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Investigating the use of Chenopodium quinoa to improve rumen biofermentability and reduction of methane and carbon dioxide production

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ABSTRACT

Quinoa forage can be used as a sustainable source of ruminants to reduce environmental pollution. This study aimed to assess the chemical composition, *in vitro* fermentation and *in situ* degradability of quinoa forage in harvestable stages and compare the nutritional value of this forage with alfalfa. Experimental treatments were: Al, alfalfa forage; Q45, Q95 and Q125, quinoa harvested 45, 95 and 125 days after planting respectively. The increment of harvesting time in quinoa increased the quantities of NDFom, ADFom and ADL but reduced the contents of CP, EE, total phenolics (TP), total tannins (TT), *in vitro* organic matter digestibility (IVOMD) and metabolizable energy (ME) ($P < 0.0001$). Total VFAs were decreased in Q125 and Q145 treatments *versus* Al treatment and this VFA decreased with increasing plant age ($P < 0.0001$). The concentration of acetate and the acetate to propionate ratio ($P < 0.0001$) in quinoa forages were lower, while the concentration of propionate was higher than that in the alfalfa ($P = 0.0002$). Applying quinoa forage reduced CH_4 production ($P = 0.0002$) and NH_3-N concentration ($P = 0.0004$), total protozoa ($P < 0.0001$), subfamilies of *Entodiniinae* ($P < 0.0001$) *Ophrioscolecinae* ($P = 0.029$) in comparison with Al. The amounts of fresh and dry quinoa forages/ha and WU and WUE increased with the quinoa growing ($P < 0.0001$). Applying quinoa forage in ruminant's diets may be a substitute answer to ecological problems in some areas where usual plants cannot grow as a result of the salinity and dryness of the soil.

1. Introduction

Global warming and environmental alterations, including drought conditions and a decline in water resources, have recently led to a decrease in the availability of essential forages for livestock, such as alfalfa, corn silage, and grain straw. This situation has also contributed to rising costs associated with livestock products (Adegbeye et al., 2020; Abarghuei & Salem, 2021). Therefore, ruminant nutrition experts have tried to use drought-resistant food sources in feeding livestock to avoid wasting national funds in addition to reducing production costs. Quinoa (*Chenopodium quinoa*) has emerged as a notable plant in recent discussions surrounding sustainable agricultural practices. Quinoa originated in the Andean region, as an important food crop about 7000 years ago (Jaikishun, Li, Yang & Song, 2019; Shitikova, Kukharenkova & Khaliluev, 2022). Because of the special characteristics of quinoa (need for little water requirements, adaptation to soil salinity conditions and difficult weather conditions), studies and development of this crop are ongoing globally (Jaikishun, Li, Yang & Song, 2019; Alkhamisi et al., 2021). There is deficient data on the nutritional properties of quinoa as ruminant feed. Barros-Rodríguez et al. (2018) investigated quinoa seed, whole plant and stem under both *in situ* and *in vitro* conditions. Their findings indicated that quinoa seed and the whole plant are applicable in ruminant diets due to proper chemical composition and digestibility. Additionally, another investigation assessed the application potential of

Abbreviations: A, asymptotic gas production; *a*, water-soluble fraction; $a + b$, the potential degradability; Al, alfalfa forage; ADFom, ash-free acid detergent fiber; ADL, lignin; ADS, apparent degraded substrate; *b*, insoluble but fermentable fraction; CP, crude protein; CPP, crude protein production; DF, dry forage; DM, dry matter; ED, the effective degradability; EE, ether extract; FF, fresh forage; GP_{24}, biogas production at the 24 h of fermentation; GY_{24}, gas yield at the 24 h of fermentation; *L*, lag time; IVOMD, *in vitro* organic matter digestibility; ME, metabolisable energy; MP, microbial protein synthesis; N, nitrogen; NDFom, ash-free neutral detergent fiber; NTP, non-tannin phenol; OM, organic matter; PF_{24}, partitioning factor, at the 24 h of incubation; PSMs, Plant secondary metabolites; PVPP, polyvinylpyrrolidone; RFV, relative forage value; TP, total phenols; TT, total tannin; WU, water use; WUE, water use efficiency; μ, fermentation rate.

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three quinoa cultivars (Giza 1, Rosada and Q102) in three stages of harvesting (start of seeding, seed milking and whole maturity) was tested using the *in vitro* method. The results suggested that these quinoa varieties could be effectively utilized for forage production during the autumn season (Kardooni, Tavoosi, Mahdavi Majd, Taheri Dezfoli & Anafjeh, 2020).

On the other hand, Currently, the role of ruminants in pollution and increasing the temperature of the environment (ruminal NH_3-N loss and production of greenhouse gases including CH_4 and CO_2) is very important (Króliczewska, Pecka-Kiełb & Bujok, 2023; Wang et al., 2024). Various strategies are being implemented to reduce the production of these gases in livestock. One of these suggested strategies is the use of forage management, which involves the selection of forage varieties with enhanced digestibility and the incorporation of plants that contain plant secondary metabolites (PSMs) to improve rumen fermentation. The quinoa contains PSMs such as phenolics, tannins and saponins. In research reported that quinoa forage contains 1.5–7.0 g/kg DM total phenolic and 0.5–6.0 g/kg DM total tannin (Ranjbar, Rouzbehan & Abarghuei, 2024). Research indicates that these metabolites can improve livestock performance by influencing rumen microorganisms and optimizing fermentation processes, such as decreasing NH3-N loss and reducing the production of CH_4 and CO_2 (Attri et al., 2020; Cardoso-Gutierrez et al., 2021; Karimi, Abarghuei, Amiri Ghanatsaman, Agah & Boostani, 2023).

Quinoa may be regarded as an appropriate forge to achieve sustainable agriculture in many regions. However, there is a scarcity of information regarding the impact of using quinoa forage and their PSMs on the ruminal ecosystem, as well as its environmental implications compared to the use of alfalfa as a forage alternative. So, this study was conducted to determine the chemical composition, *in vitro* biofermentation and degradability of quinoa forage (Sjama variety) in harvestable stages (budding stage, 10% flowering stage, 125 days of growth, 145 days of growth) and comparing this forage with alfalfa.

2. Materials and methods

2.1. Preparation of quinoa forages

The research was conducted in two main phases. The first phase consisted of planting, cultivating and harvesting the quinoa plant of Sjama variety at the research farm of Agricultural and Natural Resources Research and Education Center, Shiraz (Latitude 30 ° and 3 mins north, longitude 53 ° and 7 mins east and with a height of 1892 m above sea level). The second phase was conducted *in vitro* experiments. The region has mild, cold weather with a usual annual rainfall of 300 mm per year. The temperature ranges from a minimum of minus 4 ° Celsius to a maximum of 38 ° Celsius.

The quinoa plant (Sjama variety) was cultivated and harvested at the expected stages. The seeds were planted in March in the middle of the crop rows at a distance of 1–2 cm in the soil depth. Six plots were considered for each treatment. A total of 200 kg of urea fertilizer was used per hectare. Urea fertilization was carried out at the stages of 4 to 6 leaves, budding and flowering.

Each plot included three lines at a distance of 70 cm from each other and with a length of four meters, a surface equivalent to 12 m^2. The first irrigation was done immediately after the seed was planted, and subsequent irrigations were done every 15 days.

Harvestable stages were 1- Q45 (budding stage, quinoa harvested 45 days after planting), 2- Q95 (10% flowering stage, quinoa harvested 95 days after planting), 3- Q125 (before milk stage, quinoa harvested 125 days after planting) and 4- Q145 (before milk stage, quinoa harvested 145 days after planting).

To assess plant performance at various harvest stages, the plots were completely harvested and the green forage weight of each plot was determined in the field. Sampled forages were crushed. A sample of 100 g of green forage was collected from each plot, and the quantity of DM was determined in the laboratory using an oven (48 h and 60 °C). The forages were dried, and powdered to pass a 1 mm mesh and stored for subsequent analyses.

2.2. Biofermentation study

The experiment was carried out according to The Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) guidelines. All procedures and guidelines involving animals were approved by the Animal Experiment Committee at Research Institute of Animal Science, Iran. A trial was conducted to assess the parameters of *in vitro* biofermentation, such as determining IVOMD and ME of the samples and using biogas production syringes. For the biofermentation study, 3 runs of *in vitro* biogas production and three adult rumen cannulated sheep were used. The sheep were fed with a diet containing alfalfa hay, barley grain, soybean meal and vitamins/minerals supplement, administered twice daily, 08:30 and 17:30 h using fresh water. Rumen fluid was collected one hour prior to the morning meal, subsequently filtered with four sheets of cheesecloth and maintained under CO_2 gas using magnetic stirrer (at 39 °C). Two sets of syringes were prepared and 500 mg of each test sample (alfalfa and quinoa forages) was poured into each syringe (4 syringes as replication). Syringes were pre-heated at 39 °C for a duration of one hour. Subsequently, 40 mL of rumen buffer combination was dispensed in the syringes and positioned in a water tank at a temperature of 39 °C (Makkar, 2010). The volume of gas produced was recorded at fermentation times (3, 6, 8, 12, 16, 24, 48, 72 and 96 h) for the first set of syringes (Menke & Steingass, 1988). At 24 hours after incubation, the volume of gas created in the second series of syringes was measured, and their contents were centrifuged (20,000 × *g* for 20 min at 4 °C). The amount of 1 mL of HCl 0.2 N was mixed with 5 mL of supernatant and k stored at –20 °C in preparation for the analysis of NH3-N, as outlined by Broderick and Kang (1980). A volume of 1 mL of supernatant was combined with 0.20 mL of 25% metaphosphoric acid. This solution was subsequently stored at -20 °C for VFAs measuring. For the quantification of VFAs, a volume of 1 µl of supernatant was introduced into a gas chromatograph (Nucon-5765) equipped with a dual flame ionization detector (FID) and a chromosorb glass column measuring 4 mm in length and 1.8 mm in diameter. The flow rates of the gases utilized, specifically nitrogen, hydrogen, and air, were maintained at 30, 30, and 320 ml/min, respectively. The thermal conditions of the injector oven, column oven, and detector were set at temperatures of 270, 172, and 270 °C, respectively (Cottyn & Boucque 1968). For drying fermentation remains, the oven (at 60 °C for 48 h) was used. The amount of digested DM was equal to the weight loss of the sample after fermentation. The DM degradability at 24 h of fermentation (ADS) was determined using the following formula (Makkar, 2010).

ADS (mg/g DM) = DM amount (mg) of substrate beforehand fermentation - undegradable DM (mg) afterward fermentation

For measuring the protozoa population, a subsample was diluted with a formalin solution. To dilute the rumen fluid, 4 mL of fluid was mixed with 20 mL of formalinized physiological saline. The counting of protozoa was conducted across 30 microscopic fields at a magnification of 20 × utilizing a Haemocytometer (Neubauer improved, Marienfeld, Germany) (Dehority, 2003).

2.3. In situ degradability

Degradability was conducted using polyester bags (53 ± 10 µm pore size; Bar Diamond, Inc., Parma, ID) and cannulated sheep. A total of 5 g of forages were weighed and were put in polyester bags in 4 repetitions and were placed in the rumen. After the required time, the bags were taken out of the rumen and was leached in the washing machine for 1 hour and dried for 48 h at 60 °C. For the zero time, 4 bags were leached in the washing machine for one hour using cold water. The amounts of

DM and CP in each bag were determined.

The ruminal degradability (Y) of DM and CP at time (t) was calculated by the following formula (Ørskov & McDonald, 1979):

$$Y = a + b(1 - e^{(-ct)})$$

The a is a soluble degradable section and b is an insoluble but possibly degradable section and c is a degradation rate of b (/h). The ED of DM and CP in each sample was assessed by the equation: ED (g/kg DM) = a + bc/c + k.

The k is the outflow rate in rates of 0.02, 0.04 and 0.06 per h (Ørskov & McDonald, 1979).

2.4. Calculations

For a further accurate calculation of GP in the time of *in vitro* fermentation, the following non-linear equation was used to evaluate the data (France, Dijkstra, Dhanoa, Lopez & Bannink, 2000).

$$G = A \times \left(1 - e^{-\mu(t-L)}\right)$$

The A is the amount of GP at time t; A is the asymptotic GP (mL/g DM); μ is the rate of GP (/h), and L is the lag time.

The subsequent equations (Menke et al., 1979) were used for ME and *in vitro* organic matter disappearance.

$$ME(MJkg / DM) = 2.20 + 0.136G + 0.057CP + 0.0029CP^2$$

$$OMD(g / kgOM) = 148.8 + 8.89G + 4.5CP + 0.651ASH$$

Where CP is crude protein (g/100 g DM); ASH is ash (g/100 DM) and G is the net gas production (mL/200 mg) of forage.

Gas yields (GY_{24}) were estimated with following equation:

$$GY_{24} = mLgas / gADS$$

The ratio of organic matter truly degraded (mg) to gas production (mL) after 24 h of incubation was used to the calculation of the partitioning factor (Makkar, 2010).

CH_4 and CO_2 are calculated by stoichiometric equation from VFAs production (Makkar 2010).

$$CO_2\ (mmol) = (Acetate\ (mol)/2) + (Propionate\ (mol)/4) + (1.5Butyrate\ (mol))$$

$$CH_4\ (mmol) = Acetate\ (mmol) + 2Butyrate\ (mmol) - CO_2\ (mmol)$$

The RFV is an estimation of the total value of forage and is determined from the absorption and digestibility of DM (Atis, Konuskan, Duru, Gozubenli & Yilmaz 2012; Shah et al., 2020). This index is calculated from the following equations.

$$RFV = DDM\% \times DMI\% \times 0.775$$

$$DDM = 88.9 - -(0.77 \times ADF\%)$$

$$DMI = 120/NDF\%$$

DDM is a digestible DM and DMI is a DM intake.

2.5. The WU and WUE

From the planting to harvesting, the amount of WU and the WUE were determined using the behind equation (Taaime et al., 2022).

$$WUE = Y/WU$$

WUE is a water use efficiency (kg/m^3 water use), Y is a forage performance (kg DM/ha) and WU is a water use (m^3/ha).

2.6. Chemical composition and PSMs of forages

To determine the DM, the AOAC, 1990; method 930.15 and the oven device were used at a temperature of 60 °C for 48 h. A muffle furnace at a temperature of 550–600 °C was used to obtain Ash (AOAC, 1990 method 942.05). Technique no. 988.05 of the AOAC (1990) was used for total nitrogen. Soxhlet apparatus was used to obtain EE no (AOAC, 1990 method 920.39). The NDFom was determined based on the method of Van Soest et al. (1991) and ADFom was determined based on the method of AOAC (1990; method 973.18). The amount of ADL was measured based on the method of Robertson and Van Soest, (1981).

Measuring TP in forages was done by the Folin–Ciocalteau reaction. For determination of total tannins (TT), the amount of 100 mg Insoluble polyvinyl polypyrrolidone (PVPP) was added to 1 mL distilled water and vortexes. The mixture was kept at 4 °C for 15 min. Then, the blend was vortexed another time and centrifuged (3000 × g for 10 min) and the supernatant was gathered. The TP amount was measured and known as the non-tannin phenolic (NTP). The TT was calculated by the difference between TP and NTP (Makkar, 2000).

2.7. Statistical analyses

One-way analysis of variance by the "GLM" option of SAS (2002) was used for analysis of data (chemical composition, fermentation factors, protozoa and *in situ* parameters). Duncan's multiple-range test was used for chemical composition and *in situ* parameters by distinct means.

$$Yij = \mu + T_i + e_{ij}$$

Yij is the general observation, μ_{ij} is the general mean, T_i is the ith effect of forages treatments and e_{ij} is the standard error term.

Biofermentation was conducted out in three distinct *in vitro* runs with four replicates. The data related to the *in vitro* parameters of three runs were averaged for each sample. Tukey's multiple-range test was used for fermentation parameters by distinct means. The *in vitro* data were analyzed as repeated measures utilizing the model outlined below:

$$Y_{ijk} = \mu + S_i + R_j + TR_{ij} + e_{ijk}$$

Y_{ijk} is the general observation, μ is the general mean, T_i is the ith effect of forages treatments, R_j is the run effect, and TR_{ij} is the interaction between forage treatment and run and e_{ij} is the standard error term. The interaction effects of treatment and run were removed because they were not significant.

3. Results

3.1. Chemical composition and PSMs of the alfalfa and quinoa forages

The chemical composition quantities are presented in Table 1. As the quinoa plant matures, the amounts of DM (g/kg fresh weight) and OM of quinoa forage increased ($P < 0.0001$), but these values were lower than the dry matter of alfalfa forage. The ash content in quinoa forage was between 169.02 and 244.90 g/kg DM and at all harvest stages was higher than that of alfalfa forage ($P < 0.0001$).

In quinoa forage, it was observed that as the harvest stage progressed, the amounts of CP and EE decreased, while the levels of NDFom, ADFom and ADL exhibited a significant increase ($P < 0.0001$). The CP and EE contents only in Q45 and Q95 treatments were more than Al treatment ($P < 0.0001$). The amount of NDFom in Q45 and Q95 treatments was lower than those observed in the Al treatment, while the Q125 and Q145 treatments exhibited higher levels compared to the Al treatment ($P < 0.0001$). The amount of ADFom of quinoa harvested at 45, 95 and 125 days was lower than that of alfalfa forage, while at 145 days, it was comparable to alfalfa forage ($P < 0.0001$). The amounts RFV, TP and TT decreased as the harvest period extended ($P < 0.0001$, $P = 0.0007$ and $P < 0.0001$, respectively). The amounts of TP and TT in

Table 1
Chemical composition and PSMs levels (g/kg of DM) of the alfalfa and quinoa forages.

	Forage						
	Al	Q45	Q95	Q125	Q145	SEM	*P*-value
Chemical composition							
DM (g/kg fresh)	337.50^{a}	160.83^{e}	169.68^{d}	201.71^{c}	250.00^{b}	2.115	< 0.0001
Ash	97.67^{d}	244.90^{a}	215.61^{b}	177.53^{c}	169.02^{c}	3.469	< 0.0001
CP	144.50^{c}	199.52^{a}	183.31^{b}	141.53^{c}	112.00^{d}	2.731	< 0.0001
EE	16.10^{c}	26.32^{a}	22.83^{b}	16.23^{c}	15.45^{d}	0.139	< 0.0001
NDFom	408.33^{c}	291.03^{e}	376.57^{d}	455.65^{b}	515.83^{a}	8.441	< 0.0001
ADFom	333.67^{a}	155.06^{d}	215.00^{c}	286.10^{b}	338.08^{a}	3.059	< 0.0001
ADL	85.33^{a}	33.30^{e}	45.23^{d}	53.40^{c}	60.00^{b}	0.457	< 0.0001
PSMs							
TP	6.60^{d}	22.13^{a}	18.71^{b}	14.94^{c}	14.75^{c}	0.148	< 0.0001
TT	4.20^{d}	13.28^{a}	11.22^{b}	8.97^{c}	8.85^{c}	0.083	< 0.0001
NTP	2.40^{d}	8.85^{a}	7.48^{b}	5.98^{c}	5.90^{c}	0.161	< 0.0001
RFV	143.29^{c}	245.63^{a}	178.39^{b}	136.24^{c}	112.83^{d}	3.357	< 0.0001

Al: alfalfa forage; Q45: budding stage, quinoa harvested 45 days after planting; Q95: 10% flowering stage, quinoa harvested 95 days after planting; Q125: before milk stage, quinoa harvested 125 days after planting; Q145: before milk stage, quinoa harvested 145 days after planting; NDFom = ash-free NDF; ADFom = ash-free ADF; ADL = lignin; TP = Total phenolics; TT = Total tannins; NTP = non tannins phenolics; RFV = relative forage value; SEM = Standard error of the mean; Means within a row with different superscripts differ ($P < 0.05$).

quinoa forages were greater than those in alfalfa forage.

3.2. In Vitro biofermentation parameters of alfalfa and quinoa forages

The biogas production volumes of alfalfa and quinoa forages harvested in different stages are given in Table 2 and Fig. 1. The GP before 24 h of fermentation for quinoa forages was lower than that of alfalfa forage. However, at 24 h of fermentation, the GP for quinoa forage, especially for Q45 and Q95 was nearly equivalent to that of alfalfa forage ($P < 0.0001$). At 96 h of fermentation, the quantity of GP in Q45 and Q95 treatments had a tendency to increase compared to Al treatment.

The *A* value was not different between the alfalfa and Q45, Q95 and Q125 treatments, but it was lower in Q145 than in Al treatment. With the exception of the 145-day harvest period, extending the harvest duration did not influence this index ($P = 0.003$). The content of *L* in alfalfa forage was lower than quinoa forages and harvesting time did not affect the value of this index ($P = 0.011$). The GP_{24} value only decreased in Q125 and Q145 treatments compared to Al treatment ($P < 0.0001$). The highest and lowest quantities of IVOMD and ME were observed in Q45 and Q145 treatments respectively. As the age of the plant increased, the value of these parameters decreased ($P < 0.0001$). The PF_{24} in alfalfa forage was lower than quinoa forages, and the highest values for this parameter was detected in Q95 and Q145 treatments ($P < 0.0001$). The highest quantity of MP was observed in Q95 treatment ($P < 0.0001$). The GY_{24} was the highest in Al treatment and the lowest in Q45 treatment ($P < 0.0001$). The level of ADS in alfalfa forage was found to be less than that in quinoa forages ($P < 0.0001$).

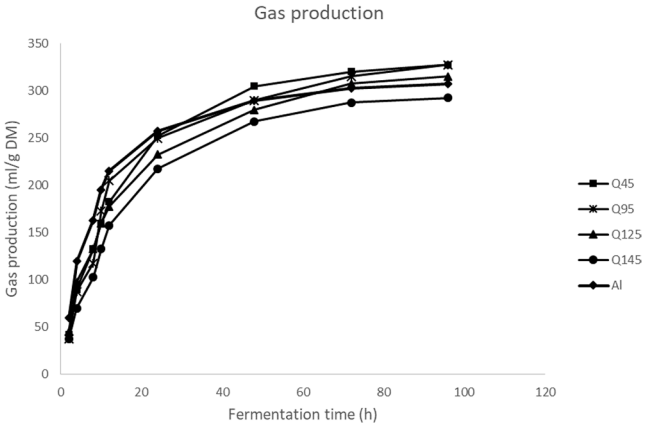

Fig. 1. gas produced (mL/g DM) during the fermentation times of forages alfalfa and quinoa harvested at different stages.
Al: alfalfa forage; Q45: budding stage, quinoa harvested 45 days after planting; Q95: 10% flowering stage, quinoa harvested 95 days after planting; Q125: before milk stage, quinoa harvested 125 days after planting; Q145: before milk stage, quinoa harvested 145 days after planting.

3.3. Concentrations of VFAs, NH_3-N and CH_4 and CO_2 production

Total VFAs were decreased in Q125 and Q145 treatments compared to the Al treatment. Additionally, there was a significant reduction in this parameter with the advancing age of the plant ($P < 0.0001$). The

Table 2
Gas produced (mL/g DM) during the fermentation times of forages alfalfa and quinoa harvested at different stages.

	Forage						
Time	Al	Q45	Q95	Q125	Q145	SEM	*P*-value
2	60.00^{a}	42.50^{b}	37.50^{b}	45.00^{b}	37.50^{b}	2.141	0.0001
4	120.00^{a}	90.00^{b}	87.50^{b}	97.52^{b}	70.50^{c}	3.291	< 0.0001
8	162.50^{a}	132.55^{b}	117.45^{bc}	132.60^{b}	102.50^{c}	3.476	< 0.0001
10	195.00^{a}	160.00^{b}	172.50^{b}	160.00^{b}	132.50^{c}	4.564	< 0.0001
12	215.00^{a}	182.50^{bc}	250.00^{ab}	232.50^{dc}	217.40^{d}	5.123	< 0.0001
24	257.50^{a}	252.55^{a}	250.00^{a}	232.50^{b}	217.50^{c}	2.582	< 0.0001
48	290.00^{ab}	305.00^{a}	290.00^{ab}	280.00^{ab}	267.50^{b}	5.809	0.012
72	302.50	320.00	315.00	307.50	287.50	9.916	0.247
96	307.50	327.50	325.25	315.00	292.50	10.547	0.178

Al: alfalfa forage; Q45: budding stage, quinoa harvested 45 days after planting; Q95: 10% flowering stage, quinoa harvested 95 days after planting; Q125: before milk stage, quinoa harvested 125 days after planting; Q145: before milk stage, quinoa harvested 145 days after planting; NDFom ash-free NDF; ADFom = ash-free ADF; RFV = relative forage value; SEM = Standard error of the mean; Means within a row with different superscripts differ ($P < 0.05$).

application of quinoa forages resulted in a decreased concentration of acetate ($P < 0.0001$). The utilization of quinoa forages increased propionate concentration ($P = 0.002$). The concentration of butyrate was decreased in the Q145 treatment compared to the Al treatment ($P = 0.134$). The amount of isovalerate declined in the Q45 and Q95 treatments compared to the Al treatment ($P = 0.004$). The acetate propionate ratio was decreased when quinoa forages were utilized in comparison to alfalfa forage ($P < 0.0001$). The NH_3-N concentration was decreased in the Q45, Q95 and Q125 treatments compared to the Al treatment ($P = 0.0004$). The volumes of CH_4 and CO_2 production were decreased in quinoa forages *versus* alfalfa forage but harvesting time did not influence on these parameters ($P = 0.0002$ and $P = 0.0001$).

3.4. Protozoa counts

The utilization of quinoa forage resulted in a reduction of total protozoa and subfamilies of *Entodiniinae* compared to alfalfa forage. The population of these protozoa decreased with the vegetative growth of the plant ($P < 0.0001$). The population of *Isotricha* spp. and subfamilies of *Diplodiniinae* and *Ophrioscolecinae* were decreased by using quinoa forage compared to alfalfa forage ($P = 0.018$, $P = 0.055$ and $P = 0.029$ respectively).

3.5. In situ DM and CP degradability of alfalfa and quinoa forages

The *a* parameter for DM degradability in quinoa forage was more significant than that of alfalfa forage, and this parameter decreased with increasing harvest time in quinoa forage ($P < 0.0001$). The amount of *b* fraction of DM degradability in the Al treatment was higher than in the Q45, Q125 and Q145 treatments, and his parameter's value diminished as the quinoa matured ($P < 0.0001$). The parameters of $a + b$ and ED of dry matter with flow rates of 0.02, 0.04 and 0.06 in the Al treatment were lower than Q45 and Q95 treatments, but more than Q125 and Q145 treatments ($P < 0.0001$).

Table 6

The information on *in situ* CP degradability is given in Table 7. The values of *a* fraction in quinoa forages, with the exception of forage harvested for 45 days, were lower than alfalfa forage ($P < 0.0001$). The *b* fraction in the Al treatment was lower than that of the Q95 and Q125 treatments. As the plant matured, this parameter (except for the Q45 treatment) decreased in quinoa forage ($P < 0.0001$). The values of $a + b$ and ED for CP degradability in the Al treatment were found to be lower than those of quinoa forages ($P < 0.0001$). The *c* parameter of CP in alfalfa forage was greater than that of quinoa forages ($P = 0.005$). The ED value decreased with increasing quinoa growth ($P = 0.0001$).

3.6. Fresh and dry forage, CPP, WU and WUE of alfalfa and quinoa harvested at different stages

The production values of fresh forage and DM of quinoa forages are presented in Table 8. In this study, it was observed that the yield of both fresh and dry quinoa forages per hectare increased as the plants matured ($P < 0.0001$). The highest and lowest extents of CPP estimated per hectare were recorded for the Q95 and Q45 treatments, respectively. This value in quinoa forage harvested at various growth stages was less than that of alfalfa forage ($P < 0.0001$). The amount of WU in quinoa plant increased with maturation, however, this measure was lower in quinoa than in the alfalfa plant. The WUE was higher in quinoa plant compared to the alfalfa plant. Notably, the efficiency in the quinoa plant improved as the harvest age increased ($P < 0.0001$).

4. Discussion

4.1. Chemical composition and PSMs of the alfalfa and quinoa forages.

The chemical composition of the plant correlated to plant variety, climatic conditions during planting, plant accessibility, harvest time, the nutritional conditions of the plant (extent and time of fertilization) and plant storage conditions (Uke, Kale, Kaplan & Kamalak, 2017; Ahmed et al., 2023). In the current study, the highest amount of DM was observed at the 145-day harvesting stage. It has been shown in various research that different harvest times have significant influences on morphological characteristics and forage quality parameters. According to the present research, Yilmaz, Ertekin and Atis (2021) and Temel and Yolcu (2020) also showed that DM of quinoa forage improved as the harvest age increases. The ash content of quinoa forages was more significant than that of alfalfa forage. This increase can be attributed to quinoa has a 4-carbon metabolism and carbon absorption (Abbasi, Rouzbehan & Rezaei, 2012). Also, this increase is probably due to the absorption of cations and their accumulation within the plant (Masters, Bennes & Norman, 2007; Shakeri, Dayani, Asadi Korom, Najafi Neghad & Aghashahi, 2019). Consistent with present research, Liu, Yang and Yang (2021) described that the ash content quinoa forage varies according to phonological periods. Similarly, other works have shown that quinoa forage had noticeably high CP concentrations (Fang et al., 2022; Ahmed et al., 2023). The reduction in the CP and increase of NDFom and ADFom in quinoa forage with the increase in the harvesting time can be attributed to a decrease in the leaf proportion within the forage, which have more CP in the plant biomass and reduction of leaves to stems fraction (Ahmed et al., 2023). Additionally, this phenomenon is a result of the plant's aging process and the subsequent development of lignin (Nielsen, Stødkilde, Jørgensen & Lærke, 2021; Yilmaz, Ertekin & Atis, 2021). Plants that are sown earlier and harvested later experience a longer growth period, which is advantageous for ecological issues such as light, water and nutrients. Consequently, their stems tend to be denser, resulting in an increased concentration of lignin and cellulose within their cell walls (Temel & Yolcu, 2020). In research conducted in China, the nutrients structure of quinoa forage harvested in the stages of flowering and seed ripening was compared with alfalfa forage. The findings indicated that the CP content in quinoa forage was higher than that of alfalfa forage, while the amounts of NDFom and ADFom in quinoa forage was lower than those in alfalfa forage (Shah et al., 2020). In other studies, it was demonstrated that as the age of the plant increased, there was a reduction in protein levels, while the concentrations of NDFom and ADFom rose. (Peiretti, Gai & Tassone, 2013; Casini, 2019; Shah et al., 2020). In another research, Kardooni, Tavoosi, Mahdavi Majd, Taheri Dezfoli and Anafjeh (2019) investigated the quantitative and qualitative value of quinoa genotypes (Giza 1, Rosada and Q102) in 3 phases of harvest (start of seeding, seed milking and whole maturity). Their findings exhibited that the quantity of CP in three stages of the harvest was not different from each other and the amount of this nutrient was more affected by the genotype. Also, these researchers exhibited that the NDFom content increased as the plant matures. Contrary to the results of this study, Yilmaz, Ertekin and Atis (2021) indicated that the quantity of NDFom was not affected by the harvest stage. The findings regarding the impact of harvest age on EE content have varied across studies. Similar to our results, Peiretti, Gai and Tassone (2013) and Uke, Kale, Kaplan and Kamalak (2017) displayed that the EE content decreased with increasing plant age. Conversely, Yilmaz, Ertekin and Atis (2021) indicated an increase in EE content. These discrepancies are probably correlated to the relational quantity of plant organs at the time of harvest (Liu, Yang & Yang, 2021).

A lot of evidence is about quinoa forge PSMs and more studies have been done on quinoa seeds. The total phenolic and total tannin values in 8 quinoa forage varieties were determined between 1.5 to 7.0 and 0.5 to 6.0 g/kg DM respectively (Ranjbar, Rouzbehan & Abarghuei, 2024), which were less than the results of current work. In research, the amounts of TP and total TT in alfalfa hay and quinoa crop residues were determined as 21.7, 6.7 and 44.4, 22.1 g/kg DM, respectively (Ghavipanjeh, Fathi Nasri, Bashtani & Farhangfar, 2021) which was less than the consequences of the current research. According to the research conducted by Li, Lietz and Seal (2021), the TP contents of the 13 quinoa

seed varieties were estimated to between 2.18 ± 0.45 mg gallic acid/g DM. Variations in the concentration of PSMs in different studies may be attributed to vegetative growth period, the procedure of storing, drying conditions, species differences (Makkar & Singh, 1993), the geographical location of plant cultivation and different extraction methods (Li, Lietz & Seal, 2021), Also, PSMs are correlated to the metabolic stability among plant biosynthesis, catabolism and environmental disorders (Karimi, Mirzaei, Emam-Djomeh, Sadeghi Mahoonak & Khomeiri, 2013).

The RFV indicates the whole quality of forage and is designed as of the absorption and digestibility of DM, and its reference value is 100, which equivalent to alfalfa in the entire flowering stage (Atis, Konuskan, Duru, Gozubenli & Yilmaz, 2012; Shah et al., 2020). Horrocks and Vallentine (1999) exhibited that forages with RFV ranging from 125–151 are considered excellent forages. The RFV value was reported as 150 units for alfalfa forage (with 150 g/kg DM CP and 510 g/kg DM NDFom), which was slightly higher than the RFV value calculated (143.29) in our research. This index has a negative relationship with the contents of NDFom and ADFom (Table 1). In the current study, the value of this index for quinoa forage ranged from 112.83–245.63, which is comparable to the results of quinoa harvested in the flowering stage (162.2–225.7) and lower than those for quinoa harvested in the seed ripening stage (149.9–273.3) in the research conducted by Shah et al. (2020). In another research, the RFV for different cultivars of quinoa (harvested at the seed ripening stage) ranged from 147.60–134.36 (Kaya & Aydemir, 2020). Differences among these studies can be due to the variety used, maturity stage, weather conditions, planting conditions and harvest stage (Sahoo, Ogra, Sood & Ahuja, 2010).

4.2. *In Vitro* biofermentation parameters of alfalfa and quinoa forages

Studies suggested that the structure and composition of the cell wall, rumen microbial ecosystem and forage chemical composition correlate with fermentation rate (Ammar, Lopez, Gonzalez & Ranilla, 2004; Sahoo, Ogra, Sood & Ahuja, 2010). The *L* value represents the time period for hydration and colonization of the feedstuff particles by ruminal microbes. This value is influenced by the characteristics of the fermented substrate as well as the diversity and quantity of microbes present in the inoculation environment. Antinutritional components such as phenolic compounds, tannins (as illustrated in Table 1) and saponins may also increase the lag time by obstructing and reducing the attachment of ruminal microorganisms to feedstuff particles (Dehority, 2003; Noordar, Malecky, Jahanian Najafabadi & Navidshad, 2017). In the early hours of fermentation (before 24 hours), the *L* value observed in quinoa forage surpassed that of alfalfa forage, which can be seen in gas production, probably due to mention the factors.

Factors that affect fermentation parameters include leaf-to-stem ratio in forage and nutritional compounds such as CP, NDFom and lignin. The higher amounts of IVOMD and ME in quinoa forage harvested at 45 and 95 days compared to alfalfa can be due to higher protein content and lower concentrations of NDFom and lignin (Van Soest, 1994; McDonald, Edwards, Greenhalgh & Morgan, 1995). A positive correlation exists between CP and GP during fermentation. The hydrolysis of CP in the rumen leads to the release of NH_3-N, which supplies the nitrogen needed for the growth and proliferation of cell wall fermenting microbes and nutrients. This process creates an optimal environment for fermentation, thereby enhancing gas production (Norton & Poppi, 1995). The decrease in CP and the increment of NDFom associated with extended harvesting periods can be the reason for the lower amounts of GP, IVOMD and ME in quinoa forages harvested at 125 and 145 days, in comparison to other forage types. The digestibility of forages depends on the ratio of contents inside the cell and components of its cell wall. As quinoa develops, like all plants, it creates xylem tissue to transport water and accumulate cellulose and further carbohydrates. These tissues are connected by the process of lignin formation, which prevents the digestion of cell wall polysaccharides in the rumen (Hoffman, Lundberg, Bauman & Shaver, 2003). The any difference in the GP_{24} and μ values between the alfalfa treatment and the Q45 and Q95 treatments indicates that the quality of these forages is comparable to alfalfa. Consequently, these forages may serve as viable alternatives to alfalfa in the diets of ruminants. The results of our research are in agreement with some investigations that have displayed the quality of quinoa forage is high, because it has large amounts of protein and is highly digestible (Peiretti, Gai & Tassone, 2013; Barros-Rodríguez et al., 2018; Asher, Galili, Whitney & Rubinovich, 2020). Ebeid et al. (2022) investigated the effect of replacing quinoa forage at levels of 15, 30 and 45% of DM instead of clover forage in the diet using the gas production test. Their findings indicated that GP remained unchanged and suggested that quinoa forage can be used for up to 45% of the ruminant's diet. Kardooni, Tavoosi, Mahdavi Majd, Taheri Dezfoli and Anafjeh (2019) studied the nutritional value of 3 genotypes of quinoa (Giza 1, Rosada and Q102) across three distinct harvest stages (start of seeding, seed milking and whole ripeness). Their findings indicated that for the Rosada and Q102 genotypes, there was a decline in IVOMD as the harvest time progressed; however, the ME remained unchanged. Anyway, in research by Shakeri, Najafi Neghad, Aghashahi and Shakeri (2023), the Sjama variety was harvested at the time of dough of seeds stage and the parameters of GP_{24}, IVOMD and ME were determined 145.65 mL/g DM, 482.20 g/kg DM and 1.67 Mcal/kg DM, respectively, which were lower than those observed in the current research. These differences between research results are probably attributable to the different genotypes used, season, climatic conditions, plant maturity (which influences the composition of cell wall components and proteins), harvest stage and the presence of PSMs (which affect the degradability and digestibility of the feed) (Meza-Bone et al., 2022).

The PF_{24} represents the ratio of the actual decomposition of the substrate to the volume of gas produced during the fermentation periods and is an indicator for the separation of the digested organic matter between the pathways of fermentation (production of gas and VFAs or efficiency of MP synthesis) *in vitro* (Blümmel, Karsli & Russell, 2003). The amount of PF_{24} in this research was in the advised range (2.70–4.40 digested substrate per mL of produced gas) as established in previous research involving various feedstuffs (Blümmel, Karsli & Russell, 2003; Al-Sagheer, Elwakeel, Ahmed & Sallam, 2018; Abarghuei & Salem, 2021). The increase in the value of this index in quinoa forage compared to alfalfa forage, can be attributed to the utilization of nutrients for the synthesis of MP and the decrease in the concentration of VFAs (Table 3). Microbial protein is a critical resource of protein for ruminants as it provides more than 50 % of the total protein requirements (Hackmann & Firkins, 2015). Accessibility of NH_3-N and energy is an important issue in MP production. The increase in MP in quinoa forage compared to alfalfa forage can be due to the higher synchronization in the availability of carbohydrate and nitrogen sources (Abarghuei & Salem, 2021). Research suggests that amounts less than 5% of PSMs, depending on their structural characteristics, can increase the amount of MP and PF_{24} (Jiménez-Peralta et al., 2011; Abarghuei, Rouzbehan, Salem & Zamiri, 2013), which is similar to the values of metabolites measured in the current research (Table 1). Therefore, less GP along with a higher PF_{24} usually indicates a higher efficiency of MP production (Bhatt, Soni & Sahoo, 2019). Contrary to the current investigation, Yacout, Salama, Elgzar and Awad (2021) examined the effects of the consumption of quinoa forage and silage harvested for 90 days (before setting the seeds), clover forage and corn silage on Barki ewes. Their findings showed that the quantity of MP in ewes fed with quinoa forage and silage was lower than those in ewes that were provided with clover forage and corn silage.

4.3. *Concentrations of VFAs, NH_3-N and CH_4 and CO_2 production*

The final products of ruminal microorganisms are VFAs. The concentrations of VFAs in the rumen commonly show the degradation

Table 3
Variables of *in vitro* rumen fermentation of forages alfalfa and quinoa harvested at different stages.

Parameters	Forage					SEM	*P*-value
	Al	Q45	Q95	Q125	Q145		
A	300.00[a]	303.33[a]	301.00[a]	286.67[ab]	266.67[b]	5.164	0.003
μ	0.10	0.10	0.10	0.10	0.10	0.00	> 0.05
L	0.10[b]	1.10[a]	1.10[a]	0.77[a]	1.43[a]	0.211	0.011
GP_{24}	257.50[a]	252.55[a]	250.00[a]	232.50[b]	217.50[c]	2.582	< 0.0001
ADS	696.25[e]	885.75[a]	863.00[b]	770.00[c]	723.75[d]	3.140	< 0.0001
IVOMD	685.03[b]	703.46[a]	689.83[ab]	636.94[c]	596.52[d]	4.591	< 0.0001
ME	10.71[b]	11.36[a]	11.02[b]	9.90[c]	9.11[d]	0.070	< 0.0001
PF_{24}	2.41[d]	2.63[a]	2.76[bc]	2.64[ab]	2.72[d]	0.020	< 0.0001
MP	54.75[c]	108.10[b]	138.05[a]	103.20[b]	113.65[b]	4.139	< 0.0001
GY_{24}	369.86[a]	285.07[c]	286.79[c]	301.92[b]	300.51[b]	2.502	< 0.0001

Al: alfalfa forage; Q45: budding stage, quinoa harvested 45 days after planting; Q95: 10% flowering stage, quinoa harvested 95 days after planting; Q125: before milk stage, quinoa harvested 125 days after planting; Q145: before milk stage, quinoa harvested 145 days after planting; *A* = asymptotic GP (mL); μ = fermentation rate (/h); L = lag time (h); GP_{24} = Gas production at 24 h of fermentation (mL); ADS = Apparent degraded substrate (mg/g DM); IVOMD = *in vitro* organic matter disappearance (g/kg); ME = metabolizable energy (MJ/kg DM); PF_{24} = partitioning factor at 24 h of fermentation (mg ADS/mL gas); MP = microbial protein synthesis (mg/g DM); GY_{24} = gas yield at 24 h (mL gas/g ADS); SEM = Standard error of the mean; Means within a row with different superscripts differ ($P < 0.05$).

patterns of carbohydrates by theses microorganisms (Rong-zhen, Yi, Hai-xia, Min & Dao-wei, 2016). These fatty acids are the key source of ME for the ruminant. Decrement in VFAs probably has harmful nutritive consequences on livestock (Van Soest, 1994). The absence of difference in total VFAs using quinoa forages harvested at 45 and 95 days can be due to meeting the needs of rumen microorganisms as a result of the breakdown of nutrients, especially carbohydrates, in the fermentation environment (Rong-zhen, Yi, Hai-xia, Min & Dao-wei, 2016) and also as a result of the lack of difference in the digestibility of these forages (Table 3). The reduction of these acids at harvest times of 125 and 145 days can be the result of reduced fermentability and digestibility compared to alfalfa forage (Table 3). The VFAs profile can be changed by the nutritional quality of the forage (carbohydrate fermentation and small part of feed protein fermentation), presence of PSMs, the composition of the rumen microbial population and the nature of the fermented substrate (Ningrat, Zain, Erpomen & Suryani, 2017; Brutti, Canozzi, Sartori, Colombatto & Barcellos, 2023). With the physiological growth of the plant, there is a corresponding increase in carbohydrate content, which subsequently influences the composition of VFAs (Meza-Bone et al., 2022). In the rumen, acetate is predominantly generated by the activity of bacteria and protozoa, and this fatty acid is the greatest main end product of fermentation by protozoa (Dehority, 2003). The reduction in acetate concentration could be due to the detrimental effects of PSMs on these microorganisms (Table 5) (El-Zaiat & Abdalla, 2019). The observed increase in acetate levels in Q125 and Q145, in contrast to Q45 and Q95, could be linked to the reduction of PSMs as plant growth progresses. The increase in propionate concentration is due to the inhibitory effect of PSMs, which is similar to earlier works (Abarghuei & Salem, 2021; Kholif et al., 2023). Notably, the most significant increase occurred during the initial stages of plant development (Q45 treatment), a while the enhancement of this fatty acid diminished as the plant matured. Gram-positive ruminal bacteria usually produce acetate and butyrate and are further sensitive to PSMs than propionate-producing gram-negative bacteria (Vasta et al., 2019). In the current research, decrement of acetate to propionate ratio may be due to the impact of PSMs present in quinoa forages on acetate and propionate production (Wu et al., 2018; Kinley et al., 2020). Additionally, the decrement of acetate can be due to the inhibition of protozoa responsible for the production of acetate (Table 5) (El-Zaiat & Abdalla, 2019). In alignment with our results, Al-Sagheer, Elwakeel, Ahmed and Sallam (2018) revealed that the inclusion of guava leaves instead of berseem hay, which contains 1.4, 12.6, 15.61, and 38.65 g TT/kg DM, resulted in a reduction of total VFAs and acetate production. In a study, a mixture of *Moringa oleifera* leaf silage (contains 4.9 % of DM total phenol and 1.9 % of DM tannin) and *Chlorella vulgaris* microalgae (at levels of 1, 2 and 3 % of DM) replaced at different levels of concentrate feed mixture in the diets. These researchers concluded that *Moringa oleifera* rations with 1% and 2% *Chlorella vulgaris* improved the concentrations of total VFAs acetate and propionate, while these fatty acids were not affected at 3% *Chlorella vulgaris* amount (Kholif et al., 2023). In other study, Abarghuei and Salem (2021) illustrated that using 150 and 300 g/kg DM of *Glycyrrhiza glabra* leaves and pulp in lambs fattening diet decreased concentrations of total VFAs and acetate. Furthermore, the concentration of propionate was only diminished when 300 g/kg DM of pulp was included, while the acetate to propionate ratio only decreased in a diet that contained 300 g/kg DM of leaves. In another research, the potential of either chestnut or quebracho tannins (20 mg tannin/g diet) on rumen fermentation was investigated. The findings showed that the use of these compounds led to a reduction in the total VFAs, while simultaneously increasing the levels of propionate, but did not affect the amount of acetate (Foggi et al., 2022). The use of plants containing PSMs had different effects on butyrate concentration. Research indicates that PSMs influencing the rumen microbial community, particularly protozoa, may lead to a decrease in butyrate concentration (Abarghuei & Salem, 2021). Similar to our results, El-Zaiat and Abdalla (2019) and Kholif et al. (2023) indicated that with reducing total protozoa amounts, butyrate concentration was not affected by the addition of PSMs. The variability in VFAs profile in studies could be attributed to the used substrate, the amount and type of PSMs, other dietary components, such as available N and microbial adaptation (Ugbogu et al., 2019; Brutti, Canozzi, Sartori, Colombatto & Barcellos, 2023).

Ruminants play an important role in the production of greenhouse gases. The production of these gases in ruminants, not only have detrimental impacts on environmental quality but also result in a reduction of feed energy efficiency (lose 2–12% of gross energy with emissions of CH_4, CO_2, and H_2) (Johnson & Johnson, 1995; Cardoso-Gutierrez et al., 2021). Therefore, it is very necessary to find suitable solutions to reduce this loss by ruminants. The use of quinoa forages reduced the production of CH_4 and CO_2 by 3.96–5.52% and 1.91–2.95% compared to alfalfa forage. The reduction of CH_4 production in the rumen in the presence of PSMs will be done through several mechanisms. Direct impact on methane-producing bacteria and indirect reduction in CH_4 emissions by impairing nutrient's digestibility, particularly fiber degradability (Parra-Garcia et al. 2019; Battelli et al., 2023). Approximately 25% of methane-producing bacteria have a symbiotic relationship with protozoa in the rumen (Adegbeye et al., 2019). The PSMs can also play a role in reducing CH_4 production by inhibiting protozoa in the rumen. Cellulolytic bacteria in the rumen produces VFAs, especially acetate, H_2 and CO_2. The inhibition of this bacterial activity, along with a decrease in acetate production by PSMs (as shown in Table 4), leads to a reduction in the availability of H_2 and CO_2, which are essential for the proliferation of methanogens (Cardoso-Gutierrez et al., 2021; Batelli et al.,

Table 4
Variables of VFA profiles, NH_3-N, CO_2 and CH_4 of forages alfalfa and quinoa harvested at different stages.

	Forage						
Parameters	Al	Q45	Q95	Q125	Q145	SEM	*P*-value
Total VFA (mmol/L)	78.90^{a}	78.99^{a}	78.71^{a}	67.87^{b}	61.80^{c}	0.075	< 0.0001
Individual VFA (mmol/100 mmol)							
Acetate	69.94^{a}	68.80^{c}	68.89^{c}	69.02^{c}	69.40^{b}	0.060	< 0.0001
Propionate	18.68^{b}	20.00^{a}	19.98^{a}	19.76^{a}	19.48^{a}	0.136	0.0002
Butyrate	9.68	9.55	9.47	9.27	9.24	0.125	0.134
Isovalerate	1.32^{b}	1.30^{b}	1.29^{b}	1.56^{a}	1.48^{a}	0.033	0.0004
Valerate	0.37^{ab}	0.36^{ab}	0.35^{b}	0.38^{ab}	0.39^{a}	0.008	0.025
Isobutyrate	3.62	3.58	3.55	3.86	4.03	0.356	0.839
Acetate / Propionate	3.74^{a}	3.44^{b}	3.44^{b}	3.49^{b}	3.56^{b}	0.027	< 0.0001
NH_3-N (mg/L)	32.56^{a}	29.49^{bc}	29.04^{bc}	26.53^{c}	31.75^{ab}	0.971	0.0004
CH_4 (mmol)	3.94^{a}	3.72^{b}	3.73^{b}	3.73^{b}	3.79^{b}	0.023	0.0002
CH_4 (mL/g OMD)	101.04^{a}	95.50^{b}	95.46^{b}	95.62^{b}	97.04^{b}	0.582	0.0002
CO_2 (mmol)	8.31^{a}	8.15^{b}	8.13^{b}	8.06^{b}	8.08^{b}	0.022	0.0001
CO_2 (mL/g OMD)	212.76^{a}	208.69^{b}	208.19^{b}	206.49^{b}	206.95^{b}	0.571	0.0001

Al: alfalfa forage; Q45: budding stage, quinoa harvested 45 days after planting; Q95: 10% flowering stage, quinoa harvested 95 days after planting; Q125: before milk stage, quinoa harvested 125 days after planting; Q145: before milk stage, quinoa harvested 145 days after planting; VFAs: volatile fatty acids; NH_3-N: ammonia concentration at 24 h; CH_4 (mmol): Methane gas (mmol); CH4 (mL/g OMD): mL methane gas per g organic matter degraded in media; CO_2 (mmol): Carbon dioxide gas (mmol); CO_2 (mL/g OMD): mL carbon dioxide gas per g organic matter degraded in media; SEM = Standard error of the mean; Mean values in rows which do not have a common superscript letter are significantly different ($P < 0.05$).

Table 5
Protozoa counts (log_{10} /mL media) of forages alfalfa and quinoa harvested at different stages.

	Forage						
Protozoa	Al	Q45	Q95	Q125	Q145	SEM	*P*-value
Total	6.10^{a}	5.90^{d}	5.96^{c}	5.99^{c}	6.04^{b}	0.009	< 0.0001
Isotricha	5.04^{a}	4.70^{b}	4.77^{ab}	4.77^{ab}	4.85^{ab}	0.064	0.018
Dasytricha	4.88	4.70	4.77	4.71	4.85	0.066	0.293
Entodiniinae	5.91^{a}	5.77^{d}	5.82^{c}	5.86^{bc}	5.88^{ab}	0.010	< 0.0001
Diplodiniinae	5.09	4.77	4.77	4.85	4.97	0.079	0.055
Ophrioscolecinae	5.04^{a}	4.69^{b}	4.77^{ab}	4.85^{ab}	4.85^{ab}	0.067	0.029

Al: alfalfa forage; Q45: budding stage, quinoa harvested 45 days after planting; Q95: 10% flowering stage, quinoa harvested 95 days after planting; Q125: before milk stage, quinoa harvested 125 days after planting; Q145: before milk stage, quinoa harvested 145 days after planting; a = water-soluble fraction (g/kg DM); b = insoluble but fermentable fraction (g/kg DM); $a + b$ = the potential degradability (g/kg DM); c = the degradation rate of b (/h); ED = the effective degradability of crude protein calculated for an outflow rate (K = 0.02, 0.04 and 0.06/h) (g/kg DM); SEM = Standard error of the mean; Means within a row with different superscripts differ ($P < 0.05$).

Table 6
In situ dry matter degradability parameters of forages alfalfa and quinoa harvested at different stages.

	Forage						
Parameters	Al	Q45	Q95	Q125	Q145	SEM	*P*-value
a	291.07^{d}	522.87^{a}	387.73^{b}	338.37^{c}	330.87^{c}	4.355	< 0.0001
b	442.20^{a}	248.43^{c}	426.33^{a}	349.33^{b}	349.77^{b}	7.783	< 0.0001
$a + b$	733.27^{c}	771.30^{b}	814.07^{a}	687.70^{d}	680.63^{d}	5.882	< 0.0001
c	0.109	0.102	0.090	0.093	0.094	0.011	0.744
ED (K0.02)	663.75^{b}	730.60^{a}	734.73^{a}	623.50^{c}	616.30^{c}	3.680	< 0.0001
ED (K0.04)	613.70^{c}	701.37^{a}	680.57^{b}	579.67^{d}	572.43^{d}	6.461	< 0.0001
ED (K0.06)	575.45^{c}	679.37^{a}	641.20^{b}	547.67^{d}	540.43^{d}	8.172	< 0.0001

Al: alfalfa forage; Q45: budding stage, quinoa harvested 45 days after planting; Q95: 10% flowering stage, quinoa harvested 95 days after planting; Q125: before milk stage, quinoa harvested 125 days after planting; Q145: before milk stage, quinoa harvested 145 days after planting; a = water-soluble fraction (g/kg DM); b = insoluble but fermentable fraction (g/kg DM); $a + b$ = the potential degradability (g/kg DM); c = the degradation rate of b (/h); ED = the effective degradability of dry matter calculated for an outflow rate (K = 0.02, 0.04 and 0.06/h) (g/kg DM); SEM = Standard error of the mean; Means within a row with different superscripts differ ($P < 0.05$).

2023). Similar to our study, Abarghuei and Salem (2021) demonstrated using pulp and leaves of *Glycyrrhiza glabra* in levels of 150 and 300 g/kg DM in diet led to reductions in CH_4 and CO_2 by 3.97–18.46% and 2.93–12.76%, respectively. Another study investigated the ensiling of *Neolamarckia cadamba* leaves at varying proportions of 0, 10, 30 and 50% with corn stalk, revealing a decrease in CH_4 and CO_2 production without any significant negative impacts on rumen fermentation (Zhou, Pian, Yang, Chen & Zhang, 2021). It is reported that differences in harvesting time can effect on CH_4 production which maybe related to the variation in physical-chemical characteristics of the plant, the passage rate and PSMs content. The production of CH_4 increases with a higher amount of structural carbohydrates, while it diminishes with higher concentration of soluble carbohydrates (Meza-Bone et al., 2022). In the present research, the various harvesting stages had no effect on CH_4 and CO_2 production. This lack of effect may be attributed to the minimal variations observed in the concentrations of acetate, propionate, and butyrate.

The minimum concentration of NH_3-N required for the growth of

Table 7
In situ crude protein degradability parameters of forages alfalfa and quinoa harvested at different stages.

Parameters	Forage					SEM	*P*-value
	Al	Q45	Q95	Q125	Q145		
a	540.33^{b}	607.17^{a}	470.47^{c}	493.27^{c}	531.70^{b}	11.257	< 0.0001
b	353.83^{cd}	332.97^{d}	487.10^{a}	433.97^{b}	371.87^{c}	10.083	< 0.0001
a + *b*	894.67^{d}	940.13^{b}	957.57^{a}	927.23^{c}	903.57^{d}	3.490	< 0.0001
c	0.146^{a}	0.131^{ab}	0.111^{c}	0.117^{bc}	0.115^{bc}	0.005	0.005
ED (K0.02)	776.37^{e}	895.97^{a}	882.40^{b}	863.67^{c}	848.30^{d}	2.972	< 0.0001
ED (K0.04)	693.77^{c}	862.13^{a}	827.47^{b}	816.36^{b}	807.30^{b}	4.408	< 0.0001
ED (K0.06)	627.30^{c}	835.40^{a}	785.50^{b}	779.73^{b}	775.77^{b}	5.502	< 0.0001

Al: alfalfa forage; Q45: budding stage, quinoa harvested 45 days after planting; Q95: 10% flowering stage, quinoa harvested 95 days after planting; Q125: before milk stage, quinoa harvested 125 days after planting; Q145: before milk stage, quinoa harvested 145 days after planting; *a* water-soluble fraction (g/kg DM); *b* = insoluble but fermentable fraction (g/kg DM); *a* + *b* = the potential degradability (g/kg DM); *c* = the degradation rate of *b* (/h); ED = the effective degradability of crude protein calculated for an outflow rate (K = 0.02, 0.04 and 0.06/h) (g/kg DM); SEM = Standard error of the mean; Means within a row with different superscripts differ ($P < 0.05$).

rumen microorganisms is 50 mg/L of rumen fluid. If the level of this parameter is too low in the rumen, there will be a lack of available nitrogen for bacteria and feed digestibility will decrease (Harun & Sali, 2019). In the current work, the NH_3-N concentration in forages of alfalfa and quinoa at different stages of harvest was in the appropriate range in the rumen (85 to 300 mg/L of rumen fluid) (McDonald, Edwards, Greenhalgh & Morgan, 1995). The decrease in NH_3-N production observed in quinoa forages is probably due to the presence of PSMs. The NH_3-N production in the rumen correlated to the concentration of degradable protein and energy availability, diet composition especially the concentration and structure of PSMs and endogenous factors (age, species, physiological situation and sex) (Harun & Sali, 2019; Kapp--Bitter, Dickhoefer, Kreuzer & Leiber, 2021). Various researches have shown PSMs reduce the NH_3-N concentration in the rumen by inhibiting the proteolytic activity of microorganisms. This inhibition is attributed to decreased penetrability, reduced activity, and the breakdown of microbial cell membranes (McIntosh et al. 2003; Abarghuei & Salem, 2021; Moheghi, Ghoryar & Ataei, 2022), a finding that is corroborated by the results of the present research. The obstruction of protozoa may lead to a decrease in NH_3-N concentration because of declining bacterial lysis (Williams & Coleman 1991; Holtshausen et al., 2009). A further possible cause for the reduction in NH_3-N production could be utilization of NH_3-N for MP synthesis (Table 3) (Makkar, 2003).

4.4. Protozoa counts

Protozoa make up about 50% of the rumen microbial population. They have a symbiotic relationship with archaea, consuming organic matter particles and bacteria, and significantly contribute to the digestion of fiber, carbohydrates, proteins, and lipids (Newbold, De La Fuente, Belanche, Ramos-Morales & McEwan, 2015; Vasta et al., 2019). Different factors influence on ruminal protozoa population including feed composition, water, salivation, the passage rate of the digesta through the rumen, fermentative activity, the production of acids in the rumen and some PSMs (Guimaraes et al., 2023). The antiprotozoal impacts of PSMs may be influenced by their dose and structure in feed. Applying quinoa forage reduced total protozoa (0.98–3.28%) and subfamilies of *Entodiniinae* (0.51–2.37%) compared to alfalfa forage. Different harvest stages had diverse effects on the population of *Isotricha* spp., subfamilies of *Diplodiniinae* and *Ophrioscolecinae*. Phenolics and tannins disturb protozoa membrane, deactivate enzymes and depletion of essential nutrients for the metabolism of these organisms (Patra & Saxena, 2011; Demirtaş, Öztürk& Pişkin, 2018). Nevertheless, the effects of PSMs on protozoa counts are vary in many studies and are influenced by factors such as diet substrates, PSMs concentrations and structures, degradation of PSMs by rumen microorganisms, animal differences and sampling methods (Patra & Saxena, 2011; Abarghuei & Salem, 2021). Studies regarding the effects of PSMs on ruminal protozoa were not constant. Benchaar (2020) and Rajabi, Rouzbehan and Rezaei (2017) reported no significant impact. While Raghuvansi et al. (2007) showed an increase, and Romero et al. (2023) and Ashkvari, Rouzbehan, Rezaei and Boostani (2023) indicated a decrease in the protozoan population.

4.5. In situ DM and CP degradability of alfalfa and quinoa forages

The difference in *a* value of dry matter can be due to the timing of plant harvesting and the zero-time calculation method. Also, forages that contain a higher concentration of mineral elements have more soluble dry matter (Bashtani, Seifi, Naemipour Yonesi & Farzadmehr, 2012). Probably, the higher content of ash in quinoa forage (Table 1) could be the cause for the more significant *a* value compared to alfalfa forage. The DM degradability parameters are influenced by chemical composition, solubility, physical structure and cell wall construction (Givens, Owen, Auford & Omend, 2000). Degradability is also indirectly associated with cell wall carbohydrates and has a direct correlation with CP (Ghavipanjeh, Fathi Nasri, Bashtani & Farhangfar, 2019). The differences in values of *a* + *b* and ED of dry matter for quinoa forages compared to alfalfa forage may be attributed to the differing contents of NDFom and ADFom. In contrast with current research, the amounts of *a, b* and *c* of DM for *Chenopodium album* plant, which is the same genus of quinoa plant, were determined 267.40 and 340.50 g/kg DM and 0.194/h, respectively (Hoseini Nejad, Yoosefollahi & Fazaeli, 2012). Barros-Rodríguez et al. (2018) reported the degradability potential of the DM of the whole quinoa plant harvested in 180 days, 80.86% DM, which was higher than the results of our research. The difference between the present research and previous studies can be due to the quinoa variety, conditions of planting and harvest, harvest time, chemical composition and cell wall of the experimental plant and animals (Shahbazi, et al., 2012; Ma, et al., 2021).

Fig. 2

Determining forage protein degradability is essential in the ruminant's diets balancing because it may improve the nitrogen utilization efficiency of the diet (McCarthy et al., 2023). However, there is a scarcity of data regarding the protein degradability of harvestable quinoa forage at different growth stages. The higher *a* value of the CP observed in Q45 treatment may be attributed to the youngness of the plant and the lower contents of NDFom, ADFom and lignin at this growth stage. The content of CP in quinoa forage harvested between 45 and 95 days was greater than that of alfalfa forage (199.52 and 183.31 *vs* 144.50 g/kg of DM respectively). This higher protein is probably the reason for the more *a* + *b* and ED parameters of quinoa forage. Also, the decrease in the content associated with the growth of quinoa forage may contribute to a decrease in protein degradability. Reduction in *c* parameter for quinoa forages compared to alfalfa forage, suggests an extended time for the protein ruminal fermentation. This phenomenon may be attributed to the presence of PSMs such as tannins. The results of the present study showed that the degradability of alfalfa forage was lower than that of

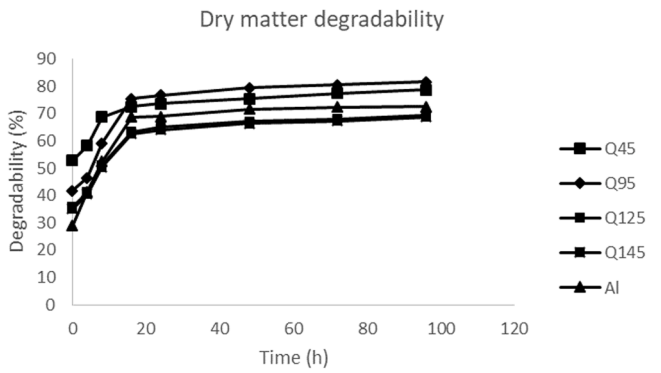

Fig. 2. *In situ* dry matter degradability parameters of forages alfalfa and quinoa harvested at different stages.
Al: alfalfa forage; Q45: budding stage, quinoa harvested 45 days after planting; Q95: 10% flowering stage, quinoa harvested 95 days after planting; Q125: before milk stage, quinoa harvested 125 days after planting; Q145: before milk stage, quinoa harvested 145 days after planting.

quinoa forage (Fig. 3). This suggests a diminished protein digestion value, attributable to reduced proteolysis occurring in the rumen before the material reaches the intestine. These PSMs are known to either reduce protein degradation or inhibit the activity of microorganisms involved in protein degradation (Loregian et al., 2023). Therefore, increasing the degradability and decreasing the fermentation rate in quinoa forage can have an important influence on the normal NH_3-N production and increase the synthesis of MP in the rumen (Table 3). Barros-Rodríguez et al. (2018) reported that the values of the CP degradability parameters of the quinoa seed were the highest, while those for quinoa stems were at their lowest. Also, the value of $a + b$ parameter in the whole quinoa plant harvested for 180 days was 71.71% of DM, which was lower than the findings presented in our study. However, forage CP degradability in the rumen is influenced by several factors, including the duration of storage, the difficulty of fermentation, the livestock used, the type of forage, the forage physiognomies (the composition of CP, non-protein nitrogen, the actual protein content, the physical and chemical properties of the actual protein and PSMs) (Ma et al., 2021).

4.6. Fresh and dry forage, CPP, WU and WUE of alfalfa and quinoa harvested at different stages

The yield of quinoa forage was different compared to that of alfalfa

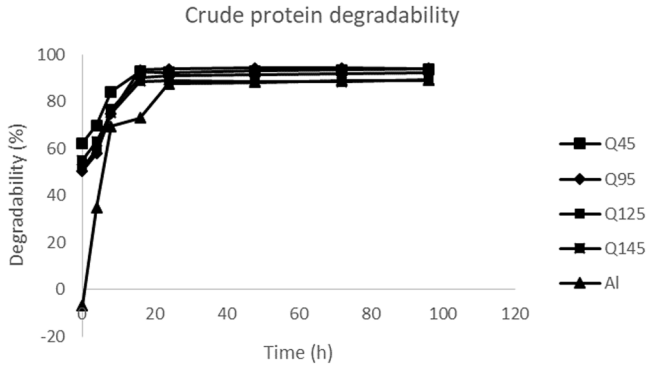

Fig. 3. *In situ* crude protein degradability parameters of forages alfalfa and quinoa harvested at different stages.
Al: alfalfa forage; Q45: budding stage, quinoa harvested 45 days after planting; Q95: 10% flowering stage, quinoa harvested 95 days after planting; Q125: before milk stage, quinoa harvested 125 days after planting; Q145: before milk stage, quinoa harvested 145 days after planting.

plant (equal to alfalfa planting conditions). The findings of the study indicated that the accumulation of DM increases until the final stages of harvesting. Numerous studies have demonstrated that the yield of quinoa forage is affected by genotype, planting conditions, planting season and geographical region (Kardooni, Tavoosi, Mahdavi Majd, Taheri Dezfoli & Anafjeh, 2019; Yilmaz, Ertekin & Atis, 2021). In a study, Temel and Yolcu (2020) found that the content of DM produced in the quinoa plant varies from 5.33 to 22.7 t/ha according to the planting date and harvesting steps. Tavosi, Kardooni and Mahdavi Majd (2018) planted three quinoa genotypes (Giza 1, Rosada and Q102) and reported the dry forage production of the plant in winter season was more than 5 t/ha. In another study, six quinoa plants were cultivated during two distinct winter seasons for two years via a small volume of irrigation. When the dry matter of the plant reached 28 to 30%, the plants were harvested and the DM yield was 5270 and 12,710 kg/ha (Asher, Galili, Whitney & Rubinovich, 2020). The lower CPP in quinoa treatments compared to Al treatment may be attributed to several factors, including the type of plant, the content of DM and CPP of forage and the quantity of forage harvested per hectare. In research, the effects of various nitrogen fertilizer applications on CPP/ha of quinoa forage was investigated and the amount of CPP was reported between 1250 and 2481 kg/ha (Kakabouki et al., 2014). The findings indicated that under the conditions of this research, it was possible to yield over 12 tons per hectare of dry feed from quinoa plants harvested after a growth period of 145 days.

Recently, droughts and the lack of water resources have prompted global experts to seek solutions aimed at enhancing water consumption efficiency in agricultural practices. One way to improve this productivity could possibly be to cultivate plants adapted to water stress, such as quinoa. By enhancing the production output per unit of water utilized in agriculture, it is possible to significantly improve water productivity. The amount of WU in quinoa forage increased with increasing harvest age (Table 8). The results showed that quinoa forage had 26.89 to 205.52 percent more WUE compared to alfalfa forage. This enhancement can be attributed to the plant's growth and the time of harvest, resulting in greater water requirements. The volume of WU in quinoa plant was lower than that of alfalfa plant. The quinoa plant exhibits low water consumption as a result of its intrinsic traits, which include minimal water absorption, tolerance to saline conditions, and resilience to drought (Jaikishun, Li, Yang & Song, 2019). Also, quinoa's root systems are efficient in deeper soil layers, enhancing water uptake and productivity, especially under deficit irrigation strategies (Mirsafi, Sepaskhah & Ahmadi, 2024). There is no research on WU and WUS for quinoa forage production. In research reported that the volume of WU in quinoa plant was 3330 to 13,600 m^3/ha and the WUE was between 0.24 and 0.62 kg/m^3 (Beyrami, Yazdani Biouki, Rahimian & Salehi, 2019). In another research, Yazar, Sezen, Çolak, Kaya and Tekin (2017) mentioned the efficiency of WUE in quinoa plant harvested at seeding was between 1.00 and 1.57 kg/m^3. Another study reported that the amount of WU the Giza 1 quinoa variety ranged from 2645 and 4970 m^3/ha, while the WUE varied between 1.00 and 1.38 kg/m^3 (Jamali and Ansari, 2021). The differences between the studies for WU and WUE can be attributed to the variety of quinoa cultivated and the conditions of planting, harvesting and harvesting.

5. Conclusion

Quinoa forages, especially those harvested at the stage of 45 and 95 days of growth, can be used as a food with a fast fermentable energy source for ruminants, due to having the potential of degradability and effective degradability of CP more than alfalfa forage. Additionally, quinoa forage harvested at these stages without effect on digestibility, has the potential to decrease the production of CH_4, CO_2, and NH_3-N, thereby representing a more environmentally sustainable approach to mitigating pollution. The results showed that quinoa forage had higher WUE compared to alfalfa forage. Quinoa forage can be beneficial in

Table 8
Fresh and dry forage, CP, WU and WUE of alfalfa and quinoa forages harvested at different stages.

	Forage						
	Al	Q45	Q95	Q125	Q145	SEM	*P*-value
FF	45.71^{c}	10.30^{d}	45.10^{c}	47.50^{c}	49.37^{a}	0.228	< 0.0001
DF	16.00^{a}	1.66^{e}	7.98^{d}	9.58^{c}	12.34^{b}	0.089	< 0.0001
CPP	2312.39^{a}	330.48^{d}	1461.99^{b}	1355.83^{c}	1382.30^{bc}	25.892	< 0.0001
WU	11000^{a}	900^{e}	1800^{d}	2700^{c}	3150^{b}	13.292	< 0.0001
WUE	1.45^{e}	1.84^{d}	4.43^{a}	3.55^{c}	3.92^{b}	0.033	< 0.0001

Al: alfalfa forage; Q45: budding stage, quinoa harvested 45 days after planting; Q95: 10% flowering stage, quinoa harvested 95 days after planting; Q125: before milk stage, quinoa harvested 125 days after planting; Q145: before milk stage, quinoa harvested 145 days after planting; FF = fresh forage (t/ha); DF = dry forage (t/ha); CPP = crude protein production (kg/ha); WU = water use (m^3); WUE = water use efficiency (kg DF/m^3 WU); SEM = Standard error of the mean; Means within a row with different superscripts differ ($P < 0.05$).

regions facing forage scarcity, offering a sustainable option for livestock feeding. However, it is essential to explore additional quinoa cultivars that have a reduced growth duration, and better results can be achieved by using the forage of this plant *in vivo* studies.

Ethical Statement

The experiment was carried out according to The Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) guidelines. All procedures and guidelines involving animals were approved by the Animal Experiment Committee at Research Institute of Animal Science, Iran.

Ethical Statement for Solid State Ionics

Hereby, I /insert author name/ consciously assure that for the manuscript /insert title/ the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.

2) The paper is not currently being considered for publication elsewhere.

3) The paper reflects the authors' own research and analysis in a truthful and complete manner.

4) The paper properly credits the meaningful contributions of co-authors and co-researchers.

5) The results are appropriately placed in the context of prior and existing research.

6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.

7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

The violation of the Ethical Statement rules may result in severe consequences.

I agree with the above statements and declare that this submission follows the policies of Solid State Ionics as outlined in the Guide for Authors and in the Ethical Statement.

Date: 22/10/2024

Corresponding author's signature: Mohammad Javad Abarghuei

CRediT authorship contribution statement

Mohammad Javad Abarghuei: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Alidad Boostani:** Project administration, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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