

Lavender (*Lavandula angustifolia*) volatile compounds as a functional feed additive: improving rumen fermentation and reducing pro-inflammatory cytokines in calves

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ABSTRACT. The aim of this study was to demonstrate the effectiveness of lavender (*Lavandula angustifolia*) volatile compounds (LVCs) when supplemented to calf milk to assess their effect on performance, ruminal metagenomics, fermentation end-products and immune variables. Calves received milk, starter feed and wheat straw until weaning at 65 days of age. Milk was supplemented with 0 µl/day (control), 60 µl/day (LAV60), or 120 µl/day (LAV120) of LVCs per calf. Feed intake, body weight at days 40 and 65, ruminal concentrations of acetic acid (AcA), propionic acid (PA) and ammonia-N, and the relative abundance of *Prevotella_7*, *Erysipelotrichaceae_UCG_002*, *Succiniclasticum*, *Ruminococcus* and *Bifidobacterium* were all increased by LVCs ($P < 0.05$). In contrast, serum amyloid-A (SAA), tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) concentrations decreased linearly with LVC supplementation ($P < 0.05$). Overall, LVCs improved feed intake and body weight during the liquid and solid feeding period, and reduced pro-inflammatory cytokines at weaning. The active components of LVCs appear to promote the formation of a bacterial ecosystem required for solid feed digestion and development of rumen functions characteristic of adult ruminant.

Introduction

Alternative substances such as volatile compounds (VCs) and prebiotics, supplemented to calves as feed additives during the liquid and solid feeding period before weaning, can support immune function. For instance, immunoglobulin A (IgA) supplementation has been shown to elevate IgA titres, improving the response to bovine respiratory disease (Swedzinski et al., 2020). VCs or aromatic oils, extracted from aromatic plants, possess functional compounds with important biological effects, including the potential to reduce antibiotic use (Soković et al., 2010). Support-

ing this, Liu et al. (2020) demonstrated that a combination of VCs (including carvacrol, caryophyllene, *p*-cymete, cineole, terpinene, and thymol) and a prebiotic (arabino-galactons), when added to calf starter feed, increased dry matter (DM) intake, feed conversion ratio (FCR), body weight gain and morphometric traits.

Studies on VCs from the members of the family *Lamiaceae* such as *Thymus vulgaris* (thyme), *Origanum vulgare* (oregano), and *Rosmarinus officinalis* (rosemary) have mostly focused on their antioxidant, immunomodulatory, growth-promoting, and meat quality enhancing properties in poultry

(Popović et al., 2016; Puvača et al., 2022). However, evidence on the effectiveness of VCs in calf nutrition, particularly their impact on health, growth performance, immune function, rumen microbiota, and fermentation parameters, remains limited (Akbarian-Tefaghi et al., 2018; Swedzinski et al., 2020). Addition of sage (*Salvia officinalis*, family *Lamiaceae*) VCs (100 or 200 µl/day) to calf milk increased live weight, feed intake, and daily body weight gain (Kara and Pirici, 2024). The same study reported that sage VCs reduced serum concentrations of tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) at weaning, while increasing the relative abundance of beneficial ruminal bacteria such as *Bifidobacterium*, *Acidaminococcus*, *Prevotella*, *Prevotellaceae_NK3B31_group*, and *Prevotella_9* (Kara and Pirici, 2024). These findings suggest that LVCs will likely exert similar effects during the milk-feeding period in calves.

Lavender (*Lavandula angustifolia*), another member of the family *Lamiaceae*, is widely cultivated in Anatolia and the Mediterranean Levant. LVCs are obtained from flowers and leaf parts using steam distillation, with flowers containing at least 1% VCs. The oil is rich in monoterpenoids, linalool, linalyl-acetate, camphor, 1,8-cineole and terpinen-4-ol compounds (Sandner et al., 2020). Linalool and linalyl-acetate compounds are key quality markers and contribute to the functional and aromatic properties of this oil (Elsharif et al., 2015). Although VCs show promising benefits in improving calf performance and health, reported outcomes remain inconsistent, likely due to variation in dosage and chemical composition, diet formulation, and animal physiology across studies (Schären et al., 2017).

Neonatal calves are protected against environmental pathogens by passive immunity from maternal antibodies received through colostrum. However, their own active immunity is not fully formed in the first weeks of life, and the stress of the weaning period, which involves immune materials such as inflammatory cytokines and acute phase proteins, makes calves vulnerable to diseases (Kim et al. 2011). The hypothesis of the study was that supplementation of milk with lavender volatile compounds (LVCs), which contain biologically active substances, during the critical pre-weaning feeding period would affect growth performance, rumen parameters, rumen microbiota and immune system variables. The objective was to determine the effects of incrementing LVC doses added to milk until weaning on growth traits, immune responses, ruminal metagenomics and fermentation end-products.

Material and methods

LVC analyses

LVCs were obtained from *Lavandula angustifolia* harvested at the flowering stage and produced by the Medicinal and Aromatic Plants Centre (Afyon, Turkey). LVCs were extracted by hydrodistillation and stored in amber bottles. Samples of 100 µl were diluted to a final volume of 2000 µl with chromatographic-grade methanol. The relative percentages of chemical components in LVCs were detected using gas chromatography-mass spectrometry (GC-MS; Shimadzu, GC MS-QP2010 SE, Nakagyo-ku, Kyoto, Japan) with a 5% diphenyl/95% dimethyl polysiloxane column. The GC-MS analysis was conducted following the method of Mothana et al. (2013), and compounds were identified by comparison with the Wiley 9-Nist 11 Mass Spectral Database.

Study design and calf nutrition

This study was approved by the Local Ethics Committee for Animal Experiments of Erciyes University, Province of Kayseri, Türkiye (Approval date and number: 02.06.2021 and 21/128).

Purebred Holstein calves from a disease-free dairy farm in the Bünyan district of Kayseri province were used. The calves were offspring of Holstein dairy cattle that had reached at least their third lactation. All calves were born via normal delivery, had similar live weights and colostrum consumption volumes, and received no antibiotics during the study. Feeding with LVCs was conducted in April–May 2023. A synchronisation protocol was applied to the dairy herd to ensure simultaneous calving, providing a sufficient number of calves of the same age, all of which were included in the study at the same time.

Eighteen Holstein calves, housed in individual boxes, were divided into three groups of 6 calves each with similar initial live weights (51.38 ± 1.1 kg), age (15 days), and gender (3 females, 3 males per group). The groups consisted of a control group (LAV0) and two experimental groups receiving LVCs at 60 µl/day (LAV60) or 120 µl/day (LAV120) per calf in whole milk. LVC levels were based on previous studies (Coelho et al., 2023; Kara and Pirici, 2024). All calves had *ad libitum* access to water and a solid feed mixture consisting of 90% calf starter and 10% wheat straw from 15 days of age until weaning at 65 days. Milk feeding consisted of 6 l/day (38 °C) from day 15 to 55, and 3 l/day from day 56 to 65, provided in three meals using a nipple bottle. Milk was obtained from the Holstein herd on the same farm. The daily LVC dose for each

calf was pre-measured into microcentrifuge tubes, stored at +4 °C on the farm, and added directly to the milk in the nipple bottle immediately before feeding. LVCs was readily soluble in milk due to its oil- and water-soluble nature, aided by natural emulsifiers present in milk, such as polar lipids and milk proteins (Braun et al., 2019). All milk offered was completely consumed throughout the study, with no refusals attributed to LVCs or any other reason. Faecal consistency was monitored daily from day 15 to 65 day using a 1–5 scoring scale (1 = normal, 2 = soft, 3 = soft-watery, 4 = very soft-watery, 5 = watery), under the supervision of the on-farm veterinarian.

Determination of chemical compositions of starter feed and wheat straw and milk

Milk was sampled at the beginning, middle, and end of the study, with 20 ml of homogeneous sample collected on each occasion. Analyses were carried out in triplicate using a MilkoScan FT2 milk analyser (Foss Electric, Hillerod, Denmark). Calf starter feed and wheat straw were dried in an oven (Binder, Binder GmbH, Germany) for DM determination. Dried samples were ground to pass through a 1-mm sieve and subsequently analysed for ash, crude protein (CP), and diethyl ether extract (EE) according to AOAC International (1995). Neutral detergent fibre (NDF) was determined following the method of Van Soest et al. (1991). Total starch content of calf starter and wheat straw was determined using a commercial assay kit (Megazyme, Bray Business Park, Bray, Co. Wicklow, Ireland) (Kara et al., 2019). All chemical analyses were carried out in triplicate.

Growth performance

Daily intake of whole milk (liquid feed) and calf starter plus wheat straw (solid feeds) was recorded daily. All calves were weighed at the beginning (day 15) and end (day 65) of the study. Body measurements, including wither height, rump height, body depth, hearth girth and body length were also taken at both time points.

Determination of short chain fatty acids and ammonia-N in rumen fluid

Rumen fluid samples (~30 ml) were collected from all calves in each group 2 h after feeding on the weaning day using a rumen tube. Of the collected fluid, 20 ml was used for short-chain fatty acid (SCFA) and ammonia-N analyses, while 10 ml was reserved for metagenomic analyses. Ruminal SCFA analysis detected acetic acid (AcA), butyric acid (BA), propionic acid (PA), valeric acid (VA),

iso-valeric acid (IVA), iso-butyric acid (IBA), iso-caproic acid (ICA), hexanoic acid (HA) and heptanoic acid (HEPA). The fluid samples were filtered and centrifuged at 13 000 rpm for 15 min using a Gyrozen 1524 microcentrifuge (GYROZEN, Seoul, South Korea). The resulting supernatants were mixed with a 25% meta-phosphoric acid at a 5:1 ratio. SCFA concentrations in these mixtures were analysed using a gas chromatograph equipped with a flame ionisation detector (GC-FID) (Thermo Trace 1300, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), following the procedure described by Ersahince and Kara (2017). Ammonia-N concentration was determined using the Megazyme K-AMIAR 02/20 assay (Neogen-Megazyme, Ireland).

Metagenomics rumen fluid analyses

Rumen fluid from each calf (10 ml per animal) was used for metagenomics analysis (n = 6 per group). In total, 18 rumen fluid samples (6 calves × 3 groups) were analysed. Samples were collected into tubes free from RNase, DNase, and endotoxins (Labsolute, Geyer GmbH & Co. KG, Rellingen, Germany). Total DNA was extracted using a commercial kit (EURx Molecular Biology Products, Gdańsk, Poland). The V3 and V4 regions of the 16S ribosomal RNA (rRNA) gene were amplified for metagenomic species identification, using primers from Klindworth et al. (2013):

Forward Primer:

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Reverse Primer:

5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

Metagenomic sequencing was performed using the Illumina NovaSeq 6000 platform (San Diego, CA, USA). Deoxynucleoside triphosphates (dNTPs) were used to minimise sequencing errors and reduce the incorporation of incorrect bases, ensuring high-accuracy results. Image processing and base calling were performed using the NovaSeq Control Software and Real-Time Analysis v1.18. The raw base calls were converted to FASTQ format with bcl2fastq (v1.8.4). Non-specific adapter sequences were removed using Scythe (v0.994 BETA) and Sickle. For bioinformatics analysis, taxonomic classification was performed by aligning reads to reference sequences. VSEARCH within QIIME 2 was used for taxonomic assignment at 97% similarity. The taxonomic distributions presented in figures represent the average values of six rumen samples per group.

Immune parameters

Blood samples were collected from the *V. jugularis* of each calf at weaning (08:00, day 65) into silicone tubes. Samples were centrifuged at 4 000 rpm for 5 min (Nüve NF 400R, Nüve, Ankara, Türkiye) to obtain serum, which was stored at -20°C for up to 2 months and thawed at $+4^{\circ}\text{C}$ before analysis. Serum immunoglobulin E (IgE) was measured using a bovine ELISA kit (Catalogue No: 201040151; SunRed, Shanghai, China; detection range 10–3000 $\mu\text{g/ml}$). IL-1 β (Catalogue No. 201040157, 1.5–400 pg/ml), IL-6 (Catalogue no. 201-04-0008, 30–6000 pg/ml), TNF- α (Catalogue no. 201-04-0007, 15–4000 ng/ml), and serum amyloid A (SAA) (Catalogue no. 201-04-0126, 0.15–40 $\mu\text{g/ml}$) were analysed following the manufacturer's protocols.

Statistical analyses

The experimental data were first subjected to Levene's test to assess homogeneity of variance, and normality confirmed using the Shapiro-Wilk test. A General Linear Model procedure was used to compare variables. All statistical analyses were performed using a software package (IBM SPSS Modeler 17.0). The effect of LVC supplementation at different doses on performance, body measurements, faecal scores, ruminal short-chain fatty acids, ammonia-N concentrations, and relative bacterial abundances were evaluated using one-way ANOVA. Linear and quadratic polynomial contrast analyses were performed to examine dose-effect relationships. The effects of different LVC doses on the variables were evaluated using one-way ANOVA. The statistical model applied was:

$$Y_i = \mu + S_i + e_i,$$

where: Y_i – measured variables (e.g., growth values, rumen fluid end-products etc.), μ – overall mean, S_i – effect of LVC supplementation, and e_i – residual error. Differences between group means were assessed using Tukey's multiple range test ($P < 0.05$).

Results

Chemical composition of LVCs and the milk

The main components of LVCs were linalool, linalyl acetate, camphor, eucalyptol, terpinene-4-ol, p-cymene, β -caryophyllene, β -farnesene and anthranilic acid (Table 1). Milk contained 3.84% fat, 3.18% protein, 4.9% lactose, 12.56% DM, and a somatic cell count of 95×10^3 cells/ml. The calf starter feed contained 19.01% CP, 29.47% NDF and 27.79% starch on a DM basis, while wheat straw contained 5.14% CP, 78.04% NDF, and 1.01% starch in DM (Table 2).

Table 1. Chemical composition of lavender (*Lavandula angustifolia*) volatile compounds (LVCs)

Chemical class	Compound	% abundance	
Monoterpenes	α -pinene	0.38	
	camphene	0.36	
	2-beta-pinene	0.64	
	β -myrcene	0.69	
	trans- β -ocimene	0.98	
	β -cis-ocimene	0.61	
	β -ocimene	0.78	
	γ -terpinene	0.59	
	α -terpinolene	0.51	
	p-cymene	6.26	
	Oxygenated monoterpenoids	linalool	19.46
		linalyl acetate	16.85
		eucalyptol (1,8-cineole)	9.12
		camphor	13.11
terpinen-4-ol		9.48	
r- α -terpinyl acetate		0.38	
iso-borneol		0.47	
lavandulyl acetate		0.97	
cis-farnesol		0.15	
caryophyllene oxide		0.66	
Sesquiterpenes		α -zingiberene	0.42
		trans-caryophyllene	0.59
		β -copaene	0.57
		α -bergamotene	0.14
	β -cis-farnesene	2.04	
	germacrene d isomer	2.00	
	α -bisabolol	0.16	
	Alcohols	1-hexanol	0.11
1-octen-3-ol		0.34	
Phenolic compounds	phenol, 4-ethyl-3-methyl	0.27	
Acids and derivatives	acetic acid, hexyl ester	0.49	
	butanoic acid	0.65	
	hexanoic acid, hexyl ester	0.44	
	anthranilic acid, linalyl ester (vit L1)	3.47	
	N-hexadecanoic acid	0.44	
	palmitamide	0.82	
	oleic acid, methyl ester	0.48	
	octadecanoic acid, methyl ester	0.24	
	9,12-octadecadienic acid	1.39	
	octadecanoic acid	0.28	
	9-octadecenamide	0.22	
	Esters	geraniol butyrate	0.14
		hexyl iso-valerate	0.26
		hexyl tiglate	0.21
neryl (s)-2-methylbutanoate		0.64	
Aldehydes	benzaldehyde (p-cumic aldehyde)	0.19	
	Sulphonic compound	propane sulfone	0.42
Fatty acid methyl esters		methyl palmitate	0.24

% abundance – area percentage excluding solvent peak in chromatograms

Table 2. Chemical composition of starter feed and wheat straw

	Starter feed*	Wheat straw
DM, % feed basis	90.81	95.38
Ash, % DM	8.83	7.01
CP, % DM	19.01	5.14
NDF, % DM	29.47	78.04
EE, % DM	4.15	0.74
Starch, % DM	27.79	1.01
NFC, % DM	38.54	9.07

DM – dry matter, CP – crude protein, NDF – neutral detergent fibre, EE – diethyl ether extract; NFC – non-fibrous carbohydrates, NFC = 100 – (NDF + CP + EE + ash); * produced by Kayseri Feed Factory, Kayseri, Turkey

Performance and body measurements

Calves received 753.6 g/day DM, 230.4 g/day fat and 190.8 g/day protein from milk between 15 and 55 days of age, alongside solid feed (starter feed + forage). From day 56 to 65 (weaning), calves received 376.8 g/day DM, 115.2 g/g fat and 95.8 g/day protein in milk, in addition to solid feed. Average daily DM intake of calves in the LAV120 group was higher compared to LAV0 ($P < 0.05$). Protein and ME intakes from solid and liquid feeds did not differ between control and treatment groups ($P > 0.05$). At weaning, body weight in LAV60 was higher than in LAV0 ($P < 0.05$), while DM intake on the day of weaning was similar in all groups. Body length, wither height, body depth, rump

height and hearth girth were not affected by LVC supplementation. Average faecal scores were similar among all groups (Table 3).

Ruminal short chain fatty acid and ammonia-N concentrations

The concentrations of total SCFA (T-SCFA), AcA and PA in the rumen fluid were higher in the LAV60 and LAV120 groups compared to LAV0 ($P < 0.05$). In contrast, BA concentrations were lower in LAV60 and LAV120 than in LAV0 ($P < 0.05$). IVA and IBA concentrations decreased linearly with increasing LVC supplementation ($P < 0.05$). HEPA concentration increased in LAV120 compared to LAV0 ($P < 0.05$), while VA, HA, and ICA concentrations were unaffected by LVC addition. Ruminal ammonia-N concentrations increased with LVC supplementation ($P < 0.05$) (Table 4).

Ruminal microbiome profile

Metagenomic analysis indicated that 93.70% of species in LAV0, 93.66% in LAV60, and 95.95% in LAV120 belonged to the Bacteria kingdom. The relative abundance of archaea in the rumen fluid of LAV120 (2.95%) was lower than in LAV0 (3.74%) and LAV60 (3.62%) ($P < 0.05$). At the phylum level, the dominant bacteria were *Firmicutes*, *Bacterioidota*, and *Actinobacteriota* (Figure 1). The relative

Table 3. Performance values and body measurements of calves fed milk containing lavender volatile compounds (LVCs)

Parameter	LVC addition			SD	SEM	P-value	
	LAV0	LAV60	LAV120			L	Q
Body weight, kg							
initial (15 days of age)	51.40	51.80	50.96	4.74	1.11	0.883	0.810
final (65 days of age)	80.42 ^b	84.06 ^a	81.48 ^{ab}	2.52	1.54	0.043	0.020
DM intake, g/day							
average of study*	473.07 ^b	485.05 ^{ab}	542.56 ^a	27.10	7.85	0.040	0.245
average at weaning	1226.60	1314.66	1237.60	92.05	73.01	0.956	0.615
protein intake, g/day							
average of study*	93.98	77.37	90.53	7.40	4.12	0.240	0.478
ME intake, kcal/day							
average of study*	4238.99	4176.72	4184.34	42.78	10.35	0.341	0.578
faeces score							
average of study	1.10	1.20	1.10	0.15	0.03	0.983	0.235
Body measurements, cm							
body length	91.66	89.83	92.16	5.29	1.24	0.877	0.462
withers height	93.83	92.00	94.83	2.48	1.01	0.688	0.523
body depth	57.00	55.83	58.83	2.78	0.65	0.246	0.134
rump height	94.83	94.66	96.00	3.29	0.78	0.566	0.670
hearth girth	99.33	97.66	99.16	3.10	0.73	0.929	0.338

LVC – lavender volatile compound, LAV0 – calves fed milk without LVCs, LAV60 – calves fed milk with 60 µl/calf/day of LVC; LAV120 – calves fed milk with 120 µl/calf/day of LVCs; values are means with standard deviation (SD) and standard error of the mean (SEM); DM – dry matter; * average dry matter intake from 15 to 65 days of age; ME – metabolizable energy, calculated as ME (Mcal/kg) = 0.93 × (0.057 × % protein + 0.092 × % fat + 0.0395 × % lactose (NRC, 2001)); L – linear contrast; Q – quadratic contrast; ^{ab} – means within a row with different superscripts are significantly different at $P < 0.05$

Table 4. Ruminal short chain fatty acid and ammonia-N concentrations in calves fed milk containing lavender volatile compounds (LVCs)

	LVC addition			SD	SEM	P-value	
	LAV0	LAV60	LAV120			L	Q
Ammonia-N, mg/l rumen fluid	25.53 ^b	27.12 ^{ab}	30.21 ^a	2.12	0.59	0.002	0.875
Short chain fatty acids, mmol/l rumen fluid							
T-SCFA	89.24 ^b	103.59 ^a	103.88 ^a	10.94	3.98	0.041	0.414
SCFA							
AcA	47.00 ^b	54.87 ^a	62.48 ^a	4.19	2.54	0.009	0.978
PA	26.31 ^b	37.72 ^a	28.78 ^a	1.35	2.08	0.584	0.022
BA	9.96 ^a	6.72 ^b	7.14 ^b	3.19	0.79	0.052	0.286
VA	4.07	2.72	2.58	1.45	0.36	0.099	0.433
HA	0.58 ^{ab}	0.43 ^b	1.50 ^a	0.78	0.19	0.033	0.101
HEPA	0.26 ^b	0.23 ^b	0.38 ^a	0.11	0.02	0.040	0.121
BSCFA							
IVA	0.48 ^a	0.31 ^b	0.45 ^{ab}	0.009	0.05	0.052	0.244
IBA	0.50 ^a	0.36 ^b	0.43 ^b	0.03	0.04	0.012	0.313
ICA	0.08	0.21	0.11	0.10	0.02	0.705	0.057

LVC – lavender volatile compound, LAV0 – calves fed milk without LVCs, LAV60 – calves fed milk with 60 µl/calf/day of LVC; LAV120 – calves fed milk with 120 µl/calf/day of LVCs; T-SCFA – total short chain fatty acids, SCFA – short-chain fatty acids, AcA – acetic acid, PA – propionic acid, BA – butyric acid, VA – valeric acid, HA – hexanoic acid, HEPA – heptanoic acid, BSCFA – branched short-chain fatty acids; IVA – iso- valeric acid, IBA – iso-butyric acid, ICA – iso-caproic acid, SD – standard deviation; L – linear contrast; Q – quadratic contrast; SEM – standard error of the mean; ^{ab} – means within a row with different superscripts are significantly different at $P < 0.05$

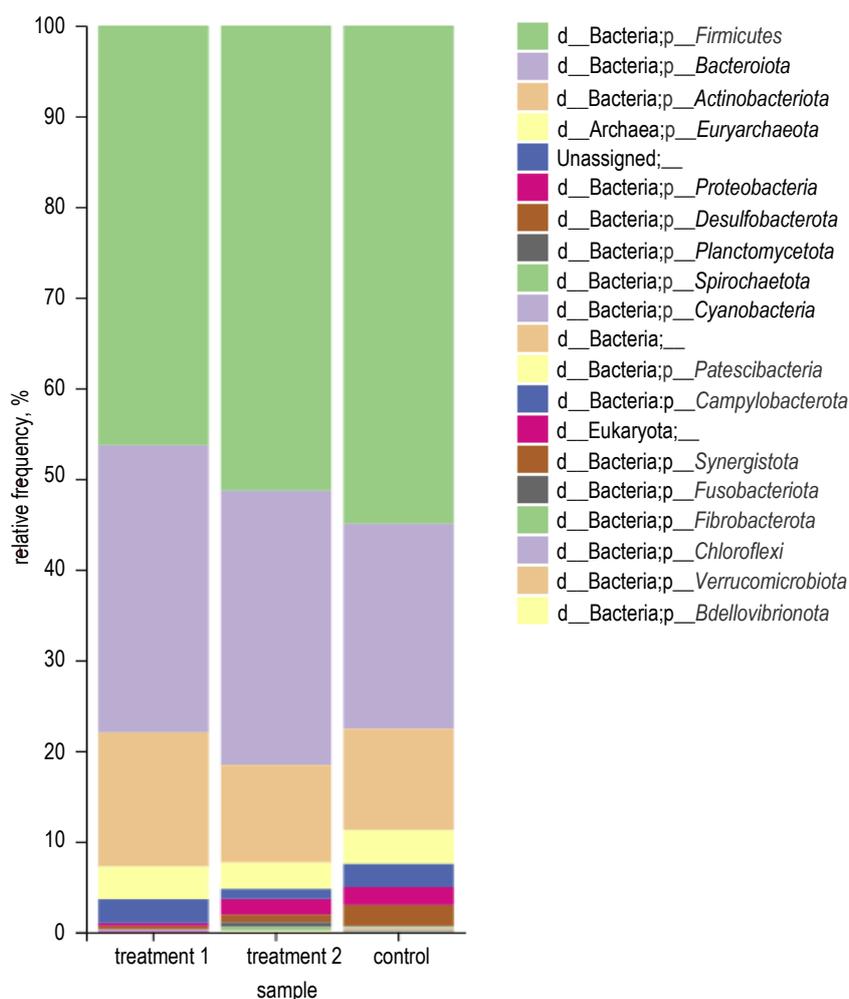
**Figure 1.** Relative abundance of bacterial and archaeal phyla in the rumen fluid of calves fed milk containing lavender volatile compounds (LVCs). A total of 6 x 3 = 18 rumen fluid samples (6 calves x 3 groups) were analysed, and the values represent the average of 6 samples per group

Table 5. Relative abundance of dominant taxa with an average relative abundance $\geq 0.5\%$ in rumen fluid of calves fed milk containing lavender volatile compounds (LVCs)

Taxon	VC addition to milk			SD	SEM	P-value	
	LAV0	LAV60	LAV120			L	Q
p__Firmicutes	54.23 ^a	45.69 ^b	50.62 ^b	3.75	1.25	<0.001	<0.001
p__Bacteroidota	22.28 ^b	31.25 ^a	29.83 ^a	4.18	1.39	<0.001	<0.001
p__Actinobacteriota	11.02 ^b	14.56 ^a	10.59 ^b	1.88	0.62	<0.001	<0.001
o__Bacteroidales	22.25 ^b	31.17 ^a	29.79 ^a	4.16	1.38	<0.001	<0.001
o__Lachnospirales	20.44 ^a	16.24 ^b	10.16 ^b	4.47	1.49	<0.001	<0.001
o__Erysipelotrichales	17.37 ^a	13.02 ^b	14.05 ^b	1.97	0.65	<0.001	<0.001
o__Coriobacteriales	9.82 ^a	6.71 ^b	6.87 ^b	1.51	0.50	<0.001	<0.001
p__Euryarchata	3.69 ^a	2.91 ^b	3.57 ^a	0.36	0.12	<0.001	<0.001
c__Bacilli	18.53 ^a	13.30 ^b	14.13 ^b	2.44	0.81	<0.001	<0.001
c__Negativicutes	10.30 ^a	7.77 ^b	11.50 ^a	1.65	0.55	<0.001	<0.001
c__Coriobacteriia	9.82 ^a	6.71 ^b	6.87 ^b	1.51	0.51	<0.001	<0.001
c__Actinobacteria	1.19 ^b	7.84 ^a	3.71 ^{ab}	2.90	0.96	<0.001	<0.001
c__Methanobacteria	3.69 ^a	3.57 ^{ab}	2.91 ^b	0.36	0.12	<0.001	<0.001
c__Desulfovibrionia	2.30 ^a	0.23 ^b	0.82 ^b	0.92	0.30	<0.001	<0.001
g__Prevotella_7	13.62 ^b	28.24 ^a	19.66 ^a	3.19	2.06	<0.001	<0.001
g__Erysipelotrichaceae_UCG_002	0.03 ^b	9.82 ^a	11.11 ^a	5.25	1.75	<0.001	<0.001
g__Olsenella	8.77 ^a	6.45 ^b	6.46 ^b	1.16	0.38	<0.001	<0.001
f__Lachnospiraceae	20.68 ^a	16.18 ^b	10.27 ^b	3.68	1.22	<0.001	<0.001
g__Sharpea	13.36 ^a	0.10 ^b	2.01 ^b	6.20	2.06	<0.001	<0.001
g__Succiniclasticum	2.47 ^{ab}	1.90 ^b	9.49 ^a	3.66	1.22	<0.001	<0.001
g__Lachnospiraceae_NK3A20_group	4.44 ^a	2.03 ^b	4.99 ^a	1.36	0.45	<0.001	<0.001
g__Methanobrevibacter	3.54 ^a	2.88 ^b	2.78 ^b	0.36	0.12	<0.001	<0.001
g__Ruminococcus_gauvreauii_group	6.98 ^a	1.23 ^b	1.36 ^b	2.84	0.94	<0.001	<0.001
g__Dialister	4.73 ^a	3.50 ^b	0.67 ^b	1.80	0.60	<0.001	<0.001
g__Ruminococcus	0.14 ^b	1.18 ^b	7.44 ^a	3.41	1.13	<0.001	<0.001
g__Prevotella	0.73 ^b	0.52 ^b	5.49 ^a	2.43	0.81	<0.001	<0.001
g__Rikenellaceae_RC9_gut_group	3.90 ^a	0.47 ^b	1.35 ^b	1.54	0.51	<0.001	<0.001
g__Shuttleworthia	2.78 ^a	1.38 ^b	0.82 ^b	0.87	0.29	<0.001	<0.001
g__Bifidobacterium	0.02 ^b	1.04 ^a	3.21 ^a	1.41	0.47	<0.001	<0.001
g__Desulfovibrio	2.30 ^a	0.35 ^b	0.82 ^b	0.88	0.29	<0.001	<0.001

LVC – lavender volatile compound, LAV0 – calves fed milk without LVCs, LAV60 – calves fed milk with 60 $\mu\text{l}/\text{calf}/\text{day}$ of LVC; LAV120 – calves fed milk with 120 $\mu\text{l}/\text{calf}/\text{day}$ of LVCs; SD – standard deviation; L – linear contrast; Q – quadratic contrast; SEM – standard error of the mean; ^{ab} – means within a row with different superscripts are significantly different at $P < 0.05$

abundance of the phylum *Firmicutes* was higher in LAV0 than in LAV60 and LAV120 ($P < 0.001$; Table 5), while *Bacteroidota* in LAV60 and LAV120 abundance was higher in LAV60 and LAV120 than in non-supplemented group ($P < 0.001$; Table 5). At the class level, major bacterial taxa included *Bacteroidia*, *Clostridia*, *Bacilli*, *Negativites*, *Coriobacteriia*, *Actinobacteria*, *Gamma-proteobacteria*, *Desulfovibrionia* and *Planctomycetes* (Figure 2). The most abundant orders of bacteria and archaea were *Bacteroidales*, *Lachnospirales*, *Erysipelotrichales*, *Coriobacteriales*, *Veillonellales-Selenomonadales*, *Acidaminococcales*, *Oscil-*

lospirales, *Bifidobacteriales*, *Clostridia_UCG-014*, *Methanobacteriales*, *Desulfovibrionales* and *Enterobacteriales* (Figure 3). The relative abundance of the order *Bacteroidales* in the rumen fluid of calves in the LAV60 and LAV120 groups was higher than in the control group ($P < 0.05$; Table 5). In contrast, the relative abundance of orders *Lachnospirales* and *Erysipelotrichales* from the phylum *Firmicutes*, and the order *Coriobacteriales* from the phylum *Actinobacteriota* linearly decreased with increasing LVC supplementation ($P < 0.001$; Table 5). The relative abundance of archaea (genus *Methanobrevibacter*), class *Desulfovibrio-*

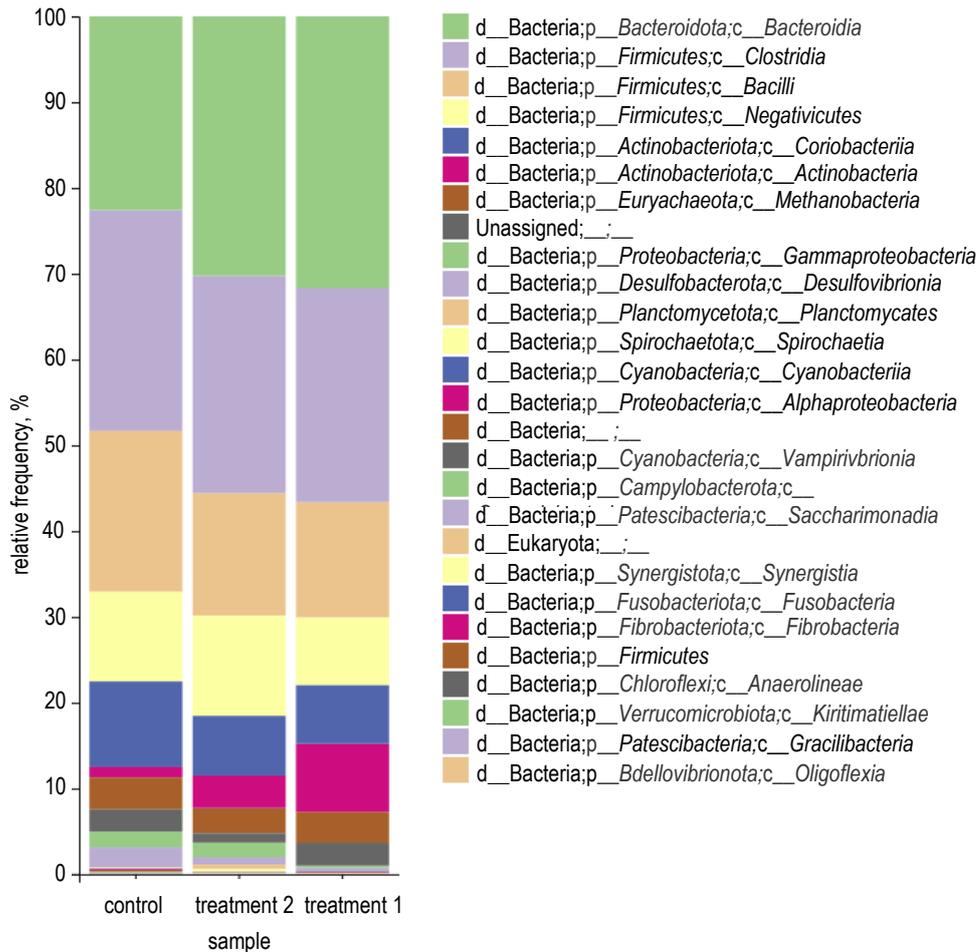


Figure 2. Relative abundance of bacterial and archaeal classes in the rumen fluid microbiome of calves fed milk containing lavender volatile compounds (LVCs). A total of 18 rumen fluid samples (6 calves × 3 groups) were analysed, and values represent the average of 6 samples per group

nia, genus *Rikenellaceae_RC9_gut_group*, genus *Ruminococcus_gauvreauii_group* decreased linearly with LVC supplementation ($P < 0.05$). In contrast, the relative abundance of the genera *Prevotella_7*, *Erysipelotrichaceae_UCG_002*, *Succiniclaticum*, *Ruminococcus* and *Bifidobacterium* increased linearly with incrementing LVC levels ($P < 0.05$; Table 5).

Blood serum levels of immunoglobulin E, pro-inflammatory cytokines and amyloid-A

Serum IgE titres were not affected by LVC supplementation. In contrast, the concentrations of the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, decreased linearly with increasing LVC levels ($P < 0.05$; Table 6).

Table 6. Immunoglobulins, pro-inflammatory cytokines and amyloid-A values in serum of calves fed with milk containing (LVCs)

	LVC addition to milk			SD	SEM	P-value	
	LAV0	LAV60	LAV120			L	Q
IgE, mg/l	92.86	89.74	97.46	17.57	4.26	0.663	0.572
TNF- α , pg/ml	687.49 ^a	525.99 ^b	477.24 ^b	136.77	26.34	0.004	0.307
IL-1 β , pg/ml	14.38 ^a	10.51 ^b	11.61 ^b	6.19	1.46	<0.001	0.448
IL-6, pg/ml	1.66 ^a	1.42 ^b	1.36 ^b	0.22	0.05	0.014	0.352
SAA, mg/l	4.09 ^a	2.95 ^b	3.18 ^b	0.44	0.23	0.026	0.026

LVC – lavender volatile compound, LAV0 – calves fed milk without LVCs, LAV60 – calves fed milk with 60 μ l/calf/day of LVC; LAV120 – calves fed milk with 120 μ l/calf/day of LVCs; IgE – immunoglobulin E, TNF- α – tumour necrosis factor-alpha, IL-1 β – interleukin-1 beta, IL-6 – interleukin-6, SAA – serum amyloid A; SD – standard deviation; L – linear contrast; Q – quadratic contrast; SEM – standard error of the mean; ^{ab} – means within a row with different superscripts are significantly different at $P < 0.05$

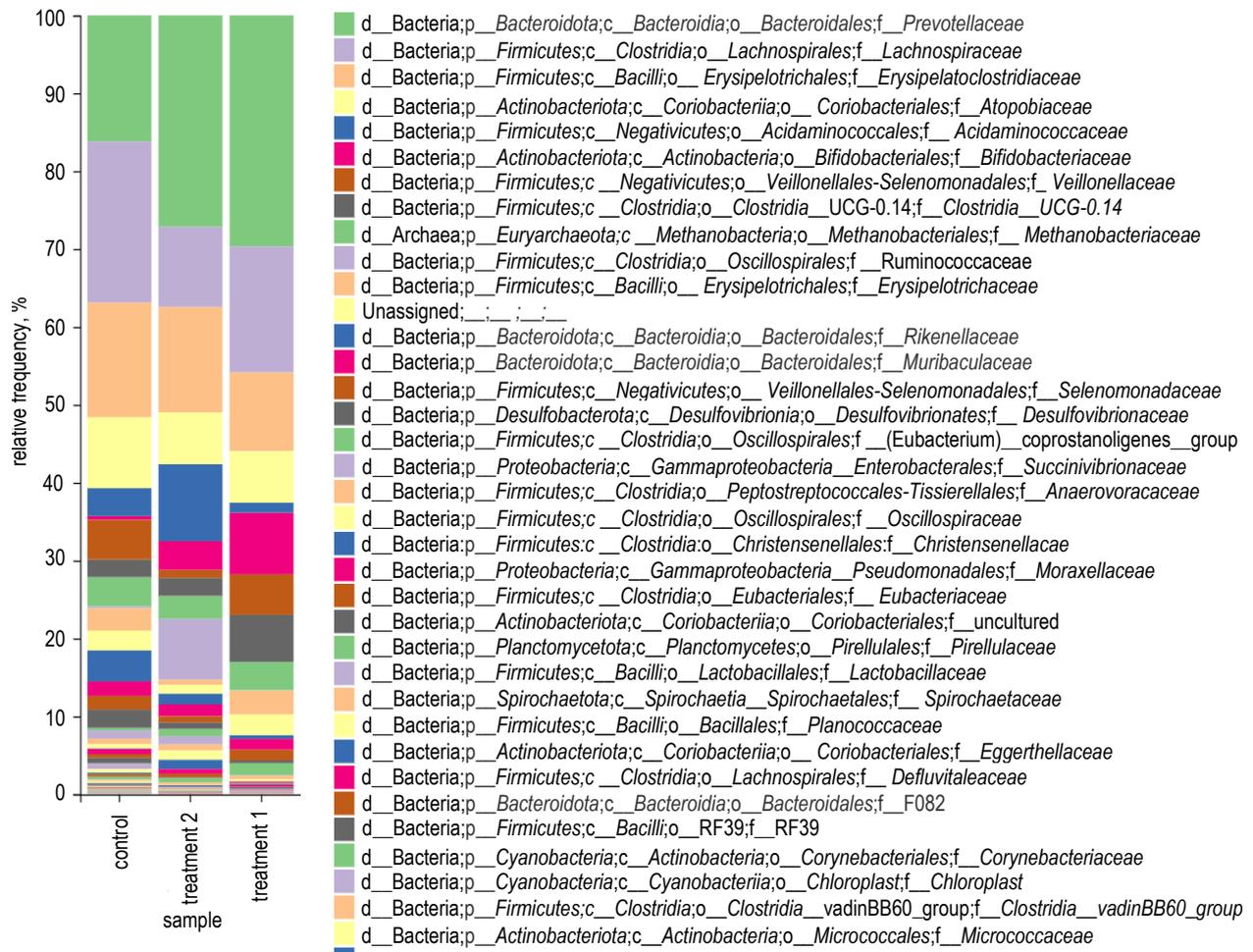


Figure 3. Relative abundance of bacterial and archaeal families in the rumen fluid microbiome of calves fed milk containing lavender volatile compounds (LVCs). A total of 18 rumen fluid samples (6 calves × 3 groups) were analysed, and values represent the average of 6 samples per group

Discussion

Performance parameters

The efficacy of *Lamiaceae* plant-derived essential oils on performance is influenced by the composition and proportions of bioactive compounds, including monoterpenes, monoterpene phenols, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes (Estrada-Angulo et al., 2021; Ramos da Silva et al., 2021). The increased feed intake observed in this study could be related to the odorous substances in lavender volatile compounds (LVCs) acting as flavour enhancers (Spence, 2022). Feed intake and body weight increased linearly with LVC supplementation, likely due to the positive effects on ruminal TS-FCA, AcA, P, ammonia-nitrogen, and modifications in the relative abundance of rumen bacteria and archaea. No further significant differences in body weight were observed at the high LVC dose at the end of the study, and body measurements at both low and high doses of LVCs have been limited by the small sam-

ple size (6 calves per group). Similarly, Akbarian-Tefaghi et al. (2018) reported that average daily gain and feed efficiency remained unaffected by dietary VCs (thyme, eucalyptus, and celery) during the calf pre-weaning period. The increase in calf body weight with LVC supplementation corresponded with an increased relative abundance of *Prevotella*, a genus involved in both protein and carbohydrate fermentation (Betancur-Murillo et al., 2022), and a lower relative abundance of *Methanobrevibacter*, a methane-producing archaeon in the rumen (Danielsson et al., 2017). The addition of LVCs during the milk-feeding period had no effect on the growth parameters in the present study. Values for wither height, body depth, body length and hearth girth were similar to those reported by Benetton et al. (2019) for Holstein calves weaned at 75 days.

Rumen parameters

Linalool and linalyl acetate were the most abundant terpenes in LVCs used in this study. The proportions of these compounds within the total

terpenes and hydrocarbons were higher than those previously reported for LVCs (Popović et al., 2019). These compounds have been demonstrated to exert strong antimicrobial effects (Kivrak, 2018). The majority of the rumen bacterial population consists of Gram-negative bacteria, while feeding animals with concentrated diets increases the number of methanogenic Gram-positive bacteria (Castillo-Gonzalez et al., 2014). The antimicrobial effects of volatile compounds (VCs) vary; some are more effective against Gram-positive bacteria, others against Gram-negative bacteria, and a limited number is effective against both. For instance, Aljaafari et al. (2021) reported that VCs from *Echinophora platyloba* and thyme inhibited Gram-positive bacteria. On the other hand, lavender and thyme belong to the same family, which produce citronellol and carveol compounds that can inhibit Gram-negative bacteria. In addition, both Gram-positive and Gram-negative bacteria were affected by citral, eugenol, carvacrol and thymol terpenes (Aljaafari et al., 2021). Hydrophobicity of VCs allows them to penetrate bacterial cell walls, disrupting membrane integrity, increasing permeability, and causing the release of intracellular materials (Lopez-Romer et al., 2015). In a related study, the addition of sage VCs containing 1.8-cineole, α -thujone, cis-cimene, camphene, β -pinene, β -caryophyllene and camphor terpenoids to calf milk was found to modify the rumen microbiome (Kara and Pirici, 2024). The specific composition of active ingredients in LVCs varies and may explain discrepancies in effects on rumen microbiota. To our knowledge, no previous studies have investigated the effects of supplementing LVCs in the feed or milk of pre-weaning calves. The higher ammonia-N concentration observed in LAV120 calves could be associated with the increased relative abundance of the genus *Prevotella*, a dominant rumen bacterium important for protein and peptide metabolism (Wallace et al., 1997).

Ruminal microbiota plays a key role in the fermentation of feedstuffs and the production of fermentation end-products, including SCFAs, BCFAs and ammonia-N (Kara, 2024). In the present study, the rumen bacterial community of weaned calves was mainly composed of *Bacteroidota* and *Firmicutes*, which together accounted for approximately 90% of the microbiome. This is consistent with previous studies reporting the same abundance of 90% in cattle rumen microbiota based on the 16S rRNA gene (Delgado et al., 2019). LVC supplementation in milk influenced the relative number of several bacterial families in calf rumen fluid, including *Prevotellaceae*, *Lachnospiraceae*, *Erysipelotrichaceae*, *Atopobiaceae*, *Acidaminococcaceae*, *Bifidobacteriaceae*, *Veillonellace-*

ae, *Ruminococcaceae* and *Desulfovibrionaceae*. The population of the family *Ruminococcaceae* of cellulolytic bacteria increased in response to LVC supplementation, particularly in the LAV120 group, while *Lachnospiraceae* decreased. LVCs also elevated the relative abundance of *Prevotella*, which contributes to protein and carbohydrate fermentation, potentially improving nutrient utilisation. This likely explains the linear increase in ruminal ammonia-N concentration observed in calves fed LVC-supplemented milk.

At the genus level, LVC supplementation increased the population size of *Prevotella_7* (13.8% vs. 20.0–28.2%) and *Erysipelotrichaceae_UCG-002* (0.2% vs. 9.96–11.26%) in rumen fluid of weaned calves. In adult ruminants, cellulose- and lignocellulose-degrading bacteria, including *Prevotella*, *Rikenellaceae RC9 gut group*, *Ruminococcus*, *Saccharofermentans*, *Butyrivibrio*, *Succiniclasticum*, *Selenomonas*, and *Streptococcus*, are predominant in the rumen, particularly under grass-based feeding regimes (Rabee et al., 2022). The higher number of *Prevotella_7* in calves receiving LVCs may have enhanced the fermentation of plant cell wall carbohydrates in the starter feed and wheat straw (Kara and Pirici, 2024).

The increase in ruminal AcA, PA, and T-SCFA concentrations observed with LVC supplementation could be linked to the higher abundance of *Prevotellaceae*, which produce AcA and PA through fermentation of structural carbohydrates such as xylan, xyloglucan, and pectin (Seshadri et al., 2018). In addition, the genus *Succiniclasticum* ferments succinate almost exclusively to PA (van Gylswyk, 1995) and its relative abundance in the rumen microbiome rose from 2.5% in LAV0 to 6.6% in LAV120, which corresponded with the increase in PA concentration from 26.31 to 37.71 mmol/l.

The ruminal archaeal community of weaned calves in the current study was mainly composed of *Methanobrevibacter* and *Methanosphaera*, both belonging to the order *Methanobacteriales* within the phylum *Euryarchaeota*. These archaea utilise H_2/CO_2 or H_2 /methanol produced by fermentative bacteria during the ruminal degradation of plant material (Mackie et al., 2024). *Methanobrevibacter* spp. in particular, dominate the hydrogenotrophic pathway, catalysing the reduction of CO_2 to methane (Danielsson et al., 2017). LVC supplementation reduced the relative abundance of methane-producing archaea, suggesting an anti-methanogenic effect. This effect is consistent with the enhanced growth performance observed in the weaned calves, as reduced methane production is often associated with improved feed efficiency and energy retention.

Immune status

In the present study, LVC supplementation decreased the concentrations of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6), indicating an immunomodulatory rather than a stimulatory effect. This outcome is consistent with the findings of Giovannini et al. (2016). The reduction in cytokine production may help mitigate the inflammatory response typically observed during weaning and reduced milk intake (Kim et al., 2011). The immunomodulatory activity of LVCs could be attributed in part to eucalyptol (1,8-cineole) monoterpene with well-documented anti-inflammatory properties (Juergens et al., 2004; Serafino et al., 2008). SAA, a major acute-phase protein, whose levels increase during stress and infection of calves (Tóthová et al., 2015), linearly decreased with LVC addition. Specifically, LVC supplementation before weaning reduced SAA concentrations at weaning by 25%, indicating a protective effect against inflammatory agents. The primary components of LVCs, linalool and linalyl acetate, are not inherently allergenic. However, when exposed to air, they can undergo autoxidation and form allergenic degradation products (Sköld et al., 2008). In the current study, LVC was absorbed into milk without air exposure, which was reflected in lack of changes in serum IgE titre, indicating no allergenic response. Importantly, none of the tested LVC doses exerted adverse effects on calf health.

Conclusions

The addition of lavender volatile compounds (LVCs) to the milk of pre-weaning calves has the potential to improve their feed intake and body weight. Supplementation at 60 or 120 μ l/day enhanced ruminal fermentation by modifying the rumen microflora, promoting the growth of bacteria involved in the degradation of protein, cellulose, lignocellulose and starch, thereby supporting solid feed fermentation after weaning. Both doses also demonstrated an anti-methanogenic effect by reducing the abundance of methanogenic archaea in the rumen. Furthermore, LVC supplementation at these levels mitigated weaning-related stress and strengthened immune function by lowering pro-inflammatory cytokine concentrations.

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Conflict of interests

The Authors declare that there is no conflict of interest.

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