HARVEST MATURITY AND ENSILING DURATION MODULATE FERMENTATION QUALITY, NUTRIENT PROFILE, RUMINAL FERMENTATION, AND METHANE PRODUCTION POTENTIAL OF PERENNIAL RYEGRASS (*Lolium perenne L.*) SILAGE

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ABSTRACT

Optimal forage utilization is vital for sustainable ruminant production. This study evaluated effects of harvest maturity (vegetative, boot, early-heading) and ensiling duration (7, 21, 42 days) on fermentation characteristics, nutritional profile, energy value, *in vitro* ruminal fermentation, and methane (CH_4) production potential of perennial ryegrass (*Lolium perenne L.*) silage under subtropical conditions. Advancing maturity at harvest, from vegetative to early-heading, increased ($P \leq 0.001$) the contents of dry matter (DM; 24.8 to 30.5%), neutral detergent fiber (48.0 to 57.2% DM), and acid detergent fiber (30.3 to 41.2% DM), and decreased ($P \leq 0.001$) the contents of crude protein (18.9 to 10.2% DM) and water-soluble carbohydrates (18.5 to 10.6% DM). These compositional changes resulted in a significant decrease in the *in vitro* DM digestibility (64.4 to 55.9%), total gas production (246 to 201 mL/g organic matter [OM]) and metabolizable energy (9.66 to 8.39 MJ/kg DM), causing a significant increase in CH_4 production (45.6 to 63.2 mL/g OM; $P \leq 0.001$). Vegetative-stage silage exhibited superior fermentation quality with greater lactic acid (7.16% DM), lower pH (4.19), and higher Flieg scores (87.0) compared with early-heading silage ($P \leq 0.05$). Ensiling for 21 days achieved stable fermentation, whereas prolonged storage reduced digestibility and energy values. Significant harvest stage × ensiling duration interactions ($P \leq 0.05$) for various parameters indicated that prolonged storage exacerbated nutrient losses in mature forage. Harvesting at the vegetative to early-boot stage and ensiling for 21 days optimized silage quality while minimizing CH_4 production potential under subtropical conditions.

Keywords: ryegrass silage, fermentation quality, ensiling duration, methane production potential, nutrient digestibility, subtropical conditions, sustainable livestock production.

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INTRODUCTION

Forages are natural, low-cost, and often a major source of nutrients for ruminant livestock under most dietary regimes. Good quality forages are not only the central component and driver of the productivity and profitability of small and medium scale dairy farms in the tropics and subtropics, but also provide the foundation for achieving high efficiency in commercial dairy farms (Jiang et al., 2022; Khan et al., 2023; Haq et al., 2026). In Pakistan, ruminant livestock are predominantly (>80%) reared under subsistence small and medium scale production systems, where the limited availability and poor nutritional quality of forages, particularly during the prolonged winter and summer scarcity periods, severely constrain their productivity (Farooq et al., 2024; Khan et al., 2024a). Moreover, in recent years intensifying climatic challenges have further exacerbated forage production and nutritional quality, creating critical feed gaps and marked fluctuations in quality that threaten the sustainability and profitability of smallholder livestock systems (Khan et al., 2025a; Haq et al., 2026). This background provides strong impetus for the development of good-quality forage resources in terms of high yield, nutritional value and year-round availability.

Ryegrass (*Lolium perenne L.*) produces a high biomass yield of highly digestible, palatable and nutritious forage, and is easy to establish under diverse soil and environmental conditions (Özelçam *et al*., 2015; Guo et al., 2025). When harvested in the late vegetative to early boot stage of maturity, ryegrass provides biomass with superior dry matter (DM) digestibility (> 65%), and higher contents of crude protein (CP; >15%), energy, minerals and vitamins (Taweel, 2004). Its high digestibility value, nutrient composition, and rapid growth rate make it valuable for year-round feeding strategies in intensive dairy systems (Shi et al., 2023). Ryegrass is particularly valued for silage production due to its high yield, excellent nutritional and fermentation characteristics (Guo et al., 2025). These agronomic and nutritional advantages, coupled with high intake potential, make perennial ryegrass silage one of the most widely used forages for high-

producing dairy animals, for which good-quality fiber and an adequate energy and protein supply are critical for sustaining milk yield, growth, and overall performance.

However, the nutritional quality of ryegrass silage is highly variable, being strongly influenced by harvest maturity stage, growing regions, ensiling condition and storage duration (Khan et al., 2012; Ali et al., 2015). Maturity stage at harvest plays an important role in determining the nutritive value of forage. Early vegetative stages typically yield herbage with greater concentrations of CP, water-soluble carbohydrates (WSC), and digestibility. Advancing harvesting to boot or early-heading stages increases biomass yield; however, this is accompanied by a marked increase in neutral detergent fiber (NDF) and lignin contents, leading to lower digestibility, metabolizable energy (ME) and DM intake (Ferreira et al., 2025; Temoche-Socola et al., 2025). Prolonged ensiling durations may enhance lactic acid dominance and pH stability but at the expense of CP degradation, and may increase losses due to extended fermentation and effluent, particularly under hot conditions (Ali et al., 2015; Wang et al., 2023; Winichayakul et al., 2026). Mature ryegrass, with lower soluble carbohydrate content and higher buffering capacity, is more vulnerable to nutrient losses during extended ensiling, often resulting in higher pH and reduced CP preservation (Gordo et al., 2023). The hot summer conditions of the tropics and subtropics are expected to further exacerbate these effects (Ali et al., 2015; Khan et al., 2023). Conversely, early-harvest ryegrass ensiles more efficiently due to greater availability of fermentable substrates, supporting rapid acidification and stable silage quality (Fu et al., 2022).

However, most published evidence on ryegrass ensiling derives from temperate maritime climates of Europe, New Zealand, and south-eastern Australia, where ambient temperature during growth and ensiling, sward buffering capacity, and WSC dynamics differ substantially from the semi-arid subtropical conditions of Pakistan and neighboring South Asian production zones. The harvest-maturity and ensiling-duration thresholds established under temperate conditions have therefore not been validated under the warmer temperatures and shorter, more variable harvest windows of the tropics and subtropics. Specifically, tropical forages generally contain lower concentrations of WSC, higher moisture and fiber contents, and are ensiled under warmer conditions than temperate forages; their fermentation dynamics may therefore respond differently to harvest maturity and ensiling conditions, yet these interactions remain poorly quantified, particularly in South Asian dairy systems. We hypothesized that (i) advancing harvest maturity from the vegetative to early-heading stage would progressively reduce fermentation quality, nutrient value, and in vitro digestibility while increasing methane (CH_4) production potential, and that (ii) these maturity-related declines would be exacerbated, rather than offset, by prolonged ensiling under warm subtropical conditions. To test these hypotheses, this study evaluated three harvest maturity stages and three ensiling durations for their effects on the nutrient profile, fermentation quality, energy value, and *in vitro* ruminal fermentation and CH_4 production potential of perennial ryegrass silage produced under subtropical conditions in Pakistan, with the aim of establishing maturity-by-duration benchmarks and practical recommendations for forage conservation in South Asian dairy systems.

MATERIALS AND METHODS

Experimental site and ryegrass cultivation: The ryegrass was grown in four replicate plots at the research field (34°01′N latitude and 71°32′E longitude) of the University of Agriculture Peshawar, located within the semi-arid subtropical zone of Khyber Pakhtunkhwa province, Pakistan. The climate of the area is characterized by mild winters (average minimum temperature 4–7°C) and hot summers (average maximum temperature 35–40°C), and annual rainfall of about 400–500 mm, concentrated during the monsoon season (July–September). According to regional recommendations, perennial ryegrass seed was sown at 15kg/ha, into plots measuring 2.5 m × 10 m (25 m² total plot area). Basal fertilizer was applied at establishment at a rate of 30–40–40 kg/ha of N–P_2O_5–K_2O, supplemented by seasonal nitrogen top-dressings totaling 120 kg N/ha over the active growth period. The supplementary irrigation was provided as needed to maintain sward establishment. The first two cuts were excluded from experimental silage preparation to allow the sward to establish fully and to avoid the high variability in forage quality and insufficient biomass yield typically observed during the establishment phase. The experimental treatments consisted of three harvest maturities based on regrowth intervals: vegetative stage at 21–28 days regrowth (pre-stem elongation), boot stage at 35–45 days regrowth (inflorescence enclosed in the flag leaf), and early-heading stage at 50–60 days regrowth (inflorescence fully visible). At each stage, grass was cut at approximately 5 cm above ground level to ensure uniformity. A 3-m border area around each plot was discarded to minimize edge effects and within-plot variability.

Ensiling of ryegrass: Harvested forage was immediately chopped to a theoretical length of 2 cm using a mechanical forage cutter to facilitate compaction and anaerobic fermentation. Representative samples from fresh forage were collected at each harvest stage for DM and chemical analysis to determine baseline nutritional composition prior to ensiling. Silage was prepared in laboratory-scale silos at a compaction density of 260 kg DM/m^3, as reported earlier (Khan et al., 2023). Briefly, chopped ryegrass was packed layer by layer into 1-L polyethylene terephthalate bottles, and

compacted manually after each layer of freshly added forage to ensure anaerobic conditions, and achieve the targeted compaction density. While laboratory-scale silos allow precise control of experimental variables, we acknowledge that they may differ from commercial farm-scale silos in oxygen ingress, temperature dynamics, compaction uniformity and fermentation characteristics; therefore, results should be extrapolated to field conditions with caution. Ensiling was carried out under ambient laboratory temperature (25–30°C) for three predetermined durations: 7, 21 and 42 days. Ambient temperature within the ensiling room was continuously monitored throughout the storage period using a calibrated digital data logger. Each treatment (harvest stage × ensiling duration) was replicated six times, resulting in a total of 54 experimental silos (3 maturities × 3 ensiling durations × 6 replicates), with each silo serving as the experimental unit in the statistical analysis. At the end of each storage period, silos were opened, and representative samples were collected for chemical and fermentation analyses.

Silage sampling and chemical analysis: Upon opening the silos, the silage of each replicate silo was mixed, and representative 200 g samples were extracted from each container, thoroughly homogenized, and partitioned into two subsamples. One subsample was immediately analyzed for pH and fermentation characteristics, while the other portion was oven-dried at 60°C for 48 h to a constant weight for subsequent proximate and fiber fraction analyses.

The chemical composition of ryegrass silage was determined according to standard guidelines of AOAC (2000). DM content was determined by oven drying samples at 105°C until constant weight (method 934.01), ash content was obtained by incineration at 550°C in a muffle furnace (method 942.05), and CP was determined using the Kjeldahl method (method 984.13). Ether extract (EE) was determined by Soxhlet extraction with petroleum ether (method 920.39). The NDF, acid detergent fiber (ADF), and acid detergent lignin (ADL) contents were determined sequentially using an ANKOM fiber analyzer (ANKOM Technology, Macedon, NY, USA) adapted from Van Soest et al. (1991). NDF was assayed with heat-stable amylase and expressed inclusive of residual ash. The ADL was determined by sequential solubilization of ADF residues in 72% sulfuric acid. Hemicellulose (HC) was calculated as the difference between NDF and ADF, while cellulose (CEL) was determined by subtracting ADL from ADF. The indigestible NDF (iNDF) fraction was estimated using the equation as described by Traxler et al. (1998) while digestible NDF (dNDF) was computed following the methodology of Soufizadeh et al. (2018).

Silage fermentation characteristics and organic acid profile: For analysis of silage fermentation characteristics 20 g subsample of fresh silage was homogenized with 180 mL of distilled water for 60 s using a laboratory stomacher (BagMixer 400, Interscience, France), and the resulting suspension was filtered through four layers of cheesecloth to obtain a clear extract. The silage pH was determined immediately in the filtrate using a calibrated glass electrode pH meter (SevenEasy, Mettler-Toledo, Switzerland). The concentration of WSC was subsequently quantified from the same aqueous extract following the anthrone colorimetric method (Yemm and Willis, 1954). Briefly, aliquots of the extract were reacted with anthrone reagent in concentrated sulfuric acid at 100°C for 10 min, after which absorbance was measured spectrophotometrically at 630 nm against a glucose standard curve, with results expressed as a percentage of DM.

The concentrations of lactic acid, acetic acid, and butyric acid were quantified using high-performance liquid chromatography (Hitachi, L-2400, Ibaraki, Japan). The silage extract was initially centrifuged at 10,000 × g for 10 min and subsequently filtered through a 0.45 µm polyethersulfone membrane filter. Chromatographic separation was achieved using an Aminex HPX-87H ion-exclusion column (300 × 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA) maintained at an oven temperature of 60°C, with 5 mM H_2SO_4 as the isocratic mobile phase at a flow rate of 0.6 mL/min. Organic acids were detected using a UV detector at 210 nm (Hitachi L-2400) with sample injection handled via an autosampler (Hitachi L-2200).

Flieg score: The Flieg fermentation quality score was calculated using the modified formula of Zimmer and Wermke (1986):

$$\text{Flieg score} = 205 + (2 \times \text{DM\%}) - (40 \times \text{pH})$$

Fermentation quality was categorized as bad (≤40), medium (40–59), good (60–79), or excellent (≥80). We acknowledge that this equation was originally developed for maize silages with lower DM contents and that its applicability to higher-DM grass silages (>25% DM) remains debated; therefore, Flieg scores in the present study should be interpreted alongside pH, organic acid profiles, and WSC concentrations to provide a comprehensive assessment of fermentation quality.

***In vitro* ruminal fermentation, total gas and methane production:** *In vitro* DM digestibility (IVDMD) was determined using ANKOM DAISY incubator system (ANKOM Technology, Macedon, NY, USA) following a modification of the two-stage technique of Tilley and Terry (1963). Briefly, samples were incubated in the rumen fluid buffer matrix for 48 h, followed by a 1 h extraction with a neutral detergent solution (NDS) to remove microbial cell walls and soluble matter, yielding true rather than apparent digestibility. All animal procedures were approved by the

Table 1. Main effects of harvest stage and ensiling duration on chemical composition of ryegrass silage

Parameters	DM (% of FM)	Chemical composition (% DM)								
		CP	NDF	ADF	ADL	Ash	EE	WSC	HC	CEL
Harvest stage										
Vegetative	24.8^{c}	18.9^{a}	48.0^{c}	30.3^{c}	3.64^{c}	9.80^{a}	3.17^{a}	18.5^{a}	17.6^{a}	26.6^{c}
Boot	27.5^{b}	14.1^{b}	53.9^{b}	35.4^{b}	5.01^{b}	7.40^{b}	2.72^{b}	14.8^{b}	18.5^{a}	30.5^{b}
Early heading	30.5^{a}	10.2^{c}	57.2^{a}	41.2^{a}	7.18^{a}	5.08^{c}	2.18^{c}	10.6^{c}	15.9^{b}	34.0^{a}
SEM	0.20	0.21	0.30	0.26	0.120	0.281	0.115	0.34	0.42	0.29
Ensiling duration (days)										
7	25.4^{c}	16.3^{a}	50.6^{c}	33.8^{c}	4.16^{c}	8.49^{a}	2.83	17.1^{a}	16.8^{b}	29.6^{b}
21	27.5^{b}	14.3^{b}	52.2^{b}	35.8^{b}	5.31^{b}	7.47^{b}	2.74	14.1^{b}	16.4^{b}	30.5^{ab}
42	30.0^{a}	12.6^{c}	56.3^{a}	37.4^{a}	6.36^{a}	6.31^{c}	2.45	12.8^{c}	18.9^{a}	31.0^{a}
SEM	0.22	0.21	0.30	0.26	0.120	0.281	0.115	0.34	0.42	0.29
Significance										
HS	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	0.0013	≤.0001
ED	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	0.0001	0.1309	≤.0001	0.0010	0.0095

[abc]Means within the same column within harvest stage (HS), ensiling duration (ED) bearing different superscripts differ at $P \leq 0.05$; SEM, standard error of mean

HS, harvest stages; ED, ensiling duration;

DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; HC, hemicellulose; CEL, cellulose; EE, ether extract; WSC, water-soluble carbohydrates; FM, fresh matter.

Table 2. Interaction effects of harvest stage × ensiling duration on chemical composition of ryegrass silage

Harvest Stage	ED (days)	DM (% of FM)	Chemical composition (% DM)								
			CP	NDF	ADF	ADL	Ash	EE	WSC	HC	CEL
Vegetative	7	22.2^{e}	21.2	43.4^{f}	28.9	2.59	11.2	3.37	21.3	14.4^{c}	26.3
	21	24.2^{d}	18.5	47.7^{e}	30.3	3.56	9.81	3.15	17.4	17.4^{bc}	26.7
	42	28.0^{c}	16.9	52.8^{d}	31.7	4.74	8.35	2.99	16.8	21.2^{a}	26.9
Boot	7	25.1^{d}	16.1	53.3^{cd}	33.9	4.26	8.18	2.90	17.7	19.4^{ab}	29.6
	21	27.3^{c}	14.3	52.3^{d}	35.2	5.24	7.54	2.79	14.5	17.2^{bc}	29.8
	42	30.2^{ab}	12.1	56.2^{b}	37.2	5.48	6.48	2.47	12.3	19.1^{ab}	31.7
Early heading	7	28.9^{bc}	11.7	55.1^{bc}	38.5	5.63	6.07	2.34	12.3	16.6^{bc}	32.8
	21	30.9^{a}	10.2	56.6^{b}	42.0	7.04	5.07	2.18	10.3	14.6^{bc}	34.9
	42	31.9^{a}	8.80	59.9^{a}	43.3	8.87	4.10	2.03	9.3	16.6^{bc}	34.4
SEM		0.35	0.36	0.52	0.45	0.346	0.49	0.199	0.58	0.73	0.50
Significance											
HS × ED		0.0035	0.2552	≤.0001	0.0913	0.0846	0.7846	0.9101	0.2607	0.0007	0.1355

[abcde]Means within the same column within harvest stage (HS), ensiling duration (ED) or HS×ED bearing different superscripts differ at $P \leq 0.05$; SEM, standard error of mean

HS, harvest stages; ED, ensiling duration; HS × ED; interaction of harvest stage and ensiling duration

DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; EE, ether extract; HC, hemicellulose; CEL, cellulose; WSC, water soluble carbohydrates; FM, fresh matter

institutional ethical committee. Rumen fluid was harvested prior to morning feeding from two rumen-fistulated Holstein-Friesian cattle (450 ± 25 kg body weight) maintained on a total mixed ration consisting of 60% maize silage, 20% alfalfa hay, 15% concentrate, and 5% mineral-vitamin supplement (DM basis). Animals were adapted to the experimental ryegrass silages for 14 days prior to rumen fluid collection to ensure microbial acclimation to the test substrates. Rumen fluid was strained through four layers of cheesecloth and homogenized with McDougall's artificial saliva buffer (pH 6.9) in a 1:4 (v/v) ratio under continuous CO_2 flushing. Subsamples (0.5 g DM) of dried, ground forage or silage were sealed in F57 filter bags and incubated at 39°C for 48 h, followed by a secondary 48-h incubation in neutral detergent solution at 100°C using an ANKOM Fiber Analyzer. Bags were dried at 105°C to determine residual DM and combusted at 550°C for ash determination. In vitro total gas production (IVGP) and CH_4 production during 72-h rumen fermentation

in buffered rumen fluid were measured using an automated pressure transducer system (ANKOM Technology) calibrated against standard CH_4: CO_2 mixtures (60:40 v/v). Blanks (buffer + rumen fluid without substrate) were included in each analytical run and subtracted from sample values to correct for background gas production. Gas volumes were corrected to standard temperature and pressure.
IVDMD was calculated as follows:

$$IVDMD\ (\%)\ =\ 100\ -\ \left[\frac{W_3 - (W_1 \times C_1)}{W_2} \times 100\right]$$

where W_1 is the bag tare weight (g), W_2 is initial sample DM weight (g), W_3 is final bag plus residue DM weight after the complete two-stage incubation (g), C_1 is blank bag correction factor (blank bag weight after incubation divided by original blank bag weight). The values of digestible energy (DE), metabolizable energy (ME), net energy for lactation (NEL) and total digestible nutrients (TDN) were determined according to NRC (2001). The *in vitro* total gas (IVGP) and CH_4 production during 72 h rumen fermentation in buffered-rumen fluid followed Khan et al. (2021).

Statistical analysis: Data on the effects of harvest stage, ensiling duration, and their interaction on silage fermentation characteristics, nutrient profile, IVDMD, IVGP, and CH_4 production were analyzed using the PROC MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA). The statistical model was:

$$Yijk = \mu + HSi + EDj + (HS \times ED)ij + \varepsilon ijk,$$

where μ is the overall mean, HSi is the fixed effect of harvest stage i, EDj is the fixed effect of ensiling duration j, $(HS \times ED)_{ij}$ is the fixed interaction effect between harvest stage and ensiling duration, and εijk is the random error associated with the *k*th experimental unit within the *ij*th treatment combination. For the 3 × 3 factorial arrangement with six replicate silos per treatment combination (54 experimental units), the F-tests for harvest stage, ensiling duration, and their interaction had numerator and denominator degrees of freedom of 2 and 45, 2 and 45, and 4 and 45, respectively. The residual error had 45 degrees of freedom. For parameters with significant ($P \leq 0.05$) F-tests, post hoc pairwise comparisons among least-squares means were performed using the Tukey-Kramer adjustment. Mean separation letters were generated using the PDMIX800 SAS macro (Saxton, 1998). All results are reported as least-squares means with standard errors of the mean (SEM), and significance was declared at $P \leq 0.05$ unless otherwise stated.

RESULTS

Effects of maturity and ensiling duration on nutritional profile of ryegrass silage: The effect of harvest maturity, ensiling duration, and their interaction on the nutritional profile of ryegrass silage is shown in Tables 1 and 2. Advancing maturity from the vegetative to the early-heading stage altered ($P \leq 0.01$) the contents of all measured chemical components in ryegrass silage (Table 1). Notably, with advancing maturity from vegetative to early-heading stage, the contents of DM (24.8 to 30.5%), NDF (48.0 to 57.2%), ADF (30.3 to 41.2%) and ADL (3.64 to 7.18%) increased ($P \leq 0.05$), whereas the contents of CP (18.9 to 10.2%) and WSC (18.5 to 10.6%) decreased ($P \leq 0.05$). Likewise, the EE and ash also decreased progressively with advancing maturity ($P \leq 0.05$). Prolonged ensiling (42 *vs.* 7 d) increased the contents of DM, NDF, ADF, ADL, and HC, and decreased the contents of CP and WSC ($P \leq 0.05$; Table 1). Except for EE ($P = 0.1309$), ensiling duration affected all measured chemical components ($P \leq 0.0095$; Table 1). Moreover, Table 2 shows that significant harvest stage × ensiling duration interactions were detected for DM ($P = 0.0035$), NDF ($P \leq 0.0001$), and HC ($P = 0.0007$), indicating that the magnitude of compositional changes in these chemical components was dependent on both maturity at harvest and ensiling duration.

Effects of Harvest Maturity and Ensiling Duration on Ryegrass Silage Fermentation Quality: Fermentation quality was strongly modulated by harvest maturity and ensiling duration (Tables 3 and 4). Except for the lactic acid to acetic acid ratio (LA:AA), all measured fermentation parameters were significantly affected by maturity ($P \leq 0.01$; Table 3). Silage prepared from crop harvested at vegetative stage exhibited the highest ($P \leq 0.05$) concentrations of lactic acid (7.16% DM) and acetic acid (1.47% DM), resulting in the lowest ($P \leq 0.05$) pH (4.19) and highest ($P \leq 0.05$) Flieg score

Table 3. Main effects of harvest stage and ensiling duration on fermentation characteristics of ryegrass silage

Parameters	Organic acids (% DM)			LA:AA	Flieg score	pH
	LA	AA	BA			
Harvest stage						
Vegetative	7.16^{a}	1.47^{a}	0.08^{b}	5.17	87.0^{a}	4.19^{c}
Boot	6.02^{b}	1.11^{b}	0.16^{a}	5.60	82.5^{b}	4.44^{b}
Early heading	5.03^{c}	0.98^{c}	0.17^{a}	5.26	79.8^{c}	4.66^{a}
SEM	0.176	0.026	0.016	0.185	0.42	0.009
Ensiling duration (days)						
7	4.79^{c}	0.80^{c}	0.13	6.10^{a}	75.0^{c}	4.52^{a}
21	7.72^{a}	1.24^{b}	0.15	6.22^{a}	82.6^{b}	4.43^{b}
42	5.70^{b}	1.52^{a}	0.14	3.72^{b}	91.6^{a}	4.33^{c}
SEM	0.177	0.026	0.017	0.186	0.42	0.010
Significance						
HS	≤.0001	≤.0001	0.0027	0.2641	≤.0001	≤.0001
ED	≤.0001	≤.0001	0.8300	≤.0001	≤.0001	≤.0001

^{abc}Means within the same column within harvest stage (HS), ensiling duration (ED) bearing different superscripts differ at $P \leq 0.05$; SEM, standard error of mean
LA: lactic acid, AA: Acetic acid, BA: butyric acid, LA:AA, lactic acid to acetic acid ratio

Table 4. Interaction effects of harvest stage × ensiling duration on fermentation characteristics of ryegrass silage

HS	ED (days)	Organic acids (% DM)			LA:AA	Flieg score	pH
		LA	AA	BA			
Vegetative	7	5.69	1.06^{d}	0.08	5.37^{ab}	78.8^{d}	4.26
	21	8.69	1.29^{bc}	0.09	6.73^{a}	86.1^{c}	4.18
	42	7.09	2.07^{a}	0.08	3.43^{c}	96.1^{a}	4.12
Boot	7	4.96	0.75^{e}	0.18	6.64^{a}	74.1^{e}	4.53
	21	7.87	1.33^{b}	0.15	5.94^{a}	81.1^{d}	4.46
	42	5.23	1.24^{bcd}	0.16	4.21^{bc}	92.3^{b}	4.33
Early heading	7	3.72	0.60^{e}	0.14	6.28^{a}	72.0^{e}	4.77
	21	6.59	1.10^{cd}	0.20	5.99^{a}	80.7^{d}	4.65
	42	4.77	1.25^{bc}	0.19	3.51^{c}	86.6^{c}	4.55
SEM		0.306	0.045	0.029	0.322	0.73	0.017
Significance							
HS × ED		0.3700	≤.0001	0.6015	0.0384	0.0218	0.1548

Means within the same column within HS×ED bearing different superscripts differ at $P \leq 0.05$; SEM, standard error of mean; harvest stage (HS), ensiling duration (ED)
LA: lactic acid, AA: Acetic acid, BA: butyric acid, LA:AA, lactic acid to acetic acid ratio;

(87.0). As maturity advanced to early heading, lactic acid decreased by 29.7% ($P \leq 0.001$), pH increased by 0.47 units ($P \leq 0.001$). Butyric acid remained very low across all treatments (≤0.20% DM), with the lowest concentration in vegetative silage (0.08% DM) and slightly higher values in boot and early-heading silages (0.16–0.17% DM; $P \leq 0.05$). The consistently low butyric acid concentrations can be attributed to the rapid pH decline (pH ≤ 4.26 within 7 days) in vegetative coupled with adequate DM content (22.2%) and high initial WSC, which effectively suppressed clostridial proliferation. The Flieg score improved markedly from 75.0 at 7 days to 91.6 at 42 days ($P \leq 0.05$), with a concomitant pH reduction from 4.52 to 4.33, indicating that primary fermentation was largely complete by 21 days and that extended storage enhanced microbial stability. The LA:AA ratio was unaffected by harvest stage ($P = 0.2641$), averaging 5.17, 5.60, and 5.26 for vegetative, boot, and early-heading stages, respectively.

A significant harvest stage × ensiling duration interaction was observed for AA, LA:AA ratio, and Flieg score ($P \leq 0.0384$, Table 4), reflecting maturity-dependent shifts in fermentation dynamics during storage. For instance, the

increase in acetic acid from 7 to 42 days was more pronounced in vegetative (1.06% to 2.07% DM) than in early-heading silage (0.60% to 1.25% DM), consistent with greater initial WSC availability may reflect heterofermentative metabolism in younger herbage.

Effect of harvest maturity and ensiling duration on energy values, ruminal fermentation and methane production potential of ryegrass silage: Data on the effect of harvest maturity and ensiling duration on NDF digestibility, energy values, *in vitro* ruminal fermentation and CH_4 production potential of ryegrass silage are summarized in Tables 5 and 6. Harvest stage was the primary determinant of NDF digestibility, energy values, *in vitro* fermentability and CH_4 production ($P \leq 0.01$; Table 5). The proportion of iNDF increased nearly two-fold, from 8.74% DM in vegetative silage to 17.2% DM at early heading stage. As a result, IVDMD decreased from 64.4 to 55.9% and IVGP from 246 to 201 mL/g OM. In contrast, the CH_4 production increased from 45.6 to 63.2 mL/g OM. The DE (11.6 to 10.1 MJ/kg DM), ME (9.66 to 8.39 MJ/kg DM), and NEL (5.50 to 4.53 MJ/kg DM) also exhibited marked ($P \leq 0.05$) decrease from vegetative to early heading stage. Similarly, RFV decreased from 125.9 to 92.0 as maturity advanced from vegetative to early heading stage.

Table 5. Digestibility, energy values and methane production potential of ryegrass silage as affected by harvest stage, ensiling duration

Parameters	Digestibility (%)				Energy parameters (MJ/kg DM)				TDN (%)	RFV
	iNDF	dNDF	IVDMD	IVGP (mL/g OM)	CH_4	DE	ME	NEL		
Harvest Stage										
Vegetative	8.74^{c}	39.2^{b}	64.4^{a}	246^{a}	46.4^{c}	11.6^{a}	9.66^{a}	5.50^{a}	65.3^{a}	125.9^{a}
Boot	12.0^{b}	41.9^{a}	60.4^{b}	220^{b}	55.2^{b}	10.9^{b}	9.07^{b}	5.05^{b}	61.3^{b}	104.4^{b}
Early heading	17.2^{a}	39.9^{ab}	55.9^{c}	201^{c}	63.2^{a}	10.1^{c}	8.39^{c}	4.53^{c}	56.7^{c}	92.0^{c}
SEM	0.479	0.56	0.60	5.67	2.34	0.04	0.030	0.023	0.20	0.86
Ensiling duration (days)										
7	9.98^{c}	40.6	61.7^{a}	233^{a}	50.7^{b}	11.1^{a}	9.26^{a}	5.20^{a}	62.6^{a}	115.4^{a}
21	12.7^{b}	39.5	60.1^{b}	221^{b}	54.7^{ab}	10.8^{b}	9.02^{b}	5.01^{b}	61.0^{b}	108.1^{b}
42	15.3^{a}	41.0	58.9^{c}	213^{b}	58.9^{a}	10.6^{c}	8.83^{c}	4.87^{c}	59.8^{c}	98.8^{c}
SEM	0.48	0.56	0.60	5.67	2.34	0.04	0.030	0.023	0.20	0.86
Significance										
HS	≤.0001	0.0088	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001
ED	≤.0001	0.1489	≤.0001	0.0063	0.0063	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001

Means within the same column within harvest stage (HS), ensiling duration (ED) bearing different superscripts differ at $P \leq 0.05$;
SEM, standard error of mean, HS, harvest stages; ED, ensiling duration;
IVDMD, in vitro dry matter digestibility; IVGP, *in vitro* total gas production; iNDF, indigestible neutral detergent fiber; dNDF, digestible neutral detergent fiber; TDN, total digestible nutrients; CH_4, methane
RFV, relative feed value (RFV = DDM × DMI / 1.29; Linn and Martin, 1989), where DDM = 88.9 − (0.779 × ADF%) and DMI = 120 / NDF%; ME, metabolizable energy; DE, digestible energy; NEL, net energy for lactation.

Except for dNDF, ensiling duration also had a significant influence on *in vitro* fermentability and CH_4 production, and estimated energy values of ryegrass silage ($P \leq 0.01$; Table 5). With the increase in ensiling duration from 7 to 42 days, the iNDF and CH_4 production increased, whereas IVDMD, IVGP, DE, ME and NE values decreased ($P \leq 0.05$). A significant interaction of harvest stage × ensiling duration was detected for dNDF ($P = 0.0081$, Table 6) and RFV ($P \leq 0.001$), indicating that the combined effects of harvest maturity and ensiling duration significantly influenced fiber digestibility and overall feed value.

Table 6. Interaction effects of harvest stage × ensiling duration on digestibility, energy values, and methane production potential of ryegrass silage

Harvest Stage	ED (days)	Digestibility (%)				Energy parameters (MJ/kg DM)				TDN (%)	RFV
		iNDF	dNDF	IVDMD	IVGP	CH_4	DE	ME	NEL		
Vegetative	7	6.2	37.2^{b}	65.5	260	42.2	11.8	9.82	5.63	66.4	140.5^{a}
	21	8.6	39.1^{ab}	64.4	243	46.7	11.6	9.66	5.50	65.3	125.6^{b}
	42	11.4	41.5^{ab}	63.3	233	50.3	11.4	9.50	5.38	64.2	111.5^{c}
Boot	7	10.2	43.1^{a}	61.6	228	51.1	11.1	9.24	5.19	62.5	107.6^{c}
	21	12.7	39.6^{ab}	60.6	219	54.3	10.9	9.09	5.07	61.5	107.8^{c}
	42	13.2	43.1^{a}	59.0	210	59.2	10.6	8.86	4.89	59.9	97.7^{d}
Early heading	7	13.5	41.6^{ab}	58.1	211	58.7	10.4	8.71	4.78	58.9	98.1^{d}
	21	16.9	39.7^{ab}	55.3	201	63.2	9.96	8.30	4.46	56.2	90.9^{de}
	42	21.3	38.6^{b}	54.3	195	67.3	9.77	8.15	4.34	55.1	87.2^{e}
SEM		0.83	0.96	1.35	8.75	3.02	0.063	0.053	0.040	0.053	1.49
Significance											
HS × ED		0.0850	0.0081	0.0922	0.4231	0.4231	0.0932	0.0811	0.0937	0.0922	≤.0001

Means within the same column within HS×ED bearing different superscripts differ at $P \leq 0.05$; SEM, standard error of mean
HS, harvest stages; ED, ensiling duration; HS × ED; interaction of harvest stage and ensiling duration
IVDMD, in vitro dry matter digestibility; IVGP, *in vitro* total gas production; iNDF, indigestible neutral detergent fiber; dNDF, digestible neutral detergent fiber; TDN, total digestible nutrients; CH_4, methane
RFV, relative feed value (RFV = DDM×DMI/1.29, Linn & Martin, 1989); ME, metabolizable energy; DE, digestible energy; NEL, net energy for lactation.

DISCUSSION

The present study provides comprehensive data on the effects of harvest maturity and ensiling duration on in-silo fermentation characteristics, nutrient profile, fiber digestibility, energy values, and *in vitro* ruminal fermentation and CH_4 production potential of perennial ryegrass silage produced under subtropical conditions. Our findings demonstrate that both harvest maturity and ensiling duration substantially influence silage quality. However, maturity at harvest emerged as the dominant factor determining fermentation quality, nutritional value, and environmental impact.

The present study demonstrated that advancing harvest maturity from the vegetative to the early-heading stage markedly influenced all chemical constituents of perennial ryegrass silage. The DM content increased progressively from 24.8% at the vegetative stage to 27.5% at boot, and 30.5% at early-heading, reflecting progressive lignification of cell walls and reduced moisture content as plants transitioned from vegetative to reproductive growth (Colas et al., 2022; Leddin et al., 2022). In contrast, the amount of CP declined sharply with advancing maturity, falling from 18.9% at the vegetative stage to 10.2% at early heading, which arises from the dilution of leaf protein by stem expansion and the redirection of nitrogen from photosynthetic enzymes and soluble proteins to structural and reproductive tissues (Stirling et al., 2022). Similar reductions in CP have been reported in King grass (Long et al., 2022) and small-grain grasses under Mediterranean and temperate climates, underscoring the trade-off between yield and nutritive quality (Ferreira et al., 2025). Simultaneously, NDF, ADF, and ADL increased due to the decrease in leaf-to-stem ratio, progressive deposition of secondary cell-wall polymers, and thickening of sclerenchyma and xylem tissues (Colas et al., 2022; De Zutter et al., 2024).

From a mechanistic perspective, the most critical quality determinant is the increased recalcitrance of the cell-wall matrix with maturity. Lignin forms covalent ester and ether cross-links with hemicellulose ferulic acid residues, physically occluding cellulose microfibrils and rendering them inaccessible to rumen microbial cellulases and hemicellulases (Tedeschi et al., 2023; Khan et al., 2024b; Khan et al., 2025b). The decrease in CP and increase in NDF content and cell wall recalcitrance could explain the reduction in IVDMD and IVGP and increase in CH_4 production potential with advancing maturity of ryegrass from vegetative to early heading stage in the present study (Khan et al., 2015; Khan et al., 2021). Lignin forms covalent cross-links with hemicellulose, creating a physical barrier that restricts microbial enzyme access to fermentable polysaccharides (Sufyan et al., 2024; Khan et al., 2025b). This structural matrix decreases overall digestibility while simultaneously increasing the indigestible NDF fraction. The structural barrier is considered a major driver of reduced nutritive value in mature cool-season grasses. Collectively, the results confirm that delayed harvest increases DM and fiber fractions while reducing CP, WSC, and overall digestibility. Harvesting ryegrass at the vegetative or early boot stage represents the most effective management strategy to maximize protein and energy supply for ruminants (Coblentz et al., 2018; De Zutter et al., 2023).

Extending ensiling from 7 to 42 d produced modest compositional shifts, with DM rising largely from fermentation-driven loss that concentrated the residual mass. Comparable patterns have been reported in maize silages, where epiphytic microbiota activity and effluent losses elevate DM concentration during prolonged storage (Gordo et al., 2023; Wróbel et al., 2023). Fiber fractions increased due to concentration effects, induced by the loss of WSC, and other fermentable nutrients. In the present study, WSC declined from 17.1% to 12.8% DM and CP from 16.3 to 12.6% DM between day 7 and 42 of ensiling, due to fermentation and proteolysis, resulting in the indirect increase in fiber fractions. Comparable reductions in CP have been observed with extended storage, despite stable fermentation in Jinmu grain grass and alfalfa silages (Liu et al., 2024a; Tang et al., 2025). Prolonged ensiling duration results in extensive proteolysis with concurrent increase in DM content due to effluent loss, particularly in low DM silages. Therefore, prolonged ensiling results in protein degradation and lower nutritional value, if the ensiling conditions are not optimized. These findings demonstrate that the chemical composition and fermentation dynamics of ryegrass silage are shaped by harvest stage and ensiling duration, with forage quality at harvest playing a decisive role in determining stability and nutritive value during ensiling.

Among the harvest stages, superior fermentation characteristics were observed for the forage ensiled at vegetative stage, characterized by lowest terminal pH (4.19), highest lactic acid concentration (7.16% DM), and highest Flieg score (87.0). In contrast, early heading silages had high pH (4.66), coupled with lower concentration of lactic acid (5.03% DM), and preservation indices (Flieg score = 79.8). With the advancement of maturity there is progressive decrease in the concentration of WSC in ryegrass with a concomitant increase in buffering capacity associated with mineral accumulation and stem lignification during maturity (Kung Jr et al., 2018; Fu et al., 2022; Liu et al., 2024b). Due to higher amount of fermentable substrate, young forages rapidly support the growth of homofermentative lactic acid bacteria (LAB), causing rapid acidification and suppression of proteolytic and clostridial microorganisms, as shown by lower concentration (≤0.18% DM) of butyric acid in the present study (Borreani et al., 2018; Muck et al., 2018). In contrast, in mature ryegrass, the lower concentration of WSC and high DM content can limit LAB proliferation, resulting in a slower pH decline and greater reliance on secondary metabolic pathways. In agreement with our findings, Sousa et al. (2021) reported similar trends for timothy and tall fescue silages, where delayed harvest increased pH by 0.3–0.5 units and decreased lactic acid accumulation by approximately 30% due to diminished WSC availability and elevated buffering capacity with advancing maturity. Similarly, Fan et al. (2022) reported 0.4-unit higher pH and 22% lower lactic acid in perennial ryegrass harvested at early heading stage compared to boot-stage silage, confirming that lower WSC reserves and increased buffering capacity in mature forage reduces the efficiency of homofermentative LAB.

The in-silo fermentation dynamics were altered nonlinearly with the prolongation of ensiling duration from 7 to 42 days in the current study. The concentration of lactic acid peaked at 21 days (7.72% DM) before declining by day 42 (5.70% DM), acetic acid concentrations progressively increased from 0.80% to 1.52% DM with the progression of ensiling duration from 7 to 42 days. Despite the rise in acetic acid, pH continued to decline (4.52 to 4.33), and Flieg scores improved substantially (75.0 to 91.6), indicating that extended anaerobic storage facilitates the completion of primary fermentation and enhances microbial stability. The observed acetic acid accumulation likely reflects the metabolic shift toward heterofermentative LAB or the slow oxidation of residual soluble fractions under prolonged storage, a phenomenon consistently documented in extended grass silage trials where initial rapid acidification transitions to a stabilized ecosystem (Akhtar et al., 2025). This pattern is consistent with the findings of craig et al. (2024), who reported progressive acetic acid accumulation and pH stabilization in silage stored beyond 28 days, attributing these shifts to secondary microbial succession and residual carbohydrate metabolism. Collectively, these results reinforce that harvest maturity is the primary lever for optimizing silage fermentation outcomes, with ensiling duration serving as a secondary management tool to stabilize, rather than fundamentally improve, silage quality.

Next to nutrient composition, the extent of ruminal fermentation is a major determinant of forage quality (Khan et al., 2009; Khan and Habib, 2012; Bezabih et al., 2014). The rate and extent of ruminal fermentation of CP and carbohydrate fractions not only affect the ruminal microbial fermentation efficiency and supply of absorbable nutrients to ruminants (Khan et al., 2014; Huang et al., 2015; Yu et al., 2015). Irrespective of the large variability due to harvest maturity and ensiling duration, the results of the present study showed that ryegrass silage was highly fermentable, particularly when ensiled at early maturity stages. Notably, the IVGP and IVDMD are important indices that reflect the extent of ruminal fermentation, that in turn are strongly correlated with DM intake and nutritive value of forages. On the other hand, there is a strong negative relationship between forage fermentability and CH_4 production (Khan et al., 2021). In the current study, the IVGP decreased from 246 mL/g OM in vegetative-stage silage to 201 mL/g OM in early heading stage, that directly translated into substantial decreases in DE, ME, and NEL. These findings are consistent with contemporary research establishing plant ontogeny as the predominant driver of forage energy density in temperate grasses (Craig et al., 2024). The observed decline in IVDMD and IVGP aligns closely with recent meta-analyses reporting 7–10% reductions in digestibility for perennial ryegrass harvested at heading versus boot stage, attributable primarily to lignification and increased iNDF concentration (Ban et al., 2017; Raffrenato et al., 2017; Refat et al., 2017).

For example, Leddin et al. (2022) reported combined-site seasonal ME values of 10.2–11.8 MJ/kg DM and NDF concentrations of 42.1–50.3% DM for perennial ryegrass cultivars grazed under South-Eastern Australian (temperate) dairy conditions, compared with 8.39–9.66 MJ/kg DM (ME) and 48.0–57.2% NDF recorded here across the maturity gradient under subtropical conditions, indicating that even the best-quality (vegetative-stage) subtropical silage in the present study falls below typical temperate pasture ME values while carrying higher NDF, consistent with accelerated lignification under higher ambient temperature. The progressive increase in iNDF from 8.74% DM in vegetative silage to 17.2% DM in early-heading silage imposes a major constraint on digestibility and predicted DM intake, as iNDF acts as a rumen fill limit that slows passage rate and reduces voluntary intake of high-fiber forages. This mechanism explains the decline in RFV from 125.9 to 92.0 across the maturity gradient, a reduction exceeding standard forage quality thresholds (Hackmann et al., 2008). This decline indicates that delaying harvest beyond the vegetative stage shifts ryegrass silage from high-value to moderate-value feed, with direct implications for ration formulation and supplementation strategies (Wróbel et al., 2025). On the other hand, grasses with greater extent of rumen fermentation are likely to produce less CH_4 than those with a lower extent of fermentation (Khan et al., 2021). Moreover, Bezabih et al. (2014) reported that digestible NDF, cellulose, and hemicellulose are important fiber fractions influencing CH_4 production in the rumen. In the present study, these fiber fractions increased with advancing maturity and ensiling duration, which was associated with increased CH_4 production. Advancing maturity consistently reduces forage energy supply, with digestible, metabolizable and net energy values all declining as plants progress from vegetative to early-heading stages; these reductions are directly linked to the lower digestibility and high CH_4 emission (Gonzalez-Ronquillo et al., 2021). Notably, the estimated 0.97 MJ/kg DM difference in NEL between vegetative and early-heading silage represents an economically meaningful gap for dairy production. Although milk yield was not measured in the present study, assuming a conversion efficiency of 0.65 kg milk per MJ NEL, this difference could translate to approximately 0.6 kg/d lower milk yield per cow when feeding late-harvest versus early-harvest silage, if other dietary components remain unchanged (Craig et al., 2024). However, the actual milk responses depend on numerous factors, including diet composition, DM intake, animal genetics, lactation stage and energy utilization efficiency, therefore, this estimate should be interpreted cautiously.

The present results are further supported by Ozkose et al. (2024), who observed significant declines in metabolizable and net energy of Italian ryegrass with delayed harvest. Li et al. (2024) demonstrated that moderate wilting preserved total digestible nutrients and digestibility in Italian ryegrass silage. Collectively, these findings confirm that harvesting at the vegetative or early boot stage is critical for maintaining the energy value and digestibility of ryegrass silage for ruminants. Ryegrass ensiled at vegetative stage consistently exhibited superior fermentation quality, nutrient profile and energy values, enhanced ruminal fermentability, and reduced CH_4 production across all ensiling durations. In addition, the semi-arid subtropical environment of Peshawar is characterized by higher temperatures and greater evaporative demand, which accelerate stem elongation and lignification, reducing the harvest window for high-quality forage. Comparable studies under South Asian conditions report similar or more pronounced maturity effects. For example, Khan et al. (2023) observed that corn silage ensiled at 25–35°C in Pakistan exhibited faster pH decline but greater proteolysis than temperate benchmarks, while Ali et al. (2015) reported that elevated ensiling temperature accelerated WSC depletion and shifted fermentation toward heterofermentative pathways in maize silage. Our results extend these observations to perennial ryegrass, suggesting that warm ambient temperature and low WSC in mature forage may exacerbate nutrient losses during ensiling.

Practical implications: From a practical standpoint, these findings offer specific guidance for smallholder dairy farmers in Pakistan and similar subtropical regions. Harvesting perennial ryegrass at the vegetative to early-boot stage maximizes CP, WSC, digestibility and energy density while minimizing fiber recalcitrance and CH_4 production potential. An ensiling duration of approximately 21 days appears sufficient to achieve stable fermentation under warm subtropical conditions, as evidenced by peak lactic acid, adequate pH decline, and favorable Flieg scores. Extending storage beyond 21 days yields only marginal fermentation gains while further eroding nutritive and energy value, particularly in more mature forage. Farmers should therefore prioritize early harvest and avoid prolonged storage, especially during hot summer months when ambient temperatures accelerate proteolysis and nutrient losses. Where harvest timing must be delayed due to labor or weather constraints, the use of microbial inoculants or chemical additives should be considered to enhance fermentation efficiency and aerobic stability, although this requires further validation under local field conditions.

Conclusions: This study demonstrates that harvest maturity and ensiling duration significantly influence the nutrient profile, fermentation quality, energy value, and CH_4 production potential of perennial ryegrass silage under subtropical conditions. Advancing maturity increased DM, NDF, ADF, and lignin while reducing CP, WSC, and energy availability, reflecting the physiological shift from leaf protein synthesis to structural carbohydrate deposition. These compositional changes reduced IVDMD and IVGP and increased in vitro CH_4 production potential. Ensiling for 21 days achieved

stable fermentation, whereas prolonged storage to 42 days improved Flieg scores marginally but reduced CP, digestibility, and energy values, particularly in mature herbage. Harvesting at the vegetative to early-boot stage and ensiling for approximately 21 days offers the optimal balance between fermentation stability and nutrient preservation for dairy and beef production systems in subtropical South Asia. Future research should evaluate microbial inoculants, chemical additives, and genotypic variation under on-farm subtropical conditions to further mitigate maturity-ensiling interactions and enhance forage conservation outcomes.

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