J. Dairy Sci. 109:2476–2491
https://doi.org/10.3168/jds.2025-27243

Terpene transfer to milk from fresh leaves of hemp (*Cannabis sativa* L.) and savory (*Satureja hortensis* L.)

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ABSTRACT

This study aimed to investigate the transfer of terpenes from fresh aromatic plants, hemp (*Cannabis sativa* L.) and savory (*Satureja hortensis* L.), into cow milk, quantify their transfer efficiency, and explore their dynamics. Six Simmental dairy cows were allocated to a single concurrent 3 × 3 Latin square design with 3 dietary treatments: a TMR as control (CTRL) and the same ration supplemented with fresh hemp (HEMP) or savory (SAV) leaves. Each period lasted 14 d, with herb supplementation administered from d 1 to d 6 at increasing doses (0.2–0.8 kg/d DM). Milk samples were collected daily and analyzed to determine terpene concentrations and transfer kinetics. Terpenes were extracted using headspace solid-phase microextraction and analyzed by GC-MS. A total of 24 terpenes were identified across feed and milk. The HEMP had the highest terpene content (85,962 μg/kg), followed by SAV (11,313 μg/kg) and CTRL (228 μg/kg). Corresponding milk concentrations were 388.8 μg/kg (HEMP-diet), 48.7 μg/kg (SAV-diet), and 50.8 μg/kg (CTRL). Despite the lower absolute terpene content in CTRL, its relative carryover to milk was highest (15.4%) compared with HEMP-diet (8.7%) and SAV-diet (6.2%). Key terpenes such as α-farnesene, β-caryophyllene, and p-cymene were transferred to milk. The results demonstrate that terpenes can be transferred to milk in a compound-dependent manner, with hemp producing the highest absolute terpene concentration in milk. This research supports the application of terpene-rich plants in dairy diets to enhance milk quality, traceability, and value, contributing to more sustainable and differentiated dairy production systems.
Key words: aromatic plants, volatile organic compounds, gas chromatography–mass spectrometry, milk quality, terpene transfer

Received July 14, 2025.
Accepted November 15, 2025.
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INTRODUCTION

Terpenes, often studied for their role in plants, are proving to be fundamental in determining the flavor and quality attributes of cow milk. Plants produce a range of secondary metabolites, which humans have exploited for their valuable roles in numerous biological functions (Balandrin et al., 1985). Terpenes are a broad and varied group of bioactive compounds formed from a branched C5 carbon structure originating from isoprene (Srivastava et al., 2021). They are classified by the number of isoprene units, ranging from simple C5 hemiterpenes to larger structures such as C40 tetraterpenes and polyterpenes (Alamgeer et al., 2020).

Terpenes in dairy products have recently gained attention for their potential influence on the sensory characteristics of cheese and as potential indicators in milk and cheese of diverse forage types in the diets of dairy cows (Bugaud et al., 2001). Terpene compounds are present in higher concentrations in dairy milk derived from cows grazing on diverse upland grasslands compared with those from cows fed on less varied or monoculture meadows (Tornambé et al., 2006). Once forage is ingested by cows, terpenes are absorbed in the digestive system, transferred into the bloodstream, and subsequently secreted into milk via the mammary glands (Viallon et al., 2000). Moreover, Revello Chion et al. (2010) suggested that terpenes can trace dairy product origin and enhance sensory and nutritional value.

In this context, aromatic plants such as *Cannabis sativa* L. (hemp) and *Satureja hortensis* L. (savory), rich in terpenes, are gaining interest in livestock nutrition for their potential to influence the flavor, nutritional profile, and bioactive properties of milk. Hundreds of terpenes have been identified that confer to hemp sensory attributes (Sommano et al., 2020). For instance, terpenes are largely responsible for the characteristic aroma of hemp (Russo, 2011). To date, 200 terpenes have been detected in hemp (Lewis et al., 2018). The presence of several terpenes was determined in the seed oil of hemp, the most

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-26. Nonstandard abbreviations are available in the Notes.

abundant of which were β-caryophyllene and myrcene (Leizer et al., 2000). However, the complete identification and quantification of the vast majority of terpenes/terpenoids remains undetermined (Brown et al., 2019).

In addition to terpenes, hemp also contains cannabinoids such as Δ^9-tetrahydrocannabinol (**THC**) and cannabidiol, which are concentrated in the inflorescences and occur at much lower levels in leaves (Jin et al., 2020). The transfer of cannabinoids to milk in certain feeding scenarios is conceivable (Wagner et al., 2022). For this reason, the European Union limits industrial hemp to a maximum of 0.3% Δ^9-THC based on Regulation (EU) 2021/2115 (European Parliament and Council, 2021).

As for savory, previous studies have stated that the oils of Satureja species are a rich source of p-cymene, linalool, carvacrol, thymol, and β-caryophyllene (Ejaz et al., 2023). In a study conducted by Golbotteh et al. (2024), it was reported that *Satureja hortensis* L. plant inclusion increased dairy milk n-3 and CLA while reducing SFA.

Analytical techniques such as headspace solid-phase microextraction coupled with GC-MS (**HS-SPME-GC-MS**) have been widely used for the qualitative and quantitative analysis of volatile compounds in milk (Czerwiński et al., 1996). Despite the growing body of research on terpenes in dairy products, little is known about the dynamics of terpene transfer from specific feed sources to milk, particularly regarding distribution and occurrence in dairy cows.

This study aims to examine the profiles and concentrations of terpenes transferred from *C. sativa* and *S. hortensis* L. into milk, as well as the transfer kinetics in dairy cows fed these aromatic plants. By exploring terpene transfer, the research aims to improve our understanding of how feed composition can be tailored to enhance the sensory, nutritional, and bioactive qualities of milk.

MATERIALS AND METHODS

Dairy Cows and Experimental Design

This in vivo experiment involved 6 purebred Simmental dairy cows, housed at the "Lucio Toniolo" Experimental Stable of the University of Padua (Legnaro, Padua, Italy). The cows, with an average of 1.3 calvings, DIM of 120 ± 28 d, milk production of 30 ± 4.8 kg/d, BCS of 3.41 ± 0.34, BW of 658 ± 30 kg, and an age range of 30 to 46 mo, were individually housed in pens with permanent bedding. A single concurrent 3 × 3 Latin square design was employed to evaluate the effects of 3 experimental diets: a TMR without any supplementation (**CTRL**), a TMR supplemented with fresh hemp leaves (*Cannabis sativa* diet, **HEMP**-diet), or with fresh savory leaves (*S. hortensis* L. diet, **SAV**-diet). This design was chosen to ensure each cow received each dietary treatment across a different period, thus accounting for individual variations in the ruminal and metabolic utilization of bioactive compounds.

The 3 groups of 2 cows were each subjected to a series of three 14-d experimental periods in a 3 × 3 Latin square rotation. In each period, cows received 1 of the 3 dietary treatments (hemp, savory, or control), with treatments rotated among groups across periods. During the first 6 d of each period, supplementation with fresh hemp or savory leaves, in addition to the basal TMR, was progressively increased (0.2 kg/d DM on d 1 and 2, 0.4 kg/d DM on d 3 and 4, and 0.8 kg/d DM on d 5 and 6) to allow adaptation and avoid refusals, as hemp leaves are characterized by poor palatability. To favor intake, the fresh leaves were coarsely chopped and partially mixed with the morning portion of the TMR, and daily monitoring of feed distribution and refusals ensured that cows successfully ingested the planned amounts, with full intake achieved at 0.8 kg/d. From d 7 to 14 of each period, supplementation was stopped, and all cows continued on the basal diet alone, providing an 8-d washout before the next treatment period began. Thus, each cow completed 3 consecutive 14-d periods, receiving all 3 diets in rotation with a washout interval in every cycle.

Diet, Plants, and Sample Collection

The TMR was formulated to meet the nutritional requirements of cows producing 30 kg of milk (NASEM, 2021) and was individually distributed using a feed mixer wagon.

The HEMP and SAV denote the plant materials; HEMP-diet and SAV-diet denote the TMR containing these botanicals at the stated inclusion levels; CTRL denotes the supplemented basal TMR. The chemical composition of the CTRL, as well as HEMP and SAV leaves, was analyzed and is reported in Table 1. The HEMP leaves used in this study were from the Futura 75 variety, a French monoecious strain. This variety's cultivation in Italy was authorized under the provisions of Law 242 of December 2, 2016 (Italian Republic, 2016), which regulates the cultivation of *Cannabis sativa* L. for industrial purposes, including fiber and biomass production. The concentration of Δ^9-THC in HEMP leaves was determined by the Istituto Zooprofilattico Sperimentale delle Venezie and was found to be below the analytical limit of detection (**LOD**). In accordance with Italian Law 242/2016, the maximum permissible Δ^9-THC content in industrial hemp is 0.2%. The plants were cultivated at the "Research Centre of Cereal and Industrial Crops of the Council for Agricultural Research and Economics" in Rovigo (Veneto, Italy) during the summer of 2022, with

Table 1. Formulation and chemical characteristics (% DM) of TMR (CTRL) and aromatic plants involved in the test[1]

Trait	CTRL	HEMP	SAV
Formulation (% DM)			
Alfalfa	10.8		
Permanent meadow hay	4.3		
Corn gluten meal	7.3		
Soybean meal	2.2		
Energy mix	32.7		
Protein mix	14.2		
Wheat germ	1.3		
Molasses	2.4		
Grass silage	7.7		
Sorghum silage	17.2		
Chemical composition (% DM)			
DM (%)	59.60	23.20	48.40
CP	14.68	7.59	8.43
EE	3.41	7.12	5.41
CF	20.30	14.4	35.31
NDF[2]	41.44	30.49	60.54
ADF	21.12	15.81	45.62
Gross ADL	4.11	5.29	5.79
AIA	0.55	0.09	0.29
Net ADL	3.95	5.20	5.50
Ash	7.52	13.88	7.20

[1]EE: ether extract; CF: crude fiber; CTRL: control diet (TMR); HEMP: hemp leaves; SAV: savory leaves; AIA: acid insoluble ash.

[2]NDF: determined with heat-stable α-amylase and without sulfite (cellulose, hemicellulose, lignin).

an average temperature of 21.4°C. Manual harvesting was conducted, involving the selection of plants and the subsequent separation of stems and leaves. The harvested leaves were stored at −20°C, thawed as required, and used in the experimental trials. The *S. hortensis* L. leaves used in the experimental trial were sourced from a farm in Teolo (Padua, Italy) at an altitude of 300 m above sea level. Planted in 2016, the crop was cultivated without pesticide treatments (Regulation-2018/848-EN-EUR-Lex; European Union, 2018), fertilization, or irrigation, and with manual weed removal. Manual harvesting occurred after flowering in August. The harvested SAV leaves were immediately frozen and stored at −20°C until administration. The SAV leaves were harvested in the second week of September, past its balsamic period, due to an unusually dry season that accelerated the period's onset. Both plant leaves were defrosted at room temperature for 1 h before administration. Subsequently, they were coarsely chopped using a shredder and mixed with half of the daily TMR portion to ensure full intake. The remaining portion of the TMR was provided later in the day. The individual feed intake was recorded daily by weighing distributed feed and leftovers the following morning. Milk samples (10 mL) were collected from d 6 (evening milking) and d 7 (morning milking; 2 replicates) of all 3 experimental periods, coinciding with peak supplementation of each period, and promptly transported to the laboratory for chemical analysis (Department DAFNAE, Legnaro, Padua, Italy), to analyze terpene content, resulting in 36 samples (2 cows × 3 groups × 3 periods × 2 replicates). In addition, transfer kinetics were studied during the first experimental period, where all 6 cows were sampled individually over 14 consecutive days. Morning and evening milkings were pooled per cow/day, resulting in 84 individual cow samples (6 cows × 14 d).

Chemical Analysis of Milk and Aromatic Plants

Sample Preparation and SPME Sampling. For the analysis of terpenes, 2 g of sodium chloride (NaCl) were added to 8 mL of the milk sample, which was placed in a 20 mL glass vial sealed with a rubber septum. For HEMP, SAV, and CTRL, 300 mg of crushed fresh leaves were used and placed in similar 20 mL vials. The samples underwent pre-extraction equilibration by heating at 40°C for 5 min. Following this, a divinylbenzene/carboxen/polydimethylsiloxane SPME fiber was inserted into the headspace of the sample to extract the volatiles. The extraction was performed for 10 min with constant heating at 40°C. After extraction, the fiber was desorbed for 10 min in a split/splitless inlet set to 270°C in splitless mode.

Gas Chromatography Analysis. Gas chromatography analysis was carried out using an Agilent 7890A GC coupled with a 5977-mass spectrometer. The separation of the components was performed on a HP-5MS silica capillary column (5% diphenyl- and 95% dimethylpolysiloxane, 30 m × 0.25 mm, 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA). Helium was used as the carrier gas at a flow rate of 1 mL/min. The GC oven temperature program was as follows: an initial temperature of 40°C held for 2 min, increased to 160°C at a rate of 3°C/min, followed by an increase to 250°C at a rate of 10°C/min, and then held at 250°C for 5 min. The total GC run time was 56 min per sample.

Detection and Identification of Terpenes. The separated components were analyzed by the mass spectrometer, with the transfer line, ion source, and quadrupole mass analyzer temperatures set to 280°C, 230°C, and 150°C, respectively. Ionization was achieved using electron impact ionization (EI) at 70 eV, and mass detection was performed in scan mode over an m/z range of 30 to 500. Data processing was carried out using MassHunter Workstation Software, version B.07.01 (Agilent Technologies, Santa Clara, CA), in combination with the National Institute of Standards and Technology database (**NIST**), to match the mass spectra obtained from milk and feed samples to known terpene compounds. In addition, Kovats indices were calculated by comparing a compound's retention time with those of nearby n-alkanes. The identification of each terpene was confirmed based on the

Kovats index and mass spectrum comparison with the NIST library, which contains a comprehensive database of known compounds.

Quantification of Terpenes. Sample quantification was performed using the external standard addition method. Calibration curves were constructed in triplicate within the milk matrix, spiked with known concentrations of terpene standards. Terpene standards were purchased from Merck Life Science S.r.l. (Milano, Italy) and were as follows: (−)-β-pinene with a purity ≥99%, myrcene (≥90%), β-citronellene (≥99%), camphene (≥95%), α-pinene (≥98%), 3-carene (≥90%), ρ-cymene (≥97%), (r)-(+)-limonene (≥93%), (r)-(−)-α-phellandrene (≥95%), *cis*-sabinene hydrate (≥97%), linalool (≥97%), *trans*-β-farnesene (≥90%), (−)-*trans*-caryophyllene (≥98%), α-terpineol (≥96%), D-camphor (≥97%), and citral (≥95%). Stock solutions of individual terpenes were prepared by dissolving 30 ± 0.1 mg of each compound in 25 mL of ethanol (1.2 mg/mL). A mixed standard solution was prepared by combining 1 mL of each stock solution and diluting to 20 mL with ethanol (60 µg/mL per terpene). Milk samples were spiked with the mixed standard at concentrations ranging from 0.01 to 0.75 µg/mL, and 8 mL of each spiked sample was analyzed following the procedure described for test samples.

Once the terpenes were identified by comparing their retention times and mass spectra to the standards, the peak areas corresponding to the identified terpenes were extracted from the chromatogram, and the concentration of each terpene in the samples was then determined by using the previously constructed calibration curves. The analytical method was validated to ensure reliable quantification. Accuracy, assessed via recovery experiments at low, medium, and high concentrations, ranged from 92% to 107%, whereas intraday precision (repeatability) and interday precision (intermediate precision) were ≤6% and ≤8%, respectively. These results demonstrate that the method provides reproducible and robust quantification. In addition, specificity and selectivity were confirmed by analyzing blank matrices and spiked samples, ensuring no interfering peaks overlapped with the analyte signal. Carryover was examined by injecting blanks after high-concentration samples, with results showing analyte signals well below the accepted threshold.

The concentration of each terpene in the milk samples was expressed in micrograms per kilogram (µg/kg) of milk and feed.

Terpene Transfer in Milk

The terpene transfer rate (%) from diet to milk was calculated according to the formula below by determining the ratio between the total amount of each terpene excreted in the milk and the total amount ingested from the diet. The ingested terpenes (µg/d) were calculated by multiplying the terpene concentrations in the CTRL, HEMP, and SAV (µg/kg) by the corresponding DMI (kg DM/d). The excreted terpenes (µg/d) were calculated by multiplying the terpene concentrations in milk (µg/kg) by the total daily milk production (kg), considering both the evening and morning milkings:

$$\frac{\text{Terpene in milk}\,(\mu\text{g/kg})\times\text{Milk yield}\,(\text{kg/d})}{\text{Terpene in feed}\,(\mu\text{g/kg})\times\text{DMI}\,(\text{kg/d})}\times 100$$

Statistical Analysis

The data were analyzed using the SAS MIXED procedure, version 9.4 (SAS Institute Inc., Cary, NC). The following linear mixed model was used:

$$\begin{aligned} y_{ijklm} &= \mu + \text{treatment}_i + \text{period}_j + \text{group}_k \\ &+ \text{cell}_l(\text{treatment}_i \times \text{period}_j \times \text{group}_k) \\ &+ \text{cow}_m(\text{group}_k) + e_{ijklm}, \end{aligned}$$

where y_{ijklm} represents the concentration of a single terpene in milk (µg/kg) and its transfer rate (%). In this model, µ represents the overall intercept of the model, treatment_i is the ith fixed effect of diet (CTRL, HEMP-diet, and SAV-diet). The period_j is the fixed effect of the jth periods (1, 2, and 3), and group_k is the fixed effect of the kth group (1, 2, and 3). The random effect $\text{cell}_l(\text{treatment}_i \times \text{period}_j \times \text{group}_k)$ represents the lth combination of treatment, period, and group in the Latin square design (3 × 3 = 9 levels), following a normal distribution $\sim N(0, \sigma^2 \text{ cell})$. The cow_m (group_k) indicates the random effect of the mth cow within the kth group, also considered to be normally distributed $\sim N(0, \sigma^2 \text{ cell})$. The residual error e_{ijklm} is also considered normally distributed $\sim N(0, \sigma^2 \text{ cell})$. To study the effect of the treatment, orthogonal contrasts between LSM of the treatment effects (CTRL vs. Herbs, HEMP-diet vs. SAV-diet) were performed, and differences were considered significant at $P < 0.05$.

The fixed effects of the Latin square design were tested against the error term associated with the cells of the Latin square design. Data were checked for normality, and any observations falling outside ±3 SD were flagged for review. All analyses were performed in duplicate to ensure reproducibility.

Ethics Statement

The animal study protocol was approved by the Ethics Committee for the Care and Use of Experimental Animals of the University of Padua (Prot. n. 131204, 26/07/2022).

Table 2. Terpenes identified with HS-SPME/GC-SM analysis

Common name	IUPAC[1] name	Kovats[2]
α-Pinene	α-Pinene	935
β-Citroellene	1,6-Octadiene, 3,7-dimethyl-, (S)-	947
Camphene	Camphene	948
β-Pinene	β-Pinene	978
β-Myrcene	β-Myrcene	989
α-Phellandrene	α-Phellandrene	1,004
3-Carene	3-Carene	1,011
p-Cymene	1-Methyl-4-(propan-2-yl)benzene	1,027
D-Limonene	1,2 D-Limonene	1,029
Linalool	1,6-Octadien-3-ol, 3,7-dimethyl-	1,100
Camphor	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1R)-	1,139
Terpinen-4-ol	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	1,175
Thymoquinone	2,5-Cyclohexadiene-1,4-dione, 2-methyl-5-(1-methylethyl)-	1,252
α-Citral	2,6-Octadienal, 3,7-dimethyl-, (E)-	1,256
Ylangene	Ylangene	1,364
β-*cis*-Caryophyllene	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-	1,407
Thymol methyl ether	Benzene, 1-methoxy-4-methyl-2-(1-methylethyl)-	1,244
α-Humulene	(1E,4E,8E)-3,7,11-trimethyl-1,4,8-cycloundecatriene	1,495
α-Farnesene	3,7,11-Trimethyl-1,3,6,10-dodecatetraene	1,491
β-Caryophyllene	Caryophyllene	1,420
Caryophylladienol II	Bicyclo[7.2.0]undecane, 10,10-dimethyl-2,6-bis(methylene)-, [1S-(1R*,9S*)]-	1,440
trans-α-Bergamotene	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	1,430
α-Caryophyllene	α-Caryophyllene	1,445
β-Farnesene	β-Farnesene	1,458

[1]IUPAC: International Union of Pure and Applied Chemistry.

[2]Kovats index: standardized comparison of retention times using n-alkanes.

RESULTS

Terpenes Identified by HS-SPME-GC-MS Analysis

The identification of terpenes in the samples was carried out using HS-SPME coupled with GC-MS. A total of 24 terpenes were identified, as detailed in Table 2. The common names and IUPAC (International Union of Pure and Applied Chemistry) names for each terpene are listed to provide comprehensive data regarding the identification and quantification.

As indicated in Table 3, several terpenes differed across CTRL, HEMP, and SAV. For example, α-pinene was present at low concentrations in CTRL (1.0 µg/kg) and SAV (25.6 µg/kg) but reached 548.3 µg/kg in HEMP. Similarly, p-cymene was relatively low in both CTRL (1.1 µg/kg) and HEMP (9.1 µg/kg), yet very abundant in SAV (1,584.6 µg/kg).

Whereas β-caryophyllene and its isomeric forms (e.g., β-*cis*-caryophyllene) were present across all 3 matrices, the highest concentrations were observed in HEMP at 17,849.9 µg/kg and 17,835.0 µg/kg for β-*cis*-caryophyllene and β-caryophyllene, respectively. In contrast, thymoquinone, a compound only minimally present in HEMP (1.2 µg/kg), was substantially higher in SAV (1,050.0 µg/kg).

These trends are visually represented in Figure 1, where the bar chart (expressed on a logarithmic scale) illustrates how certain terpenes, such as linalool, appear

Table 3. Terpenes content (µg/kg DM) of control diet and *Cannabis sativa* L. (HEMP) and *Satureja hortensis* L. (SAV) leaves fed to the dairy cows

Item	CTRL	HEMP	SAV	Reference[1,2]
Terpenes (µg/kg DM)				
α-Pinene	1.0	548.3	25.6	a,b,d,f,g,h,i,j,k
β-Citronellene	0.2	116.9	4.4	—
Camphene	0.2	3.3	2.5	b,d,j
β-Pinene	0.2	147.4	8.1	a,b,d,f,g,h,i,j
β-Myrcene	0.9	156.2	105.4	a,b,c,d,f,g,h,i,j
α-Phellandrene	0.1	158.0	36.9	a,b,d,f,i
3-Carene	0.9	146.7	34.2	d,f,j
p-Cymene	1.1	9.1	1,584.6	f,g,h,i,j,
D-Limonene	19.8	107.6	49.7	a,b,c,f,g,h,i,j,k
Linalool	19.7	1.9	50.5	c,d,g,h,j
Camphor	1.4	3.9	23.7	f
Terpinen-4-ol	1.9	2.8	82.7	c,d
Thymoquinone	0.1	1.2	1,050.0	e
α-Citral	64.6	—	303.4	—
Ylangene	25.1	123.9	75.8	j
β-*cis*-Caryophyllene	3.3	17,849.9	2,029.7	b,j
Thymol methyl ether	0.1	0.2	2.0	d
α-Humulene	20.1	7,771.7	892.7	a,b,d,f,g,h,i,j
α-Farnesene	33.7	21,123.8	2,398.6	h
β-Caryophyllene	28.8	17,835.0	2,006.2	b,c,d
Caryophylladienol II	3.3	44.8	70.6	—
trans-α-Bergamotene	0.3	2,430.9	56.4	j,k
α-Caryophyllene	0.3	13,616.0	396.8	b,f,g,h,i,j
β-Farnesene	0.6	3,762.3	22.9	f,i,j,k

[1]Reference SAV: a: (Mahboubi and Kazempour, 2011), b: (Katar et al., 2017), c: (Shanaida et al., 2017), d: (Farzaneh et al., 2015), e: (Taborsky et al., 2012).

[2]Reference HEMP: f: (Kumeroa et al., 2022), g: (Ibrahim et al., 2023), h: (Giovannoni et al., 2023), i: (Stenerson, 2017), j: (Mediavilla, 1997), k: (Naz et al., 2023).

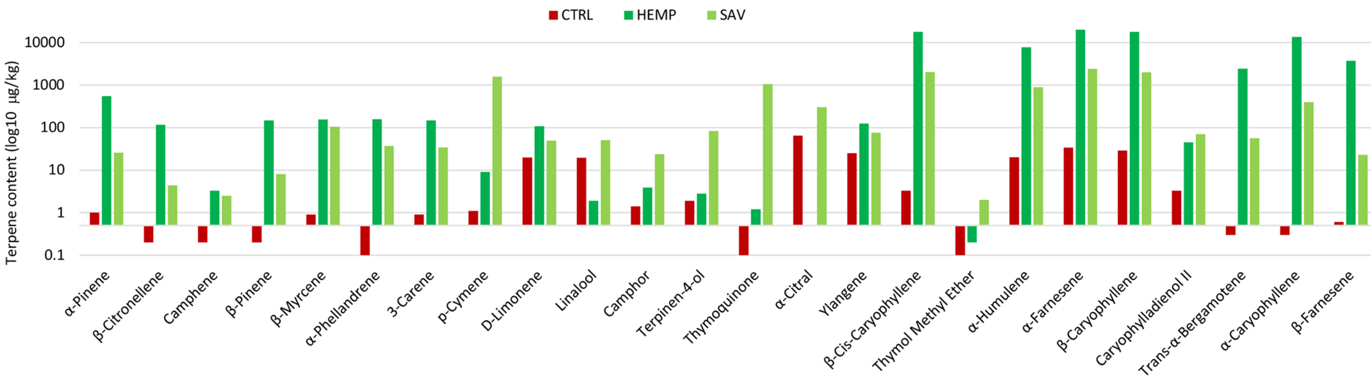

Figure 1. Terpene content (log_{10} µg/kg) of CTRL, HEMP, and SAV leaves.

at higher levels in CTRL than in HEMP, whereas others, specifically β-caryophyllene and β-*cis*-caryophyllene, are evidently elevated in HEMP. Figure 1 also shows that α-citral was detected at high concentrations in TMR and SAV, and underscores SAV's abundance of p-cymene.

Statistical Evaluation of Terpene Concentrations Under Different Dietary Treatments

The ANOVA for terpene content in milk, presented in Table 4, showed that variations in terpene concentrations were primarily driven by dietary treatments, with no significant contribution from group or period effects. Large F-values for α-caryophyllene (P = 0.001), caryophylladienol II (P = 0.001), *trans*-α-bergamotene (P = 0.005), and α-farnesene (P = 0.01) highlighted the strong influence of diet on their concentrations in milk. Similarly, β-pinene exhibited a considerable treatment effect (P = 0.05).

Variance partitioning showed that a portion of the variation in p-cymene and terpinen-4-ol concentrations (30% each) was attributable to the interaction of treatment, period, and group (cell-level variance). In contrast, individual cow differences within groups contributed more to the variability of compounds such as D-limonene (27%) and α-caryophyllene (20%). Nonetheless, dietary supplementation remained the dominant factor, as reflected by the pronounced F-values.

These findings were supported by comparisons in Table 5, which showed clear differences in terpene concentrations among the 3 dietary treatments. For example, α-pinene (P = 0.03) was higher in HEMP-diet milk (3.43 µg/kg) compared with CTRL (2.27 µg/kg) and SAV-diet (2.51 µg/kg). Similarly, β-pinene (P = 0.05; 2.70 µg/kg in HEMP-diet vs. 1.36 µg/kg in CTRL and 1.75 µg/kg in SAV-diet) and β-myrcene (P = 0.05; 2.66 µg/kg in HEMP-diet vs. 1.39 µg/kg in CTRL and 1.76 µg/kg in SAV-diet) followed the same trend.

The most significant differences were observed for α-humulene (P = 0.005), α-farnesene (P = 0.01), β-caryophyllene (P = 0.02), caryophylladienol II (P = 0.001), *trans*-α-bergamotene (P = 0.005), and α-caryophyllene (P = 0.001), all of which showed highly significant treatment effects, with HEMP supplementation yielding the highest concentrations. For instance, α-humulene reached 4.25 µg/kg in HEMP-diet milk, compared with 1.07 µg/kg in CTRL and 0.71 µg/kg in SAV-diet. Likewise, caryophylladienol II was significantly higher in HEMP-diet (4.24 µg/kg) than in both CTRL and SAV-diet (0.71 µg/kg), with strong overall and pairwise treatment effects (HEMP vs. SAV P = 0.001).

In contrast, some terpenes such as camphene (2.36 µg/kg in CTRL vs. 2.22 and 2.21 µg/kg in HEMP-diet and SAV-diet), linalool (3.04 µg/kg in CTRL vs. 3.00 and 2.90 µg/kg), and thymoquinone (2.57 µg/kg in CTRL vs. 2.51 and 2.31 µg/kg) remained slightly higher in the CTRL but did not differ significantly among treatments. Period and group effects also had no substantial effect on these compounds. Additionally, p-cymene concentrations were numerically higher in milk from SAV-diet cows (3.04 µg/kg) compared with CTRL (2.26 µg/kg) and HEMP-diet (2.44 µg/kg), although these differences were not significant. Terpinen-4-ol showed minimal variation among treatments (3.00, 2.99, and 3.04 µg/kg in CTRL, HEMP-diet, and SAV-diet, respectively), resulting in a nonsignificant treatment effect.

Animal Performance and Terpene Transfer Rates from Diet to Milk

Herb supplementation did not affect feed intake (F = 11.23, P = 0.785) or milk yield (F = 5.26, P = 0.587). Nevertheless, feed intake was numerically lower in the HEMP group compared with the SAV group (P = 0.352), without altering milk production (P = 0.457). Several terpene transfer rates displayed important treatment effects,

Table 4. ANOVA for terpene content in milk (μg/kg; n = 12)

Item	*F*-value[1]			Variance proportion		RMSE[4]
	Diet	Period	Group	Cell[2] (%)	Cow[3] (group; %)	
Performance						
DMI (kg DM/d)	11.23	5.26	2.85	5	2	2.264
Milk yield (kg/d)	5.26	3.28	1.28	2	10	1.857
Terpenes (μg/kg milk)						
α-Pinene	28.88*	1.09	0.69	4	0	0.368
β-Citronellene	13.75	1.24	0.42	0	8	0.423
Camphene	1.70	2.31	1.71	0	0	0.226
β-Pinene	20.88*	0.28	1.40	3	0	0.490
β-Myrcene	19.78*	0.15	0.80	0	0	0.509
α-Phellandrene	8.36	4.36	0.96	0	20	0.424
3-Carene	5.75	2.55	0.55	0	18	0.611
p-Cymene	10.46	1.12	0.08	30	28	0.224
D-Limonene	8.63	2.65	0.05	0	27	0.496
Linalool	0.65	1.60	0.58	0	0	0.309
Camphor	0.23	5.82	0.43	0	0	0.423
Terpinene-4-ol	0.17	0.71	0.23	30	14	0.117
Thymoquinone	1.23	8.30	0.72	23	20	0.260
α-Citral	1.21	8.85	0.36	0	0	0.205
Ylangene	0.92	1.50	1.17	0	0	1.270
β-*cis*-Caryophyllene	0.61	3.11	0.49	2	0	0.353
Thymol methyl ether	3.85	2.18	1.85	0	18	0.239
α-Humulene	194.52**	2.90	2.08	0	11	0.485
α-Farnesene	79.30*	1.29	0.49	0	10	0.805
β-Caryophyllene	59.37*	0.03	0.10	0	2	0.391
Caryophylladienol II	790.95***	1.00	0.47	6	18	0.218
trans-α-Bergamotene	194.52**	2.90	2.08	0	11	0.485
α-Caryophyllene	1,279.72***	0.46	0.18	0	20	0.210
β-Farnesene	7.84	0.16	0.21	22	21	0.959

[1]Diet: dietary treatment (TMR, HEMP, and SAVdiet); period: experimental period; group: group of cows.
[2]Cell variance proportion = (cell variance)/(cell + cow + residual variance) × 100.
[3]Cow (group) variance proportion = (cow variance)/(cell + cow + residual variance) × 100, where "cow" is nested within group.
[4]RMSE: root mean square error.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

as indicated by high *F*-values in Table 6. In particular, α-humulene ($F = 17.72$, $P < 0.05$), α-farnesene ($F = 20.98$, $P < 0.05$), α-caryophyllene ($F = 19.90$, $P < 0.05$), thymol methyl ether ($F = 15.16$, $P < 0.05$), and caryophylladienol II ($F = 14.06$, $P < 0.05$) exhibited evident responses to feed supplementation.

The variance partitioning further illustrated that p-cymene (43%), terpinen-4-ol (38%), and thymoquinone (34%) had relatively large proportions of cell-level variance, whereas camphene (46%) and 3-carene (27%) were more affected by individual cow variation nested within groups. In contrast, period and group exhibited no significant effects on transfer percentages.

Furthermore, few terpenes exceeded a 100% transfer rate, especially for terpenes characterized by very low concentrations in CTRL. This pattern was most apparent for α-citral, thymoquinone, and thymol methyl ether. Beyond these higher values, most terpenes displayed more moderate transfer rates, generally below 50%.

For instance, α-pinene remained close to 18% across all diets, whereas α-phellandrene decreased from 69.0% in CTRL to 3.4% in HEMP-diet and 11.5% in SAV-diet.

Additionally, α-caryophyllene, caryophylladienol II, α-farnesene, and α-humulene showed significant contrasts between HEMP-diet and SAV-diet. For α-caryophyllene, the overall treatment effect was significant, with transfer rates of 1.2% in the CTRL, 6.2% under HEMP-diet, and 0.0% under SAV-diet, and a significant HEMP-diet vs. SAV-diet contrast ($P = 0.03$; Table 7). For caryophylladienol II, the pairwise comparison between HEMP-diet and SAV-diet ($P = 0.04$) demonstrated a marked increase from 0.0% to 64.8% in HEMP-diet-fed cows. A similar pattern occurred for α-humulene, which rose from 0.6% (CTRL) and 0.0% (SAV-diet) to 6.8% under HEMP-diet, although effect was not significant, the HEMP-diet vs. SAV-diet contrast remained significant ($P = 0.03$).

Kinetics of Terpene Transfer into Milk

To better understand how terpenes behaved in response to feeding, we monitored their daily concentrations in milk throughout the supplementation period. This approach, based on individual milk samples collected each day, allowed us to observe how terpene levels changed

Table 5. Effect of experimental diets (CTRL, HEMP, and SAV) on performance and milk content of terpenes (μg/kg; n = 12)

Terpene	Diet LSM[1]			Contrasts (*P*-values)	
	CTRL	HEMP-diet	SAV-diet	CTRL vs. herbs[2]	HEMP-diet vs. SAV-diet[3]
Performance					
DMI (kg DM/d)	17.8	16.0	18.1	0.785	0.352
Milk yield (kg/d)	23.5	24.0	23.8	0.587	0.457
Terpenes (μg/kg milk)					
α-Pinene	2.27	3.43	2.51	0.037*	0.030*
β-Citronellene	1.69	2.59	2.19	0.042*	0.149
Camphene	2.36	2.22	2.21	0.207	0.983
β-Pinene	1.36	2.70	1.75	0.043*	0.047*
β-Myrcene	1.39	2.66	1.76	0.045*	0.049*
α-Phellandrene	1.50	2.20	1.83	0.074	0.161
3-Carene	2.39	3.19	2.55	0.156	0.125
p-Cymene	2.26	2.44	3.04	0.090	0.078
D-Limonene	2.72	3.47	2.77	0.146	0.075
Linalool	3.04	3.00	2.90	0.502	0.510
Camphor	2.06	2.18	2.12	0.610	0.784
Terpinene-4-ol	3.00	2.99	3.04	0.898	0.625
Thymoquinone	2.57	2.51	2.31	0.394	0.372
α-Citral	2.63	2.52	2.63	0.482	0.323
Ylangene	1.80	1.10	1.42	0.350	0.605
β-*cis*-Caryophyllene	3.74	3.59	3.72	0.558	0.482
Thymol methyl ether	4.46	4.70	4.47	0.293	0.140
α-Humulene	1.07	4.25	0.71	0.014*	0.003**
α-Farnesene	0.71	4.76	2.01	0.011*	0.014*
β-Caryophyllene	3.22	4.68	3.13	0.038*	0.011*
Caryophylladienol II	0.71	4.24	0.71	0.003**	0.001***
trans-α-Bergamotene	1.07	4.25	0.71	0.014*	0.003**
α-Caryophyllene	0.71	4.47	0.71	0.002**	0.001***
β-Farnesene	1.26	3.43	1.28	0.181	0.076

[1]Diet: least squares means of the dietary treatment (control diet "CTRL," TMR supplemented with *Cannabis Sativa* L. "HEMP-diet," and TMR supplemented with *Satureja hortensis* L. "SAV-diet").

[2]CTRL vs. herbs: contrast comparing TMR versus both supplemented diets (HEMP-diet + SAV-diet).

[3]HEMP-diet vs. SAV-diet: contrast comparing HEMP-supplemented versus SAV-supplemented diets.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

over time in relation to the increasing doses of HEMP or SAV leaves, illustrating their absorption and appearance in milk. Figure 2 illustrates the daily variation in terpene concentrations in milk during dietary supplementation with HEMP (HEMP-diet "a") and SAV (SAV-diet "b").

Concentrations of α-farnesene, β-caryophyllene, caryophylladienol II, *trans*-α-bergamotene, and α-caryophyllene (Figure 2a) increased progressively from d 1 through d 6, closely following the stepwise increase in supplementation doses. A sharp rise was observed between d 5 and 7, with maximum concentrations consistently occurring on d 7, one day after the highest supplementation dose (d 6). After supplementation was discontinued, concentrations declined rapidly, showing a marked decrease by d 8 to 9. By d 11, most sesquiterpenes approached baseline levels, and from d 12 to d 14, concentrations remained low and stable, indicating limited persistence in milk.

As for p-cymene (Figure 2b), concentrations increased progressively from d 1 to d 6, in parallel with the incremental supplementation. The maximum concentration was observed on d 7, corresponding to the day following the highest supplementation dose. A sharp decline occurred on d 8, followed by a minor secondary elevation on d 9, suggesting a transient redistribution before complete clearance. After d 9, concentrations decreased rapidly, reaching near-baseline values by d 11. From d 12 to 14, levels remained consistently low and close to zero, indicating a short persistence of p-cymene in milk once supplementation was withdrawn.

DISCUSSION

Terpene Content of HEMP and Savory

The terpene profile of HEMP and SAV leaves used in this study showed noticeable differences. The HEMP was particularly rich in β-caryophyllene (20%), α-caryophyllene (15%), and α-farnesene (24%), confirming its sesquiterpene-dominant nature (Tornambé et al., 2006; Sommano et al., 2020). Sesquiterpenes accounted for 98% of the total terpene content in HEMP, aligning with previous findings showing they represent 80% to 90% of HEMP's terpene profile in the Futura 75 variety

Table 6. ANOVA for terpenes transfer percentages (n = 12)

Terpene	*F*-value[1]			Variance proportion		RMSE[4]
	Diet	Period	Group	Cell[2] (%)	Cow[3] (group, %)	
α-Pinene	0.00	0.59	0.61	18	0	15.217
β-Citronellene	3.28	0.36	0.17	6	11	37.329
Camphene	9.20	1.66	0.57	21	46	18.804
β-Pinene	0.06	0.31	0.97	29	0	13.229
β-Myrcene	2.80	0.22	1.39	12	0	6.762
α-Phellandrene	5.41	1.82	0.59	14	21	39.073
3-Carene	4.51	1.66	0.21	0	27	73.303
p-Cymene	1.92	1.07	0.10	43	17	9.224
D-Limonene	7.36	1.14	0.24	0	26	22.526
Linalool	0.84	1.29	0.06	0	15	3.169
Camphor	0.18	5.84	1.01	0	0	10.964
Terpinene-4-ol	0.38	0.62	0.29	38	34	1.078
Thymoquinone	3.73	0.88	0.13	34	27	49.941
α-Citral	6.86	1.61	0.40	37	11	121.264
Ylangene	1.39	1.18	0.76	0	0	0.968
β-*cis*-Caryophyllene	2.12	3.07	1.01	5	14	9.284
Thymol methyl ether	15.16*	1.08	0.66	31	20	215.585
α-Humulene	17.72*	0.53	0.88	0	4	3.108
α-Farnesene	20.98*	0.89	0.09	0	25	3.006
β-Caryophyllene	6.35	0.36	0.07	0	5	3.897
Caryophylladienol II	14.06*	1.00	0.46	7	20	29.340
trans-α-Bergamotene	0.98	1.15	1.00	26	13	37.879
α-Caryophyllene	19.90*	0.60	0.27	0	17	2.533
β-Farnesene	0.09	0.76	0.23	18	13	50.414

[1]Diet: dietary treatment (CTRL, HEMP-diet, SAV-diet); period: experimental period; group: group of cows.

[2]Cell variance proportion = (cell variance)/(cell + cow + residual variance) × 100.

[3]Cow (group) variance proportion = (cow variance)/(cell + cow + residual variance) × 100, where "cow" is nested within group.

[4]RMSE: root mean square error.

*$P < 0.05$.

(Haczkiewicz et al., 2025). β-Caryophyllene concentrations ranged from 2,000 to 18,000 µg/kg, consistent with the levels observed in this study (Lee et al., 2023).

Other sesquiterpenes such as β-citronellene, thymoquinone, and caryophylladienol II were also detected, though rarely reported in the literature, suggesting further divergence due to genetic, environmental, or methodological factors (Fischedick et al., 2010). Conversely, α-citral was not detected, in line with its absence in previous HEMP studies. The detection of α-pinene, β-pinene, β-myrcene, and limonene in HEMP leaves aligns with prior studies identifying them as dominant volatiles in *Cannabis* spp. (Hood et al., 1973; Oswald et al., 2021; Lee et al., 2023). Although β-myrcene is often reported as the most abundant monoterpene (Sommano et al., 2020; Giovannoni et al., 2023), it appeared only at moderate concentrations in our samples. Notably, Kumeroa et al. (2022) found it was the second most abundant in Futura 75, supporting genotype-specific variation. In addition, oxidative transformations during storage and sample handling may have influenced terpene profiles. Plant material was stored at −20°C and thawed once before use, which minimizes but does not entirely prevent changes in terpene composition, in addition to being defrosted at room temperature before administration. As volatile monoterpenes can evaporate or undergo conversion into oxygenated derivatives, their relative abundances can be potentially altered (Booth and Bohlmann, 2019; Bueno et al., 2023).

In our study, p-cymene, thymoquinone, and α-citral were the most abundant, supporting previous findings that identified p-cymene as a major compound in SAV (Mahboubi and Kazempour, 2011; Katar et al., 2017). The terpene profiles of SAV and HEMP differed significantly. In SAV, the main sesquiterpenes, β-caryophyllene, α-farnesene, and α-caryophyllene, were present at much lower levels than in HEMP, though they still accounted for 70% of total terpenes, exceeding monoterpene levels. This difference may stem from the different plant maturation stages (postbloom for SAV vs. prebloom for HEMP) and drying processes, which can affect terpene retention. Monoterpenes, being more volatile, are more susceptible to loss during drying (Khangholil and Rezaeinodehi, 2008). Additionally, the unusually dry season may have shifted the optimal balsamic period. Despite this, p-cymene still constituted 14% of SAV terpenes, close to the 19% reported by Mahboubi and Kazempour (2011), and β-myrcene was similarly comparable (0.93% vs. 1.1%). Thymoquinone was detected at 1,050.0 µg/kg in our SAV

Table 7. Terpene transfer rates (%) in CTRL, HEMP-diet, and SAV-diet dietary treatments (n = 12)

	Diet LSM[1]			Contrast (*P*-value)	
Terpene	CTRL	HEMP-diet	SAV-diet	CTRL vs. herbs[2]	HEMP-diet vs. SAV-diet[3]
α-Pinene	18.1	17.6	17.5	0.953	0.993
β-Citronellene	37.1	9.2	52.9	0.726	0.127
Camphene	>100.0	48.3	56.5	0.051†	0.634
β-Pinene	14.8	11.7	13.3	0.792	0.873
β-Myrcene	4.0	9.6	1.7	0.630	0.149
α-Phellandrene	69.0	3.4	11.5	0.082	0.745
3-Carene	26.0	>100.0	27.0	0.265	0.123
p-Cymene	17.2	16.9	2.2	0.418	0.235
D-Limonene	2.1	33.3	3.5	0.176	0.084†
Linalool	5.0	4.7	3.4	0.477	0.438
Camphor	11.6	8.9	10.1	0.644	0.810
Terpinen-4-ol	5.0	4.2	5.0	0.717	0.523
Thymoquinone	>100.0	77.0	2.0	0.168	0.228
α-Citral	>100.0	>100.0	1.2	0.080†	0.244
Ylangene	0.9	0.2	0.4	0.254	0.652
β-*cis*-Caryophyllene	21.2	12.4	16.8	0.218	0.407
Thymol methyl ether	>100.0	7.5	37.1	0.032*	0.875
α-Humulene	0.6	6.8	0.0	0.122	0.033*
α-Farnesene	0.0	7.4	1.1	0.059†	0.036*
β-Caryophyllene	5.8	7.1	1.7	0.432	0.076†
Caryophylladienol II	0.0	64.8	0.0	0.118	0.044*
trans-α-Bergamotene	34.9	23.5	0.2	0.403	0.454
α-Caryophyllene	1.2	6.2	0.0	0.166	0.027*
β-Farnesene	23.7	14.0	25.9	0.896	0.725

[1]Diet: least squares means of the dietary treatment (control diet "CTRL," TMR supplemented with *Cannabis sativa* L. "HEMP-diet," and TMR supplemented with *Satureja hortensis* L. "SAV-diet").
[2]CTRL vs. herbs: contrast comparing CTRL versus both supplemented diets (HEMP-diet + SAV-diet),
[3]HEMP-diet vs. SAV-diet: contrast comparing HEMP-diet versus SAV-diet.
*$P < 0.05$. †Indicates a statistical trend ($P < 0.10$).

samples, higher than the trace levels reported in some Lamiaceae species, including Satureja (Taborsky et al., 2012). This elevated concentration may reflect cultivar-specific traits or environmental factors that enhanced its biosynthesis, as supported by Katar et al. (2017), who linked essential oil composition in SAV to solar radiation and temperature. Additionally, thymoquinone may result from oxidative transformation of thymol and carvacrol,

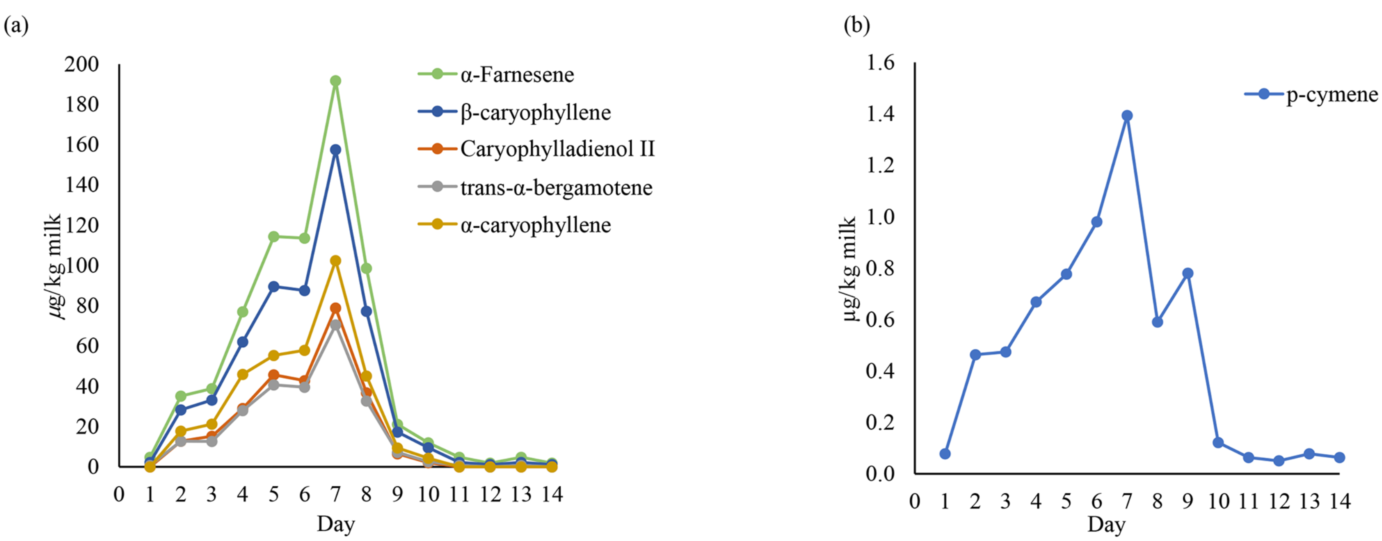

Figure 2. Daily variation of α-farnesene, β-caryophyllene, caryophylladienol II, *trans*-α-bergamotene, α-caryophyllene (a), and p-cymene (b) in the milk of a dairy cow fed with a CTRL diet supplemented with HEMP (a) or SAV (b) leaves from d 1 to 6 (µg/kg).

2 monoterpenes abundant in SAV (Fierascu et al., 2018; Krause et al., 2021). While this pathway is documented in related species such as oregano and thyme (Zhang et al., 2025), it may also occur in *Satureja hortensis* L. under suitable conditions.

Dry Matter Intake and Milk Production

The inclusion of hemp leaves in the diet transiently reduced feed intake, but this effect disappeared at the peak of supplementation (d 6–7), when intake was comparable with that of the other groups.

Schwerdtfeger et al. (2025) similarly reported that feeding hemp leaves at 7% to 8% of diet DM reduced DMI by 1.7 kg/d and milk yield by 1.1 kg/d. Previous observations of bovine aversion to hemp leaves and sorting behavior against hemp-containing feeds have been attributed to terpene-associated sensory load (Fruge et al., 2025). Consistent with these findings and with common practice when supplementing essential-oil–rich botanicals, we adopted a step-up regimen (0.2, 0.4, 0.8 kg/d over 6 d) to minimize palatability shocks, allow sensory adaptation to the aromatic profile, and safeguard intake while reaching the target inclusion level. Because the primary objective of this trial was to evaluate terpene transfer into milk, ensuring complete ingestion of the offered leaves was essential. Although the amounts of plant fed to the cows were relatively small, the high terpene concentrations in fresh leaves ensured detectable transfer into milk.

In contrast, savory, despite its intense aromatic profile (commonly referred to as “pepper herb”), showed good palatability (Golbotteh et al., 2024). This observation is consistent with the fact that savory and related Lamiaceae species are naturally found in pastures and forages and are therefore familiar to ruminants. Previous studies have reported that exposure to aromatic plants in grazing systems may facilitate acceptance and reduce aversion compared with less familiar botanicals. The high palatability of savory observed in this trial suggests that its strong sensory traits did not impair intake and may even reflect an adaptive familiarity of cows with such aromatic forages.

Influence of Herb Supplementation on Terpene Concentrations in Milk

This trial showed that variations in milk terpene concentrations were mainly driven by dietary treatments, reflecting the high terpene content of the HEMP leaves used. Although data on α-humulene, caryophylladienol II, and *trans*-α-bergamotene in milk are limited, β-caryophyllene has been previously detected in dairy products after terpene-rich feed supplementation. Koczura et al. (2021) reported β-caryophyllene as one of the most abundant terpenes in the milk of grazing cows, confirming its transfer from feed. Similarly, Tornambé et al. (2006) identified β-caryophyllene as a dominant sesquiterpene in milk from cows grazing on aromatic-rich pastures. In line with these findings, the concentration of several monoterpenes, including α-pinene, also increased in the milk of cows fed HEMP, with higher levels observed in HEMP-diet milk compared with CTRL. β-Pinene and β-myrcene followed a similar trend, with higher concentrations in HEMP-diet milk compared with CTRL. Similarly, Tornambé et al. (2006) reported α-pinene and β-pinene as prominent monoterpenes in milk from cows grazing on pastures rich in officinal plants. The detection of the same terpenes in our study and previous research supports the conclusion that monoterpenes from terpene-rich plants can be incorporated into milk through dietary intake. Viallon et al. (2000) similarly reported the presence of β-myrcene, α-pinene, and camphene in milk after cows were fed diets enriched with *Achillea millefolium*, reinforcing the link between feed composition and milk terpene profile. Lejonklev et al. (2013) demonstrated that volatile terpenes from essential oils can rapidly transfer into milk following gastrointestinal exposure. Similarly, Viallon et al. (2000) confirmed the transfer of monoterpenes and sesquiterpenes from forages into milk fat, highlighting the dietary influence on milk terpene composition.

In our study, several terpenes found in SAV leaves were also detected in the milk of cows fed the SAV-diet. For example, p-cymene, one of the most abundant terpenes in SAV, was detected at higher levels in SAV-diet milk compared with CTRL milk, though the difference was not significant. Other terpenes present in SAV and recovered in SAV-diet milk included β-citronellene, β-pinene, β-myrcene, and α-farnesene. Their presence in milk supports a dietary origin, despite the lack of significant treatment effects for most compounds. Compared with HEMP, SAV supplementation appeared to have a lower impact on the terpene profile of milk. Whereas compounds such as camphene, α-phellandrene, linalool, and α-citral were present, their concentrations did not differ significantly from those in the CTRL group.

Our study revealed that the terpene composition in milk does not always directly mirror the composition found in the feed, suggesting that metabolic and transfer processes play a significant role. For instance, Poulopoulou and Hadjigeorgiou (2021) reported that only ~50% of camphene added to rumen fluid was recovered after incubation, suggesting that camphene and similar terpenes may undergo ruminal degradation or transformation, limiting their transfer to milk despite being present in the feed. Although SAV leaves were rich in thymoquinone, its concentration in SAV-diet milk was similar to that in

CTRL and HEMP-diet milk. This could suggest minimal transfer or possible cross-contamination, though strict precautions, separate housing, dedicated collection buckets, and thorough cleaning were followed. Given the high volatility of terpenes, airborne transfer cannot be fully ruled out, but its effect is likely negligible compared with dietary intake. Similarly, compared with the CTRL diet, HEMP and SAV leaves contained higher concentrations of β-*cis*-caryophyllene. Although β-*cis*-caryophyllene levels in the diets differed greatly for CTRL, HEMP-diet, and SAV-diet, no significant differences were found in milk concentrations. This suggests poor transfer efficiency, likely due to degradation or biotransformation during rumen or liver metabolism (Coppa et al., 2011). Also, De Noni and Battelli (2008) reported high variability in milk terpene levels from cows on terpene-rich pasture, attributing it to ruminal degradation and hydrogenation processes. Moreover, some sesquiterpenes, such as β-*cis*-caryophyllene, are highly lipophilic and tend to accumulate in body fat after ingestion (Serrano et al., 2007; Takemoto et al., 2021), reducing their availability for transfer into milk. These findings highlight that milk terpene levels depend not only on feed content but also on absorption, ruminal metabolism, and fat partitioning, reflecting complex digestive and systemic processes.

Feed-to-Milk Transfer Dynamics of Volatile Terpenes

To further understand the dietary effect on milk terpene profiles, this study evaluated the transfer rate (%) of individual terpenes from feed to milk, calculated as the ratio between the daily excreted amount in milk and the daily ingested amount through the diet. Calculating the transfer rate of terpenes is essential to quantify their carryover from feed into milk, enabling an accurate assessment of their bioavailability, metabolic fate, and potential implications for product authenticity or flavor. Despite the ecological and nutritional relevance of these volatile compounds, systematic evaluations of their transfer kinetics remain scarce, with most literature focusing on qualitative detection rather than quantitative transfer (Tornambé et al., 2006; Lejonklev et al., 2013).

Some compounds show an inverse relationship between the amount of a terpene in the feed and its transfer rate (%) to milk, meaning higher concentration in feed often corresponds to lower transfer efficiency. Among the terpenes analyzed, β-pinene, α-phellandrene, and α-pinene illustrate the inverse relationship.

Although β-pinene concentrations varied across diets, with higher levels in the HEMP-diet, the corresponding transfer rates to milk remained relatively consistent (11.7%–14.8%). This suggests that beyond dietary concentration, other factors such as absorption efficiency or metabolic processing may limit β-pinene transfer to milk.

Interestingly, α-phellandrene showed an inverse relationship between dietary concentration and transfer rate. Despite being barely detectable in the CTRL diet, it exhibited a high transfer rate (69.0%), whereas higher dietary levels in the HEMP-diet and SAV-diet corresponded with lower transfer rates. A similar pattern was observed for α-pinene, where increased dietary concentrations did not translate into higher transfer rates, which remained stable across treatments (~17%–18%).

Although not yet documented for terpenes, similar inverse relationships have been demonstrated for mycotoxins, indicating that transfer efficiency may decrease at higher dietary inputs due to saturation, metabolism, or absorption dynamics (Battacone et al., 2009; Zentai et al., 2023). These patterns suggest that hepatic metabolism may reduce transfer efficiency after absorption. Glucuronidation in the liver represents a major conjugation pathway for lipophilic compounds, producing polar metabolites that are more readily excreted in urine or bile (Hulin et al., 2026). Such conjugated derivatives would not have been detected by our analytical approach, which focused on free terpenes, and may partly explain the low or inconsistent transfer rates observed. In addition, metabolism is not independent for each compound. Cytochrome P450 enzymes, for instance, are key players in terpene modification. In plants, CYP71, CYP72, and CYP85 families are known to hydroxylate and rearrange terpene structures (Hamberger and Bak, 2013). In cattle, functional CYP3A isoforms are expressed in the liver: their activity varies by breed and sex (Dacasto et al., 2005), and CYP3A28 has been shown to be active in bovine liver slices (Maté et al., 2015). Whereas direct evidence of terpene metabolism by bovine CYPs is not yet available, these findings strongly suggest that CYP-mediated oxidation in cows contributes to reduced systemic terpene availability and therefore to the limited transfer observed in our study.

A particular observation in this study was the unexpectedly high transfer percentages, especially in the CTRL for certain terpenes such as α-citral and thymol methyl ether. Such values are biologically implausible, and they need to be interpreted cautiously. For instance, low analyte concentrations inherently show higher relative variability, so small feed values can greatly inflate transfer ratios. This effect is magnified in ratio-based calculations where small denominators increase variance. These factors provide a clear methodological explanation for the implausibly high transfer rates observed, rather than true biological carryover (Mertens, 2009).

Although transfer rates above 100% are biologically implausible and most likely reflect analytical artifacts, a limitation we have acknowledged and that warrants further investigation, possible metabolic transformations may also contribute to the unexpectedly high concentrations observed in milk. As discussed by Sahoo et al. (2021), thymol serves as a versatile pharmacological support that readily undergoes structural modifications, including O-methylation, to yield more stable or bioactive derivatives. The formation of thymol methyl ether could thus result from enzymatic methylation of thymol or thymol-like precursors present in the basal diet or environment. Additionally, Sahoo et al. (2021) highlight that thymol derivatives such as methyl ethers exhibit enhanced biological stability and membrane permeability, which could facilitate accumulation in milk fat.

Given the hydrophobic nature of thymol methyl ether and its potential for endogenous formation through methylation of residual thymol, the elevated milk levels observed in control cows may not reflect dietary intake alone but also postabsorptive metabolic conversion. Interestingly, α-citral was already detectable in the CTRL group at 64.6 μg/kg, indicating a basal presence of this compound in the diet even without specific supplementation. Whereas this concentration alone does not fully explain the high transfer rate observed (>100%), it supports the hypothesis that α-citral may originate from both direct dietary intake and endogenous transformation of precursor monoterpenes. In fact, studies in microbial systems such as *Castellaniella defragrans* (Lüddeke et al., 2012) have shown that compounds such as geraniol and β-myrcene, commonly present in forages, can be metabolized into α-citral via efficient enzymatic pathways. Given the anaerobic nature of the rumen and the known capability of rumen microbes to degrade and transform terpenes, it is possible that the α-citral detected in milk reflects both direct feed-derived input and ruminal biotransformation of structurally related monoterpenes present at low but biologically relevant levels.

Finally, quantifying trace volatiles in milk is challenging, and some variability is unavoidable, as evidenced by the very marked fluctuations observed, raising questions about the precision and challenging the reliability of terpene-based tracers over time (Tornambé et al., 2006).

Kinetics of Terpene Transfer into Milk

Appearance/disappearance kinetics were estimated from period 1 only; thus, time constants reflect that period's standardized schedule and are presented as mechanistic estimates rather than cross-period comparisons.

In our study, terpene concentrations in milk rose rapidly during supplementation and declined sharply once feeding ceased, indicating that these compounds are highly transient. As noted by Lejonklev et al. (2013), the presence of specific terpenes in milk following essential oil administration suggests that several of these compounds can be transferred into milk in recognizable forms, despite possible transformation or partial metabolism along the way.

Similar disappearance kinetics were noted by Tornambé et al. (2006), who found that monoterpene levels in milk decreased rapidly once the terpene-rich forage was removed, reaching similar or lower than initial baseline values. Likewise, Viallon et al. (2000) observed that within 4 d after stopping *Achillea millefolium* supplementation, terpene concentrations in milk returned to pre-treatment levels, confirming the short persistence of both monoterpenes and sesquiterpenes in the animals' body (Viallon et al., 2000). Lejonklev et al. (2013) further supported this notion, stating that whereas terpene transfer to milk is fast, it is also transient, with concentrations diminishing soon after the compound is no longer ingested, thereby exhibiting no memory effect (Viallon et al., 2000). This rapid depletion is consistent with previous findings in sheep, where it was observed that, after a short lag phase of 3 d, terpenes reached measurable blood levels that fluctuated markedly between sampling days and declined sharply once oral administration ended (Poulopoulou et al., 2012). Also, similar to many xenobiotics, terpenes are distributed through the cardiovascular system to peripheral tissues, where they undergo enzymatic conversion into metabolites. These metabolites may then re-enter circulation and be eliminated in urine or be processed in the liver and secreted into bile for excretion in the feces (McLean and Duncan, 2006).

Whereas most terpenes showed a rapid decline after withdrawal, p-cymene displayed a minor secondary elevation around d 9. This transient rebound suggests that, in addition to direct clearance, certain terpenes may undergo temporary redistribution. Once sequestered in fat, terpenes may gradually re-enter circulation and reach the mammary gland even after dietary exposure ends (Serrano et al., 2007); however, the overall trend continues downward. Moreover, THC, hemp cannabinoids similar in lipophilicity to terpenes, can be stored in adipose tissue, and lipolysis enhances their release from fat stores back into blood (Gunasekaran et al., 2009).

To further evaluate the persistence of selected terpenes in milk following dietary withdrawal, we fitted their concentrations to an exponential decay model described by the equation: $y = y_0 .\ e^{-kx}$, where y is the terpene concentration at time x (days), y_0 is the initial concentration, and k is the elimination rate constant (d^{-1}). From this model, we calculated the decay constants and corresponding half-lives ($t1/2 = \ln(2)/k$) and we obtained $k = 0.348$ d^{-1} for p-cymene, $k = 0.943$ d^{-1} for α-farnesene, and $k = 1.03$ d^{-1} for β-caryophyllene, resulting in half-lives of ~1.99 d (≈48 h), 0.73 d (≈17.5 h), and 0.67 d (≈16.1 h), respectively.

The rapid disappearance of terpenes from milk underscores their volatile nature following dietary withdrawal. For comparison within hemp-derived lipophiles, unlike our rapidly vanishing terpenes, cannabinoids remained detectable after an 8-d hemp-free depuration in dairy cows, with milk levels showing a rapid initial decline after feeding ceased followed by a slower, prolonged decrease consistent with biphasic elimination (Wagner et al., 2022). Consistent with recent spent hemp biomass feeding data, milk THC fell below the LOD by d 12 after withdrawal, but it remained detectable in the adipose tissue until 30 d after SHB withdrawal (Irawan et al., 2025).

CONCLUSIONS

This study examines the transfer of terpenes from HEMP (*C. sativa* L.) and SAV (*S. hortensis* L.) leaves to cow milk, offering new insights into their kinetics, bioavailability, and metabolic fate. The HEMP supplementation, rich in sesquiterpenes, resulted in higher concentrations and transfer rates of compounds such as α-caryophyllene, α-farnesene, α-humulene, and caryophylladienol II compared with SAV. In contrast, SAV contributed more monoterpenes, such as p-cymene and thymoquinone, though their transfer was less efficient. Terpenes appeared rapidly in milk post-supplementation and declined sharply after withdrawal, confirming their short-lived, diet-dependent nature. Transfer rates, generally below 10%, varied by compound, influenced by lipophilicity, molecular weight, and volatility. Apparent transfer rates >100% for compounds such as thymol methyl ether and α-citral suggest possible endogenous transformation or analytical artifacts. Future research should investigate rumen microbial metabolism and postingestion transformations and employ targeted metabolomics for improved traceability. Overall, this study advances strategies to enhance the nutritional and sensory quality of dairy products.

NOTES

The authors declare that the research was cofunded by the European Union's Horizon Europe 101136346 EU-PAHW and by the University of Padova with the project BIRD213117/2021. The data presented in this study are available from the corresponding author upon reasonable request. The animal study protocol was approved by the Ethics Committee for the Care and Use of Experimental Animals (OPBA) of the University of Padua (Prot. n. 131204, 26/07/2022). The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: AIA = acid insoluble ash; CF = crude fiber; CTRL = TMR without any supplementation; EE = ether extract; HEMP = TMR supplemented with fresh hemp leaves; HS-SPME-GC-MS = headspace solid-phase microextraction coupled with GC-MS; LOD = limit of detection; NIST = National Institute of Standards and Technology database; RMSE = root mean square error; SAV = TMR supplemented with fresh savory leaves; THC = Δ^9-tetrahydrocannabinol.

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