Indian Journal of Animal Sciences **95** (9): 833–837, September 2025/Article
https://doi.org/10.56093/ijans.v95i9.170329

In vitro gas production profiles of corn silage: The function of bacterial inoculants

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Received: 16 August 2025; Accepted: 22 January 2026

ABSTRACT

This study was carried out to evaluate the effect of bacterial inoculants on the quality of corn silage and its *in-vitro* digestibility. Whole-plant corn, specifically the brown mid rib, was chopped and treated with five different microbial inoculants including *Lactobacillus buchneri, Lactobacillus keifiri, Lactobacillus oris, Lactobacillus hilgardii,* and *Lactobacillus rhamnosus*. The uninoculated silage served as the control treatment, and all samples were ensiled in PVC containers. The silos were subjected to fermentation for durations of 14, 21, and 28 days at ambient temperature, after which they were opened for the examination of fermentation products and nutrient composition. *In vitro* gas production was assessed using 100ml calibrated glass syringes at intervals of 3, 6, 9, and 24 hours with the dry ground silage. The gas production observed with silage treated bacterial inoculants exceeded that of the control, and no significant differences were noted in total gas production values at the 24-hour mark among the various groups. The results also indicate that LAB quickly reduces the pH of silage by converting water-soluble carbohydrates (WSC) into lactic acid, thereby improving preservation. Therefore, the objective of this study was to investigate the influence of selected lactic acid bacteria on fermentation quality and in vitro digestibility of corn silage.

Keywords: Bacterial inoculants, Digestibility, Corn silage, *In vitro* gas production, Fermentation

In tropical developing countries such as Malaysia, maintaining a consistent supply of forage crops for ruminant production presents a significant challenge, particularly during the dry season when the availability of fresh forage is limited. To overcome this constraint, many farmers have adopted the use of alternative feed resources such as silage, which is both cost-effective and relatively easy to prepare (Suntara *et al*. 2020; Pholsen *et al*. 2016). However, the nutritional value of forage crops preserved as silage can vary considerably, and in some cases, may be inadequate, leading to poor digestibility and reduced feed efficiency (Polyorach and Wanapat, 2015). These nutritional limitations can negatively affect the productivity and growth performance of ruminant animals.

To address this issue, biological methods particularly the use of microbial inoculants have been proposed as an environmentally sustainable strategy to enhance the nutritional quality of silage. Previous studies have demonstrated that such approaches can improve feed palatability, nutrient digestibility, and overall animal performance compared to untreated feed materials (Suntara *et al*. 2020; Cherdthong *et al*. 2020; Wanapat *et al*. 2009). Therefore, exploring the application of biological treatments in silage production holds potential to support more efficient and sustainable ruminant livestock systems in regions with seasonal forage shortages. In ruminant nutrition, ensiling is a widely adopted method for forage preservation, primarily aimed at improving feed quality and, consequently, enhancing animal performance (Fan, 2016). Modern agricultural practices increasingly incorporate additives such as molasses, enzyme enhancers, and bacterial inoculants particularly lactic acid bacteria (LAB) to optimize silage quality. These additives not only enhance the fermentation process but also contribute to improved livestock productivity and long-term silage stability (Muck 2010; Khota *et al*. 2017; Cherdthong *et al*. 2020).

Extensive research has demonstrated that the inclusion of LAB during silage production positively influences the digestive health of ruminants, thereby supporting better animal performance (Guo *et al.* 2020; Weinberg *et al.* 2003). According to So *et al.* (2020) and Eun and Beauchemin (2008), additives like molasses and enzyme cellulase can increase lactic acid concentrations and water-soluble carbohydrate (WSC) content, both of which are crucial for promoting effective rumen function. Similarly, studies by Linna *et al*. (2021), Tian *et al*. (2014), and Cherdthong *et al.* (2020) have shown that LAB inoculants significantly improve silage quality over time and support *in vitro* digestibility. In the current context of erratic

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climate conditions and escalating feed costs, silage has become a vital alternative feed resource. LAB strains, which have been extensively studied and characterized, are now routinely employed to maintain silage quality during storage. Moreover, their use helps reduce potential nutrient losses during large-scale ensiling operations (Blajman *et al*. 2020).

The evaluation of forage digestibility and fermentation kinetics can be effectively conducted using *in-vitro* fermentation techniques. The method developed by Menke and Steingass (1988), based on *in-vitro* gas production, has become a standard procedure for estimating the fermentative potential of various feedstuffs. This technique simulates rumen fermentation under controlled conditions and provides valuable information on the kinetics of gas production, substrate degradability, and potential energy yield. It also serves as a rapid and cost-effective tool for screening the efficacy of microbial or enzymatic treatments on feedstuffs. Given the limited studies on LAB inoculants in tropical corn silage systems, especially in Malaysia, there is a critical need to evaluate their effects on fermentation quality and digestibility using reliable in-vitro techniques.

MATERIALS AND METHODS

Sample preparation: This study was conducted in accordance with ethical guidelines for the care and use of animals and received approval from the Ethics Committee for Animal Use in Scientific Research at Universiti Putra Malaysia. Corn silage samples were prepared by weighing approximately 200 ± 10 mg using a small polypropylene spoon, ensuring the samples passed through a 1 mm sieve. Each sample was weighed in triplicate to ensure consistency. For each incubation batch, three syringes served as blanks (without feedstuff) and three as reference standards containing a commercial concentrate. Following sample loading, Vaseline was applied to the syringe pistons before insertion to ensure airtight seals. The syringes were then placed in a water bath maintained at 39°C for pre-incubation.

Rumen fluid collection: Rumen fluid was collected from a rumen-fistulated goat prior to the morning feeding. The animal, approximately two years of age and weighing an average of 40 kg, had been adapted to a mixed diet of corn silage and native grass in a 1:1 ratio, administered twice daily for three weeks before sampling. Approximately 1 L of rumen contents was withdrawn and immediately transferred into a pre-warmed thermos flask to preserve microbial activity. The flask was then promptly transported to the laboratory. Upon arrival, the rumen contents were homogenized using a kitchen blender and filtered through four layers of muslin cloth to obtain a particle-free fluid. During the entire sampling and preparation process, carbon dioxide (CO_2) was continuously flushed through the rumen fluid to maintain strict anaerobic conditions, ensuring the viability of obligate anaerobic microbes for subsequent *in vitro* incubation.

Preparation of syringe and sample for analyses: Preparation of the incubation solution: The media was prepared following the methodology outlined by Menke and Steingass (1988). The substrates, which included corn silage inoculated with five species of bacterial treatments as well as those without any bacterial inoculants, were dried in an oven overnight at 60˚C. They were then ground using a 1-mm screen sieve and stored at -20˚C for *in-vitro* studies. The dry matter (DM) content of the corn silage, both treated and untreated, was calculated. The 100ml syringes were evaluated for their suitable pistons, washed with acetone, and maintained at 39˚C for *in-vitro* studies. Prior to each experiment, approximately 200 mg of the substrates were weighed and placed into 100ml calibrated glass syringes (FORTUNA® Hiberle Labortechnik, Germany). Once the sample was transferred, the piston of the syringes was lubricated with Vaseline, inserted into the syringe, and then placed in the incubator at 39˚C.

Preparation of incubation medium: The buffered medium that was prepared the previous day in a water bath was maintained at a temperature of 39ºC, with carbon dioxide being introduced slowly for a duration of 15 to 20 minutes. The medium was kept in constant motion using a magnetic stirrer. A reducing solution was formulated and incorporated into the medium. The medium is expected to change to pink and ultimately become colorless. Rumen fluid was measured to a volume of 650 ml and added to the medium. Carbon dioxide continued to be bubbled through the medium. Rumen inoculums were dispensed into a syringe, with 30 ml being transferred through a silicone tube attached to the nozzle. The gas bubble was expelled, and the silicone tube was sealed with clamps. The volume was documented, and the syringe was placed on the rotor of the incubator or in a water bath maintained at 39ºC.

Chemical analyses: Gas production was measured after 24 hours of incubation following the method described by Menke and Steingass (1988). The pH values of each treatment were determined immediately post-incubation using a Corning pH meter (Model 240). Syringes were agitated every 4 hours, and after 24 hours, incubation was terminated. The medium from each syringe was collected, and 10 ml of each sample was transferred to test tubes and stored at –20ºC for volatile fatty acid (VFA) analysis. Residual contents were collected to determine dry matter loss (DM). This was achieved by rinsing the residues with hot water and filtering them under vacuum using crucibles. The crucibles were then dried in an oven at 105ºC until a constant weight was reached. Measurements of gas production were recorded at 1, 3, 6, 9, 12, 18, and 24 hours of incubation. VFA analysis followed a modified procedure based on Cottyn and Boucque (1968). VFA concentrations (acetic, propionic, and butyric acids) were quantified using gas chromatography (GC; Agilent 6890 N) equipped with a flame ionization detector (FID) and a packed column (5% Thermon-3000, Shincarbon A 60/80). The column was 30 meters in length with a film thickness of 0.25 µm. Oven temperatures ranged from 90 to 180ºC,

with nitrogen as the carrier gas. The detector temperature was maintained at 250ºC.

Statistical analysis: All measured parameters, including pH, dry matter loss, VFA concentrations, and gas production were analyzed using analysis of variance (ANOVA) with SPSS STAT software version 16.0 (2000). Treatment means were compared using the least significant difference (LSD) test, with significance accepted at $p < 0.05$. Standard deviation (SD) for each treatment and the standard error of the mean (SEM) was also calculated.

RESULTS AND DISCUSSION

Table 1 summarizes the effects of bacterial inoculation on the fermentation characteristics and digestibility of corn silage, specifically focusing on pH, gas production and dry matter (DM) loss. A total of seven treatments were evaluated, with Treatment 1 serving as the uninoculated control, while the remaining treatments involved the application of different lactic acid bacteria (LAB) strains. No significant differences in pH were observed among treatments ($p > 0.05$), indicating that inoculation did not markedly influence silage acidity under the conditions of this study. However, inoculation with LAB strains enhanced silage digestibility, as reflected by increased DM loss and gas production, which aligns with findings reported by Nguyen *et al.* (2020), who demonstrated improved DM and organic matter (OM) digestibility following LAB application.

For 14-day-old corn silage, treatments CLB, CLK, and CLR resulted in significantly higher DM loss compared to the Control ($p < 0.05$), whereas CLH and CLO did not differ significantly from one another ($p > 0.05$). At 21 and 28 days, CLK continued to show significantly greater DM loss than the other treatments ($p < 0.05$), indicating sustained enhancement of silage degradation. Crops such as maize and certain grasses, which contain high levels of WSC and exhibit low buffering capacity, tend to favor LAB activity and reduce dry matter (DM) loss. Conversely, legumes such as alfalfa and red clover are more prone to undesirable clostridial fermentation due to their low WSC content and high buffering capacity (Dinic *et al*., 2010). Thus, increasing the availability of fermentable carbohydrates in silage is critical to ensure rapid acidification and prevent DM losses associated with inefficient fermentation. Oliveira *et al.* (2011) reported that the combination of homo and heterofermentative LAB strains promotes the production of ferulic acid esterase an enzyme that breaks bonds between lignin and cell wall carbohydrates thereby improving fiber digestibility. Similarly, Carvalho *et al.* (2020) emphasized the importance of selecting LAB strains that are specific to the type of forage being ensiled, due to variations in fermentation dynamics across different plant materials.

Gas production profiles, presented in Figures 1–3 for days 14, 21, and 28 respectively, revealed a similar trend. After 24 hours of incubation on day 14, gas production from CLB, CLH, CLK, and CLO exceeded that of the control, although differences were not statistically significant ($p > 0.05$), with CLB exhibiting the highest production. On days 21 and 28, all inoculated treatments, except CLK, demonstrated increased gas production compared to the control, while CLK showed a significant reduction ($p < 0.05$). Notably, CLB, CLO, and CLR consistently produced higher gas volumes than CLH and Control across all time points. The blank treatment exhibited the lowest gas production values ($p < 0.05$), confirming the necessity of substrate for fermentation. The inclusion of LAB strains, particularly in the CLB, CLO, and CLR treatments, significantly improved the *in vitro* digestibility of corn silage, as evidenced by enhanced DM loss and gas production ($p < 0.05$). The current study supports these findings, demonstrating that LAB inoculants positively influenced in vitro digestibility after 24 hours of incubation, consistent with results reported by Filya and Weinberg *et al.* (2007). These differences in gas production may be attributed to the enzymatic breakdown of structural

Table 1. Mean of the pH, dry matter loss and gas production using corn silage substrate inoculation with lactic acid bacteria

Treatment	pH	DM Loss (%)	Gas (ml)
14 Day			
Blank	7.4^{a}	$40.8±0.4^{b}$	$2±0.0^{d}$
Control + Rumen microflora	7.4^{a}	$42.0±0.5^{b}$	$31.6±0.5^{b}$
CLB + Rumen microflora	7.3^{a}	$44.9±0.5^{a}$	$32.9±0.4^{ab}$
CLH + Rumen microflora	7.3^{a}	42.5±0.3b	$31.9±0.1^{ab}$
CLK + Rumen microflora	7.3^{a}	$45.6±0.4^{a}$	$29.1±0.1^{c}$
CLO + Rumen microflora	7.3^{a}	$42.1±0.5^{b}$	$32.2±0.6^{ab}$
CLR + Rumen microflora	7.3^{a}	$44.3±0.8^{a}$	$33.0±0.15^{a}$
21Day			
Blank	7.4^{a}	$40.0±0.5^{d}$	$2±0.0^{d}$
Control + Rumen microflora	7.4^{a}	$42.1±0.5^{c}$	$32.3±0.1^{b}$
CLB + Rumen microflora	7.3^{a}	$43.0±0.5^{c}$	$33.4±0.3^{ab}$
CLH + Rumen microflora	7.3^{a}	$43.3±0.3^{c}$	$32.2±0.1^{b}$
CLK + Rumen microflora	7.3^{a}	$47.0±0.5^{a}$	$29.5±0.4^{c}$
CLO + Rumen microflora	7.3^{a}	$45.6±0.3^{ab}$	$34.0±0.5^{a}$
CLR + Rumen microflora	7.3^{a}	$45.1±0.5^{b}$	$34.5±0.3^{a}$
28 Day			
Blank	7.4^{a}	$39.7±0.4^{c}$	$2±0.0^{d}$
Control + Rumen microflora	7.4^{a}	$42.5±0.5^{b}$	$32.6±0.2^{b}$
CLB + Rumen microflora	7.3^{a}	$42.9±0.5^{b}$	$34.0±0.5^{ab}$
CLH + Rumen microflora	7.3^{a}	$42.5±0.3^{b}$	$33.3±0.3^{ab}$
CLK + Rumen microflora	7.3^{a}	$46.5±0.4^{a}$	$29.7±0.4^{c}$
CLO + Rumen microflora	7.3^{a}	$44.6±0.5^{ab}$	$35.0±0.6^{a}$
CLR + Rumen microflora	7.3^{a}	$45.4±0.8^{a}$	$35.2±0.4^{a}$

^{a,b,c} = Means in the same column with different subscripts are different significantly ($p<0.05$). Control: corn silage only, CLB: corn silage + *L. buchneri* , CLH: corn silage + *L. hilgardii*, CLK: corn silage + *L. keifiri*, CLO: corn silage + *L. oris*, CLR: corn silage + *L. rhamnosu*

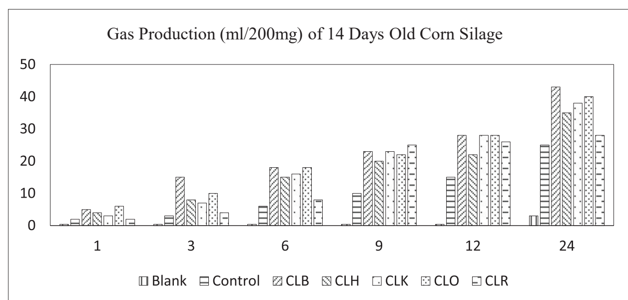

Fig. 1 Gas productions (ml/200mg) of 14 day old corn silage

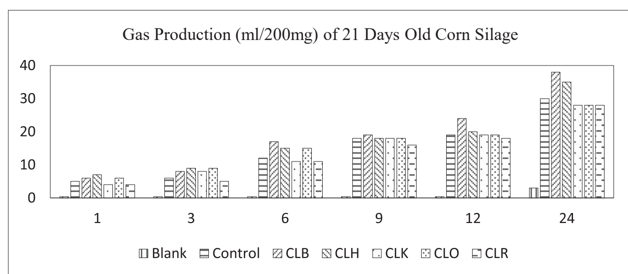

Fig. 2 Gas productions (ml/200mg) of 21 day old corn silage

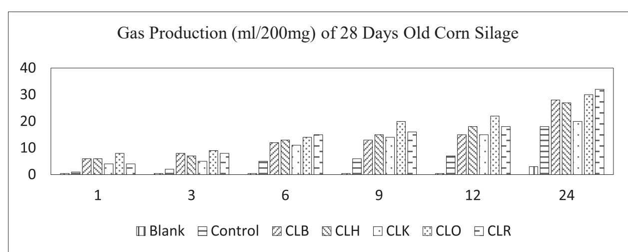

Fig. 3 Gas productions (ml/200mg) of 28 day old corn silage

carbohydrates, such as cellulose and hemicellulose by endogenous enzymes, microbial activity, and acid hydrolysis during fermentation (McDonald *et al*. 1991; Yahaya *et al*. 2000). The degradation of fiber components makes nutrients more accessible for rumen microbes during in vitro incubation, thereby enhancing fermentability and gas production.

Table 2 shows that CLB produced the highest level of acetic acid and propionic acid, with a statistically significant difference ($p < 0.05$) compared to all other treatments. In contrast, Control recorded the lowest acetic acid concentration. The levels of propionic and butyric acids were generally low across treatments. However, CLB yielded the highest concentrations of both, although propionic acid levels among CLB**,** CLH**,** CLK, and CLO did not differ significantly **(**$p > 0.05$**).** The lowest values for both acids were observed in Control. Once in the rumen, lactic acid is fermented by specific lactate-utilizing bacteria such as *Megasphaera elsdenii, Selenomonas ruminantium, Fusobacterium necrophorum,* and *Veillonella parvula,* leading to the production of propionic acid (Russel *et al.* 1997). McGinn *et al.* (2004) noted that the conversion of lactic acid into propionate reduces hydrogen levels and thus inhibits the formation of methane from CO_2 and hydrogen. Supporting this mechanism, Cao *et al*. (2010) observed that sheep fed a total mixed ration with higher lactic acid content exhibited elevated levels of ruminal propionic acid two hours post-feeding compared to those receiving a control diet. These findings align with the hypothesis that silage inoculated with LAB strains producing high lactic acid content can enhance propionate production and potentially mitigate methane emissions. This knowledge can aid in developing more targeted inoculant formulations and fermentation management practices to optimize animal performance while minimizing greenhouse gas emissions.

Overall, the results demonstrated that corn silage treated with bacterial inoculants exhibited enhanced fermentation characteristics compared to untreated silage. This improvement was evidenced by increased gas production, higher *in-vitro* digestibility, and a greater proportion of propionic acid. Notably, the inoculation with *Lactobacillus buchneri* significantly enhanced the digestibility of corn silage when combined with rumen microflora, indicating its potential to improve silage fermentation quality and nutrient availability.

Table 2. The mean value (±SE) of the acetic, propionic, and butyric acid concentration using corn silage with bacteria inoculants as substrate.

Treatment	Acetic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
Initial rumen fluid	60.61	10.54	4.2
Control + Rumen microflora	7.13±0.04c	7.3±0.24c	1.29±0.14ab
CLB + Rumen microflora	20.49±0.20a	9.3±0.24a	1.56±0.24a
CLH + Rumen microflora	16.2±0.24b	8.3±0.24ab	1.26±0.24ab
CLK + Rumen microflora	15.46±0.24b	8.9±0.24ab	1.05±0.24c
CLO + Rumen microflora	14.43±0.24b	8.880.24ab	1.01±0.13c
CLR + Rumen microflora	15.5±0.13b	7.3±0.13c	1.24±0.13ab
Rumen microflora (Blank)	13.1±0.13b	7.6±0.13c	Not detected

[a,b,c] = Means in the same column with different subscripts are different significantly ($p<0.05$). Control: corn silage only, CLB: corn silage + *L. buchneri* , CLH: corn silage + *L. hilgardii*, CLK: corn silage + *L. keifiri*, CLO: corn silage + *L. oris*, CLR: corn silage + *L. rhamnosus*

ACKNOWLEDGEMENT

The authors gratefully acknowledge the research facilities and support provided by University Putra Malaysia.

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