

PHYSIOLOGICAL RESPONSES OF SHEEP'S MILK TO FORAGE-BASED DIETS PRODUCED USING TREATED WASTEWATER

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Supporting Information



ABSTRACT: The study aimed to investigate the effects of different feeding strategies and irrigation methods of forage on sheep milk yield and composition. Specifically, it evaluated the impact of alfalfa (*Medicago sativa* L.) and guinea grass (*Panicum maximum*)-based diets irrigated with either treated wastewater (TWW), after physical, biological, and chemical methods to remove contaminants or fresh water. 30 lactating ewes were assigned to different feeding groups, and milk yield and composition parameters including fat, solids-not-fat (SNF), lactose, protein, and total solids were analyzed over a 25-day period. Results indicated that while milk yield remained stable across feeding groups, significant differences ($P \leq 0.05$) were observed in milk composition. The highest milk yield (3.5 kg/day) was recorded in ewes supplemented with 100% *M. sativa* irrigated with TWW, while the lowest yield (1.5 kg/day) was observed in the 50% straw irrigated with freshwater treatment. Milk fat percentage and lactose did not significantly differ between feeding treatments, meanwhile SNF, density, total solids, and protein content were significantly affected. Ewes consuming *Panicum maximum* irrigated with TWW had higher SNF and protein levels, suggesting a nutritional advantage of drought-resistant grasses in dairy production. A significant decline ($P \leq 0.05$) in milk fat percentage was observed over time, particularly in ewes consuming 100% *M. sativa* irrigated with TWW. These findings suggest that feeding strategy plays a more decisive role in milk composition than irrigation type, with *P. maximum* improving SNF and protein levels. Irrigated with treated wastewater did not negatively impact milk yield or major composition parameters, indicating its potential as a sustainable irrigation source for forage plants.

Keywords: Feeding strategies, *Medicago sativa*, *Panicum maximum*, Sheep, Wastewater.

INTRODUCTION

Milk production and quality are key determinants of both livestock management efficiency and the long-term sustainability of the dairy industry (Priyashantha, 2025). Among the many factors influencing milk yield and composition, feeding strategies play a particularly important role (Mukasafari et al., 2025). The choice of forage type, along with its cultivation conditions, impacts not only the efficiency of milk production but also its nutritional composition (Casper et al., 2025). However, some studies have explored how different forage species, particularly alfalfa, *Medicago sativa* L. and guinea grass, *Panicum maximum*, influence milk composition when irrigated with treated wastewater (TWW) (Guzatti et al., 2018; Neto et al., 2018). Understanding these interactions is crucial for optimizing dairy production in water-scarce regions while promoting sustainable agricultural practices.

The *M. sativa* is widely recognized as a high-quality forage legume, often referred to as the "queen of forages" due to its high protein content, digestibility, and drought resistance. It is extensively used in ruminant feeding systems to improve milk yield and quality. Studies have reported that *M. sativa*-based diets can enhance milk composition, particularly fatty acid profiles, without significantly altering milk yield (Babiker et al., 2017; Guzatti et al., 2018). On the other hand, *P. maximum* is a perennial C4 grass known for its high biomass yield and adaptability to tropical and subtropical climates. Studies have indicated that *P. maximum*-based diets can influence milk yield and composition, though their impact on specific milk parameters remains underexplored (De Souza et al., 2014; Neto et al., 2018). Despite their agronomic advantages, comparative studies on the effects of *M. sativa*- and *P. maximum*-based diets on milk composition are limited, particularly under different irrigation systems considering indigenous sheep such as Awassi sheep (Al-Atiyat et al., 2012 and 2014).

Water scarcity is a major constraint in forage production for small ruminants, leading to an increasing reliance on alternative irrigation sources, such as TWW (Benaafi et al., 2024). The reuse of TWW in agriculture has gained attention as a sustainable solution to freshwater shortages, providing essential nutrients for plant growth while reducing environmental pollution (Ganjegunte et al., 2017; El Moussaoui et al., 2019). Previous studies have confirmed that wastewater irrigation enhances soil fertility and forage biomass production (Mohammad and Mazahreh, 2003; Ganjegunte et al., 2017), but concerns remain regarding potential heavy metal accumulation, microbial

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contamination, and effects on animal health (Sdiri et al., 2023). Despite the impacts of TWW on plant physiology and soil health have been widely studied, its effects on milk production and composition remain largely unexplored. Addressing this gap is essential to determine the feasibility of TWW-irrigated forage as a viable feed source in dairy production.

This study investigates the effects of different feeding strategies and irrigation methods on the milk yield and composition of sheep. Specifically, it evaluates how *M. sativa*- and *P. maximum*-based diets irrigated with TWW or freshwater influence key milk parameters, including milk yield, fat percentage, lactose, solids-not-fat (SNF), protein content, total solids and density. It is hypothesized that forage type may significantly affect milk composition, while wastewater irrigation may indirectly influence these parameters through its impact on forage nutrient content. By integrating agronomic and animal performance perspectives, this research offers new insights into sustainable feeding practices of small ruminants, particularly for regions facing water scarcity and increasing dependence on wastewater irrigation.

MATERIALS AND METHODS

Experimental site and ethical considerations

This study was carried out in National Agriculture Research Center of Ministry of Agriculture, Palestine from January to November 2021. This study was approved by the Institutional Review Board (IRB) / ethical committee at An-Najah National university, Nablus, Palestine. The approval letter (No. IRB-12/2021) ensures that the procedures for sample collection, sheep feeding trials, and wastewater handling conform to safety and welfare regulations.

Feeding trial

Two experimental fields were established for the cultivation of *M. sativa* and *P. maximum* under TWW and freshwater irrigation. Three random samples were collected to enable a comparative assessment of nutritional composition, including dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), crude fat, and mineral content (calcium and phosphorus). These parameters provided a comprehensive assessment of the productivity and nutritional value of the forages, enabling a direct comparison between TWW-irrigated and fresh water-irrigated crops. The wastewater was tertiary treated by physical, biological, and chemical methods to remove contaminants and then disinfection was made. A total of 30 lactating ewes (initial body weight: 43.2 ± 0.97 kg, age: 4 years old), each nursing a single lamb of third party, were selected for the feeding trial. To ensure reproductive synchronization, ewes underwent estrous synchronization using intravaginal sponges. The animals were then randomly assigned to five experimental dietary groups (n = 10 ewes per group) as follows: group 1: Control diet (straw as a traditional ration used in local ewe farms), group 2: 50% of traditional roughage replaced with *M. sativa*, group 3: 100% of traditional roughage replaced with *M. sativa*, group 4: 50% of traditional roughage replaced with *P. maximum*, and group 5: 100% of traditional roughage replaced with *P. maximum*.

The ewes were individually housed in shaded pens (0.75 × 1.5 m), each equipped with plastic feed and water buckets. Diets were formulated according to NRC (NRC, 2007) guidelines to meet the nutritional requirements of lactating ewes. Feed was offered ad libitum at 08:00 AM with a 10% refusal margin, and fresh water was available at all times. Nutrient intake was daily recorded, and lambs relied exclusively on their dams' milk for nourishment during the trial. At the end of the experimental period, lambs were gradually transitioned to concentrate- and roughage-based diets to initiate the weaning process. The feeding trial lasted eight weeks, with the first week serving as an adaptation period and data collection were conducted during the subsequent seven weeks. Feed refusals were weighed, recorded, and sampled daily to determine nutrient intake accurately. The ewe body weight was measured at the beginning of the trial and thereafter bi-weekly. A digestion trial was conducted at the end of the feeding study. Three ewes from each dietary group were randomly selected for the digestion experiment. A one-week adaptation period was followed by a 7-day total fecal collection period to estimate nutrient digestibility. Fecal samples were collected, weighed, and analyzed for DM, CP, fiber fractions (NDF, ADF), and ether extract to determine nutrient utilization efficiency. The treatments were: 1) STRW1: 100% straw irrigated with fresh water; 2) STRW2: 50% straw irrigated with fresh water; 3) FST50: 50% *M. sativa* irrigated with TWW, 50% straw; 4) FT100: 100% *M. sativa* irrigated with TWW; 5) FFW50: 50% *M. sativa* irrigated with fresh water, 50% straw; 6) FFW100: 100% *M. sativa* irrigated with fresh water; 7) PST50: 50% *P. maximum* irrigated with treated water, 50% straw; 8) FSFW50: 50% *P. maximum* irrigated with fresh water, 50% straw, and 9) PFW100: 100% *P. maximum* irrigated with fresh water.

Milk yield and composition analysis

Milk yield was recorded bi-weekly at 08:00 AM using hand-milking techniques. To standardize the process, lambs were separated from their dams 12 hours before milking. The recorded yield was extrapolated to a 24-hour basis for accurate comparison. From each ewe, a 125 ml milk sample was collected and analyzed for total solids by drying at 55 °C in a forced-air oven. Milk fat content was determined using the Gerber method, while CP was calculated via the Kjeldahl procedure using a nitrogen-to-protein conversion factor of $N \times 6.38$.

Chemical analysis of diets and feed refusals

To determine the chemical composition of diets and feed refusals, samples were processed as follows: for the DM the samples were oven-dried at 105 °C overnight (AOAC, 2006). The CP was measured using the Kjeldahl method ($N \times 6.25$ conversion factor). The NDF and ADF were analyzed using an ANKOM2000 fiber analyzer. Ether extract (fat content) was determined using the Soxhlet procedure, while the metabolizable energy (ME) was estimated using NRC (NRC, 2007) tabular values. The DM and nutrient intakes were calculated by subtracting feed refusals from total feed offered.

Statistical analysis

The statistical analysis was performed using the SAS statistical software package (version 9.2) (SAS, 2012). Data normality was assessed using the Kolmogorov-Smirnov and Shapiro-Wilk tests. As the data did not follow normal distribution, a non-parametric test was applied. Comparisons of milk yield and composition among feeding strategy groups were conducted using the least significant difference (LSD) test at a probability level of $P \leq 0.05$ (Zar, 1999).

RESULTS

The chemical composition of forage grown under different irrigation methods is presented (Table 1). The main difference between *M. sativa* (alfalfa) and *P. maximum* (Guinea grass) lies in their CP content. The *M. sativa* contains a high protein level (23.6-24.8% of DM), whereas *P. maximum* provides a moderate protein content (11.5-13.9% of DM). Other components, such as crude fiber, NDF, and ADF, are also reported (Table 1).

The milk yield and composition (mean \pm SEM) of lactating ewes under different feeding strategies and irrigation methods (Table 2). Milk yield varied significantly among feeding treatments ($P = 0.001$), ranging from 1.5 to 3.5 kg per ewe per day. The highest yield (3.5 kg/day) was recorded in ewes supplemented with FT100, while the lowest yield (1.5 kg/day) was observed in the STRW2 treatment. These findings suggest that partially replacing straw under freshwater irrigation with *M. sativa* under TWW irrigation improved milk yield by approximately 2.5-fold, potentially due to enhanced digestibility or reduced metabolic imbalances. In contrast, no significant differences were observed between ewes fed forages irrigated with fresh water and those irrigated with TWW, indicating that irrigation source alone had no effect on total milk yield. Rather, the composition of the diet appeared to play the dominant role in determining production levels.

Milk composition analysis showed considerable variation in fat percentage, SNF, density, lactose, solids, and protein content across treatments. Fat percentage ranged from 4.10% to 9.09%, with the highest values observed in ewes receiving the FT100 treatment, followed by those supplemented with *P. maximum* irrigated with FSFW50 (8.43%) and PST50 (8.42%). The lowest fat percentage (4.10%) was recorded in ewes supplemented with PFW100, suggesting that legume-based diets may be associated with reduced milk fat content. Nevertheless, differences in fat percentage among feeding treatments were not statistically significant ($P = 0.089$), indicating that factors beyond diet composition, such as metabolic adaptations, may play a role in regulating milk fat content over time (Table 2).

The SNF content differed significantly among feeding treatments ($P = 0.029$), ranging from 8.41 to 9.62. The highest SNF values were observed in ewes supplemented with STRW2, suggesting a potential link between fiber-rich diets and elevated SNF levels, while the lowest values were recorded in ewes fed FSFW50. Milk density also varied significantly ($P = 0.040$), with the highest values in ewes fed FFW50 (30.93) and STRW2 (30.84), and the lowest in those fed FSFW50 (26.26). In contrast, lactose content remained relatively stable across treatments (4.87–5.50%), with no significant differences detected ($P = 0.470$), indicating that dietary interventions had little influence on lactose synthesis. Total solids were significantly affected by diet ($P = 0.030$), ranging from 0.73 to 1.12, with STRW2 showing the highest values and FFW50 the lowest. Similarly, protein percentage varied significantly ($P = 0.036$), with the highest levels in STRW2 (3.17%) and FFW50 (3.16), while FSFW50 yielded the lowest protein content (2.41%). These findings highlight the role of forage type and diet composition in shaping milk quality traits, particularly SNF, density, total solids, and protein content (Table 2).

The results show that four parameters, i.e. milk yield, fat, total solids, and protein content were significantly affected ($P < 0.05$) by both feeding strategy and irrigation method (Figure 1). The LS-means test indicated that ewes fed alfalfa irrigated with treated water (FT100) produced a significantly higher milk yield. A similar trend was observed for milk fat, with the highest fat content also recorded under the FT100 strategy. For total solids, the STRW2 strategy resulted in significantly higher values. Regarding protein content, the highest levels were reported for STRW2, FFW50, and FT100 feeding strategies (Figure 1).

Table 1 - Chemical composition of offered fodder of different irrigation methods.

Feed type	Sample replicate	Fiber (%)	Moisture	Ash (%)	Protein (%)	ADF (%)	NDF (%)
<i>M. sativa</i> fresh water irrigated	1	14.1	82.0	10.06	24.8	19.7	24.6
<i>M. sativa</i> fresh water irrigated	2	14.5	82.1	10.0	24.2	20.2	25.3
<i>M. sativa</i> fresh water irrigated	3	15.2	83.0	10.1	23.9	21.2	26.5
<i>M. sativa</i> TWW irrigated	1	19.1	81.2	10.3	23.6	26.7	33.4
<i>M. sativa</i> TWW irrigated	2	18.2	81.0	10.1	23.96	25.4	31.8
<i>M. sativa</i> TWW irrigated	3	18.6	81.1	10.12	24.6	26.02	32.5
<i>P. maximum</i> fresh water irrigated	1	16.0	82.0	15.8	11.5	22.4	28.0
<i>P. maximum</i> fresh water irrigated	2	16.1	82.1	16.9	11.8	22.5	28.17
<i>P. maximum</i> fresh water irrigated	3	17.0	81.1	16.0	12.09	23.6	29.7
<i>P. maximum</i> TWW irrigated	1	13.0	84.0	14.3	12.8	18.0	22.7
<i>P. maximum</i> TWW irrigated	2	13.1	82.0	14.0	13.0	18.3	22.9
<i>P. maximum</i> TWW irrigated	3	13.2	83.1	14.5	13.9	18.4	23.1

TWW: treated wastewater; ADF: acid detergent fiber; NDF: neutral detergent fiber,

Table 2 - Milk yield and composition (mean ± SEM) of lactating ewes fed under different feeding strategies and irrigation methods.

Treatment	Yield	Fat content (%)	SNF (%)	Total solids (%)	Lactose (%)	Protein (%)
FFW100	2.00±0.19 ^b	6.71±0.78 ^{bc}	8.87±0.38	28.20±1.70	5.10±0.21	2.56±0.15 ^c
FFW50	2.00±0.19 ^b	7.39±0.78 ^b	9.36±0.38	30.93±1.70	5.27±0.21	3.16±0.15 ^a
FSFW50	3.00±0.19 ^a	8.43±0.78 ^a	8.41±0.38	26.26±1.70	4.87±0.21	2.41±0.15 ^d
FST50	3.00±0.19 ^a	6.15±0.78 ^c	8.67±0.38	28.58±1.70	4.89±0.21	2.93±0.15 ^b
FT100	3.50±0.19 ^a	9.09±0.78 ^a	8.95±0.38	28.26±1.70	5.16±0.21	3.09±0.15 ^a
PFW100	2.60±0.13 ^{ab}	4.10±0.55 ^d	8.90±0.27	29.83±1.21	4.98±0.15	2.92±0.11 ^b
PST50	2.00±0.19 ^b	8.42±0.78 ^a	8.94±0.38	28.20±1.70	5.16±0.21	2.90±0.15 ^b
STRW1	2.00±0.19 ^b	5.98±0.78 ^c	9.25±0.38	28.99±1.70	5.35±0.21	2.98±0.15 ^b
STRW2	1.50±0.19 ^c	5.77±0.78 ^c	9.62±0.38	30.84±1.70	5.50±0.21	3.17±0.15 ^a
P-Value	*: 0.001	*: 0.0007	NS: 0.520	NS: 0.63	NS: 0.408	*: 0.0199

FFW100: 100% *M. sativa* irrigated with fresh water; FFW50: 50% *M. sativa* irrigated with fresh water, 50% straw; FSFW50: 50% *P. maximum* irrigated with fresh water, 50% straw; FST50: 50% *M. sativa* irrigated with TWW, 50% straw; FT100: 100% *M. sativa* irrigated with TWW; PFW100: 100% *P. maximum* irrigated with fresh water; PST50: 50% *P. maximum* irrigated with treated water, 50% straw; STRW1: 100% straw irrigated with fresh water; STRW2: 50% straw irrigated with fresh water; SNF solids-not-fat.*: Indicates significant differences among the different treatments with the same column (LSD, $p \leq 0.05$). NS: Indicates no significant differences among the different treatments with the same column (LSD, $p > 0.05$).

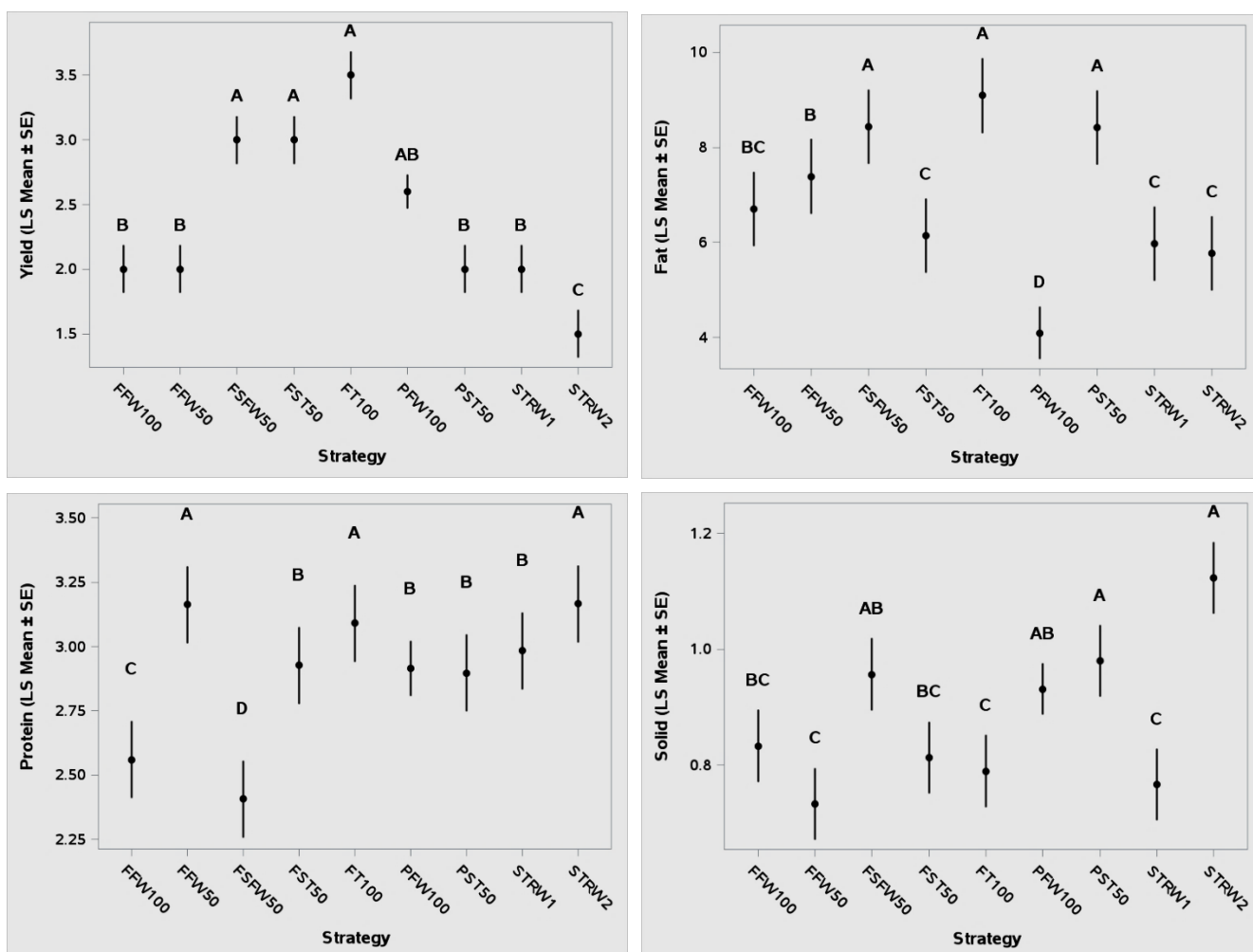


Figure 1 - Long-term effects of different feeding strategies on sheep milk yield and composition. FFW100: 100% *M. sativa* irrigated with fresh water; FFW50: 50% *M. sativa* irrigated with fresh water, 50% straw; FSFW50: 50% *P. maximum* irrigated with fresh water, 50% straw; FST50: 50% *M. sativa* irrigated with TWW, 50% straw; FT100: 100% *M. sativa* irrigated with TWW; PFW100: 100% *P. maximum* irrigated with fresh water; PST50: 50% *P. maximum* irrigated with treated water, 50% straw; STRW1: 100% straw irrigated with fresh water; STRW2: 50% straw irrigated with fresh water

DISCUSSION

The findings of this study provide essential insights into the effects of different feeding strategies and irrigation methods on milk yield and composition in sheep. Over the 25-day experimental period, specific feeding combinations resulted in notable variations in milk composition, while overall milk yield remained largely stable. These results indicate that both diet composition and irrigation type can influence milk quality, particularly regarding fat content and total solids percentage.

It is important to first consider the general chemical composition of both forage species. Alfalfa (*M. sativa*) is notably richer in protein, bioactive compounds (such as isoflavones, phenolics, and saponins), vitamins, and minerals compared to Guinea grass (*P. maximum*) (Horvat et al., 2022). The protein content of *M. sativa* typically ranges between 18-25% of DM and is accompanied by higher concentrations of essential amino acids, particularly lysine. Moreover, its protein is more degradable in the rumen, although tannins and saponins present in the plant can influence its utilization (Liu et al., 2024). In contrast, *P. maximum* contains higher levels of carbohydrates and fiber, but lower protein concentrations (8-15% of DM), and it provides far fewer bioactive phytochemicals (Johnson et al., 1967). The NDF and ADF contents typically range from 35-45% and 25-35% for *M. sativa*, and 55-70% and 35-45% for *P. maximum*, respectively (Johnson et al., 1967; Horvat et al., 2022). Furthermore, the ME and TDN values are relatively similar between the two species, ranging from ~9-11 MJ/kg DM and ~55-65%, respectively (Kumara et al., 2022; Liu et al., 2024). Overall, these reported findings on the chemical composition of both species are consistent with the results obtained in our study.

The effects of *M. sativa*- and *P. maximum*-based diets on milk yield and composition have been widely studied in the literature for sheep and goat (Al-Araimi et al. 2017; Babiker et al. 2017; Guzzati et al. 2018). In this regard, Guzzati et al. (2018) stated that diets containing *M. sativa* and red clover silage did not significantly affect milk yield but altered the fatty acid profile of milk. Similarly, Babiker et al. (2017) found that the addition of Moringa leaves to *M. sativa*-based diets increased milk yield without a significant effect on the composition of milk. Consistent with these findings, our study demonstrates that *M. sativa*-based diets do not significantly impact milk yield but induce specific changes in milk components. Previous research on *P. maximum*-based feeding strategies also supports our results. De Souza et al. (2014) found that *P. maximum*-based diets increased milk yield but had no major effects on milk composition. Our findings align with this, indicating significant differences in milk fat content but minimal changes in milk yield when *P. maximum* was included in the diet. These results confirm that forage composition can influence milk quality, whereas its effect on milk production remains limited.

A key aspect of this study was the evaluation of TWW as an irrigation source for forage plants. Prior research has demonstrated that TWW can enhance soil nutrient levels and improve forage growth (Ganjegunte et al., 2017; El Moussaoui et al., 2019). However, Sdiri et al. (2023) suggested that TWW irrigation may lead to metal accumulation in the soil, potentially altering plant nutritional composition. Our findings indicate that sheep consuming TWW-irrigated forage exhibited variations in milk fat content, but no significant differences were observed in protein, lactose, or total solids percentages compared to sheep fed with fresh water-irrigated forage. These results are consistent with previous studies suggesting that TWW enhances forage nutritional content; however, further research is necessary to evaluate its potential long-term impact on milk quality.

This study represents one of the few investigations that simultaneously assess the effects of *M. sativa*- and *P. maximum*-based diets alongside TWW irrigation. While previous studies have focused on the effects of TWW on soil properties and plant growth (Bedbabis et al., 2014; Soufan et al., 2019), fewer studies have explored its influence on animal nutrition and milk production. By examining the combined effects of feeding strategies and irrigation methods, this study fills an important gap in literature. Moreover, Bevilacqua et al. (2014) and Ceci et al. (2023) have examined the effects of TWW-irrigated forage on animal health and milk safety. Our results indicate that the impact of TWW-irrigated forage on milk composition is limited, with no significant negative effects detected. However, long-term monitoring is necessary to assess the potential risks of metal accumulation and microbial contamination in milk.

Despite these valuable contributions, this study has some limitations. The small sample size (three animals per group), the specific geographic conditions, and the relatively short duration (25 days) may restrict the generalizability of the findings. Furthermore, the long-term effects of TWW irrigation on micronutrient profiles in forage were not fully assessed. Future research should address these limitations by focusing on the following points: 1) conducting studies with larger sample sizes to improve statistical power; 2) evaluating feeding strategies across diverse geographic regions to assess environmental impacts; 3) performing detailed chemical and microbiological analyses of milk composition to ensure food safety; 4) implementing long-term follow-up studies to monitor the potential effects of TWW irrigation on livestock health; and 5) investigating genetic and environmental factors that may influence milk yield and composition.

CONCLUSION

This study examined the effects of different feeding strategies and irrigation methods on sheep milk yield and composition. The results indicate that *M. sativa*- and *P. maximum*-based diets can influence milk composition, particularly

fat content and total solids, while overall milk yield remained largely unaffected. Regarding irrigation, no adverse effects on milk yield were observed in sheep consuming TWW-irrigated forage, though minor variations in milk composition were noted, suggesting that TWW may be a viable irrigation source for forage production. As one of the few studies assessing both feeding strategies and TWW irrigation, this work provides valuable insights into the interplay between animal nutrition, milk quality, and sustainable sheep production. Nevertheless, future research with larger sample sizes and longer durations is needed to fully evaluate the nutritional and safety implications of these practices. Overall, optimizing feeding strategies and selecting appropriate forage types and irrigation methods are critical for enhancing milk yield and composition while supporting sustainable small ruminant production and mitigating potential risks associated with TWW irrigation. However, further research is needed to assess long-term impacts on animal health and milk safety, particularly in regions where water scarcity necessitates alternative irrigation sources.

DECLARATIONS

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Data availability

The data are available upon request from corresponding author

Authors' contribution

All authors have read and approved the final version of the manuscript and agree to its submission to the journal.

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Ethical considerations

This study was ethically approved by the Institutional Review Board (IRB) and ethical committee at An-Najah National university, Nablus, Palestine (Approval No. IRB-12/2021).

Declaration of generative AI in scientific writing

The authors declare that no generative artificial intelligence (AI) tools were used in the writing and editing of this manuscript except for grammar correction, which was conducted under the authors' supervision. The authors are responsible for all intellectual contributions, data analysis, and interpretations this manuscript presents.

Competing interests

The authors declare no conflict of interest.

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