* (k, g25), and *Pediococcus* (m, g2) genera. The families found in the control were mainly involved in plant component degradation and SCFA production, such as Mycobacteriaceae, Microbacteriaceae (Yeager *et al.*, 2017), Lachnospiraceae (Vacca *et al.*, 2020), and Oxalobacteraceae (Adrangi *et al.*, 2010; Du *et al.*, 2021; Wilhelm *et al.*, 2019). It should be noted that the dominant genera found in the control showed significantly positive correlations with acetic acid, including *Lactiplantibacillus* (h, g1 in Supplementary Table S3), *Lentilactobacillus* (j, g7), *Mycobacterium* (b, g46), *Lachnoclostridium* (p, g32), *Devosia* (s, g28), Rhizobiaceae gen. (v, g42), *Aminobacter* (y, g31), and *Massilia* (a1, g51) at rho values of 0.50 ($P = 0.007$), 0.46 ($P = 0.015$), 0.61 ($P = 0.001$), 0.61 ($P = 0.001$), 0.44 ($P = 0.081$), 0.49 ($P = 0.008$), 0.49 ($P = 0.008$), and 0.46 ($P = 0.013$), respectively. Studies have shown that some of these genera, such as *Lactiplantibacillus* (Fonseca *et al.*, 2021; García-Núñez *et al.*, 2022; Yan *et al.*, 2023) and *Lentilactobacillus* (Benjamim da Silva *et al.*, 2023; Nishino *et al.*, 2003), must have contributed to a significant increase in the acetic acid concentration of control (Table 1).

Generally, the microbial community of silage shifts during the ensiling process associated with LAB starters, most of which produce two moles of lactic acid from one mole of glucose, known as homofermentative LAB whereas heterofermentative LAB produces one mole of lactic acid, one mole of CO_2, and either one mole of ethanol or acetic acid (Muck, 2010). Both Lactobacilli

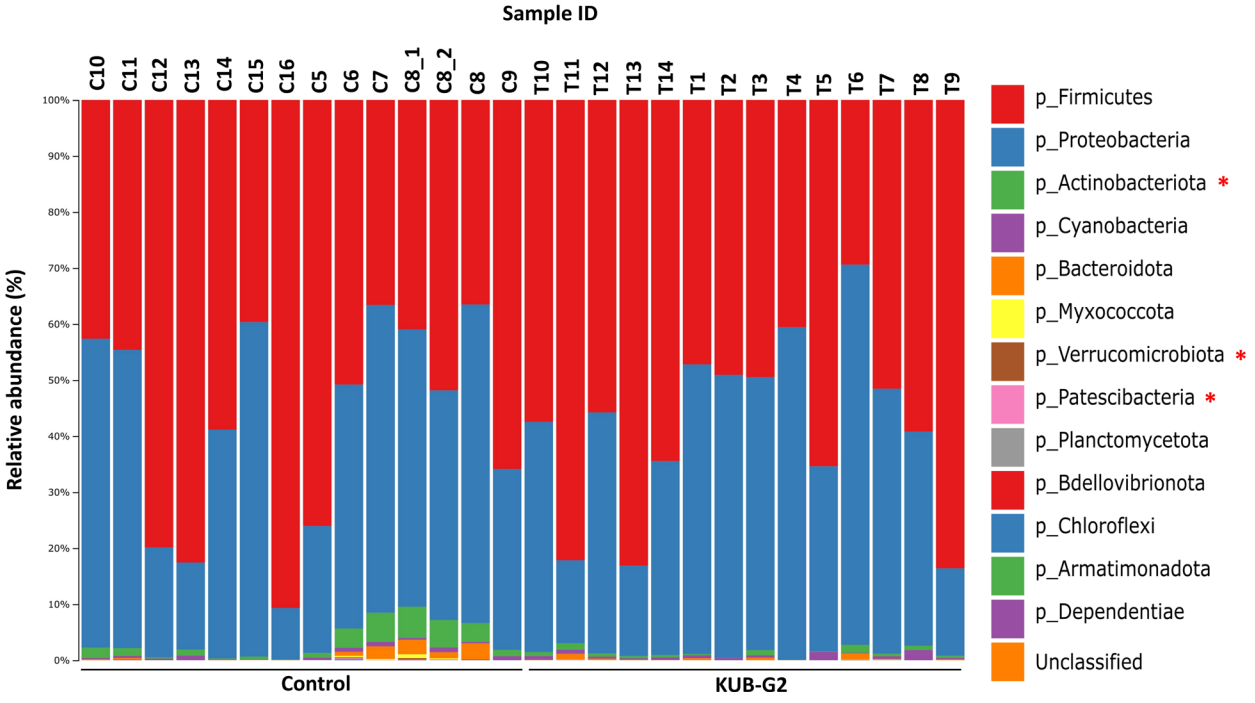

Figure 5. Bacterial phyla found in silage post-ensiling. *Significant different abundance of phyla between KUB-G2 and control groups at $P < 0.05$ (Wilcoxon rank-sum test).

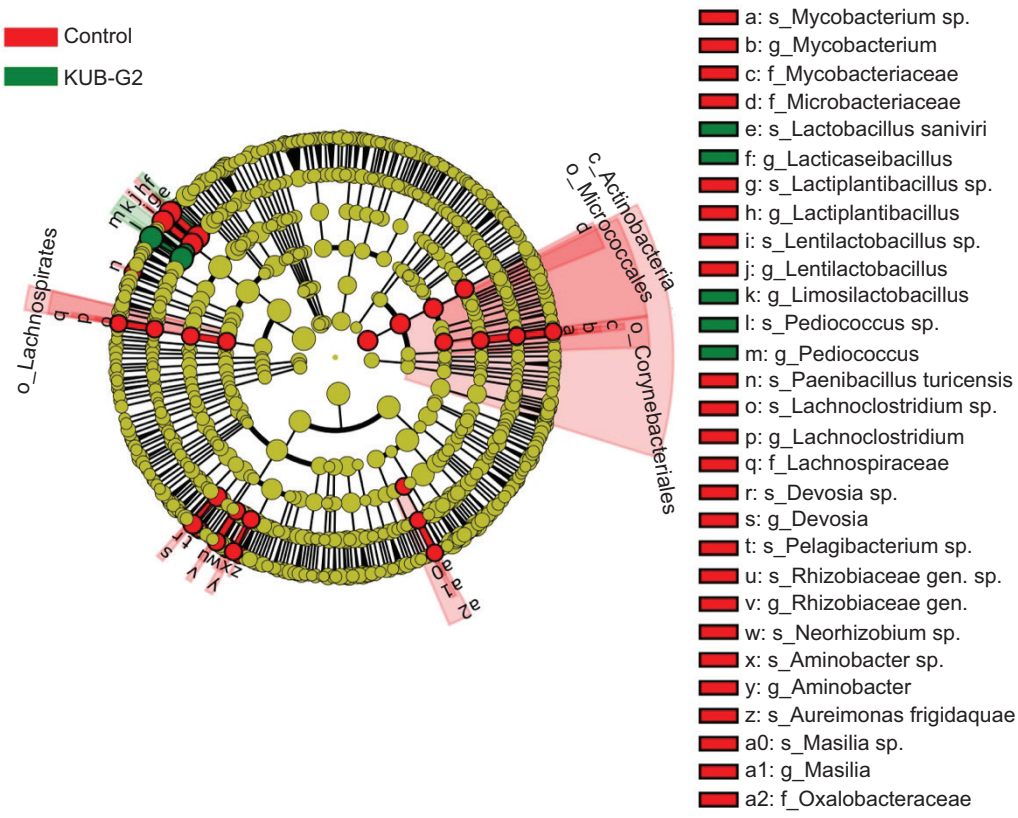

Figure 6. Silage microbiome features associated with LAB starters, control, and KUB-G2 post-ensiling. The LEfSe analysis shown in cladogram was performed using bacterial composition data of silage samples from the level of phylum to species (Supplementary Table S1). LDA scores were calculated by using Wilcoxon rank-sum test. Taxonomic groups, showing LDA scores >3.0 with $P < 0.05$, are highlighted in cladogram by the indicated colors. Bacterial taxa include c_: class, o_: order, f_: family, g_: genus, s_: species, gen.: unclassified genus, and sp.: unclassified species.

and Pediococci are well-known lactic acid producers. However, in the present study, no genera were significantly correlated with lactic acid levels (Supplementary Table S5). KUB-G2 showed a higher diversity of LAB, compared to the control, including *Lactobacillus saniviri* (e, s22 in Supplementary Table S4), *Lacticaseibacillus* (f, g17), *Limosilactobacillus* (k, g25), *Pediococcus* (m, g2), and *Pediococcus* sp. (l, s2). A lower pH level because of organic acid production in KUB-G2 (Table 1) resulted in a narrow microbial community with highest dominance in *Pediococcus* (m, g2) and *Pediococcus* sp. (l, s2) at 32.70% and 31.80%, respectively, compared to 14.56% and 14.56% in the control silage, although *Lb. plantarum* KUB-SP1-3 starter was also added. This could be because Pediococci was more aggressive and produced lactic acid faster than Lactobacilli (Abedi and Hashemi, 2020; Zhang *et al.*, 2020). In contrast, a lower diversity of LAB was observed in the control, such as *Lactiplantibacillus* (h, g1), *Lactiplantibacillus* sp. (g, s1), *Lentilactobacillus* (j, g7), and *Lentilactobacillus* sp. (i, s7) at 18.69%, 18.69%, 11.14%, and 11.14%, respectively. This suggested that additional starter in each treatment provided bacterial groups involved in organic acid production but differed in taxa, especially in the KUB-G2 treatment, which supported the high abundance of Pediococci.

Nutritional values and appearance of silage treated with KUB-G2

The nutritional value of the post-ensiling silage was analyzed to study the productivity of microbial starters. Similar contents of crude protein (6.2–6.8%), acid detergent fiber (ADF; 32.4–32.7%), neutral detergent fiber (NDF; 52.0–53.0%), and gross energy of 4,450–4,503 cal/g from both treatments were obtained. This implied that the starter source did not affect the nutritional value. This was compared to the study done by Patterson *et al.* (2021), who investigated the nutritional values of grass silage on Northern Ireland farms from 1998 to 2017 and both inoculant silages in the study indicated lower crude protein but similar trends in ADF and NDF. This indicated that the nutritional value of plant silages could vary in different bioclimatic areas (Lee, 2018) whereas different starters used in this study had no influence. Moisture contents of silage were stable post-ensiling at

54.71 ± 4.07% and 55.93 ± 1.49% in the control and KUB-G2, respectively. It played a key role in silage quality, which varied based on raw materials and field environment. High moisture content is conducive to LAB fermentation (Franco *et al.*, 2017). However, it promotes the growth of acetate and butyrate producers (Kung *et al.*, 2018). Generally, the recommended moisture content for grass silage should be less than 70% to prevent clostridial activity (Muck *et al.*, 2003). This implies that low moisture contents in both treatments carried out in this study were well-designed without detection of butyric acid.

The color of silage indicated the quality of preservation. Yellow and yellow-green as distinct colors appeared on day 21 silage performed by both control and KUB-G2 treatments, respectively. This was a well-preserved silage according to the study conducted by Kung *et al.* (2018).

Conclusion

A local LAB starter, KUB-G2, containing *Lb. plantarum* KUB-SP1-3 and *Pd. Acidilactici* KUB-M6, was investigated for its ability to signal grass silage in 140-ton plastic bag silos to replace the import of starters for silage production. KUB-G2 produces high-quality silage by controlling microbial growth during fermentation, resulting in no significant differences in viable microbes, organic acids, or nutrients, compared to a commercial starter. Moreover, butyric acid was not detected in the silage ensiled using these starter sources. Different starter sources affected microbial community and improved fermentation quality. Microbiome profile analysis suggested that KUB-G2 drives a narrow microbial community but a higher diversity of LAB, especially the colonization of Pediococci, whereas the commercial strain showed a broad range of microbial communities but less LAB diversity with the colonization of *Lactiplantibacillus* and *Lentilactobacillus*. To the best of our knowledge, this study is the first to investigate a local LAB starter for large-scale silage production in Thailand. One limitation of this study was the cost of negative control (silage with an uninoculated starter). Effects on silage stability after exposure to aerobic conditions and shelf life require further study.

Author Contributions

Conception and design of study: Sunee Nitisinprasert, Chaowarit Mapato, and Phatthanaphong Therdtatha. KUB-G2 starter preparation by spray drying: Phatthanaphong Therdtatha. Silage ensiling: Phatthanaphong Therdtatha, Chaowarit Mapato, and Sunthorn Rungruang. Microbial and chemical analyses: Phatthanaphong Therdtatha. Next-generation sequencing: Orawan La-ongkham, Massalin Nakphaichit, and Jiro Nakayama. Nutritional analysis: Chaowarit Mapato and Sunthorn Rungruang. Data analysis and visualization: Phatthanaphong Therdtatha and Sunee Nitisinprasert. Manuscript writing: Phatthanaphong Therdtatha and Sunee Nitisinprasert. All authors read and agreed to the published version of the manuscript.

Funding

This study was supported by the National Research Council of Thailand to Sunee Nitisinprasert and partially supported by Chiang Mai University to Phatthanaphong Therdtatha.

Data Availability Statement

All data generated and analyzed in this study are included in this article.

Acknowledgments

We thank all workers at Pakthongchai Dairy Farm who helped us to ensile all 140 tons of silage. We also thank the Center for Advanced Instrumental and Educational Supports, Faculty of Agriculture, Kyushu University to support the next-generation sequencing for use of Illumina MiSeq sequencer.

Conflicts of Interest

The authors declared no conflict of interest.

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Supplementary

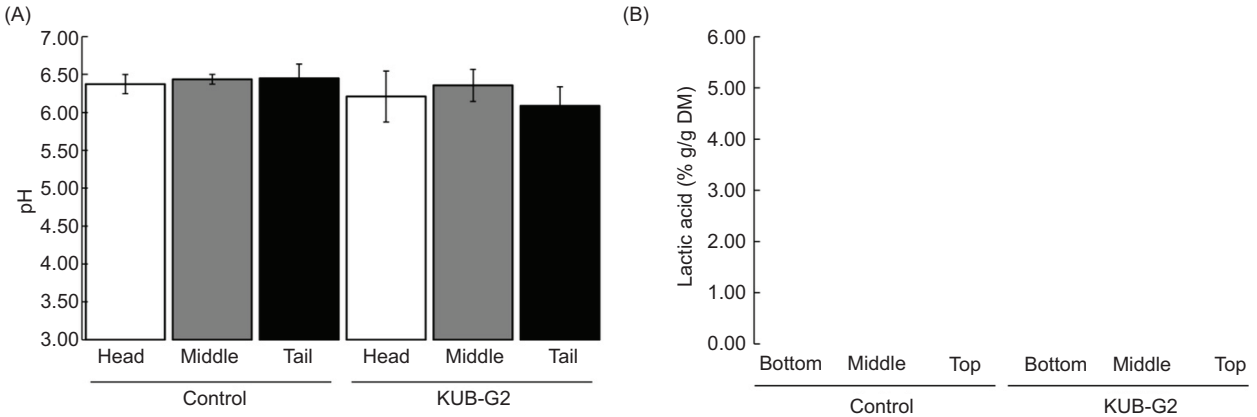

Figure S1. Acidic properties of silage on day 0 fermentation. (A) pH value. (B) % Lactic acid (g/g DM). Uppercase letters indicate significant difference of each position between starters at *P*< 0.05 (paired *t*-test). Lowercase letters indicate significant differences between positions in each starter at *P* < 0.05 (Tukey's multiple pairwise comparison).

Table S1. Bacterial taxa with their relative abundance found in silages treated with LAB starters after post-ensiling.
Click here

Table S2. Abundance of bacterial phyla found in grass silage ensilaged by LAB starters.
Click here

Table S3. Abundance of bacterial genera found in grass silage ensilaged by LAB.
Click here

Table S4. Abundance of bacterial species found in grass silage ensilaged by LAB starters.
Click here

Table S5. Spearman's rank correlation of each genus to organic acid concentration.
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